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Integrating artificial with natural cells

Tutor

Prof. Sheref S. Mansy

Armenise-Harvard Laboratory of synthetic and reconstructive biology

CIBIO (Centre for Integrative Biology)

Ph.D. Thesis of

Roberta Lentini

Armenise-Harvard Laboratory of synthetic and reconstructive biology

CIBIO (Centre for Integrative Biology)

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Declaration

I, Roberta Lentini, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

PhD candidate

Abstract

Previous attempts to control cellular behavior were mainly based on genetic engineering. While useful, such an approach suffers from several complications. Living cells grow and evolve which could lead to modifications of the engineered circuits, causing not only the loss of their functions but also an altering of the environment. However, other methods are possible. All living cells can naturally sense and respond to their environment and to each other. Thus, artificial, non-living cells can be engineered to activate already existing natural cellular pathways. In this way, the genetic engineering component moves from the natural to completely artificial, laboratory-made cells. Moreover, synthetic systems operating in living organisms also depend on elements with unknown function, leaving many gaps in the understanding of how living cells work. Building life-like systems with non-living components could help reveal unrecognized but necessary cellular mechanisms. However, the design of functional, genetically encoded cell-free systems is difficult, because biological parts have been evolved to function optimally inside of living cells. *In vitro* conditions are different.

First, some practical rules for the construction of functional synthetic circuits *in vitro* were defined. The Influences of the organization of genetic elements within a synthetic operon on protein expression levels were studied and optimal sequence compositions and lengths between genes to assemble genetic circuits were found. Then, artificial cells that can control the behavior of living systems were built. The artificial cells were able to sense a molecule that *Escherichia coli* cannot sense on its own and translate that molecule into a chemical message that *E. coli* can sense and respond to. The natural sensing of *E. coli* was expanded without genetically modifying the bacteria. Finally, to better integrate artificial with natural cells, a complete communication pathway was constructed. Bacteria speak to each other by quorum sensing. Such mechanisms mediate cell-cell communication among bacteria and regulate several cell density related processes, such as virulence. Various synthetic quorum sensing mechanisms were constructed *in vitro* within artificial cells. Artificial cells able to sense the presence of living cells were built. In addition, the artificial cells were capable to synthesize quorum sensing molecules for *E. coli*, *Vibrio harveyi*, *Vibrio fischeri* and *Pseudomonas aeruginosa*. When integrated together, artificial cells successfully mediated interspecies communication with natural cells. Such artificial systems could be useful as therapeutic tools to defeat pathogenic infections. Moreover, the achievement of such functions represents a new way to better understand the potential of the artificial cells to mimic cellular life.

Table of contents

Abbreviation list

3OC4 HSL = 3-oxo-N-(2-oxotetrahydrofuran-3-yl)butanamide

3OC6 HSL = N-3-(oxohexanoyl) homoserine lactone

3OC12 HSL = N-(3-oxododecanoyl)-l- homoserine lactone

32P-ATP = 32P- alpha adenosine triphosphate

ACP = acyl carrier protein

αHL = alpha-hemolysin

AHLs = acyl homoserine lactones

AI-2 = autoinducer 2

C4 HSL = N-butanoyl-l-homoserine

C8 HSL = N-octanoyl-L-Homoserine lactone

CRP = cAMP receptor protein

DOPG = 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

DPD = 4,5- dihydroxy- 2,3- pentanedione

DSPE = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine

DTNB = 2,2'-dinitro-5,5'-ditiodibenzoico

DTT = dithiothreitol

FACS = fluorescence-activated cell sorting

HLPT = His-LuxS-Pfs-Tyr

IPTG = isopropyl ß-D-1-thiogalactopyranoside

PCR = polymerase chain reaction

PE = phosphatidylethanolamine

PEG = polyethylene glycol

PG = phosphatidylglycerol

POPC = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

QS = quorum sensing

RBS = ribosome binding site

RT-qPCR = reverse transcription quantitative PCR

SAH = S-adenosylhomocysteine

SAM = S-adenosylmethionine

SRH = S-ribosylhomocysteine

Chapter 1.
Cellular imitations

This chapter was adapted from:

Cellular imitations

Michele Forlin, **Roberta Lentini**, Sheref S. Mansy

Curr Opin Chem Biol, 2012, 16, 5-69: 586-592[1]

Attached at the end of the chapter.

The main approach used in synthetic biology relies on the construction of new behavior within already existing cells through genetic engineering. Living cells are genetically modified to accomplish specific tasks. For example, biological parts are assembled in predictable systems and inserted in natural cells building organisms that can detect pollutants[2] or produce pharmaceuticals[3]. A large variety of standardized biological parts are now available[4, 5] to create new complex genetic circuits within living cells[6, 7]. Several studies show engineered bacteria which could be used as a diagnostic tool[8], for both the prevention and treatment of different diseases. For example, programmed bacteria are able to recognize and invade cancer cells[9] or to act as biosensors to sense and report exposures to chemicals[10]. In this view, the engineered bacteria will be applied to the patients. However, the control of living cellular behavior through genetic intervention could lead to various complications. Engineered pathways have to be stable also in complex environments different from the controlled conditions found in the laboratory. Living systems grow and evolve which could lead to the modification of the engineered circuits, causing not only the loss of their functions but also to the altering of the environment. To avoid such complications, the genetically engineered parts could be moved from the natural cells to artificial, laboratory-made cells. Artificial cells can be made to only contain the elements needed for a specific task and disappear from the environment once that task has been accomplished.

Moreover, the construction of new biological function could lead to significant advances to the knowledge of how life works. However, synthetic systems operating in living organisms also depend on elements with unknown function, leaving many gaps in the understanding of how living cells work. Building life starting from scratch, using non-living components will bring to a better understanding and help to move from traditional engineering. There is no clear definition of life and no defined criteria to describe what is alive. Thus, efforts are made in imitate something that resembles extant cells and in mimicking some common features of living systems[11, 12].

1.1 Reconstructing the parts and organization of life

Cellular mimics are typically constructed from the same building blocks as natural living cells, (Fig. 1.1) including a compartment of some type to distinguish the cell from the surrounding environment, nucleic acids to store genetic information, and some type of machinery to synthesize proteins. Each part can be functionally produced in the laboratory. The construction of the compartment can be easily achieved since lipids spontaneously assemble in aqueous solutions forming vesicles. Non-lipid defined compartments can be made with nonbiological polymers and proteins[13]. Transcription and translation can be carried out *in vitro* with reconstituted systems made from purified components[14] or from cell extracts[15]. Moreover, protein synthesis is amenable to the conditions inside of a vesicle[16].

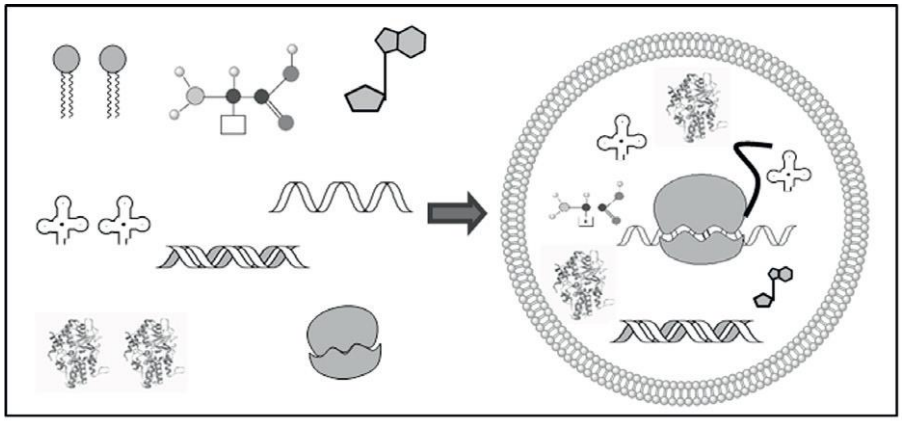

Fig. 1.1 Minimal components of artificial cells.

Artificial cells often comprise a compartment made of lipids to separate the inside from the outside, DNA or RNA to store genetic information and transcription-translation machinery to synthesize proteins (adapted from Forlin et al.[1]).

To further organize the interior of the artificial cells, polymers, such as dextran and polyethylene glycol, can be added to form distinct aqueous phases[17] in which molecules can preferentially partition. Aqueous phase separation mimics the segregation properties of intracellular organelles inside of living cells. In fact, aqueous phase separated systems are compatible with protein synthesis[18] resulting in preferential partitioning of the hydrophilic proteins to the dextran rich phase. However, recent studies suggest an inability of aqueous phase separated systems to efficiently segregate RNA molecules[19]. Vesicle organization can also be improved by reconstructing a cytoskeleton[20] with bacterially derived cytoskeletal elements that self-assemble into filamentous structures within phospholipid membranes[21]. Such structures can also be used to drive shape changes. For example, actin filaments anchored to liposome membranes[22] contract upon the addition of myosin[23].

The construction of something that physically looks like a cell is not enough to be perceived as a living cell. Living organisms are able to reproduce, evolve, communicate with the external environment, move and adapt. So far, most of the effort put into mimicking cellular life has been on replication and evolution, although a few studies have explored other features of life.

1.2 Artificial reproduction

Cellular mimics need a way to replicate their genetic information and their compartment. One fundamental cellular function is represented by replication. While DNA replication *in vitro* is

easy, the reconstruction of the replication system of a cell is challenging. Bacterial isothermal DNA replication machinery based on thermophilic helicase is able to replicate DNA when encapsulated inside phospholipid vesicles[24]. Moreover, a four protein based replication system based on phage Phi29 allows for the replication of the entire viral genome *in vitro*[25] [26]. To reduce the amount of elements needed for replication, RNA based systems were constructed inside vesicles in a way that the RNA molecule acts as a template both for replication and protein synthesis[27]. Moreover, such systems presented the ability to evolve, while performing long-term replication errors are introduced and highly replicable mutants prevailed over the population, following Darwinian evolution[28].

It may be possible to simplify cellular mimics by removing the dependence upon proteins, and instead exploit RNA catalytic activity. RNA polymerase ribozymes functionally replicate a wide range of RNAs sequences[29] and even catalyze the accurate synthesis of RNA sequences longer than themself[30]. Perhaps it will eventually be possible to fully replicate genomes with a replicase ribozyme in the absence of proteins. Alternatively, replication could be further simplified by removing the catalyst all together. For example, imidazole activated-nucleotides can diffuse into fatty acids vesicles and mediate template-directed polymerization[31] [32].

Several advancements in *in vitro* vesicle replication were made. Vesicle budding and division were achieved without proteins due to the presence of different lipid micro domains and the application of osmotic pressure[33]. In addition, systems encapsulating two different aqueous phases were able to undergo one cycle of division[34]. While when both mechanisms were joined together, even daughter vesicles maintained the correct asymmetry needed to divide[35]. Vesicles composed by phospholipids and fatty acids have the capacity to grow into unstable filaments that then split apart with slight mechanical agitation[36] or through internal chemical mechanisms dependent on thiol oxidation-reduction reactions[37].

It is also possible to divide vesicles based on the activity of proteins. Recent studies focus on the reconstruction *in vitro* of minimal cell division machinery[38] composed of the elements necessary for the generation of a constriction force. In particular, FtsZ forms *in vivo* a constriction ring, the Z ring, together with other proteins that eventually leads to cell division. When inserted within liposomes, an engineered FtsZ is able to reach the membrane and form multiple Z rings that exert constriction in vesicles[39] but are not able to divide the lipid compartment. The incorporation of the partner protein FtsA that helps mediate interactions between FtsZ and the lipid membrane apparently leads to complete liposome scission[40]. Another key division element is the Min protein system that oscillates *in vivo* along the bacterial cell to select the division site in the center of the cell. *In vitro* reconstitutions form waves on flat membranes[41, 42] and oscillate *in vitro* in synthetic systems mimicking bacterial cell shape[43]. When integrated in a single *in vitro* system, Min proteins

form a gradient that targets FtsZ to the middle of the cell-like compartment, regulating the localization of the protein[44].

Sugawara developed instead an alternative method for vesicles division dependent upon intravesicular DNA replication. By coupling two different fundamental processes plus a few specific membrane interacting molecules, the replication of DNA through PCR inside the lipid compartment lead to ionic interactions between the DNA and the membrane resulting in the division of the vesicle[45].

1.3 Life-like behavior

Several advances were made in the construction *in vitro* of cellular division, organization and replication. However, such features are not enough to perceive artificial cells as living. Natural cells sense and respond to their internal and external environment. Recent studies show a step forward through the construction of *in vitro* sense-response systems with the development of several sensing pathways. Synthetic genetic circuits can be used *in vitro* to sense small molecules, such as IPTG, and activate or repress different pathways[46]. Multiple step cascades[47], logic gates and feedback loops[48] can be implemented in cell-free systems and controlled by the availability of small molecules. In addition, *in vitro* systems were built to sense molecules secreted from living cells, such as bacterial homoserine lactones, that can be used to activate gene expression[49]. Communication with the external environment can be established through the diffusion of activators and repressors among connected silicon compartments[50].

Other than proteins, *in vitro* transcription and translation can be controlled by RNA regulatory elements, such as riboswitches[51]. Riboswitches reside in the untranslated region of mRNA and act as transcriptional or translational controlled elements, changing their conformation upon the binding of specific molecules and in response regulating protein expression. Such regulatory elements can be exploited within artificial cells to build systems able to sense the external environment. For example, a theophylline riboswitch functionally can control protein production inside of phospholipid vesicles[52].

In addition, non-genetically encoded sensing mechanisms are possible. In two aqueous phase systems, proteins localization can change in response to pH fluctuations[53] and chemical systems can move towards food molecules[54].

The formation of pores into the lipid membrane allows the continuous exchange of nutrients between the inside and the outside of the artificial cells[55]. Not only can energy sources be exchanged between the artificial cells and their surroundings, but also small molecules can traverse the membrane, which could be exploited to send chemical messages to living systems.

Autoinducers, small molecules naturally secreted from bacteria, can be synthesized within vesicles and can escape through pores to bacterial cells[56]. Pore forming protein activity in artificial systems can be also increased through *in vitro* evolution performed directly inside liposomes to generate highly functional membrane proteins[57].

The capacity to sense the external environment, elaborate the information and send an output to the outside can be unified in a single artificial system. This could lead to an encapsulated, artificial system that senses a chemical secreted by living cells, the synthesis of a new signaling molecule inside the vesicle in response to the sensed signal, release of the newly synthesized molecule, and finally detection by natural cells[58].

1.4 Conclusion

Artificial cells able to sense and respond to their surroundings could better represent the complexities of life and may come closer to being perceived as living. Pursuing this approach, it will be possible to construct artificial systems that are better integrated with natural cells. The achievement of such functions could be useful to better understand the potential of the artificial cells to mimic cellular life. Although several life-like behaviors were successfully reconstructed *in vitro*, there is not a clear idea of what to build if the goal is to build a living cell. This is partly because there is no agreed upon definition of life. The result has been a somewhat subjective evaluation of artificial cells in terms of their relatedness to known living systems. One path forward that could potentially remove such obstacles would be to construct artificial cells with the ability to perceive the presence of living organisms and in response send chemical messages to the organism. Since, in this case, the artificial cell would be engaging the natural cell in a manner analogous to another natural cell, the ability of the natural cell to distinguish between the two can be evaluated. The evaluation of the life-like properties of the artificial cell would, therefore, be moved from us, the observers, to natural cells. Similar to that described by Turing to evaluate the intelligence of a machine[59], natural cells interacting with artificial cells could be used to evaluate how life-like the cellular mimic are without conclusively passing judgment on whether something is actually alive or not[60]. In other words, a useful metric would become available that could help push the field forward.

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Cellular imitations

Michele Forlin, Roberta Lentini and Sheref S Mansy

Synthetic biologists typically construct new pathways within existing cells. While useful, this approach in many ways ignores the undefined but necessary components of life. A growing number of laboratories have begun to try to remove some of the mysteries of cellular life by building life-like systems from non-living component parts. Some of these attempts rely on purely chemical and physical forces alone without the aid of biological molecules, while others try to build artificial cells from the parts of life, such as nucleic acids, proteins, and lipids. Both bottom-up strategies suffer from the complication of trying to build something that remains undefined. The result has been the development of research programs that try to build systems that mimic in some way recognized living systems. Since it is difficult to quantify the mimicry of life, success often times is evaluated with a degree of subjectivity. Herein we highlight recent advances in mimicking the organization and behavior of cellular life from the bottom-up.

Address
CIBIO, University of Trento, via delle Regole 101, 38123 Mattarello (TN), Italy

Corresponding author: Mansy, Sheref S. (mansy@science.unitn.it)

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Introduction

The term synthetic biology was intended simply to denote the assembly of biological parts into larger systems, just as synthetic chemists build larger molecules from smaller molecules [1]. From this perspective, synthetic biology has grown into a wide spectrum of research programs (Figure 1) incorporating elements from engineering, biology, chemistry, physics, design, and art. The predominant way in which synthetic biology is practiced is to engineer subsystems within the larger framework of a cell that was not engineered. Individual, mostly natural, biological parts are thoroughly characterized, that is standardized, so that predictable (sub)systems consisting of these parts can be built. Just as the same set of Lego pieces can be used to build many different structures, standardized biological parts can be put together in many ways giving organisms that manufacture fuel, produce pharmaceuticals, or detect environmental pollutants. The exercise of building biological behavior, in turn, contributes to our understanding of how natural biological systems function. However, the construction of systems that operate within a host that is dependent upon genes with unknown function, as is the case for all known life, leaves many gaps in our knowledge untouched.

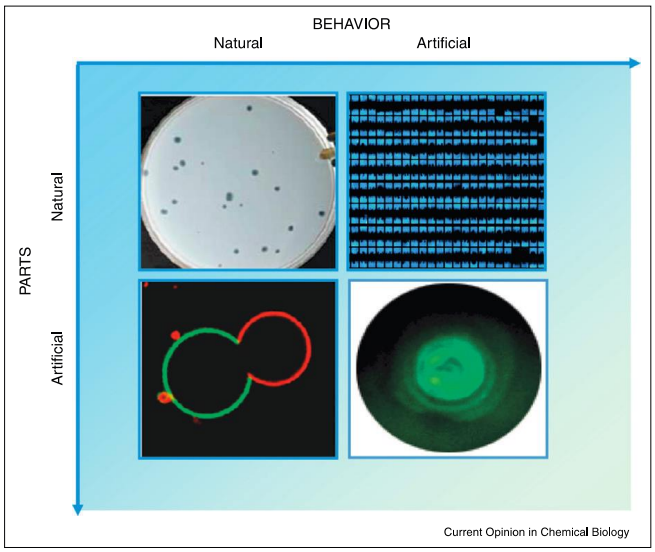
The engineering of life does not solely rely on the use of previously existing natural biological parts. Instead, new cellular pathways can be built with artificial components. Because of the difficulties associated with engineering proteins with new functionality, artificial RNA rather than protein molecules are more commonly exploited. For example, Gallivan and colleagues built a ligand responsive artificial RNA to engineer Escherichia coli to swim towards a pollutant molecule [2]. In this case, the artificial RNA was integrated with natural RNA and protein components to elicit the new behavior. Conversely, entire artificial systems can be made to exist within a natural host cell. For instance, orthogonal ribosomes can be engineered to not recognize natural host transcripts and only translate sequences containing orthogonal ribosome binding sites [3].

The de novo engineering of cellular life

The examples described above fit broadly within the engineering paradigm. In other words, life is treated as a machine in which characterized parts are assembled in various ways to generate systems with desired function. This is possible because the chassis, that is the host of the engineered genetic elements, is used to provide the ill-understood properties of life. If, however, the desired function is life itself built from non-living component parts, then we begin to move away from traditional engineering. This is because we do not have a clear idea of what is to be built. There is no satisfactory definition of life. Nevertheless, it is generally agreed that biological parts alone are not alive, but the properties that emerge from their cooperation are collectively referred to as living.

Without clear criteria that can be objectively fulfilled for a system to be considered living, the available path forward is simply to build systems that imitate the common features of life. For example, living things generally reproduce, move, adapt to changing environmental conditions, and interact with each other. Of these features of life, reproduction has attracted the most attention, which is understandable since replication and evolution form the foundation of life as we know it. However, a machine, even a machine that is built with natural biological parts,

Figure 1



Different ways in which synthetic biology is practiced. (Top-left) Natural parts can be used to build natural behavior. A refactored T7 genome supports the infection of *E. coli* (adapted with permission from Macmillan Publishers Ltd. [41]). (Top-right) Natural parts can be used to construct unnatural behavior. For example, natural sensory pathways were constructed in such a way as to give synchronous fluorescence of *E. coli* in a microfluidic device (adapted with permission from Macmillan Publishers Ltd. [42]). (Bottom-left) Artificial components can be used to mimic natural behavior. Poly(ethylene glycol) and dextran aqueous phases inside of phospholipid vesicles can divide (adapted with permission from [31]). (Bottom-right) An artificial part can be used to encode unnatural behavior. Here an artificial riboswitch was used to make *E. coli* swim towards a molecule that the bacterium does not naturally swim towards (adapted with permission from Macmillan Publishers Ltd. [2]).

that is programmed to copy DNA and to split into two probably would not be confused with a living system. Perhaps this is because the decision of whether something is alive or not is the result of a subjective comparison between what was previously agreed upon as living with the system in question. The successful mimicking of a single trait when compared against the complexity of a living cell would be perceived as an inadequate representation of cellular life. Additionally, the programming of repetitive behavior in itself misses another aspect of life, which is error. Cellular function is largely based on stochastic processes and even the fundamental event of genomic replication proceeds with error. A system that mimics a trait of life too well, probably would be perceived more as a machine rather than life.

The lack of clearly objective means of evaluating the outcome of experimental efforts in building a cell has slowed progress. A potential solution to this problem would be to shift the responsibility of determining whether something is alive or not away from us and towards natural cells. In this way, the interaction between the interrogator and the artificial system would be mediated by sensory pathways of similar scale. Such an approach is similar to that described by Turing in evaluating artificial intelligence in the absence of an agreed upon definition of intelligence [4]. The translation of such a Turing test to a cellular scale, as previously suggested [5], could allow for a more direct and unbiased way to evaluate success in building cell-like systems with life-like the behavior. A starting point for an artificial system that could pass the cellular Turing test could be the construction of a synthetic quorum pathway between an artificial and a natural cell [6].

The inability to define what is being built poses some problems, but also provides room for a variety of different research avenues. Mimics that morphologically resemble a cell, others that carryout similar chemical transformations as natural cells, and artificial systems engineered to pass a Turing-like test all will deepen our understanding of life. Thus far, most of the progress has been in building bottom-up replication and division mechanisms, but complementary studies are beginning to point to a more

exciting phase of bottom-up synthetic biology that better captures the complexities of life.

Reconstituting the parts and organization of life

To build something that looks like an extant cell, DNA, RNA, protein, and lipids should be assembled in a manner that gives a genetically encoded system with a cytoskeleton and a lipid membrane (Figure 2a). Each of these molecular components can be functionally reconstituted in the laboratory. However, the lack of knowledge concerning the way the biological parts fit together to give life is obvious when one considers that the successful synthesis of an entire genome [7] required genes of unknown function and a recipient host cell to provide additional components with unknown function.

When provided with the required monomeric building blocks, the information stored within a DNA molecule can be used to direct the synthesis of RNA through the activity of a single protein *in vitro*. Although the synthesis of protein from an RNA template is much more complex, after the pioneering work of Ueda and co-workers, it is now rather straightforward to carryout translation *in vitro* [8,9]. Similarly, the construction of a membrane-defined body to house a cell-like system is achieved easily *in vitro*. Many lipids spontaneously form vesicle membranes in aqueous solution that efficiently retain large molecules, allow for the selective exchange of small molecules, and are compatible with growth and division. The interior of a vesicle can be further organized. Polymer solutions, such as polyethylene glycol and dextran, can form distinct aqueous phases to which some molecules preferentially partition depending on their hydrophobicity [10].

Since protein synthesis proceeds efficiently in vesicles [11], vesicle structure and organization can be reinforced by the formation of cytoskeletal mimics (Figure 2b and c). Actin polymer filaments can be anchored to lipid membranes [12] and bacterially derived cytoskeletal elements can be assembled inside of vesicles [13]. It should be noted, however, that while active RNA polymerases can be produced through *in vitro* transcription–translation reactions, the *in vitro* production of translation machinery has not been achieved to date. Therefore, current bottom-up constructions of cellular mimics make use of bacterially derived translation components.

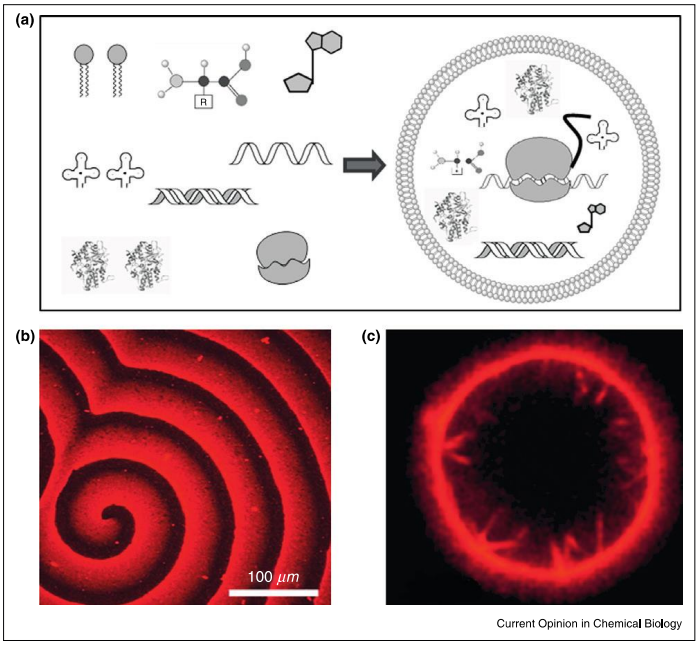
Artificial reproduction

At a minimum, cell-like reproduction consists of genomic replication and the division of the vesicle body [14]. The replication of DNA *in vitro* is easy, but to do so in a fashion amenable to the construction of a cell is challenging. A typical cell uses ten to twenty proteins to synthesize RNA primers, copy the leading and lagging DNA strands, substitute the RNA primer sequences with DNA, and ensure that no regions are left uncopied. Several isothermal DNA replication strategies have been developed that fulfill many of these needed activities [15,16]. However, thus far only the phi29 replication machinery has proven effective in copying entire genomic sequences end-to-end *in vitro* [17•]. Remarkably, only four phi29 proteins are necessary to copy viral genomes *in vitro*. Considering the small size of the phi29 bacteriophage genome, it will be important to determine whether the system in its current form will be capable of copying genomes with greater than 20 encoded genes.

Attempts to further simplify the construction of a cell have sought at times to remove some of the perceived redundancies of the DNA to RNA to protein pathway that pervades life. Since RNA and DNA are both capable of storing information, *in vitro* systems guided by RNA encoded information rather than DNA have been constructed in which the same RNA molecule acts as both the template for replication and the template for protein synthesis [18]. While this apparent simplification does reduce the number of needed components, it is unclear if an artificial, autonomous cell ultimately could be built with an RNA genome. DNA based life, that is all known life, is able to more easily separate genomic replication from the production of protein, whereas an organism that relies on an RNA genome would have to cope with the influences of RNA folding on replication and translation efficiencies [19] and on competition between RNA polymerases and ribosomes for the same template [20]. One potential solution would be to simplify the RNA genome-based organism even further by removing the need for protein function. Not only would this remove complications arising from coordinating replication and translation, it would also greatly simply the genome itself. This is because few genes are required for DNA and RNA synthesis, whereas protein synthesis necessitates over 100 genetically encoded elements [21]. Since RNA can possess catalytic activity and can replicate segments of RNA templates [22•], it is conceivable that a self replicating cell-like system could be built with an RNA genome and without proteins. Nevertheless, significant advances are required in RNA replicase processivity before such a goal can be accomplished.

The lack of a sufficiently processive RNA replicase could be circumvented by building systems that do not depend on catalysts. While the complexities of extant life probably require high activation energy barriers for metabolic processes to ensure proper control and coordination through enzyme activity, simpler cells may not require such regulatory mechanisms. For example, the incorporation of better leaving groups in nucleotides allows for template guided nucleic acid polymerization [23] that is compatible with lipid vesicles [24]. Other non-enzymatic mechanisms exist, too, such as those that exploit intercalators [25] or altered backbone connectivities [26].

Figure 2

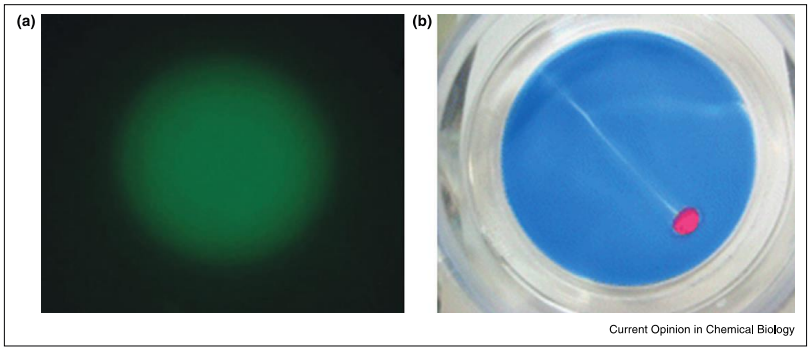


Structural organization of a cell. **(a)** Cellular mimics are often constructed from the basic parts of life, such as DNA, RNA, protein, amino acid, nucleotide, and lipid. **(b)** Some proteins can self-organize on lipid surfaces, such as those of the cell division Min system (adapted with permission from AAAS [28]). **(c)** A synthetic cytoskeleton built with polymerized actin inside of a vesicle (adapted with permission from Macmillan Publishers Ltd. [43]).

Impressively, several advances in *in vitro* vesicle division mechanisms have been reported. One such system relies on the bacterial division pathway consisting of Fts and Min proteins. In particular, focus has been placed on FtsZ, which forms a constricting ring *in vivo* localized to the midcell that divides the cell into two. The Min proteins help guide the placement of the Z-ring by inhibiting FtsZ polymerization at the poles of the cell. Although over fifteen proteins are believed to be involved in bacterial division, much simpler versions have begun to be built in the laboratory. For example, the tubulin homologue FtsZ was engineered to insert directly into membranes by Erickson and colleagues. This engineered protein polymerized into rings within tubular liposomes and generated noticeable indentations within the membrane [27], suggestive of the first steps of division. Although less work has been reported on the Min system, Min proteins self organize into protein waves on supported lipid bilayers consistent with their *in vivo* behavior [28]. To date, the Min and Fts systems have not been integrated into a single *in vitro* system.

Vesicle division mechanisms that do not depend on protein activity have proved easier to build *in vitro*. In fact, membranes consisting of three different lipids that phase separate into liquid ordered and liquid disordered domains can result in membrane curvature, budding, and division facilitated by osmotic pressures [29]. More recently an alternative system that exploits encapsulated aqueous two phase systems was shown to similarly induce budding and division in hypertonic solution [30]. While impressive, both methods only allow for a single cycle of division since the needed asymmetries are not retained in the daughter vesicles. However, when both mechanisms were integrated in such a way as to create a mismatch between the surface area of the two lipid domains with the volume of the two aqueous phases, the daughter vesicles maintained a level of asymmetry sufficient to allow for a second cycle of division [31••]. If this remarkable lipid domain – aqueous two phase system were coupled with a vesicle growth mechanism, then a self sustained growth – division cycle could be envisaged.

Figure 3



Examples of artificial systems that mimic cellular behavior. **(a)** Cell-like systems can be built to sense their surroundings. Here a riboswitch is used to sense the extravesicular addition of theophylline and responds by synthesizing a fluorescent protein (reproduced with permission from the Royal Society of Chemistry [38•]). **(b)** Just as cells move, droplets can be formulated to move down concentration gradients (adapted with permission of the Royal Society [40]).

An unrelated non-protein based system does just that, couples vesicle growth with division. Vesicles composed of single chain fatty acids have a broader range of accessible dynamics than vesicles made from the types of diacyl lipids that are typically found in biological membranes. Although the details of the mechanism are unclear, if fatty acid micelles are added to multilamellar vesicles, the vesicles grow into unstable thread-like filaments [32]. Division into daughter vesicles can be induced either by mild agitation or through the oxidation of thiol containing compounds that interact with the membrane when oxidized [33•]. The fluid shear force division mechanism can go through multiple growth and division cycles through forces imparted by the environment. The latter thiol oxidation mechanism suggests that if a metabolic-like oxidation–reduction cycle were reconstituted within the vesicle, then multiple rounds of growth and division could be mediated by internal processes rather than by external forces.

An alternative pathway developed by the Sugawara laboratory uses DNA replication to drive vesicle division. The lipid composition is more complex, including a mixture of natural and unnatural lipids plus a catalyst that converts precursor molecules into more lipid [34••]. During intravesicular DNA replication through PCR, ionic interactions between DNA and the membrane results in the division of the vesicle. Not only does this system couple two processes crucial for constructing cellular life, that is genomic replication and compartment division, the molecular components used are compatible with biological machinery, suggesting that cellular mimics that more closely resemble life as we know it could be built. However, the lipid composition of the membrane changes over the course of the reaction so that multiple rounds of division are not possible.

Life-like behavior

There are now available many mechanisms for vesicle division that could be exploited for the construction of a cell. However, as noted above, the construction of a self-replicating system in the absence of other distinguishing features of life is unlikely to be perceived as living. A more convincing cellular mimic would sense and respond to internal and external stimuli in order to coordinate different physiological processes and to adapt to changing environmental conditions. For example, natural cells ensure that division only occurs after genomic replication, and natural organisms adapt to fluctuating temperatures by modulating membrane compositions and protein chaperone levels. Interestingly, some of the environmental fluctuations that a cell must cope with arise from the cell itself, since living systems modify their environment by acquiring food and releasing waste. Although examples of *in vitro* constructed sense–response systems are few, recent developments suggest viable routes forward in exploiting sensory pathways for the building of cellular mimics.

In vitro genetic systems can be constructed to sense and respond to the availability of small molecules. An *in vitro* cascading genetic network, for example, was built to control the production of protein in response to IPTG [35]. More recently, *in vitro* negative feedback loops exploiting tetracycline [36] and arabinose transcriptional repressors [37] were built. Rather than using natural

protein transcriptional repressors, protein production can be controlled by the activity of artificial RNA sequences, such as that displayed by the theophylline riboswitch [38•] (Figure 3a). Riboswitches are regulatory elements residing in the untranslated regions of mRNA that control translation through direct ligand binding. The advantage of riboswitches is that they are much simpler to engineer than proteins. Of the systems described above, the arabinose sensing [37] and the theophylline sensing [38•] systems were reconstituted in phospholipid vesicles, thus allowing for the development of cellular mimics capable of responding to the chemical composition of their extravesicular surroundings.

Non-genetically encoded sensing mechanisms are a potential complement to the use of protein and RNA sensors. The aqueous two phase system developed by Keating and colleagues can be used to control the localization of molecules in response to environmental fluctuations. This is because many biological molecules undergo structural changes that affect their surface charge distribution upon shifts in pH or temperature [39•]. Sensing that results in the movement of a chemical system is also possible [40] (Figure 3b). Hanczyc and colleagues built a chemical system that moves away from depleted nutrients and towards molecules that sustain movement.

Now that it possible to build cellular mimics that sense and respond to changing chemical conditions, it seems that the time is right to begin to more deeply probe non-replication aspects of life. Sensory pathways are required for the construction of systems that better represent the complexities of extant life. Unlike life, machines are programmed to act in a very defined manner, performing a designated task regardless of external conditions. Cellular mimics with sense–response capabilities, therefore, probably would come closer to being perceived as living than a machine. Further, the incorporation of sense–response pathways allows for a more objective means of evaluating success through the implementation of a cellular Turing test.

Conclusion

Many of the features of cellular life now can be built in the laboratory. However, the individually reconstituted features of life may not be compatible with each other in their present form. Their integration into a system that better represents the complexity of life poses a significant challenge. It may be that the purely chemical approaches and those that make use of biological molecules will continue to proceed on separate tracks, which would be unfortunate. DNA replication is easier to achieve with the aid of proteins and vesicle division is simpler through purely chemical–physical means. If these two branches of bottom-up synthetic biology found a way to merge, perhaps the synthesis of an artificial cell would be much nearer.

Bottom-up synthetic biology has largely focused on self-replication and in the process has developed a wide variety of ways to copy nucleic acids and divide vesicles. However, life is not simply a machine that divides. Instead, life is integrated with its surroundings, both on a cellular and a chemical level. The recent advances in building cellular mimics capable of sensing and responding to small molecules opens an exciting alternative to the prevalent attempts at building bottom-up cells. Perhaps it is time to allow a bacterium to judge our work.

Acknowledgements

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Chapter 2.

In vitro genetic organization for cell-free synthetic biology

This chapter has been adapted from:

Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology [61]

Roberta Lentini, Michele Forlin, Laura Martini, Cristina Del Bianco, Amy C. Spencer, Domenica Torino and Sheref S. Mansy

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Attached at the end of the chapter. Supporting information attached in the appendix.

My contribution to this work concerns the design, cloning and mutagenesis of the genetic constructs, the setting and collecting of all the experimental measurements, manuscript writing and editing.

Many efforts were made in the standardization of biological parts finding methods to identify and characterize various genetic elements. For example, a set of standard biological parts is now available in the registry of standard biological parts[62]. However these parts are manly characterized in living organisms, and synthetic regulatory networks are well studied *in vivo* in both prokaryotic[63, 64]and eukaryotic[65, 66]. Living systems are not fully understood. The fact that new circuits are implemented in unknown environments makes difficult the realization of the desired behavior in extant cells[67]. Conversely, cell-free synthetic biology avoids the interferences derived from the complexity of living cells. In addition, building artificial cells that mimic cellular functions without the complexity of living cells could help contribute to our understanding of how living organisms work. However, the use of biological parts well characterized *in vivo* within the cell-free chassis is challenging. Biological parts evolved to function optimally inside of living cells, while *in vitro* conditions are undoubtedly different. Thus, the design of programmable genetically encoded cell-free systems is difficult because of the lack of a complete knowledge of *in vivo* genetics and of data relative to the cell-free chassis.

Artificial cells are constructed using minimal components and are characterized by a compartment, made of lipids or polymers, and transcription-translation machinery to synthesize proteins starting from nucleic acids. Transcription and translation can be carried out using cell-free extracts[15] such as the S30 *E. coli* translation system or with purified, fully defined components[14]. The system developed by Ueda, referred to as the PURE system, consists of T7 RNA polymerase and *E. coli* translation machinery. Studies have shown the suitability of both systems within liposomes[16, 55]. However, little is known regarding genetic organization influences on protein production in cell-free systems. Nevertheless, few recent studies attempted to define the influences of different ribosome binding site (RBS) in both PURE system and S30 *E. coli* extract[48], transcriptional repressors[47], promoters[49] and gene organization inside a synthetic operon[68].

The work described in this chapter sought to define simple and practical rules to build genetically encoded cell-free systems. First, a set of fluorescent proteins were characterized *in vitro* with the PURE system, then a ratiometric assay was developed to quantify in a precise way the influences of genetic organization in synthetic operons. Finally, the method was applied in a series of synthetic operons that differed in sequence compositions and spacing between the genetic elements within the operons.

2.1 *In vitro* expression of fluorescent proteins

Seventeen different fluorescent proteins were expressed with the PURE system at 37 °C to test their functionality within the cell-free systems. Four of the fluorescent proteins contained a substitution of the alanine at position 206 to a lysine to inhibit dimerization[69]. Most of the proteins

tested gave rise to easily detectable fluorescent signals above the background, except for CyPet and monomeric CyPet (mCyPet) (Fig. 1a Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). However, the two cyan fluorescent proteins were able to give rise to a slightly higher fluorescent signal when expressed at 30 °C, presumably because the lower temperature helped the proteins to fold properly. All kinetic experiments showed a sigmoidal shaped curve, except for Tag-RFP-T and T-sapphire, which did not reach their maximal fluorescence within 6 h. Kinetic data were fit to a logistic model to determine the time needed for each fluorescent protein to reach half of its maximal fluorescence ($t_{f/2}$). $T_{f/2}$ value includes all the steps needed to give rise to the final fluorescent output starting from the DNA and, therefore, this term is influenced by the time it takes for transcription, translation, protein folding, and maturation of the chromophore. GFP mut3b showed the shortest $t_{f/2}$ while Tag-RFP-T the slowest, with a time of 79 min and 300 min, respectively. The average $t_{f/2}$ values for red, yellow, green and cyan fluorescent proteins were 245, 122, 122 and 105 min, respectively (Fig. 1c Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). As previous studies reported[70], yellow fluorescent proteins were the brightest proteins tested, followed by green, cyan and red fluorescent proteins. The dimeric version of Venus was 40 % more intense than the monomeric Venus, while between monomeric and dimeric versions of Cerulean and YPet only 5 % of difference was shown. However, the error arising from the measurements of fluorescence signals for individual fluorescent proteins was too high to make any conclusions.

To reduce the experimental error due to pipetting, lamp performance and DNA quality a ratiometric assay was developed based on an operon containing two different fluorescent proteins. To construct such a system, a red fluorescent protein was ideal due to its fluorescent spectrum that is well separated from the spectra of other fluorescent proteins. Thus, super folder GFP (sfGFP) and mCherry were cloned within a bicistronic operon. Synthetic operons contained a T7 promoter, a ribosome binding site (RBS), *sfGFP* gene followed by *mCherry* and a T7 transcriptional terminator. Both fluorescent proteins gave rise to a detectable fluorescent signal. The fluorescent ratio was calculated by dividing the fluorescent intensity of sfGFP by the fluorescent intensity of mCherry. The kinetic profile of the ratiometric value after an initial increase, stabilized at 3 h and remained constant until the end of the kinetic experiment. Then, six bicistronic operons were built coupling mCherry with different fluorescent proteins. The profiles obtained at the end of the reactions were similar to the values obtained for the single constructs. Moreover, the experimental errors were dramatically reduced from 60 % of the single fluorescent proteins to the 8 % of the bicistronic operons (Fig. 1d Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). The mVenus – mCherry pair was chosen for the next experiments thank to its greater stability and higher

fluorescent ratio intensity. mCherry was placed in the first position of the operon to better function as reference for fluorescent signal.

2.2 Influence of the sequences upstream and downstream of the ribosome binding site

The resulted bicistronic operon contained a T7 promoter, mCherry followed by mVenus and a T7 terminator was used to determine influences due to spacer length and sequence composition immediately upstream and downstream of the second gene RBS. First, different spacer lengths between the stop codon of mCherry and the RBS of mVenus were tested. Then the nucleotides at the 5' of mVenus RBS were substituted with the most common used restriction sites. No correlation between different spacer length and variations in protein expression levels were found (Fig. 2 Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). However, a stronger effect is shown when sequence composition was modified. In particular, the presence of a NotI site upstream of the RBS reduced protein level by 70 % (Fig. 3 Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). Subsequently, the region at the 3' of mVenus RBS was modified. To monitor influences due to spacing, the length of the sequence between the RBS and the start codon was tested by adding one nucleotide at time. The results showed a Gaussian distribution in which the optimal length resulted between 4 and 9 bp (Fig. 4 Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489). As previously described, also the sequence composition between the RBS and the start codon of mVenus was modified. Sequences containing different restriction sites were placed immediately downstream of the RBS. In addition, a C-rich sequence and mutations that introduce additional base pairing with the 16S rRNA were tested. A strong influence of sequence composition was shown, in particular NotI site reduced protein expression by 87 % and the C-rich sequence decreased expression of 98 %. A longer, more extensive base-pairing region complementary to 16S rRNA did not affect protein production (Fig. 5 Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489).

Finally, non-AUG start codons were tested *in vitro* with the PURE system. *In vivo*, GUG and UUG function as start codons at a frequency of 14 % and 3 %, respectively[71]. Thus, the start codon AUG was substituted with GUG, UUG and CUG within the R027A construct. All start codons allowed protein production with a significantly reduced level comprised between 27 % and 12 %, respectively (Fig. 6 Lentini et al. Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology. ACS Synth Biol 2013, 2(9): 482-489).

2.3 Conclusions

The ratiometric assay developed to quantify the influences of the sequence between two proteins in an operon cannot discriminate between effects due to a lower production of the first gene or to a higher expression of the second gene. However, the assay could measure the influences of the sequence in the ratio of two encoded proteins in the same operon. The results obtained in this study showed that the influence of the sequence placed between two genes in a bicistronic operon is not uniform. The region at 5' of the RBS of the second gene affected less the protein ratio between the first and second gene, whereas the sequence at the 3' strongly influenced protein production. Both spacer length and sequence composition changes downstream of the RBS resulted in different outputs. For example, a high G content negatively correlated with protein ratio while the presence of a UA rich sequence lead to higher protein ratio. The optimal spacer length between the RBS and the second gene is comprised between 4 and 9 nucleotides. Taken together the results showed some simple rules to follow during the construction of synthetic circuits for *in vitro* transcription-translation. Which restriction site to use, where to place the restriction site, and what sequences between the RBS and the gene of interest are amenable to protein expression were all determined. Nevertheless, certainly several additional factors influence transcription-translation *in vitro*. A better knowledge regarding how synthetic devices work in cell-free systems will facilitate the construction of artificial cells.

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Fluorescent Proteins and *in Vitro* Genetic Organization for Cell-Free Synthetic Biology

Roberta Lentini, Michele Forlin, Laura Martini, Cristina Del Bianco, Amy C. Spencer, Domenica Torino, and Sheref S. Mansy*

CIBIO, University of Trento, via delle Regole 101, 38123 Mattarello (TN), Italy

Supporting Information

ABSTRACT: To facilitate the construction of cell-free genetic devices, we evaluated the ability of 17 different fluorescent proteins to give easily detectable fluorescence signals in real-time from *in vitro* transcription-translation reactions with a minimal system consisting of T7 RNA polymerase and *E. coli* translation machinery, i.e., the PUREsystem. The data were used to construct a ratiometric fluorescence assay to quantify the effect of genetic organization on *in vitro* expression levels. Synthetic operons with varied spacing and sequence composition between two genes that coded for fluorescent proteins were then assembled. The resulting data indicated which restriction sites and where the restriction sites should be placed in order to build genetic devices in a manner that does not interfere with protein expression. Other simple design rules were identified, such as the spacing and sequence composition influences of regions upstream and downstream of ribosome binding sites and the ability of non-AUG start codons to function *in vitro*.

KEYWORDS: *cell-free, fluorescent protein, transcription-translation, ribosome binding site, synthetic biology*

The majority of synthetic biology research makes use of a living chassis that provides for the necessary but poorly characterized biological components required for life. Conversely, a smaller community of synthetic biologists has begun to build cell-like systems with a nonliving, cell-free chassis.[1−7] Although the cell-free branch of synthetic biology has progressed more slowly, success could provide for new technologies with several beneficial features. For example, the resulting cellular mimics would consist of fully defined components. Therefore, it should be possible to build a complete mathematical model describing the cellular mimic that could aid in designing new features. Additionally, potentially technologically problematic features of life, such as evolution, could be intentionally removed by building systems that do not replicate.

A significant step forward in allowing for the construction of such well-defined, bottom-up systems came from Ueda and colleagues, who showed that coupled transcription and translation reactions can be mediated by fully defined components *in vitro*.[8] Their system, hereafter referred to as the PUREsystem, consisted of T7 RNA polymerase and *Escherichia coli* translation machinery. Subsequent work demonstrated the compatibility of the PUREsystem with liposomes[9,10] and with the expression of gene networks.[11] Nevertheless, there has been little attempt to better define the influences of genetic organization on protein output with purified transcription-translation machinery. Recently, a S30 *E. coli* cell extract translation system and the PUREsystem were used to determine the influences of different ribosome binding sites and transcriptional repressors on the synthesis of eGFP.[12,13]

Although much is known about natural, *in vivo* genetics, much still remains unresolved. For example, the refactoring of the T7 genome was successful in the sense that viable bacteriophage were produced; however, the refactored bacteriophage was significantly less infective.[14] Similar challenges are routinely encountered when genetic elements are inserted into organisms to engineer new circuitry. Typically, many permutations are required before desired function is achieved.[15] The situation is perhaps even more challenging for systems that exploit a cell-free chassis since biological parts are evolved to function optimally under the chemical conditions found *in vivo*. *In vitro* conditions are undoubtedly different. Further, unidentified molecular components necessary for activity *in vivo* may be missing from *in vitro* constructions. The design and implementation of predictable, genetically encoded cell-free systems is difficult because of the lack of cell-free chassis data coupled with an incomplete understanding of natural, *in vivo* genetics.

Here we sought to identify some practical rules for the construction of genetically encoded, cell-free systems. First, 17 different fluorescent proteins were screened for their ability to generate easily detectable fluorescence signals after *in vitro* transcription and translation with the PUREsystem. Fluorescent proteins then were expressed from a bicistronic construct to identify fluorescent protein pairs that could be used to quantify the influences of genetic organization on protein production. A series of synthetic operons that differed in the spacing and sequence between the two encoded genes, the spacing and sequence between the ribosome binding site and the start codon, and the influence of the first nucleotide position of the start codon on *in vitro* expression levels was assessed with the developed ratiometric fluorescence assay. We

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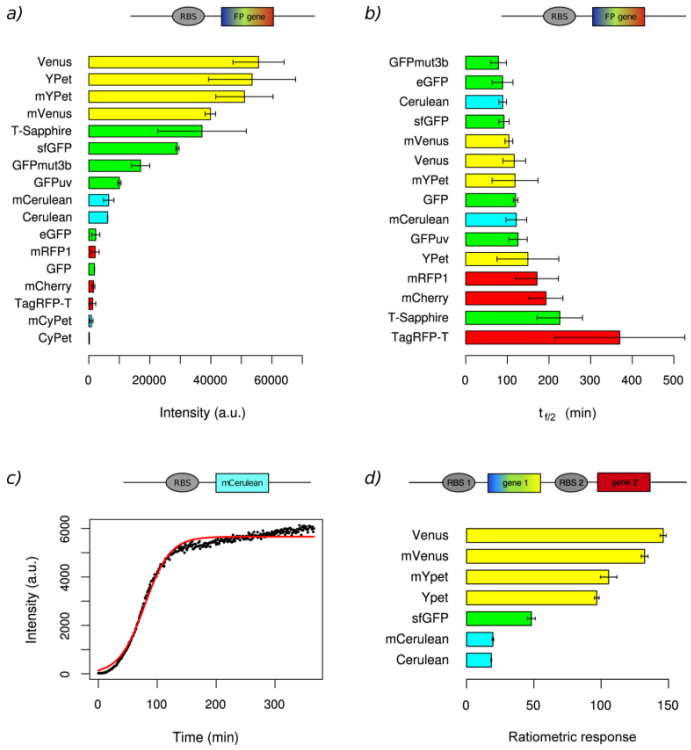


Figure 1. Fluorescence profiles of *in vitro* expressed genetic constructs at 37 °C with the PUREsystem. (a) Fluorescence intensities after 6 h of *in vitro* expression for 17 different fluorescent proteins. (b) The $t_{f/2}$ of each fluorescent protein was calculated by fitting the kinetic data to a logistic model as described in the Methods section. The $t_{f/2}$ represents the time at maximum growth. (c) The fitting of mCerulean kinetic data is shown as a representative example. The logistic model estimation is shown in red, while the black points represent measured values. A control reaction without plasmid showed no fluorescence. (d) The ratiometric response of bicistronic constructs after 6 h of *in vitro* expression. The ratiometric response was calculated by dividing the fluorescence arising from the protein encoded by gene 1 by the fluorescence resulting from the gene product of gene 2. Here gene 2 always encoded mCherry. A cartoon above each panel gives a schematic representation of the used constructs. The data shown in panels a and b are from constructs RL001A-RL013A and CD100A-CD103A. Panel c used RL005A, and panel d used RL015A-RL021A. More information on each construct is provided in Supplementary Table S1.

found that a high guanosine content inhibited translation, that sequences 5′ to the ribosome binding site were more amenable to the incorporation of restriction sites for cloning, and that ribosome binding sites were most efficient when separated from the start codon by 4−9 nucleotide positions. GUG, UUG, and CUG were functional as start codons in minimal, reconstituted translation systems, although their associated expression levels were significantly reduced.

RESULTS AND DISCUSSION

***In Vitro* Expression of Fluorescent Proteins.** A total of 17 different fluorescent proteins were tested individually for their ability to give easily detectable fluorescence signals from *in vitro* transcription-translation reactions with the PUREsystem at 37 °C. Of these 17 proteins, four (mCerulean, mCyPet, mVenus, and mYPet) contained a A206K substitution to inhibit dimerization. As seen in Figure 1a, all of the tested constructs produced easily detectable signals above background arising from the fluorescent protein except for CyPet and mCyPet. These two cyan fluorescent proteins gave slightly increased fluorescence when expressed at 30 °C (Supplementary Figure S1). Consistent with the reported brightness of each fluorescent protein,[16] the yellow fluorescent proteins were associated with the most intense fluorescence, followed by the green, cyan, and red fluorescent proteins (Figure 1a). Monomeric versions of Cerulean and YPet gave fluorescence intensities within 5% of their dimeric parent construct. *In vitro* transcribed and translated Venus was 40% more intense than mVenus; however, the error associated with the single fluorescent protein measurements was too large to make meaningful conclusions. This issue was resolved by using a ratiometric method described below. After 6 h of *in vitro* transcription-translation, the mVenus concentration reached 8 μM.

Most of the constructs gave sigmoidal shaped kinetic profiles and were complete within 6 h. The exceptions were T-Sapphire and TagRFP-T (Supplementary Figure S2), both of which did not reach their maximal fluorescence within 6 h. The fitting of the kinetic data to a logistic model was used to determine the time point at which the rate of fluorescence increase was maximal, which corresponded to the time required to reach half maximal fluorescence ($t_{f/2}$) (Figure 1b,c). Note that the $t_{f/2}$ includes all of the steps involved in converting the information encoded in DNA to a fluorescence signal and does not solely describe the last oxidation step of chromophore formation.[17] The shortest $t_{f/2}$ value was 79 min for GFPmut3b, and the longest $t_{f/2}$ was over 300 min for TagRFP-T (Supplementary Table S4). The average $t_{f/2}$ values for the expression of cyan, green, yellow, and red fluorescent proteins were 105, 122, 122, and 245 min, respectively. The $t_{f/2}$ was 40% larger for mCerulean than Cerulean, whereas mVenus and mYPet had $t_{f/2}$ values 12% and 26% smaller than Venus and YPet, respectively. On the basis of fluorescence intensity and kinetic data, Cerulean, mCerulean, super folder GFP (sfGFP), Venus, mVenus, YPet, mYPet, mRFP1, and mCherry were selected for further analysis.

To reduce experimental error, we pursued the construction of a ratiometric fluorescence system based on synthetic operons that encoded two fluorescent proteins. In this way the influences of pipetting, lamp performance, and DNA template quality and concentration, among other difficult to control variables, would be removed. To build such a ratiometric system, a red fluorescent protein was desirable because the excitation and emission spectra of red fluorescent proteins are better separated from the fluorescence spectra of other fluorescent proteins. mRFP1 and mCherry were, therefore, tested in bicistronic constructs that additionally encoded sfGFP to evaluate their utility in characterizing expression levels. More specifically, small synthetic operons containing a standard T7 transcriptional promoter, a ribosome binding site (RBS), a gene encoding sfGFP followed by a sequence that encoded the red fluorescent protein and a T7 transcriptional terminator were assembled. All of the fluorescent proteins in these constructs gave reproducible and easily detectable fluorescence signals. After 6 h of expression with purified transcription-translation machinery, the ratio of sfGFP fluorescence to mRFP1 and to mCherry fluorescence was 115.1 ± 6.9 and 49.9 ± 2.4, respectively (Supplementary Figure S3). We chose to use mCherry for the remaining experiments, because mCherry showed more intense fluorescence from the bicistronic construct and because mCherry was shown to be more photostable than mRFP1.[16]

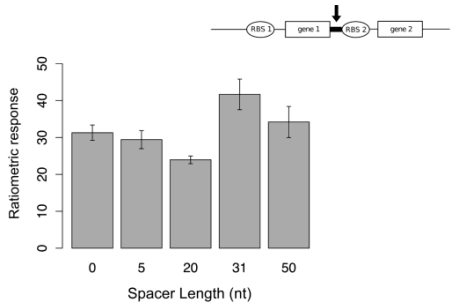
We next assembled six additional synthetic operons that encoded different fluorescent proteins followed by a sequence coding for mCherry. After *in vitro* transcription and translation, the fluorescence profiles were similar to those obtained with the single fluorescent protein constructs in that the yellow fluorescent proteins were the most intense, followed by green, and cyan fluorescent proteins (Figure 1d). However, the error of each ratiometric measurement was significantly reduced (relative standard error <8%) in comparison to the data obtained from the monocistronic, single fluorescent protein constructs (relative standard error <60%, excluding TagRFP-T). The A206K substitution that inhibits protein dimerization had a small effect on fluorescence intensity. More specifically, the ratiometric response, i.e., the fluorescence intensity of the fluorescent protein tested divided by the fluorescence intensity of mCherry, for mVenus, mYPet, and mCerulean were within 10% of the values measured for Venus, YPet, and Cerulean, respectively. The ratiometric response over time showed that stable readings could be taken after 3 h for all constructs tested (Supplementary Figure S4).

It was not clear from the outset which fluorescent proteins would perform well *in vitro* with minimal transcription-translation machinery. Although the physical characteristics of individually purified proteins, such as brightness and photostability, are useful in deciding if a protein could be suitable for a specific application, these parameters are not enough to understand if *in vitro* expression will give a robust, reproducible signal. For example, if *in vitro* produced protein is insoluble, folds slowly, or requires a long period of time for chromophore formation, then that protein would be less useful as an *in vitro* genetic reporter. Even within cells, differences in fluorescent protein behavior have been noted, particularly for multidomain proteins.[18] Despite these difficulties, we found that most of the fluorescent proteins tested function satisfactorily in *in vitro* transcription-translation reactions with the PUREsystem at 37 °C. One exception is CyPet, which fails to give a significant fluorescent output. The fact that CyPet expression at 30 °C gives a better fluorescence signal is consistent with previous reports on the poor folding properties of CyPet.[16] If a fluorescent protein with cyan spectral properties were desired, cerulean would be a better choice. The green fluorescent proteins are generally bright and rapidly give rise to fluorescence signals, e.g., the $t_{f/2}$ of sfGFP is 92 min. sfGFP is particularly amenable to *in vitro* transcription-translation; however, GFPmut3b performs similarly well. GFPmut3b is one of the more common fluorescent proteins used in synthetic biology. Two of the tested green fluorescent proteins fluoresce upon excitation with near-UV light. Of these two, T-Sapphire has a $t_{f/2}$ approximately 100 min longer than that of GFPuv. Therefore, GFPuv would be better for real-time detection assays than T-Sapphire. The yellow fluorescent proteins Venus and YPet are the brightest fluorescent proteins that we tested and have $t_{f/2}$ values below 150 min. Venus and YPet are excellent choices to monitor *in vitro* reactions particularly when low protein output is expected, e.g., when expressing inside of vesicles.[19] YPet is more photostable,[16] which could be important depending upon the nature of the planned experiments. The red fluorescent proteins mCherry and mRFP1 perform similarly well in *in vitro* transcription-translation reactions, but mCherry is more photostable. Although TagRFP-T is a highly photostable red fluorescent protein alternative, the long $t_{f/2}$ of TagRFP-T limits its usefulness.

All of the seven tested double fluorescent protein constructs performed well, and so the choice of fluorescent protein pairs depends on the specifics of the experimental setup. We found that the mVenus-mCherry pair gives easy to detect fluorescence signals and reproducible data without interference between the emission of mVenus and the emission of mCherry. Therefore, the subsequent experiments that probed the effects of genetic organization on protein production were performed with synthetic operons encoding mVenus and mCherry. However, for the remaining experiments the order of the genes was reversed so that mCherry was encoded first followed by mVenus in the bicistronic message. In this way, the lower intensity fluorescent protein, i.e., mCherry, could be used to provide the reference fluorescence signal and the influences of the region between the two genes on the expression of the

brighter fluorescent protein, i.e., mVenus, could be more easily assessed. Nevertheless, care should be taken in interpreting the resulting data. The assay can be used to characterize how changes in DNA sequence influence the ratio of the two encoded proteins. However, the assay does not differentiate between the decrease of expression of gene 1 or the increase of expression of gene 2. In other words, multiple mechanisms can give indistinguishable results.

