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Synthetic biology – characterizing promoters and evaluating the methods

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Title (English)

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Abstract

Synthetic biology is a new genetic engineering approach built on: (i) a complexity hierarchy of parts (e.g. promoters), devices and systems, and (ii) standardization. I have evaluated fluorescence imaging, microscopy imaging and Northern blotting as methods to characterize and quantify the strength of individual promoters. Three different promoters were assembled with fluorescent protein constructs, using the BioBrick system, and characterized. Quantifications revealed that the promoters behaved like the theoretical models predicted and fluorescence imaging was found to be the most robust quantification method.

Keywords

Synthetic biology, BioBrick, genetic engineering, promoters, promoter characterization, fluorescence imaging, fluorescence microscopy, Northern blotting.

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Synthetic biology – characterizing promoters and evaluating the methods

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Sammanfattning

Syntetisk biologi är modern biologisk ingenjörskonst som utifrån ett systematiskt arbetssätt öppnar upp för stora framtidsmöjligheter. Man använder små DNA-element, t.ex. gener, med olika egenskaper som byggstenar. Dessa sätts sedan ihop för att producera ett biologiskt system med önskad funktion. Den syntetiska biologin är uppbyggd kring en komplexitetshierarki; små genetiska element kombineras för att bygga upp en större del, vilken i sin tur kombineras med andra delar till ett helt system, t.ex. en energiproducerande bakterie. En analogi är mikroelektroniken där små delar som transistorer kombineras med t.ex. resistorer, tillsammans skapar de något större som en logisk krets. Sedan kombineras dessa kretsar med andra kretsar och till slut har man skapat ett helt system – en dator. Precis som i mikroelektroniken måste olika biologiska delar vara standardiserade, dvs. de ska passa med varandra och deras egenskaper måste vara kända.

Jag har undersökt hur väl tre olika metoder passar för att karaktärisera små standardiserade genetiska delar, promotorer, som driver uttrycket av en gen. Genom att använda ett standardiserat tillvägagångssätt, BioBricksystemet, kopplades dessa promotorer till gener för fluorescenta proteiner. Sedan mättes genuttrycket från promotorerna och resultaten jämfördes mellan de tre metoderna. Två av metoderna, fluorescensavbildning samt fluorescensmikroskopi, mäter fluorescensen från de bildade proteinerna som ett mått på hur stark en promotor är. Den tredje metoden, Northern blotting, mäter promotorstyrkan några steg innan proteinerna bildas. Det visade sig att resultaten från alla tre metoderna stödjer varandra och stämmer överens med vad som förväntades av promotorerna teoretiskt. Fluorescensavbildning är den mest pålitliga metoden av de tre för promotorkaraktärisering eftersom den är lättanvänd och minst känslig för störningar.

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1. Introduction

1.1 A new era of biological engineering

In some areas of molecular biology, especially genetic engineering, many procedures are inefficient and resource consuming. Nonetheless, it is likely that some laboratories have devised brilliant solutions making these procedures better and more efficient. Unfortunately, many other labs will never hear of them. Genetic engineering is a good example of a molecular biology trial and error process where routine cloning of a DNA sequence can prove almost impossible. However, it is quite possible that this sequence already has been cloned and that it lies frozen in somebody's freezer. Considering these obstacles, is there a way to make the slow process of genetic engineering faster and more predictable so time and resources can be spent better? The answer could be Synthetic biology. This new concept does not only aim to solve genetic engineering issues, but to take engineering principles into molecular biology with the purpose of understanding complex biological circuits and building new ones (Endy 2005). By using standard methods and well characterized genetic parts available in registries, methods and constructs can be shared, and with the ever-cheaper possibility of synthesizing DNA sequences it is enough to obtain the sequence of a wanted construct and order it (Pleiss 2006). Synthetic biology can be used for a plethora of applications in addition to making genetic engineering more efficient. Using genetic network and protein interaction computer simulations to aid in construction, one can envision complex molecular machines with specific functions being built. With further development in the field, such engineered multi-protein machines could be tailor-made to achieve greater goals in medicine or environmental remediation such as improved healing of damaged tissue or neutralization of toxic chemicals. At present time though, a successful area of applied Synthetic biology is metabolic engineering, with the production of the anti-malarial drug precursor artemisinin being a prime example of one of the first demonstrated applications (Ro *et al.* 2006).