Influence of Sequences Upstream of the Ribosome Binding Site. The first question we sought to answer was whether the number of nucleotides separating the stop codon of gene 1 from the ribosome biding site of gene 2 influenced gene expression. Therefore, constructs containing 0, 5, 20, 31, and 50 bp spacer sequences between the UAA stop codon of gene 1 and the AAGGAG RBS of gene 2 were tested (Figure 2). Although differences in expression levels were observed, the

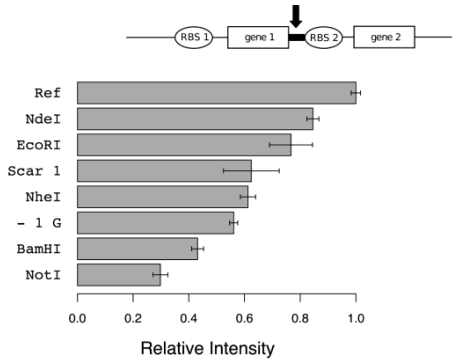


feature	name	RNA sequence
0	RL025A	
5	RL026A	GCGGA
20	RL024A	GCGGAUCCGAAUUCAAUUAG
31	RL027A	GCGGAUCCGAAUUCAAUUAGUUUGAACUUAU
50	RL030A	GCGGAUCCGAAUUCAAUUAGUUUGAACUUAUCAUAGUCAUAUCUUUCAAU

Figure 2. Influence of spacer length between an upstream gene and a downstream ribosome binding site on expression levels. The ratiometric response represents the fluorescence arising from mVenus (encoded by gene 2) divided by the fluorescence of mCherry (encoded by gene 1). Spacer lengths of 0, 5, 20, 31, and 50 nucleotides were tested. The corresponding RNA sequence for the region of interest of each construct is shown below the graph. Each bicistronic construct was expressed *in vitro* with the PURExpress at 37 °C for 6 h.

differences did not correlate with the length of the spacer. For example, the 5 bp and the 31 bp spacer containing constructs both resulted in higher relative expression of gene 2 when compared with the 20 bp spacer. This suggested that the variance in fluorescence ratios resulted from something other than spacer length, such as sequence composition. For the remainder of the experiments, the 31 bp spacer construct (RL027A) was used as the reference.

Since the length of the spacer between gene 1 and RBS 2 did not appear to be correlated with the expression of gene 2, we wondered if the sequence composition rather than the length was responsible for the observed differences in expression. We decided to investigate the influences of sequence composition by incorporating different restriction sites immediately upstream to RBS 2. In this way we hoped to additionally identify restriction sites useful for the assembly of genetically encoded devices. Therefore, in each of the tested constructs, the 31 bp spacer length was maintained, and sequences containing a NdeI, *Bam*HI, NheI, *Eco*RI, NotI, or a scar site were incorporated. The scar site represented the sequence that results from standard BioBrick assembly in which complementary XbaI and SpeI digested products are ligated.[20] Additionally, the U before the AAGGAG RBS was mutated to a G, since a U residue is capable of base-pairing with 16S rRNA. A significant effect of sequence composition on the amount of protein produced was observed (Figure 3). The

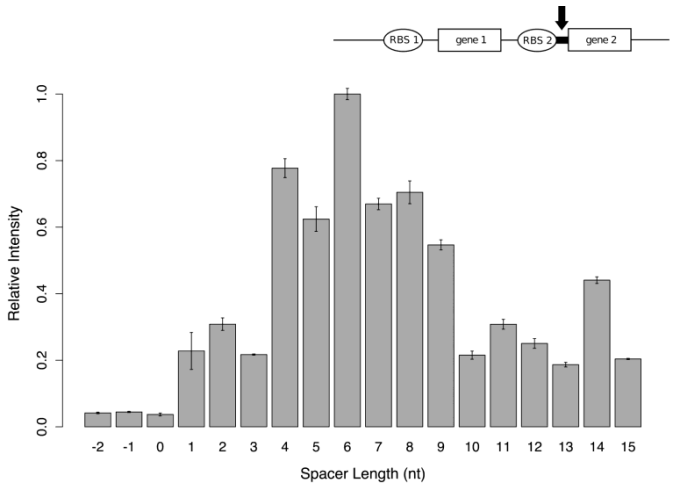


feature	name	RNA sequence
Ref	RL027A	GCGGAUCCGAAUUCAAUUAGUUUGAACUUAU
NdeI	RL036A	GCGGAUCCGAAUUCAAUUAGUUUGACAUAUG
EcoRI	RL039A	GCGGAUCCGAAUUCAAUUAGUUUGAGAATTC
Scar 1	RL041A	GCGGAUCCGAAUUCAAUUAGUUUUACUAGAG
NheI	RL038A	GCGGAUCCGAAUUCAAUUAGUUUGAGCUAGC
- 1 G	LM021A	GCGGAUCCGAAUUCAAUUAGUUUGAACUUAG
BamHI	RL037A	GCGGAUCCGAAUUCAAUUAGUUUGAGGATCC
NotI	RL040A	GCGGAUCCGAAUUCAAUUAGUUUGCGGCCGC

Figure 3. Influence of sequence composition upstream of the ribosome binding site on *in vitro* expression levels. The corresponding RNA sequence for the region of interest of each construct is shown below the graph. Underlined positions refer to the introduced feature. Ref refers to the reference construct RL027A, Scar 1 indicates the standard BioBrick scar sequence, and −1 G refers to the introduction of a G immediately prior to RBS 2. Each bicistronic construct was expressed *in vitro* with the PURExpress at 37 °C for 6 h. Gene 1 encoded mCherry, and gene 2 encoded mVenus. Data are plotted relative to RL027A.

introduction of a NotI site was the most inhibitory, bringing relative expression down by 70% in comparison to the reference RL027A construct. Of the restriction sites tested, NdeI and *Eco*RI restriction site sequences were the most conducive to high expression (84% and 77% relative expression, respectively). Removing the additional base-pair of the RBS, i.e., the U to G mutation, decreased protein production by 44%, consistent with the observed decrease in expression from the 20 bp spacer construct described above that contained the same nucleotide at this position.

Influence of Sequences Downstream of the Ribosome Binding Site. Having probed the influences of the region 5′ to RBS 2, we next investigated the impact of the region 3′ to RBS 2. First, we altered the spacing between RBS 2 and the start codon of gene 2 one nucleotide at a time from −2 to 15 bp. Here the spacing nomenclature followed the aligned spacing described by Chen et al.[21] in which the RBS was aligned with



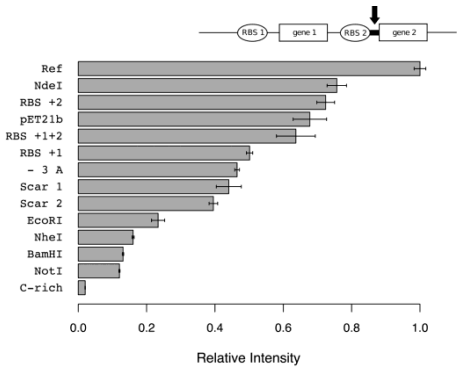
feature	name	RNA sequence	feature	name	RNA sequence
−2	RL032A		7	RL033A	AAUAAUCUA
−1	AS006A	A	8	LM003A	AAUAAUCUAC
0	DT007A	AA	9	RL034A	AAUAAUCUACU
1	DT008A	AAU	10	AS007A	AAUAAUCUACUU
2	DT006A	AAUA	11	LM022A	AAUAAUCUACUUC
3	RL031A	AAUAA	12	LM023A	AAUAAUCUACUUCA
4	AS002A	AAUAAU	13	LM024A	AAUAAUCUACUUCAA
5	AS001A	AAUAAUC	14	RL043A	AAUAAUCUACUUCAAU
6	RL027A	AAUAAUCU	15	RL044A	AAUAAUCUACUUCAAUU

Figure 4. Ribosome binding site spacing. The influence of the aligned spacing between the ribosome binding site and the start codon is shown. The corresponding RNA sequence for the region of interest of each construct is reported below the graph. Each bicistronic construct was expressed *in vitro* with the PURExpress system at 37 °C for 6 h. Gene 1 encoded mCherry, and gene 2 encoded mVenus. Data are plotted in reference to RL027A.

the anti-RBS sequence of the 16S rRNA and the position across from the last position of the anti-RBS was taken as 0 (Supplementary Figure S5). The results were consistent with previous *in vivo* studies,[22] which showed a Gaussian distribution of activity with optimal aligned spacing between 4 and 9 bp (Figure 4). Spacer lengths shorter or longer than this range generally resulted in dramatically decreased protein production. For example, the 3 bp spacer produced 72% less protein than the 4 bp spacer. Similarly, the 10 bp spacer reduced protein synthesis by 60% when compared to the 9 bp spacer construct. For the specific constructs tested in this study, the 6 bp spacer produced the most protein. Since protein expression was detected with the shortest spacer tested on both sides of RBS 2, we also made a minimal construct with a 0 bp spacer between the UAA stop codon of gene 1 and RBS 2 and −2 aligned spacing between RBS 2 and the start codon of gene 2. The synthesis of mVenus from this minimally spaced construct was low but still detectable (3% relative to RL027A).

Next, we evaluated the effect of sequence composition of the region between RBS 2 and the AUG start codon of gene 2 on expression levels. This region of the reference sequence RL027A was designed to be high in A-U content and low in G content because a sequence that is known to facilitate gene expression, i.e., the T7 phage gene 10 leader sequence,[23] has similar characteristics. Sequences that contained the same restriction sites tested above for the region upstream of RBS 2 were placed immediately upstream of the start codon of gene 2. An additional BioBrick scar site also was screened that was shorter and thus thought to interfere less with translation. The presence of an A three nucleotides upstream of the start codon was evaluated since an A at this position is frequently found in prokaryotic and eukaryotic sequences.[24,25] A C-rich sequence was evaluated since a previous *in vitro* study[26] found increased expression associated with high C-content. Finally, mutations that introduced additional base-pairing with the 16S rRNA were added. The data showed a strong influence of sequence composition on protein yields with the NotI restriction site being the most inhibitory, decreasing expression by 87% (Figure 5). The NdeI restriction site was the most conducive to protein synthesis (76% relative expression). Both scar sequences resulting from BioBrick assembly performed similarly, decreasing translation by over 50%. The C-rich sequence greatly decreased protein expression by 98% relative to RL027A. Neither an A residue three nucleotides preceding the start codon nor the expansion of the RBS-anti-RBS base-pairing region increased protein production in the tested constructs.

Finally, we investigated whether other codons could substitute for the AUG start codon. In *E. coli*, GUG and UUG function as start codons at a frequency of 14% and 3%,



feature	name	RNA sequence	feature	name	RNA sequence
Ref	RL027A	AAUAAUCU	Scar 1	LM010A	UACUAGAG
NdeI	LM005A	AAUAACAU	Scar 2	LM011A	AAUACUAG
RBS +2	LM014A	AUUAAUCU	EcoRI	LM008A	AAGAAUUC
pET21b	LM016A	AUAUACAU	NheI	LM007A	AAGCUAGC
RBS +1+2	LM015A	GUUAAUCU	BamHI	LM006A	AAGGAUCC
RBS +1	LM013A	GAUAAUCU	NotI	LM009A	GCGGCCGC
- 3 A	LM012A	AAUAAACU	C-rich	RL050A	CCCCCUCC

Figure 5. Influence of sequence composition between the ribosome binding site and the start codon on expression levels. The corresponding RNA sequence for the region of interest of each construct is shown below the bar graph. Ref indicates the reference construct RL027A. Scar 1 is the standard BioBrick scar sequence. Scar 2 is the shorter, alternate scar sequence. −3 A indicates the introduction of an A three positions upstream of the start codon. pET21b is the same spacer sequence found in the expression vector pET21b (Novagen). RBS +1, RBS +2, and RBS +1+2 indicate RBS expansions. Each introduced feature is underlined in the corresponding sequence. Note that only half of the NdeI restriction site is shown since the remaining half overlaps with the start codon. Each bicistronic construct was expressed *in vitro* with the PURExpress system at 37 °C for 6 h. Gene 1 encoded mCherry, and gene 2 encoded mVenus. Data are plotted in reference to RL027A.

respectively.[25] If non-AUG codons can function as start codons in minimally reconstituted systems, then these alternate start codons could be used to control protein levels. Also, knowledge regarding the functionality of non-AUG start codons could help to identify internal RBS-start codon pairs that could potentially interfere with the intended activity of genetic devices. We therefore substituted a GUG, UUG, and CUG in place of the AUG start codon and measured the production of mVenus. All of the alternate start codons produced protein, albeit at a significantly reduced level between 12% and 27% relative to the AUG start codon containing reference construct (Figure 6).

Considerations for the Assembly of *in Vitro* Genetic Systems. To determine if simple rules could be formulated that would facilitate the construction of genetically encoded, cell-free devices, the collected data were statistically analyzed. First, we sought to determine which regions were more amenable to the incorporation of restriction sites. A paired *t* test showed that sequences upstream of RBS 2 had less influence on the protein fluorescence ratios than the sequence between RBS 2 and the start codon (p-value = 0.0145). Next, sequences immediately 5′ and 3′ to RBS 2 (8 bp each) were considered. The resulting data from 22 synthetic operons were fit to multiple regression models that searched for first and second order interactions between base composition that

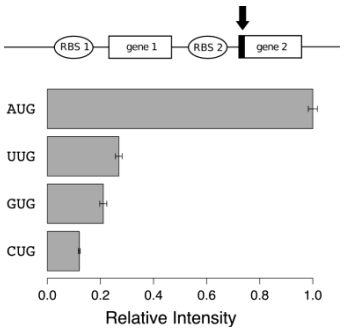


Figure 6. Alternate start codons. The ability of UUG, GUG, and CUG to function as start codons *in vitro* was evaluated. Relative intensities are averages of three replicates and plotted in reference to the AUG start codon containing construct. Each bicistronic construct was expressed *in vitro* with the PURExpress system at 37 °C for 6 h. Gene 1 encoded mCherry, and gene 2 encoded mVenus. The AUG, UUG, GUG, and CUG start codon constructs were RL027A, LM019A, LM018A, and LM020A, respectively.

correlated with the measured fluorescence intensity ratios. The resulting model was statistically significant (F-test p-value = 8.79×10^{-7}) and described almost 75% of the data variability (adjusted r^2 = 0.7453). The estimated parameters (Supplementary Table S1) revealed a strong effect of the G content in sequence composition of the region 5′ to RBS 2 (p-value <0.001). More specifically, a high G content negatively correlated with the fluorescence ratio, whereas combined A-U-rich sequences in the region 3′ to RBS 2 positively correlated with the fluorescence intensity ratio (p-value <0.001).

Taken together, the data indicate that the nucleotide sequence between genes 1 and 2 influence protein production, but not uniformly. The spacing upstream of the RBS is not as strong of a determinant of expression levels as the spacing downstream of the RBS. The one construct that deviates from this trend (RL024A) contains a mutation that decreases the number of potential base-pairs between the mRNA and the 16S rRNA. Most of the constructs tested here contain six to seven potential base-pairing interactions between the Shine-Dalgarno (RBS) and the anti-Shine-Dalgarno site of the ribosome. The introduction of additional base-pairing does not facilitate expression, consistent with previous studies that show that on average *E. coli* mRNA RBS sequences interact with the ribosome via six base-pairs and that the strengthening of the interaction often decreases rather than increases protein synthesis.[27] The optimal aligned spacing between the RBS and the start codon and the functionality of alternate start codons is the same for *in vitro* protein production with the PURExpress system and for natural *E. coli* expression.

Taken together, a few simple rules for the construction of *in vitro* genetic systems can be formulated from the acquired data. Restriction sites should either be placed before the RBS, since this region is more amenable to sequence modification, or a NdeI site that overlaps with the start codon should be exploited. If high protein levels are desired, then the aligned RBS spacing should be between four and nine nucleotides and the spacer sequence should be high in A and T content and low in G content. The use of alternate start codons can be used to

significantly reduce protein synthesis, when needed, and the spacing between the end of one gene and the RBS of the next gene is not crucial. Nevertheless, the complexity of transcription and translation ensures that there are many more factors that influence gene expression than was probed here. mRNA can interact with regions of the ribosome other than the 3′-terminus of the 16S rRNA[26−29] and the folding of mRNA significantly affects protein synthesis.[19,30−33] Further studies with purified, *in vitro* systems likely will aid in better understanding these processes and in facilitating the synthesis of more complex cellular mimics.

METHODS

Genetic Constructs. Genes encoding the fluorescent proteins were synthesized by Genscript or Mr. Gene, except for super folder GFP (BBa_I746916), GFPmut3b (BBa_E0040), and mRFP1 (BBa_E1010), which were from the registry of standard biological parts (http://partsregistry.org), and eGFP, which was from Roche. Mutagenesis was either performed by Genscript or through the use of phusion site-directed mutagenesis (Finnzymes). All genes were subcloned into pET21b by restriction digestion and ligation with NdeI and *Bam*HI, except for super folder GFP and GFPmut3b, which used NheI and *Bam*HI sites. All constructs were confirmed by sequencing at Genechron or Eurofins MWG Operon. The DNA sequences of all the constructs used are provided in the Supporting Information (Table S2).

Transcription-Translation Reactions. Plasmids were amplified in *E. coli* DH5α or NovaBlue and purified with Wizard Plus SV Minipreps DNA Purification System (Promega) or QIAprep Spin Miniprep Kit (Qiagen). Subsequently, the DNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in deionized and diethylpyrocarbonate (DEPC) treated water. A 250 ng portion (2 nM final concentration) of DNA was used for each transcription-translation reaction with the PURExpress *in vitro* protein synthesis kit (New England BioLabs) supplemented with 20 units of human placenta RNase inhibitor (New England BioLabs). The final volume of each reaction was 25.5 μL. Reactions were monitored by fluorescence spectroscopy with a Photon Technology International (PTI) QuantaMaster 40 UV−vis spectrofluorometer equipped with two detectors (T-format). Excitation and emission wavelengths were specific for each fluorescent protein (Supplementary Table S3). The reaction components, except for the DNA template, were assembled on ice and then incubated at 37 °C in the spectrofluorometer. Subsequently, the reaction was initiated by the addition of DNA template. Mineral oil was layered on top of each sample to inhibit evaporation during the course of the experiment. Control experiments with GFPmut3b showed that mineral oil did not influence the appearance of fluorescence. Each reaction was repeated at least three times. An Agilent 8453 UV−vis spectrophotometer was used to quantify mVenus protein concentration by using an extinction coefficient at 515 nm of 92,200 M^{-1} s^{-1}.[16]

Data analysis. All statistical analyses used R statistical computing software.[34] The single protein construct fluorescent data were fit to

$$I(t) = \frac{K}{1 + e^{-B(t-t_{f/2})}} \quad (1)$$

where K, B, and $t_{f/2}$ were the upper asymptote, growth rate, and time of maximum growth, respectively (Supplementary Table S4). The parameters were estimated by using a nonlinear least-squares analysis with the Gauss−Newton algorithm. The mean values and standard errors were then calculated from data from three replications. The influence of spacer nucleotide composition on the fluorescence intensity was determined with multiple regression models. The models were estimated and reduced by using stepwise regression with a penalty term that was selected by minimum predictive mean squared error based on repeated cross-validation (10% leave-out). The best predictive models were obtained by using a stringent criterion (twice the Bayesian Information Criterion, BIC). We then estimated the model with such a penalty term on the whole set of operon spacer data. Paired t tests were used to test whether the restriction sites 5′ or 3′ to RBS 2 affected differently fluorescence intensity ratios.

ASSOCIATED CONTENT

Supporting Information

Supplementary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +39 0461 28 3438. Fax: +39 0461-283091. E-mail: mansy@science.unitn.it.

Notes

The authors declare no competing financial interest.

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RL015A, RL016A, RL018A, RL020A, and RL027A are available through Addgene. Versions of RL027A modified by the 2012 Trento iGEM team to be BioBrick compatible were deposited as BBa_K731700 and BBa_K731710 in the Registry of Standard Biological Parts (http://partsregistry.org/). We thank the Armenise-Harvard Foundation, the autonomous province of Trento (Ecomm), the Marie-Curie Trentino COFUND (ACS), and CIBIO for funding.

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Chapter 3.
Artificial cells to control *E. coli* behavior

This chapter has been adapted from:

Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behavior.

Roberta Lentini, Silvia Perez Santero, Fabio Chizzolini, Dario Cecchi, Jason Fontana, Marta Marchioretto, Cristina Del Bianco, Jessica L. Terrell, Amy C. Spencer, Laura Martini, Michele Forlin, Michael Assfalg, Mauro Dalla Serra, William E. Bentley & Sheref S. Mansy

Nature communications, 2014, 5:4012[58]

Attached within the chapter. Supplementary information attached in the appendix.

My contribution to this work concerns the design, cloning and mutagenesis of the genetic constructs, the setting and collecting of all the experimental measurements, data analysis manuscript writing and editing.

The main approach in synthetic biology relies on the genetic engineered of living cells to control their behavior. The genetic content of extant cells is modified to acquire new functions. Many studies focus on the construction of complex genetic circuits inside living cells[72, 73, 74, 75]. However, the genetic engineering of natural cells suffers from some limitations. For example, living systems can evolve leading to alterations of the engineered pathways over time. However, it may not be necessary genetically modify living cells. Natural cells evolved several systems to sense their environment and to modify their behaviors to adapt to external and internal changes[76, 77]. Thus, extant cells naturally own the capability to sense various stimuli and to accomplish many tasks.

The work described in this chapter presents an alternative method to modify the behavior of a living cell without using genetic engineering. Targeting the sensory pathway of extant cells, the genetically engineering moves away from the natural cells to artificial, laboratory-made cells. The artificial cells built in this study act as chemical translators sensing a molecule that *E. coli* does not sense on its own. In response, the artificial cells send a message that *E. coli* can naturally sense and respond to. In other word, the artificial cells sense an unrecognized message and translate it to a recognized message. *E. coli* sensing is expanded without genetically modify the natural cells. To sense a molecule that *E. coli* cannot naturally sense, the artificial cells were constructed using a phospholipid compartment, transcription-translation machinery made of purified components[14], a genetically encoded element controlled by theophylline, a molecule that *E. coli* cannot sense on its own, and loaded with IPTG, a molecule that *E. coli* can naturally sense. The genetic element codes for a previously selected riboswitch responsive for theophylline[78]. Only in the presence of theophylline, the riboswitch allows the expression of the pore forming protein alpha-hemolysin (αHL). The protein goes to the membrane of the artificial cells, creates pores and IPTG is released to the outside (Fig. 3.1). The response of *E. coli* to IPTG released from the artificial cells was assessed in two different ways. First, the expression of GFP in *E. coli* carrying an IPTG responsive plasmid was used to detect the released IPTG. Then, the expression of the *lac* operon in non-genetically modified *E. coli* through reverse transcription quantitative PCR (RT-qPCR) was monitored.

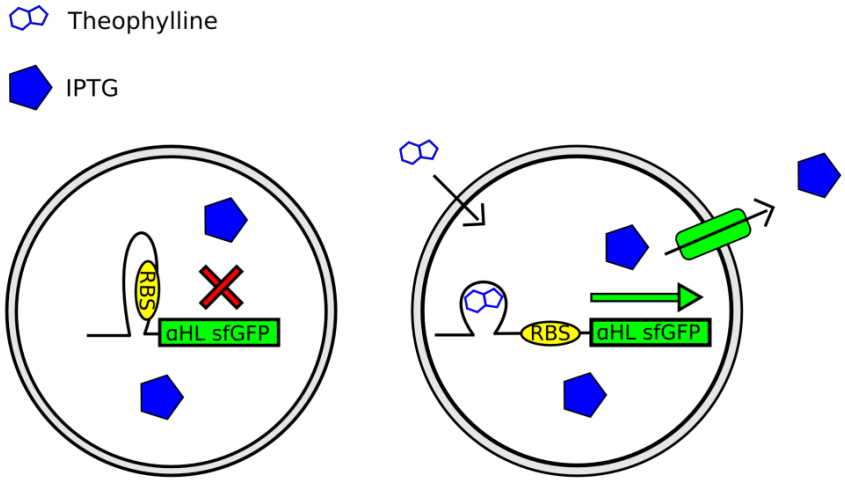

Fig. 3.1 Artificial cells.

The artificial cells made of phospholipids contain cell-free transcription-translation machinery, genetically encoded theophylline riboswitch, and IPTG. In the absence of theophylline, the conformation of the riboswitch does not allow protein expression (left panel). In the presence of theophylline, the riboswitch changes conformation and protein expression can occur. αHL forms pores in the membrane, and the IPTG is released outside (right panel).

3.1 The artificial cells can sense a molecule that *E. coli* cannot sense on its own

To build artificial cells that sense theophylline, a theophylline-sensing device was constructed. The genetic device was made of a T7 promoter, theophylline riboswitch and *αHL* gene. To facilitate the monitoring of the functionality of the artificial system, αHL was fused to super folder GFP (sfGFP) at the carboxyl terminus. Thus, only when theophylline is present, αHL is expressed and the reaction could be monitored by fluorimetry. However, when expressed *in vitro,* the theophylline-sensing device gave rise to similar fluorescent signals both in the presence and in the absence of theophylline. Since previous work showed the functionality of the same riboswitch *in vitro*[52], the sequence of αHL-sfGFP was further investigated. Putative ribosome binding site (RBS) and ATG pairs were found within αHL-sfGFP gene, compatible with the expression of truncated αHL isoforms but in frame with the encoded region of sfGFP. After the confirmation of the presence of internal RBS in the αHL portion, one of the putative RBS was removed from the αHL coding sequence. When tested *in vitro,* the resulted theophylline-sensing device showed a clear difference in protein expression, in the presence and in the absence of theophylline (Fig. 2a-c Lentini R et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014, 5: 4012).

Subsequently, the correct functionality of αHL to form active pores *in vitro* was tested. The theophylline-sensing device was expressed *in vitro* in the presence and in the absence of

theophylline. An aliquot of each reaction was mixed with rabbit red blood cells and hemolysis was measured. Only in the presence of theophylline was active αHL produced (Fig. 2d Lentini R et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014, 5: 4012).

3.2 Artificial cells translate a message for *E. coli*

Once the theophylline-sensing device was shown to work *in vitro*, artificial cells containing all the necessary components were built. The theophylline-sensing device, transcription-translation machinery and IPTG were encapsulated inside liposomes. Phospholipid vesicles are known to be permeable to theophylline[52] while IPTG cannot cross the lipid membrane (Supplementary figure 1. Lentini R et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014, 5: 4012). Artificial cells were then purified by using dialysis and mixed with *E. coli* Bl21(DE3) pLysS cells carrying an IPTG responsive plasmid coding for sfGFP. IPTG induces expression of T7 RNA polymerase (T7 RNAP) and derepresses sfGFP expression in the plasmid. Artificial cells were incubated at 37 °C together with *E. coli.* Expression of sfGFP was monitored by flow cytometry. Theophylline alone was not able to induce a response in *E. coli*, IPTG could not cross the vesicles in the absence of αHL. When artificial cells were incubated with *E. coli* and theophylline, 69±3 % of the bacteria emitted fluorescence after 3 h, while in the same conditions but in the absence of theophylline, only 24±5 % of bacteria emitted fluorescence (Fig. 3ab Lentini R et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014, 5: 4012). Then, artificial cells were incubated together with untransformed *E. coli* to determine whether the artificial cells could elicit a natural response in non-genetically modified cells. RT-qPCR was used to monitor *lac* operon expression. IPTG is well known to induce expression of *lacZYA* genes. *E. coli* was grown in LB to reduce background expression from the operon and then transferred to minimal medium. Artificial cells were added to the bacteria and incubated at 37 °C. RNA was then extracted and reverse transcribed. Bacteria incubated with artificial cells and theophylline showed a 20 fold higher expression of *lacZYA* genes (calculated from AC/(AC + theo) than samples incubated with artificial cells alone (Fig. 3c Lentini R et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014, 5: 4012). The artificial cells were able to translate a message to *E. coli* and induced a response in natural cells.

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Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour

Roberta Lentini[1], Silvia Perez Santero[1,2], Fabio Chizzolini[1], Dario Cecchi[1], Jason Fontana[1], Marta Marchioretto[3], Cristina Del Bianco[1], Jessica L. Terrell[4,5], Amy C. Spencer[1], Laura Martini[1], Michele Forlin[1], Michael Assfalg[2], Mauro Dalla Serra[3], William E. Bentley[4,5] & Sheref S. Mansy[1]

Previous efforts to control cellular behaviour have largely relied upon various forms of genetic engineering. Once the genetic content of a living cell is modified, the behaviour of that cell typically changes as well. However, other methods of cellular control are possible. All cells sense and respond to their environment. Therefore, artificial, non-living cellular mimics could be engineered to activate or repress already existing natural sensory pathways of living cells through chemical communication. Here we describe the construction of such a system. The artificial cells expand the senses of *Escherichia coli* by translating a chemical message that *E. coli* cannot sense on its own to a molecule that activates a natural cellular response. This methodology could open new opportunities in engineering cellular behaviour without exploiting genetically modified organisms.

[1] CIBIO, University of Trento, via delle Regole 101, 38123 Mattarello (TN), Italy. [2] Department of Biotechnology, University of Verona, 37134 Verona, Italy. [3] National Research Council—Institute of Biophysics & Bruno Kessler Foundation, Via alla Cascata 56/C, 38123 Trento, Italy. [4] Fischell Department of Bioengineering, University of Maryland, College Park, Maryland 20742, USA. [5] Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, Maryland 20742, USA. Correspondence and requests for materials should be addressed to S.S.M. (email: mansy@science.unitn.it).

Synthetic biology thus far has relied upon the engineering of new cellular function through the insertion and deletion of genetic information in living cells. This genetic engineering based approach has progressed rapidly. There is now available a set of well-characterized biological parts[1–3] that can be used to build complex genetic circuitry within and between the living cells[4–6]. Further, entire genomes can be edited[7] and synthesized[8], suggesting that fully designed organisms with heretofore unseen capabilities are likely in the future.

Despite the wide range of technologies and target pathways exploited, the desire to control microorganisms to date has always employed direct genetic intervention. The limitations of these prevalent methods are due to the difficulties of engineering living systems, including evolutionary pressures that may alter engineered pathways over time and the potential long-term consequences of altering ecosystems with engineered organisms. However, it may not be necessary to genetically modify living cells. Extant life is already extremely complex, endowed with numerous sensory and metabolic pathways tuned by billions of years of evolution to be efficiently responsive to changing intracellular and extracellular conditions. A simple change in pH, for example, results in the up and downregulation of nearly 1,000 genes in *Escherichia coli*[9]. In other words, cells are already capable of sensing many different stimuli and capable of performing many tasks. Therefore, it should be possible to exploit these existing cellular pathways to control cellular behaviour without changing the genetic makeup of the cells.

Here we explore this idea of engineering *E. coli* through alternative means by targeting the sensory pathways of *E. coli*. To do so without altering the genetic content of the bacterium, we instead construct artificial cells that could interact with natural cells in order to evoke a behavioural response. The artificial cells in this system function as chemical translators that sense molecules that *E. coli* alone cannot sense. In response, the artificial cells release a molecule that *E. coli* can naturally respond to, thereby translating an unrecognized chemical message into a recognized chemical message. In this way, the sensory capabilities of *E. coli* are expanded without altering the genetic content of the bacterium. The artificial cell is built with a phospholipid vesicle containing isopropyl β-D-1-thiogalactopyranoside (IPTG), DNA, and transcription–translation machinery. The DNA template codes for a previously selected riboswitch that activates translation in response to the presence of theophylline[10]. The theophylline riboswitch controls the synthesis of the pore forming protein α-hemolysin (αHL). Therefore, in the presence, but not the absence, of theophylline a pore forms that releases entrapped IPTG. *E. coli* alone does not respond to theophylline, and IPTG does not cross the vesicle membrane of the artificial cell in the absence of the pore. The ability of *E. coli* to receive the chemical message sent by the artificial cells is assessed in two ways. First, the fluorescence of *E. coli* carrying a plasmid encoding a fluorescent protein behind an IPTG-responsive, *lac* operator sequence is evaluated. Second, the gene expression of untransformed *E. coli* is monitored by reverse transcription quantitative PCR (RT–qPCR). To our knowledge, this is the first artificial, cell-like system capable of translating unrecognized signals into a chemical language that natural cells can recognize. The integration of artificial translator cells with natural cells represents a new strategy to introduce synthetic features to a biological system while circumventing the need for direct genetic manipulation.

Results

The theophylline-sensing device is functional *in vitro*. To build artificial cells that sense theophylline and in response release IPTG (Fig. 1), a theophylline-sensing genetic device was built with a T7 transcriptional promoter, a theophylline riboswitch and a gene encoding a fusion between αHL and super folder GFP at the carboxy terminus. If functioning properly, this arrangement should result in the expression of protein and thus green fluorescence only in the presence of theophylline. However, cell-free expression in the presence and absence of theophylline showed similar levels of fluorescence (Fig. 2a). Since this same riboswitch was previously shown to function *in vitro*[11], the sequence of the αHL-GFP gene was more closely examined. Multiple pairs of potential ribosome binding sites (RBS) and start codons were identified within the αHL portion of the gene that were in-frame with the GFP-encoding region. The theophylline riboswitch controls translation, meaning that sequences behind the theophylline riboswitch are always transcribed. Translation from the RBS within the riboswitch is activated by direct binding of theophylline to the messenger RNA. Therefore, if additional sequences outside of the riboswitch but within the αHL portion of the gene were recognized by the ribosome, then regardless of the theophylline concentration, the expression of truncated peptide products with fluorescently active GFP would have been possible. To test if such internal RBSs were present, the theophylline riboswitch and thus the RBS preceding the αHL-GFP sequence was deleted. *In vitro* transcription–translation of this construct showed the accumulation of fluorescence over time similar to the riboswitch containing construct (Fig. 2b). Sequence analysis revealed three potential RBS-start codon pairs within the αHL coding portion of the gene. Of these, a putative RBS of

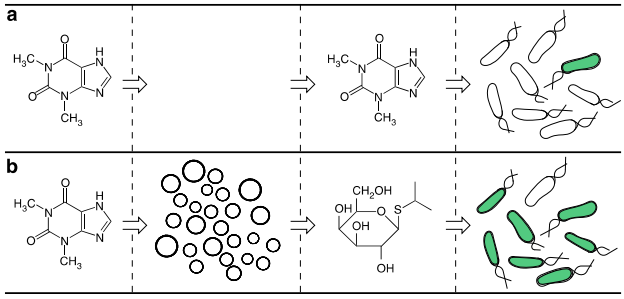

Figure 1 | Artificial cells translate chemical signals for *E. coli*. (**a**) In the absence of artificial cells (circles), *E. coli* (oblong) cannot sense theophylline. (**b**) Artificial cells can be engineered to detect theophylline and in response release IPTG, a chemical signal that induces a response in *E. coli*.

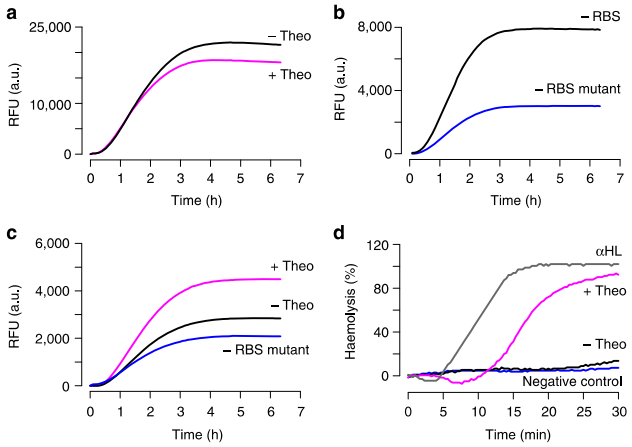

Figure 2 | *In vitro* characterization of the theophylline-sensing device and αHL. (**a**) The cell-free expression of αHL–GFP behind a theophylline riboswitch gives rise to similar levels of fluorescence both in the presence (+theo) and absence (−theo) of theophylline at 37 °C. (**b**) The removal of the theophylline riboswitch and thus, the RBS preceding the start codon of αHL–GFP shows production of a fluorescent protein product when incubated with transcription–translation machinery (−RBS). The removal of a putative internal RBS within the αHL coding portion of the fusion construct significantly decreases the production of the fluorescent protein product (−RBS mutant). (**c**) The activity of the theophylline-sensing device is observable by fluorescence when an internal RBS is removed. The top and middle curves are the *in vitro* expression of αHL–GFP behind the theophylline riboswitch in the presence (+theo) and absence of theophylline (−theo), respectively. Background fluorescent protein production is shown with the same construct lacking the theophylline riboswitch (−RBS mutant) used in **b**. (**d**) The cell-free expression of theophylline riboswitch-controlled αHL-degraded red blood cells (RBCs) in the presence (+theo) but not the absence of theophylline (−theo). Control reactions include the expression of an αHL construct lacking the theophylline riboswitch (αHL) and RBCs alone (negative control). RBC degradation was monitored by attenuance at 22 °C. The exploited constructs were SP011A for panel A, SP002A and AS014A for panel B, RL069A and AS014A for **c**, and RL067A and JF001A for **d** (Supplementary Table 1). Data are averages of three independent reactions.

AAAGAA was selected as the most likely candidate for giving fluorescent protein expression based on sequence composition and spacing[12]. The putative internal RBS was removed by mutation to TCTACC, resulting in a carboxy-terminal GFP tagged K30S E31T αHL construct. Fluorescence from this mutated construct was reduced threefold, consistent with the removal of an internal RBS (Fig. 2b). Finally, K30S E31T αHL-GFP was placed behind the theophylline riboswitch to test the activity of the cell-free sensing device. A clear difference was observed between protein expression in the presence and absence of theophylline (Fig. 2c), and the fluorescence arising in the absence of theophylline was within 20% of the construct lacking an RBS upstream of the full gene. The data were consistent with a functioning riboswitch sensor with background fluorescent protein expression arising from internal RBS within αHL. Therefore, the final artificial cellular mimic described below was built with αHL lacking a GFP-tag to avoid complications arising from the expression of truncated fluorescent protein product.

Active αHL is produced in response to theophylline *in vitro*. To ensure that the cell-free expressed αHL was active as a pore, the ability of αHL to degrade rabbit red blood cells was assessed through a standard haemolysis assay[13]. Each construct was expressed *in vitro* at 37 °C for 6 h after which, an aliquot was removed and added to red blood cells. Haemolysis was quantified by measuring attenuance at 650 nm. In the presence of theophylline, 90% haemolysis was observed when the genetic construct containing a riboswitch-controlled αHL was expressed. The cell-free expression of the same construct in the absence of theophylline gave haemolysis levels similar to the negative control reactions (Fig. 2d), as was expected for a functioning theophylline riboswitch that controls the production of αHL. Control reactions with commercial αHL-purified protein and *in vitro*-expressed αHL and αHL–GFP all were fully active (Fig. 2d, Supplementary Table 2), whereas aliquots from *in vitro*-expressed GFP alone and αHL with a carboxy-terminal His-tag were inactive (Supplementary Table 2). αHL with a carboxy-terminal His-tag was previously shown to have reduced activity[14]. Also, comparison of the riboswitch activity fluorescence data with the haemolysis assay data was consistent with the production of GFP containing protein fragments from an internal RBS without an active αHL domain. For example, the αHL–GFP construct lacking one of the putative internal RBSs failed to produce protein with haemolysis activity (Supplementary Table 2), despite giving rise to fluorescence during *in vitro* transcription–translation (Fig. 2b).

Artificial cells can translate chemical messages for *E. coli*. After demonstrating that the riboswitch was able to control the *in vitro* expression of αHL in response to theophylline and that the expressed αHL molecules formed functional pores, the component parts were next assembled inside of phospholipid vesicles to build artificial cells. Theophylline is capable of passing through the membrane of vesicles[11]. Phospholipid vesicles were generated in the presence of IPTG, transcription–translation machinery and DNA encoding αHL under the control of the theophylline riboswitch. The vesicles were then purified by dialysis at 4 °C to remove unencapsulated molecules. The receiver bacterial cells were mid-exponential phase *E. coli* BL21(DE3) pLysS carrying a

plasmid encoding GFP behind a T7 promoter and a *lac* operator sequence. In this commonly exploited system, IPTG induces the expression of a chromosomal copy of T7 RNA polymerase in *E. coli* BL21(DE3) and derepresses the expression of GFP from the plasmid. Background expression is typically low with such an arrangement because of the presence of constitutively expressed lysozyme from pLysS, a natural inhibitor of T7 RNA polymerase.

To test if the artificial cells could function as chemical translators for *E. coli*, the artificial cells were incubated with *E. coli* BL21(DE3) pLysS carrying the GFP-encoding plasmid at 37 °C, and the fluorescence of *E. coli* was evaluated by flow cytometry. A control reaction in which theophylline was directly added to *E. coli* in the absence of artificial cells failed to show green fluorescence after 3 h (Fig. 3a). Similarly, IPTG loaded vesicles that did not contain the machinery necessary to form pores did not induce fluorescence in *E. coli*. Therefore, theophylline was not able to induce a detectable response in *E. coli*, and IPTG could not cross the vesicle membrane in the absence of αHL, which was consistent with permeability measurements (Supplementary Fig. 1). However, when *E. coli* was incubated with artificial cells and theophylline, 17 ± 10% and 69 ± 3% of the bacteria fluoresced green after 0.5 and 3 h, respectively. When the same experiment was repeated in the absence of theophylline, 3 ± 1% and 24 ± 5% of the bacteria were fluorescent after 0.5 and 3 h, respectively (Fig. 3a,b). Longer incubations resulted in diminishing differences between the two samples suggesting the presence of low levels of αHL expression in the absence of theophylline. Also, the GFP response was encoded within a medium copy number plasmid. Therefore, higher background levels of GFP were to be expected in comparison with gene expression from the chromosome. The flow cytometry experiments were consistent with the ability of artificial cells to translate an unrecognized chemical signal (theophylline) into a signal (IPTG) that *E. coli* could respond to.

Although the artificial cells were capable of communicating with *E. coli*, the induction of GFP synthesis, as observed above, exploited an engineered response. To assess whether artificial cells could elicit a natural, chromosomally encoded response, RT–qPCR was used to measure gene expression from the *lac* operon of *E. coli*. The *lac* operon is one of the most thoroughly characterized sensory pathways[15]. The presence of allolactose (or the non-hydrolyzable analogue IPTG) induces the expression of *lacZ*, *lacY* and *lacA*. To facilitate detection of *E. coli* responding to the chemical message sent from the artificial cells, *E. coli* BL21 (DE3) pLysS were grown in LB supplemented with glucose to decrease the background expression of the *lac* operon and then transferred to M9 minimal media prior to incubation with artificial cells. The artificial cells were prepared as described for the GFP induction experiments above. After incubating together artificial cells with *E. coli* in the presence and absence of theophylline for 4 h, aliquots were collected for RNA isolation. The RNA was then reverse transcribed and *lacZ*, *lacY*, and *lacA* expression quantified by qPCR. The RNA isolated from bacteria incubated with artificial cells plus theophylline showed on average over 20-fold higher *lacZYA* expression than samples incubated with artificial cells alone (calculated from AC/(AC + theo) as shown in Fig. 3c). Taken together, the data are consistent with the ability of artificial cells to translate chemical messages and induce both engineered and natural pathways in *E. coli*.

Discussion

Direct genetic engineering of living cells is not needed to control cellular behaviour. It is possible, instead, to coerce desired activity through communication with artificial cells. The foundation for such technologies has already been laid by both cell-free and *in vivo* studies. Engineered communication paths between living cells have been constructed to coordinate cellular activities in response to external stimuli[6,16] and are being developed for therapeutic purposes[17]. In these systems, sender cells often can process information and in response release molecules that affect other cells. What has been shown herein builds on these past efforts but does so by integrating reconstituted, non-living systems with living cells. This allows for the genetic engineering component of the system to be moved from the living, evolving, replicating cells to the more controllable, ephemeral artificial

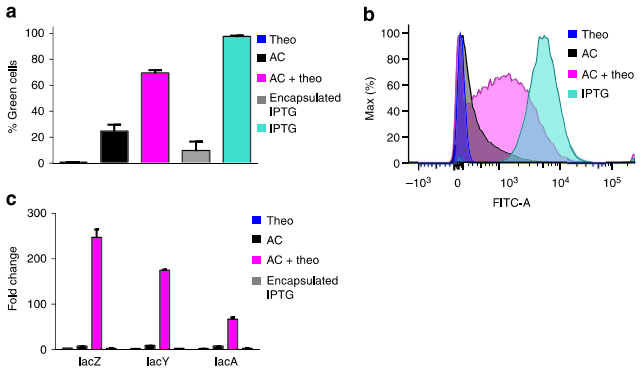

Figure 3 | The artificial translator cells are functional. (**a**) Artificial cells can induce the expression of a plasmid encoded gene within *E. coli* in response to a molecule that *E. coli* cannot naturally sense. BL21(DE3) pLysS carrying a plasmid encoding GFP behind a *lac* operator sequence was incubated with the following components at 37 °C for 3 h: theophylline (theo), artificial cells (AC), artificial cells plus theophylline (AC + theo), IPTG encapsulated inside of vesicles (encapsulated IPTG), and unencapsulated IPTG (IPTG). *E. coli* fluorescence was quantified by flow cytometry. The reported averages and s.e.m. were calculated from three separate reactions run on three different days from independently assembled artificial cells. (**b**) A histogram of a subset of the FACS data used in panel **a** shows a clear shift in the *E. coli* population in the presence of artificial cells plus theophylline. (**c**) Artificial cells can induce the expression of chromosomally encoded genes of *E. coli*. After 4 h of incubation of artificial cells with *E. coli* at 37 °C, the messenger RNA encoding *lacZ*, *lacY* and *lacA* was quantified by RT–qPCR. Data are reported as averages of three measurements and error bars represent s.e.m.

cells. When the artificial cells degrade, the natural cells go back to their original state, thereby diminishing the possibility of unintended long-term consequences. For example, rather than engineering bacteria to search for and clean up environmental contaminants, artificial cells could be built to sense the contaminant molecules and in response release chemoattractants that bring natural bacteria capable of feeding on the contaminants[18] to the affected site.

Several recent reports have described the engineering of seek-and-destroy bacteria for the eradication of tumours or bacterial infections[19–22]. However, these methods ultimately rely on administering living bacteria to the patient. Artificial cells could be built to carry out similar tasks if the sensor module of the artificial cell was designed to detect the chemical conditions associated with the ailment. For instance, rather than spraying engineered bacteria into the lungs of cystic fibrosis patients, artificial cells could be built to detect the presence of *Pseudomonas aeruginosa* biofilms through the quorum signalling molecules that are naturally secreted by the organism, such as *N*-(3-oxododecanoyl)-L-homoserine lactone, a molecule capable of crossing membranes without the aid of transporters. Subsequently, the artificial cells could release small molecules, for example, D-amino acids[23], to disperse the biofilm and thus clear the infection. Moreover, the use of dispersion rather than killing would decrease the probability of the bacteria developing resistance. Similar strategies with artificial cells could be developed to substitute for engineered probiotics that integrate with gut microbiota[24] and prevent disease[25,26].

There are several limitations to these first generation artificial cells. First, heterogeneity in membrane lamellarity and in encapsulation efficiency[27] results in a mixture of artificial cells with varying degrees of activity. Microfluidic-based methods for compartment formation and solute encapsulation would likely alleviate many of the complications associated with vesicle-to-vesicle and batch-to-batch variability. Also, a system fully dependent upon the permeability properties of the membrane limits the types of molecules that can be sensed and released. The development of specific membrane-associated sensors and transporters will likely be necessary as the complexity of artificial cells increase. Finally, the simple release of encapsulated molecules means that release could result from compartment degradation as opposed to an engineered response to the detection of a specific molecule. It is, therefore, important to develop an output that is mediated by synthesis so that compartment degradation would only result in the release of inactive starting molecules. An example of such a system is the biological nanofactory described by Fernandes *et al.*[28] that synthesizes a signalling molecule from *S*-(5′-deoxyadenosin-5′)-L-homocysteine via two enzymatic steps.

The absence of a living chassis opens up greater opportunities to assemble or biofabricate various mechanisms or functions that would be difficult to implement with living cells. For example, chemical systems housed within inorganic and peptide-based compartments are capable of sensing the environment through, in part, the gating behaviour of the non-lipid compartment[29,30]. Further, artificial cells can synthesize and release signalling molecules sensed by living cells without exploiting genetically encoded parts[31,32]. The possibility of merging advances with non-genetically encoded and genetically encoded parts may lead to the construction of artificial cells that are better able to imitate natural cellular life[33,34].

Methods

Genetic constructs. The gene encoding *Staphylococcus aureus* αHL was synthesized by Genscript. Super folder GFP (BBa_I746916) was from the registry of standard biological parts (http://parts.igem.org). The theophylline riboswitch sequence was from Lynch and Gallivan[10] and was amplified from a previously described construct[11]. All genes were subcloned into pET21b (Novagen) with NdeI and XhoI restriction sites. Mutagenesis was performed by Phusion site-directed mutagenesis (Thermo Scientific). All constructs were confirmed by sequencing at Genechron or Eurofins MWG Operon. Sequences of all the exploited constructs are listed in Supplementary Table 1. All experiments were repeated at least three times. Data are reported as averages with standard error, or representative runs are shown.

***In vitro* characterization of the riboswitch.** Plasmids were amplified in *E. coli* Novablue (Novagen) and purified with Wizard Plus SV Minipreps DNA Purification System (Promega). Plasmid DNA was phenol–chloroform extracted, ethanol precipitated and resuspended in deionized and diethyl pyrocarbonate-treated water. PCR products were purified with Wizard Plus SV Gel and PCR Clean-Up Systems (Promega). Transcription–translation reactions used the PURExpress *In Vitro* Protein Synthesis Kit (New England Biolabs) supplemented with 20 units of Human Placenta RNase Inhibitor (New England Biolabs). Reactions were monitored by fluorescence with a CFX96 Touch real-time PCR (Bio-Rad) using the SYBR green filter set.

α-hemolysin activity. Each construct was expressed with the PURExpress *In Vitro* Protein Synthesis Kit at 37 °C in a final volume of 25 μl either in the presence or absence of 1.5 mM theophylline for 6 h. Rabbit red blood cell (RBC) suspensions (adjusted to $D = 0.1$ at 650 nm) were added to a microplate where the reaction mixtures were serially diluted. Changes in attenuance of the RBC suspension were measured at 650 nm with a microplate reader (UVmax, Molecular Devices) for 30 min at 22 °C as reported in Laventie *et al.*[35] The results are reported as percentage of haemolysis or as the time necessary to reach 50% of haemolysis.

Preparation of *E. coli* receiver cells. Mid-exponential *E. coli* BL21(DE3) pLysS transformed with a plasmid encoding super folder GFP behind a T7 promoter and a *lac* operator sequence (CD101A[12]) were grown in LB supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol to an optical density of 0.5 at 600 nm. A quantity of 200 μl aliquots in 10% (vol/vol) glycerol were flash frozen with liquid nitrogen and stored at −80 °C for later use. Aliquots were rapidly thawed and mixed with 2 ml LB supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol and incubated for 2 h at 37 °C with 220 r.p.m. shaking. Finally, the cells were gently pelleted and resuspended in 1 ml M9 minimal media.

Preparation of artificial cells. Vesicles were prepared as previously described[36,37]. Briefly, 12.5 mg 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 12.5 mg cholesterol (Avanti Polar Lipids) in chloroform were mixed in a round bottom flask. A thin lipid film was made through rotary evaporation with a Buchi Rotovapor R-210 equipped with a Buchi Vacuum Pump V-700 for 5 h. A quantity of 2 ml DEPC-treated deionized water was then added to the thin lipid film and vigorously vortexed. The resulting liposome dispersion was homogenized with an IKA T10 basic homogenizer at a power setting of 4 for 1 min. A quantity of 100 μl aliquots were frozen in liquid nitrogen or dry ice and lyophilized overnight in a vacuum concentrator (Centritrap DNA concentrator, Labconco) at 40 °C. The lyophilized empty liposomes were stored at −20 °C. A quantity of 100 μl aliquots of freeze-dried liposomes were hydrated with 25 μl of 100 mM IPTG (Sigma) dissolved in 50 mM HEPES pH7.6, 25 μl of the PURE system, 500 ng DNA and 20 units of human placenta RNase inhibitor (final volume of 50 μl), unless otherwise noted. Solutions were gently mixed for 30 s.

To remove extravesicular material, the vesicles were dialyzed following a method previously described by Zhu and Szostak[38]. The original membranes of 500 μl Slide-a-Lyzer dialysis cassettes (Pierce) were exchanged with 25 mm diameter polycarbonate track-etched membranes with a 1 μm pore size (Whatman). A quantity of 50 μl of unpurified vesicles were loaded onto the center of the dialysis system with a 100 μl Hamilton syringe and dialyzed against 250 ml of buffer A (50 mM HEPES, 10 mM $MgCl_2$, 100 mM KCl, pH 7.6) with stirring. The first four rounds of dialysis were for 10 min each. Two more rounds of dialysis in which the buffer was changed after 30 min incubations were further performed. All dialysis steps were carried out at 4 °C.

Artificial–natural cell communication. Purified vesicles containing DNA, the PURE system, and IPTG were incubated with *E. coli* BL21(DE3) pLysS transformed with CD101A in M9 minimal media supplemented with 1 mg ml^{-1} of Proteinase K and 5 mM theophylline at 37 °C in a final volume of 40 μl. Control reactions did not contain theophylline. At different time points, 1 μl was removed and diluted 1:100 in PBS. The sample was then analysed by flow cytometry with a FACSCanto A (BD Biosciences). The FITC filter was used for the detection of positive cells. The incident light was at 488 nm for forward scatter (FSC), side scatter (SSC) and fluorescence. Detection for SSC and fluorescence was at 488 ± 10 nm and 530 ± 30 nm, respectively. The threshold parameters were 200 for both FSC and SSC. The PMT voltage settings were 525 (FSC), 403 (SSC) and 600 (FITC). The flow rate was set to 'low'. For each sample 30,000 events were

collected. Reactions were repeated three times on three separate days. Data were analysed using FlowJo software (TreeStar, USA).

Samples were also evaluated by RT–qPCR. Here, the dialyzed vesicles and *E. coli* were incubated as described above for 4 h at 37 °C. Subsequently, the total RNA was extracted with the RNeasy Mini kit (Qiagen). A quantity of 10 µl of 500 ng of RNA was reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific). cDNA was quantified with a CFX96 Touch real-time PCR (Bio-Rad) with SYBR green detection. Each sample was diluted to 5 ng and measured in triplicate in a 96 wells plate (Bio-Rad) in a reaction mixture containing SsoAdvanced SYBR green supermix (Bio-Rad) and 180 nM of each primer in a 10 µl finale volume. The primers used to quantify *lacZ*, *lacY* and *lacA* expression were lacZ FW: 5′-TACGATGCGCCCATCTACAC-3′, lacZ REV: 5′-AACAACCC GTCGGATTCTCC-3′, lacY FW: 5′-GGTTTCCAGGGCGCTTATCT-3′, lacY REV: 5′-TTCATTCACCTGACGACGCA-3′, lacA FW: 5′-GCGTCACCATC GGGGATAAT-3′, lacA REV: 5′-CCACGACGTTTGGTGGAATG-3′. Gene expression was normalized to the expression of *idnT*[39] with the following primers: 5′-CTGCCGTTGCGCTGTTTATT-3′ and 5′-GATTTGCTCGATGGTGCGTC-3′.

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Author contributions

Design, cloning and mutagenesis of genetic constructs were done by R.L., A.C.S., J.F., S.P.S., M.F., and C.D.B. *In vitro* riboswitch activity was investigated by R.L., S.P.S., C.D.B., L.M., M.F. and A.C.S. αHL activity was measured by R.L., S.P.S., M.M., and M.D.S. R.L., J.L.T., D.C., F.C. and S.P.S. ran the cell flow cytometry experiments, and RT–qPCR was performed by R.L. and J.F. S.S.M. supervised the project. All authors analysed and interpreted the data and contributed to the writing of the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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3.3 Artificial cells as a tool to reduce AI-2 uptake

In the work presented in this chapter it has been shown the ability of artificial cells to control bacterial behavior through the release of IPTG. However, artificial cells could be built to send other types of molecules and be exploited as tools to defeat pathogens. For example, artificial cells could be constructed to sense the presence of *Pseudomonas aeruginosa* and release inhibitors of biofilm formation such as D-amino acids[79] known to disperse biofilm or analogs of quorum sensing molecules to block virulence[80, 81]. Bacteria communicate through a process called quorum sensing (QS). The process depends on the diffusion of small molecules, called autoinducers, and control various behaviors, among them biofilm formation and virulence[82]. Autoinducer 2 (AI-2) is an interspecies signaling molecule sensed by both Gram negative and positive bacteria[83]. Previous studies showed that AI-2 plays a role in biofilm formation in *E. coli*[84]. AI-2 accumulates extracellularly in the mid-late exponential phase and declines in the stationary phase because of uptake by a transporter cassette included in the *lsr* operon. The operon contains *lsrACDBFG* genes and is activated by AI-2 itself. Next to the *lsr* operon, *lsrR*, a repressor, and *lsrK*, a kinase, are divergently transcribed. The LsrR repressor binds an intergenic region of 250 bp in length adjacent to the *lsr* operon thereby repressing the transcription of the operon[85]. It has been shown that the presence of glucose in the media reduces the internalization of AI-2[86] due to a link between catabolite repression and AI-2 transport[18]. Catabolite repression influences AI-2 accumulation through the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex. In the presence of glucose, low intracellular levels of cAMP-CRP are present, *lsr* operon is not transcribed and AI-2 import is not possible. Thus, the construction of artificial cells able to send glucose should indirectly block the AI-2 pathway in *E. coli* and reduce biofilm formation.

Artificial cells were built as previously described[58], except that they were loaded with glucose instead of IPTG. The theophylline-sensing device controls the production of αHL. In the presence of theophylline, αHL forms pores on the membrane and glucose is released. The presence of glucose represses the expression of CRP leading to a downregulation of the *lsr* operon and absence of AI-2 internalization. The inhibition of AI-2 uptake was monitored in two ways. First, the higher amount of AI-2 in the media of *E. coli* incubated together with artificial cells was monitored by flow cytometry by using an AI-2 *E. coli* reporter strain. Then, the level of *lsr* transcript was monitored through RT-qPCR.

3.4 Methods

3.4.1 Vesicle permeability to glucose

A shrink-swell assay[87] was performed to assess whether glucose was capable of crossing vesicle membranes. 1:2 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC): cholesterol vesicles with 10 mM calcein were prepared in buffer A (10 mM MgCl2, 100 mM KCl, 50 mM HEPES, pH 7.6) and purified by gel filtration chromatography with sepharose-4b. Purified vesicles were diluted two-fold with 1.0 M glucose (final concentration = 0.5 M) at 37 °C. The reaction was monitored by spectrofluorimetry with excitation and emission wavelengths of 495 nm and 515 nm, respectively.

3.4.2 *E. coli* W3110 AI-2 reporter strain as receiver cells

Mid-exponential *E. coli* W3110 transformed with a plasmid encoding GFPuv behind a T7 promoter and a plasmid encoding T7 RNA polymerase (T7 RNAP) under the control of AI-2 were grown in LB supplemented with 100 µg mL^{-1} ampicillin and 50 µg mL^{-1} kanamycin to OD_{600} 0.5. Cells were then harvested and resuspended in fresh LB. Finally, cells were added to the artificial cells at a final OD_{600} 0.1.

3.4.3 RT-qPCR analysis

Vesicles were dialyzed as previously described[58]. Then, vesicles and *E. coli* were incubated for 6 h at 37 °C. Subsequently, the total RNA was extracted with the RNeasy Mini kit (Qiagen). A quantity of 500 ng of RNA was reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific). cDNA was quantified with a CFX96 Touch real-time PCR (Bio-Rad) with SYBR green detection. Each sample was diluted to 5 ng and measured in triplicate in a 96 wells plate (Bio-Rad) in a reaction mixture containing SsoAdvanced SYBR green supermix (Bio-Rad) and 180 nM of each primer in a 10 µl finale volume. The primers used to quantify *crp, lsrB* and *lsrD* expression were crp FW: 5′-AGACTCTGCTGAATCTGGCAA-3′, crp REV: 5′-TCTGACCAATTTCCTGACGGG -3′, lsrB FW: 5′- CACGGTGAAAGAATTTGGCCT -3′, lsrB REV: 5′- TCAATAATGCATCCGCGACATACA -3′, lsrD FW: 5′- CGATGGCGTTTACAGATTTCGC -3′, lsrD REV: 5′- AGCCAGAAAACGAGGAGACAT -3′. Gene expression was normalized to the expression of idnT by using idnT FW: 5′-CTGCCGTTGCGCTGTTTATT-3′ and idnT REV: 5′-GATTTGCTCGATGGTGCGTC-3′.