As fossil energy sources are predicted to become scarce in the future, and since the utilization of them exacerbate global warming, there is a need for alternative and renewable energy sources (IPCC Working Group III report 2007). Hydrogen is a clean and storable energy carrier that can be produced by sustainable energy sources including solar, wind, geothermal, nuclear and hydropower (Elam *et al.* 2003). Some Cyanobacteria, which are the photosynthetic bacteria responsible for our oxygen-rich atmosphere, have the ability of producing small amounts of hydrogen gas if the conditions are right. Considering these factors, the improvement of hydrogen production in Cyanobacteria could become a lucrative and important application of Synthetic biology. Therefore, this project is carried out to lay the path for a large scale European project concerning the use of Synthetic biology for obtaining a cyanobacterium that produces significant amounts of hydrogen, BioModularH_2 (BioModularH_2 2006).

1.2 Synthetic biology

1.2.1 Definition

Synthetic biology is a new concept in molecular biology and can be defined as:
A. The design and construction of new biological parts, devices, and systems, and
B. The re-design of existing, natural biological systems for useful purposes (Synthetic biology community).

1.2.2 Standardization and hierarchy

Two important ideas reside in the heart of Synthetic biology: standardization and an abstract hierarchy, which both confer characteristic engineering features to this new field.

Standardization of parts involves surrounding them with standardized sequences containing restriction sites and storing them in standardized plasmids. The functions of the parts are characterized, and they or the plasmids must not contain certain restriction sites that are used in part manipulations. By surrounding parts with standard restriction sites they may be assembled arbitrarily, which gives many advantages. Required parts may be synthesized or acquired directly from a part repository, and these parts can be directly assembled in a construct or added to an existing construct by means of a standard procedure involving repeated restriction cleavage and ligation. Furthermore, by having parts with well-characterized functions, one immediately gets an idea about how they will function in an assembly, and an aid in the engineering of composite devices and systems.

Parts and assemblies are divided in a hierarchical abstraction according to their complexity level. The first is the part level, which consists of single genetic parts, e.g. promoters. On the second level are assemblies of parts called devices, e.g. an oscillating genetic circuit. The third and most complex level is the system level, which consists of assemblies of devices and parts, together creating e.g. a whole metabolic pathway (Fig. 1). Because of part standardization parts may be arbitrarily assembled into devices and systems, and thanks to the hierarchical abstraction people working on specific levels does not have to be experts in other levels. This means that the hierarchical abstraction together with standardization creates level specialization, and makes parallel work on different levels possible.

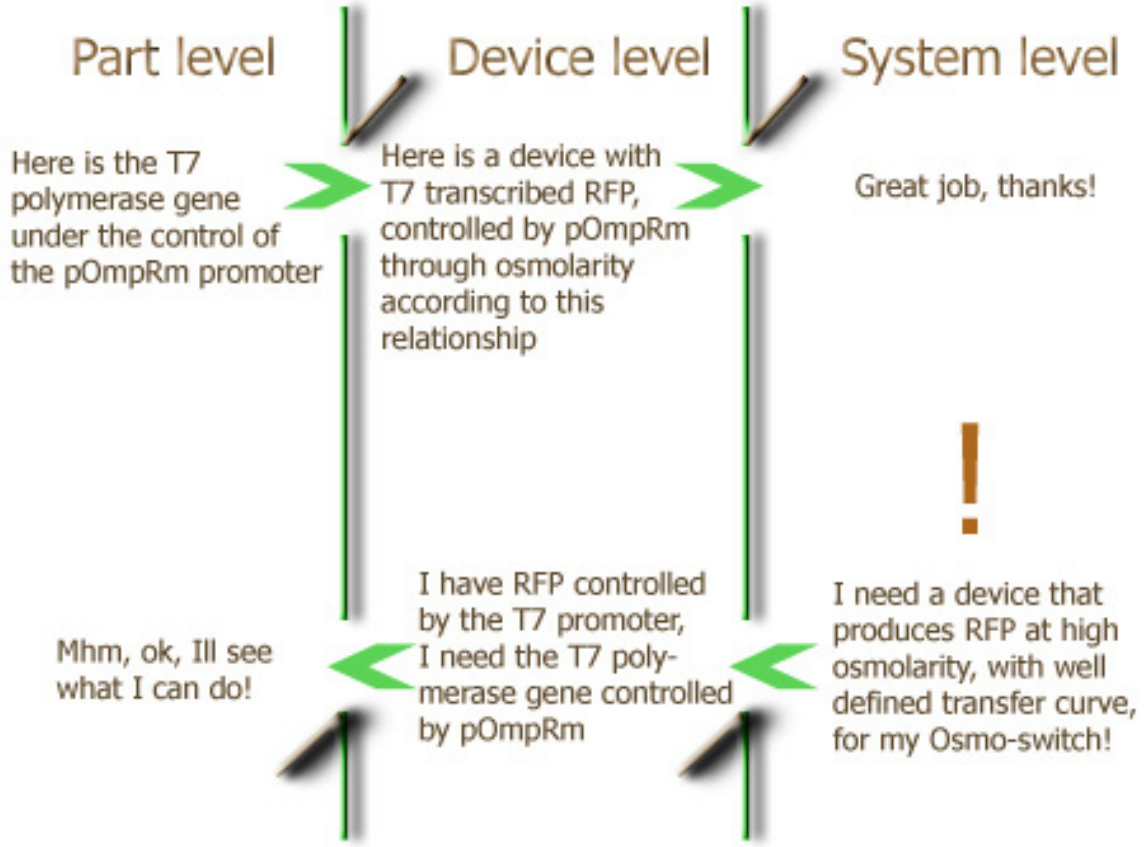

Fig. 1. Hierarchy levels in Synthetic biology. A fictional line of events that might take place between three biological engineers working at different hierarchy levels.

1.2.3 The standard BioBrick interface

In 2001, a vector carrying a standard cloning site sequence named "Standard BioBrick Sequence Interface" was introduced. This BioBrick interface was developed to simplify the normally tedious trial and error process of cloning and assembling genetic parts, and to enable arbitrary part assembly (Knight 2001). Since then, several BioBrick interface carrying vectors containing lots of different BioBrick parts have been produced; the sequences are all available on an open source basis through the Registry of Standard Biological Parts (Registry of Standard Biological Parts). At this time, all BioBrick parts are surrounded by either of two standard genetic interfaces, depending on if the part is protein coding or not. Common for both interfaces are the five recognition sites for the restriction enzymes EcoRI, NotI, XbaI, SpeI and PstI. These standard sequences implement the concept of standardization and make arbitrary assembly of parts possible.

1.2.3.1 Non-coding parts

This interface contains the standard restriction enzyme recognition sites and a few nucleotides as spacers (Fig. 2).

```
GAATTC GCGGCCGC T TCTAGA G "part1" T ACTAGT A GCGGCCG CTGCAG
CTTAAG CGCCGGCG A AGATCT C "part1" A TGATCA T CGCCGGC GACGTC
 EcoRI   NotI      XbaI               SpeI      NotI   PstI
  E               X                  S              P
```

Fig. 2. The standard DNA interface of the Synthetic biology BioBrick standard for non-coding parts, restriction enzyme recognition sites are marked with name. Different BioBrick parts can be arbitrarily assembled by digestion and ligation. Digestion occurs between the nucleotides marked in green.

1.2.3.2 Coding parts

The interface sequence of the coding parts differs slightly from the non-coding interface. It contains a small modification upstream the insert, designed to eliminate possible problems related to the distance between the ribosomal binding site (RBS) and the coding part start codon (Registry of Standard Biological Parts: RBS-CDS Issues). When using the ordinary non-coding interface for coding parts the distance between an upstream RBS and the coding part would become too long, hindering translation. Additionally, the coding part interface also contains a stop-codon duplicate immediately upstream of the part (Fig. 3).

```
GAATTC GCGGCCGC T TCTAG "ATG part1" TAATAA T ACTAGT A GCGGCCG CTGCAG
CTTAAG CGCCGGCG A AGATC "TAC part1" ATTATT A TGATCA T CGCCGGC GACGTC
 EcoRI   NotI        XbaI           2*STOP    SpeI      NotI   PstI
  E               X                    S              P
```

Fig. 3. The standard DNA interface of the Synthetic biology BioBrick standard for coding parts, restriction enzyme recognition sites are marked with name. Different BioBrick parts can be arbitrarily assembled by digestion and ligation. Digestion occurs between the nucleotides marked in green. The coding interface differs from the non-coding interface (Fig. 2) in that the sequence upstream of the part is engineered to make the distance between an inserted RBS and the coding part optimal, and that two stop codons are situated directly downstream of the part.