3.5 Results

3.5.1 Vesicle permeability to glucose

In the artificial cells the release of glucose has to be controlled by pore formation upon the addition of theophylline. To assess phospholipid vesicle permeability to glucose, a shrink-swell assay[87] was performed as previously described[58]. 1:2 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC): cholesterol vesicles were formed with 10 mM calcein diluted in buffer A (50mM HEPES, 10mM MgCl2, 100mM KCl, pH 7.6) and unencapsulated dye was removed through gel filtration. 1 M glucose in buffer A was added 1:1 to the vesicle suspension. The fluorescent signal was monitored by fluorimetry. Calcein is a self-quenching dye. Upon the addition of the solute, water leaks out from the vesicles to equilibrate the inner and the outside solutions. Therefore, the concentration of calcein increases and the fluorescent signal decreases. If the molecule enters within the vesicles to reach equilibrium, calcein would be diluted and the fluorescence signal would increase. No increase of signal was detected within 10 h of incubation at 37 °C. 1:2 POPC: cholesterol vesicles are not permeable to glucose (Fig. 3.2).

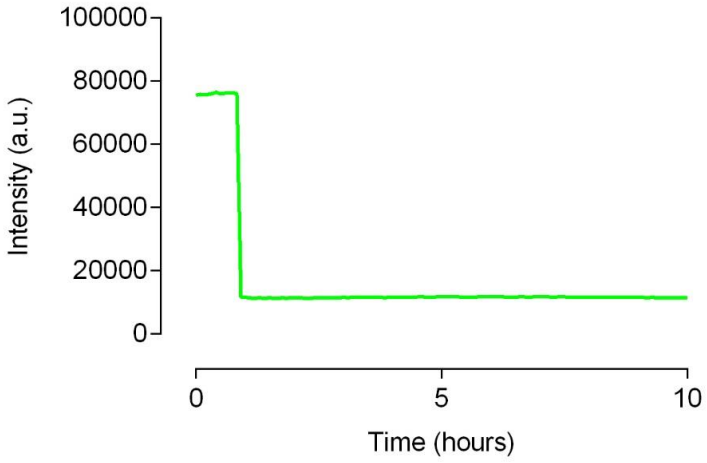


Fig. 3.2 Glucose does not cross 1:2 POPC: cholesterol vesicles.

Fluorescent profile of 1:2 POPC: cholesterol vesicles contained 10 mM of calcein in the presence of glucose. A shrink-swell assay was performed to assess whether glucose was capable of crossing 1:2 POPC: cholesterol membranes. 1:2 POPC: cholesterol vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) were mixed with 1 M of glucose (final concentration 0.5 M). The fluorescent signal was monitored by fluorimetry (ex 495 nm, em 515 nm) at 37 °C for 10 h. The decrease in fluorescence was due to both dilution with the glucose solution and calcein self-quenching. The solute was incapable of crossing the membrane, as shown by no recovery of fluorescence.

3.5.2 The effects of glucose on *E. coli*

To assess whether glucose has an effect on AI-2 uptake, *E. coli* W3110 containing pTC6 and pET GFPuv plasmids was used as an AI-2 reporter strain. The pTC6 plasmid contains the *T7 RNA polymerase* (T7 RNAP) under the control of the intergenic region of *lsr* operon, while pET GFPuv contains GFPuv under the control of a T7 promoter. In the presence of AI-2, T7 RNAP is produced and drives the expression of GFPuv. When glucose is present in the media, it shuts down CRP expression, leading to low expression of the *lsr* operon. In the absence of the lsrACDB transport system, AI-2 produced from the reporter strain itself cannot enter the *E. coli* cells. 100 mM of glucose were added to LB media of the AI-2 reporter strain, and the cells were incubated at 37 °C. Each hour a few microliters were collected and analyzed by flow cytometry. When *E. coli* grew in LB without glucose, an increase over time of green positive cells was recorded until approximately 60 % were positive, whereas the percentage of green positive cells in samples treated with glucose remained stable at 5 % for each time point (Fig. 3.3a). In addition, RT-qPCR was performed to monitor the effects of glucose on *E. coli.* 4 mM glucose were added to *E. coli* Bl21(DE3) pLysS in LB and incubated at 37 °C. After 6 h, the cells were collected and the RNA was isolated and reverse transcribed. RT-qPCR was performed on *crp* and *lsrBD* genes as representatives of the *lsr* operon. As expected, samples treated with glucose showed a low expression of both CRP and *lsr* operon genes (Fig. 3.3b).

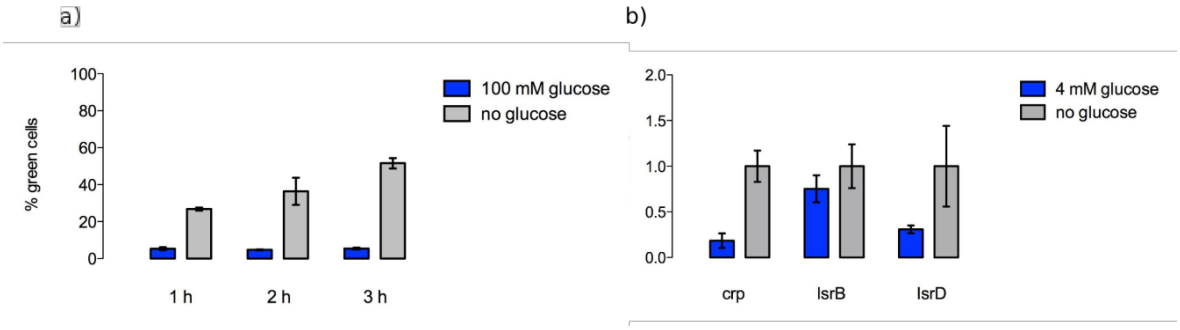


Fig. 3.3 Effects of glucose on AI-2 uptake.

Glucose effects on *E. coli* were assessed in two ways. a) Flow cytometry analysis of *E. coli* W3110 AI-2 reporter strain grown in the presence or in the absence of 100 mM glucose in LB. Glucose represses CRP expression which cannot control expression of the *lsr* operon. AI-2 cannot enter the cells. As expected, a low percentage of green positive cells was shown in samples treated with glucose (blue bars), while control cells showed an increase over time of positive events (grey bars). b) RT-qPCR analysis of *crp* and *lsrBD* in the presence and in the absence of 4 mM glucose. *E. coli* Bl21(DE3) cells grown in the presence of glucose showed downregulation of *crp, lsrB* and *lsrD* (blue bars) compared to control cells (grey bars).

3.5.3 Artificial cells control AI-2 uptake in *E. coli* through the release of glucose

Once it was established the suitability of the two methods to monitor the effects of glucose on *E. coli*, artificial cells loaded with glucose were tested for their ability to control AI-2 uptake in *E.*

coli cells through glucose release. Artificial cells were built as previously described[58], except that IPTG was substituted with glucose. Only in the presence of theophylline, αHL is produced and forms pores in the lipid membrane of artificial cells. Glucose is then released to *E. coli*. Mid-exponential cultures of *E. coli* BI21(DE3) pLysS cells were incubated with artificial cells at 37 °C in the presence or in the absence of 5 mM theophylline. To obtain a global understanding on the effects of the system, RT-qPCR was performed. *crp* and two genes of *lsr* operon, *lsrB* and *lsrD,* were monitored. After 6 h the cells were collected and RT-qPCR was used to monitor *crp*, *lsrB* and *lsrD* levels. Artificial cells in the presence of 5 mM theophylline showed a downregulation of 5.0±0.6 and 1.3±0.1 in *crp* and *lsrD* compared to samples in which theophylline was not present, respectively. No effect was shown in *lsrB* expression (Fig. 3.4ab). However, the effect reported in *crp* levels was higher than in the controls. Even cells incubated with 100 mM glucose showed a higher *crp* expression than in the presence of artificial cells. 5 mM of theophylline affected *crp* gene expression, leading to an upregulation of the gene, while mild effects were seen for *lsrB* and *lsrD* genes. The results obtained were difficult to interpret. Theophylline had an opposite effect than glucose on *crp* expression. Theophylline upregulated *crp* expression, while glucose downregulated the expression of *crp*. When used together within the system, theophylline and glucose led to no effect or minimal effect of artificial cells action on *lsr* operon. Moreover, the presence of artificial cells in general seemed to affect CRP, resulting in downstream regulation of the *lsr* operon.

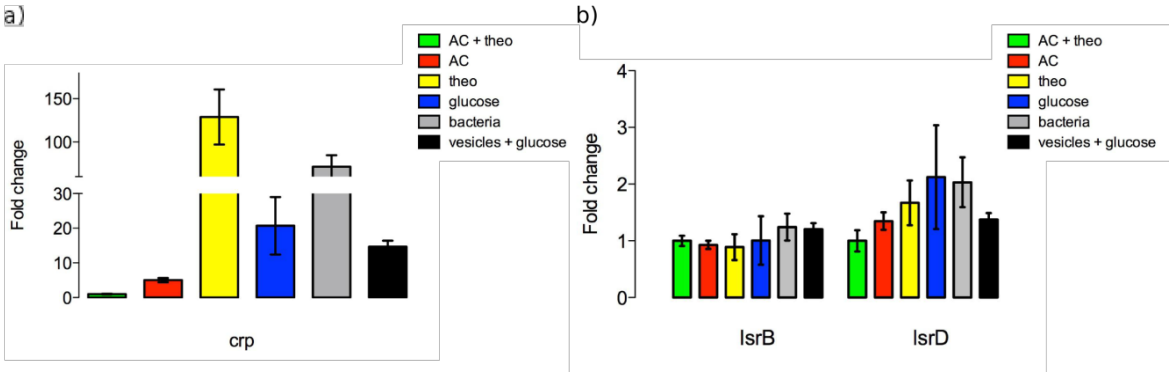


Fig. 3.4 Artificial cells partially failed to control *crp* and *lsr* expression through the release of glucose.

RT-qPCR analysis performed on *crp* and *lrs* operons in the presence of artificial cells and theophylline. Artificial cells were incubated with *E. coli* BI21(DE3) pLysS in the presence or in the absence of 5 mM theophylline. Theophylline allows αHL expression and the release of glucose. Glucose downregulates the *crp* gene and in turn *lsr* operon transcription. a) In cells incubated with 5 mM of theophylline alone, *crp* expression was upregulated (yellow bar). Mild differences are shown from cells treated with artificial cells in the presence or in the absence of theophylline (green and red bars, respectively). b) The presence of theophylline with artificial cells did not affect *lsrB* and *lsrD* expression. AC = artificial cells, theo = 5 mM theophylline.

To further investigate the action of artificial cells when loaded with glucose, AI-2 uptake was measured directly by using the *E. coli* W3110 AI-2 reporter strain. If the artificial cells are able to send glucose in the presence of theophylline, a lower GFPuv expression is expected due to the

absence of AI-2 uptake from the *E. coli* reporter strain. Artificial cells were built as previously described[58] and incubated at 37 ºC with the *E. coli* W3110 AI-2 reporter strain. Each hour few microliters were collected and GFPuv expression was monitored by flow cytometry. Bacteria incubated with 100 mM of glucose showed a lower GFPuv expression compared to bacteria grown in LB not supplemented with 100 mM glucose (Fig. 3.5). Glucose does not cross phospholipid vesicles. After 3 h no clear difference was observed when artificial cells and bacteria were incubated in the presence or in the absence of 5 mM theophylline, with a percentage of 15 % and 26 %, respectively. However, a higher percentage of cells (60 %) expressed GFPuv when incubated with theophylline alone.

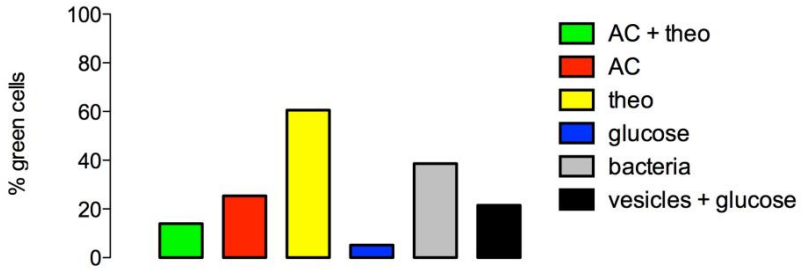


Fig. 3.5 No clear effects of artificial cells loaded with glucose on *E. coli*.

Flow cytometry analysis of artificial cells loaded with glucose. Artificial cells were incubated with *E. coli* W3110 AI-2 reporter strain at 37 ºC in the presence or in the absence of theophylline. After 3 h cells were collected and analyzed by flow cytometry. If glucose is released from the artificial cells, a decrease in AI-2 uptake, and thus a decrease in green positive cells would be expected. No clear difference was observed in the presence or in the absence of theophylline, 15 % and 26 % of green positive cells were recorded, respectively (green and red bars). Control cells grown in LB supplemented with 100 mM glucose showed a low percentage of green positive events (blue bar), while control cells grown in LB showed 40 % of green cells (grey bar). Vesicles loaded with glucose showed a lower percentage of green positive cells compared to control cells (black bar), probably due to vesicle breakage. Unexpectedly, cells grown in LB supplemented with 5 mM theophylline showed 60 % of positive events (yellow bar), a percentage higher than control cells. AC = artificial cells, theo = 5 mM theophylline.

To further investigate the effect shown by theophylline on AI-2 uptake, the *E. coli* AI-2 reporter strain was incubated with different amounts of theophylline. When 5 mM of theophylline were present in the media, an increment of GFPuv positive cells was detected. Samples treated with lower amounts of theophylline did not show any differences when compared to control cells grown in LB media (Fig. 3.6). Theophylline has been proposed to act as a cAMP phosphodiesterase inhibitor raising intracellular levels of cAMP[88], thus it could have an effect on genes related to the cAMP-CRP pathway. Moreover, the high concentration of theophylline used could increase these effects.

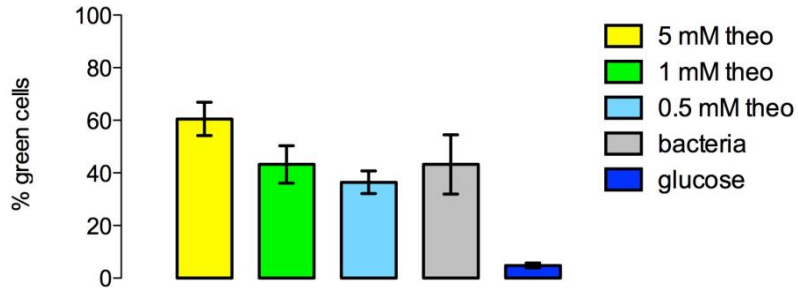


Fig. 3.6 Theophylline effects on AI-2 uptake.

Flow cytometry analysis of *E. coli* W3110 AI-2 reporter strain in the presence of various theophylline concentrations. *E. coli* cells were grown at 37 ºC in LB supplemented with 5 mM, 1 mM and 0.5 mM of theophylline. Cells grown in LB and in LB supplemented with 100 mM glucose were used as controls. After 3 h cells grown in LB supplemented with 5 mM of theophylline showed a percentage of 60 % green positive cells (yellow bar), higher than control cells (grey bar). Cells treated with lower amounts of theophylline showed similar percentages of positive cells compared control.

Experiments were then set up using 1 mM theophylline, a concentration that seemed to have not affect *lsr* operon expression. *E. coli* W3110 AI-2 reporter strain was incubated with artificial cells at 37 °C. When a lower amount of theophylline was added, the unwanted effect on AI-2 uptake was reduced (Fig. 3.7). However, no differences were observed in samples incubated with artificial cells in the presence or in the absence of theophylline. Moreover, both samples showed a low percentage of positive cells compared to control cells with glucose. The amount of theophylline could have been too low to activate the artificial cells. Glucose release over time could be due to leaky expression of the theophylline riboswitch, leading to the presence of the same glucose concentration in both samples.

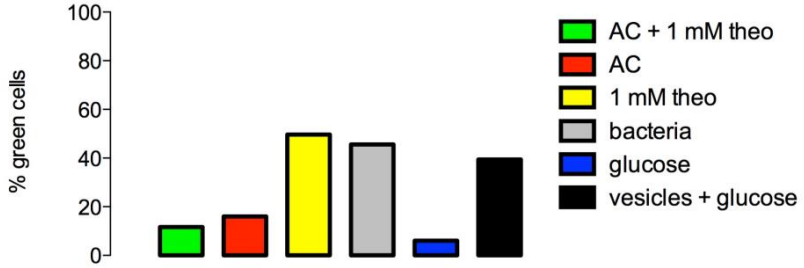


Fig. 3.7 Artificial cells failed to control AI-2 uptake through glucose release.

Flow cytometry analysis of artificial cells in the presence of 1 mM theophylline. Artificial cells were incubated with *E. coli* W3110 AI-2 reporter strain at 37 ºC in the presence or in the absence of 1 mM theophylline and analyzed by flow cytometry. No difference was observed in the presence or in the absence of 1 mM theophylline, 12 % and 16 % of green positive cells were recorded, respectively (green and red bars). Control cells grown in LB supplemented with 100 mM glucose showed a low percentage of green positive events (blue bar). 1 mM of theophylline did not affect AI-2 uptake showing a similar percentage of green cells compared to control cells (yellow and blue bars, respectively). Glucose did not cross phospholipid membrane, resulting in a similar percentage of positive events compared to the control (black bar). AC = artificial cells.

3.6 Conclusions

The results obtained showed the possibility to control natural cell behavior without direct genetic manipulation of the living cells. The genetic engineered components were moved from the living cells to more controllable artificial cells. Contrary to natural cells, the artificial cells were made with just known components, and the artificial cells could not grow or evolve. Once the artificial cells accomplish their tasks, the artificial systems degrade and the living cells go back to normality, thereby avoiding unwanted long-term consequences. However, this first generation of artificial cells suffers from several limitations, such as low encapsulation efficiency, which results in a population of artificial cells with different degrees of activity. In addition, the synthesis of active molecules starting from precursors should be integrated in the artificial system to avoid the release of active molecules from compartment degradation.

Moreover, the work presented in this chapter showed the construction of a one-way communication path between artificial and natural cells. The artificial cells sense a molecule that *E. coli* cannot sense on its own and release a molecule that *E. coli* can sense. The proof-of-concept developed in this study could be exploited to control other pathways within natural cells. Unfortunately, attempts to affect *E. coli* QS through glucose release presented difficulties due to the combination of theophylline and glucose, which showed opposite effects on CRP. Thus, the effect of the artificial cells on the targeted *lsr* operon resulted in unclear data.

However, artificial cells could be constructed to better imitate natural cellular life. Engineered communication between living cells have be established to coordinate bacterial populations[89, 90] and artificial QS pathways were constructed within natural cells[91, 92]. It could be possible to construct similar pathways within artificial cells to reach a two-way communication between artificial and natural systems. The artificial cells could sense the living cells through QS and then send in return QS messages. The construction of such a system would represent a more complete integration between artificial and living cells. Moreover, acting on QS pathway, artificial cells could potentially be exploited as tools to reduce biofilm formation and defeat infections.

Chapter 4.

Integrating artificial with natural cells through quorum sensing

4.1 Quorum sensing

Bacteria are not just autonomous unicellular organisms. Instead, bacteria participate in cell-to-cell communication processes referred to as quorum sensing (QS), a term first introduced by Fuqua in 1994.[93] QS is a mechanism used by bacteria to coordinate gene expression in such a way that specific genes are either up or downregulated when a high cell density is reached. The process depends on small diffusible molecules, called autoinducers, that are released by bacteria into the external environment. As the bacterial density increases, the concentration of autoinducers rises until a critical threshold is reached. The internalization of the small molecules is either through passive diffusion or via the action of specific membrane transporters and ultimately regulates several physiological processes, including biofilm formation[94], bioluminescence[95] and virulence[96].

A large variety of bacteria species are known to communicate and coordinate their behavior through this language. One of most studied QS pathways is of is *Vibrio fischeri*. In the 1970s it was noticed that *V. fischeri* produces bioluminescence only at high cell density[97], and depended on the production of a small molecules referred to as N-3-(oxohexanoyl)homoserine lactone (3OC6 HSL)[98]. At low cell density, 3OC6 HSL diffuses out of the cells into the external environment, and when the concentration increases the quorum molecule diffuses into the cells[99]. 3OC6 HSL is recognized by a cytoplasmic receptor LuxR. The 3OC6 HSL-LuxR complex then binds a specific region of the DNA, thus activating the expression of the *lux* operon and leading to luminescence. The genes involved in the process are composed of two divergent transcriptional units, one contains the *luxR* gene and the other is constituted by the lux operon, *luxICDABE.* Between the two units is a 150 bp region[100] that includes a specific sequence recognized by the LuxR-3OC6 HSL complex, referred to as the lux box[101]. The *luxI* gene codes for a synthase which drives the synthesis of 3OC6 HSL, while *luxCDABE* are genes dedicated to light production[102]. LuxI and LuxR regulate bioluminescence in correlation to cell density[103]. At low density, LuxI is expressed at a basal level and produces 3OC6 HSL which is released to the outside. When the cell density is high, the molecule diffuses into the cells, binds LuxR, and activates light production and LuxI expression in a positive feedback loop[99].

The LuxI-LuxR signal response mechanism is exploited by a large number of Gram-negative species to control a variety of density related functions. Many homologous of LuxI synthase and the LuxR receptor were found, such as CepI-R in Burkholderia cepacia[104] or PpuI-R in *Pseudomonas putida*[105]. Furthermore some species are characterized by more than one LuxI-LuxR system that act together to control different behaviors. For example, *Pseudomonas aeruginosa* contains both LasI-LasR[106] and RhlI-RhlR[107] QS systems that are controlled by N-(3-oxododecanoyl)-l- homoserine lactone (3OC12 HSL) and N-butanoyl-l-homoserine (C4 HSL), respectively.

The autoinducer molecules that characterize the LuxI-LuxR systems are homoserine lactones (AHLs). The structure of AHL (Table 4.1) comprises a homoserine lactone ring and an acyl chain (from 4 to 18 carbons)[108]. Each QS pair is characterized by a different autoinducer molecule which shares a common moiety and differs from the length of the acyl chain and the possible substitution of a carbonyl in the third carbon[109]. Each AHL is species-specific and is synthesized by a specific synthase and can be recognized by a specific receptor.

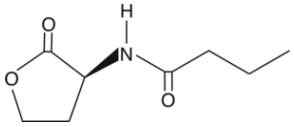
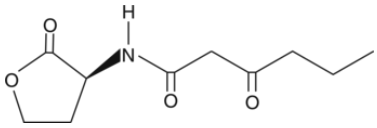
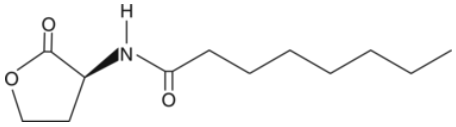
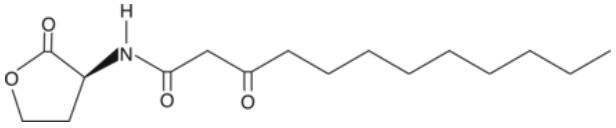
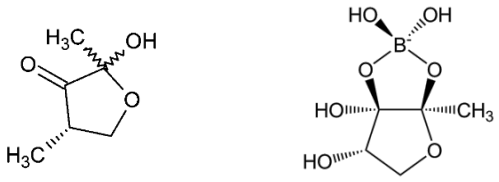
NAME	STRUCTURE	luxI-luxR	BACTERIA
C4 HSL		rhlI-rhlR	*P. aeruginosa*
306C HSL		luxI-luxR	*V. fischeri*
C8 HSL		ainS-luxR	*V. fischeri*
3OC12 HSL		lasI-lasR	*P. aeruginosa*
AI-2		luxS	*E. coli* *V. harveyi*

Table 4.1 AHLs and autoinducers used in this study.

C4 HSL and 3OC12 HSL were from *P. aeruginosa.* 3OC6 HSL and C8 HSL were from *V. fischeri.* Different AI-2 structures from *E. coli* and *V. harveyi* are shown.

Bacteria are characterized also by an interspecies QS molecule called autoinducer 2 (AI-2). First described in *Vibrio harveyi*[110], AI-2 is produced by LuxS in both Gram-negative and Gram-

positive bacteria[111] (Table 4.1). LuxS homologous have been found in more than 60 different species[112]. Different roles have been proposed for AI-2, including as an additional layer of control for biofilm formation in *E. coli*[84] and virulence genes in *Vibrio cholerae*[96].

4.2 Construction of an *in vitro* quorum sensing mechanism

To integrate artificial with natural cells, the artificial cells need to sense the living cells. Living cells are able to sense their environment and to modify their behavior to adapt to external changes. Many external stimuli are recognized by bacterial cells, such pH[113], temperature[114] and stress[115], leading to the regulation of thousands of genes[77]. Moreover bacteria release various small molecules such as metabolic end products[116], iron chelators[117] and autoinducers. Through these small molecules, living cells can determine optimal survive strategies and monitor their own population density[118]. Thus, bacterial QS can be exploited in an artificial system to sense the presence of living cells and to communicate with them.

To construct a synthetic QS pathway, artificial cells able to recognize the QS molecules that are synthesized and secreted by bacteria are needed. Therefore, the *E. coli* AI-2 sensory pathway was reconstructed *in vitro*. *E. coli* produces AI-2 through the activity of the LuxS enzyme. AI-2 accumulates extracellular in the mid-late exponential phase and declines in the stationary phase as a consequence of uptake by a transporter cassette included in the *lsr* operon[119]. The operon contains *lsrACDBFG* genes and is activated by AI-2 itself[120]. *lsrACDB* genes code for the AI-2 transport system, while *lsrFG* genes are involved in AI-2 degradation[121]. Next to the *lsr* operon, *lsrR*, a repressor, and *lsrK*, a kinase, are divergently transcribed. The LsrR repressor binds an intergenic region of 250 bp in length adjacent to the *lsr* operon thereby repressing the transcription of the operon[85]. After internalization, AI-2 is phosphorylated by LsrK. LsrR complexed with phosphorylated AI-2 leads to the derepression of the *lsr* operon.

To mimic the AI-2 sensory pathway *in vitro*, a genetically encoded device in which a T7 promoter drives the expression of *lsrR, lsrK* and *T3 RNA polymerase* (T3 RNAP) was built to constitutively express the three genes with cell-free transcription-translation machinery containing T7 RNA polymerase (T7 RNAP). A second synthetic construct expresses αHL under a T3 promoter placed behind the intergenic region of *lsr* operon controlled by LsrR. A promoter cascade was introduced to avoid basal expression of αHL. Thus, in the absence of AI-2, LsrR represses αHL expression. When AI-2 is added, AI-2 is phosphorylated by LsrK, and phosphorylated AI-2 disrupts LsrR binding, thereby allowing αHL transcription with T3 RNAP.

To complete the construction of *in vitro* QS able to communicate with *E. coli* a synthetic device that synthesizes AI-2 was built to send a message to the bacteria. *E. coli* produces AI-2 starting from S-adenosylmethionine (SAM), a central metabolite used as a methyl donor dependent on methyltransferases during the biosynthesis of nucleic acids and proteins. The

reaction produces a toxic intermediate, S-adenosylhomocysteine (SAH), which is rapidly hydrolyzed to S-ribosylhomocysteine (SRH) and adenine by Pfs, a nucleosidase. Subsequently, SRH is cleaved by LuxS into homocysteine and 4,5- dihydroxy- 2,3- pentanedione (DPD)[122]. DPD spontaneously cyclizes, giving AI-2. Different arrangements are possible leading to various AI-2 molecules that can be recognized by different bacteria[123]. For example, AI-2 in *V. harveyi* is a cyclic borate diester[110]. It has been shown that AI-2 can be enzymatically produced starting from SAH with purified LuxS and Pfs,[122, 124] and AI-2 production can be further optimized using LuxS and Pfs in a fusion protein, His-LuxS-Pfs-Tyr (HLPT)[125]. Thus, a genetically encoded HLPT to synthesize *in vitro* AI-2 was constructed.

Other than *E. coli* QS, also several LuxI-LuxR QS pairs can be reconstituted *in vitro*. To mimic *V. fischeri* a synthetic device that comprises the *luxR* gene under a tet promoter was used. When 3OC6 HSL is present, the quorum molecule is recognized by LuxR and activates the transcription of the *luxI* gene under the correspondent responsive promoter, promoting 3OC6 HSL production. In the same manner the two *P. aeruginosa* QS systems, LasI-LasR and RhlI-RhlR controlled by 3OC12 HSL and C4 HSL, respectively, were constructed.

4.3 Methods

4.3.1 Bacterial strains and growth conditions

E. coli and *P. aeruginosa* were grown in LB. *V. fischeri* was grown either in LBS (tryptone 10 g/L, yeast extract 5 g/L, NaCl 20 g/L) or photobacterium broth (ammonium chloride 0.3 g/L, calcium carbonate 1 g/L, casein enzymic hydrolisate 5 g/L, ferric chloride 0.01 g/L, magnesium sulfate 0.3 g/L, monopotassium dihydrogen phosphate 3 g/L, sodium chloride 30 g/L, sodium glycerophosphate 23.5 g/L, yeast extract 2.5 g/L). *V. harveyi* was grown in marine broth supplemented with 2 % of casamino acids or autoinducer bioassay (AB) medium (NaCl 17.5 g/L, MgSO4 12.3 g/L, casamino acids 2 g/L, 1M potassium phosphate, 0.1 M arginine and 1% glycerol v/v, pH 7.5) . When necessary, media were supplemented with the correct antibiotic (100 µg/mL ampicillin, 50 µg/mL kanamycin, or 34 µg/mL chloramphenicol).

4.3.2 Genetic constructs

Plasmids used in this study are listed in the appendix. To build *E. coli* synthetic QS pathway, *lsrR*, *lsrK* and *lrs* operon intergenic region were amplified from the *E. coli* MG1655 genome by PCR. αHL and SP6 RNA polymerase were synthesized by Genscript, and T3 RNA polymerase was from the registry of standard biological parts. For *E. coli*, AHL sensor strains were generated with K575024, K575037 and T9002, which were taken from the registry of standard biological parts. To build LuxI-LuxR type QS, genes were taken from the registry of standard biological parts, while pLasRL was extracted from the *P. aeruginosa* PA14 genome by PCR.

4.3.3 *In vitro* cell-free transcription-traslation reactions

Synthetic *E. coli* QS sensor device reactions were expressed using the PURE system (New England BioLabs) transcription-translation cell-free machinery. Each reaction comprises 10 µL solution A, 7.5 µL solution B, 20 U RNase inhibitor and 250 ng DNA, unless specifically stated otherwise. Different amounts of enzymatically produced AI-2 were added to activate the QS system. Reactions were incubated at 37 ºC and sfGFP expression was monitored with a PTI QuantaMaster 40 UV VIS spectrofluorometer (fluorimeter) for 6 h.

Reconstituted *P. aeruginosa* and *V. fischeri* QS sensor systems used an *E. coli* cell-free S30 extract for circular DNA (Promega). Each reaction contains, unless stated otherwise, 20 µL premix, 15 µL S30 extract, 5 µL amino acids mix, 40 U of RNase inhibitor and 2 µg of DNA. From 0.1 µM to 10 µM of 3OC12 HSL, N-octanoyl-L-Homoserine lactone (C8 HSL), 3OC6 HSL or C4 HSL were added to activate protein expression. Reactions were monitored at 37 ºC using a spectrofuorometer or CFX96 Touch™ Real-Time PCR Detection System (BioRad) machine for 6 h.

In vitro QS sender devices were expressed either with the PURE system or the S30 T7 High-Yield Protein Expression System (Promega). PURE system reactions contained 10 µL solution A, 7.5 µL solution B, 20 U RNase inhibitor 250 ng DNA and 0.5 mM of SAH. S30 T7 high yield *E. coli* S30 cell-free extract reactions were composed by 20 µL S30 premix, 18 µL T7 S30 extract, 40 U of RNase inhibitor and 1ug of DNA. For AI-2 production, 1 mM of SAH was added. To synthesize AHLs, 100 µM of acetyl-CoA and 0.5 mM of SAM were added. Reactions were carried out at 37 ºC from 4 h to 6 h.

4.3.4 AI-2 extraction from culture fluids

E. coli cells were grown overnight from a 10% glycerol stock in LB supplemented with 0.8% of glucose. The day after, cells were diluted 1:100 in fresh LB 0.8 % glucose and grown for 8 h at 37 ºC at 220 RPM. Cells were removed by centrifugation at 5000 RPM for 10 min, and the supernatant was filtered through 0.2 µM filters and stored at -20 ºC.

4.3.5 Enzymatic production of AI-2

AI-2 was enzymatically produced *in vitro* using 12 µM of purified HLPT and 5 mM of SAH. Reactions were incubated overnight at 37 ºC 220 RPM. Subsequently, reactions were chloroform extracted to remove proteins. The aqueous phase contained AI-2. Since the enzymatic reaction produces in a 1:1 ratio AI-2 and homocysteine, AI-2 was indirectly quantified using 2,2'-dinitro-5,5'-ditiodibenzoico (DTNB) reagent. The solution for quantification contained 10 µL of sample, 100 µL of Tris-HCl, pH 8, 50 µL working solution 2 mM DTNB 50 µM NaAc, and 840 µL of water. After 5 min of incubation at room temperature, the absorbance at 412 nm was measured and the concentration of homocysteine calculated using molar extinction coefficient (13,600 M-1 cm-1) of the reaction product 5-thio-2-nitrobenzoic acid (TNB).

4.3.6 *V. harveyi* bioluminescence assay

To measure AI-2 production with HLPT or *in vitro* synthesis *V. harveyi* BB170 was used. This strain can naturally sense AI-2 but it is engineered to not sense its own autoinducer 1 (3-oxo-N-(2-oxotetrahydrofuran-3-yl)butanamide, 3OC4 HSL). The assay was performed as described by Vilchez et al.[126]. Briefly, *V. harveyi* was grown overnight from 200 µL glycerol stock in AB medium supplemented with kanamycin, at 30 °C 220 RPM. The day after, bacteria were diluted to OD_{600} 0.7 and grown for 1 h and 30 min (until OD_{600} 1.1) in AB medium. The culture was then diluted 1:5000 and 90 µL were loaded into 96 well white plate for luminescence assay. 10 µL of sample was added to the wells. AB medium was used as blank, and 50 µM of enzymatically produced AI-2 was used as a positive control. Plates were incubated at 30 °C and luminescence was monitored with Infinite200 PRO plate reader (Tecan) every hour.

4.3.7 LsrK phosphorylation activity test

Expression of LsrK was carried out in PURE system reaction containing 10 µL solution A, 7.5 µL solution B, 20 U RNase inhibitor and 250 ng DNA. Reaction was supplemented with 60 µM of ATP, 0.2 uCi of [γ-32P]ATP and 0.8 mM of AI 2 and incubated at 37 °C for 6 h. Hydrolyzed ATP (incubated at 100 ºC in 2 M NaOH) was added as a control. Reactions were then chloroform extracted to remove proteins and 2 µL aliquots were loaded on silica TLC plates, dried, and developed with 4:2:2 butanol: water: acetic acid. Plates were visualized with a phosphorimaging screen at Typhoon phosphoimager (Amersham Biosciences).

4.3.8 The resistance of vesicles to bacteria

To test whether bacteria could break phospholipid vesicles, a dye leakage assay[127] was performed. Different vesicles compositions were formed as from thin lipid film and resuspended in 10 mM of calcein dissolved in buffer A containing 50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6. Vesicles were extruded through a polycarbonate membrane of 1 µm pore size and loaded into a sepharose 4B column. The first three yellow drops containing vesicles were loaded into a quartz cuvette and monitored at fluorimeter. After 15 min, 1:1 volume of bacteria at OD_{600} 0.5 was added to the cuvette. Reactions were monitored for 10 h. The temperature was set accommodate bacterial growth. Then 0.3% of Triton X-100 was added as a control to break the vesicles.

4.3.9 Artificial cells that sense 3OC6 HSL

Aliquots of 1:2 (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) POPC: cholesterol vesicles were formed as previously described[128]. 100 µL aliquots were rehydrated with 50 µL of S30 *E. coli* extract reaction containing 20 µL S30 premix, 15 µL S30 extract, 5 µL amino acids mixture, 40 U of RNase inhibitor and 4 µg of DNA (RL082A). Vesicles were diluted 1:1 with LB, supplemented with 1 mg/mL proteinase K and 3 mg/mL RNase A. Reactions were incubated at 30 °C for 5 h. For external 3OC6 HSL sensing, 10 µM of synthetic 3OC6 HSL was added to the artificial cells. For *V. fischeri* sensing, bacteria were first grown overnight from 200 µL glycerol stock at room temperature in LBS medium supplemented with ampicillin. The day after, bacteria were diluted 1:100 in fresh LBS medium supplemented with ampicillin and grown until OD_{600} 1.2. 1.8 mL of culture were loaded into 2 mL dialysis tubes, while artificial cells were added onto the dialysis cap. 1.8 mL of LBS were used as negative control instead of *V. fischeri* culture. Tubes were incubated at 30 °C for 5 h, then artificial cells were collected and loaded into 96 well white plates. 0.3 % of Triton X-100 was added to break vesicles and 100 µL of luciferase assay reagent (Promega) were added to the samples. After less than 5 min, the luminescence was monitored with a plate reader.

4.3.10 Artificial sender cells

Frozen aliquots of 1:2 POPC: cholesterol vesicles were hydrated with 50 µL of 30 T7 High-Yield Protein Expression System supplemented with 4 µg of DNA encoding the correspondent synthase under a T7 promoter (MC001A, MC002A, MC003A, JF005A). For the production of AHLs, 1 mM of SAM and 300 µM of acetyl-CoA were added, whereas for AI-2 production 1 mM of SAH was added. Artificial cells were diluted 1:3 in buffer A and 1 mg/mL proteinase K were added externally. Reactions were incubated at 37 °C for 6 h. In the meantime, the correspondent *E. coli* reporter strain was grown from one colony in LB supplemented with the correct antibiotic until OD_{600} 0.5. Cells were harvested, resuspended in fresh LB and added to artificial cells to a final OD of 0.1. Samples were incubated at 37 °C, each hour few µL were collected, diluted in PBS and monitored by flow cytometry with a FACS canto A (BD biosciences).

4.3.11 Artificial cells that sense and send chemical signals (*V. fischeri – E. coli, V. harveyi*)

E. coli reporter strains correspondent to QS molecule were grown from one colony in LB supplemented with the correct antibiotic until OD_{600} 0.5. Cells were then harvested and resuspended in fresh LB. Frozen aliquots of 1:2 POPC cholesterol vesicles were hydrated with 50 µL S30 *E. coli* extract for circular template supplemented with 4 µg of DNA encoding 3OC6 HSL sensing device and the correspondent synthase (JF008A, RL079A or RL080A). For the production of AHLs, 1 mM of SAM and 700 µM of acetyl-CoA were added, whereas for AI-2 production 1 mM of SAH was added. In the synthetic 3OC6 HSL sensing, 1 µM of 3OC6 HSL was added to 50 µL of artificial cells mixed with 50 µL of the correspondent *E. coli* reporter strain, final OD 0.1. 1 mg/mL of proteinase K were added. Samples were incubated at 37 °C, aliquots collected after each hour, diluted in PBS, and monitored by flow cytometry. For *V. fischeri* sensing, bacteria were grown at 28 °C in LBS medium supplemented with ampicillin until OD_{600} 1.2. 1.8 mL of culture were loaded into 2 mL dialysis tubes, while 50 µL of LB were added onto the dialysis cap. Samples were incubated at room temperature for 2 h to equilibrate the 3OC6 HSL concentration between the two sides of the dialysis apparatus. Then, 50 µL of artificial cells and 50 µL of *E coli* reporter strain were added onto the dialysis cap. Samples were incubated at 37 °C. Aliquots were collected each hour, diluted in PBS, and monitored by flow cytometry.

4.4 Results

4.4.1 Synthetic AI-2 sensory pathway

The first AI-2 sensory pathway mimic was constructed *in vitro* to respond to AI-2 produced by *E. coli*. As a proof-of-concept, the synthetic system was developed to sense AI-2, control αHL expression upon the presence of the QS molecule AI-2 and release IPTG as message to *E. coli.* To reconstitute *in vitro* the AI2 sensory pathway of *E. coli* inside of artificial cells a genetically encoded device, RL028K, was built. A T7 promoter drives the expression of the *lsrR* repressor and the kinase *lsrK*. A second synthetic device, named RL023K, in which αHL expression is regulated by the intergenic region of *E. coli lsr* operon was built. The intergenic region comprised two divergent promoters: pLsrR and pLsrA. Both promoters contain a specific binding site for LsrR[85]. In the absence of AI-2, LsrR forms a tetramer, binds the intergenic region and represses αHL gene expression. To avoid unspecific expression of αHL before the binding of LsrR to its operators, a promoter cascade was inserted. Thus, the *SP6 RNA polymerase* (SP6 RNAP) gene is transcribed by T7 RNAP along with *lsrR* and *lsrK* in a polycistronic operon, and a SP6 promoter is placed behind the intergenic region of the *lsr* operon. When AI-2 is added, it is phosphorylated by LsrK and disrupts the LsrR tetramer, allowing αHL expression from the SP6 promoter. To test the synthetic sensory device, αHL was tagged at the C-terminus with super folder GFP (sfGFP), to monitor gene expression through fluorescence.

To check whether SP6 RNAP functionally drives αHL expression under a SP6 promoter a control reaction was performed with the PURE system. The RL029A plasmid containing SP6 RNAP was constitutively expressed from a T7 promoter, which was observed to promote the expression of sfGFP tagged αHL (Fig. 4.1).

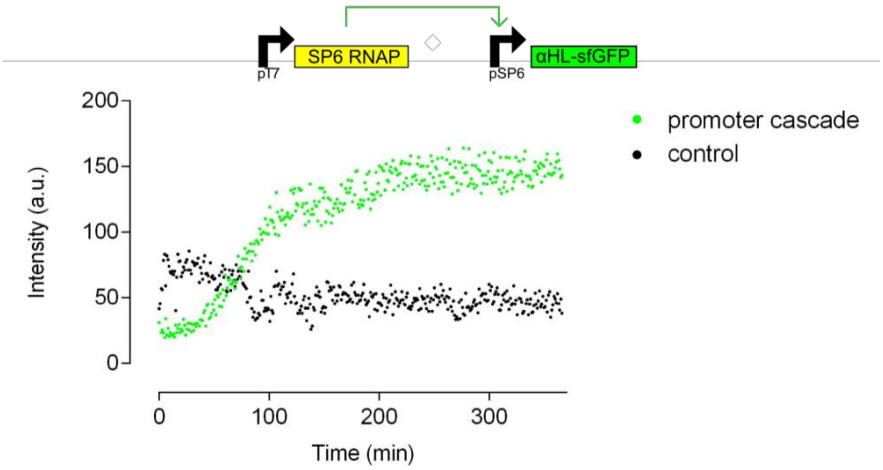


Fig. 4.1 The T7-SP6 promoter cascade.

The T7-SP6 promoter cascade was expressed *in vitro* with the PURE system at 37 °C. Kinetic experiments were monitored by fluorescence for 6 h (ex 485 nm, em 510 nm). T7 RNAP drives the expression of SP6 RNAP, which in turn allows for the translation of sfGFP (green circles). To show that SP6 RNAP is necessary to express sfGFP in this cascade, a control reaction without SP6 RNAP was performed. No signal is shown when SP6 RNAP is not added to into the cell-free reaction (black circles). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from constructs RL029A and RL042K.

To test the *in vitro* system in the presence of LsrR, the *lsr* operon intergenic region was placed between the SP6 promoter and sfGFP tagged αHL (RL023K) and expressed in a cell-free reaction together with LsrR (RL028K). In the presence of LsrR, the repressor functionally bound the intergenic region and repressed αHL expression (Fig. 4.2).

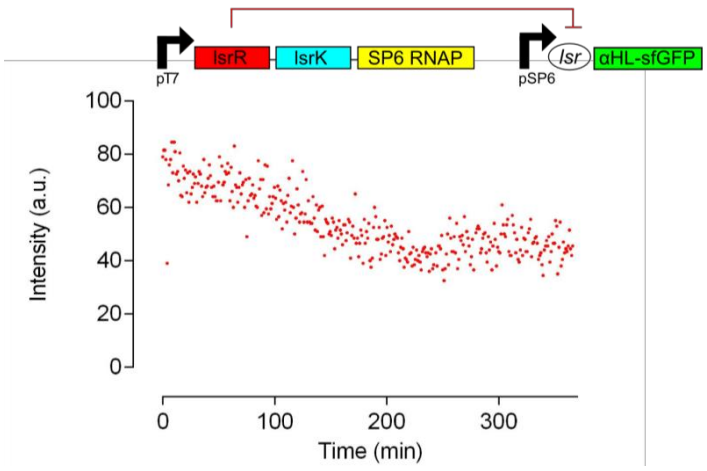


Fig. 4.2 LsrR functionally repressed gene expression *in vitro.*

LsrR was expressed *in vitro* with the PURE system at 37 °C. Reaction was monitored for 6 h (ex 485 nm, em 510 nm). The repressor binds the *lsr* intergenic region placed between SP6 promoter and sfGFP tagged αHL and shuts down gene expression (red circles). A cartoon above the graph gives a schematic representation of the used genetic circuit. *lsr* indicates the intergenic region of the *lsr* operon. Data shown are from constructs RL028K and RL023K.

Since the promoter cascade was functional and LsrR was able to repress gene expression, the next step was to add AI-2 to the *in vitro* reaction to release LsrR repression and induce gene expression. *E. coli* produces and secretes AI-2 in the mid exponential phase and the signaling molecule is degraded during the stationary phase. Therefore, it is possible to collect AI-2 in cell-free culture fluids from *E. coli.* To monitor the presence of AI-2 in culture fluids, the *V. harveyi* reporter strain BB170 was used. The strain has the quorum sensing phenotype sensor 1- and 2+, meaning that it can sense AI-2 but not AI-1 and in response emit light. After 8 h of growth in LB, 10% of the culture fluids from different *E. coli* strains were added to *V. harveyi* cultures. *E. coli* JM109 elicited luminescence in the reporter strain, while *E. coli* DH5 alpha was not able to synthesize the AI-2 because of the lack of *luxS*[111] and thus did not show any AI-2 production (Fig. 4.3).

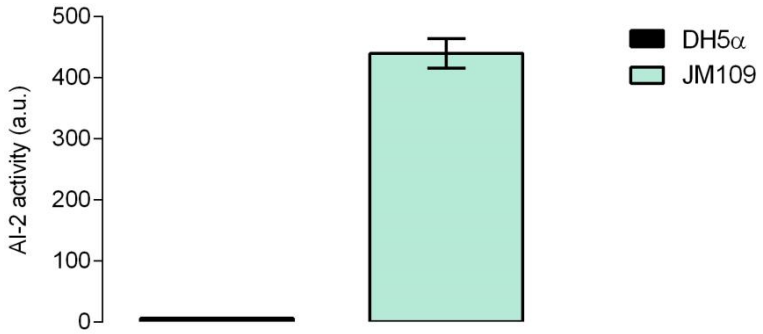


Fig. 4.3 AI-2 extraction from *E. coli* culture.

DH5α and JM109 *E. coli* cells were grown at 37 °C until mid-exponential phase. Free culture fluids were filtered and mixed together with *V. harveyi* BB170 reporter strain. After 4 h, the AI-2 present in JM109 free culture fluid elicited *V. harveyi* luminescent response (light blue bar). No signal is shown from DH5α culture fluid due to absence of AI-2 (black bar). AB medium was used as blank, AI-2 activity is calculated dividing luminescence outputs by the signal produced for the blank sample.

AI-2 extracted from *E. coli* JM109 culture fluid was added to the *in vitro* reaction containing the reconstituted AI-2 sensory pathway. AI-2 should be phosphorylated by LsrK and phospho AI-2 should bind LsrR, disrupting the tetramer. SP6 RNAP should then allow for the transcription of sfGFP tagged αHL. The kinetic experiment was carried out at 37 °C and fluorescence was monitored for 6 h. The synthetic AI-2 pathway did not respond to the presence of AI-2, i.e. no gene expression was observed upon the addition of AI-2 (Fig. 4.4).

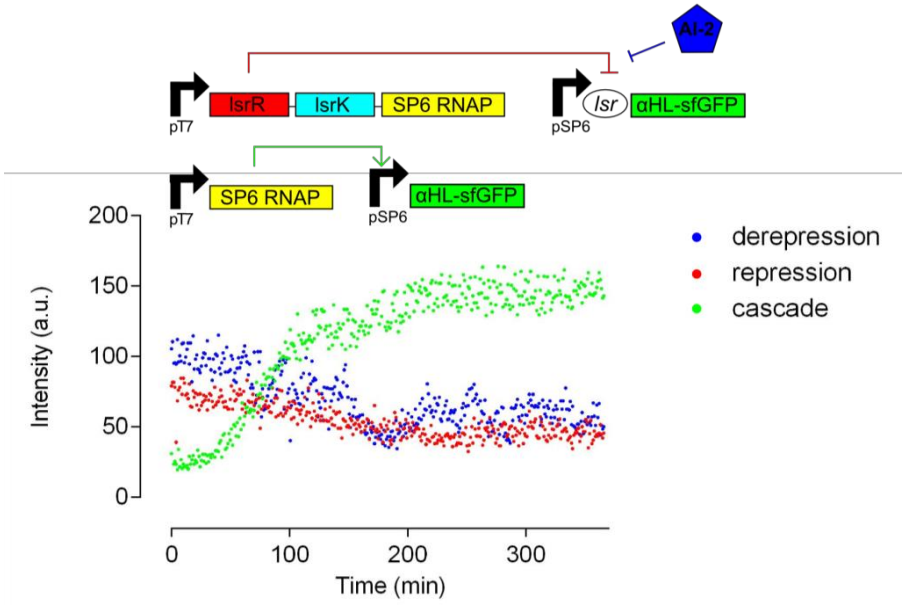


Fig. 4.4 AI-2 collected from cell-free culture fluids did not derepress the synthetic AI-2 sensory pathway.

A synthetic AI-2 sensory pathway was expressed with the PURE system at 37 °C. Kinetic experiments were monitored by fluorescence for 6 h (ex 485 nm, em 510 nm). The T7-SP6 promoter cascade functionally produced sfGFP tagged αHL (green circles). When LsrR was present, gene expression was repressed (red circles). However, when AI-2 produced by *E. coli* JM109 cells was added to the synthetic AI-2 sensory pathway, no derepression occurred (blue circles). Cartoons above the graph give a schematic representation of the used genetic circuits. *lsr* indicates the intergenic region of the *lsr* operon. Repression and derepression data shown are from constructs RL028K and RL023K, while cascade data are from RL029A and RL042K.

Since is not possible to directly quantify AI-2 concentration in the medium, the amount of AI-2 added to the *in vitro* reaction could have been too low to derepress gene expression in our synthetic circuit. AI-2 could be also enzymatically synthesized through His-LuxS-Pfs-Tyr (HLPT), a fusion protein made of LuxS and Pfs enzymes, starting from S-adenosylhomocysteine (SAH) precursor. Thus HLPT was overexpressed in *E. coli* Bl21 cells and purified. Then the protein was added to its substrate SAH and incubated at 37 °C. AI-2 concentration was indirectly quantified using 2,2'-dinitro-5,5'-ditiodibenzoico (DTNB), which measures the free thiols group of homocysteine, the side product of the Pfs enzyme, produced in a 1:1 ratio homocysteine: AI-2. After 8 h at 37 ºC about 2 mM of AI-2 was produced (Fig. 4.5).

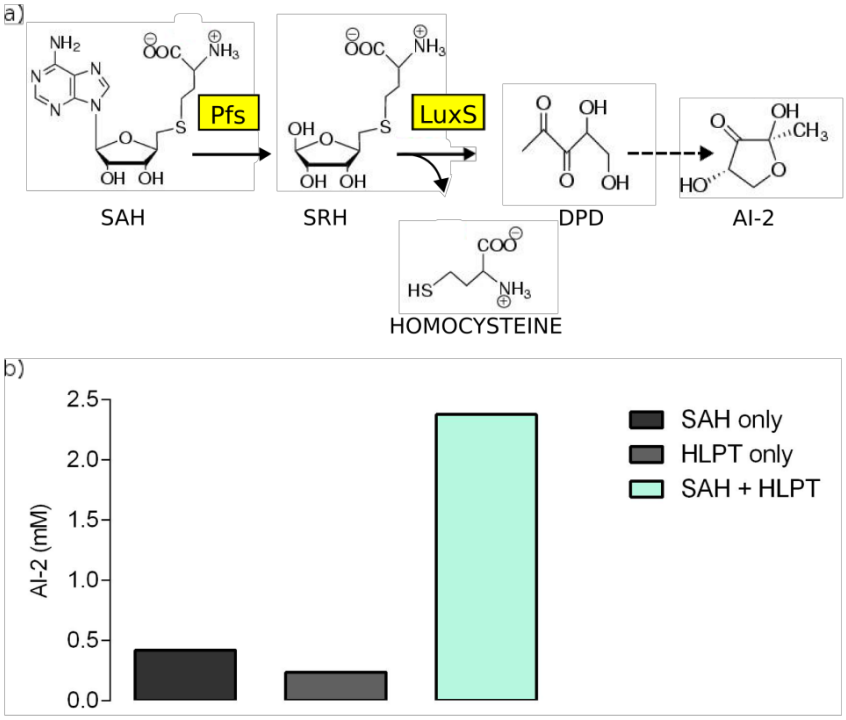


Fig. 4.5 Enzymatic production of AI-2.

Purified HLPT synthesized AI-2 starting from the precursor SAH after overnight incubation at 37 °C. a) A scheme of AI-2 synthesis through HLPT. SAH is hydrolyzed to SRH and adenine by Pfs. Subsequently, SRH is cleaved by LuxS into homocysteine and DPD in a 1:1 molar ratio. DPD spontaneously cyclizes, giving AI-2. b) AI-2 was indirectly quantified with DTNB, measuring the free thiol groups of homocysteine. Values were calculated with the extinction coefficient of TNB^{2-} at 412 nm (13,600 M^{-1} cm^{-1}).

Enzymatically produced AI-2 was then added at various concentrations to the *in vitro* reaction expressing the synthetic AI-2 sensory pathway. Kinetic experiments were monitored with a plate reader for 6 h. In all of the conditions, no sfGFP expression was observed upon the addition of synthetic AI-2 (Fig. 4.6).

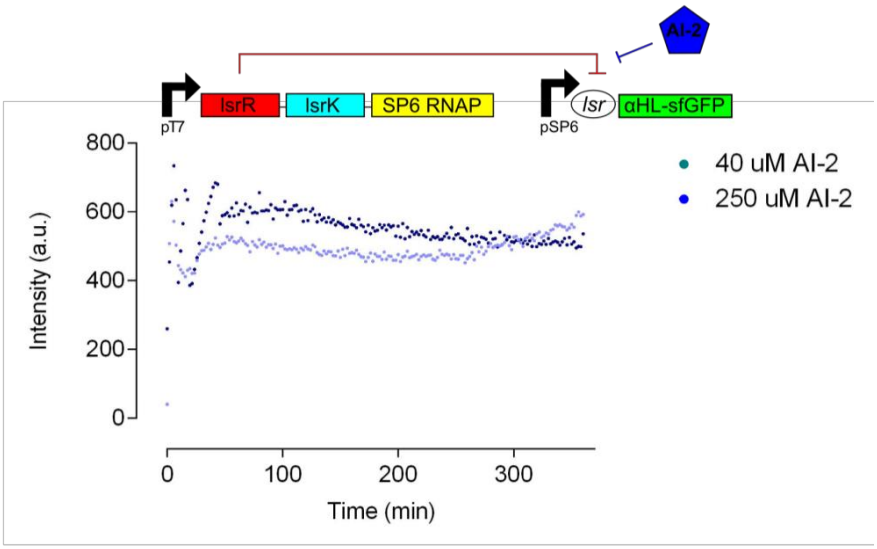


Fig. 4.6 Enzymatically produced AI-2 did not derepress the synthetic AI-2 sensory pathway.

The synthetic AI-2 sensory pathway was expressed with the PURE system at 37 °C. Kinetic experiments were monitored by fluorescence for 6 h (ex 485 nm, em 510 nm). 40 μM (blue circles) and 260 μM (light blue circles) were added at the beginning of the reactions. No derepression occurred in the presence of AI-2. A cartoon above the graph gives a schematic representation of the used genetic circuit. *lsr* indicates the intergenic region of the *lsr* operon. Data shown are from constructs RL028K and RL023K.

Neither cell-free culture fluids nor enzymatically produced AI-2 were able to restore gene expression. Taking in account that the synthetic AI-2 sensory pathway is made of different modules, each component was further investigated to ensure the functionality of each single part. LsrK is a kinase that should be constitutively expressed by T7 RNAP present in the cell-free transcription-translation reaction. LsrK should recognize and phosphorylate AI-2. Only phospho AI-2 can bind LsrR and disrupt the tetramer. To investigate LsrK functionality, the construct containing the LsrR, LsrK and SP6 RNAP was expressed with the PURE system for 6 h at 37 ºC. AI-2 was then added to the reaction and incubated for 1 h at 37 ºC. The reaction was then placed together with *V. harveyi* BB170 reporter. The reporter strain can sense AI-2 but not phospho AI-2 since phosphorylated AI-2 cannot cross the bacterial membrane. Enzymatically produced AI-2 and cell-free culture fluids from *E. coli* JM109 were used as positive controls, culture fluid from *E. coli* DH5α was used as a negative control. No AI-2 activity was observed when AI-2 was incubated with LsrK, meaning that the vast majority of the AI-2 was converted to phospho AI-2 by the kinase LsrK (Fig. 4.7).

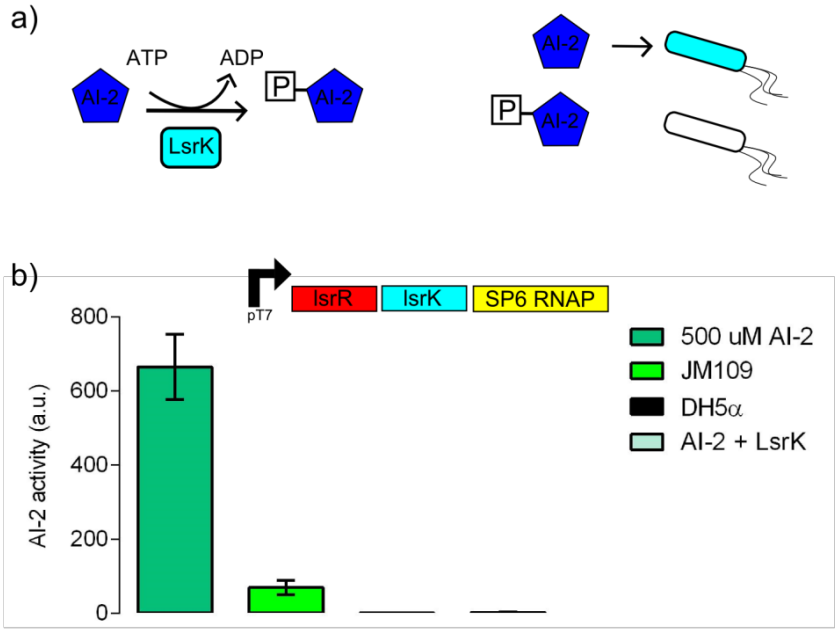


Fig. 4.7 LsrK phosphorylated AI-2 into phospho AI-2.

LsrK was expressed *in vitro* with the PURE system at 37 °C. a) A schematic representation of the experiment performed. LsrK phosphorylates AI-2 in phospho AI-2. *V. harveyi* can sense AI-2 and emits luminescence, but not phospho AI-2. b) AI-2 was added to the reaction and incubated for 1 h at 37 °C. The reaction was mixed together with *V. harveyi* BB170 reporter strain. Phospho AI-2 cannot cross *V. harveyi* membrane. No luminescence output was detected from the reporter strain (light blue bar). Enzymatically produced AI-2 (dark green bar) and *E. coli* JM109 cell-free culture fluid (green bar) were used as positive controls. *E. coli* DH5α cell-free culture fluid was used as negative control. A cartoon above the graph gives a schematic representation of the construct used (RL028K).

To confirm LsrK activity, thin layer chromatography (TLC) was performed. LsrK catalyzes the conversion of AI-2 into phospho AI-2 with the simultaneous conversion of ATP to ADP. Therefore, LsrK was expressed *in vitro* as previously described, y32P-ATP and AI-2 were added to the reaction and incubated for 6 h at 37 ºC. A few microliters were spotted onto a silica TLC plate and run in 4:2:2 butanol: water: acetic acid. After incubation with LsrK, ATP was no longer present in the reaction and a lower spot that did not migrate on the TLC plate appeared (Fig. 4.8). Based on previous studies performed by Xavier et al.[121] the non-migrating spot was identified as phospho AI-2. Both the TLC and the *V. harveyi* bioluminescence assays confirmed that LsrK is working properly under the *in vitro* conditions exploited.