1.3 The BioModularH_2 project

Starting in the beginning of 2007, this long-term EU team project aims at ultimately producing a photosynthetic bacterium capable of competitive hydrogen production. In order to achieve this, standardized molecular parts will be designed and assembled according to a hierarchical engineering methodology. Already encompassing two key concepts of Synthetic biology, standardization and a hierarchy of parts, the BioModularH_2 project will also make use of protein engineering, metabolic modeling, molecular evolution methodologies and the growing knowledge made available by systems biology (BioModularH_2 2006). This work of

promoter characterization and evaluation of characterization methodologies, utilizing the methods of Synthetic biology, will pave the way for additional research about part characterization and standardization within the BioModularH_2 project.

1.4 Promoters

As gene expression promoting elements, promoters are critical for all biological systems. There are many different promoters that vary in their ability to promote transcription, i.e. their strength, and differ in other characteristics such as binding sites for transcription factors. In order to engineer a composite genetic system with clearly defined features, all parts have to be well characterized. Having the crucial characteristics mentioned above, promoters are important parts in any composite genetic system and their function must be thoroughly investigated. In this study, three different promoters have been chosen for characterization: the pLacI promoter, the pOmpR and the pOmpRm promoter.

1.4.1 The *lac* promoter, pLacI

This is the wild type *E. coli* lacZYA operon promoter, which has been closely scrutinized ever since Jacob and Monod explained the function of the *lac* operon in 1961 (Jacob & Monod 1961). The transcriptional activity is positively regulated by the binding of a cyclic-AMP dependent catabolite gene activator protein (CAP) and negatively regulated by the binding of the *lac* repressor (Fig. 4) (Eron & Block 1971, Dickson *et al.* 1975).

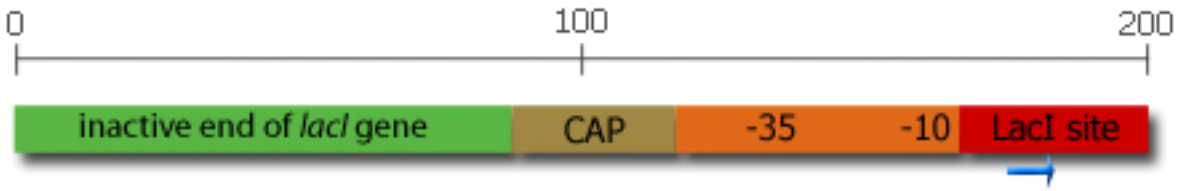

Fig. 4. Schematic picture of the *lac* promoter including the binding sites for the CAP and the *lac* repressor. The promoter comes from the *E. coli* lacZYA operon and includes the end of the upstream *lacI* gene, which contains one LacI binding site, and one LacI site downstream of the –10 box. The transcription starting point is indicated by arrow, the -10 and -35 boxes by position.

The CAP protein up-regulates transcription by binding its recognition site on the promoter, effectively stabilizing the binding of the RNA polymerase (Zubay *et al.* 1970). Repression is mediated through the binding of a homo-tetramer of the *lac* repressor protein to its binding site, the *lac* operator. In order to repress transcription, it must bind to another recognition site simultaneously and thus bend the DNA. The wild type *lac* operon contains two such secondary operators; one upstream of the promoter in the end of the *lacI* gene, coding for the repressor, and one far downstream in the *lacZ* gene. All three operators are required for maximum repression (Oehler *et al.* 1990). Repression is abolished by isopropyl-β-D-1-thiogalactopyranoside (IPTG), an analogue of the natural inducer allolactose, which binds to the *lac* repressor and induces a conformational change, leading to decreased binding affinity for the operator DNA (Jacob & Monod 1961, Pace *et al.* 1990).