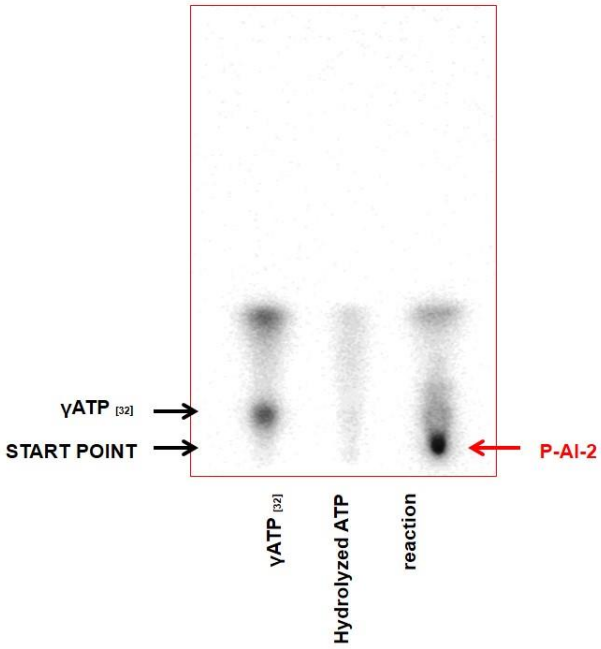


Fig. 4.8 TCL confirmed LsrK activity.

LsrK was expressed *in vitro* with the PURE system at 37 °C supplemented with 0.2 uCi [γ32P]-ATP and 0.8 mM of AI-2. Samples were run on a TLC plate with 4:2:2 butanol: water: acetic acid. The lower spot present in the third row corresponds to AI-2 that does not migrate under the conditions used here. [γ32P]-ATP and hydrolyzed ATP were added as controls. Data shown are from construct RL028K.

The second component of the *in vitro* AI-2 sensory pathway is the repressor LsrR. Previous analysis showed the absence of gene expression when LsrR is present but no release of repression upon the addiction of AI-2. To make sure that the absence of gene expression was due to LsrR repression and not to depletion of resources as a consequence of transcription-translation of LsrR itself, LsrR was mutated to inactivate the protein. It was previously shown that Y26H and Q33A mutations reduce LsrR DNA binding activity[129]. LsrR was mutated and tested *in vitro*. As expected, Y26H Q33A LsrR was not able to repress sfGFP tagged αHL expression, underlining that the absence of signal in the reconstituted system is due to the correct functionality of wild type (WT) LsrR (Fig. 4.9).

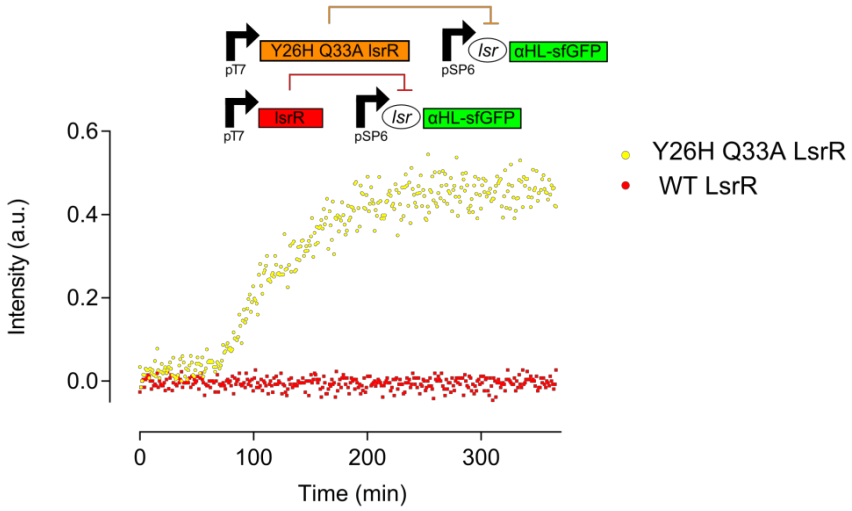


Fig. 4.9 Y26H Q33A LsrR was not able to repress gene expression.

Y26H Q33A LsrR was expressed *in vitro* with the PURE system at 37 °C together with sfGFP tagged αHL under the control of SP6 promoter and the *lsr* operon intergenic box. The LsrR variant was not able to bind the *lsr* intergenic region and repress gene expression (yellow circles). WT LsrR was added as control (red circles). Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). Cartoons above the graph give a schematic representation of the used genetic circuit. *lsr* indicates the intergenic region of the *lsr* operon. LsrR variant data shown are from constructs RL064K and RL023K, WT LsrR data are from and CD200A and RL023K.

A synthetic version of the *E. coli* AI-2 sensing pathway was implemented with a promoter cascade in order to introduce a delay in target gene expression and thus to avoid leaky expression. In the RL028K plasmid *lsrR, lsrK* and *SP6 RNAP* were expressed as a polycistronic operon. Gene position within a synthetic operon can influence the amount of protein produced by the system[68]. In particular, since the *SP6 RNAP* was placed in the last position, expression of SP6 RNAP was reduced in comparison to LsrR. Moreover, the PURE system was optimized for T7 RNAP expression, meaning that SP6 RNAP could potentially need other conditions to work in an efficient manner. To test SP6 RNAP efficiency, the *lsr* intergenic region was deleted to allow the expression of sfGFP tagged αHL also in the presence of LsrR. The construct, referred as RL042K, was expressed with the PURE system together with RL028K. The data confirmed that low protein yield was obtained with SP6 RNAP in the synthetic system (Fig. 4.10).

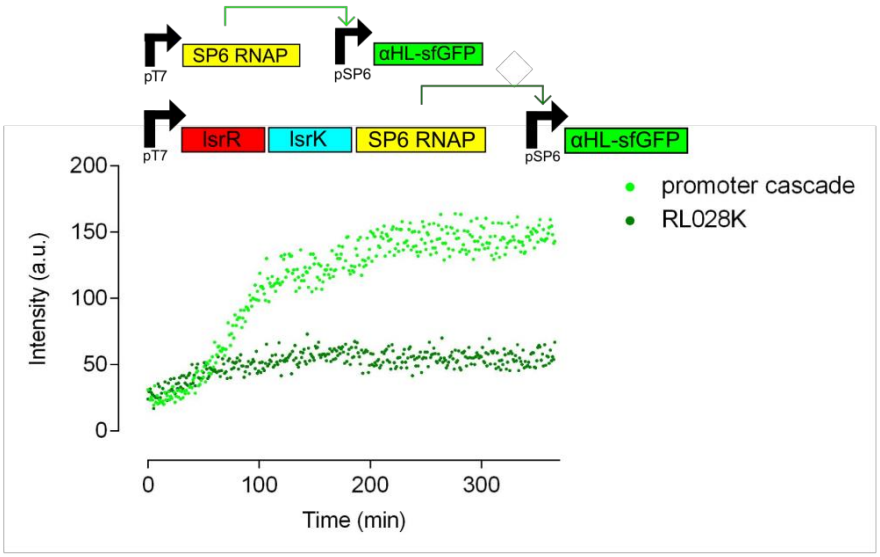


Fig. 4.10 Low protein expression is driven from SP6 RNAP.

SP6 RNAP placed in RL028K polycistronic operon was expressed with the PURE system at 37 °C. A plasmid encoded sfGFP tagged αHL from SP6 promoter without the Isr intergenic region was added. The T7-SP6 promoter cascade produced a very low amount of protein when SP6 RNAP is transcribed from the polycistronic operon (dark green circles, RL028K). The T7-SP6 RNAP promoter cascade in which SP6 RNAP is transcribed from a single gene construct was added as control (green circles). Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). Cartoons above the graph give a schematic representation of the used genetic circuits. Promoter cascade data shown are from constructs RL029A and RL042K, Data shown as RL028K are from RL028K and RL042K.

To improve expression from SP6 RNAP, a third plasmid (RL049A) containing a SP6 promoter behind the *SP6 RNAP* gene was inserted to create a positive loop and increase the SP6 RNAP concentration within the cell-free reaction. However, no difference was observed in the presence of the extra SP6 RNAP plasmid (Fig. 4.11).

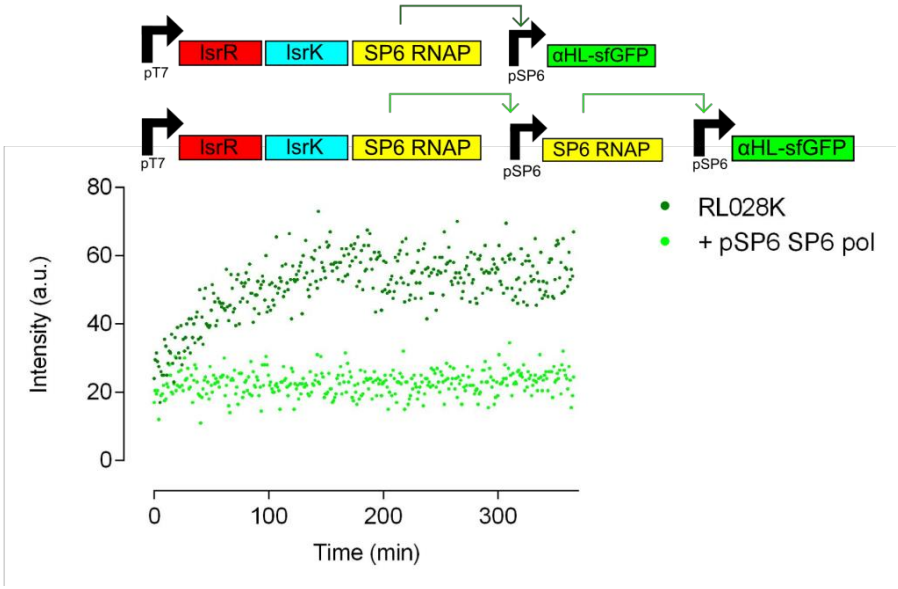


Fig. 4.11 SP6 RNAP positive loop did not increase protein yield.

RL049A containing SP6 RNAP under SP6 promoter control was expressed with the PURE system together with RL028K and a construct encoded sfGFP tagged αHL from SP6 promoter without the *lsr* operon intergenic region (dark green circles, RL028K). No increment of fluorescence output was shown when compared to T7-SP6 RNAP promoter cascade into the polycistronic operon (green circles, + pSP6 SP6 pol). Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). Cartoons above the graph give a schematic representation of the used genetic circuits. Data shown as RL028K are from RL028K and RL042K. Data shown as +pSP6 SP6 pol are from RL028K, RL042K and RL049A.

Better conditions could be necessary to improve the expression resulting from SP6 RNAP. Therefore, cell-free reactions were optimized to help both the polymerase and the transcription-translation machinery. Dithiothreitol (DTT) was shown to enhance RNA polymerase activity. Similarly, the availability of NTPs was shown to be an important determinant of the yield of RNA[130]. Upon the addition of 1 mM ATP or 1 mM fresh DTT, only a slightly increment in protein production was achieved in the transcription-translation reaction. While the addition of both NTPs and DTT completely shut down the expression (Fig. 4.12).

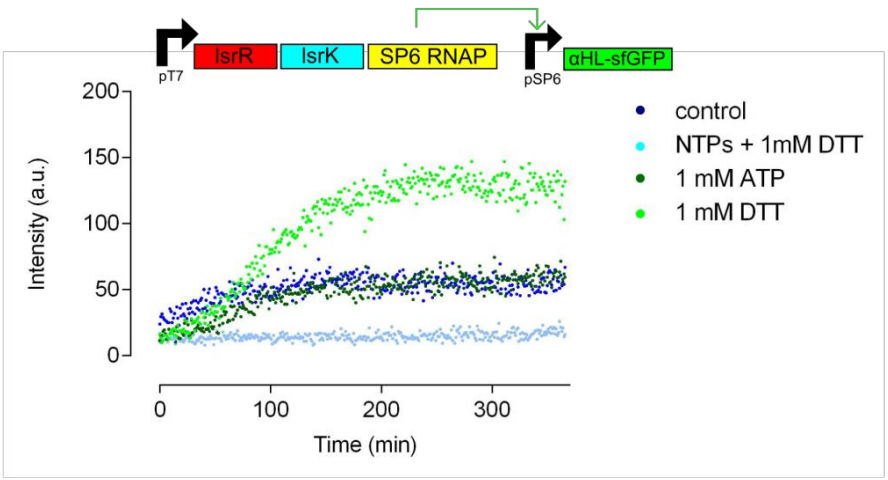


Fig. 4.12 DTT helped SP6 RNAP activity.

T7-SP6 RNAP promoter cascade in a polycistronic operon was expressed with the PURE system at 37 °C (blue circles, control). 1 mM of DTT was added to the reaction to enhance SP6 RNAP activity, which showed a higher protein production (green circles, 1mM DTT) when compared with the standard PURE system reaction (blue circles). No increment in fluorescence was shown when 1 mM ATP was added (dark green circles, 1 mM ATP) or both DTT and NTPs were added (light blue circles, NTPs + 1 mM DTT). Reactions were monitored for 6 h (ex 485 nm, em 510 nm). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from RL028K and RL042K.

The negative results obtained with the T7-SP6 cascade lead to the substitution of the SP6 RNAP with T3 RNAP to better improve the synthetic circuit. To construct a T7-T3 cascade, a plasmid in which a T7 promoter drives the expression of the T3 RNAP (RL053A) was built, while a T3 promoter was placed in front of sfGFP tagged αHL (RL054K) in a second construct. As previously described, a T7-T3 cascade was tested to assess its functionality *in vitro.* The new promoter cascade was functional, and T3 RNAP was more efficient than SP6 RNAP within the synthetic circuit (Fig. 4.13).

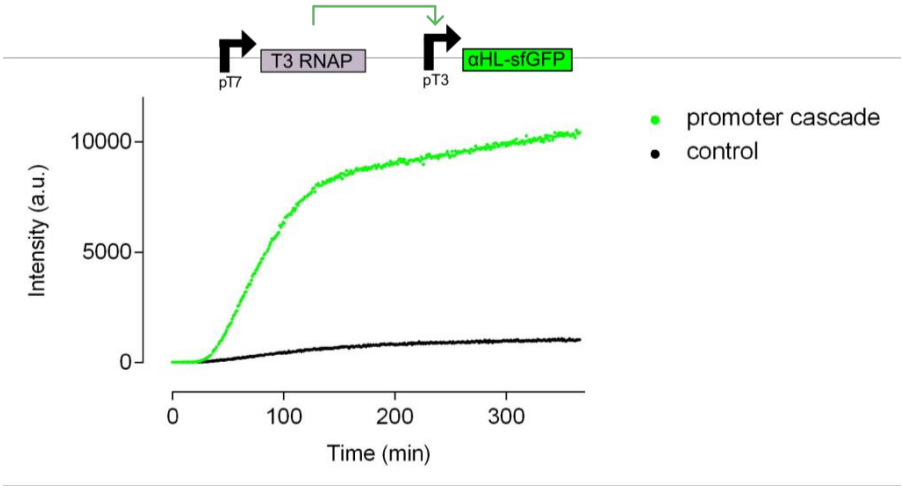


Fig. 4.13 T7-T3 RNAP cascade.

T7-T3 promoter cascade was expressed *in vitro* with the PURE system at 37 °C. Kinetic experiments were monitored by fluorimetry for 6 h (ex 485 nm, em 510 nm). T7 RNAP drives the expression of T3 RNAP, which in turn induces expression of sfGFP (green circles). To determine that T3 RNAP is necessary to express sfGFP, a control reaction without T3 RNAP was performed. No signal is shown when T3 RNAP is not added to into the cell-free reaction (black circles). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from RL053A and RL054K.

However, a lower unspecific expression was observed when synthesis was driven by T7 RNAP from T3 promoter, that is, T7 RNAP weakly recognizes the promoter of T3 RNAP. Next, the *SP6 RNAP* gene in the synthetic operon was substituted with *T3 RNAP* (RL060K), and the new circuit was tested in the presence and in the absence of AI-2. A construct in which the *lsr* intergenic region was absent was used as control. LsrR repressed transcription of αHL even if a low background expression is shown (Fig. 4.14); however, gene expression was not recovered upon the addition of AI-2. The substitution of the RNA polymerase within the reconstructed AI-2 sensory pathway did not help the synthetic system in sensing the presence of the molecule.

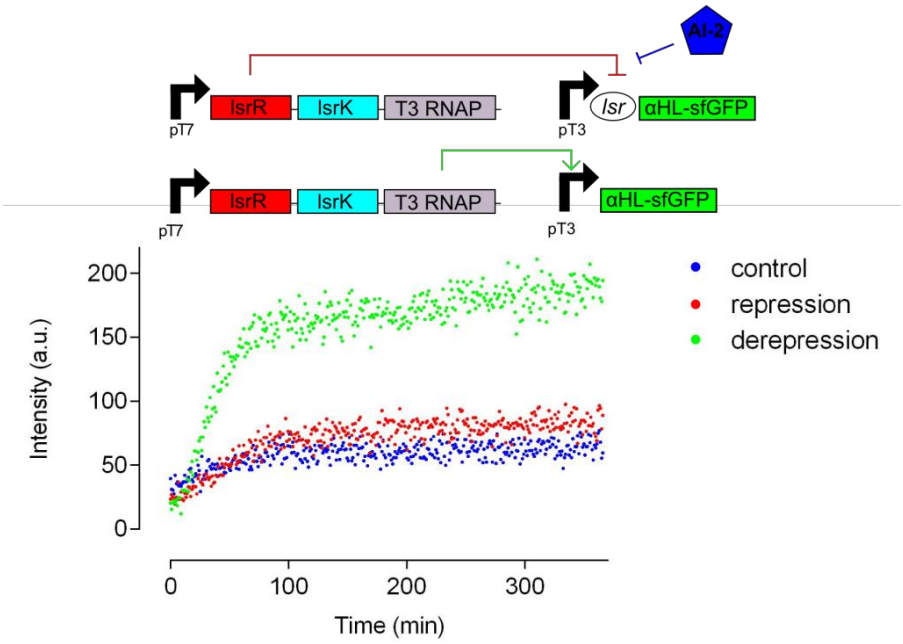


Fig. 4.14 AI-2 did not derepress the synthetic AI-2 sensory pathway constructed with T3 RNAP.

The synthetic AI-2 sensory pathway was expressed with the PURE system at 37 °C. The kinetic experiments were monitored by fluorimetry for 6 h (ex 485 nm, em 510 nm). The T7-T3 promoter cascade functionally produced sfGFP tagged αHL (green circles). When LsrR was present, LsrR repressed gene expression (red circles). However, when AI-2 was added to the synthetic AI-2 sensory pathway expressed with the PURE system, no derepression occurred (blue circles). Cartoons above the graph give a schematic representation of the used genetic circuits. *lsr* indicates the intergenic region of the *lsr* operon. Repression and derepression data shown are from constructs RL059K and RL060K, while cascade data are from RL054K and RL060K.

Another complication was represented by the high concentration of repressor produced from the synthetic circuit. It has been shown that the amount of protein can vary upon the presence of different start codon both *in vivo*[131] and in a cell-free transcription-translation system[61]. In the attempt to reduce the amount of LsrR protein present in the system, the ATG start codon was mutated to TTG in the resulting plasmid RL061K. However, the lower amount of LsrR did not facilitate the release of repression (Fig. 4.15).

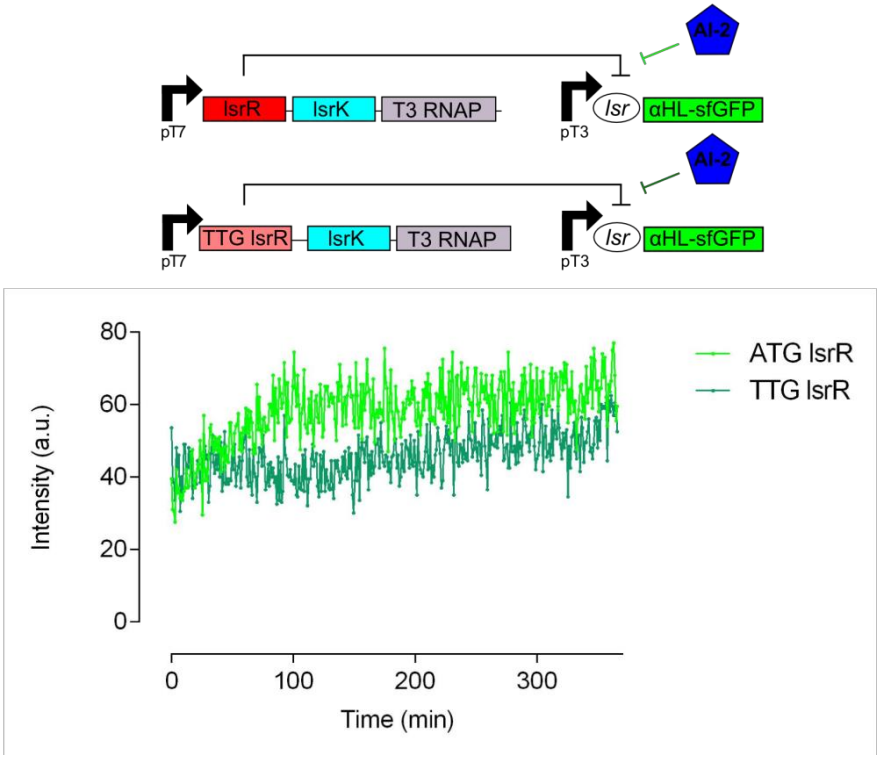


Fig. 4.15 A lower amount of LsrR did not allow derepression from AI-2.

A synthetic AI-2 sensory pathway was expressed with the PURE system at 37 °C. Reactions were monitored for 6 h (ex 485 nm, em 510 nm). The ATG start codon of *lsrR* was replaced by a TTG. AI-2 did not release repression even when a lower amount of the repressor was present (dark green circles, TTG lsrR). A derepression reaction with LsrR expressed from an ATG start codon containing gene was performed as control (green circles, ATG lsrR). Cartoons above the graph give a schematic representation of the used genetic circuits. *lsr* indicates the intergenic region of the *lsr* operon. ATG lsrR data shown are from constructs RL059K and RL060K, while TTG lsrR data are from RL059K and RL061K.

In *E. coli*, LsrR binds a 260 bp region upstream of the *lsr* operon. This region is composed of two divergent promoters, pLsrR and pLsrA. Different groups studied the minimum number of nucleotides necessary for LsrR binding,[85] and four putative binding sites were found. Inside this region, four sequences were found to be recognized by the repressor in its tetrameric form, and each promoter contained two of these identified sequences. In order to test whether a shorter intergenic region could help release the repression *in vitro,* the entire *lsr* intergenic region was substituted with shorter parts. Two different intergenic regions were then tested, named pLsrA (RL063K) and pLsrR (RL065K). Only pLsrR gave expression when tested with the T7-T3 promoter cascade. However, LsrR was no longer able to repress gene expression (Fig. 4.16).

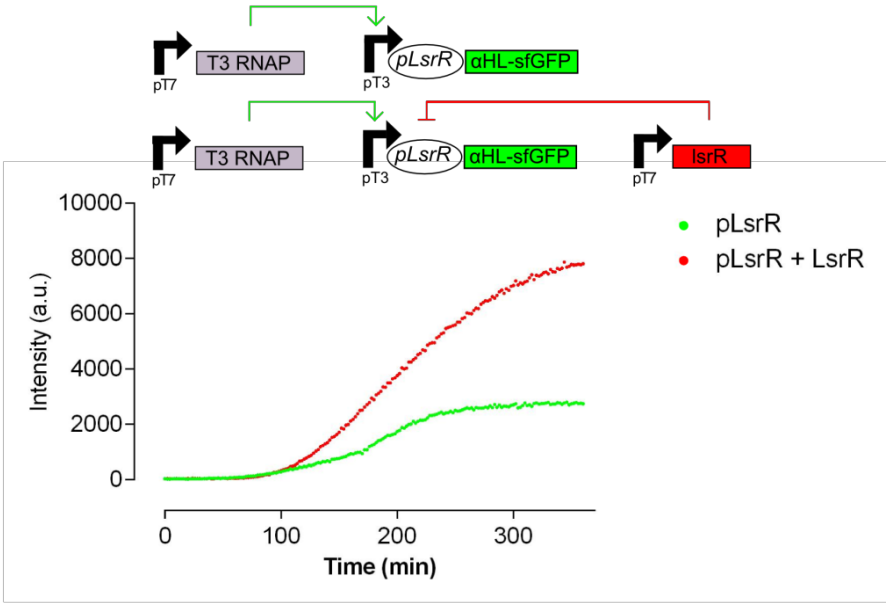


Fig. 4.16 LsrR did not bind pLsrR promoter and failed to repress gene expression.

A synthetic AI-2 sensory pathway was expressed with the PURE system at 37 °C. Protein expression occurred in the control reaction where pLsrR was placed between pT3 and sfGFP tagged αHL (green circles). LsrR was not able to bind pLsrR and repress gene expression (red circles). Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). Cartoons above the graph give a schematic representation of the used genetic circuits. *plsrR* indicates part of the intergenic region of the *lsr* operon. plsrR data shown are from constructs RL053A and RL065K, while pLsrR + LsrR data are from RL053A, RL065K and CD200K.

Recently a LsrR variant was obtained through directed evolution[132]. Enhanced LsrR (eLsrR) showed *in vivo* a higher repression activity and also lead to higher expression in the presence of AI-2. Thus, LsrR was mutated into eLsrR and tested in with the *in vitro* AI-2 sensory pathway. As expected, the eLsrR variant was able to bind to the pLsrR region and repress αHL expression; however, expression was not restored when AI-2 was added to the reaction (Fig. 4.17).

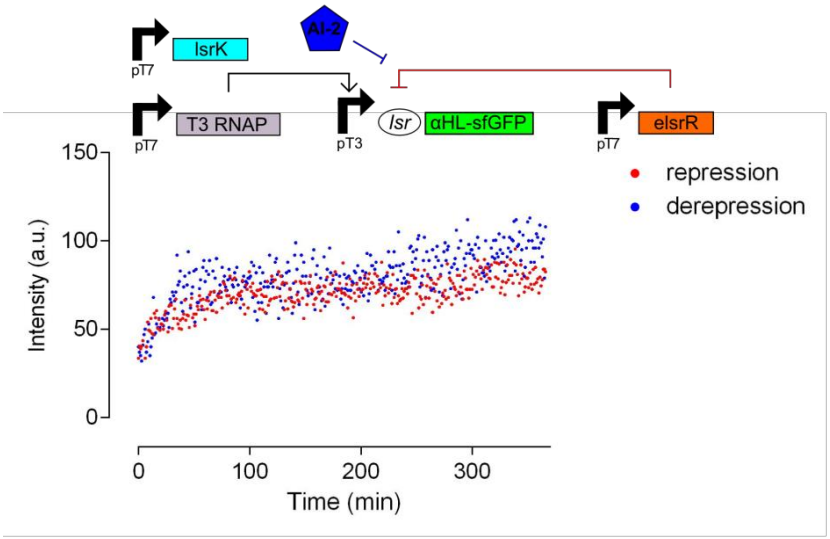


Fig. 4.17 AI-2 did not derepress the synthetic AI-2 sensory pathway made with eLsrR.

A synthetic AI-2 sensory pathway was expressed *in vitro* with the PURE system at 37 °C. Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). WT LsrR was replaced with eLsrR. eLsrR functionally bound the *lsr* intergenic region and repressed protein expression (red circles). When AI-2 was added, no derepression was observed (blue circles). Cartoons above the graph give a schematic representation of the used genetic circuits. *lsr* indicates the intergenic region of *lsr* operon. Data shown are from constructs RL053A. RL059K, RL068K, CD200K and CD201K.

In the previous genetic system tested, the *lsr* intergenic region was placed in a way that allowed expression from a pLsrA promoter. Since no expression was shown when only pLsrA was used within a cell-free reaction, this region was inverted, leading to a construct which was under the control of pLsrR. LsrR was not able anymore to tightly bind the region and partially failed to repress gene expression (Fig. 4.18).

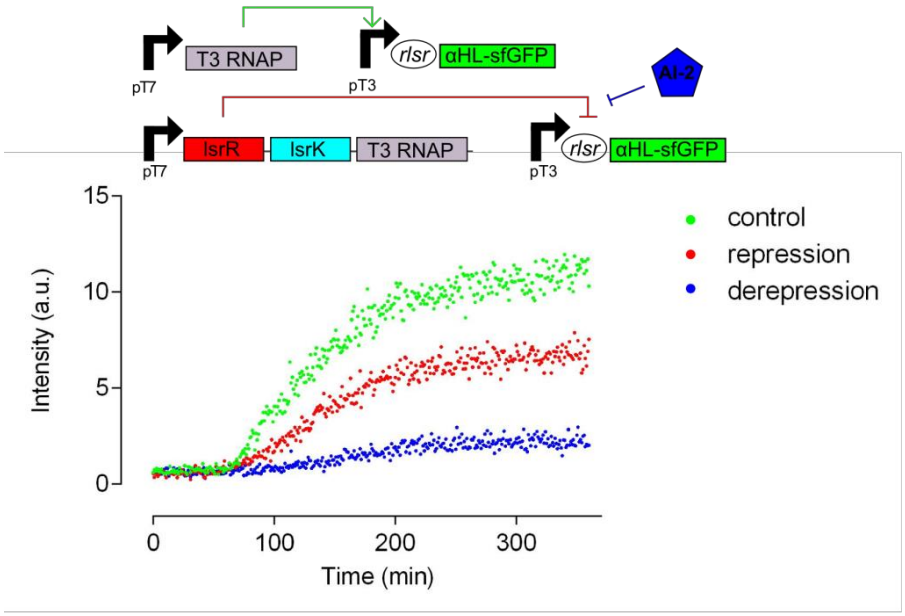


Fig. 4.18 Reverting *lsr* intergenic region did not lead to AI-2 derepression.

A synthetic AI-2 sensory pathway was expressed *in vitro* with the PURE system at 37 °C. Reactions were monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). The *lsr* operon intergenic region was reverted to allow expression from a pLsrR promoter. The T7-T3 promoter cascade functionally produced sfGFP tagged αHL (green circles). LsrR partially failed to repress gene expression (red circles). However, when AI-2 was added to the synthetic AI-2 sensory pathway, no derepression occurred (blue circles). Cartoons above the graph give a schematic representation of the used genetic circuits. *rlsr* indicates the inverted intergenic region of the *lsr* operon. Repression and derepression data shown are from constructs RL060K and RL070K, while cascade data are from RL053K and RL070K.

Since two binding sites for cAMP receptor protein (CRP) were previously identified within the *lsr* intergenic region,[119] we explored the use of CRP in our genetic circuit. Mutations in both CPR binding sites were previously shown to drastically reduce expression from both pLsrR and plsrA. The CRP protein seems to be fundamental for the correct functionality of the AI-2 sensory pathway. Thus, a plasmid encoded CRP behind a T7 promoter (RL076A) was inserted into the synthetic circuit. 1 mM of cAMP was added and the reactions were monitored at 37 ºC for 6 h. Finally, upon the addition of AI-2, a slight elevation of αHL expression was observed (Fig. 4.19). Later experiments described in this chapter lead to the conclusion that the cell-free transcription-translation machinery of the PURE system was not compatible with a functional genetic circuit that was responsive to AI-2. The synthetic AI-2 sensory pathway was thus tested with the S30 T7 High-Yield Protein Expression System.

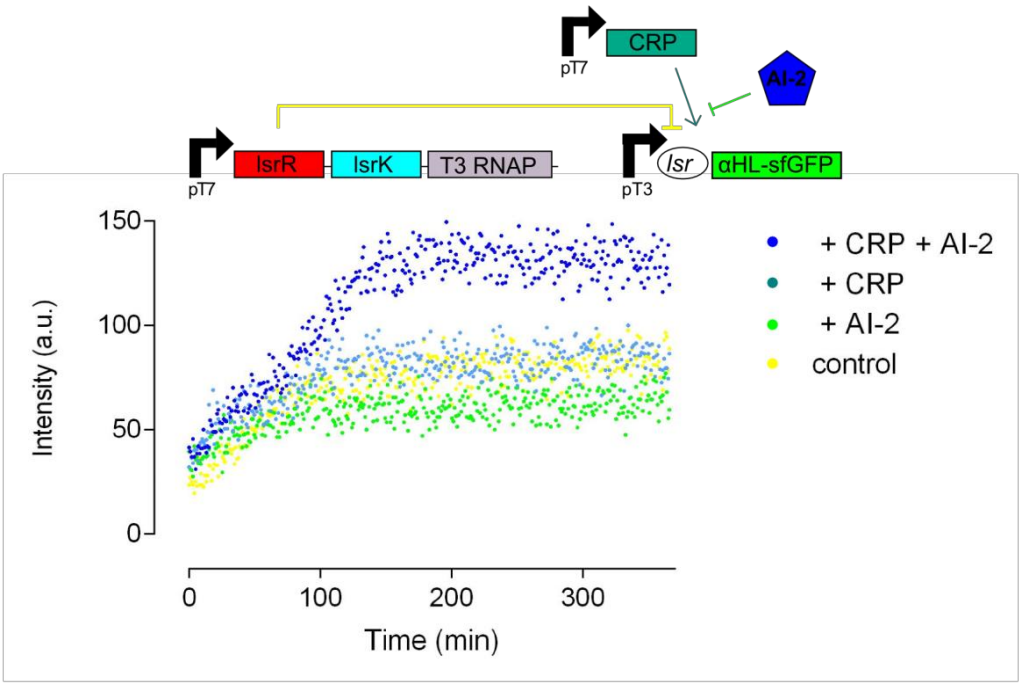


Fig. 4.19 CRP facilitated AI-2 induced derepression.

The synthetic AI-2 sensory pathway was expressed *in vitro* with the PURE system at 37 °C. Reactions were monitored for 6 h (ex 485 nm, em 510 nm). CRP and cAMP were added to the cell-free reaction. When LsrR was present, LsrR repressed gene expression (yellow circles, control). When AI-2 was added to the synthetic AI-2 sensory pathway in the absence of CRP, no expression was observed (green circles, AI-2). CRP did not derepress gene expression when present alone (light blue, CRP). When AI-2 was added to the synthetic AI-2 sensory pathway in the presence of CRP, low level derepression was observed (blue circles, CRP + AI-2). A cartoon above the graph gives a schematic representation of the used genetic circuit. *lsr* indicates the intergenic region of the *lsr* operon. Data shown are from constructs RL059K and RL060K, RL076A contains CRP.

The presence of the CRP protein helped the synthetic AI-2 sensory pathway in responding to AI-2; however, the expression obtained was low. The CRP protein is known to facilitate the binding of *E coli* RNA polymerase (*E. coli* RNAP) to the promoter [133]. Thus, another cell-free transcription-translation machinery containing *E coli* RNAP was tested. The S30 T7 High-Yield Protein Expression System consists of an *E. coli* cell-free extract containing both *E. coli* RNAP and T7 RNAP. The synthetic AI-2 sensory pathway was tested with this new cell-free system to investigate if the presence of *E coli* RNAP together with cAMP-CRP could lead to higher protein production in response to AI-2. However, when the QS molecule was added to the reaction, no protein expression was observed with the S30 T7 High-Yield Protein Expression System (Fig. 4.20).

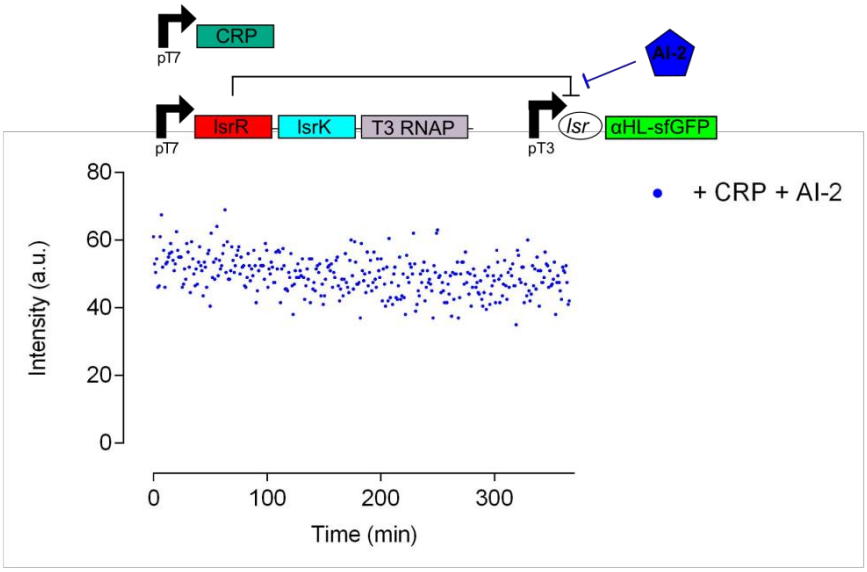


Fig. 4.20 Synthetic AI-2 sensory pathway did not work in a S30 *E. coli* extract.

The synthetic AI-2 sensory pathway was expressed *in vitro* with the S30 T7 high yield protein expression system at 37 °C with cAMP-CRP. When AI-2 was added, no derepression was observed (blue circles). Reaction was monitored for 6 h by fluorimetry (ex 485 nm, em 510 nm). A cartoon above the graph gives a schematic representation of the used genetic circuit. *lsr* indicates the intergenic region of the *lsr* operon. Data shown are from constructs RL059K and RL060K, RL076A contains CRP.

4.4.2 AI-2 artificial sender cells

E. coli synthesizes its own QS molecule through LuxS synthase. The pathway consists of three enzymes. First, a methyltransferase transforms SAM (S-adenosylmethionine) into SAH (S-adenosylhomocysteine), a toxic bioproduct which is rapidly converted to SRH (S-ribosylhomocysteine) by the enzyme Pfs. SRH is then used as a substrate by LuxS to obtain AI-2 and homocysteine in a 1:1 ratio. It has been shown that purified HLPT is able to produce *in vitro* AI-2 starting from the SAH precursor. To reconstitute in a cell-free system the synthesis of AI-2, HLPT was genetically encoded in JF006A in which a T7 promoter drives the expression of the fusion protein. HLPT was expressed with the PURE system for 6 h at 37 °C together with 0.5 mM of SAH. The reaction was then added to the *V. harveyi* BB170 reporter strain to monitor the production of AI-2. No response from the reporter strain was observed when the PURE system was used as the transcription-translation system (Fig. 4.21). Therefore, the HLPT plasmid was next expressed with the S30 T7 High-Yield Protein Expression System. After 6 h of reaction together with 0.5 mM of SAH, a bioluminescence signal was detected from *V. harveyi*, showing the production of AI-2 within the reaction.

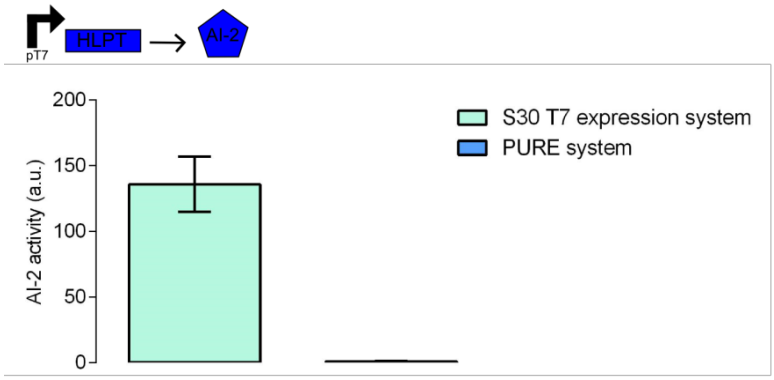


Fig. 4.21 AI-2 was produced *in vitro* with the S30 T7 high yield protein expression system.

JF006A contained HLPT under pT7 was expressed *in vitro* at 37°C with SAH precursor. Product reactions were mixed together with *V. harveyi* BB170 reporter strain. When expressed with the PURE system, no AI-2 was synthesized (blue bar). AI-2 was produced with the S30 T7 high yield protein expression system (light blue bar). A cartoon above the graph gives a schematic representation of the used construct.

AI-2 does not freely cross the membrane of *E. coli*, but AI-2 is internalized through a specific transport system encode by *lsrACDB*. However, previous studies showed a slow permeability of AI-2 to phospholipid membranes which could be enhanced by the addition of the pore forming protein αHL[56]. Thus the permeability of AI-2 to phospholipid vesicles was tested. Purified HLPT was encapsulated inside of phospholipid vesicles made by different lipid compositions together with SAH precursor. After vesicles formation, reactions were left at 37 °C for 6 h. Protease K was added to the outside of the vesicles to avoid the external production of AI-2. Vesicles were then added to *V. harveyi* BB170 reporter strain to measure the presence of AI-2 released outside of the vesicles. Both 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1:1 POPC: cholesterol vesicles were permeable to AI-2 produced inside of the lipid compartments (Fig. 4.22).

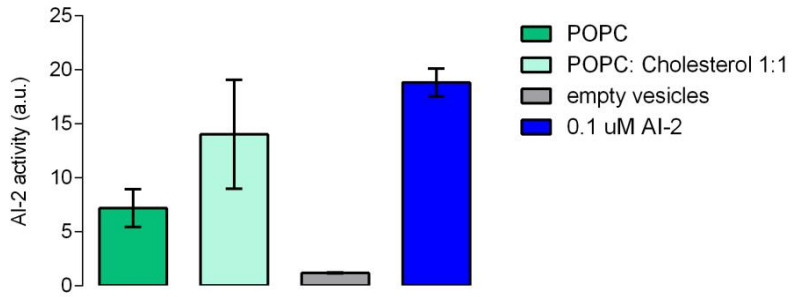


Fig. 4.22 Phospholipid membranes were permeable to AI-2.

AI-2 was enzymatically produced inside different vesicles compositions. Samples were mixed together with *V. harveyi* BB170 reporter strain and luminescence output was monitored. Both POPC and POPC: cholesterol vesicles were permeable to AI-2 (green and light blue bars, respectively). AI-2 was added as a positive control. Empty vesicles were used as a negative control.

Next, genetically encoded HLPT was expressed inside of phospholipid vesicles. 1:1 POPC cholesterol lyophilized vesicles were hydrated with S30 T7 high yield expression system containing DNA and SAH precursor. After 6 h of incubation at 37 ºC, the samples were mixed with *V. harveyi BB170* reporter strain and luminescence was monitored. Artificial cells failed to elicit a response in the reporter strain, consistent with no AI-2 production after 6 h (Fig. 4.23).

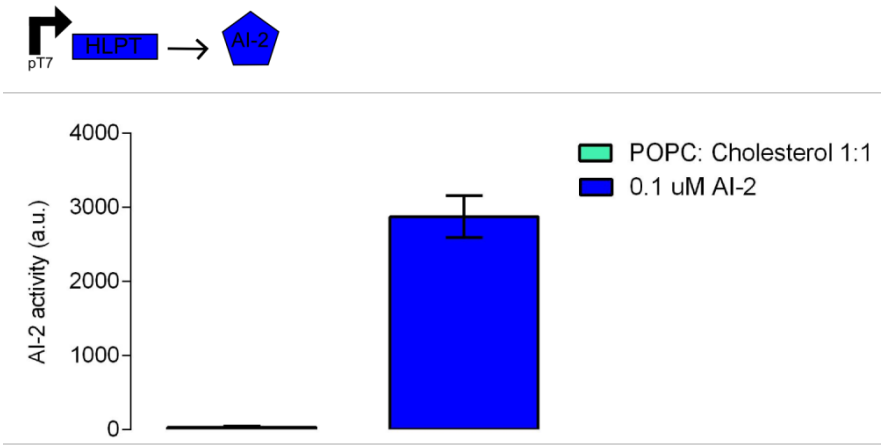


Fig. 4.23 No AI-2 was produced in 1:1 POPC: cholesterol vesicles.

JF006A was encapsulated together with in S30 T7 high yield protein expression system at 37 °C. After 6 h, artificial cells were added to *V. harveyi* BB170 reporter strain and luminescence output was monitored. No AI-2 is produced in the artificial cells (light blue bar). Enzymatically produced AI-2 was added as positive control (blue bar). A cartoon above the graph gives a schematic representation of the used construct within vesicles.

Therefore another composition was evaluated to assess whether another compartment could facilitate AI-2 synthesis and release. 1:2 POPC: cholesterol vesicles were hydrated with HLPT plasmid, S30 T7 high yield expression system and SAH precursor. Samples were left at 37 °C for 6 h. To determine the permeability properties associated with the lower concentrations of AI-2 produced from cell-free expression, the vesicles were either untreated or treated with the addition of Triton X-100, sonication, or purified αHL that was added from the outside of the vesicles after 6 h incubation. Samples were then added to the reporter strain and luminescence output was measured. Artificial cells made of 1:2 POPC: cholesterol allowed AI-2 production and release to the external environment (Fig. 4.24).

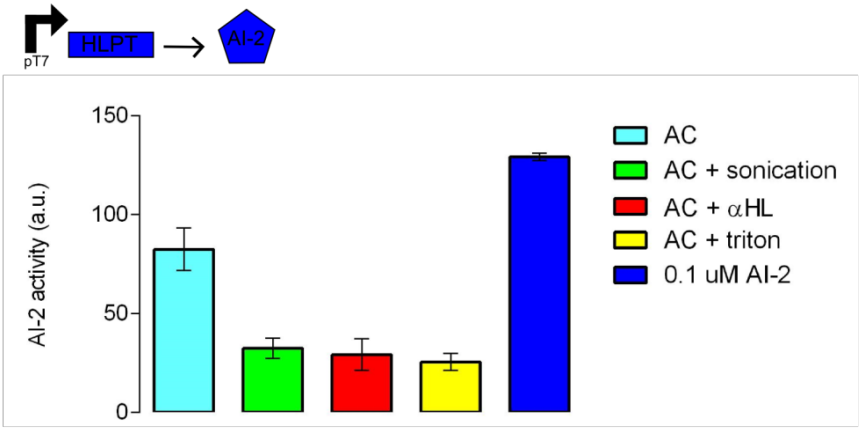


Fig. 4.24 AI-2 is produced in 1:2 POPC: cholesterol vesicles.

JF006A was encapsulated together with the S30 T7 high yield protein expression system at 37 °C. After 6 h, artificial cells were added to *V. harveyi* BB170 reporter strain and luminescence was monitored. 1:2 POPC: cholesterol artificial cells produced AI-2, which was released to the outside (light blue bar). No differences are shown in sample treated after 6 h incubation with sonication (green bar), αHL (red bar), Triton X- 100 (yellow bar). Enzymatically produced AI-2 was added as positive control (blue bar). A cartoon above the graph gives a schematic representation of the used construct within vesicles.

No improvements were shown in samples treated with αHL, sonication or Triton X-100. The results obtained showed that AI-2 is produced by the artificial cells and can freely cross the phospholipid membrane.

4.4.3 *In vitro* reconstruction of LuxR sensory QS

After the construction *in vitro* of the *E. coli* AI-2 QS pathway, the LuxI-LuxR QS pathway used by *V. fischeri* and *P. aeruginosa* were built *in vitro*. The LuxI-LuxR QS system is an intraspecies communication module exploited by a large number of Gram-negative bacteria. Various LuxI-LuxR homologous are found in bacteria that are able to recognize specific AHLs molecules. *P. aeruginosa* is characterized by two LuxI-LuxR QS systems, which act together to coordinate QS process. The first QS system comprises the receptor LasR and the synthase LasI. LasI synthesizes N-(3-oxododecanoyl)-l- homoserine lactone (3OC12 HSL), a quorum molecule that is recognized by LasR. When 3OC12 HSL binds its receptor, the complex stabilizes LasR, binds a specific DNA binding site, named the *las* box, and allows transcription from a specific promoter. The second QS system behaves in the same manner and involves RhlR and RhlI.

In order to build *in vitro* a 3OC12 HSL sensing device, *lasR* was placed downstream of an *E. coli* promoter derived from a synthetic promoter library, named J23100. Whereas GFP mut3b was cloned in order to be transcribe from pLasRL promoter to monitor the functionality of the

system. pLasRL promoter is a 3OC12 HSL responsive promoter which drives *in vivo* the expression of the elastase gene in *P. aeruginosa*. The construct, RL073C, was adapted from a previous study by Freemont et al.[49] that showed the *in vitro* reconstruction of a functional 3OC12 HSL sensing system. LasR is constitutively expressed from J23100, only in the presence of 3OC12 HSL the complex LasR-3OC12 HSL binds pLasRL and activates expression of GFP mut3b. The sensing device was expressed in S30 T7 high yield expression system, in the presence and in the absence of 3OC12 HSL. After 6 h at 37 ºC no expression was observed in the system when 3OC12 HSL was added (Fig. 4.25).

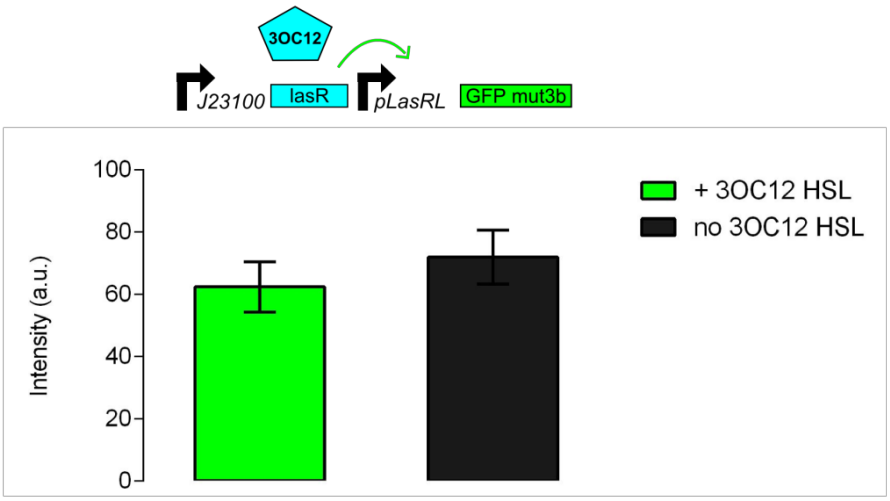


Fig. 4.25 3OC12 HSL did not activate protein expression.

Fluorescent intensities after 6 h of expression of RL073C *in vitro* with the S30 T7 high yield protein expression system at 37 °C. Fluorescence was monitored with ex 501 nm and em 511 nm. When 10 µM of 3OC12 HSL no signal is shown from the reaction (green bar) compared to the negative control (black bar). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct RL073C.

The LasRL promoter comprises the complete region upstream of the elastase gene. The shorter version, named pLasB, is known to functionally respond to 3OC12 HSL *in vivo*. Thus pLasRL was substituted with pLasB within the genetic construct (K575024) and tested *in vitro.* Again, the shorter responsive promoter failed to drive GFP expression when 3OC12 HSL was present (Fig. 4.26).

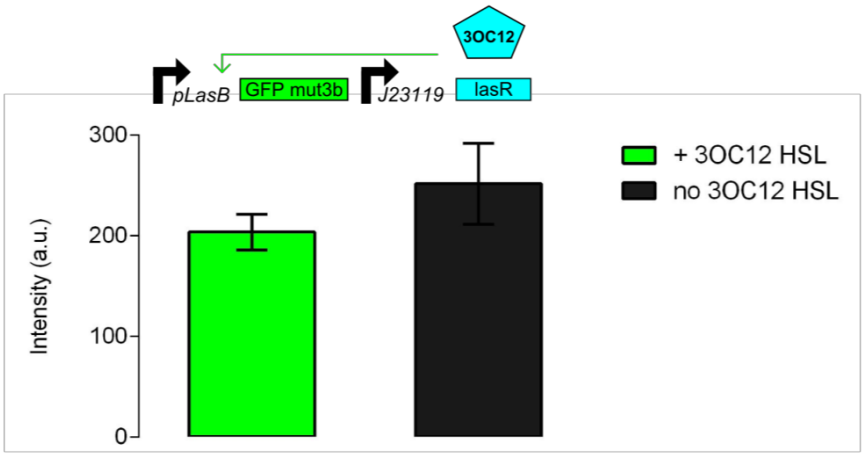


Fig. 4.26 3OC12 HSL did not activate protein expression from pLasB responsive promoter.

Fluorescent intensities after 6 h of expression of the 3OC12 HSL sensing part *in vitro* with a S30 T7 high yield protein expression system at 37 °C (ex 501 nm, em 511 nm). When 10 µM of 3OC12 HSL was added, no signal was present (green bar) compared to the negative control (black bar). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct K575024.

Data collected in our lab showed a low expression yield derived from J23100 promoter library, thus the synthetic promoter was replaced in both constructs with a tet promoter to assess whether the lack of protein production in the synthetic 3OC12 HSL sensing system was due to a low expression of LasR. RL083C contains *lasR* behind a tet promoter and GFP mut3b controlled by pLasRL. RL084C comprises *lasR* behind the tet promoter and GFP mut3b controlled by pLasB. Both genetic devices were expressed with the S30 T7 high yield expression system in the presence and in the absence of 3OC12 HSL. After 6 h no differences in GFP signal were recorded upon the addition of 3OC12 HLS (Fig. 4.27).

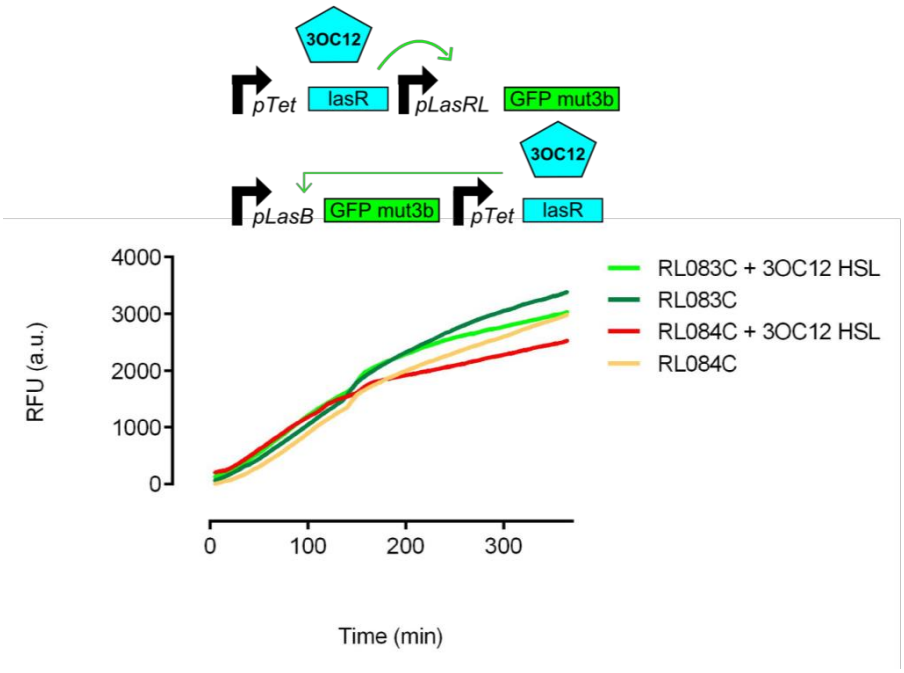


Fig. 4.27 3OC12 HSL did not activate protein expression when the tet promoter constitutively drove LasR expression.

RL083C and RL084C were expressed *in vitro* with the S30 T7 high yield protein expression system at 37 °C. Kinetic experiments were monitored at CFX 96 Real-Time PCR machine for 6 h using SYBR green filter. No differences were observed in the presence and in the absence of 3OC12 HSL in both constructs. A cartoon above the graph gives a schematic representation of the used genetic circuits.

Previous studies showed the ability of the 3OC12 HSL-LasR complex to recognize and bind *in vivo* to the *lux* box region contained in the promoter of LuxR (pLuxR), similar to that seen for *V. fischeri* LuxI-LuxR QS[134]. Since it has been demonstrated that *E. coli* RNAP and pLuxR genetic elements are compatible in the transcription of downstream genes[135], the pLasRL was substituted with pLuxR in the synthetic 3OC12 HSL sensing device. NY009A contains LasR constitutively expressed by tet promoter and pLuxR behind GFP mut3b. However, the new genetic construct failed to sense 3OC12 HSL and no expression was detected upon the addition of the molecule (Fig. 4.28).

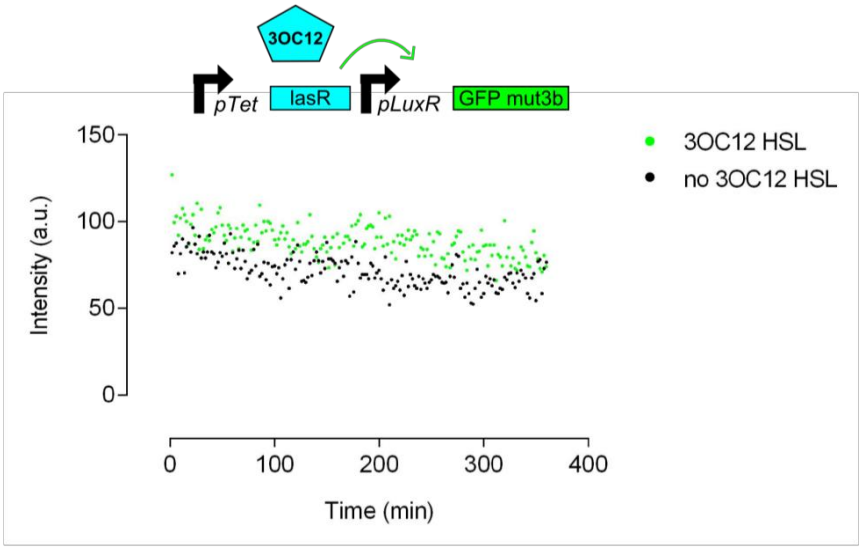


Fig. 4.28 3OC12 HSL did not activate protein expression from a pLuxR responsive promoter.

NY009A was expressed *in vitro* with the S30 T7 high yield protein expression system at 37 °C. Kinetic experiments were monitored by fluorimetry for 6 h (ex 501 nm, em 511 nm). No protein expression was observed when 10 μM of 3OC12 HSL was added to the *in vitro* reaction (green circles). A negative control in the absence of 3OC12 HSL was performed (black circles). A cartoon above the graph gives a schematic representation of the used genetic circuit.

Even if various promoters were tested, no success was obtained in the reconstruction of 3OC12 HSL sensing device *in vitro.* However *P. aeruginosa* also contains a second quorum pathway made of RhlI and RhlR that responds to C4 HSL. *rhlR* was cloned downstream of the *E. coli* promoter J23119 while pRhlAB was used as a C4 HSL responsive promoter to control GFP mut3b expression. In *P. aeruginosa*, pRhlAB drives the expression of rhamnolipid synthesis genes and is activated by the C4 HSL-RhlR complex[136]. The synthetic C4 HSL sensing device (K575037) was expressed with a S30-T7 system in the presence and in the absence of the QS molecule. After 6 h at 37 ºC, GFP mut3b was produced at low levels both in the presence and in absence of C4 HSL, showing the lack of control of RhlR over transcription from pRhlAB (Fig. 4.29).

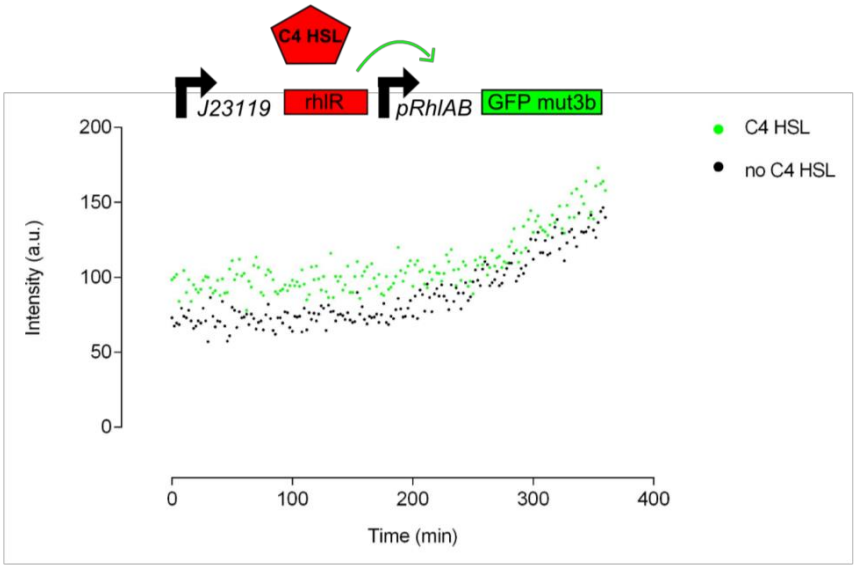


Fig. 4.29 C4 HSL did not activate protein expression from the pRhlAB responsive promoter.