1.4.2 The *ompC* promoter, pOmpR

Osmoregulation is a critical feature of *E. coli* that enables it to live in environments that differ widely in osmolarity, nutrients and temperature. An important signalling pathway for

osmoregulation is the EnvZ-OmpR histidyl-aspartyl phospho-relay (Fig. 5) (Egger *et al.* 1997).

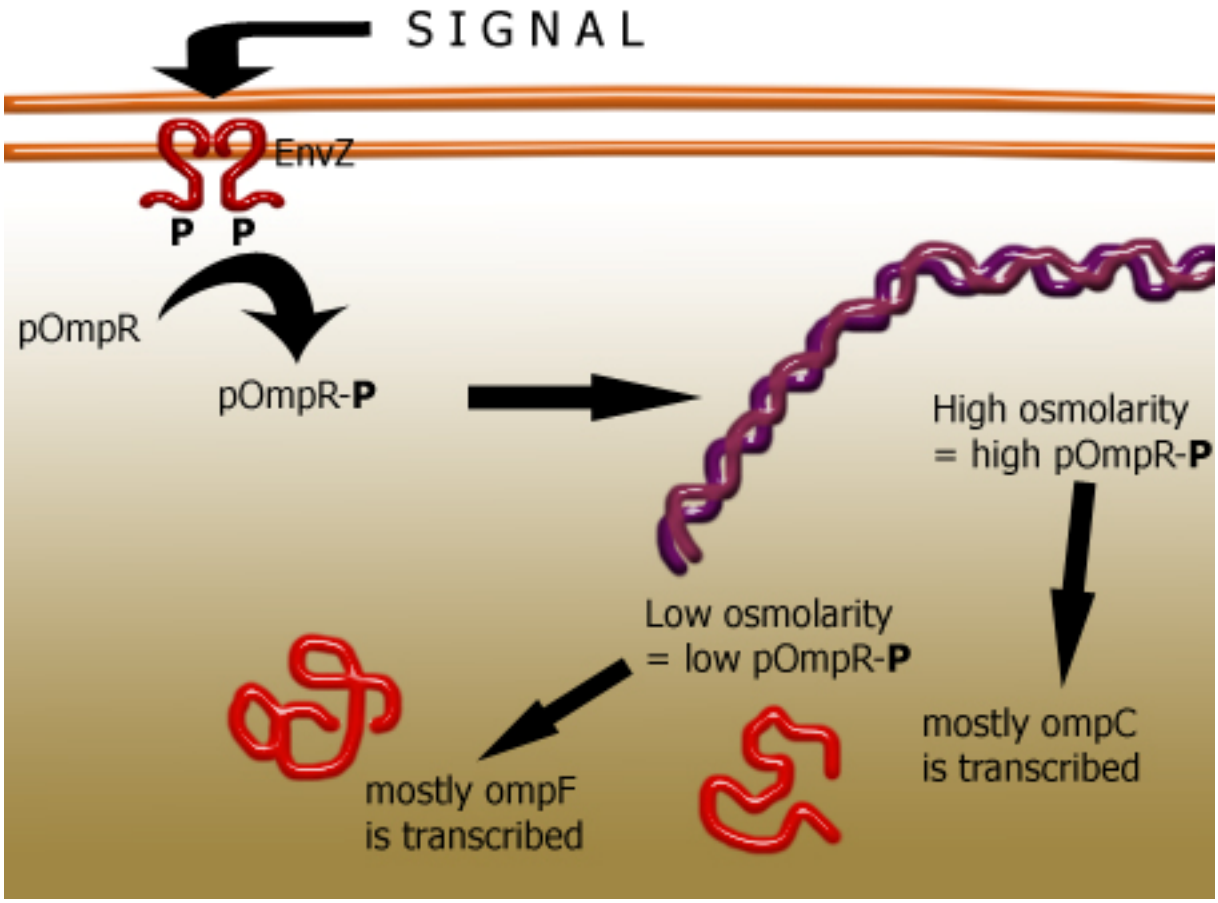

Fig. 5. The EnvZ-OmpR His-Asp phosphorelay. The inner membrane histidine kinase EnvZ senses an osmotic signal and is autophosphorylated, whereupon it acts as a phospho-donor for the cytoplasmic transcription factor OmpR (Roberts *et al.* 1994). The phosphorylation induces a conformational change in OmpR-P, which may then bind its recognition sites upstream the porin genes *ompF* and *ompC*. At high osmolarity there are high levels of OmpR-P resulting in transcriptional activation of *ompC* and repression of *ompF*. At low osmolarity only low levels of OmpR-P exist and mostly *ompF* is transcribed. The differential expression of these porins helps controlling the osmolarity of the bacteria in different environmental conditions (Egger *et al.* 1997).