The synthetic C4 HSL sensing device was expressed with a S30 T7 high yield system in the presence and in the absence of 10 μM C4 HSL. No protein expression was observed when 10 μM of C4 HSL was added to the *in vitro* reaction (green circles) compared with negative control (black circles). Kinetic experiments were monitored by fluorimetry for 6 h (ex 501 nm, em 511 nm). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct K575037.

4.4.4 AHL artificial sender cells

Although the *in vitro* reconstituted *P. aeruginosa* sensing systems failed to sense 3OC12 HSL and C4 HSL, attempts were made to construct artificial cells able to synthesize and send AHLs. LuxI-LuxR QS comprises a synthase which produces AHL molecules starting from a central metabolite SAM. The synthase interacts with a specific acyl carrier protein (ACP) loaded with the acyl chain of the specific length needed to synthetize the corresponding QS molecule. For example, SAM binds RhlI followed by butanoyl-ACP, an amide bond is formed between SAM and the acyl group, and both apo ACP and C4 HSL are released. In the same manner, LasI synthesizes 3OC12 HSL from SAM and acylated ACP.

To produce *in vitro* 3OC12 HSL, a plasmid containing *lasI* under the control of a T7 promoter (MC001A) was expressed with a S30 T7 high yield expression reaction containing 0.5 mM of SAM. To monitor the production of the AHL, a 3OC12 HSL *E. coli* reporter strain carrying the K575024 plasmid was built. The reporter strain was demonstrated to respond to 3OC12 HSL expressing GFP mut3b. After 6 h at 37 °C, the *in vitro* 3OC12 HSL synthesis reaction was added to the 3OC12 HSL reporter strain. The cells were incubated at 37 °C and every hour a few microliters were diluted in PBS and analyzed by flow cytometry. In the same manner a plasmid, named MC003A, containing *rhlI* synthase behind a T7 promoter was expressed with the S30 T7

high yield reaction to produce C4 HSL molecules *in vitro*, starting from SAM. To monitor the presence of AHL, a C4 HSL *E. coli* reporter strain was engineered using the K575037 plasmid. The reporter cells express GFP mut3b only in the presence of C4 HSL. After 6 h of incubation of the reactions together with *E. coli* reporter strains, no green cells were found (Fig. 4.30). Both synthetic sending devices failed to produce 3OC12 HSL and C4 HSL.

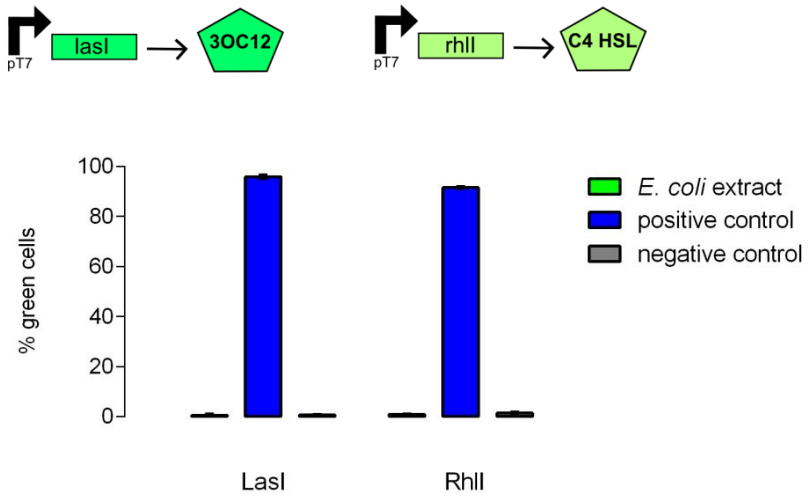


Fig. 4.30 AHLs sending devices failed to produce QS molecules *in vitro*.

LasI and RhlI were expressed *in vitro* with a S30 T7 high yield protein expression system at 37 °C. Reactions were mixed together with the corresponding AHL *E. coli* reporter strain and GFP expression was monitored by flow cytometry. No green positive cells were counted (green bars). Positive controls were performed by adding 0.1 μM of the correspondent AHL to *E. coli* (blue bars). No AHLs were added in the negative controls (grey bars). Cartoons above the graph give a schematic representation of the used genetic constructs. Data shown are from MC001A and MC003A.

To check whether the genetically encoded devices for 3OC12 HSL and C4 HSL were at least able to produce the AHLs molecules *in vivo*, *E. coli* Bl21(DE3) were transformed with MC001A and MC003A. Cells were grown in LB until OD_{600} 0.5 and harvested. The supernatant was then mixed with AHL *E. coli* reporter strains and incubated at 37 ºC. Both 3OC12 HSL and C4 HSL were successfully produced *in vivo*, with 40% and 45% of green positive cells counted through flow cytometry, respectively (Fig. 4.31).

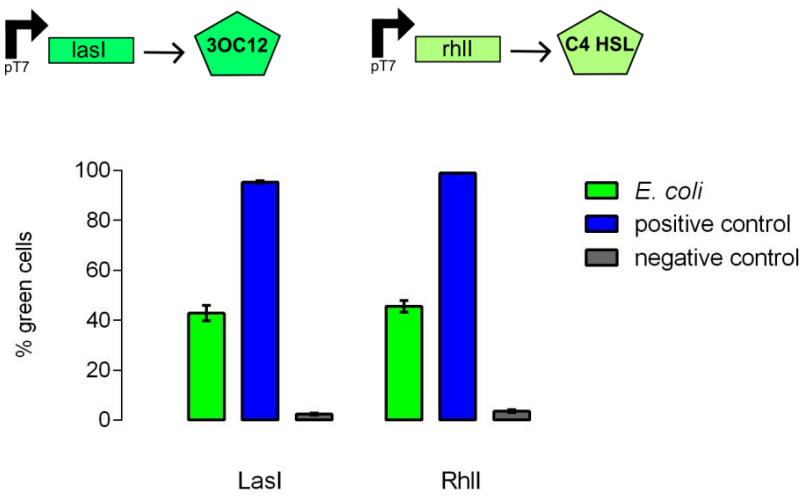


Fig. 4.31 AHLs sending device were functional *in vivo*.

LasI and RhlI were expressed in *E. coli* Bl21(DE3) . Cell-free culture fluids were added to the correspondent AHL *E. coli* reporter strain and GFP expression was monitored by flow cytometry. 40% green events were counted for both *E. coli* expressing cells. Positive controls were performed by adding 0.1 μM of the correspondent AHL to the *E. coli* cells (blue bars). No AHLs were added in the negative controls (grey bars). Cartoons above the graph give a schematic representation of the used genetic constructs. Data shown are from MC001A and MC003A.

Since the syntheses of AHLs was fully functional *in vivo*, it is possible that the cell-free *E. coli* extract used in the *in vitro* reaction lacked some of the components necessary to produce the AHLs. Previous studies showed that the *in vitro* synthesis of C4-HSL molecules from purified RhlI and the appropriately charged coenzyme A derivative, n-butanoyl-CoA[137]. Thus, the *in vitro* production of QS molecules was tested with the addition of acetyl-CoA as precursor. Reactions were performed as previously described, except for the addition of 100 μM acetyl-CoA. After 6 h at 37 ºC, reactions were collected and added to the *E. coli* reporter strains. After overnight incubation at 37 ºC, the *E. coli* reporter strains were analyzed by flow cytometry. The 3OC12 HSL reporter strain responded to 3OC12 HSL produced *in vitro* with a percentage of 50% of green positive cells, while C4 HSL reporter strain showed 87 % of green events (Fig. 4.32). Thanks to the presence of acetyl-CoA, both RhlI and LasI synthases were able to produce C4-HSL and 3OC12-HSL, respectively.

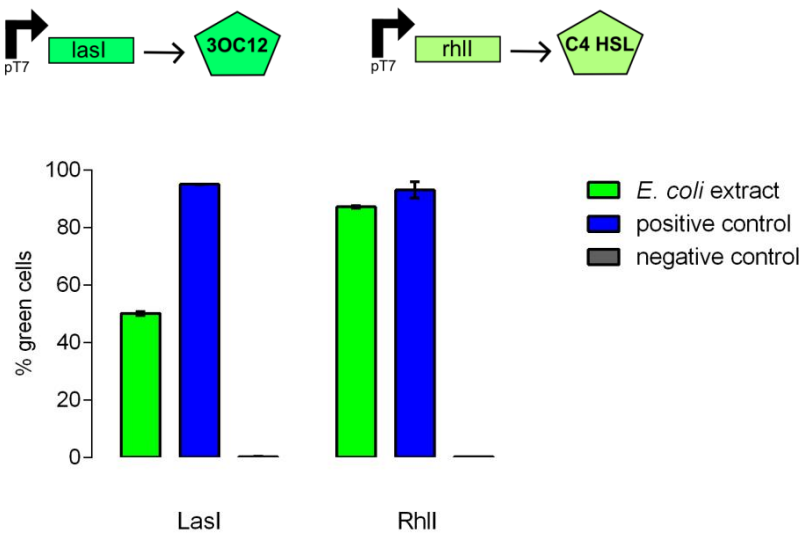


Fig. 4.32 AHLs sending devices produced QS molecules *in vitro* when acetyl-CoA was added.

LasI and RhlI were expressed *in vitro* with the S30 T7 high yield protein expression system at 37 °C together with 100 μM of acetyl-CoA. Reactions were mixed together with the correspondent AHL *E. coli* reporter strain, and GFP expression was monitored by flow cytometry. A percentage of 50% and 90% of green positive cells were counted (green bars) for LasI and RhlI expressing reactions, respectively. Positive controls were performed by adding 0.1 μM of the correspondent AHL to *E. coli* cells (blue bars). No AHLs were added in the negative controls (grey bars). Cartoons above the graph give a schematic representation of the used genetic constructs. Data shown are from MC001A and MC003A.

In order to build artificial cells able to synthesize and send AHL molecules, the next step was to encapsulate the synthetic sender devices within phospholipid vesicles. Relying on previous results obtained with the AI-2 sender device, 1:2 POPC-cholesterol vesicles were rehydrated with T7 S30 *E. coli* cell extract supplemented with 300 μM of acetyl-CoA, 1 mM of SAM and 2 μg of MC001A or MC003A to produce C4 HSL or 3OC12 HSL, respectively. Protease K was added to the outside of the formed vesicles to avoid unwanted protein production. Vesicles were mixed with AHL reporter strains at 37 ºC to allow the expression of the synthases, the production of the QS molecules and the resulting response of *E. coli* cells. Aliquots were analyzed by flow cytometry at various time points. Artificial cells were able to synthesize and send 3OC12 HSL, as shown in figure 4.33. 35% of green positive cells were recorded after 6 h of incubation. Instead, no signal arose from the C4 HSL reporter strain.

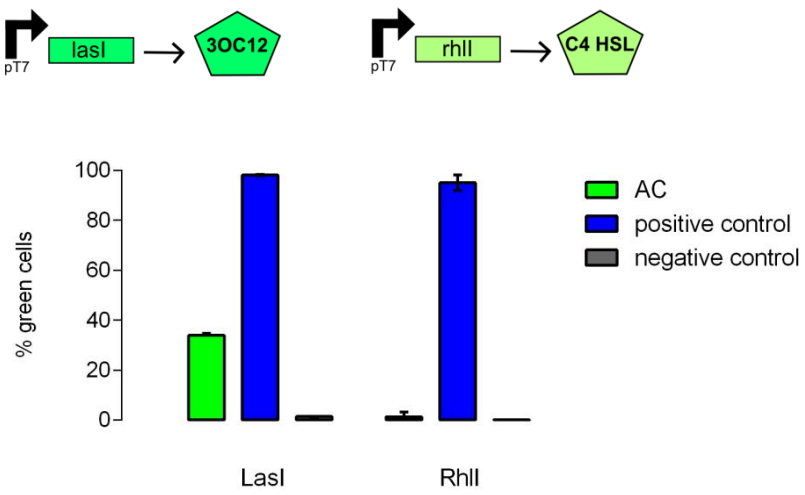


Fig. 4.33 3OC12 HSL artificial sender cells produced 3OC12 HSL.

LasI and RhlI were encapsulated in phospholipid vesicles together with the S30 T7 high yield protein expression system. Artificial cells were incubated at 37 °C together with the correspondent AHL *E. coli* reporter strain and GFP expression was monitored by flow cytometry. 34% of green positive cells were counted in 3OC12 HSL artificial sender cells and no green events were observed with the artificial cells producing C4 HSL (green bars). Positive controls were performed by adding 0.1 μM of the correspondent AHL to *E. coli* cells (blue bars). No AHLs were added to the negative controls (grey bars). Cartoons above the graph give a schematic representation of the used genetic constructs within vesicles. Data shown are from MC001A and MC003A.

To test whether C4 HSL was produced inside of the artificial cells but failed to cross the phospholipid compartment, another reaction was set up. Vesicles were incubated alone at 37 °C to allow for the production of C4 HSL. Then the samples were divided and one aliquot was treated with sonication to disrupt the vesicles and release any C4 HSL molecules trapped inside. Samples were mixed together with C4 HSL *E. coli* reporter strain and incubated at 37 °C. No green cells were present in both treated and untreated samples (Fig. 4.34). In other words, the artificial cells failed to produce C4 HSL.

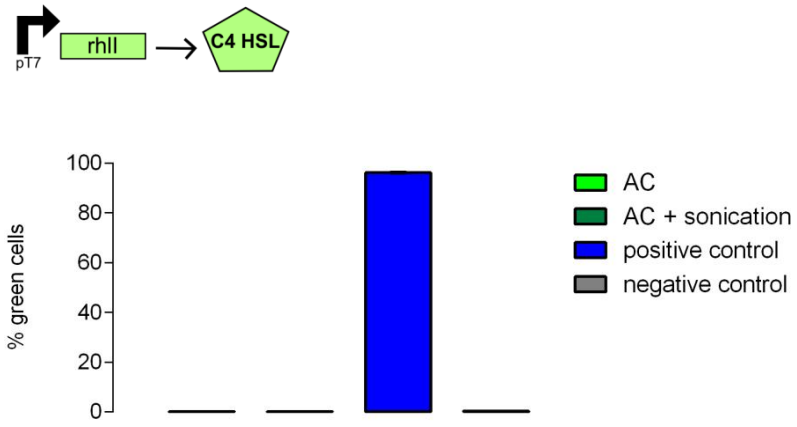


Fig. 4.34 C4 HSL artificial sender cells failed to produce C4 HSL.

RhlI was encapsulated in phospholipid vesicles together with the S30 T7 high yield protein expression system. After 6 h of incubation, artificial cells were mixed together with the correspondent AHL *E. coli* reporter strain and GFP expression was monitored by flow cytometry. No green positive events were found in both intact (green bar) or sonicated vesicles (dark green bar). Positive controls were performed by adding 0.1 μM of the correspondent AHL to *E. coli* cells (blue bars). No AHLs were added in the negative controls (grey bars). A cartoon above the graph gives a schematic representation of the used genetic construct within vesicles. Data shown are from MC003A.

4.4.5 *In vitro* reconstruction of *V. fischeri* QS

To build an *in vitro V. fischeri* QS device, a genetically encoded *luxR* gene was placed under a tet promoter to constitutively express the 3OC6 HSL receptor. To monitor the specific response, GFP mut3b was cloned downstream of pLuxR, a 3OC6 HSL responsive promoter. The pLuxR used here was composed of the *lux* box region of the intergenic region of the *lux* operon. The synthetic 3OC6 HSL sensing device, named T9002, was expressed with an *E. coli* S30 extract in the presence and in the absence of 10 μM 3OC6 HSL. Reactions were incubated at 37 °C and monitored for 6 h. Kinetic experiments showed the specific expression of GFP only in the presence of the *V. fischeri* AHL molecule (Fig. 4.35).

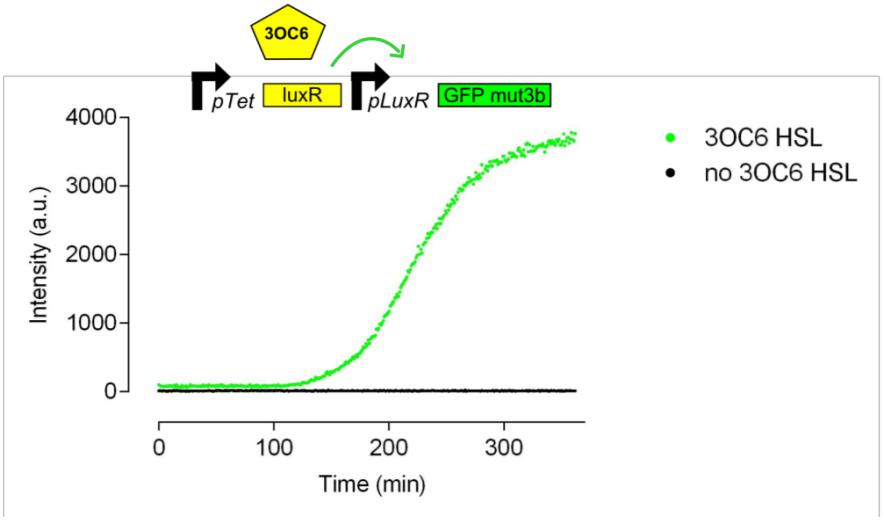


Fig. 4.35 3OC6 HSL activated protein expression *in vitro*.

A synthetic 3OC6 HSL sensing device was expressed with a S30 *E. coli* cell extract at 37 °C. Kinetic experiments were monitored by fluorimetry for 6 h (ex 501 nm, em 511 nm). In the presence of 10 μM 3OC6 HSL, the synthetic device expressed GFP mut3b (green circles), while no signal was observed when this QS molecule was absent (black circles). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct T9002.

The concentration of 3OC6 HSL used in the cell-free reaction was higher than the physiological concentration found in natural cells. Thus, the activity of the synthetic 3OC6 HSL system was tested also with lower amounts of the QS molecule. Reactions were set up as previously described except for the concentration of 3OC6 HSL, which varied from 10 μM to 0.1 μM. All concentrations gave rise to the expression of the reporter gene (Fig. 4.36), showing that the synthetic sensing device can sense 3OC6 HSL also at natural concentrations.

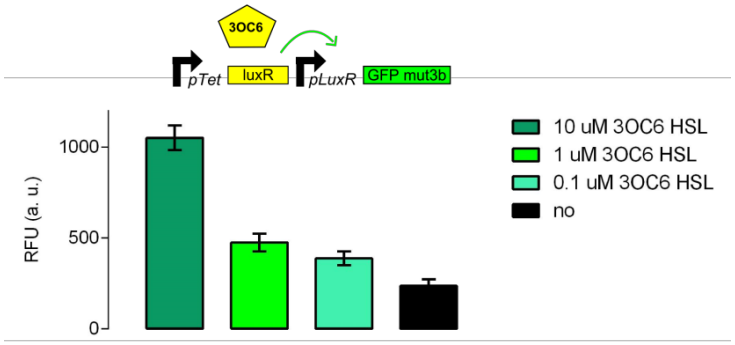


Fig. 4.36 Natural concentrations of 3OC6 HSL activate protein expression *in vitro*.

Fluorescent intensities after 6 h of expression of the 3OC6 HSL sensing part *in vitro* with a S30 *E. coli* cell extract at 37 °C. Different concentrations of 3OC6 HSL were added. 10 µM 3OC6 HSL activated protein expression (dark green bar). Both 1 µM and 0.1 µM activated protein expression (green and light blue bars, respectively). No 3OC6 HSL was added in the negative control (black bar). Kinetic experiments were monitored at CFX 96 Real-Time PCR machine for 6 h using SYBR green filter. A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct T9002.

V. fischeri also contains a second QS system, which involves N-octanoyl-L-Homoserine lactone (C8 HSL) molecules[138]. C8 HSL is synthesized by AinS and binds LuxR with a lower affinity than 3OC6 HSL. Since C8 HSL is produced at low cell density it can bind LuxR and induce the production of 3OC6 HSL. A synthetic 3OC6 HSL sensing device was tested also in the presence of C8 HSL. However when T9002 was expressed with the S30 *E. coli* extract with 10 µM of C8 HSL, no protein production was detected, probably due to the lower affinity of this QS molecule for LuxR (Fig. 4.37).

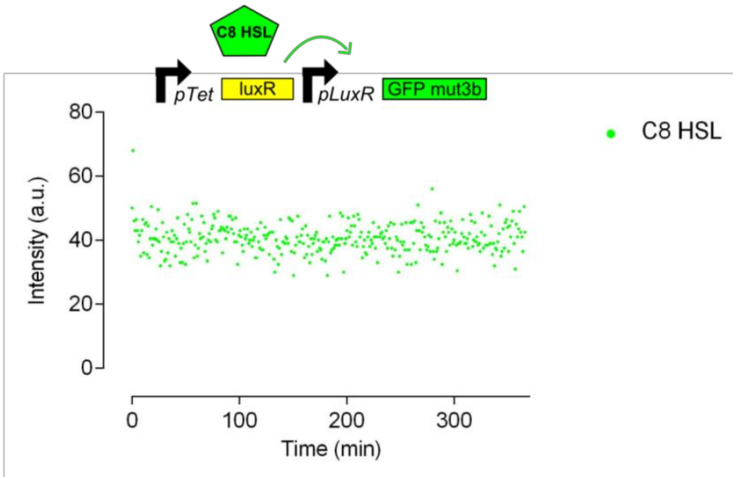


Fig. 4.37 C8 HSL failed to activate protein expression *in vitro*.

A synthetic 3OC6 HSL sensing device was expressed with a S30 *E. coli* cell extract at 37 °C. Kinetic experiments were monitored by fluorimetry for 6 h (ex 501 nm, em 510 nm). In the presence of 10 µM C8 HSL, no protein expression was observed (green circles). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct T9002.

Greenberg[139] showed how LuxR could be mutated to sense *in vivo* both 3OC6 HSL and C8 HSL with high affinity, and the authors engineered a version of LuxR capable sensing only C8 HSL. To assess if such LuxR variants could work in the same manner also *in vitro*, WT LuxR in T9002 was mutated. A first construct containing T33A S116A S135I LuxR was tested to sense both 3OC6 and C8 HSL within a cell-free reaction. GFP mut3b expression was activated in the presence of both AHLs without background signal in their absence (Fig. 4.38).

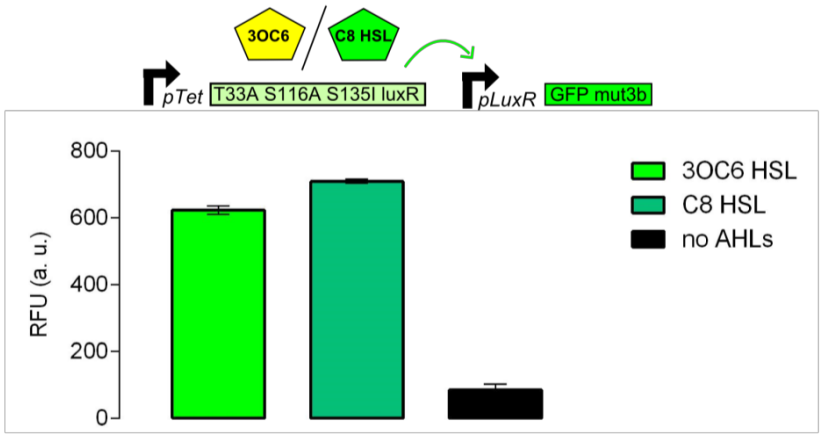


Fig. 4.38 The T33A S116A S135I LuxR variant activates protein expression in response to 3OC6 HSL and C8 HSL.

Fluorescent intensities after 6 h of expression of the LuxR variant sensing part *in vitro* with a S30 *E. coli* cell extract at 37 °C. Reactions were monitored at CFX 96 Real-Time PCR machine for 6 h using SYBR green filter. 10 μM 3OC6 HSL and C8 HSL were added. Both QS molecules could activate protein expression when T33A S116A S135I LuxR is used in the synthetic sensing device (green and dark green bars, respectively). No AHLs were added in the negative control (black bar). A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct RL086A.

An additional mutation in LuxR causes the protein to lose the ability to sense 3OC6 HSL, and thus specifically respond to C8 HSL. T33A M65R S116A S135I LuxR was tested *in vitro* with a S30 *E. coli* cell extract with or without 3OC6 or C8 HSL. As showed *in vivo*, the LuxR mutant failed to recognize 3OC6 HSL and activated protein production in the presence of C8 HSL (Fig. 4.39).

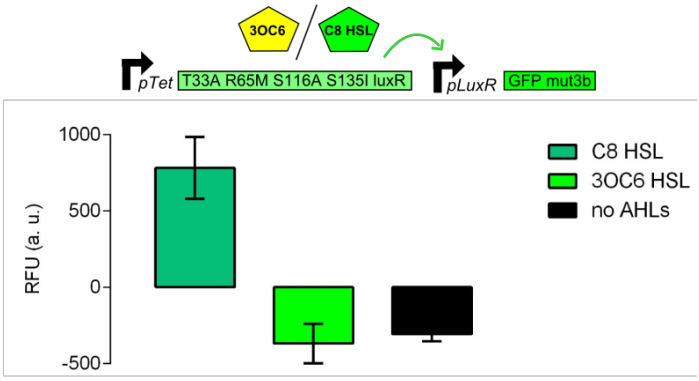


Fig. 4.39 The T33A M65R S116A S135I LuxR variant activated protein expression only in the presence of C8 HSL.

Fluorescent intensities after 6 h of expression of the LuxR variant sensing part *in vitro* with a S30 *E. coli* cell extract at 37 °C. 10 µM 3OC6 HSL and C8 HSL were added. C8 HSL activated protein expression (dark green bar). 3OC6 HSL was not recognized by the LuxR variant (green bar). No AHLs were added to the negative control (black bar). Reactions were monitored at CFX 96 Real-Time PCR machine for 6 h using SYBR green filter. A cartoon above the graph gives a schematic representation of the used genetic circuit. Data shown are from construct RL087A.

Since the 3OC6 HSL synthetic sensing device successfully sensed *V. fischeri* QS molecules, the following step was to encapsulate the device inside a lipid compartment to build artificial cells that can sense living cells. First, the possibility of monitoring GFP production inside phospholipid vesicles was tested. 1:2 POPC: cholesterol vesicles were rehydrated with a plasmid encoded GFP mut3b under the control of a T7 promoter together with the S30 T7 high yield expression system. GFP production was monitored over time with a fluorimeter. No signal was detected after 6 h of incubation at 37 °C (Fig. 4.40).

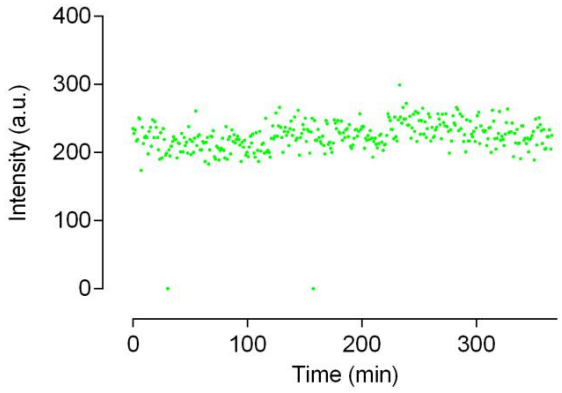


Fig. 4.40 GFP mut3b production inside phospholipid vesicles could not be detected with a fluorimeter.

Kinetic profile of GFP mut3b expression with the S30 T7 high yield protein expression system inside of phospholipid vesicles (ex 501 nm, em 511 nm). No fluorescent signal was detected using a spectrofluorimeter to monitor GFP production.

Vesicles scatter light, leading to difficulties in monitoring the fluorescent signal. GFP expressing phospholipid vesicles were therefore next analyzed by flow cytometry. Vesicles were incubated at 37 °C and at different time points 2 µL were collected and analyzed by flow cytometry. The data revealed a small percentage (2%) of active vesicles (Fig. 4.41).

Fig. 4.41 GFP mut3b production inside of phospholipid vesicles could not be detected by flow cytometry.

Flow cytometry analysis of GFP mut3b expressed with the S30 T7 high yield protein expression system inside of phospholipid vesicles. No green positive events were counted.

To actively express GFP, vesicles need to contain all of the transcription-translation machinery components and a sufficient amount of plasmid DNA. The low amount of GFP expressing vesicles could be due to low encapsulation efficiency. To monitor artificial cells able to sense living cells, a stronger output signal was necessary. Instead of monitoring the production of a fluorescent protein, the enzyme luciferase could be used. In this way, once the enzyme is expressed it can continuously produce the signal. GFPmut3b in T9002 was then substituted with the firefly luciferase gene. 1:2 POPC: cholesterol vesicles were hydrated with RL082A and S30 *E. coli* cell extract. Formed vesicles were incubated at 37 °C for 5 h in the presence and in the absence of 10 µM 3OC6 HSL. After the reaction, ATP and luciferin were added as an energy source and a substrate for luciferase. Vesicles were broken with 0.3% Triton X-100 to allow luciferin to interact with the produced enzyme. Only in the presence of 3OC6 HSL in the external environment were vesicles found to produce the luciferase enzyme and emit light (Fig. 4.42).

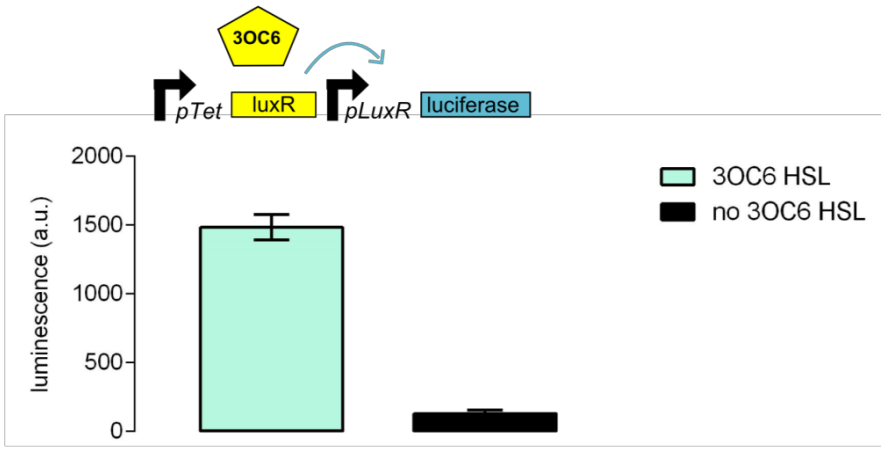


Fig. 4.42 Artificial cells sense 3OC6 HSL.

RL082A was encapsulated in 1:2 POPC: cholesterol vesicles together with a S30 *E. coli* cell extract. 10 μM of 3OC6 HSL was added to the external solution and vesicles were incubated at 37 °C for 5 h. Luminescence was monitored with a plate reader. Artificial cells sensed 3OC6 HSL and emitted light (light blue bar). No 3OC6 HSL was added to the negative control (black bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

The artificial cells were able to sense 3OC6 HSL added to the outside of the compartment. The following step was to test whether artificial cells could sense living cells. Thus, a culture of *V. fischeri* was placed together artificial cells. At high cell density, *V. fischeri* releases in the outside 3OC6 HSL and emits light itself. Since also artificial cells emit light in the presence of 3OC6 HSL, the bacteria were separated from the vesicles (artificial cells) so that only light produced from the artificial cells was detected. A *V. fischeri* culture at OD 1.2 was placed on one side of a dialysis apparatus, while 1:2 POPC: cholesterol vesicles containing RL082A and S30 *E. coli* cell extract were inserted on the other side. In this manner, 3OC6 HSL produced by *V. fischeri* could freely diffuse through the membrane and reach the artificial cells, but bacteria cannot cross the membrane. After 5 h of incubation, the artificial cells were removed from the dialysis apparatus, broken with Triton X-100, and ATP and luciferin were added. The artificial cells successfully sensed the 3OC6 HSL produced from *V. fischeri* and emitted light only in the presence of the bacteria (Fig. 4.43).

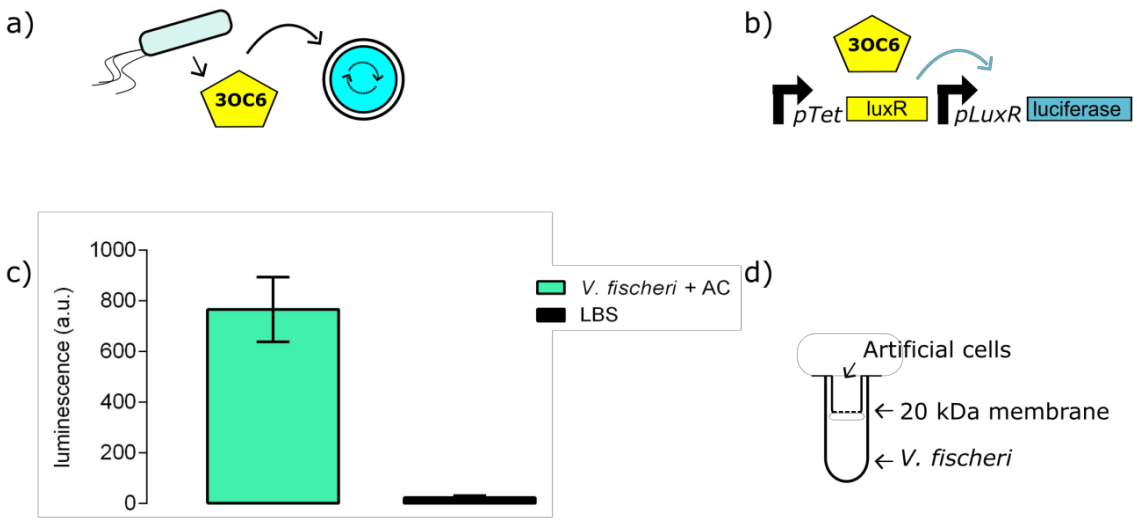


Fig. 4.43 Artificial cells sense *V. fischeri*.

a) A graphic representation of the experiment performed. In the presence of *V. fischeri,* artificial cells sense 3OC6 HSL and emit light through luciferase expression. b) A cartoon gives a schematic representation of the used genetic circuit inside artificial cells (RL82A). c) RL082A was encapsulated in 1:2 POPC: cholesterol vesicles together with a S30 *E. coli* cell extract. Artificial cells were incubated with a culture of *V. fischeri* for 5 h. Luminescence was monitored with a plate reader. Artificial cells sensed 3OC6 HSL from *V. fischeri* and emitted light (light blue bar). Vesicles were incubated with LBS in the negative control (black bar). d) A scheme of the dialysis apparatus used in the experiments. *V. fischeri* is separated from artificial cells through a 20 kDa membrane. The membrane does not allow the transit of bacterial cells, whereas 3OC6 HSL produced from *V. fischeri* can freely cross the membrane and reach the artificial cells.

V. fischeri produces 3OC6 HSL through LuxI synthase, starting from SAM and acyl-ACP. To construct artificial cells able to synthesize and release 3OC6 HSL, the *luxI* gene was cloned into pET21b. The construct named MC002A expressed LuxI behind a T7 promoter. As previously described, first the activity of LuxI was tested when expressed with the S30 T7 high yield expression system. MC002A was added to the reaction together with SAM and acetyl-CoA and incubated for 6 h at 37 °C. The reaction was stopped and mixed together with a 3OC6 HSL *E. coli* reporter strain. The reporter strain carried the plasmid T9002 and produced GFPmut3b only in the presence of 3OC6 HSL. The mixture was left overnight at 37 °C and monitored by flow cytometry. Cells responded to the presence of 3OC6 HSL showing 95% of green positive events (Fig. 4.44).

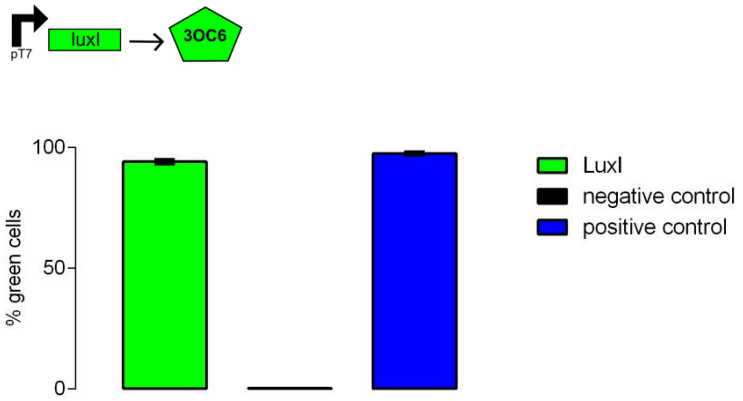


Fig. 4.44 3OC6 HSL sender device produced 3OC6 HSL *in vitro*.

LuxI was expressed *in vitro* with the S30 T7 high yield protein expression system at 37 °C together with 100 μM of acetyl-CoA. Reactions were mixed together with the correspondent 3OC6 HSL *E. coli* reporter strain, and GFP expression was monitored by flow cytometry. A percentage of 95% was counted (green bar). Positive controls were performed by adding 0.1 μM of 3OC6 HSL to *E. coli* (blue bar). No molecule was added in the negative controls (grey bar). A cartoon above the graph gives a schematic representation of the used genetic construct. Data shown are from MC002A.

The 3OC6 HSL *in vitro* sending device was then encapsulated into 1:2 POPC: cholesterol vesicles. Frozen vesicle aliquots were rehydrated with MC002A, S30 T7 high yield expression system, SAM and acetyl-CoA. Protease K was added to the outside of the formed vesicles (artificial cells) and the artificial cells were mixed with a 3OC6 HSL *E. coli* reporter strain. Few microliters were analyzed by flow cytometry at different time points to monitor 3OC6 HSL production. After 6 h, artificial cells were able to synthesize and send 3OC6 HSL to *E. coli* cells with almost 90% of green positive events recorded (Fig. 4.45).

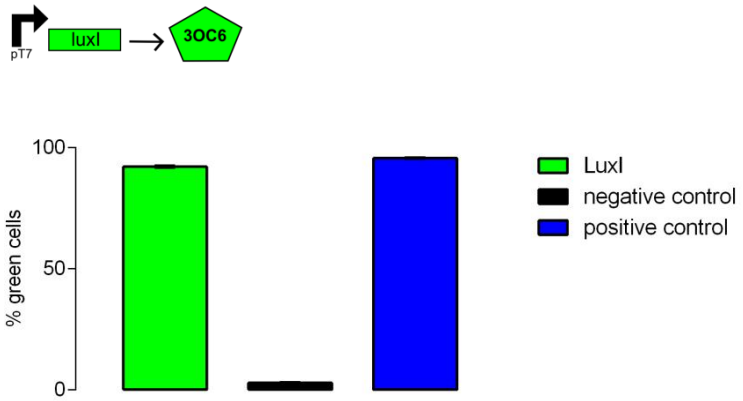


Fig. 4.45 3OC6 HSL artificial sender cells produced 3OC6 HSL.

LuxI was encapsulated in phospholipid vesicles together with the S30 T7 high yield protein expression system. Artificial cells were incubated at 37 °C with a 3OC6 HSL *E. coli* reporter strain, and GFP expression was monitored by flow cytometry. 90% of green positive cells were counted with the 3OC6 HSL artificial sender cells (green bar). Positive controls were performed by adding 0.1 μM of 3OC6 HSL to *E. coli* cells (blue bar). No AHLs were added to the negative controls (grey bars). A cartoon above the graph gives a schematic representation of the used genetic construct within vesicles. Data shown are from MC002A.

4.4.6 Mixing together artificial and natural cells

Artificial sensor cells able to sense living cells through QS and artificial sender cells able to synthesize and send QS molecules to living cells were successfully built. To integrate the artificial cells together with living cells it is necessary assess whether living bacteria can be mixed with the artificial systems without interference or damage. For example, *P. aeruginosa* is a pathogen known to produce phospholipases, toxins and biosurfactants[140] which could break the phospholipid compartment. Thus, a dye leakage assay was used to test the resistance of phospholipid vesicles when placed together with bacteria. The lipid film was rehydrated with 10 mM calcein to form vesicles. After homogenization and overnight tumbling, the vesicles were loaded onto a sepharose 4b column to remove non-encapsulated dye. Purified vesicles were then mixed 1:1 with bacterial culture and monitored by fluorimetry. If the bacteria could break the phospholipid membrane, calcein would be released, resulting in an increased fluorescence signal due to diminished self-quenching.

Since 1:2 POPC: cholesterol vesicles gave the best results in the previous experiments, this same lipid composition was tested together with different bacterial strains. When vesicles were placed in contact with *V. fischeri* cells, no increment of signal was detected within 10 h of incubation (Fig. 4.46). *V. fischeri* does not release lipolytic factors and, therefore, does not seem able to break phospholipid vesicles. As a control, Triton X-100 was added after 10 h, resulting in immediate breaking of the vesicles and an increment of fluorescence.

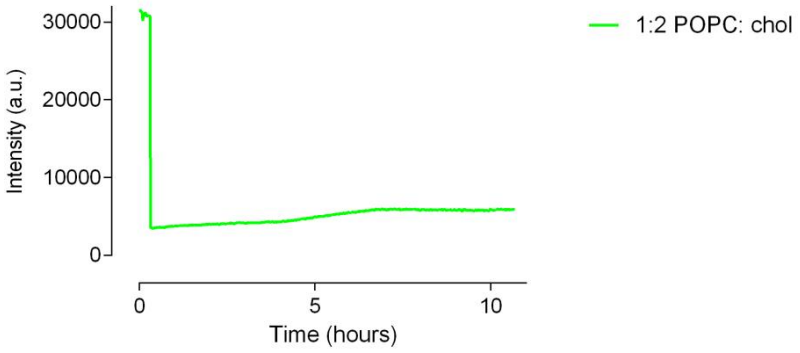


Fig. 4.46 *V. fischeri* did not damage phospholipid vesicles.

Fluorescent profile of 1:2 POPC: cholesterol vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) were mixed with a culture of *V. fischeri*. Fluorescent signal was monitored by fluorimetry (ex 495 nm, em 515 nm) at 28 °C. After 10 h, no breakage was observed in phospholipid vesicle.

1:2 POPC: vesicles were then mixed with *P. aeruginosa* PA14 cells. As shown in figure 4.47, a slow but constant increase of signal was detected, meaning that the bacteria secreted factors that could break the phospholipid membrane resulting in 20% of lysis within 4 h.

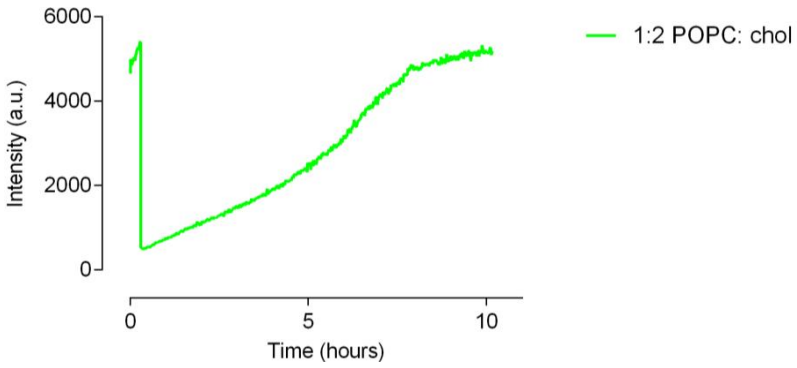


Fig. 4.47 *P. aeruginosa* damages phospholipid vesicles.

Fluorescent profile of 1:2 POPC: cholesterol vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) were mixed with a culture of *P. aeruginosa*. Vesicles were monitored for 10 h by fluorimetry (ex 495 nm, em 515 nm) at 37 °C. *P. aeruginosa* damaged the phospholipid membrane of the vesicles and calcein was released to the outside.

Since *P. aeuroginosa* was able to easily destroy 1:2 POPC: cholesterol membranes other lipid compositions were tested to find vesicles resistant to pathogens. *P. aeruginosa* secretes phospholipases which target phosphatidylcholine,[141] thus vesicles of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-5000] DSPE-PEG(5000) with 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) were made. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the major components of Gram-negative inner membranes[142] and should serve as substrates to the lytic enzymes of *Pseudomonas*. The polyethylene glycol lipid conjugate DSPE-PEG(5000) should protect the membrane of the vesicle by creating a steric barrier around the vesicle, potentially inhibiting phospholipid hydrolysis[143]. Thus, the higher concentration of PEG possible to not preclude vesicles formation was used. 9:1 DOPG: DSPE PEG vesicles were then incubated with *P. aeruginosa* cells. However, 10% of PEG conjugate lipid was not sufficient to avoid vesicles breakage (Fig. 4.48).

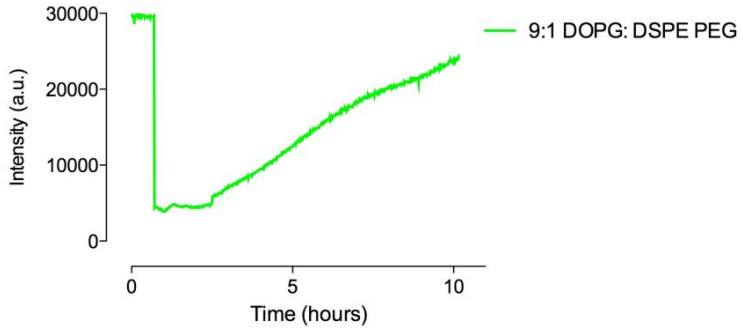


Fig. 4.48 1:9 DOPG: DSPE PEG vesicles were damaged by *P. aeruginosa* cells.

Fluorescent profile of 9:1 DOPG: DSPE PEG vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) mixed with *P. aeruginosa* culture. Vesicles were monitored for 10 h by fluorimetry at 37 °C (ex 495 nm, em 515 nm). PEG coated vesicles were destroyed by the pathogen. The increment in fluorescence is due to the release of calcein in the outside.

Pseudomonas recognizes the long chain fatty acids moiety of phospholipids as an attractant, and the phospholipases are active against such lipids[144]. Therefore, vesicles composed of lipids of a different structure, i.e. sphingomyelin, were tested. Unfortunately, *P. aeruginosa* was able to break also vesicles made with this composition (Fig. 4.49).

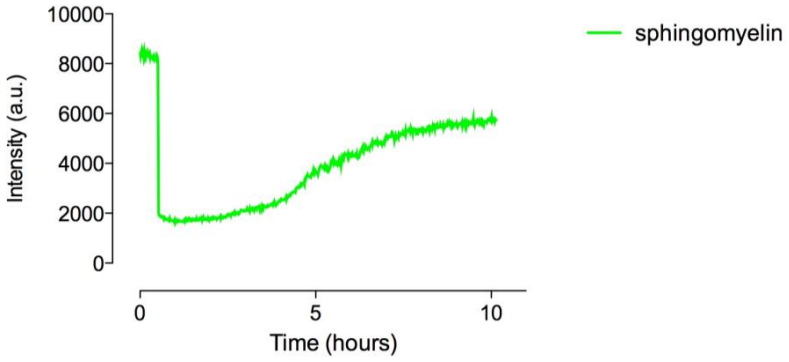


Fig. 4.49 *P. aeruginosa* destroyed sphingomyelin vesicles.

Fluorescent profile of sphingomyelin vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) mixed with *P. aeruginosa* culture. Vesicles were monitored for 10 h by fluorimetry at 37 °C (ex 495 nm, em 515). Vesicles broke when in contact with *P. aeruginosa.*

Several PA14 mutants were then used to assess whether deleting phospholipase genes would result in a more stable bacterial - vesicle system. Sphingomyelin vesicles were mixed together with various PA14 single mutants and monitored for 10 h. No improvements in vesicles stability were observed for any of the PA14 mutants (Fig. 4.50), and no *Pseudomonas* resistant lipid compositions were found.

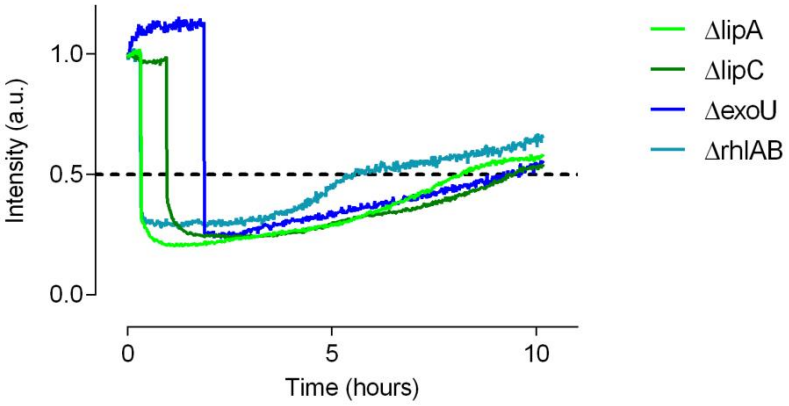


Fig. 4.50 *P. aeruginosa* PA14 mutants destroyed sphingomyelin vesicles.

Fluorescent profile of sphingomyelin vesicles containing 10 mM calcein in buffer A (50 mM HEPES, 10 mM MgCl2, 100 mM KCl, pH 7.6) when mixed with different *P. aeruginosa* PA14 single mutants. Vesicles were monitored for 10 h at 37 °C by fluorimetry (ex 495 nm, em 515 nm). Vesicles were destroyed by all the *P. aeruginosa* mutants tested.

The results obtained so far demonstrated that the construction of artificial cells able to sense *V. fischeri*, and artificial cells able to synthesize and send various QS molecules for *V. fischeri*, *E. coli, V. harveyi* and *P. aeruginosa* are possible. The next step was to join together the sensing and sending devices to build a complete communication pathway between the artificial and bacterial cells. To better understand if artificial cells could sense a QS molecule and respond by producing a QS molecule, two different QS systems were placed together within the artificial cells.

First, a synthetic circuit able to sense *V. fischeri* and send a message to *E. coli* or *V. harveyi* through AI-2 was set up. GFP cloned into T9002 was replaced with the HLTP module in order to produce AI-2 in response to 3OC6 HSL. The synthetic device, named JF008A, was then tested in solution using a S30 *E. coli* cell extract expression system. Reactions were set up as previously described, adding 0.5 mM of SAH precursor with or without 10 µM of 3OC6 HSL, and incubated at 37 °C for 6 h. Different methods were then exploited to monitor AI-2 production. To measure *V. harveyi* response to AI-2, 10 µL of reactions were added to 90 µL of *V. harveyi* BB170 reporter strain and luminescence was monitored after 4 h. No differences were observed between samples incubated with or without 3OC6 HSL (Fig. 4.51), and both samples resulted in very low luminesce output.

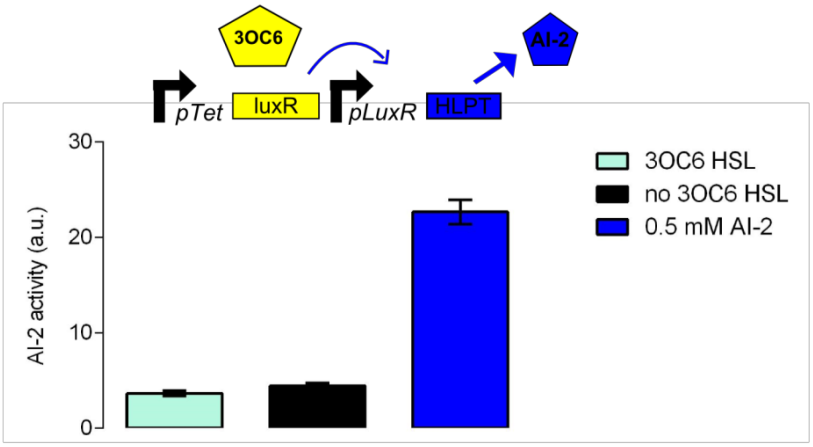


Fig. 4.51 Synthetic 3OC6 HSL sensing AI-2 sending circuit failed to elicit a response in *V. harveyi*.

JF008A was expressed *in vitro* with a S30 *E. coli* cell extract at 37 °C in the presence and in the absence of 3OC6 HSL. Samples mixed with the *V. harveyi* BB170 reporter strain did not elicit luminescence in the reporter strain when 3OC6 HSL was present in the reaction (light blue bar). 50 µM of AI-2 was added to the reporter strain as a positive control (blue bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

The presence of AI-2 could be measured also through flow cytometry exploiting an AI-2 *E. coli* reporter strain. The strain cannot produce its own AI-2, because of the lack of a functional *luxS* gene, and this strain was engineered to sense external autoinducer and express GFPuv in response. Reactions were set up as previously described and mixed with *E. coli* after 6 h. At

different time points a few microliters of cells were collected and analyzed by flow cytometry. No GFP positive cells were counted after 6 h of incubation (Fig. 4.52).

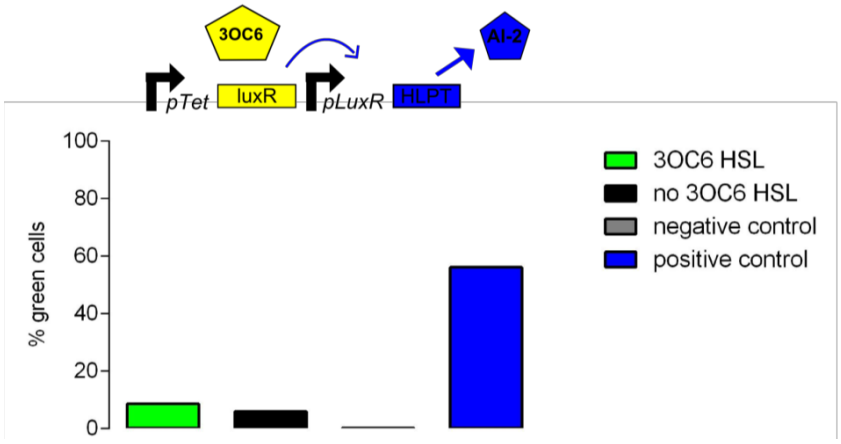


Fig. 4.52 Synthetic 3OC6 HSL sensing AI-2 sending circuit failed to elicit a response with an AI-2 *E. coli* reporter strain.

JF008A was expressed *in vitro* with a S30 *E. coli* cell extract at 37 °C in the presence and in the absence of 3OC6 HSL. Samples were mixed with the AI-2 *E. coli* reporter strain and monitored by flow cytometry. No green positive events were counted when 3OC6 HSL was present (green bar) or absent (black bar) in the reaction. 50 μM AI-2 was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

Both *V. harveyi* and *E. coli* reporter strains for AI-2 are not optimized to measured small amounts of autoinducer and to discriminate between small differences in concentrations. The *V. harveyi* bioassay is known to be more qualitative than quantitative,[126] and the reporter gene, GFPuv, is not optimal for flow cytometry. That is because the excitation wavelength of GFPuv (395 nm) is not highly compatible with the FITC filter of the flow cytometer. To quantify the response of the *E. coli* cells to AI-2, a RT-qPCR assay was exploited. AI-2 is known to activate transcription of the *lsr* operon, thus overexpression of *lsrACDB* genes was monitored in the presence of AI-2. Cell-free reactions expressing JF008A in the presence and in the absence of 3OC6 HSL were added to the *E. coli* reporter strain. After 6 h of incubation, *E. coli* was harvested and the RNA was extracted and retrotranscribed. RT-qPCR analysis showed no overexpression of *lsr* genes when 3OC6 HSL was added (Fig. 4.53).

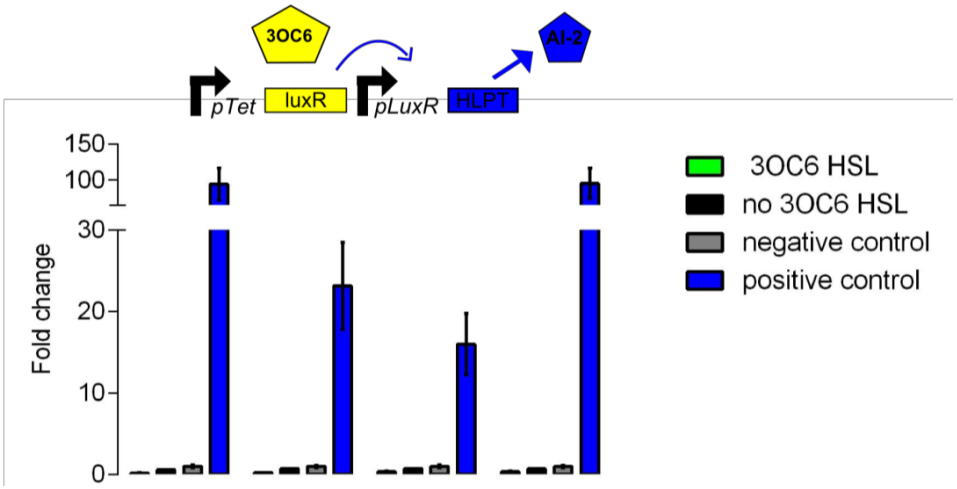


Fig. 4.53 Synthetic 3OC6 HSL sensing, AI-2 sending circuit failed to elicit a response in *E. coli*.

JF008A was expressed *in vitro* with the S30 *E. coli* extract at 37 °C in the presence and in the absence of 3OC6 HSL. Samples were mixed with AI-2 *E. coli* reporter strain. No overexpression of lsrACBD genes was observed by qPCR in the presence of 3OC6 HSL (green bars) compared to samples in which 3OC6 HSL was absent (black bars). 50 µM AI-2 was added to *E. coli* as a positive control (blue bar), and LB was added as negative control (grey bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

To understand if AI-2 was being produced at low concentrations without reaching the necessary amount to elicit a response from the bacteria, AI-2 was quantified by using 2,2'-dinitro-5,5'-ditiodibenzoico (DTNB). After 6 h at 37 °C, the cell-free expression reactions were stopped and chloroform extracted to remove the proteins. Samples were then added to the DTNB reagent and measured by UV-VIS spectroscopy. No differences were observed between the samples incubated with or without 3OC6 HSL (Fig. 4.54).

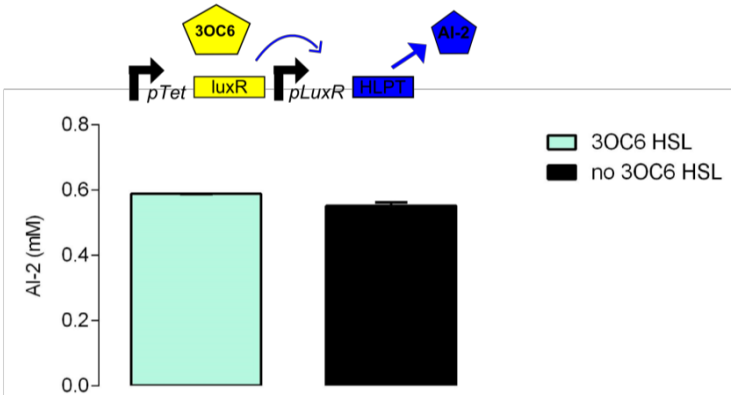


Fig. 4.54 Synthetic 3OC6 HSL sensing AI-2 sending circuit failed to produce AI-2.

JF008A was expressed *in vitro* with a S30 *E. coli* cell extract at 37 °C for 6 h in the presence and in the absence of 10 µM 3OC6 HSL. Reactions were then chloroform extracted to remove proteins. Purified reactions were added 1:100 to DTNB solution and AI-2 was quantified. No differences were observed in the presence and in the absence of 3OC6 HSL (light blue and black bars, respectively). A cartoon above the graph gives a schematic representation of the used genetic circuit.

Even if the sensing and the sending parts worked as separate units, when joined together the synthetic circuit failed to synthesize AI-2 in response to 3OC6 HSL. Other than AI-2, artificial cells were able to synthesize and send *P. aeruginosa* QS molecules. Therefore, the 3OC6 HSL sensing module was joined with either 3OC12 HSL or C4 HSL sending devices. Similarly, for HLPT, the gene coding for GFP in T9002 was replaced with *lasI* or *rhlI* genes.

RL080A contains *luxR* constitutively expressed from a tet promoter and *rhlI* controlled by the 3OC6 HSL responsive promoter pLuxR. This synthetic device produces C4 HSL in response to 3OC6 HSL. Reactions expressing RL080A with a S30 *E. coli* cell extract were incubated with or without 1 µM 3OC6 HSL for 6 h at 37 °C. Samples were then added to C4 HSL the *E. coli* reporter strain and analyzed by flow cytometry at different time points. After 10 h, the cells showed 70% and 0.1% of green positive events when incubated with or without 3OC6 HSL, respectively (Fig. 4.55). A control in which 1 µM of 3OC6 HSL was directly added to the C4 HSL reporter strain was performed in order to exclude GFP expression due to unspecific recognition of 3OC6 HSL instead of C4 HSL. The control sample gave rise to 25 % of green positive events, meaning that a lower percentage of positive cells in the positive reaction sample derived from 3OC6 HSL added to activate C4 HSL production.

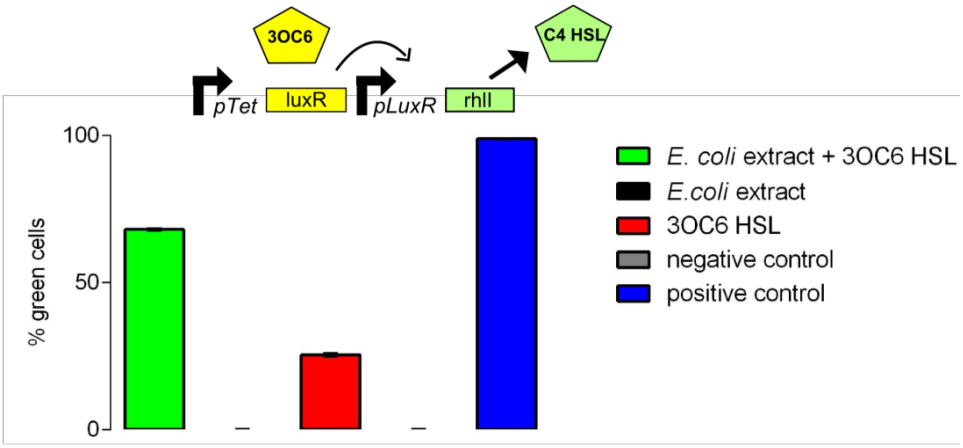


Fig. 4.55 A synthetic 3OC6 HSL sensing, C4 HSL sending circuit elicits a response in an *E. coli* reporter strain.

RL080A was expressed *in vitro* with a S30 *E. coli* cell extract at 37 °C in the presence and in the absence of 3OC6 HSL. Samples were mixed with the C4 HSL *E. coli* reporter strain and monitored by flow cytometry. 70 % of green positive events were counted when 3OC6 HSL was present in the reaction (green bar). No GFP expression was observed in the absence of 3OC6 HSL (black bar). 0.1 µM C4 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

RL080A and S30 *E. coli* cell extracts were then encapsulated within phospholipid vesicles to build artificial cells able to sense 3OC6 HSL and produce C4 HSL. Vesicles were mixed 1:1 with

the C4 HSL *E. coli* reporter strain and incubated at 37 °C with or without 3OC6 HSL added to the outside of the artificial cells. After 16 h of monitoring, no difference in percentage of positive GFP cells were counted by flow cytometry between samples containing artificial cells and control reactions for 3OC6 HSL (Fig. 4.56). As previously shown for the C4 HSL sending part, when the reaction was encapsulated inside of the lipid compartments the amount of C4 HSL produced by the artificial cells was not enough to elicit a response from the bacteria.

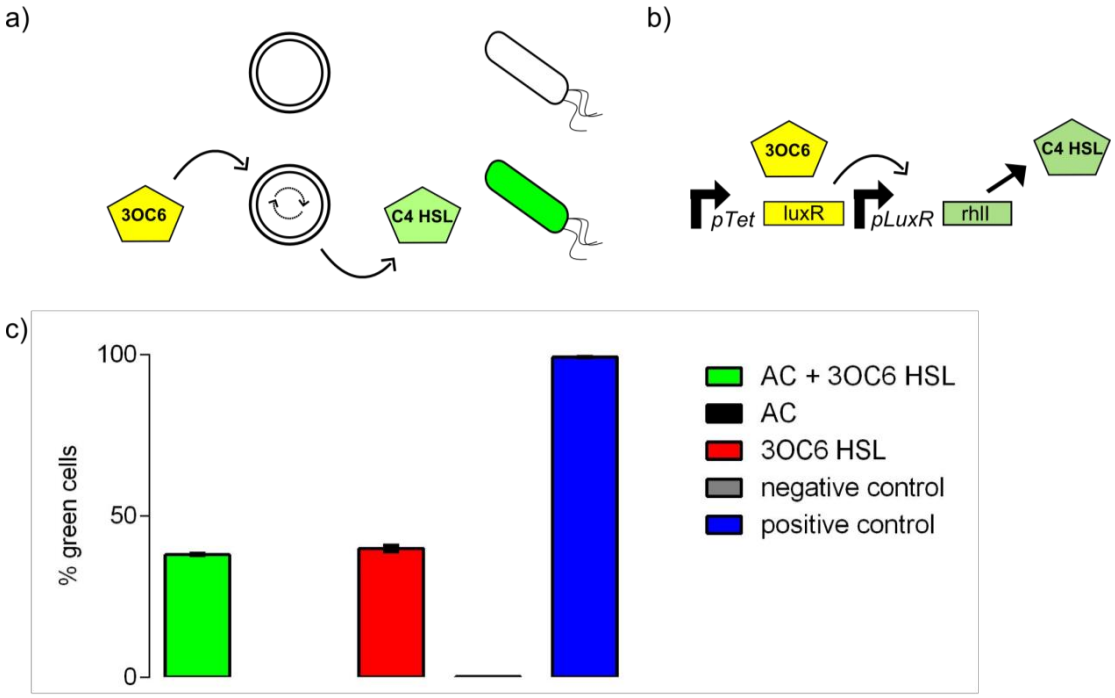


Fig. 4.56 Artificial cells were not able to produce C4 HSL in response to 3OC6 HSL.

A S30 *E. coli* cell extract supplemented with RL080A, SAM and acetyl-CoA were encapsulated in phospholipid vesicles. a) A graphic representation of the experiment performed. In the absence of 3OC6 HSL no reaction occurs inside artificial cell. When 3OC6 HSL is added outside, artificial cell senses the molecule and synthesizes C4 HSL. *E. coli* responds to C4 HSL expressing GFP. b) A cartoon gives a schematic representation of the used genetic circuit inside artificial cells (RL080A). c) Artificial cells were incubated with the C4 HSL *E. coli* reporter strain and monitored by flow cytometry. No difference in the percentage of green positive events were observed when 1 µM 3OC6 HSL was added to the artificial cells (green bar) or directly to the *E. coli* cells as control (red bar). 0.1 µM C4 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar).

However, as shown above, artificial cells can produce the other *P. aeruginosa* QS molecule, 3OC12 HSL. In RL079A, the 3OC6 HSL-LuxR complex controls LasI expression and thus 3OC12 HSL production. First, this circuit was tested with the S30 *E. coli* cell extract system plus or minus the addition of 3OC6 HSL. After 6 h at 37 °C, the reactions were mixed together with the 3OC12 HSL *E. coli* reporter strain. After 6 h 50 % of positive events were found upon the addition of 3OC6 HSL, while less than 1% of GFP expressing cells were observed in the negative reaction (Fig. 4.57).

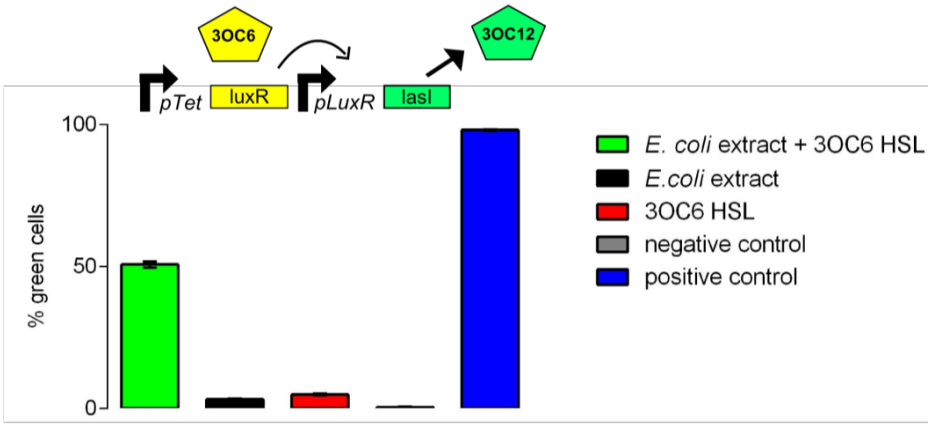


Fig. 4.57 A synthetic 3OC6 HSL sensing, 3OC12 HSL sending circuit elicits a response from the *E. coli* reporter strain.

RL079A was expressed *in vitro* with a S30 *E. coli* cell extract at 37 °C in the presence and in the absence of 1 μM 3OC6 HSL. Samples were mixed with the 3OC6 HSL *E. coli* reporter strain and monitored by flow cytometry. 50 % of green positive events were counted when 3OC6 HSL was present in the reaction (green bar). No GFP expression was present in the absence of 3OC6 HSL (black bar). 0.1 μM 3OC12 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). 4 % of positive events were counted in the sample where 1 μM 3OC6 HSL was added directly to the reporter strain to check for unspecific fluorescence (red bar). A cartoon above the graph gives a schematic representation of the used genetic circuit.

The next step was to encapsulate the reaction inside of 1:2 POPC: cholesterol vesicles. Reactions were set up as previously described for the C4 HSL module. After 10 h almost 40 % of positive cells were counted upon the addition of 1 μM 3OC6 HSL to the exterior of the artificial cells. No positive cells were present in the negative control (Fig. 4.58).

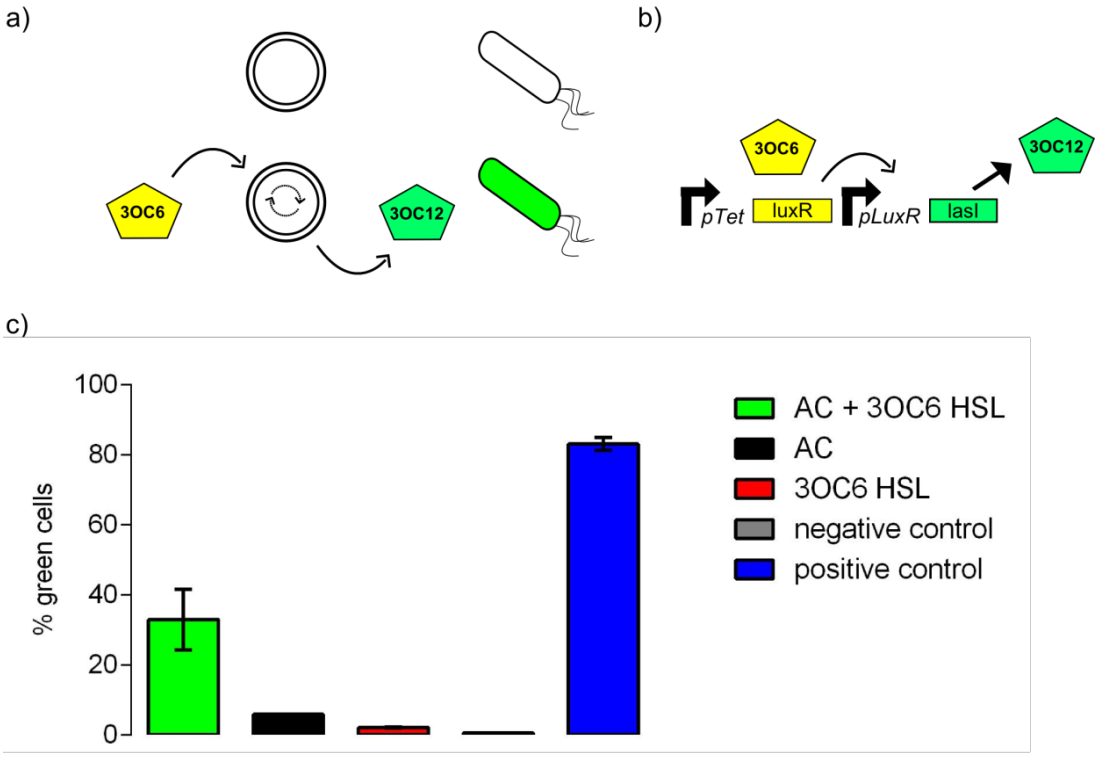


Fig. 4.58 Artificial cells produce 3OC12 HSL in response to 3OC6 HSL.

A S30 *E. coli* extract supplemented with RL079A, SAM and acetyl-CoA were encapsulated in phospholipid vesicles. a) A graphic representation of the experiment performed. In the absence of 3OC6 HSL no reaction occurs inside artificial cell. When 3OC6 HSL is added outside, artificial cell senses the molecule and synthesizes 3OC12 HSL. *E. coli* responds to 3OC12 HSL expressing GFP. b) A cartoon gives a schematic representation of the used genetic circuit inside artificial cells (RL79A). c) Artificial cells were incubated with the 3OC12 HSL *E. coli* reporter strain and monitored by flow cytometry. 33 % of green positive events were counted when 3OC6 HSL was added to the artificial cells (green bar). No GFP expression was observed in the absence of 3OC6 HSL (black bar). 0.1 μM 3OC12 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). 2 % of positive events were counted in samples where 1 μM 3OC6 HSL was added directly to the reporter strain to check for unspecific fluorescence (red bar).

Artificial cells were able to sense 3OC6 HSL and in response synthesize 3OC12 HSL, eliciting a response in *E. coli*. However, 3OC6 HSL was added to the outside of the vesicles. To set up a communication path between two different bacterial strains, the artificial cells need to sense directly the presence of the living cells. Therefore, artificial cells expressing RL079A were incubated with *V. fischeri* grown until OD 1.2. To avoid the counting of *V. fischeri* cells by flow cytometry, a dialysis apparatus was set up. Artificial cells and *E. coli* were placed on the top of a dialysis cap while the *V. fischeri* culture was put on the other side of a 20 kDa membrane. 3OC6 HSL produced by *V. fischeri* could freely cross the membrane and activate 3OC12 HSL production in the artificial cells. Upon the release of 3OC12 HSL from the artificial cells, *E. coli* expresses GFPmut3b. As a negative control, the *V. fischeri* culture was substituted with LBS medium.

Positive control contained LBS supplement with 0.1 µM of 3OC12 HSL. Also, a control in which the *E. coli* reporter strain and empty vesicles were incubated with the *V. fischeri* culture was performed to monitor GFP expression due to 3OC6 HSL instead of 3OC12 HSL. Reactions were incubated at 28 °C to facilitate the growth of *V. fischeri*. Every 2 h a few microliters of *E. coli* were collected and analyzed by flow cytometry. After 14 h the *E. coli* cells incubated with artificial cells and *V. fischeri* resulted in more than 30 % of positive green cells (Fig. 4.59). No positive events were found in negative control reaction, while 10 % of unspecific GFP production was recorded in the control sample.

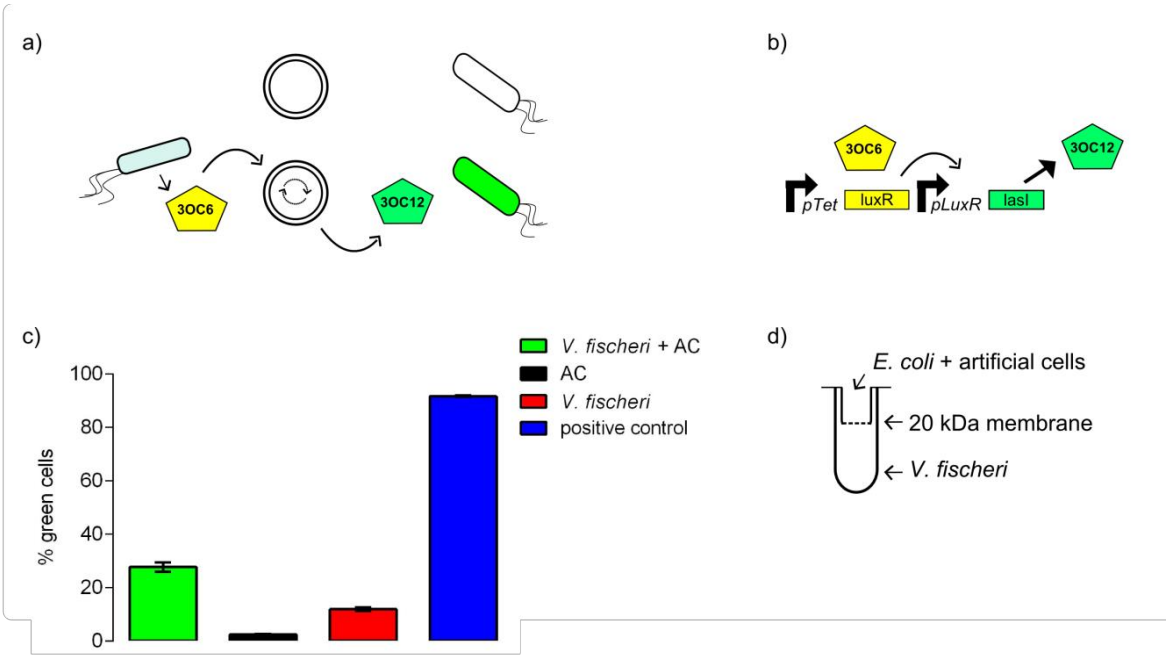


Fig. 4.59 Artificial cells sense *V. fischeri* and produce 3OC12 HSL, eliciting a response from the *E. coli* reporter strain.

a) A graphic representation of the experiment performed. In the absence of *V. fischeri* no reaction occurs inside artificial cell. When mixed with *V. fischeri,* artificial cells sense 3OC6 HSL and synthesize 3OC12 HSL. *E. coli* responds to 3OC12 HSL expressing GFP. b) A cartoon gives a schematic representation of the used genetic circuit inside artificial cells (RL79A). c) Artificial cells expressing RL079A were incubated with *V. fischeri* at 28 °C and the 3OC12 HSL *E. coli* reporter strain. Reporter cells were monitored by flow cytometry. After 14 h, the artificial cells sensed 3OC6 HSL from *V. fischeri* and sent 3OC12 HSL to the *E. coli* reporter strain, which showed 28 % of green positive events (green bar). No positive events were counted in the negative control where the artificial cells were incubated with LBS (black bar). 0.1 µM 3OC12 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). 10 % of positive events were counted in samples where 1 µM 3OC6 HSL was added directly to the reporter strain to test for unspecific fluorescence (red bar). d) A scheme of the dialysis apparatus used in the experiments. *V. fischeri* is separated from *E. coli* reporter strain and artificial cells from a 20 kDa membrane. The membrane does not allow the transit of bacterial cells, whereas 3OC6 HSL produced from *V. fischeri* can freely cross the membrane and reach the artificial cells.

Unfortunately, these results were difficult to replicate. Changes in encapsulation efficiency could lead to minimal 3OC12 HSL production and consequently a poor response from the *E. coli*

reporter cells. Reaction conditions were then optimized to improve 3OC12 HSL production. For example, in the previous experiments, reactions were incubated at 28 °C to favor the growth of *V. fischeri*. However, 28 °C is not the optimal temperature for the S30 *E. coli* cell extract to work efficiently. Moreover, 3OC6 HSL was produced on one side of the dialysis apparatus, and the molecule had to cross the membrane to equilibrate the concentration across the compartments. The process was likely slow and therefore 3OC6 HSL may have reached the artificial cells when the transcription-translation machinery within the artificial cells was no longer active. Thus, *V. fischeri* was grown until OD 1.2 and placed in the dialysis apparatus on the other side. 50 µL of LB was inserted in the dialysis cap. Samples were left at room temperature for 2 h to allow 3OC6 HSL to equally distribute between the two compartments. Artificial cells and *E. coli* were then added to the dialysis cap, and the reactions were incubated at 37 °C. At different time points a few microliters were collected and measured by flow cytometry. After 8 h, more than 40 % of the cells were positive in the sample in which the artificial cells were incubated with *V. fischeri* (Fig. 4.60).

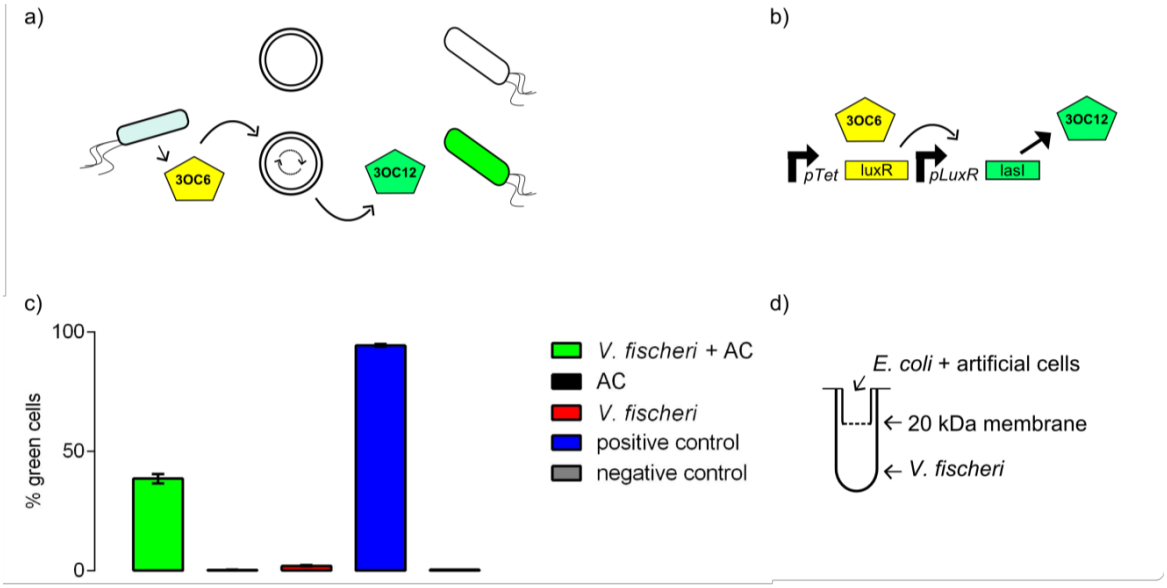


Fig. 4.60 Under optimal conditions artificial cells sense *V. fischeri* and produce 3OC12 HSL, eliciting a greater response from the *E. coli* reporter strain.

a) A graphic representation of the experiment performed. In the absence of *V. fischeri* no reaction occurs inside artificial cell. When mixed with *V. fischeri*, artificial cells sense 3OC6 HSL and synthesize 3OC12 HSL. *E. coli* responds to 3OC12 HSL expressing GFP. b) A cartoon gives a schematic representation of the used genetic circuit inside artificial cells (RL79A). c) Artificial cells expressing RL079A were incubated with *V. fischeri* at 37 °C and the 3OC12 HSL *E. coli* reporter strain. *V. fischeri* was pre-incubated with LB for 2 h at room temperature. Reporter cells were monitored by flow cytometry. After 8 h, the artificial cells sensed 3OC6 HSL from *V. fischeri* and sent 3OC12 HSL to the *E. coli* reporter strain, which showed 40 % of green positive events (green bar). No positive events were counted in the negative control where artificial cells were incubated with LBS (black bar). 0.1 µM 3OC12 HSL was added to *E. coli* as a positive control (blue bar). LB was added as a negative control (grey bar). 2 % of positive events were counted in samples where 1 µM 3OC6 HSL was added directly to the reporter strain to test for unspecific fluorescence (red bar). d) A scheme of the dialysis apparatus used in the experiments. *V. fischeri* is separated from *E. coli* reporter strain and artificial cells from a 20 kDa membrane. Bacterial cells cannot transit the membrane, whereas 3OC6 HSL produced from *V. fischeri* can freely cross the membrane and reach the artificial cells.

Artificial cells could successfully mediate the communication between two different bacterial strains that naturally cannot interact with each other through QS.

4.5 Conclusions

To better integrate artificial with natural cells, it is not sufficient for the artificial system to send messages to control the behavior of the living cells. What is needed is also a system able to sense and respond to living cells in order to establish a complete communication cycle. To reach this goal it could be possible to take advantage of the natural QS processes that bacteria use to communicate with each other. By reconstructing *in vitro* various QS pathways deriving from different bacteria, artificial cells that mimic bacterial communication were built. Similar artificial systems could be functionally adopted together with living cells. First artificial cells able to sense the presence of living cells were constructed. Then artificial cells capable of synthesizing and releasing various QS molecules to *E. coli, V. harveyi, V. fischeri* and *P. aeruginosa* were built. Finally, artificial cells that mediate interspecies communication were shown to be functional. However, the complete, two-way communication cycle was thus far only built with artificial cells and engineered *E. coli*. Future experiments will be set up in order to substitute the *E. coli* reporter strain with *P. aeruginosa* to demonstrate that artificial cells can mediate communication with natural and not genetically modified cells. Moreover, the artificial cells can sense and send the same QS molecule (3OC6 HSL from *V. fischeri*). Therefore, we will also attempt to integrate artificial cells within a single bacterial population in order to determine if living cells are able to distinguish between artificial and natural cells. This approach could lead to a new way to evaluate how life-like artificial chemical systems are.

Chapter 5.
Conclusions

The work presented in this dissertation describes the construction of artificial cells able to integrate with natural cells. Synthetic biology mainly relies on the genetic engineering of living cells. Natural cells are genetically modified to acquire new functions. While useful, such an approach could lead to several complications. The addition of new and complex genetic circuits within living cells has to face the fact that multiple elements inside of cells are still unknown. This could lead to the loss of function of the new circuits. Moreover, living cells grow and evolve. The implementation of new behaviors could lead to consequences on the cellular environment and alter the ecosystem. A way to avoid such difficulties is represented by cell-free synthetic biology. The construction of artificial cells with known purified components will help to avoid some of the uncertainty associated with living cells. Artificial cells built in this work exploited phospholipids (as a compartment to divide the inside and the outside), transcription-translation machinery (either from a cell-free extract or from minimal purified components in PURE system), and DNA (as genetic information). Synthetic circuits built to obtain a certain function in artificial cells were made with various biological parts, joined together to construct several genetic circuits. To reach the construction of artificial cells with useful activity, a preliminary study on how to combine genetic elements within synthetic circuits was performed. Many efforts were made on the characterization of biological parts and in the functionality of a great variety of *de novo* constructed genetic circuits *in vivo*. However, the implementation of such systems in a cell-free chassis was difficult. Simple and clear rules were defined at the beginning of this work to build genetic circuits that function with *in vitro* transcription-translation systems. For example, not all fluorescent proteins are suitable in cell-free chassis, and the way genetic circuits are assembled together could strongly influence their functionality in artificial cells. Just the use of a wrong sequence between two genes inside the same synthetic operon can result in minimal protein expression. Following the rules developed in this part of the dissertation, many genetic circuits needed for the construction of the artificial cells were optimized to control protein expression levels.

Next, efforts were made to construct artificial cells that are able to send messages to living cells. The following of such an approach moves the genetic engineering from living to artificial cells. The artificial system is built to carry out a specific function. After its action, the artificial system vanishes and natural cells resume to their original state. Such system does not have the possibility to evolve and mutate, avoiding long-term consequences. The work presented showed the possibility to control living cells through communication with artificial cells. Extant cells developed several pathways to sense their surroundings and modify their behavior to adapt to changes in the external environment. Thus, natural pathways can be exploited to control natural cells through communication with artificial cells. The artificial cells built in this study sense a molecule that *Escherichia coli* cannot sense on its own and translate it to a message that *E. coli* can sense. The artificial cells sense theophylline, which activates the expression of a pore forming protein, alpha hemolysin (αHL). αHL forms pores in the membrane of the artificial cells, and so

encapsulated IPTG is released. The ability of artificial cells to communicate with *E. coli* was assessed through the expression of GFP in *E. coli* receiver cells. Next, the ability of the artificial cells to elicit a transcriptional response in non-engineered *E. coli* was tested by monitoring expression from the *lac* operon. The result is that the natural ability of *E. coli* to sense small molecules was expanded without genetic intervention. Moreover, this work demonstrated the integration between natural and artificial systems and the construction of a one-way communication.

Artificial cells can be better integrated with natural cells through the construction of an artificial system able to sense and respond to natural cells. Such a system not only needs to send messages to living cells but also to sense them. Bacteria communicate with each other through quorum sensing (QS). Small diffusible molecules are sensed and produced from bacterial cells. Through such system, bacteria can sense the presence of other bacteria and determine an overall population count. When the cellular density is high enough, the bacteria begin to act as a coordinated population. Many processes are regulated by QS, such as biofilm formation, virulence and bioluminescence. In this work we described an attempt to build a synthetic QS within artificial cells to integrate artificial and natural cells. Many sensing pathways were tested *in vitro*; however, only the *Vibrio fischeri* QS pathway was found to be functional *in vitro*. Nevertheless, artificial sender cells were successfully built to synthesize several QS molecules starting from their precursors. Artificial cells were capable of sending autoinducer 2 (AI-2) for *E. coli* and *Vibrio harveyi,* N-(3-oxododecanoyl)-L-homoserine lactone (3OC12 HSL) and N-butanoyl-L-homoserine (C4 HSL) for *Pseudomonas aeruginosa* and N-3-(oxohexanoyl)homoserine lactone (3OC6 HSL) for *V. fischeri.* Finally, sensing and sending devices were joined together in a complete system within artificial cells. The resulting combinations of parts were tested by using *E. coli* reporter strains responsive to each QS molecules produced by the artificial cells in response to *V. fischeri*. Artificial cells were able to sense *V. fischeri* and to synthesize 3OC12 HSL. Thus, a complete communication module between one bacterial population, the artificial cells and a second bacterial population was successfully established. The subsequent step will be the substitution of the *E. coli* reporter strain with *P. aeruginosa* to assess a complete communication between two non-engineered bacterial populations through the artificial cells.

The artificial systems built in this study exploited an *E. coli* extract to provide the cell-free transcription-translation machinery. While functional, cellular extracts contain unknown components which leads to an incomplete understanding of the functionality of the synthetic system. To better construct a minimal system made of known components, other transcription-translation systems could be used. The PURE system is a cell-free system which allows for protein expression. The machinery is based on T7 RNA polymerase and is composed only of known, purified components. Our first attempts made with the PURE system failed to produce the AI-2 molecule. Since the PURE system lacks molecular chaperones, it is possible that HLPT did not

fold properly when expressed with the PURE system. Also, the genetic circuits designed to detect the AHLs could not use the PURE system, because the AHL responsive promoters used in the genetic devices were not recognized by T7 polymerase. Additionally, the production of AHL molecules requires the presence of acetylated ACP. In the artificial cells, the synthesis of AHLs starts from acetyl-CoA, which is a substrate of protein enzymes involved in fatty acid elongation to charge the ACPs. Therefore, to exploit the PURE system, modifications of the system would be needed. For example, for the synthesis of AHLs, purified acetylated ACPs could be used and a promoter cascade in which T7 polymerase is produced in response to AHLs could be implemented in the genetic circuit.

5.1 Future perspective

The construction of a communication pathway mediated by the artificial cells could be used as a therapeutic tool to defeat pathogenic infections. *P. aeruginosa* is an opportunistic pathogen that colonizes the lungs of cystic fibrosis patient. QS in this species mediates biofilm formation that counteracts the effect of antibiotics, making it difficult to eradicate the pathogen, which ultimately leads to the death of the patient[145]. Several studies developed seek-and-destroy bacteria to eradicate infection;[146, 147] however, these methods require the administration of engineered bacteria to the patient. The use of artificial cells capable of the same function would lead to a safer therapeutic. *P. aeruginosa* virulence genes are controlled both by QS molecules and growth related factors[148]. The artificial cells can be built to sense and release QS molecules when bacteria population has not reached yet the optimal cell density for biofilm formation. This would lead to the formation of disaggregate and incomplete biofilm structures that would be easier to disrupt. It has been shown that several bacteria naturally produce enzymes to destroy QS molecules of other species[149]. For example, *Bacillus* species are known to produce Aiia, an AHL lactonase, which hydrolyzes the homoserine lactone ring of both short and long acyl chain AHLs[150], such as C4 HSL and 3OC12 HSL. Other bacteria are known to produce AHL acylase which hydrolyze the amide bond of AHLs producing fatty acids and homoserine lactone[151]. Artificial cells could be built to express such enzymes in response to pathogens and attenuate QS. In this manner, QS molecules can cross the phospholipid membrane and be degraded by the enzymes produced inside the vesicles. Moreover, several studies focused on the synthesis of QS molecule analogs[80, 152], and so artificial cells could respond to pathogen by synthesizing and releasing such inhibitors of QS. Recently, Carbonell et al.[153] developed a web-based pathway analysis platform based on retrosynthesis[154] to search for biochemical transformations needed to obtain a specific compound starting from the chosen molecule to synthesize. The platform searches for all possible pathways found in diverse organisms that can be functional imported into the *E. coli* chassis. Such a tool could be used to determine which enzymes and precursors are needed to construct the synthetic

pathway within artificial cells composed, in part, by an *E. coli* cell extract. A different approach could be represented by targeting the *P. aeruginosa* outer membrane protein lectins. These proteins, in particular lectin A and B, present fucose and galactose binding sites involved in the binding of the pathogen to the epithelium[155]. Recent studies showed how the administration of fucose and galactose can diminish adhesion and thus biofilm formation, blocking the binding sites of lectins[156, 157] . Artificial cells containing fucose and galactose could be constructed to release sugars through αHL expression in the presence of AHLs. We experienced much difficulty in constructing an *in vitro* AI-2 sensing device. However, LsrK was functional *in vitro*. LsrK is a kinase involved in the AI-2 pathway that phosphorylates AI-2 to phospho AI-2. A recent study showed[158] how the external presence of LsrK decreases biofilm formation in *E. coli* through its natural function. When AI-2 is released from *E. coli,* it is phosphorylated by the external LsrK to phospho AI-2, and phosphorylated AI-2 cannot be taken up by *E. coli*. A similar system could be implemented with artificial cells. AI-2 is permeable to phospholipid vesicles. LsrK and ATP would be retained within and protected by the compartment, which could act as a delivery system. Phospho AI-2 produced inside the artificial cells could be used to activate the production of pore forming proteins to release the phosphorylated molecule to the outside. In this way, the action of LsrK would be dependent on the presence of *E. coli*.

The functionality of the artificial cells as QS molecule senders was demonstrated. In addition to promoting biofilm formation, QS molecules also act as chemoattractants for bacteria[159]. Thus, artificial systems could also be used to control bacterial behavior by targeting motility pathways. The bacterial population could be forced to move and to concentrate in a specific area. Such a possibility could be exploited in environment for remediation. Artificial cells could attract oil eating bacteria, for example, to sites of contamination. Subsequently, the artificial cells would degrade, and the ecosystem would return to normality.

On the other hand, the integration of artificial cells within natural cells represents a new way to evaluate how life-like artificial chemical systems are. The construction of artificial cells able to sense *V. fischeri* through 3OC6 HSL, and the ability of artificial cells to synthesize the same QS molecules were demonstrated. The union of such sensing and sending systems allows for communication between artificial cells natural bacteria. Bacteria use the QS process to recognize themselves. The presence of a certain amount of AHLs in the surrounding means for the cells the presence of their counterparts. From the reception of such signals, bacterial behavior dramatically changes. The living cell stops its behavior as single unit and starts acting as part of a coordinated population. The switch leads to a series of modifications in gene expression which are translated into several biological processes. Targeting such mechanisms through the activity of artificial cells able to mimic QS processes could be a strategy to construct an artificial system that is perceived as living by the bacteria. Due to the variety of processes that could derive from the communication between artificial cells and bacteria through QS, different ways to evaluate the bacterial response

are possible, from luminescence output to gene expression. We could then ask if the living cells are able to distinguish between the natural and artificial cells. Such an experiment would be conceptually similar to the way the Turing test is used to evaluate the intelligence of a machine. A cellular Turing test may give us a new angle from which to approach the boundary between living and nonliving chemical systems.

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Appendix

Supporting information: Fluorescent proteins and *in vitro* genetic organization for cell-free synthetic biology

SUPPORTING INFORMATION

Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology

Roberta Lentini, Michele Forlin, Laura Martini, Cristina Del Bianco, Amy C. Spencer, Domenica Torino, and Sheref S. Mansy*

Table S1. Multiple regression model parameters.

Coefficients	Estimate	Std. Error	t value	Pr (>|t|)
(Intercept)	10.343	6.656	1.554	0.137
%G_5'to RBS 2	-460.799	63.143	-7.298	6.38e-07
%U 3' to RBS 2:%A 3' to RBS 2	364.405	45.752	7.965	1.79e-07

Table S2. DNA sequences used in this study. Each sequence was immediately preceded by a T7 transcriptional promoter (TAATACGACTCACTATA) and immediately followed by a T7 transcriptional terminator (CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG) without additional residues. Start and stop codons are in bold and in bigger font and the ribosome binding site is underlined.

NAME	NOTE	SEQUENCE
RL001A	mYpet	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGTCCAAAGGCGAAGAACTGT TTACCGGTGTGGTTCCGATTCTGGTGGAACTGGATGGCGACGTTAACGGTCATAAATTTAGTGTGTCCGGCGAAGGTGAAGGCGATGCGACCTATG GCAAACTGACGCTGAAACTGCTGTGCACCACCGGTAAACTGCCGGTCCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGCGTGCAGTGTTTCG CGCGCTACCCGGACCACATGAAACAACACGATTTCTTTAAAAGTGCCATGCCGGAAGGCTATGTTCAGGAACGTACCATCTTTTTCAAAGATGACGG TAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTCAACCGTATTGAACTGAAAGGTATCGACTTCAAAGAAGATGGCAATAT TCTGGGTCATAAACTGGAATATAACTACAATAGCCACAACGTGTATATTACCGCGGATAAACAGAAAAACGGCATCAAAGCCAACTTCAAAATCCGC CATAACATCGAAGACGGCGGTGTTCAACTGGCCGATCACTACCAGCAAAACACCCCGATTGGTGATGGTCCGGTCCTGCTGCCGGATAATCATTATC TGTCATACCAGTCGAAACTGTTTAAAGACCCGAATGAAAAACGTGATCACATGGTGCTGCTGGAATTTCTGACCGCGGCCGGCATTACGGAGGGTA TGAACGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA
RL002A	Ypet	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGTCCAAAGGCGAAGAACTGT TTACCGGTGTGGTTCCGATTCTGGTGGAACTGGATGGCGACGTTAACGGTCATAAATTTAGTGTGTCCGGCGAAGGTGAAGGCGATGCGACCTATG GCAAACTGACGCTGAAACTGCTGTGCACCACCGGTAAACTGCCGGTCCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGCGTGCAGTGTTTCG CGCGCTACCCGGACCACATGAAACAACACGATTTCTTTAAAAGTGCCATGCCGGAAGGCTATGTTCAGGAACGTACCATCTTTTTCAAAGATGACGG TAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTCAACCGTATTGAACTGAAAGGTATCGACTTCAAAGAAGATGGCAATAT TCTGGGTCATAAACTGGAATATAACTACAATAGCCACAACGTGTATATTACCGCGGATAAACAGAAAAACGGCATCAAAGCCAACTTCAAAATCCGC CATAACATCGAAGACGGCGGTGTTCAACTGGCCGATCACTACCAGCAAAACACCCCGATTGGTGATGGTCCGGTCCTGCTGCCGGATAATCATTATC TGTCATACCAGTCGGCACTGTTTAAAGACCCGAATGAAAAACGTGATCACATGGTGCTGCTGGAATTTCTGACCGCGGCCGGCATTACGGAGGGTA TGAACGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA
RL003A	Venus	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**AGCAAAGGCGAAGAACTGTTCA CGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGC AAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCAC GTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAA CTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCT GGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCAC AACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTG AGTTACCAGAGCGCGCTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATG GATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCT GCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA

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RL004A!	mCyPet!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTCTCTAAAGGCGAAGAACTGT TTGGCGGTATTGTGCCGATCCTGGTTGAACTGGAAGGTGATGTCAACGGCCATAAATTTAGCGTGTCTGGCGAAGGTGAAGGCGATGCAACCTATG GTAAACTGACGCTGAAATTCATTTGCACCACCGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTCACCACCCTGACCTGGGGTGTGCAGTGTTTTTC GCGCTACCCGGATCACATGAAACAACACGACTTTTTCAAAAGCGTGATGCCGGAAGGTTATGTTCAGGAACGTACCATTTTCTTTAAAGATGACGGC AACTACAAAACCCGCGCCGAAGTTAAATTTGAAGGTGATACGCTGGTCAACCGTATTGAACTGAAAGGCATCGATTTCAAAGAAGACGGTAATATC CTGGGCCATAAACTGGAATACAACTACAACTCTCACATCGTTTACATCACCGCGGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAGCTCGCC ATAACATCACCGATGGCTCCGTGCAACTGGCCGACCACTATCAGCAAAACACGCCGATTGGTGATGGCCCGGTTATCCTGCCGGACAATCATTACCT GAGTACCCAGTCCAAACTGTCAAAAGATCCGAATGAAAAACGTGACCACATGGTCCTGCTGGAATTTGTGACGGCGGCCGGTATTACCCTGGGCAT GGATGAACTGTATAAAGGCAAAGAAAACCTGGATTTCCAAGGC**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCA CCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL005A!	mCerulean!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAAGAGCTG TTCACAGGGGTTGTTCCGATTCTGGTCGAACTGGACGGGGACGTTAATGGTCACAAATTCAGCGTTAGCGGTGAGGGCGAGGGTGATGCCACTTAT GGTAAACTGACCCTGAAATTCATCTGTACCACCGGCAAACTGCCTGTTCCTTGGCCTACACTGGTTACAACACTGACTTGGGGTGTTCAATGTTTTGC TCGCTATCCGGATCACATGAAACAGCACGATTTCTTCAAAAGCGCCATGCCTGAAGGTTATGTCCAAGAGCGTACGATCTTCTTTAAAGACGACGGC AACTATAAAACCCGTGCCGAGGTGAAATTCGAAGGTGATACCCTGGTAAACCGTATCGAACTGAAAGGGATCGACTTCAAAGAGGACGGGAACAT TCTGGGCCATAAACTGGAGTATAACGCCATCAGCGATAATGTGTATATTACCGCCGACAAACAGAAAAACGGGATCAAAGCCAACTTCAAAATCCG CCACAACATCGAGGATGGTAGCGTTCAACTGGCCGATCACTATCAACAGAATACCCCGATTGGTGATGGTCCTGTTCTGCTGCCTGATAACCACTAT CTGAGCACCCAGTCTAAACTGTCCAAAGACCCGAACGAGAAACGTGATCACATGGTTCTGCTGGAGTTTGTTACCGCTGCCGGCATTACTCTGGGTA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL006A!	TagJRFPJT!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGTCCAAAGGCGAAGAACTGA TTAAAGAAAACATGCACATGAAACTGTATATGGAGGGTACGGTGAACAATCATCACTTTAAATGCACCAGTGAAGGCGAAGGTAAACCGTACGAAG GCACCCAGACGATGCGTATCAAAGTGGTTGAAGGCGGTCCGCTGCCGTTTGCTTTCGATATTCTGGCGACCAGCTTTATGTATGGTTCTCGCACCTTC ATTAATCATACGCAGGGCATCCCGGATTTCTTTAAACAGAGCTTTCCGGAAGGTTTCACGTGGGAACGTGTCACCACGTATGAAGATGGCGGTGTGC TGACCGCCACGCAGGATACCAGCCTGCAAGACGGCTGTCTGATTTACAACGTTAAAATCCGCGGTGTCAACTTTCCGTCTAATGGCCCGGTGATGCA GAAGAAAACCCTGGGTTGGGAAGCCAATACGGAAATGCTGTATCCGGCAGATGGCGGTCTGGAAGGCCGTACCGACATGGCACTGAAACTGGTTG GCGGTGGCCATCTGATCTGCAACTTCAAAACCACGTACCGCAGCAAAAAACCGGCGAAAAATCTGAAAATGCCGGGTGTGTATTACGTTGATCACC GTCTGGAACGCATTAAAGAAGCCGACAAAGAAACCTATGTGGAACAGCATGAAGTGGCGGTTGCCCGCTACTGTGATCTGCCGTCTAAACTGGGTC ACAAACTGAACGGCATGGACGAACTGTACAAG**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACC ACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL007A!	mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTA ACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL008A!	mRFP1!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GCTTCCTCCGAAGACGTTATCAA AGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTAC CCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTT AAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTA CCGTTACCCAGGACTCCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCGGTTATGCAGAA AAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAAGACG GTGGTCACTACGACGCTGAAGTTAAAACCACCTACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTTACAAAACCGACATCAAACTGGACATCAC CTCCCACAACGAAGACTACACCATCGTTGAACAGTACGAACGTGCTGAAGGTCGTCACTCCACCGGTGCT**TAA**GCGGATCCGAATTCGAGCTCCGT CGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACC GCTGAGCAATAA!

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RL009A!	mVenus!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**AGCAAAGGCGAAGAACTGTTCA CGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGC AAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCAC GTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAA CTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCT GGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCAC AACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTG AGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATG GATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCT GCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL010A!	TJsapphire!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAAGAGCTG TTCACAGGGGTTGTTCCGATTCTGGTCGAACTGGACGGGGACGTTAATGGTCACAAATTCAGCGTTAGCGGTGAGGGCGAGGGTGATGCCACTTAT GGTAAACTGACCCTGAAATTCATCTGTACCACCGGCAAACTGCCTGTTCCTTGGCCTACACTGGTTACGACCTTCTCGTATGGGGTAATGGTTTTTGC TCGCTATCCGGATCACATGAAACAGCACGATTTCTTCAAAAGCGCCATGCCTGAAGGCTATGTCCAAGAGCGTACGATCTTCTTTAAAGACGACGGC AACTATAAAACACGTGCCGAGGTGAAATTCGAAGGCGATACCCTGGTAAACCGTATTGAACTGAAAGGGATTGACTTCAAAGAGGACGGGAACAT TCTGGGCCATAAACTGGAGTATAACTTCAACTCTCATAATGTGTATATCATGGCCGACAAACAGAAAAACGGGATCAAAGCCAACTTCAAAATCCGC CACAACATCGAGGATGGAGGCGTTCAGCTGGCAGATCACTATCAACAAAACACCCCGATTGGTGATGGTCCTGTACTGCTGCCTGATAATCACTATC TGAGCATCCAGAGTGCCCTGTCTAAAGATCCGAACGAAAAACGTGATCACATGGTGCTGCTGGAGTTTGTTACAGCTGCCGGCATTACGCTGGGTA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL011A!	eGFP!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGCAAGGGCGAGGAGCTG TTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTC AGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACG GCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAA CATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGAT CCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCA CTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCT CGGCATGGACGAGCTGTACAAG**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGA TCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL012A!	GFP!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTCTCTAAAGGCGAAGAACTGT TTACGGGTGTCGTGCCGATTCTGGTTGAACTGGATGGCGATGTTAATGGTCACAAATTCTCCGTTTCTGGCGAAGGTGAAGGCGATGCGACCTATG GTAAACTGACGCTGAAATTTATTTGCACCACGGGTAAACTGCCGGTGCCGTGGCCGACCCTGGTTACCACGTTTTCCTATGGTGTTCAGTGTTTCTCA CGCTACCCGGATCACATGAAACAACACGACTTTTTCAAATCCGCGATGCCGGAAGGTTATGTCCAGGAACGTACCATTTTCTTTAAAGATGACGGCA ACTACAAAACCCGCGCCGAAGTCAAATTTGAAGGTGATACGCTGGTGAACCGTATTGAACTGAAAGGCATCGATTTCAAAGAAGACGGTAATATCC TGGGCCATAAACTGGAATATAACTACAATTCGCACAACGTTTACATTATGGCAGATAAACAGAAAAACGGTATCAAAGTCAACTTCAAAATCCGCCA TAACATCGAAGATGGCAGCGTGCAACTGGCTGACCACTATCAGCAAAACACCCCGATCGGTGATGGCCCGGTTCTGCTGCCGGACAATCATTACCTG AGCACGCAGTCTGCACTGAGTAAAGATCCGAACGAAAAACGTGACCACATGGTGCTGCTGGAATTTGTTACGGCGGCTGGTATTACGCACGGTATG GATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCT GCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL013A!	GFPuv!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTCAGCAAAGGTGAAGAACTGT TTACGGGCGTTGTGCCGATCCTGGTGGAACTGGACGGTGATGTGAATGGTCATAAATTTTCGGTGAGCGGCGAAGGTGAAGGCGATGCGACCTAT GGTAAACTGACGCTGAAATTTATTTGCACCACGGGTAAACTGCCGGTGCCGTGGCCGACCCTGGTTACCACGTTTTCCTATGGTGTGCAGTGTTTCTC ACGTTACCCGGATCACATGAAACGCCACGACTTTTTCAAATCGGCAATGCCGGAAGGTTATGTTCAAGAACGTACCATTAGCTTTAAAGATGACGGC AATTACAAAACCCGCGCTGAAGTCAAATTCGAAGGTGATACGCTGGTGAACCGTATTGAACTGAAAGGCATCGATTTTAAAGAAGACGGTAATATC CTGGGCCATAAACTGGAATACAACTACAACTCTCACAACGTCTACATTACCGCGGATAAACAGAAAAACGGTATCAAAGCCAACTTCAAAATCCGCC ATAACATCGAAGATGGCTCCGTTCAACTGGCGGACCACTATCAGCAAAACACCCCGATCGGTGATGGTCCGGTCCTGCTGCCGGACAATCATTACCT GAGCACGCAGTCTGCCCTGAGTAAAGATCCGAACGAAAAACGTGACCACATGGTTCTGCTGGAATTTGTTACGGCGGCGGGTATCACGCACGGTAT GGATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGC TGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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CD100A!	GFPmut3b!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**CGTAAAGGAGAAGAACTTTTCA CTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAA AACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGAT ACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTA CAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGA CACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACA TTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACA CAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAAC TATACAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAA AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
CD101A!	sfGFP!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**CGTAAAGGCGAAGAGCTGTTCA CTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTA AACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGT TATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGGCACGT ACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTG GGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACA ACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGA GCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGG ATGAACTGTACAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGC TAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
CD102A!	CyPet!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTCTCTAAAGGCGAAGAACTGT TTGGCGGTATTGTGCCGATCCTGGTTGAACTGGAAGGTGATGTCAACGGCCATAAATTTAGCGTGTCTGGCGAAGGTGAAGGCGATGCAACCTAT GGTAAACTGACGCTGAAATTCATTTGCACCACCGGTAAACTGCCGGTTCCGTGGCCGAACCCTGGTCACCACCCTGACCTGGGGTGTGCAGTGTTTTT CGCGCTACCCGGATCACATGAAACAACACGACTTTTTCAAAAGCGTGATGCCGGAAGGTTATGTTCAGGAACGTACCATTTTCTTTAAAGATGACGG CAACTACAAAACCCGCGCCGAAGTTAAATTTGAAGGTGATACGCTGGTCAACCGTATTGAACTGAAAGGCATCGATTTCAAAGAAGACGGTAATAT CCTGGGCCATAAACTGGAATACAACTACAACTCTCACATCGTTTACATCACCGCGGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAGCTCGC CATAACATCACCGATGGCTCCGTGCAACTGGCCGACCACTATCAGCAAAACACGCCGATTGGTGATGGCCCGGTTATCCTGCCGGACAATCATTACC TGAGTACCCAGTCCGCACTGTCAAAAGATCCGAATGAAAAACGTGACCACATGGTCCTGCTGGAATTTGTGACGGCGGCCGGTATTACCCTGGGCA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
CD103A!	Cerulean!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAAGAGCTG TTCACAGGGGTTGTTCCGATTCTGGTCGAACTGGACGGGGACGTTAATGGTCACAAATTCAGCGTTAGCGGTGAGGGCGAGGGTGATGCCACTTAT GGTAAACTGACCCTGAAATTCATCTGTACCACCGGCAAACTGCCTGTTCCTTGGCCTACACTGGTTACAACACTGACTTGGGGTGTTCAATGTTTTGC TCGCTATCCGGATCACATGAAACAGCACGATTTCTTCAAAAGCGCCATGCCTGAAGGTTATGTCCAAGAGCGTACGATCTTCTTTAAAGACGACGGC AACTATAAAACCCGTGCCGAGGTGAAATTCGAAGGTGATACCCTGGTAAACCGTATCGAACTGAAAGGGATCGACTTCAAAGAGGACGGGAACAT TCTGGGCCATAAACTGGAGTATAACGCCATCAGCGATAATGTGTATATTACCGCCGACAAACAGAAAAACGGGATCAAAGCCAACTTCAAAATCCG CCACAACATCGAGGATGGTAGCGTTCAACTGGCCGATCACTATCAACAGAATACCCCGATTGGTGATGGTCCTGTTCTGCTGCCTGATAACCACTAT CTGAGCACCCAGTCTGCACTGTCCAAAGACCCGAACGAGAAACGTGATCACATGGTTCTGCTGGAGTTTGTTACCGCTGCCGGCATTACTCTGGGTA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGG CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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RL014A!	sfGFP! mRFP1!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGC**ATG**CGTAAAGGCGAAG AGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAA CTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTG CTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGAT GACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGG CAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAA ATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATC ACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGC ATGGTATGGATGAACTGTACAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GCTTCCTCCGAAGACGTTATCA AAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTA CCCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGT TAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTT ACCGTTACCCAGGACTCCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCGGTTATGCAGA AAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAAGAC GGTGGTCACTACGACGCTGAAGTTAAAACCACCTACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTTACAAAACCGACATCAAACTGGACATC ACCTCCCACAACGAAGACTACACCATCGTTGAACAGTACGAACGTGCTGAAGGTCGTCACTCCACCGGTGCT**TAA**CTCGAGCACCACCACCACCAC CACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL015A!	sfGFP! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GCTAGCATGCGTAAAGGCGAAG AGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAA CTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTG CTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGAT GACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGG CAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAA ATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATC ACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGC ATGGTATGGATGAACTGTACAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGA CAATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAG GTCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTA TGGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTT GAGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGT GATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACA GCGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGT TAACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGA TGAGCTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCG CTGAGCAATAA!
RL016A!	mYPet! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGTCCAAAGGCGAAGAACTGT TTACCGGTGTGGTTCCGATTCTGGTGGAACTGGATGGCGACGTTAACGGTCATAAATTTAGTGTGTCCGGCGAAGGTGAAGGCGATGCGACCTATG GCAAACTGACGCTGAAACTGCTGTGCACCACCGGTAAACTGCCGGTCCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGCGTGCAGTGTTTCG CGCGCTACCCGGACCACATGAAACAACACGATTTCTTTAAAAGTGCCATGCCGGAAGGCTATGTTCAGGAACGTACCATCTTTTTCAAAGATGACGG TAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTCAACCGTATTGAACTGAAAGGTATCGACTTCAAAGAAGATGGCAATAT TCTGGGTCATAAACTGGAATATAACTACAATAGCCACAACGTGTATATTACCGCGGATAAACAGAAAAACGGCATCAAAGCCAACTTCAAAATCCGC CATAACATCGAAGACGGCGGTGTTCAACTGGCCGATCACTACCAGCAAAACACCCCGATTGGTGATGGTCCGGTCCTGCTGCCGGATAATCATTATC TGTCATACCAGTCGAAACTGTTTAAAGACCCGAATGAAAAACGTGATCACATGGTGCTGCTGGAATTTCTGACCGCGGCCGGCATTACGGAGGGTA TGAACGAACTGTATAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATAT GGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTC CGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTC CAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGAT GGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGC CCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCT GAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACAT CAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCT!GAGTTGGCTGCTGCCACCGCTGAGC AATAA!

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RL017A!	YPet! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGTCCAAAGGCGAAGAACTGT TTACCGGTGTGGTTCCGATTCTGGTGGAACTGGATGGCGACGTTAACGGTCATAAATTTAGTGTGTCCGGCGAAGGTGAAGGCGATGCGACCTATG GCAAACTGACGCTGAAACTGCTGTGCACCACCGGTAAACTGCCGGTCCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGCGTGCAGTGTTTCG CGCGCTACCCGGACCACATGAAACAACACGATTTCTTTAAAAGTGCCATGCCGGAAGGCTATGTTCAGGAACGTACCATCTTTTTCAAAGATGACGG TAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTCAACCGTATTGAACTGAAAGGTATCGACTTCAAAGAAGATGGCAATAT TCTGGGTCATAAACTGGAATATAACTACAATAGCCACAACGTGTATATTACCGCGGATAAACAGAAAAACGGCATCAAAGCCAACTTCAAAATCCGC CATAACATCGAAGACGGCGGTGTTCAACTGGCCGATCACTACCAGCAAAACACCCCGATTGGTGATGGTCCGGTCCTGCTGCCGGATAATCATTATC TGTCATACCAGTCGGCACTGTTTAAAGACCCGAATGAAAAACGTGATCACATGGTGCTGCTGGAATTTCTGACCGCGGCCGGCATTACGGAGGGTA TGAACGAACTGTATAAA**TAA**GCGGATCCTAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATATGGC GATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTCCGT ATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTCCAA AGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGATGG TGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGCCC GGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCTGA AACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACATCA AACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCTGT ATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAA TAA!
RL018A!	mCerulean! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAAGAGCTG TTCACAGGGGTTGTTCCGATTCTGGTCGAACTGGACGGGGACGTTAATGGTCACAAATTCAGCGTTAGCGGTGAGGGCGAGGGTGATGCCACTTAT GGTAAACTGACCCTGAAATTCATCTGTACCACCGGCAAACTGCCTGTTCCTTGGCCTACACTGGTTACAACACTGACTTGGGGTGTTCAATGTTTTGC TCGCTATCCGGATCACATGAAACAGCACGATTTCTTCAAAAGCGCCATGCCTGAAGGTTATGTCCAAGAGCGTACGATCTTCTTTAAAGACGACGGC AACTATAAAACCCGTGCCGAGGTGAAATTCGAAGGTGATACCCTGGTAAACCGTATCGAACTGAAAGGGATCGACTTCAAAGAGGACGGGAACAT TCTGGGCCATAAACTGGAGTATAACGCCATCAGCGATAATGTGTATATTACCGCCGACAAACAGAAAAACGGGATCAAAGCCAACTTCAAAATCCG CCACAACATCGAGGATGGTAGCGTTCAACTGGCCGATCACTATCAACAGAATACCCCGATTGGTGATGGTCCTGTTCTGCTGCCTGATAACCACTAT CTGAGCACCCAGTCTAAACTGTCCAAAGACCCGAACGAGAAACGTGATCACATGGTTCTGCTGGAGTTTGTTACCGCTGCCGGCATTACTCTGGGTA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATAT GGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTC CGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTC CAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGAT GGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGC CCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCT GAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACAT CAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!
RL019A!	Cerulean! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAAGAGCTG TTCACAGGGGTTGTTCCGATTCTGGTCGAACTGGACGGGGACGTTAATGGTCACAAATTCAGCGTTAGCGGTGAGGGCGAGGGTGATGCCACTTAT GGTAAACTGACCCTGAAATTCATCTGTACCACCGGCAAACTGCCTGTTCCTTGGCCTACACTGGTTACAACACTGACTTGGGGTGTTCAATGTTTTGC TCGCTATCCGGATCACATGAAACAGCACGATTTCTTCAAAAGCGCCATGCCTGAAGGTTATGTCCAAGAGCGTACGATCTTCTTTAAAGACGACGGC AACTATAAAACCCGTGCCGAGGTGAAATTCGAAGGTGATACCCTGGTAAACCGTATCGAACTGAAAGGGATCGACTTCAAAGAGGACGGGAACAT TCTGGGCCATAAACTGGAGTATAACGCCATCAGCGATAATGTGTATATTACCGCCGACAAACAGAAAAACGGGATCAAAGCCAACTTCAAAATCCG CCACAACATCGAGGATGGTAGCGTTCAACTGGCCGATCACTATCAACAGAATACCCCGATTGGTGATGGTCCTGTTCTGCTGCCTGATAACCACTAT CTGAGCACCCAGTCTGCACTGTCCAAAGACCCGAACGAGAAACGTGATCACATGGTTCTGCTGGAGTTTGTTACCGCTGCCGGCATTACTCTGGGTA TGGATGAACTGTATAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATAT GGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTC CGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTC CAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGAT GGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGC CCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCT GAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACAT CAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!

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RL020A!	mVenus! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**AGCAAAGGCGAAGAACTGTTCA CGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGC AAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCAC GTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAA CTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCT GGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCAC AACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTG AGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATG GATGAACTGTATAAA**TAA**GCGGATCCTGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATATG GCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTCC GTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTCC AAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGAT GGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGC CCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCT GAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACAT CAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!
RL021A!	Venus! mCherry!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**AGCAAAGGCGAAGAACTGTTCA CGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGC AAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCAC GTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAA CTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCT GGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCAC AACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTG AGTTACCAGAGCGCGCTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATG GATGAACTGTATAAA**TAA**GCGGATCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGACAATATGG CGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGGTCGTCCGT ATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTATGGCTCCAA AGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTGAGGATGG TGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTGATGGCCC GGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAGCGTCTGA AACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTTAACATCA AACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGATGAGCTGT ATAAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAA TAA!
RL024A!	mCherry! mVenus!! 20!bp! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGATCCTG GTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAACTGAT TTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACATGAAA CGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCGGAAG TTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAATACA ACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCGGTGT TCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACTGTCT AAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TAA**CTC GAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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RL025A!	mCherry! mVenus!! 0!bp! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**AAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGT GAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCC GGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTG CGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGC TGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTA CATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCA GCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACG CGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCA CTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL026A!	mCherry! mVenus!! 5!bp! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGAAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGATCCTGGTTGAACTGGATGG CGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAACTGATTTGCACCACGGGTAA ACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACATGAAACGCCATGATTTCTTTA AATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCG ATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAATACAACTACAACAGTCATAA CGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCGGTGTTCAGCTGGCCGATCA TTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAA AAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TAA**CTCGAGCACCACCACCAC CACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
RL027A!	mCherry! mVenus!! 31!bp! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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RL030A!	mCherry! mVenus!! 50lbp! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATCATAGTCATATCTTTCAATAAGGAGAATAATCT**ATG**AGCAAAGGCGAAG AACTGTTCACGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGA CCTACGGCAAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGT GTTTCGCACGTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGAT GATGGTAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGC AATATTCTGGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAA TCCGTCACAACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATC ATTATCTGAGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCA TGGTATGGATGAACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTG CTGCCACCGCTGAGCAATAA!
RL031A!	mCherry! mVenus!! 3lbplspacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAA**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTC CGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTG AAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATC ACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCG CGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACT GGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGAT GGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL032A!	mCherry! mVenus!! J2lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAG**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGATC CTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAACT GATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACATG AAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCGG AAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAAT ACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCG GTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACT GTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TA** **A**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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RL033A!	mCherry! mVenus!! 7!bp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTA**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTG GTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACG CTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGG ATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAAC CCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAA ACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAA GATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAG AGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTG TATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCA ATAA!
RL034A!	mCherry! mVenus!! 9!bp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGT GGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGAC GCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCG GATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAA CCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACA AACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGA AGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCA GAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!
AS001A!	mCherry! mVenus!! 5!bp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGT TCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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AS002A!	mCherry! mVenus!! 4!bp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAAT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTT CCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
AS006A!	mCherry! mVenus!! J1!bp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGA**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGAT CCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAAC TGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACAT GAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCG GAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAA TACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCG GTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACT GTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TA** **A**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
AS007A!	mCherry! mVenus!! 10!bp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTG TGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGA CGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCC GGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAA ACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCAC AAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCG AAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCA GAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!

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DT006A!	mCherry! mVenus!! 2lbp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATA**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCC GATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGA AACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCA CATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGC GCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTG GAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATG GCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCA AACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCCATTACGCATGGTATGGATGAACTGTATA AA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
DT007A!	mCherry! mVenus!! 0lbp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAA**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGA TCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAA CTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACA TGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGC GGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGA ATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGC GGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAAC TGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**T** **AA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!
DT008A!	mCherry! mVenus!! 1lbp!spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAAT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCG ATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAA ACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCAC ATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCG CGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGG AATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGG CGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAA CTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA **TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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LM003A!	mCherry! mVenus!! 8lbplspacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTAC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGT GGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGAC GCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCG GATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAA CCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACA AACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGA AGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCA GAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACT GTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC AATAA!
LM022A!	mCherry! mVenus!! 11lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTTC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGT GTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTG ACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACC CGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAA AACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCA CAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATC GAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTAC CAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAA CTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGA GCAATAA!
LM023A!	mCherry! mVenus!! 12lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTTCA**ATG**AGCAAAGGCGAAGAACTGTTCACGGG TGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACT GACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTAC CCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACA AAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTC ACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACAT CGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTA CCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGA ACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTG AGCAATAA!

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LM024A!	mCherry! mVenus!! 13lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTTCAA**ATG**AGCAAAGGCGAAGAACTGTTCACGG GTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAAC TGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTA CCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTAC AAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGT CACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACA TCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTA CCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGA ACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTG AGCAATAA!
RL043A!	mCherry! mVenus!! 14lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTTCAAT**ATG**AGCAAAGGCGAAGAACTGTTCACG GGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAA ACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGT TACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACT ACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGG GTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAA CATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAG TTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGA TGAACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCG CTGAGCAATAA!
RL044A!	mCherry! mVenus!! 15lbp! spacer! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCTACTTCAATT**ATG**AGCAAAGGCGAAGAACTGTTCAC GGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCA AACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACG TTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACT ACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGG GTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAA CATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAG TTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGA TGAACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCG CTGAGCAATAA!

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LM005A!	mCherry! mVenus!! 3’!NdeI!! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAACAT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM006A!	mCherry! mVenus!! 3’!BamHI!! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAAGGATCC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM007A!	mCherry! mVenus!! 3’!NheI!! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAAGCTAGC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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LM008A!	mCherry! mVenus!! 3’!EcoRI*!* !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAAGAATTC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM009A!	mCherry! mVenus!! 3’!NotI! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGGCGGCCGC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM010A!	mCherry! mVenus!! 3’!scar!1! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGTACTAGAG**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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LM011A!	mCherry! mVenus!! 3’lscarl2! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATACTAG**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM012A!	mCherry! mVenus!! JI3IA! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAAACT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM013A!	mCherry! mVenus!! RBSl+1! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGGATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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LM014A!	mCherry! mVenus!! RBS!+2! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGATTAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGT TCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM015A!	mCherry! mVenus!! RBS!+1+2! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGGTTAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM016A!	mCherry! mVenus!! 3'!pET21b! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGATATACAT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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RL050A!	mCherry! mVenus!! 3’lCJrich! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGCCCCCTCC**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGT TCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM018A!	mCherry! mVenus!! GTG! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCT**GTG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM019A!	mCherry! mVenus!! TTG! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCT**TTG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGT TCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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LM020A!	mCherry! mVenus!! CTG! !	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTATAAGGAGAATAATCT**CTG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGT TCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCT GAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGAT CACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
LM021A!	mCherry! mVenus!! 5’lG!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAACTTAGAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL036A!	mCherry! mVenus!! 5’lNdel!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGACATATGAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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RL037A!	mCherry! mVenus!! 5’!BamHI!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAGGATCCAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL038A!	mCherry! mVenus!! 5’!NheI!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAGCTAGCAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL039A!	mCherry! mVenus!! 5’!EcoRI!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGAGAATTCAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!

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RL040A!	mCherry! mVenus!! 5’!NotI!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTGCGGCCGCAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL041A!	mCherry! mVenus!! 5’!scaI!1!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**GCGGATCCGAATTCAATTAGTTTTACTAGAGAAGGAGAATAATCT**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGG TTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGTCATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGC TGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGA TCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCCGGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCC GCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAACCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAAC TGGAATACAACTACAACAGTCATAACGTGTACATTACCGCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGA TGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAACACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGC AAACTGTCTAAAGATCCGAATGAAAAACGCGATCACATGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTAT AAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA A!
RL045A!	mCherry! mVenus!! minimal!	GGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GTGAGTAAAGGCGAGGAGGAC AATATGGCGATCATCAAAGAGTTCATGCGCTTCAAAGTCCACATGGAAGGCAGCGTTAATGGTCACGAGTTCGAAATTGAGGGCGAAGGCGAAGG TCGTCCGTATGAGGGTACACAGACCGCTAAACTGAAAGTCACGAAAGGTGGTCCACTGCCATTTGCTTGGGATATTCTGAGCCCACAGTTCATGTAT GGCTCCAAAGCCTATGTGAAACATCCGGCCGATATTCCGGACTATCTGAAACTGAGCTTCCCTGAAGGGTTCAAATGGGAACGTGTGATGAACTTTG AGGATGGTGGTGTTGTGACAGTGACACAGGATTCTAGCCTGCAAGACGGTGAGTTCATCTATAAAGTGAAACTGCGTGGCACGAATTTTCCGAGTG ATGGCCCGGTTATGCAGAAAAAAACGATGGGTTGGGAGGCCTCTAGTGAGCGTATGTATCCAGAAGATGGCGCTCTGAAAGGCGAAATCAAACAG CGTCTGAAACTGAAAGATGGTGGCCACTATGATGCCGAAGTGAAAACCACGTATAAAGCCAAAAAACCTGTCCAACTGCCTGGTGCCTATAACGTT AACATCAAACTGGACATCACCTCACACAATGAGGACTATACGATCGTGGAGCAGTATGAGCGTGCTGAAGGACGTCATTCTACCGGTGGTATGGAT GAGCTGTATAAA**TAA**AAGGAG**ATG**AGCAAAGGCGAAGAACTGTTCACGGGTGTGGTTCCGATCCTGGTTGAACTGGATGGCGATGTGAACGGT CATAAATTTAGCGTGTCTGGTGAAGGCGAAGGTGATGCGACCTACGGCAAACTGACGCTGAAACTGATTTGCACCACGGGTAAACTGCCGGTTCCG TGGCCGACCCTGGTGACCACGCTGGGTTATGGTCTGATGTGTTTCGCACGTTACCCGGATCACATGAAACGCCATGATTTCTTTAAATCTGCGATGCC GGAAGGCTATGTGCAGGAACGTACCATCTTTTTCAAAGATGATGGTAACTACAAAACCCGCGCGGAAGTTAAATTTGAAGGCGATACGCTGGTGAA CCGTATTGAACTGAAAGGTATCGATTTCAAAGAAGATGGCAATATTCTGGGTCACAAACTGGAATACAACTACAACAGTCATAACGTGTACATTACC GCCGATAAACAGAAAAACGGTATCAAAGCAAACTTCAAAATCCGTCACAACATCGAAGATGGCGGTGTTCAGCTGGCCGATCATTACCAGCAGAAC ACCCCGATTGGCGATGGTCCGGTGCTGCTGCCGGATAATCATTATCTGAGTTACCAGAGCAAACTGTCTAAAGATCCGAATGAAAAACGCGATCACA TGGTTCTGCTGGAATTTGTGACCGCGGCCGGCATTACGCATGGTATGGATGAACTGTATAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATC CGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA!

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Table S3. Excitation and emission wavelengths used for each fluorescent protein. The same excitation and emission wavelengths were used for the monomeric versions of the fluorescent proteins.

Protein	Excitation wavelength (nm)	Emission wavelength (nm)
Cerulean	433	475
CyPet	435	477
GFPuv	395	509
TJSapphire	399	511
eGFP	488	507
GFP	475	509
sfGFP	485	510
GFPmut3b	501	511
Venus	515	528
YPet	517	530
TagRFPJT	555	584
mRFP1	584	607
mCherry	587	610

Table S4. Fitting of single fluorescent protein synthesis kinetic data.

Protein	Upper Asymptote (a.u.)	$t_{f/2}$ (min)	Growth Rate (intensity min^{-1})
mCerulean	6182	121.6	0.030
Cerulean	5843	88.8	0.042
GFPuv	9632	125.5	0.026
TJSapphire	40919	226.0	0.025
eGFP	2136	88.5	0.032
GFP	1765	120.0	0.024
sfGFP	27714	91.7	0.038
GFPmut3b	15967	78.7	0.040
mVenus	37556	103.6	0.031
Venus	51874	116.6	0.036
mYPet	48637	118.5	0.036
YPet	51212	149.3	0.034
TagRFPJT	1838	370.0	0.013
mRFP1	1981	171.2	0.035
mCherry	1588	192.9	0.029

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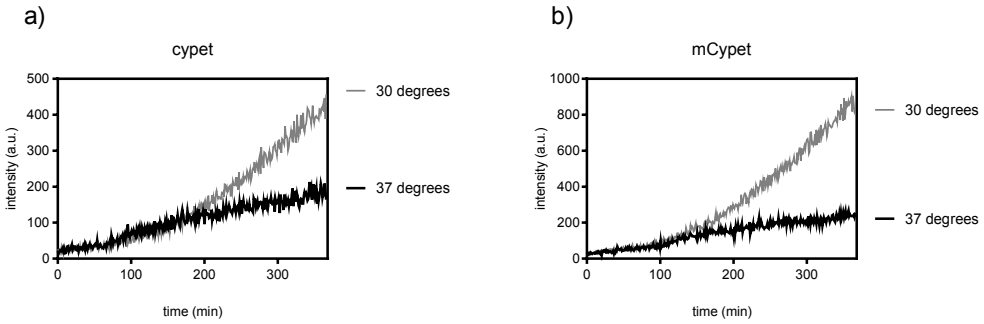

Figure S1. CyPet (a) and mCyPet (b) *in vitro* expression at 30 °C and 37 °C with the PURExpress system. !!
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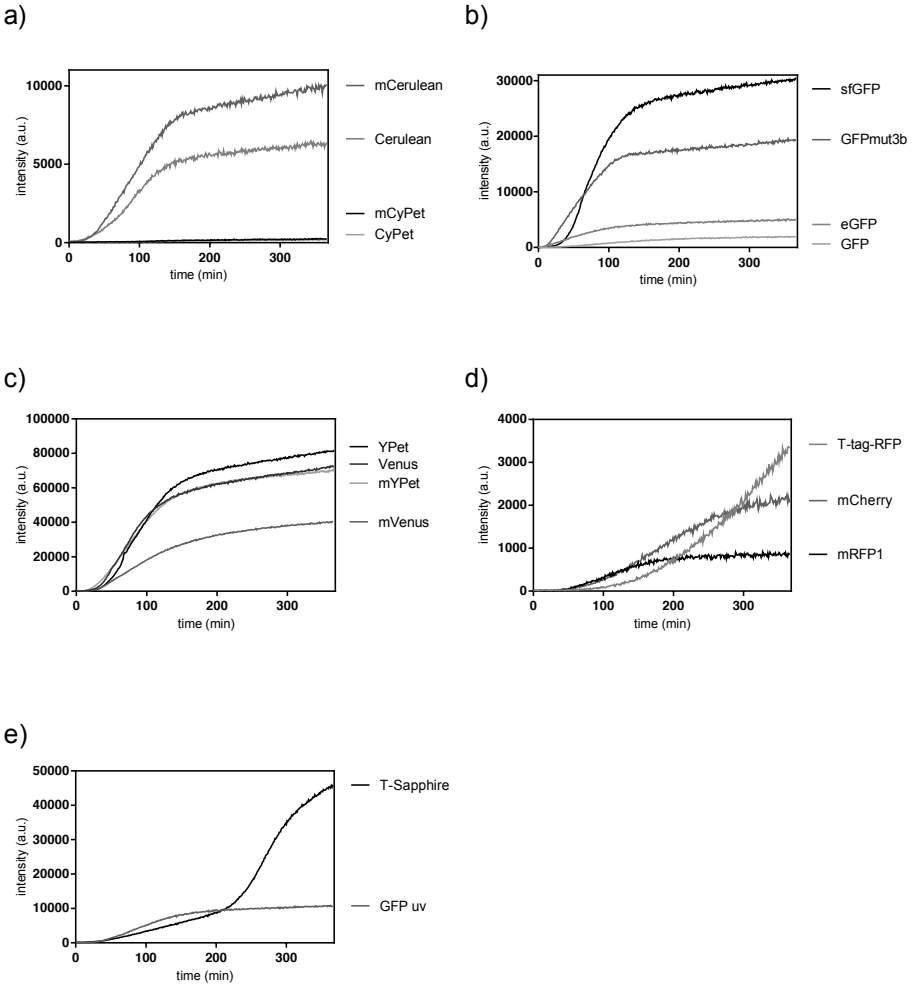

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Figure*S2.The*kinetics*profiles*of*the*17*different*fluorescent*proteins*screened.**
Cyan!fluorescent!proteins!(a),!Green!fluorescent!proteins!(b),!yellow!fluorescent!proteins!(c),!red!fluorescent!proteins!(d)!and!UVJexcitable!fluorescent!proteins!(e)!were!expressed!*in!vitro*!with!the!PURExpress!at!37!°C.!

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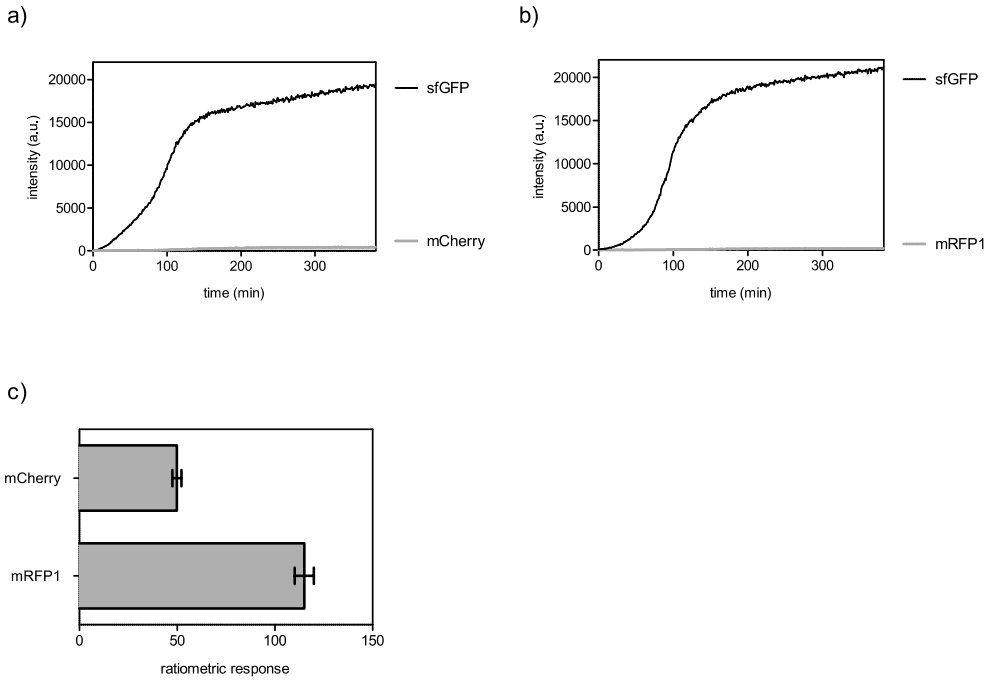


Figure S3. Green and red fluorescent proteins expressed from a bicistronic message.
Bicistronic constructs containing sfGFP and mCherry (a) and sfGFP and mRFP1 (b) were expressed *in vitro* with the PUREsystem at 37 °C. (c) The ratio of sfGFP fluorescence intensity divided by the fluorescence intensity of the red fluorescent protein after six hours shows that the sfGFP - mCherry pair is more similar in intensity.

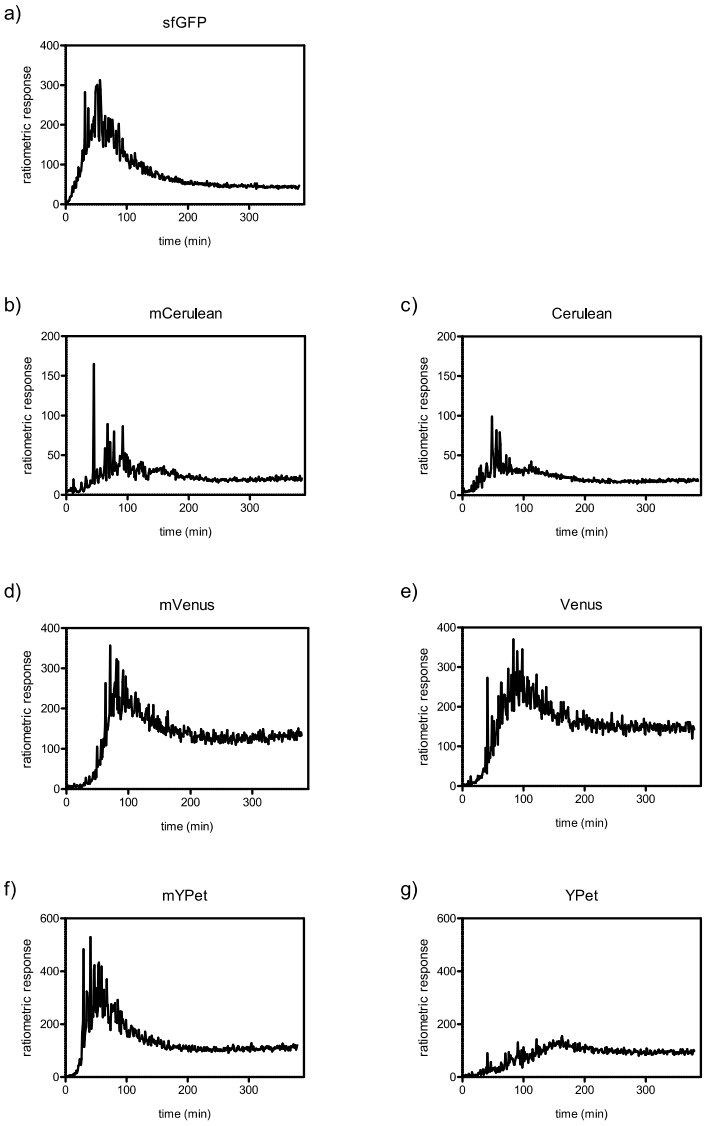
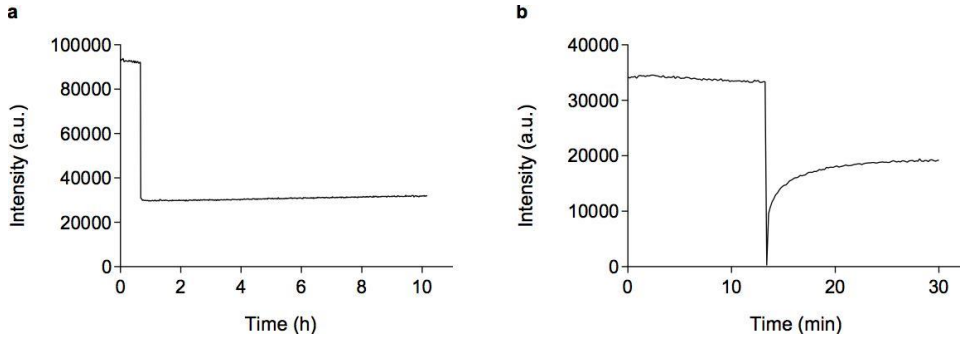


Figure S4. Representative ratiometric profiles of bicistronic constructs containing mCherry over 6 h.
The bicistronic constructs were expressed *in vitro* with the PURExpress system at 37 °C. The ratio was obtained by dividing the intensity of the first protein by the intensity of mCherry. sfGFP-mCherry (a), mCerulean-mCherry (b), Cerulean-mCherry (c), mVenus-mCherry (d), Venus-mCherry (e), mYPet-mCherry (f), and YPet-mCherry (g) all show stable readings after 3 h of expression.

Figure 55. Aligned spacing between E. coli 16S rRNA and the reference construct RL027A.

The 3' terminus of E. coli 16S rRNA that contains the anti-RBS sequence is shown base-paired with the reference construct RL027A. The boxed and grey shaded region is assigned as position 0. Therefore, in RL027A the aligned spacing between the RBS and the start codon is 6. The start codon is underlined.

Supplementary information: Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behavior.



Supplementary Figure 1. Vesicle permeability to IPTG. (A) A shrink-swell[2-4] assay was used to assess whether IPTG was capable of crossing vesicle membranes. POPC:cholesterol vesicles with entrapped calcein were prepared in 10 mM $MgCl_2$, 100 mM KCl, 50 mM HEPES, pH 7.6 as described in the methods and subsequently purified by gel filtration chromatography with sepharose-4b. An aliquot of the vesicle sample was diluted two-fold with 1.0 M IPTG (final concentration = 0.5 M) at 37 °C. The reaction was monitored by fluorescence with excitation and emission wavelengths of 495 nm and 515 nm, respectively. The rapid decrease in fluorescence was due to both dilution with the solute solution and calcein self-quenching. If IPTG were capable of crossing the membrane, a recovery of fluorescence would have been observed. (B) The permeability of POPC vesicles to ribose was observable with the shrink-swell assay. The recovery in fluorescence after two-fold dilution with 1.0 M ribose (final concentration = 0.5 M) was due to equilibration of ribose and water across the membrane.

Supplementary Table 1. DNA sequences used in this study

NAME	NOTE	SEQUENCE
AS014A	T7 promoter, K30S E31T αHL-sfGFP	ATTTAATACGACTCACTATAG**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTC CAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATTCTACCAACGGTATGCATAAAAAAGTGT TTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTG CGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTT TAAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCA TCGATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACG GGTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGG ATTTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATG GTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTAT GAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTG CTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCA GACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATT GGAAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGA AAAAGAAGAAATGACCAACGGATCCGGCAGCGGTTCT**ATG**CGTAAAGGCGAAGAGCTGTTCACTGG TGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGG GTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTA CCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATG AAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAA GGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATT GAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTA ACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGC CACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATG GTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAG AAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAAC TGTACAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGA AGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCT TGAGGGGTTTTTTG
DT101A	T7 promoter, αHL-His tag	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACT TTAAGAAGGAGATATACAT**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTCC AATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTGTT TTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGC GGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTT AAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCAT CGATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGG GTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGAT TTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGT GAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGA AAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCT GTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGA CCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGG AAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAA AAGAAGAAATGACCAACCTCGAGCACCACCACCACCACCAC**TGA**GATCCGGCTGCTAACAAAGCCCG AAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAAC GGGTCTTGAGGGGTTTTTTG
JF001A	T7 promoter, <u>theophylline riboswitch</u> αHL	AATTAATACGACTCACTATAG<u>GGTGATACCAGCATCGTCTTGATGCCCTTGGCAGCACCCTGCTAAGG TAACAACAAG</u>**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTCCAATACCACCG TTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAA AAAGTGTTTTACTCGTTT ATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCGGGTCAATA CCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCAG CTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAA

		AGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTG GCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACC ATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGA ACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCGC AACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCTGTCCAGCG GTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGACCAACATT GATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGGCAC CAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAA ATGACCAAC**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGG AAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTC TTGAGGGGTTTTTTG
RL067A	T7 promoter, αHL	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACT TTAAG*AAGGAG*ATATACAT**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTCC AATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTGTT TTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGC GGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTT AAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCAT CGATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGG GTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGAT TTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGT GAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGA AAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCT GTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGA CCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGG AAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAA AAGAAGAAATGACCAAC**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC CCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCT AAACGGGTCTTGAGGGGTTTTTTG
RL069A	T7 promoter, <u>theophylline riboswitch</u>, K30S E31T αHL	AATTAATACGACTCACTATAG<u>GGTGATACCAGCATCGTCTTGATGCCCTTGGCAGCACCCTGCTAAGG TAACAACAAG</u>**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTCCAATACCACC GTTAAAACCGGTGATCTGGTGACCTATGATTCTACCAACGGTATGCATAAAAAAGTGTTTTACTCGTTT ATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCGGGTCAATA CCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCAG CTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAA AGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTG GCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACC ATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGA ACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCGC AACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCTGTCCAGCG GTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGACCAACATT GATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGGCAC CAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAA ATGACCAACGGATCCGGCAGCGGTTCT**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTA TTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGA CGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGA CTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCAT GACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGG CACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAA GGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAA TGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGG AGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTG CTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCA TATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA**

		CTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGG CTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTT TTG
SP002A	T7 promoter, αHL-sfGFP	ATTTAATACGACTCACTATAG**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTC CAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTGT TTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTG CGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTT TAAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCA TCGATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACG GGTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGG ATTTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATG GTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTAT GAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTG CTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCA GACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATT GGAAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGA AAAAGAAGAAATGACCAACGGATCCGGCAGCGGTTCT**ATG**CGTAAAGGCGAAGAGCTGTTCACTGG TGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGG GTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTA CCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATG AAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAA GGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATT GAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTA ACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGC CACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATG GTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAG AAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAAC TGTACAAA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGA AGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCT TGAGGGGTTTTTTG
SP011A	T7 promoter, <u>theophylline riboswitch</u>, αHL-sfGFP	AATTAATACGACTCACTATAG<u>GGTGATACCAGCATCGTCTTGATGCCCTTGGCAGCACCCTGCTAAGG TAACAACAAG</u>**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGATATCGGCTCCAATACCACC GTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTGTTTTACTCGTT TATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCGGGTCAAT ACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCA GCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCA AAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATT GGCGGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAAC CATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAG AACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCG CAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCTGTCCAGC GGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGACCAACAT TGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGGCA CCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGA AATGACCAACGGATCCGGCAGCGGTTCT**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCT ATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTG ACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCG ACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCAT GACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGG CACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAA GGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAA TGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGG AGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTG

		CTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCA TATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA** CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGG CTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTT TTG

Start and stop codons are in bold and the theophylline riboswitch is underlined. The T7 promoter and T7 terminator sequences are TAATACGACTCACTATA and CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG, respectively.

5

Supplementary Table 2. The activity of cell-free expressed αHL

construct name	$t_{1/2}$ (min)	Comments
JF001A	> 30	αHL behind theophylline riboswitch in the absence of theophylline
JF001A	16.5	αHL behind theophylline riboswitch in the presence of theophylline
DT101A	> 30	αHL-His tagged
SP002A	4.5	αHL-GFP
RL067A	10.0	αHL
Sigma-Aldrich αHL	9.5	commercial αHL
CD101A[1]	> 30	GFP
AS014A	> 30	K30S E31T αHL-GFP lacking an internal RBS

Each construct was expressed *in vitro* with the PURE system and subsequently added to rabbit red blood cells. Hemolysis was measured by attenuance as described in the methods. When indicated, the theophylline concentration was 1.5 mM. Sigma-Aldrich αHL indicates purchased purified protein and was not *in vitro* expressed.

Supplementary References

1. Lentini R.*, et al.* Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology. *ACS Synth. Biol.* **2**, 482-489 (2013).

2. Bittman R. & Blau L. Permeability behavior of liposomes prepared from fatty acids and fatty acid methyl esters. *Biochim. Biophys. Acta* **863**, 115-120 (1986).

3. Chen P. Y., Pearce D. & Verkman A. S. Membrane water and solute permeability determined quantitatively by self-quenching of an entrapped fluorophore. *Biochemistry* **27**, 5713-5718 (1988).

4. Sacerdote M. G. & Szostak J.W. Semipermeable lipid bilayers exhibit diastereoselectivity favoring ribose. *Proc. Natl Acad. Sci. USA.* **102**, 6004-6008 (2005).

Table 1. DNA sequences used in chapter 4.

NAME	NOTE	SEQUENCE*
CD100A	pT7 RBS GFP mut3b T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGGA* *G*ATATACATATGGCTAGC**ATG**CGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT GATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTT ATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGA GATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATAT TTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGT TAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTAT ACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTT CAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCA CACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGA TTACACATGGCATGGATGAACTATACAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCT GAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
CD101A	pT7 RBS sfGFP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGA GATATACATATGGCTAGC**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGT GATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTT CATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCT CGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATT TCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGA GCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGT TTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGT GCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAG CACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGG GCATCACGCATGGTATGGATGAACTGTACAAA**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGAC TCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACC GCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
CD200K	pT7 RBS lsrR T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACC**ATG**GATGGCAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGG ATCGCGTGGTTTTACTATCACGACGGGCTGACCCAGAGCGAGATCAGCGATCGTCTCGGCCTGACACGTTTGAAAGTG TCGCGATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGCTGTCTGGAA TATGAAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTGCGGATGCTGATGTCGGT GGGCGACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACAACAGATGCTGGCGATTGGTTTTGG CGAGGCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCGTCACAGCAAATTCGCCTGGTCACGCTCTCCGGT GGCGTCGGTTCTTATATGACGGGAATCGGGCAGCTTAACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCG GGCATCCTCCGCTGACATTGCCCGTACGCTAAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGC GGATGTGGCGATTGTCGGCATTGGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCA GGGCGAACAGTTAATGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACG TTGTCACGAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTTG GCGTGGCAGGGGGAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTAC CGATCAGGACACAGCAGCGGCGATTTTACGTAGTCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGC**TAA**CA AAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGG GTCTTGAGGGGTTTTTTG
CD201K	pT7 RBS lsrK T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACC**ATG**GCTCGACTCTTTACCCTTTCAGAATCAAAGTACTACCTGATGGCGCTGGATGCAGGCACCGGAAG TATTCGGGCTGTGATATTCGACCTGGAAGGCAATCAAATAGCAGTGGGACAGGCGGAGTGGCGGCATCTGGCAGTA CCGGACGTTCCTGGTTCTATGGAATTTGATCTCAACAAAAACTGGCAACTGGCGTGTGAGTGTATGCGCCAGGCGCTG CACAACGCCGGCATAGCCCCGGAGTATATCGCTGCCGTTTCGGCATGTTCGATGCGTGAAGGCATTGTTTTATATAAT AATGAAGGAGCCCCGATCTGGGCCTGCGCCAATGTGGATGCCAGAGCGGCACGCGAAGTTAGCGAACTTAAAGAAC TGCACAACAATACCTTTGAAAACGAAGTTTATCGCGCGACCGGACAAACACTGGCTTTAAGTGCCATCCCCAGATTAC TTTGGCTGGCGCACCATCGTTCCGATATTTACCGTCAGGCATCAACCATCACCATGATCAGCGACTGGCTGGCCTATAT GCTCAGCGGCGAACTGGCGGTGGATCCCTCTAACGCTGGCACCACGGGACTTCTTGATCTAACCACCCGTGACTGGAA ACCTGCATTGCTGGATATGGCTGGCCTACGTGCCGATATTCTTTCTCCTGTCAAAGAAACCGGCACATTGCTGGGCGT GGTAAGTTCACAAGCGGCGGAACTCTGCGGTCTGAAGGCGGGCACTCCGGTGGTCGTTGGAGGAGGCGACGTGCAG CTTGGTTGCCTTGGGTTAGGCGTTGTGCGTCCGGCACAAACCGCGGTTCTTGGCGGCACATTCTGGCAGCAAGTTGTA AATTTAGCCGCGCCGGTGACAGACCCAGAAATGAACGTGCGCGTTAATCCTCATGTTATTCCTGGCATGGTACAAGCT GAATCTATAAGCTTTTTTACCGGACTCACCATGCGCTGGTTCCGCGATGCTTTCTGTGCCGAAGAAAAACTGATTGCCG AACGTTTAGGCATCGACACCTATACGCTGCTGGAAGAGATGGCCAGTCGGGTGCCGCCTGGGTCGTGGGGCGTAATG CCGATCTTCTCCGACAGAATGCGCTTTAAAACCTGGTATCACGCTGCGCCTTCCTTTATTAACTTGTCCATTGACCCGGA TAAATGTAACAAAGCGACATTGTTCCGTGCGCTGGAAGAAAATGCGGCGATTGTATCAGCGTGTAACTTGCAGCAAA TTGCTGATTTCTCGAATATTCATCCTTCATCGTTAGTCTTTGCAGGCGGAGGTTCAAAAGGGAAATTATGGAGTCAAAT TCTCGCTGATGTCTCGGGATTACCCGTCAATATTCCGGTGGTCAAAGAAGCCACTGCATTAGGATGTGCCATTGCAGC TGGCGTCGGTGCCGGAATTTTTTCATCAATGGCAGAAACCGGAGAACGCCTGGTTCGCTGGGAACGGACGCACACAC CAGACCCGGAAAAGCATGAACTTTATCAGGATTCACGCGATAAGTGGCAGGCAGTTTATCAGGATCAGCTGGGGCTG GTTGATCATGGACTGACGACGTCGTTATGGAAAGCGCCTGGGTTA**TAG**TCTCGAGCACCACCACCACCACCACTGAG ATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTT

		GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
JF006A	pT7 RBS HLPT T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGGA* *G*ATATACATATG**ATG**CCGTTGTTAGATAGCTTCACAGTCGATCATACCCGGATGGAAGCGCCTGCAGTTCGGGTGGCG AAAACAATGAACACCCCGCATGGCGACGCAATCACCGTGTTCGATCTGCGCTTCTGCGTGCCGAACAAAGAAGTGAT GCCAGAAAGAGGGATCCATACCCTGGAGCACCTGTTTGCTGGTTTTATGCGTAACCATCTTAACGGTAATGGTGTAGA GATTATCGATATCTCGCCAATGGGCTGCCGCACCGGTTTTTATATGAGTCTGATTGGTACGCCAGATGAGCAGCGTGT TGCTGATGCCTGGAAAGCGGCAATGGAAGACGTGCTGAAAGTGCAGGATCAGAATCAGATCCCGGAACTGAACGTC TACCAGTGTGGCACTTACCAGATGCACTCGTTGCAGGAAGCGCAGGATATTGCGCGTAGCATTCTGGAACGTGACGT ACGCATCAACAGCAACGAAGAACTGGCACTGCCGAAAGAGAAGTTGCAGGAACTGCACATCggccgaaagatccgacctcg agat**ATG**AAAATCGGCATCATTGGTGCAATGGAAGAAGAAGTTACGCTGCTGCGTGACAAAATCGAAAACCGTCAAA CTATCAGTCTCGGCGGTTGCGAAATCTATACCGGCCAACTGAATGGAACCGAGGTTGCGCTTCTGAAATCGGGCATCG GTAAAGTCGCTGCGGCGCTGGGTGCCACTTTGCTGTTGGAACACTGCAAGCCAGATGTGATTATTAACACCGGTTCTG CCGGTGGCCTGGCACCAACGTTGAAAGTGGGCGATATCGTTGTCTCGGACGAAGCACGTTATCACGACGCGGATGTC ACGGCATTTGGTTATGAATACGGTCAGTTACCAGGCTGTCCGGCAGGCTTTAAAGCTGACGATAAACTGATCGCTGCC GCTGAGGCCTGCATTGCCGAACTGAATCTTAACGCTGTACGTGGCCTGATTGTTAGCGGCGACGCTTTCATCAACGGT TCTGTTGGTCTGGCGAAAATCCGCCACAACTTCCCACAGGCCATTGCTGTAGAGATGGAAGCGACGGCAATCGCCCAT GTCTGCCACAATTTCAACGTCCCGTTTGTTGTCGTACGCGCCATCTCCGACGTGGCCGATCAACAGTCTCATCTTAGCTT CGATGAGTTCCTGGCTGTTGCCGCTAAACAGTCCAGCCTGATGGTTGAGTCACTGGTGCAGAAACTTGCACATGGC**TA** **A**GAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACTGAGATCCGGCTGCTAACA AAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGG GTCTTGAGGGGTTTTTTG
JF008A	pTet B0034 luxR B0015 pLuxR B0032 HLPT B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ATGCCGTTGTTA GATAGCTTCACAGTCGATCATACCCGGATGGAAGCGCCTGCAGTTCGGGTGGCGAAAACAATGAACACCCCGCATGG CGACGCAATCACCGTGTTCGATCTGCGCTTCTGCGTGCCGAACAAAGAAGTGATGCCAGAAAGAGGGATCCATACCC TGGAGCACCTGTTTGCTGGTTTTATGCGTAACCATCTTAACGGTAATGGTGTAGAGATTATCGATATCTCGCCAATGG GCTGCCGCACCGGTTTTTATATGAGTCTGATTGGTACGCCAGATGAGCAGCGTGTTGCTGATGCCTGGAAAGCGGCA ATGGAAGACGTGCTGAAAGTGCAGGATCAGAATCAGATCCCGGAACTGAACGTCTACCAGTGTGGCACTTACCAGAT GCACTCGTTGCAGGAAGCGCAGGATATTGCGCGTAGCATTCTGGAACGTGACGTACGCATCAACAGCAACGAAGAAC TGGCACTGCCGAAAGAGAAGTTGCAGGAACTGCACATCggccgaaagatccgacctcgagat**ATG**AAAATCGGCATCATTGG TGCAATGGAAGAAGAAGTTACGCTGCTGCGTGACAAAATCGAAAACCGTCAAACTATCAGTCTCGGCGGTTGCGAAA TCTATACCGGCCAACTGAATGGAACCGAGGTTGCGCTTCTGAAATCGGGCATCGGTAAAGTCGCTGCGGCGCTGGGT GCCACTTTGCTGTTGGAACACTGCAAGCCAGATGTGATTATTAACACCGGTTCTGCCGGTGGCCTGGCACCAACGTTG AAAGTGGGCGATATCGTTGTCTCGGACGAAGCACGTTATCACGACGCGGATGTCACGGCATTTGGTTATGAATACGG TCAGTTACCAGGCTGTCCGGCAGGCTTTAAAGCTGACGATAAACTGATCGCTGCCGCTGAGGCCTGCATTGCCGAACT GAATCTTAACGCTGTACGTGGCCTGATTGTTAGCGGCGACGCTTTCATCAACGGTTCTGTTGGTCTGGCGAAAATCCG CCACAACTTCCCACAGGCCATTGCTGTAGAGATGGAAGCGACGGCAATCGCCCATGTCTGCCACAATTTCAACGTCCC GTTTGTTGTCGTACGCGCCATCTCCGACGTGGCCGATCAACAGTCTCATCTTAGCTTCGATGAGTTCCTGGCTGTTGCC GCTAAACAGTCCAGCCTGATGGTTGAGTCACTGGTGCAGAAACTTGCACATGGC**TAA**TAATACTAGAGCCAGGCATC AAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGA GTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
K575024	pLasB B0030 mut3bGFP J23119 B0034 lasR	GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCTTTTCTGCTAGCTATTCCAGCGAAAAC ATACAGATTTCCGGCGAAATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGAAAG CTACTAGAG*ATTAAAGAGGAGAAA*TACTAG**ATG**CGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTT GAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACT TACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTC AATGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGG AAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTA ATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACT CACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAG ATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCA TTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACA GCTGCTGGGATTACACATGGCATGGATGAACTATACAAA**TAA**TAATACTAGAGTTGACAGCTAGCTCAGTCCTAGGTA TAATGCTAGCTACTAGAGATT*AAAGAGGAGAAA*TACTAG**ATG**GCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCA AGTGGAAAATTGGAGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGGATCCTGTTCGGCCT GTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGGCAACTACCCGGCCGCCTGGCGCGAGCATTACG ACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGCGTACTGCCGATTTTCTGGGAACCG TCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCAT GCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCTGAGCCTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAAC CGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGATGCTCAAGGACTACGCACTGCAAAGCGGTGCCGGACTGGCCTTC

		GAACATCCGGTCAGCAAACCGGTGGTTCTGACCAGCCGGGAGAAGGAAGTGTTGCAGTGGTGCGCCATCGGCAAGA CCAGTTGGGAGATATCGGTTATCTGCAACTGCTCGGAAGCCAATGTGAACTTCCATATGGGAAATATTCGGCGGAAGT TCGGTGTGACCTCCCGCCGCGTAGCGGCCATTATGGCCGTTAATTTGGGTCTTATTACTCTC**TAA**TAA
K575037	J23119 B0034 rhlR B0015 pRhlAB B0034 mut3bGFP B0015	TCCTGTGAAATCTGGCAGTTACCGTTAGCTTTCGAATTGGCTAAAAAGTGTTCTACTAGAG*AAAGAGGAGAAA*TACTA G**ATG**CGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAA ATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAA ACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCCAGATCATATGA AACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGA ACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTA AAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAAC AAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATC AACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAA AGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATG AACTATACAAA**TAA**TAATACTAGAGTTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCTACTAGAG*AAAGAGGAG AAA*TACTAG**ATG**AGGAATGACGGAGGCTTTTTGCTGTGGTGGGACGGTTTGCGTAGCGAGATGCAGCCGATCCACGA CAGCCAGGGCGTGTTCGCCGTCCTGGAAAAGGAAGTGCGGCGCCTGGGCTTCGATTACTACGCCTATGGCGTGCGCC ACACGATTCCCTTCACCCGGCCGAAGACCGAGGTCCATGGCACCTATCCCAAGGCCTGGCTGGAGCGATACCAGATG CAGAACTACGGGGCCGTGGATCCGGCGATCCTCAACGGCCTGCGCTCCTCGGAAATGGTGGTCTGGAGCGACAGCCT GTTCGACCAGAGCCGGATGCTCTGGAACGAGGCTCGCGATTGGGGCCTCTGTGTCGGCGCGACCTTGCCGATCCGCG CGCCGAACAATTTGCTCAGCGTGCTTTCCGTGGCGCGCGACCAGCAGAACATCTCCAGCTTCGAGCGCGAGGAAATCC GCCTGCGGCTGCGTTGCATGATCGAGTTGCTGACCCAGAAGCTGACCGACCTGGAGCATCCGATGCTGATGTCCAACC CGGTCTGCCTGAGCCATCGCGAACGCGAGATCCTGCAATGGACCGCCGACGGCAAGAGTTCCGGGGAAATCGCCATC ATCCTGAGCATCTCCGAGAGCACGGTGAACTTCCACCACAAGAACATCCAGAAGAAGTTCGACGCGCCGAACAAGAC GCTGGCTGCCGCCTACGCCGCGGCGCTGGGTCTCATC**TAA**TAA
MC001A	pT7 RBS lasI T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG AG*ATATACAT**ATG**ATCGTTCAGATCGGTCGTCGTGAAGAGTTCGACAAAAAACTGCTGGGTGAAATGCACAAACTGC GTGCTCAGGTTTTCAAAGAACGTAAAGGTTGGGACGTTTCCGTTATCGACGAAATGGAAATCGACGGTTACGACGCTC TGTCCCCGTACTACATGCTGATCCAGGAAGACACCCCGGAAGCTCAGGTTTTCGGTTGCTGGCGTATCTTCGACACCA CCGGTCCGTACATGCTGAAAAACACCTTCCCGGAACTGCTGCACGGTAAAGAAGCTCCGTGCTCCCCGCACATCTGGG AACTGTCCCGTTTCGCTATCAACTCCGGTCAGAAAGGTTCCCTGGGTTTCTCCGACTGCACCCTGGAAGCTATGCGTGC TCTGGCTCGTTACTCCTTGCAGAACGACATCCAGACCCTGGTTACCGTTACCACCGTTGGTGTTGAAAAAATGATGATC CGTGCTGGTCTGGACGTTTCCCGTTTCGGTCCGCACCTGAAAATCGGTATCGAACGTGCTGTTGCTCTGCGTATCGAAC TGAACGCTAAAACCCAGATCGCTCTGTACGGTGGTGTTCTGGTTGAACAGCGTCTGGCTGTTTCC**TAA**GCGGATCCGA ATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC CCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTT GAGGGGTTTTTTG
MC002A	pT7 RBS luxI T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG AG*ATATACAT**ATG**ACTATAATGATAAAAAAATCGGATTTTTTGGCAATTCCATCGGAGGAGTATAAAGGTATTCTAAG TCTTCGTTATCAAGTGTTTAAGCAAAGACTTGAGTGGGACTTAGTTGTAGAAAATAACCTTGAATCAGATGAGTATGA TAACTCAAATGCAGAATATATTTATGCTTGTGATGATACTGAAAATGTAAGTGGATGCTGGCGTTTATTACCTACAACA GGTGATTATATGCTGAAAAGTGTTTTTCCTGAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAAT TAAGTCGTTTTGCTGTAGGTAAAAATAGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAG CTATATATAAACACGCTGTTAGTCAAGGTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAGCGATTTTTAAA GCGTATTAAAGTTCCTTGTCATCGTATTGGAGACAAAGAAATTCATGTATTAGGTGATACTAAATCGGTTGTATTGTCT ATGCCTATTAATGAACAGTTTAAAAAAGCAGTCTTAAAT**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCG GCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCT GCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
MC003A	pT7 RBS rhlI T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGA GATATACAT**ATG**ATCGAACTGCTGTCCGAATCCCTGGAAGGTCTGTCCGCTGCTATGATCGCTGAACTGGGTCGTTAC CGTCACCAGGTTTTCATCGAAAAACTGGGTTGGGACGTTGTTTCCACCTCCCGTGTTCGTGACCAGGAGTTCGACCAG TTCGACCACCCGCAGACCCGTTACATCGTTGCTATGTCCCGTCAGGGTATCTGCGGTTGCGCTCGTCTGCTGCCGACCA CCGACGCTTACCTGCTGAAAGACGTTTTCGCTTACCTGTGCTCCGAAACCCCGCCGTCCGACCCGTCCGTTTGGGAACT GTCCCGTTACGCTGCTTCCGCTGCTGACGACCCGCAGCTGGCTATGAAAATCTTCTGGTCCTCCCTCCAGTGCGCTTGG TACCTGGGTGCTTCCTCCGTTGTTGCTGTTACCACCACCGCTATGGAACGTTACTTCGTTCGTAACGGTGTTATCCTCCA GCGTCTGGGTCCGCCGCAGAAAGTTAAAGGTGAAACCCTGGTTGCTATCTCCTTCCCGGCTTACCAGGAACGTGGTCT GGAAATGCTGCTGCGTTACCACCCGGAATGGCTCCAGGGTGTTCCGCTGTCCATGGCTGTT**TAA**GCGGATCCGAATT CGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCC GAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTG AGGGGTTTTTTG
NY008A	pTet B0034 lasR B0015 pLuxR B0032 mut3bGFP B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TA CTAGT**ATG**GCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGGAGTGGAGCGCCATCCTCCAG AAGATGGCGAGCGACCTTGGATTCTCGAAGATCCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGC CTTCATCGTCGGCAACTACCCGGCCGCCTGGCGCGAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGG TCAGTCACTGTACCCAGAGCGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCT TCGAGGAAGCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGC GCTGAGCCTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGA TGCTCAAGGACTACGCACTGCAAAGCGGTGCCGGACTGGCCTTCGAACATCCGGTCAGCAAACCGGTGGTTCTGACC AGCCGGGAGAAGGAAGTGTTGCAGTGGTGCGCCATCGGCAAGACCAGTTGGGAGATATCGGTTATCTGCAACTGCTC GGAAGCCAATGTGAACTTCCATATGGGAAATATTCGGCGGAAGTTCGGTGTGACCTCCCGCCGCGTAGCGGCCATTA TGGCCGTTAATTTGGGTCTTATTACTCTC**TAA**CACTGATAGTGCTAGTGTAGATCACTACTAGAGCCAGGCATCAAAT AAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCAC

		ACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGGTTTACGCAAGAAAA TGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**CGTAAAGGAGAAGAACTTTTCACT GGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGT GATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCA CTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCAT GCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGT TTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACA AATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCA AAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCC CTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACAT GGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAA**TAA**TAATACTAGAGCCA GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTA CTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTT
RL028K	pT7 RBS lsrR RBS lsrK RBS SP6 RNAP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG AG*ATATACC**ATG**GCAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGGATCG CGTGGTTTTACTATCACGACGGGCTGACCCAGAGCGAGATCAGCGATCGTCTCGGCCTGACACGTTTGAAAGTGTCGC GATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGCTGTCTGGAATATG AAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTGCGGATGCTGATGTCGGTGGGC GACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACAACAGATGCTGGCGATTGGTTTTGGCGAG GCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCGTCACAGCAAATTCGCCTGGTCACGCTCTCCGGTGGCG TCGGTTCTTATATGACGGGAATCGGGCAGCTTAACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCGGGCAT CCTCCGCTGACATTGCCCGTACGCTAAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGCGGATG TGGCGATTGTCGGCATTGGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCAGGGC GAACAGTTAATGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACGTTGTC ACGAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTTGGCGTG GCAGGGGGAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTACCGATCA GGACACAGCAGCGGCGATTTTACGTAGT**TAA**GCTAGCTGTTTAACTTTAAG*AAGGAG*ATATACC**ATG**GCTCGACTC TTTACCCTTTCAGAATCAAAGTACTACCTGATGGCGCTGGATGCAGGCACCGGAAGTATTCGGGCTGTGATATTCGAC CTGGAAGGCAATCAAATAGCAGTGGGACAGGCGGAGTGGCGGCATCTGGCAGTACCGGACGTTCCTGGTTCTATGG AATTTGATCTCAACAAAAACTGGCAACTGGCGTGTGAGTGTATGCGCCAGGCGCTGCACAACGCCGGCATAGCCCCG GAGTATATCGCTGCCGTTTCGGCATGTTCGATGCGTGAAGGCATTGTTTTATATAATAATGAAGGAGCCCCGATCTGG GCCTGCGCCAATGTGGATGCCAGAGCGGCACGCGAAGTTAGCGAACTTAAAGAACTGCACAACAATACCTTTGAAAA CGAAGTTTATCGCGCGACCGGACAAACACTGGCTTTAAGTGCCATCCCCAGATTACTTTGGCTGGCGCACCATCGTTCC GATATTTACCGTCAGGCATCAACCATCACCATGATCAGCGACTGGCTGGCCTATATGCTCAGCGGCGAACTGGCGGTG GATCCCTCTAACGCTGGCACCACGGGACTTCTTGATCTAACCACCCGTGACTGGAAACCTGCATTGCTGGATATGGCT GGCCTACGTGCCGATATTCTTTCTCCTGTCAAAGAAACCGGCACATTGCTGGGCGTGGTAAGTTCACAAGCGGCGGAA CTCTGCGGTCTGAAGGCGGGCACTCCGGTGGTCGTTGGAGGAGGCGACGTGCAGCTTGGTTGCCTTGGGTTAGGCGT TGTGCGTCCGGCACAAACCGCGGTTCTTGGCGGCACATTCTGGCAGCAAGTTGTAAATTTAGCCGCGCCGGTGACAG ACCCAGAAATGAACGTGCGCGTTAATCCTCATGTTATTCCTGGCATGGTACAAGCTGAATCTATAAGCTTTTTTACCGG ACTCACCATGCGCTGGTTCCGCGATGCTTTCTGTGCCGAAGAAAAACTGATTGCCGAACGTTTAGGCATCGACACCTA TACGCTGCTGGAAGAGATGGCCAGTCGGGTGCCGCCTGGGTCGTGGGGCGTAATGCCGATCTTCTCCGACAGAATGC GCTTTAAAACCTGGTATCACGCTGCGCCTTCCTTTATTAACTTGTCCATTGACCCGGATAAATGTAACAAAGCGACATT GTTCCGTGCGCTGGAAGAAAATGCGGCGATTGTATCAGCGTGTAACTTGCAGCAAATTGCTGATTTCTCGAATATTCA TCCTTCATCGTTAGTCTTTGCAGGCGGAGGTTCAAAAGGGAAATTATGGAGTCAAATTCTCGCTGATGTCTCGGGATT ACCCGTCAATATTCCGGTGGTCAAAGAAGCCACTGCATTAGGATGTGCCATTGCAGCTGGCGTCGGTGCCGGAATTTT TTCATCAATGGCAGAAACCGGAGAACGCCTGGTTCGCTGGGAACGGACGCACACACCAGACCCGGAAAAGCATGAA CTTTATCAGGATTCACGCGATAAGTGGCAGGCAGTTTATCAGGATCAGCTGGGGCTGGTTGATCATGGACTGACGAC GTCGTTATGGAAAGCGCCTGGGTTA**TAA**GAGCTCTTTAAACGAATTCGTT*AAGGAG*AATAATCT**ATG**CAAGACCTG CACGCTATTCAACTGCAACTGGAAGAAGAAATGTTTAATGGCGGTATCCGTCGCTTTGAAGCCGATCAGCAACGCCAG ATTGCGGCCGGCAGTGAATCCGATACCGCGTGGAACCGTCGCCTGCTGAGCGAACTGATTGCCCCGATGGCAGAAGG TATCCAGGCCTATAAAGAAGAATACGAAGGCAAAAAAGGTCGTGCGCCGCGTGCGCTGGCGTTTCTGCAATGCGTCG AAAACGAAGTGGCAGCTTATATCACCATGAAAGTGGTTATGGATATGCTGAATACCGACGCGACGCTGCAAGCCATT GCAATGTCTGTCGCCGAACGTATCGAAGATCAAGTGCGCTTTAGTAAACTGGAAGGCCATGCGGCCAAATACTTCGA AAAAGTGAAAAAATCTCTGAAAGCGTCTCGTACCAAAAGTTACCGCCATGCCCACAACGTTGCTGTCGTGGCGGAAAA ATCTGTCGCCGAAAAAGATGCAGACTTTGATCGTTGGGAAGCGTGGCCGAAAGAAACGCAGCTGCAAATTGGCACCA CGCTGCTGGAAATCCTGGAAGGCAGCGTGTTTTATAATGGTGAACCGGTCTTCATGCGTGCCATGCGCACCTACGGCG GTAAAACGATTTATTACCTGCAAACCTCAGAATCGGTTGGTCAATGGATCTCGGCCTTCAAAGAACACGTCGCACAGC TGAGCCCGGCTTATGCGCCGTGTGTTATTCCGCCGCGTCCGTGGCGTACCCCGTTTAACGGCGGTTTCCATACGGAAA AAGTGGCGAGCCGTATCCGCCTGGTTAAAGGTAATCGTGAACACGTCCGCAAACTGACCCAGAAACAAATGCCGAAA GTGTATAAAGCCATTAACGCACTGCAAAATACGCAGTGGCAAATCAACAAAGATGTTCTGGCGGTCATTGAAGAAGT GATCCGCCTGGACCTGGGCTACGGTGTTCCGTCCTTTAAACCGCTGATCGATAAAGAAAACAAACCGGCCAATCCGGT TCCGGTCGAATTTCAGCATCTGCGTGGCCGCGAACTGAAAGAAATGCTGAGCCCGGAACAATGGCAGCAATTTATCA ATTGGAAGGGTGAATGCGCCCGTCTGTATACCGCAGAAACGAAACGTGGCAGCAAAAGCGCGGCGGTGGTCCGTAT GGTTGGTCAGGCTCGCAAATACTCCGCGTTCGAATCAATCTACTTCGTGTACGCGATGGATAGCCGTAGCCGTGTGTA CGTTCAGAGCAGCACCCTGTCACCGCAATCGAACGACCTGGGCAAAGCACTGCTGCGTTTTACGGAAGGCCGTCCGG TGAATGGTGTTGAAGCACTGAAATGGTTCTGTATTAACGGCGCTAATCTGTGGGGTTGGGATAAGAAAACCTTTGAC GTCCGTGTGAGCAATGTTCTGGATGAAGAATTTCAGGACATGTGTCGTGATATTGCGGCCGACCCGCTGACCTTTACG CAATGGGCCAAAGCAGATGCTCCGTATGAATTTCTGGCTTGGTGTTTCGAATATGCGCAGTACCTGGACCTGGTTGAT GAAGGTCGTGCAGATGAATTTCGTACCCATCTGCCGGTGCACCAGGATGGCTCATGCTCGGGTATTCAACATTACTCC GCGATGCTGCGTGATGAAGTTGGCGCGAAAGCCGTCAACCTGAAACCGTCAGATGCGCCGCAGGACATTTATGGTGC AGTTGCTCAAGTGGTTATTAAGAAAAACGCCCTGTACATGGATGCTGATGACGCGACCACGTTTACCAGCGGCTCTGT GACCCTGTCGGGTACGGAACTGCGTGCGATGGCCTCGGCATGGGATAGCATTGGCATCACCCGCAGCCTGACGAAAA

		AACCGGTGATGACGCTGCCGTATGGTTCCACCCGTCTGACGTGTCGCGAATCAGTGATTGATTACATCGTTGACCTGG AAGAAAAAGAAGCACAGAAAGCTGTCGCGGAAGGCCGTACCGCTAACAAAGTGCACCCGTTTGAAGATGACCGCCA AGATTATCTGACCCCGGGTGCAGCTTATAATTACATGACGGCGCTGATTTGGCCGAGTATCTCCGAAGTCGTGAAAGC TCCGATTGTTGCGATGAAAATGATCCGTCAGCTGGCCCGCTTTGCGGCCAAACGCAACGAAGGCCTGATGTACACCCT GCCGACGGGTTTCATTCTGGAACAGAAAATCATGGCGACCGAAATGCTGCGTGTGCGCACGTGCCTGATGGGCGATA TTAAAATGTCTCTGCAAGTCGAAACCGACATCGTGGATGAAGCAGCTATGATGGGCGCGGCGGCACCGAATTTTGTTC ATGGTCACGATGCGAGTCATCTGATTCTGACCGTTTGTGAACTGGTGGATAAAGGCGTGACGTCTATTGCAGTGATCC ATGATAGTTTCGGTACCCACGCGGACAACACCCTGACGCTGCGTGTGGCCCTGAAAGGCCAGATGGTTGCAATGTAT ATTGATGGTAATGCTCTGCAAAAACTGCTGGAAGAACACGAAGAACGCTGGATGGTGGACACCGGTATCGAAGTCCC GGAACAAGGCGAATTTGACCTGAACGAAATCATGGATAGCGAATATGTCTTTGCG**TAA**CTCGAGCACCACCACCACC ACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCA TAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
RL029A	pT7 RBS SP6 RNAP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGA GATATACAT**ATG**CAAGACCTGCACGCTATTCAACTGCAACTGGAAGAAGAAATGTTTAATGGCGGTATCCGTCGCTTT GAAGCCGATCAGCAACGCCAGATTGCGGCCGGCAGTGAATCCGATACCGCGTGGAACCGTCGCCTGCTGAGCGAACT GATTGCCCCGATGGCAGAAGGTATCCAGGCCTATAAAGAAGAATACGAAGGCAAAAAAGGTCGTGCGCCGCGTGCG CTGGCGTTTCTGCAATGCGTCGAAAACGAAGTGGCAGCTTATATCACCATGAAAGTGGTTATGGATATGCTGAATACC GACGCGACGCTGCAAGCCATTGCAATGTCTGTCGCCGAACGTATCGAAGATCAAGTGCGCTTTAGTAAACTGGAAGG CCATGCGGCCAAATACTTCGAAAAAGTGAAAAAATCTCTGAAAGCGTCTCGTACCAAAAGTTACCGCCATGCCCACAA CGTTGCTGTCGTGGCGGAAAAATCTGTCGCCGAAAAAGATGCAGACTTTGATCGTTGGGAAGCGTGGCCGAAAGAAA CGCAGCTGCAAATTGGCACCACGCTGCTGGAAATCCTGGAAGGCAGCGTGTTTTATAATGGTGAACCGGTCTTCATGC GTGCCATGCGCACCTACGGCGGTAAAACGATTTATTACCTGCAAACCTCAGAATCGGTTGGTCAATGGATCTCGGCCT TCAAAGAACACGTCGCACAGCTGAGCCCGGCTTATGCGCCGTGTGTTATTCCGCCGCGTCCGTGGCGTACCCCGTTTA ACGGCGGTTTCCATACGGAAAAAGTGGCGAGCCGTATCCGCCTGGTTAAAGGTAATCGTGAACACGTCCGCAAACTG ACCCAGAAACAAATGCCGAAAGTGTATAAAGCCATTAACGCACTGCAAAATACGCAGTGGCAAATCAACAAAGATGT TCTGGCGGTCATTGAAGAAGTGATCCGCCTGGACCTGGGCTACGGTGTTCCGTCCTTTAAACCGCTGATCGATAAAGA AAACAAACCGGCCAATCCGGTTCCGGTCGAATTTCAGCATCTGCGTGGCCGCGAACTGAAAGAAATGCTGAGCCCGG AACAATGGCAGCAATTTATCAATTGGAAGGGTGAATGCGCCCGTCTGTATACCGCAGAAACGAAACGTGGCAGCAAA AGCGCGGCGGTGGTCCGTATGGTTGGTCAGGCTCGCAAATACTCCGCGTTCGAATCAATCTACTTCGTGTACGCGATG GATAGCCGTAGCCGTGTGTACGTTCAGAGCAGCACCCTGTCACCGCAATCGAACGACCTGGGCAAAGCACTGCTGCG TTTTACGGAAGGCCGTCCGGTGAATGGTGTTGAAGCACTGAAATGGTTCTGTATTAACGGCGCTAATCTGTGGGGTTG GGATAAGAAAACCTTTGACGTCCGTGTGAGCAATGTTCTGGATGAAGAATTTCAGGACATGTGTCGTGATATTGCGGC CGACCCGCTGACCTTTACGCAATGGGCCAAAGCAGATGCTCCGTATGAATTTCTGGCTTGGTGTTTCGAATATGCGCA GTACCTGGACCTGGTTGATGAAGGTCGTGCAGATGAATTTCGTACCCATCTGCCGGTGCACCAGGATGGCTCATGCTC GGGTATTCAACATTACTCCGCGATGCTGCGTGATGAAGTTGGCGCGAAAGCCGTCAACCTGAAACCGTCAGATGCGC CGCAGGACATTTATGGTGCAGTTGCTCAAGTGGTTATTAAGAAAAACGCCCTGTACATGGATGCTGATGACGCGACCA CGTTTACCAGCGGCTCTGTGACCCTGTCGGGTACGGAACTGCGTGCGATGGCCTCGGCATGGGATAGCATTGGCATC ACCCGCAGCCTGACGAAAAAACCGGTGATGACGCTGCCGTATGGTTCCACCCGTCTGACGTGTCGCGAATCAGTGATT GATTACATCGTTGACCTGGAAGAAAAAGAAGCACAGAAAGCTGTCGCGGAAGGCCGTACCGCTAACAAAGTGCACCC GTTTGAAGATGACCGCCAAGATTATCTGACCCCGGGTGCAGCTTATAATTACATGACGGCGCTGATTTGGCCGAGTAT CTCCGAAGTCGTGAAAGCTCCGATTGTTGCGATGAAAATGATCCGTCAGCTGGCCCGCTTTGCGGCCAAACGCAACGA AGGCCTGATGTACACCCTGCCGACGGGTTTCATTCTGGAACAGAAAATCATGGCGACCGAAATGCTGCGTGTGCGCA CGTGCCTGATGGGCGATATTAAAATGTCTCTGCAAGTCGAAACCGACATCGTGGATGAAGCAGCTATGATGGGCGCG GCGGCACCGAATTTTGTTCATGGTCACGATGCGAGTCATCTGATTCTGACCGTTTGTGAACTGGTGGATAAAGGCGTG ACGTCTATTGCAGTGATCCATGATAGTTTCGGTACCCACGCGGACAACACCCTGACGCTGCGTGTGGCCCTGAAAGGC CAGATGGTTGCAATGTATATTGATGGTAATGCTCTGCAAAAACTGCTGGAAGAACACGAAGAACGCTGGATGGTGGA CACCGGTATCGAAGTCCCGGAACAAGGCGAATTTGACCTGAACGAAATCATGGATAGCGAATATGTCTTTGCG**TAA**G GATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTA ACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAA CGGGTCTTGAGGGGTTTTTTG
RL042K	pSP6 RBS aHL sfGFP	ATTTAGGTGACACTATAGAAGAGAAATAATTTTGTTTAACTTTAAG*AAGGAG*AATAATCT**ATG**GATTCTGATATCAA TATCAAAACCGGCACCACCGATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAA CGGTATGCATAAAAAAGTGTTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAA GGCACCATTGCGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTT TAAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAA AGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGCGGTCTGAT TGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGTCCGACCGA TAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGA ATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGA ACAAAGCCTCAAGCCTGCTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCA AACAGCAGACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGA AAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAAAT GACCAACggatccggcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGAT GGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAA GTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTT GCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACG ATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCAT TGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAA TGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAG CGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTG AGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGC GGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAGGTGTCACC

		ACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL048K	pSP6 *lsr* intergenic region aHL sfGFP	ATTTAGGTGACACTATAGAAGAGAAATTCATTCTTCACTTTGAACATATTTAAATCTTTAATGCAATTGTTCAGTTCTTG CTCATTTATATCTGTGATGGCAACCACAGTTTGACTCTACGAGCATGAACAAACGCAACCGTGAAAATCAAAATAGCA TAAATTGTGATCTATTCGTCGGAAATATGTGCAATGTCCACCTAAGGTTATGAACAAATTAAAAGCAGAAATACATTTG TTCAAAACTCACCTGCAAAACTGAACG*AAGGAG*AATAATCT**ATG**GATTCTGATATCAATATCAAAACCGGCACCACC GATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTG TTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCGGGTCAAT ACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCAGCTGCAACTG CCGGATAATGAAGTGGCG CAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAAAGAATATATGAGTACCCTG ACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATT GGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAA AGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCA GCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCT GTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGACCAACATTGA TGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGGCACCAATACCAAAGA TAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAAATGACCAAC*ggatccggcagcggttc* t**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATA AGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGT AAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATA TGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACG GCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGAC TTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGAT AAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCA CTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCT GTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTA TGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTA AAACCGAAAAGATTACTTCGCGT
RL053A	pT7 RBS T3 RNAP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACAT**ATG**GCTAGCATGATGAACATCATCGAAAACATCGAAAAGAATGACTTCTCAGAAATCGAACTGGCTG CTATCCCGTTCAACACACTGGCTGACCACTACGGAAGCGCCTTGGCTAAAGAGCAGTTGGCTTTAGAACATGAGTCTT ATGAGCTAGGCGAGCGCCGCTTCCTCAAGATGCTTGAGCGTCAAGCGAAAGCTGGTGAGATTGCAGACAACGCAGCC GCTAAGCCGTTACTCGCTACGCTTCTCCCTAAGTTAACCACACGTATCGTCGAGTGGCTCGAAGAGTACGCATCGAAG AAAGGCCGCAAGCCTAGCGCATACGCACCGCTCCAGTTACTCAAGCCGGAGGCCTCCGCGTTTATCACCCTGAAAGTT ATCCTTGCGTCACTAACCAGTACGAACATGACAACCATTCAGGCCGCTGCTGGTATGCTGGGGAAAGCCATTGAGGAC GAGGCACGATTTGGGCGCATCCGTGACCTAGAAGCGAAGCACTTCAAGAAGCACGTTGAGGAACAGCTTAACAAGC GCCACGGGCAAGTCTACAAGAAAGCATTTATGCAGGTGGTCGAGGCCGATATGATTGGTCGAGGTCTGCTTGGTGGC GAGGCGTGGTCTAGCTGGGATAAAGAAACCACGATGCACGTAGGGATTCGCCTGATTGAAATGCTGATTGAATCCAC GGGTCTGGTGGAATTACAGCGCCACAACGCAGGTAACGCAGGCTCTGACCATGAGGCACTGCAACTGGCCCAAGAGT ACGTGGACGTATTAGCGAAGCGTGCAGGCGCTCTGGCGGGTATCTCTCCGATGTTCCAGCCGTGTGTCGTACCGCCG AAACCTTGGGTAGCAATCACAGGGGGCGGCTATTGGGCTAACGGTCGCAGACCTTTGGCACTCGTTCGCACTCACTCT AAGAAGGGCTTGATGCGCTACGAAGACGTTTACATGCCAGAAGTCTACAAGGCTGTGAACCTCGCGCAAAACACCGC ATGGAAAATCAACAAGAAAGTTCTTGCTGTTGTCAATGAGATTGTTAACTGGAAGAATTGCCCGGTAGCAGACATTCC ATCGCTGGAGCGCCAAGAGTTACCGCCTAAGCCTGACGACATTGACACCAACGAGGCAGCGCTCAAGGAGTGGAAG AAAGCCGCTGCTGGTATCTATCGCTTGGACAAGGCACGAGTGTCTCGCCGTATCAGCTTAGAGTTCATGCTGGAGCAG GCCAACAAGTTCGCAAGTAAGAAAGCAATCTGGTTCCCTTACAACATGGACTGGCGCGGTCGTGTGTACGCTGTGCC GATGTTCAACCCGCAAGGCAACGACATGACGAAAGGTCTGCTGACCCTTGCTAAAGGCAAGCCAATCGGTGAGGAAG GTTTCTACTGGCTGAAAATCCACGGTGCGAACTGTGCGGGTGTTGATAAGGTTCCATTCCCGGAGCGCATCGCGTTCA TTGAGAAGCACGTAGACGACATTCTGGCTTGCGCTAAAGACCCAATCAATAACACTTGGTGGGCTGAGCAGGATTCA CCGTTCTGTTTCCTCGCGTTTTGCTTCGAGTATGCAGGCGTTACGCACCACGGTCTGAGCTACAATTGCTCTCTGCCGCT GGCGTTCGACGGGTCTTGCTCTGGTATCCAGCACTTCTCCGCGATGCTCCGCGATGAGGTAGGCGGTCGTGCGGTTAA CCTGCTGCCAAGCGAAACCGTGCAGGACATTTACGGCATCGTTGCACAGAAAGTAAACGAGATTCTCAAACAGGATG CAATCAACGGCACGCCTAACGAGATGATTACCGTGACCGACAAGGACACCGGGGAAATCTCAGAGAAGCTCAAACTT GGAACCTCAACGCTGGCGCAACAGTGGCTGGCATATGGTGTAACCCGTAGCGTAACTAAACGTTCGGTCATGACGCT GGCTTACGGTTCCAAGGAGTTCGGCTTTCGTCAACAGGTATTGGATGACACCATTCAGCCTGCAATTGACAGCGGTAA GGGCTTGATGTTCACCCAACCGAACCAAGCGGCTGGCTATATGGCTAAGCTGATTTGGGATGCGGTAAGCGTGACCG TAGTTGCAGCGGTTGAGGCGATGAACTGGCTCAAATCTGCCGCTAAGCTGCTGGCTGCTGAGGTCAAGGACAAGAAG ACCAAGGAGATTCTGCGCCACCGTTGCGCGGTTCACTGGACTACGCCGGACGGCTTCCCGGTCTGGCAGGAATACCG CAAGCCACTCCAGAAGCGTCTCGATATGATTTTCTTAGGGCAATTCCGTCTGCAACCGACGATTAATACCCTCAAGGAT TCAGGCATTGACGCACACAAGCAGGAGTCTGGCATCGCTCCTAACTTTGTTCACTCACAGGACGGTAGCCACCTCCGC ATGACAGTCGTTTATGCTCACGAGAAGTATGGCATTGAGTCCTTTGCGCTCATCCATGACAGCTTTGGGACTATCCCGG CAGACGCTGGTAAGCTCTTTAAGGCTGTGCGTGAAACGATGGTTATCACCTATGAGAACAACGATGTGCTGGCAGAC TTCTACTCTCAGTTTGCCGACCAGCTACACGAGACCCAACTGGACAAGATGCCTCCGCTTCCGAAGAAAGGAAACCTG AACCTGCAAGACATTCTCAAGTCTGACTTTGCCTTTGCA**TAA**GGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGC CGACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGC CACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
RL054K	pT3 RBS aHL sfGFP	ATTAACCCTCACTAAAGGGAGAAATAATTTTGTTTAACTTTAAG*AAGGAG*AATAATCT**ATG**GATTCTGATATCAATAT CAAAACCGGCACCACCGATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGG TATGCATAAAAAAGTGTTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGC ACCATTGCGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAA GTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAAAGAA TATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGCGGTCTGATTGGC

		GCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGTCCGACCGATAAA AAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCC GGTTTATGGCAATCAGCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAA AGCCTCAAGCCTGCTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACA GCAGACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGG CACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAAATGACCA ACggatccggcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTG ATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTC ATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTC GTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTT CCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAG CTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTT TACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGT GCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAG CACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGG GCATCACGCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAGGTGTCACCACC CTGCCCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL059K	pT3 *lsr* intergenic region aHL sfGFP	ATTAACCCTCACTAAAGGGAGaAATTCATTCTTCACTTTGAACATATTTAAATCTTTAATGCAATTGTTCAGTTCTTGCTC ATTTATATCTGTGATGGCAACCACAGTTTGACTCTACGAGCATGAACAAACGCAACCGTGAAAATCAAAATAGCATAA ATTGTGATCTATTCGTCGGAAATATGTGCAATGTCCACCTAAGGTTATGAACAAATTAAAAGCAGAAATACATTTGTTC AAAACTCACCTGCAAAACTGAACGGGGGAAATACTAGA**ATG**GATTCTGATATCAATATCAAAACCGGCACCACCGAT ATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAAAAAGTGTTT TACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCGGGTCAATAC CGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCAGCTGCAACTGCC GGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAAAGAATATATGAGTACCCTGAC CTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGCGGTCTGATTGGCGCCAATGTGTCCATTGG TCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGGTTGGAAAG TTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGGCAATCAGC TGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAAGCCTGCTGT CCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAACAGCAGACCAACATTGATG TGATCTACGAACGTGTGCGTGATGATTAT CAACTGCATTGGACCTCAACCAATTGGAAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTA CAAAATTGATTGGGAAAAAGAAGAAATGACCAACggatccggcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTG GTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGT GACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTA ACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCA TGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAA ATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCC ATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATT TTAAAATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGAT GGTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGAT CATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCT GCAGTCCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL060K	pT7 RBS lsrR RBS lsrK RBS T3 RNAP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACC**ATG**GCAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGGATCG CGTGGTTTTACTATCACGACGGGCTGACCCAGAGCGAGATCAGCGATCGTCTCGGCCTGACACGTTTGAAAGTGTCGC GATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGCTGTCTGGAATATG AAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTGCGGATGCTGATGTCGGTGGGC GACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACAACAGATGCTGGCGATTGGTTTTGGCGAG GCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCGTCACAGCAAATTCGCCTGGTCACGCTCTCCGGTGGCG TCGGTTCTTATATGACGGGAATCGGGCAGCTTAACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCGGGCAT CCTCCGCTGACATTGCCCGTACGCTAAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGCGGATG TGGCGATTGTCGGCATTGGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCAGGGC GAACAGTTAATGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACGTTGTC ACGAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTTGGCGTG GCAGGGGGAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTACCGATCA GGACACAGCAGCGGCGATTTTACGTAGT**TAA**GCTAGCTGTTTAACTTTAAG*AAGGAG*ATATACC**ATG**GCTCGACTC TTTACCCTTTCAGAATCAAAGTACTACCTGATGGCGCTGGATGCAGGCACCGGAAGTATTCGGGCTGTGATATTCGAC CTGGAAGGCAATCAAATAGCAGTGGGACAGGCGGAGTGGCGGCATCTGGCAGTACCGGACGTTCCTGGTTCTATGG AATTTGATCTCAACAAAAACTGGCAACTGGCGTGTGAGTGTATGCGCCAGGCGCTGCACAACGCCGGCATAGCCCCG GAGTATATCGCTGCCGTTTCGGCATGTTCGATGCGTGAAGGCATTGTTTTATATAATAATGAAGGAGCCCCGATCTGG GCCTGCGCCAATGTGGATGCCAGAGCGGCACGCGAAGTTAGCGAACTTAAAGAACTGCACAACAATACCTTTGAAAA CGAAGTTTATCGCGCGACCGGACAAACACTGGCTTTAAGTGCCATCCCCAGATTACTTTGGCTGGCGCACCATCGTTCC GATATTTACCGTCAGGCATCAACCATCACCATGATCAGCGACTGGCTGGCCTATATGCTCAGCGGCGAACTGGCGGTG GATCCCTCTAACGCTGGCACCACGGGACTTCTTGATCTAACCACCCGTGACTGGAAACCTGCATTGCTGGATATGGCT GGCCTACGTGCCGATATTCTTTCTCCTGTCAAAGAAACCGGCACATTGCTGGGCGTGGTAAGTTCACAAGCGGCGGAA CTCTGCGGTCTGAAGGCGGGCACTCCGGTGGTCGTTGGAGGAGGCGACGTGCAGCTTGGTTGCCTTGGGTTAGGCGT TGTGCGTCCGGCACAAACCGCGGTTCTTGGCGGCACATTCTGGCAGCAAGTTGTAAATTTAGCCGCGCCGGTGACAG ACCCAGAAATGAACGTGCGCGTTAATCCTCATGTTATTCCTGGCATGGTACAAGCTGAATCTATAAGCTTTTTTACCGG ACTCACCATGCGCTGGTTCCGCGATGCTTTCTGTGCCGAAGAAAAACTGATTGCCGAACGTTTAGGCATCGACACCTA TACGCTGCTGGAAGAGATGGCCAGTCGGGTGCCGCCTGGGTCGTGGGGCGTAATGCCGATCTTCTCCGACAGAATGC

		GCTTTAAAACCTGGTATCACGCTGCGCCTTCCTTTATTAACTTGTCCATTGACCCGGATAAATGTAACAAAGCGACATT GTTCCGTGCGCTGGAAGAAAATGCGGCGATTGTATCAGCGTGTAACTTGCAGCAAATTGCTGATTTCTCGAATATTCA TCCTTCATCGTTAGTCTTTGCAGGCGGAGGTTCAAAAGGGAAATTATGGAGTCAAATTCTCGCTGATGTCTCGGGATT ACCCGTCAATATTCCGGTGGTCAAAGAAGCCACTGCATTAGGATGTGCCATTGCAGCTGGCGTCGGTGCCGGAATTTT TTCATCAATGGCAGAAACCGGAGAACGCCTGGTTCGCTGGGAACGGACGCACACACCAGACCCGGAAAAGCATGAA CTTTATCAGGATTCACGCGATAAGTGGCAGGCAGTTTATCAGGATCAGCTGGGGCTGGTTGATCATGGACTGACGAC GTCGTTATGGAAAGCGCCTGGGTTA**TAA**GAGCTCTTTAAACGAATTCGTT*AAGGAG*AATAATCT**ATG**AACATCATC GAAAACATCGAAAAGAATGACTTCTCAGAAATCGAACTGGCTGCTATCCCGTTCAACACACTGGCTGACCACTACGGA AGCGCCTTGGCTAAAGAGCAGTTGGCTTTAGAACATGAGTCTTATGAGCTAGGCGAGCGCCGCTTCCTCAAGATGCTT GAGCGTCAAGCGAAAGCTGGTGAGATTGCAGACAACGCAGCCGCTAAGCCGTTACTCGCTACGCTTCTCCCTAAGTTA ACCACACGTATCGTCGAGTGGCTCGAAGAGTACGCATCGAAGAAAGGCCGCAAGCCTAGCGCATACGCACCGCTCCA GTTACTCAAGCCGGAGGCCTCCGCGTTTATCACCCTGAAAGTTATCCTTGCGTCACTAACCAGTACGAACATGACAACC ATTCAGGCCGCTGCTGGTATGCTGGGGAAAGCCATTGAGGACGAGGCACGATTTGGGCGCATCCGTGACCTAGAAGC GAAGCACTTCAAGAAGCACGTTGAGGAACAGCTTAACAAGCGCCACGGGCAAGTCTACAAGAAAGCATTTATGCAGG TGGTCGAGGCCGATATGATTGGTCGAGGTCTGCTTGGTGGCGAGGCGTGGTCTAGCTGGGATAAAGAAACCACGAT GCACGTAGGGATTCGCCTGATTGAAATGCTGATTGAATCCACGGGTCTGGTGGAATTACAGCGCCACAACGCAGGTA ACGCAGGCTCTGACCATGAGGCACTGCAACTGGCCCAAGAGTACGTGGACGTATTAGCGAAGCGTGCAGGCGCTCTG GCGGGTATCTCTCCGATGTTCCAGCCGTGTGTCGTACCGCCGAAACCTTGGGTAGCAATCACAGGGGGCGGCTATTG GGCTAACGGTCGCAGACCTTTGGCACTCGTTCGCACTCACTCTAAGAAGGGCTTGATGCGCTACGAAGACGTTTACAT GCCAGAAGTCTACAAGGCTGTGAACCTCGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTTCTTGCTGTTGTCAA TGAGATTGTTAACTGGAAGAATTGCCCGGTAGCAGACATTCCATCGCTGGAGCGCCAAGAGTTACCGCCTAAGCCTG ACGACATTGACACCAACGAGGCAGCGCTCAAGGAGTGGAAGAAAGCCGCTGCTGGTATCTATCGCTTGGACAAGGC ACGAGTGTCTCGCCGTATCAGCTTAGAGTTCATGCTGGAGCAGGCCAACAAGTTCGCAAGTAAGAAAGCAATCTGGT TCCCTTACAACATGGACTGGCGCGGTCGTGTGTACGCTGTGCCGATGTTCAACCCGCAAGGCAACGACATGACGAAA GGTCTGCTGACCCTTGCTAAAGGCAAGCCAATCGGTGAGGAAGGTTTCTACTGGCTGAAAATCCACGGTGCGAACTG TGCGGGTGTTGATAAGGTTCCATTCCCGGAGCGCATCGCGTTCATTGAGAAGCACGTAGACGACATTCTGGCTTGCGC TAAAGACCCAATCAATAACACTTGGTGGGCTGAGCAGGATTCACCGTTCTGTTTCCTCGCGTTTTGCTTCGAGTATGCA GGCGTTACGCACCACGGTCTGAGCTACAATTGCTCTCTGCCGCTGGCGTTCGACGGGTCTTGCTCTGGTATCCAGCAC TTCTCCGCGATGCTCCGCGATGAGGTAGGCGGTCGTGCGGTTAACCTGCTGCCAAGCGAAACCGTGCAGGACATTTA CGGCATCGTTGCACAGAAAGTAAACGAGATTCTCAAACAGGATGCAATCAACGGCACGCCTAACGAGATGATTACCG TGACCGACAAGGACACCGGGGAAATCTCAGAGAAGCTCAAACTTGGAACCTCAACGCTGGCGCAACAGTGGCTGGC ATATGGTGTAACCCGTAGCGTAACTAAACGTTCGGTCATGACGCTGGCTTACGGTTCCAAGGAGTTCGGCTTTCGTCA ACAGGTATTGGATGACACCATTCAGCCTGCAATTGACAGCGGTAAGGGCTTGATGTTCACCCAACCGAACCAAGCGG CTGGCTATATGGCTAAGCTGATTTGGGATGCGGTAAGCGTGACCGTAGTTGCAGCGGTTGAGGCGATGAACTGGCTC AAATCTGCCGCTAAGCTGCTGGCTGCTGAGGTCAAGGACAAGAAGACCAAGGAGATTCTGCGCCACCGTTGCGCGGT TCACTGGACTACGCCGGACGGCTTCCCGGTCTGGCAGGAATACCGCAAGCCACTCCAGAAGCGTCTCGATATGATTTT CTTAGGGCAATTCCGTCTGCAACCGACGATTAATACCCTCAAGGATTCAGGCATTGACGCACACAAGCAGGAGTCTGG CATCGCTCCTAACTTTGTTCACTCACAGGACGGTAGCCACCTCCGCATGACAGTCGTTTATGCTCACGAGAAGTATGGC ATTGAGTCCTTTGCGCTCATCCATGACAGCTTTGGGACTATCCCGGCAGACGCTGGTAAGCTCTTTAAGGCTGTGCGT GAAACGATGGTTATCACCTATGAGAACAACGATGTGCTGGCAGACTTCTACTCTCAGTTTGCCGACCAGCTACACGAG ACCCAACTGGACAAGATGCCTCCGCTTCCGAAGAAAGGAAACCTGAACCTGCAAGACATTCTCAAGTCTGACTTTGCC TTTGCA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGC TGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
RL061K	pT7 TTG lsrR lsrK T3 RNAP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACC**TTG**GCAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGGATCG CGTGGTTTTACTATCACGACGGGCTGACCCAGAGCGAGATCAGCGATCGTCTCGGCCTGACACGTTTGAAAGTGTCGC GATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGCTGTCTGGAATATG AAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTGCGGATGCTGATGTCGGTGGGC GACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACAACAGATGCTGGCGATTGGTTTTGGCGAG GCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCGTCACAGCAAATTCGCCTGGTCACGCTCTCCGGTGGCG TCGGTTCTTATATGACGGGAATCGGGCAGCTTAACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCGGGCAT CCTCCGCTGACATTGCCCGTACGCTAAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGCGGATG TGGCGATTGTCGGCATTGGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCAGGGC GAACAGTTAATGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACGTTGTC ACGAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTTGGCGTG GCAGGGGGAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTACCGATCA GGACACAGCAGCGGCGATTTTACGTAGT**TAA**GCTAGCTGTTTAACTTTAAG*AAGGAG*ATATACC**ATG**GCTCGACTC TTTACCCTTTCAGAATCAAAGTACTACCTGATGGCGCTGGATGCAGGCACCGGAAGTATTCGGGCTGTGATATTCGAC CTGGAAGGCAATCAAATAGCAGTGGGACAGGCGGAGTGGCGGCATCTGGCAGTACCGGACGTTCCTGGTTCTATGG AATTTGATCTCAACAAAAACTGGCAACTGGCGTGTGAGTGTATGCGCCAGGCGCTGCACAACGCCGGCATAGCCCCG GAGTATATCGCTGCCGTTTCGGCATGTTCGATGCGTGAAGGCATTGTTTTATATAATAATGAAGGAGCCCCGATCTGG GCCTGCGCCAATGTGGATGCCAGAGCGGCACGCGAAGTTAGCGAACTTAAAGAACTGCACAACAATACCTTTGAAAA CGAAGTTTATCGCGCGACCGGACAAACACTGGCTTTAAGTGCCATCCCCAGATTACTTTGGCTGGCGCACCATCGTTCC GATATTTACCGTCAGGCATCAACCATCACCATGATCAGCGACTGGCTGGCCTATATGCTCAGCGGCGAACTGGCGGTG GATCCCTCTAACGCTGGCACCACGGGACTTCTTGATCTAACCACCCGTGACTGGAAACCTGCATTGCTGGATATGGCT GGCCTACGTGCCGATATTCTTTCTCCTGTCAAAGAAACCGGCACATTGCTGGGCGTGGTAAGTTCACAAGCGGCGGAA CTCTGCGGTCTGAAGGCGGGCACTCCGGTGGTCGTTGGAGGAGGCGACGTGCAGCTTGGTTGCCTTGGGTTAGGCGT TGTGCGTCCGGCACAAACCGCGGTTCTTGGCGGCACATTCTGGCAGCAAGTTGTAAATTTAGCCGCGCCGGTGACAG ACCCAGAAATGAACGTGCGCGTTAATCCTCATGTTATTCCTGGCATGGTACAAGCTGAATCTATAAGCTTTTTTACCGG ACTCACCATGCGCTGGTTCCGCGATGCTTTCTGTGCCGAAGAAAAACTGATTGCCGAACGTTTAGGCATCGACACCTA TACGCTGCTGGAAGAGATGGCCAGTCGGGTGCCGCCTGGGTCGTGGGGCGTAATGCCGATCTTCTCCGACAGAATGC GCTTTAAAACCTGGTATCACGCTGCGCCTTCCTTTATTAACTTGTCCATTGACCCGGATAAATGTAACAAAGCGACATT

		GTTCCGTGCGCTGGAAGAAAATGCGGCGATTGTATCAGCGTGTAACTTGCAGCAAATTGCTGATTTCTCGAATATTCA TCCTTCATCGTTAGTCTTTGCAGGCGGAGGTTCAAAAGGGAAATTATGGAGTCAAATTCTCGCTGATGTCTCGGGATT ACCCGTCAATATTCCGGTGGTCAAAGAAGCCACTGCATTAGGATGTGCCATTGCAGCTGGCGTCGGTGCCGGAATTTT TTCATCAATGGCAGAAACCGGAGAACGCCTGGTTCGCTGGGAACGGACGCACACACCAGACCCGGAAAAGCATGAA CTTTATCAGGATTCACGCGATAAGTGGCAGGCAGTTTATCAGGATCAGCTGGGGCTGGTTGATCATGGACTGACGAC GTCGTTATGGAAAGCGCCTGGGTTA**TAA**GAGCTCTTTAAACGAATTCGTT*AAGGAG*AATAATCT**ATG**AACATCATC GAAAACATCGAAAAGAATGACTTCTCAGAAATCGAACTGGCTGCTATCCCGTTCAACACACTGGCTGACCACTACGGA AGCGCCTTGGCTAAAGAGCAGTTGGCTTTAGAACATGAGTCTTATGAGCTAGGCGAGCGCCGCTTCCTCAAGATGCTT GAGCGTCAAGCGAAAGCTGGTGAGATTGCAGACAACGCAGCCGCTAAGCCGTTACTCGCTACGCTTCTCCCTAAGTTA ACCACACGTATCGTCGAGTGGCTCGAAGAGTACGCATCGAAGAAAGGCCGCAAGCCTAGCGCATACGCACCGCTCCA GTTACTCAAGCCGGAGGCCTCCGCGTTTATCACCCTGAAAGTTATCCTTGCGTCACTAACCAGTACGAACATGACAACC ATTCAGGCCGCTGCTGGTATGCTGGGGAAAGCCATTGAGGACGAGGCACGATTTGGGCGCATCCGTGACCTAGAAGC GAAGCACTTCAAGAAGCACGTTGAGGAACAGCTTAACAAGCGCCACGGGCAAGTCTACAAGAAAGCATTTATGCAGG TGGTCGAGGCCGATATGATTGGTCGAGGTCTGCTTGGTGGCGAGGCGTGGTCTAGCTGGGATAAAGAAACCACGAT GCACGTAGGGATTCGCCTGATTGAAATGCTGATTGAATCCACGGGTCTGGTGGAATTACAGCGCCACAACGCAGGTA ACGCAGGCTCTGACCATGAGGCACTGCAACTGGCCCAAGAGTACGTGGACGTATTAGCGAAGCGTGCAGGCGCTCTG GCGGGTATCTCTCCGATGTTCCAGCCGTGTGTCGTACCGCCGAAACCTTGGGTAGCAATCACAGGGGGCGGCTATTG GGCTAACGGTCGCAGACCTTTGGCACTCGTTCGCACTCACTCTAAGAAGGGCTTGATGCGCTACGAAGACGTTTACAT GCCAGAAGTCTACAAGGCTGTGAACCTCGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTTCTTGCTGTTGTCAA TGAGATTGTTAACTGGAAGAATTGCCCGGTAGCAGACATTCCATCGCTGGAGCGCCAAGAGTTACCGCCTAAGCCTG ACGACATTGACACCAACGAGGCAGCGCTCAAGGAGTGGAAGAAAGCCGCTGCTGGTATCTATCGCTTGGACAAGGC ACGAGTGTCTCGCCGTATCAGCTTAGAGTTCATGCTGGAGCAGGCCAACAAGTTCGCAAGTAAGAAAGCAATCTGGT TCCCTTACAACATGGACTGGCGCGGTCGTGTGTACGCTGTGCCGATGTTCAACCCGCAAGGCAACGACATGACGAAA GGTCTGCTGACCCTTGCTAAAGGCAAGCCAATCGGTGAGGAAGGTTTCTACTGGCTGAAAATCCACGGTGCGAACTG TGCGGGTGTTGATAAGGTTCCATTCCCGGAGCGCATCGCGTTCATTGAGAAGCACGTAGACGACATTCTGGCTTGCGC TAAAGACCCAATCAATAACACTTGGTGGGCTGAGCAGGATTCACCGTTCTGTTTCCTCGCGTTTTGCTTCGAGTATGCA GGCGTTACGCACCACGGTCTGAGCTACAATTGCTCTCTGCCGCTGGCGTTCGACGGGTCTTGCTCTGGTATCCAGCAC TTCTCCGCGATGCTCCGCGATGAGGTAGGCGGTCGTGCGGTTAACCTGCTGCCAAGCGAAACCGTGCAGGACATTTA CGGCATCGTTGCACAGAAAGTAAACGAGATTCTCAAACAGGATGCAATCAACGGCACGCCTAACGAGATGATTACCG TGACCGACAAGGACACCGGGGAAATCTCAGAGAAGCTCAAACTTGGAACCTCAACGCTGGCGCAACAGTGGCTGGC ATATGGTGTAACCCGTAGCGTAACTAAACGTTCGGTCATGACGCTGGCTTACGGTTCCAAGGAGTTCGGCTTTCGTCA ACAGGTATTGGATGACACCATTCAGCCTGCAATTGACAGCGGTAAGGGCTTGATGTTCACCCAACCGAACCAAGCGG CTGGCTATATGGCTAAGCTGATTTGGGATGCGGTAAGCGTGACCGTAGTTGCAGCGGTTGAGGCGATGAACTGGCTC AAATCTGCCGCTAAGCTGCTGGCTGCTGAGGTCAAGGACAAGAAGACCAAGGAGATTCTGCGCCACCGTTGCGCGGT TCACTGGACTACGCCGGACGGCTTCCCGGTCTGGCAGGAATACCGCAAGCCACTCCAGAAGCGTCTCGATATGATTTT CTTAGGGCAATTCCGTCTGCAACCGACGATTAATACCCTCAAGGATTCAGGCATTGACGCACACAAGCAGGAGTCTGG CATCGCTCCTAACTTTGTTCACTCACAGGACGGTAGCCACCTCCGCATGACAGTCGTTTATGCTCACGAGAAGTATGGC ATTGAGTCCTTTGCGCTCATCCATGACAGCTTTGGGACTATCCCGGCAGACGCTGGTAAGCTCTTTAAGGCTGTGCGT GAAACGATGGTTATCACCTATGAGAACAACGATGTGCTGGCAGACTTCTACTCTCAGTTTGCCGACCAGCTACACGAG ACCCAACTGGACAAGATGCCTCCGCTTCCGAAGAAAGGAAACCTGAACCTGCAAGACATTCTCAAGTCTGACTTTGCC TTTGCA**TAA**CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGC TGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
RL063K	pT3 pLsrA aHL sfGFP	ATTAACCCTCACTAAAGGGAGATCAAAACTCACCTGCAAAACTGAACGGGGG*AAGGAG*AATAATCT**ATG**GATTCTG ATATCAATATCAAAACCGGCACCACCGATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATA AAGAAAACGGTATGCATAAAAAAGTGTTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCC GCACCAAAGGCACCATTGCGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCG TCTGCCTTTAAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATC GATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGC GGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGT CCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGA TTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCT GGACCCGAACAAAGCCTCAAGCCTGCTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAA AGCCAGCAAACAGCAGACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAAC CAATTGGAAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAG AAGAAATGACCAACggatccggcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGG AACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTG ACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTC AGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGG AACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTA AACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAAC AGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAG GATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAAT CACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTA ACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAG GTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL064K	pT7 Y26H Q33A lsrR T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG* *AG*ATATACC**ATG**GCAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGGATCG CGT GGTTTCACTATCACGACGGGCTGACCGCGAGCGAGATCAGCGATCGTCTCGGCCTGACACGTTTGAAAGT GTCGCGATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGC TGTCTGGAATATGAAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTG CGGATGCTGATGTCGGTGGGCGACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACA

		ACAGATGCTGGCGATTGGTTTTGGCGAGGCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCG TCACAGCAAATTCGCCTGGTCACGCTCTCCGGTGGCGTCGGTTCTTATATGACGGGAATCGGGCAGCTTA ACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCGGGCATCCTCCGCTGACATTGCCCGTACGCT AAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGCGGATGTGGCGATTGTCGGCATT GGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCAGGGCGAACAGTTAA TGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACGTTGTCAC GAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTT GGCGTGGCAGGGGGAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTA CCGATCAGGACACAGCAGCGGCGATTTTACGTAGTCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGC**TAA**C AAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACG GGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGGGACG
RL065K	pT3 pLsrR aHL sfGFP	AATTAACCCTCACTAAAGGGAGATGAACAATTGCATTAAAGATTTAAATATGT*AAGGAG*AATAATCT**ATG**GATTCTG ATATCAATATCAAAACCGGCACCACCGATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATA AAGAAAACGGTATGCATAAAAAAGTGTTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCC GCACCAAAGGCACCATTGCGGGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCG TCTGCCTTTAAAGTGCAGCTGCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATC GATACCAAAGAATATATGAGTACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGC GGTCTGATTGGCGCCAATGTGTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGT CCGACCGATAAAAAAGTGGGTTGGAAAGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGA TTCCTGGAATCCGGTTTATGGCAATCAGCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCT GGACCCGAACAAAGCCTCAAGCCTGCTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAA AGCCAGCAAACAGCAGACCAACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAAC CAATTGGAAAGGCACCAATACCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAG AAGAAATGACCAACggatccggcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGG AACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTG ACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTC AGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGG AACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTA AACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAAC AGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAG GATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAAT CACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTA ACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAG GTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL068K	pT7 RBS eLsrR T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG AG*ATATACC**ATG**ACAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGAAGAAGAACAGGTCGCGCGGATCG CGTGGTTTTACTATCACGACGGGCTGACCCAGAGCGAGATCAGCGACCGTCTCGGCCTGACACGTTTGAAAGTGTCGC GATTGCTGGAGAAAGGGCATCAGTCCGGCATTATTCGCGTACAGATTAATTCTCGCTTTGAAGGCTGTCTGGAATATG AAACTCAATTACGTCGTCAGTTTTCGCTGCAACATGTCCGGGTGATCCCTGGGCTTGCGGATGCTGATGTCGGTGGGC GACTGGGGATAGGCGCGGCGCATATGTTGATGAGTTTACTTCAACCACAACAGATGCTGGCGATTGGTTTTGGCGAG GCAACCATGAATACGCTGCAACGCTTAAGTGGTTTTATTTCGTCACAGCAAATTCGCCTGGTCACGCTCTCCGGTGGCG TCGGTTCTTATATGACGGGAATCGGGCAGCTTAACGCGGCGTGCAGTGTGAATATTATTCCGGCTCCGTTGCGGGCAT CCTCCGCTGACATTGCCCGTACGCTAAAAAATGAAAATTGCGTCAAAGATGTTCTGTTAGCCGCGCAAGCAGCGGATG TGGCGATTGTCGGCATTGGTGCTGTGAGTCAACAGGACGATGCGACAATCATTCGCTCCGGTTATATCAGCCAGGGC GAACAGTTAATGATTGGCCGAAAAGGGGCGGTTGGCGACATTTTAGGCTACTTTTTTGATGCAAAAGGTGACGTTGTC ACGAATATCAAAATACATAACGAACTGATTGGCTTACCTTTAAGCGCGCTGAAGACCATACCCGTCCGGGTTGGCGTG GCAGGGGAAGAAAATAAAGCCGAAGCAATTGCCGCTGCAATGAAAGGCGGTTATATCAACGCACTGGTTATCGATCA GGACACAGCAGCGGCGATTTTACGTAGT**TAA**GCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCCGAATTCG AGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGA AAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAG GGGTTTTTTG
RL070K	pT7 reverted lsr intergenic region aHL sfGFP	AATTAACCCTCACTAAAGGGAGAATTTCCCCCGTTCAGTTTTGCAGGTGAGTTTTGAACAAATGTATTTCTGCTTTTAAT TTGTTCATAACCTTAGGTGGACATTGCACATATTTCCGACGAATAGATCACAATTTATGCTATTTTGATTTTCACGGTTG CGTTTGTTCATGCTCGTAGAGTCAAACTGTGGTTGCCATCACAGATATAAATGAGCAAGAACTGAACAATTGCATTAA AGATTTAAATATGTTCAAAGTGAAGAATGAATT*AAGGAG*AATAATCT**ATG**GATTCTGATATCAATATCAAAACCGGC ACCACCGATATCGGCTCCAATACCACCGTTAAAACCGGTGATCTGGTGACCTATGATAAAGAAAACGGTATGCATAAA AAAGTGTTTTACTCGTTTATTGACGATAAAAACCATAACAAAAAACTGCTGGTCATCCGCACCAAAGGCACCATTGCG GGTCAATACCGTGTGTACTCCGAAGAAGGTGCGAACAAAAGCGGTCTGGCTTGGCCGTCTGCCTTTAAAGTGCAGCT GCAACTGCCGGATAATGAAGTGGCGCAGATTTCAGATTATTATCCGCGTAATAGCATCGATACCAAAGAATATATGAG TACCCTGACCTATGGTTTTAATGGCAATGTTACCGGTGATGATACGGGTAAAATTGGCGGTCTGATTGGCGCCAATGT GTCCATTGGTCATACGCTGAAATACGTGCAACCGGATTTCAAAACCATTCTGGAAAGTCCGACCGATAAAAAAGTGGG TTGGAAAGTTATCTTCAACAACATGGTGAATCAGAACTGGGGTCCGTACGATCGCGATTCCTGGAATCCGGTTTATGG CAATCAGCTGTTTATGAAAACCCGCAACGGTAGTATGAAAGCGGCGGATAATTTTCTGGACCCGAACAAAGCCTCAA GCCTGCTGTCCAGCGGTTTTAGCCCGGATTTTGCCACGGTTATTACCATGGATCGCAAAGCCAGCAAAACAGCAGACCA ACATTGATGTGATCTACGAACGTGTGCGTGATGATTATCAACTGCATTGGACCTCAACCAATTGGAAAGGCACCAATA CCAAAGATAAATGGACGGATCGCAGTTCAGAACGCTACAAAATTGATTGGGAAAAAGAAGAAATGACCAACggatccg gcagcggttct**ATG**CGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAAC GGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTAC TACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCG GACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAG GATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAG

		GCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCA CCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTG GCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAA AGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCAC GCATGGTATGGATGAACTGTACAAA**TAA**ACTAGTCTGCAGTCCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCT TTTTCTTTAAAACCGAAAAGATTACTTCGCGT
RL073C	J23100 B0034 lasR B0015 pLasRL B0030 mut3bGFP B1006	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCTACTAGAG*AAAGAGGAGAAA*TACTAG**ATG**GCCTTGGTTGA CGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGGAGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTG GATTCTCGAAGATCCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGGCAACTACC CGGCCGCCTGGCGCGAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGC GTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGCCTCGGCCGCC GGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCTGAGCCTCAGCGTGGAAG CGGAAAACCGGGCCGAGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGATGCTCAAGGACTACGCACTG CAAAGCGGTGCCGGACTGGCCTTCGAACATCCGGTCAGCAAACCGGTGGTTCTGACCAGCCGGGAGAAGGAAGTGT TGCAGTGGTGCGCCATCGGCAAGACCAGTTGGGAGATATCGGTTATCTGCAACTGCTCGGAAGCCAATGTGAACTTC CATATGGGAAATATTCGGCGGAAGTTCGGTGTGACCTCCCGCCGCGTAGCGGCCATTATGGCCGTTAATTTGGGTCTT ATTACTCTC**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT CTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATAGCGCG TCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCTTTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGA AATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCCGCGGGTTCTTTTTGGTACACGAAAGCACCGTCGAAAACGGG ACCGAGCCAGGGGAGTGCAGTTCCTTCTACCCGAAGGACTGATACGGCTGTTCCGATCAGCCCACAAGGCGGCGGTA AGCGTCGGCCGAGTACTTCGGCCTGAAAAAACCAGGAGAACTGAACAAG*ATTAAAGAGGAGAAA*TACTAG**ATG**C GTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTC TGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACC TGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCCAGATCATATGAAACAG CATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTAC AAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAA GATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAG AATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAA AATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCC CAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATA CAAA**TAA**TAATACTAGAGAAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTTT
RL076A	pT7 RBS CRP T7 term	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG*AAGG AG*ATATACAT**ATG**GTGCTTGGCAAACCGCAAACAGACCCGACTCTCGAATGGTTCTTGTCTCATTGCCACATTCATAA GTACCCATCCAAGAGCACGCTTATTCACCAGGGTGAAAAAGCGGAAACGCTGTACTACATCGTTAAAGGCTCTGTGGC AGTGCTGATCAAAGACGAAGAGGGTAAAGAAATGATCCTCTCCTATCTGAATCAGGGTGATTTTATTGGCGAACTGG GCCTGTTTGAAGAGGGCCAGGAACGTAGCGCATGGGTACGTGCGAAAACCGCCTGTGAAGTGGCTGAAATTTCGTAC AAAAAATTTCGCCAATTGATTCAGGTAAACCCGGACATTCTGATGCGTTTGTCTGCACAGATGGCGCGTCGTCTGCAA GTCACTTCAGAGAAAGTGGGCAACCTGGCGTTCCTCGACGTGACGGGCCGCATTGCACAGACTCTGCTGAATCTGGC AAAACAACCAGACGCTATGACTCACCCGGACGGTATGCAAATCAAAATTACCCGTCAGGAAATTGGTCAGATTGTCGG CTGTTCTCGTGAAACCGTGGGACGCATTCTGAAGATGCTGGAAGATCAGAACCTGATCTCCGCACACGGTAAAACCAT CGTCGTTTACGGCACTCGT**TAA**GCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGACTCGAGCACCACCA CCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAAC TAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
RL078A	pTet B0034 luxR B0015 pLuxR B0032 luxI B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ACTATAATGATA AAAAAATCGGATTTTTTGGCAATTCCATCGGAGGAGTATAAAGGTATTCTAAGTCTTCGTTATCAAGTGTTTAAGCAAA GACTTGAGTGGGACTTAGTTGTAGAAAATAACCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATG CTTGTGATGATACTGAAAATGTAAGTGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTT TCCTGAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGTAGGTAAAAAT AGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAGCTATATATAAACACGCTGTTAGTCAAG GTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAGCGATTTTTAAAGCGTATTAAAGTTCCTTGTCATCGTAT TGGAGACAAAGAAATTCATGTATTAGGTGATACTAAATCGGTTGTATTGTCTATGCCTATTAATGAACAGTTTAAAAAA GCAGTCTTAAAT**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
RL079A	pTet B0034 luxR B0015 pLuxR	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG

	B0032 lasI B0015	ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ATCGTTCAGATC GGTCGTCGTGAAGAGTTCGACAAAAAACTGCTGGGTGAAATGCACAAACTGCGTGCTCAGGTTTTCAAAGAACGTAA AGGTTGGGACGTTTCCGTTATCGACGAAATGGAAATCGACGGTTACGACGCTCTGTCCCCGTACTACATGCTGATCCA GGAAGACACCCCGGAAGCTCAGGTTTTCGGTTGCTGGCGTATCTTCGACACCACCGGTCCGTACATGCTGAAAAACAC CTTCCCGGAACTGCTGCACGGTAAAGAAGCTCCGTGCTCCCCGCACATCTGGGAACTGTCCCGTTTCGCTATCAACTCC GGTCAGAAAGGTTCCCTGGGTTTCTCCGACTGCACCCTGGAAGCTATGCGTGCTCTGGCTCGTTACTCCTTGCAGAAC GACATCCAGACCCTGGTTACCGTTACCACCGTTGGTGTTGAAAAAATGATGATCCGTGCTGGTCTGGACGTTTCCCGTT TCGGTCCGCACCTGAAAATCGGTATCGAACGTGCTGTTGCTCTGCGTATCGAACTGAACGCTAAAACCCAGATCGCTC TGTACGGTGGTGTTCTGGTTGAACAGCGTCTGGCTGTTTCC**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAA GGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCA CCTTCGGGTGGGCCTTTCTGCGTTTATA
RL080A	pTet B0034 luxR B0015 pLuxR B0032 rhlI B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ATCGAACTGCT GTCCGAATCCCTGGAAGGTCTGTCCGCTGCTATGATCGCTGAACTGGGTCGTTACCGTCACCAGGTTTTCATCGAAAA ACTGGGTTGGGACGTTGTTTCCACCTCCCGTGTTCGTGACCAGGAGTTCGACCAGTTCGACCACCCGCAGACCCGTTA CATCGTTGCTATGTCCCGTCAGGGTATCTGCGGTTGCGCTCGTCTGCTGCCGACCACCGACGCTTACCTGCTGAAAGA CGTTTTCGCTTACCTGTGCTCCGAAACCCCGCCGTCCGACCCGTCCGTTTGGGAACTGTCCCGTTACGCTGCTTCCGCT GCTGACGACCCGCAGCTGGCTATGAAAATCTTCTGGTCCTCCCTCCAGTGCGCTTGGTACCTGGGTGCTTCCTCCGTTG TTGCTGTTACCACCACCGCTATGGAACGTTACTTCGTTCGTAACGGTGTTATCCTCCAGCGTCTGGGTCCGCCGCAGAA AGTTAAAGGTGAAACCCTGGTTGCTATCTCCTTCCCGGCTTACCAGGAACGTGGTCTGGAAATGCTGCTGCGTTACCA CCCGGAATGGCTCCAGGGTGTTCCGCTGTCCATGGCTGTT**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAG GCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCAC CTTCGGGTGGGCCTTTCTGCGTTTATA
RL082A	pTet B0034 luxR B0015 pLuxR B0032 luciferase B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**GAAGACGCCAA AAACATAAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGA AGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTGTGAGTATTTCTGTCTGATTTCTTTCGAGTTAACGAAATGTTCTT ATGTTTCTTTAGACAGATGCACATATCGAGGTGAACATCACGTACGCGGAATACTTCGAAATGTCCGTTCGGTTGGCA GAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATG CCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGAACGTAAGCACCCTCGC CATCAGACCAAAGGGAATGACGTATTTAATTTTTAAGGTGAATTGCTCAACAGTATGAACATTTCGCAGCCTACCGTA GTGTTTGTTTCCAAAAAGGGGTTGCAAAAAATTTTGAACGTGCAAAAAAAATTACCAATAATCCAGAAAATTATTATCA TGGATTCTAAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTACCTCCCGGTTTTAATGA ATACGATTTTGTACCAGAGTCCTTTGATCGTGACAAAACAATTGCACTGATAATGAATTCCTCTGGATCTACTGGGTTA CCTAAGGGTGTGGCCCTTCCGCATAGAACTGCCTGCGTCAGATTCTCGCATGCCAGGTATGTCGTATAACAAGAGATT AAGTAATGTTGCTACACACATTGTAGAGATCCTATTTTTGGCAATCAAATCATTCCGGATACTGCGATTTTAAGTGTTG TTCCATTCCATCACGGTTTTGGAATGTTTACTACACTCGGATATTTGATATGTGGATTTCGAGTCGTCTTAATGTATAGA TTTGAAGAAGAGCTGTTTTTACGATCCCTTCAGGATTACAAAATTCAAAGTGCGTTGCTAGTACCAACCCTATTTTCATT CTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTACACGAAATTGCTTCTGGGGGCGCACCTCTTTCG

		AAAGAAGTCGGGGAAGCGGTTGCAAAACGGTGAGTTAAGCGCATTGCTAGTATTTCAAGGCTCTAAAACGGCGCGTA GCTTCCATCTTCCAGGGATACGACAAGGATATGGGCTCACTGAGACTACATCAGCTATTCTGATTACACCCGAGGGGG ATGATAAACCGGGCGCGGTCGGTAAAGTTGTTCCATTTTTTGAAGCGAAGGTTGTGGATCTGGATACCGGGAAAACG CTGGGCGTTAATCAGAGAGGCGAATTATGTGTCAGAGGACCTATGATTATGTCCGGTTATGTAAACAATCCGGAAGC GACCAACGCCTTGATTGACAAGGATGGATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTT CATAGTTGACCGCTTGAAGTCTTTAATTAAATACAAAGGATATCAGGTAATGAAGATTTTTACATGCACACACGCTACA ATACCTGTAGGTGGCCCCCGCTGAATTGGAATCGATATTGTTACAACACCCCAACATCTTCGACGCGGGCGTGGCAGG TCTTCCCGACGATGACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAG AGATCGTGGATTACGTCGCCAGTAAATGAATTCGTTTTACGTTACTCGTACTACAATTCTTTTCATAGGTCAAGTAACA ACCGCGAAAAAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTCGACGCAA GAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAATTG**TAA**TAATACTAGAGCCAGGCATC AAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGA GTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
RL083C	pTet B0034 lasR B0015 pLasRL mut3bGFP B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TA CTAG**ATG**GCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGGAGTGGAGCGCCATCCTCCAGA AGATGGCGAGCGACCTTGGATTCTCGAAGATCCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCC TTCATCGTCGGCAACTACCCGGCCGCCTGGCGCGAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGT CAGTCACTGTACCCAGAGCGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTT CGAGGAAGCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCG CTGAGCCTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGAT GCTCAAGGACTACGCACTGCAAAGCGGTGCCGGACTGGCCTTCGAACATCCGGTCAGCAAACCGGTGGTTCTGACCA GCCGGGAGAAGGAAGTGTTGCAGTGGTGCGCCATCGGCAAGACCAGTTGGGAGATATCGGTTATCTGCAACTGCTC GGAAGCCAATGTGAACTTCCATATGGGAAATATTCGGCGGAAGTTCGGTGTGACCTCCCGCCGCGTAGCGGCCATTA TGGCCGTTAATTTGGGTCTTATTACTCTC**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAA GACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGC CTTTCTGCGTTTATAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCTTTTCTGCTAGCTATTCCAGCGAAA ACATACAGATTTCCGGCGAAATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGAA AGCACCGTCGAAAACGGGACCGAGCCAGGGGAGTGCAGTTCCTTCTACCCGAAGGACTGATACGGCTGTTCCGATCA GCCCACAAGGCGGCGGTAAGCGTCGGCCGAGTACTTCGGCCTGAAAAAACCAGGAGAACTGAACAAG*ATTAAAGA GGAGAAA*TACTAG**ATG**CGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGAT GTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATT TGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGAT ACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTT TCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTA AAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATAC ATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCA ACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACA CAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATT ACACATGGCATGGATGAACTATACAAA**TAA**TAATACTAGAGAAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTT TTT
RL084C	pLasB B0032 mut3bGFP pTet B0034 lasR	GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCTTTTCTGCTAGCTATTCCAGCGAAAAC ATACAGATTTCCGGCGAAATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGAAAG CTACTAGAG*ATTAAAGAGGAGAAA*TACTAG**ATG**CGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTT GAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACT TACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTC AATGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGG AAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTA ATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACT CACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAG ATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCA TTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACA GCTGCTGGGATTACACATGGCATGGATGAACTATACAAA**TAA**TAATACTAGAGTCCCTATCAGTGATAGAGATTGACA TCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACTAG**ATG**GCCTTGGTTGACGGTTTT CTTGAGCTGGAACGCTCAAGTGGAAAATTGGAGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTC GAAGATCCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGGCAACTACCCGGCCGC CTGGCGCGAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGCGTACTGC CGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGCCTCGGCCGCCGGCCTG GTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCTGAGCCTCAGCGTGGAAGCGGAAA ACCGGGCCGAGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGATGCTCAAGGACTACGCACTGCAAAGC GGTGCCGGACTGGCCTTCGAACATCCGGTCAGCAAACCGGTGGTTCTGACCAGCCGGGAGAAGGAAGTGTTGCAGT GGTGCGCCATCGGCAAGACCAGTTGGGAGATATCGGTTATCTGCAACTGCTCGGAAGCCAATGTGAACTTCCATATG GGAAATATTCGGCGGAAGTTCGGTGTGACCTCCCGCCGCGTAGCGGCCATTATGGCCGTTAATTTGGGTCTTATTACT CTC**TAA**TAA
RL086A	pTet B0034 T33A S116A M135I luxR B0015 pLuxR	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGGCGAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACAGCGGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATTCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA

	B0032 mut3bGFP B0015	AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ACTATAATGATA AAAAAATCGGATTTTTTGGCAATTCCATCGGAGGAGTATAAAGGTATTCTAAGTCTTCGTTATCAAGTGTTTAAGCAAA GACTTGAGTGGGACTTAGTTGTAGAAAATAACCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATG CTTGTGATGATACTGAAAATGTAAGTGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTT TCCTGAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGTAGGTAAAAAT AGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAGCTATATATAAACACGCTGTTAGTCAAG GTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAGCGATTTTTAAAGCGTATTAAAGTTCCTTGTCATCGTAT TGGAGACAAAGAAATTCATGTATTAGGTGATACTAAATCGGTTGTATTGTCTATGCCTATTAATGAACAGTTTAAAAAA GCAGTCTTAAAT**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
RL087A	pTet B0034 T33A R65M S116A M135I luxR B0015 pLuxR B0032 mut3bGFP B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGGCGAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGATGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACAGCGGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATTCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ACTATAATGATA AAAAAATCGGATTTTTTGGCAATTCCATCGGAGGAGTATAAAGGTATTCTAAGTCTTCGTTATCAAGTGTTTAAGCAAA GACTTGAGTGGGACTTAGTTGTAGAAAATAACCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATG CTTGTGATGATACTGAAAATGTAAGTGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTT TCCTGAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGTAGGTAAAAAT AGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAGCTATATATAAACACGCTGTTAGTCAAG GTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAGCGATTTTTAAAGCGTATTAAAGTTCCTTGTCATCGTAT TGGAGACAAAGAAATTCATGTATTAGGTGATACTAAATCGGTTGTATTGTCTATGCCTATTAATGAACAGTTTAAAAAA GCAGTCTTAAAT**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
T9002	pTet B0034 luxR B0015 pLuxR B0032 mut3bGFP B0015	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACTACTAGAG*AAAGAGGAGAAA*TACT AG**ATG**AAAAACATAAATGCCGACGACACATACAGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTA ATCAATGCTTATCTGATATGACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTT AAATCTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTTAATAAAATATG ATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATATTTGAAAACAATGCTGTAAATAAAAAA TCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCTTATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATG GCTTCGGAATGCTTAGTTTTGCACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACAT ACCATTAATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGATTTAACCA AAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATATTTCAAAAATATTAGGTTGCAGT GAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAAACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAG CAATTTTAACAGGAGCAATTGATTGCCCATACTTTAAAAAT**TAA**TAACACTGATAGTGCTAGTGTAGATCACTACTAGA GCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT CTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCGTACAGG TTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG*TCACACAGGAAAG*TACTAG**ATG**ACTATAATGATA AAAAAATCGGATTTTTTGGCAATTCCATCGGAGGAGTATAAAGGTATTCTAAGTCTTCGTTATCAAGTGTTTAAGCAAA GACTTGAGTGGGACTTAGTTGTAGAAAATAACCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATG CTTGTGATGATACTGAAAATGTAAGTGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTT TCCTGAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGTAGGTAAAAAT AGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAGCTATATATAAACACGCTGTTAGTCAAG GTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAGCGATTTTTAAAGCGTATTAAAGTTCCTTGTCATCGTAT TGGAGACAAAGAAATTCATGTATTAGGTGATACTAAATCGGTTGTATTGTCTATGCCTATTAATGAACAGTTTAAAAAA GCAGTCTTAAAT**TAA**TAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA

*Promoters are underlined, start and stop codons are in bold, the RBS is in italics, and linker sequences in fusion proteins are in lowercase. *lrs* intergenic regions and its variants are in red.

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