The pOmpR promoter contains the upstream region of the *ompC* porin gene, which has three binding sites for OmpR-P (Fig. 6).

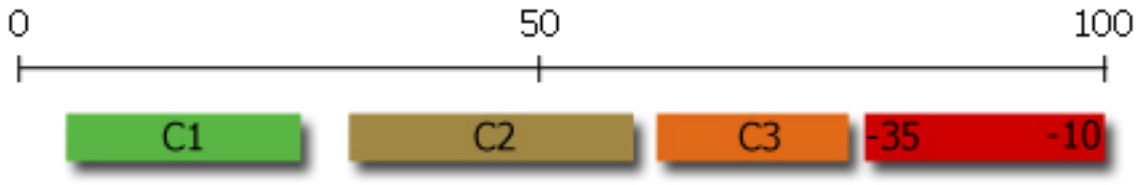

Fig. 6. The upstream region of the *ompC* porin gene. C1-C3 are the three OmpR-P binding sites where OmpR-P, a phosphorylated transcription factor, binds and stabilizes the binding of RNA polymerase through interactions with the c-terminal domain, facilitating transcription of the downstream coding element. (Egger *et al.* 1997).

1.4.3 The truncated *ompC* promoter, pOmpRm

This promoter is a truncated version of the pOmpR and lacks two of the three pOmpR-P binding sites (Fig. 7).

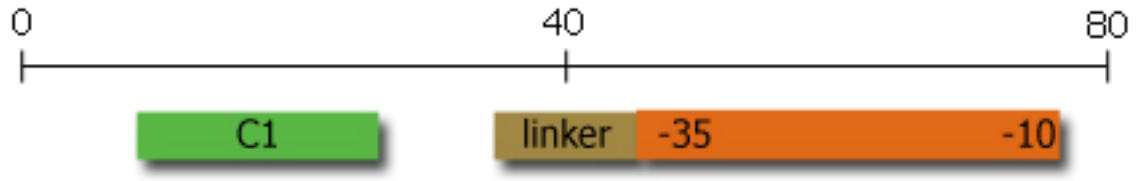

Fig. 7. The truncated upstream region of the *ompC* porin gene. Only the C1 pOmpR-P recognition site is present, just upstream of a linker sequence. The phosphorylated transcription factor pOmpR-P binds the C1 site and stabilizes the binding of RNA polymerase, facilitating transcription of the downstream coding element. This promoter responds weaker than pOmpR due to the two missing pOmpR recognition sites C2 and C3 (Maeda & Mizuno 1990).

1.5 Project description and aims

Using the concepts and methodology of Synthetic biology, BioBrick parts carrying pLacI, pOmpR and pOmpRm promoters will be assembled with BioBrick parts carrying fluorescent protein reporter constructs. Bacteria containing these assemblies will be grown in standardized cultivation conditions designed for providing appropriate amounts of reporter. The relative amounts of reporter will be quantitated using fluorescence imaging, fluorescence microscopy and Northern blotting.

1.5.1 Characterization of promoters

Reporter quantification data will be used to calculate each promoter's relative ability to promote transcription. Furthermore, the pLacI promoter will be characterized at different levels of the inducer IPTG.

1.5.2 Evaluation of characterization methodology

The three utilized methods fluorescence imaging, fluorescence microscopy and Northern blotting will be compared and their suitability for promoter characterization will be evaluated.

2. Materials and Methods

2.1 Bacterial strains

All transformation and cloning work required for assembling the promoter-reporter constructs were performed using competent DH5α cells (Invitrogen). For the quantification experiments, plasmids containing the assemblies were transformed into XL1-Blue cells (Stratagene) that were made competent prior to transformation. The XL1-Blue cells contain the *lacI*^{q} gene, a mutant of the *lac* repressor that is overproduced, which makes this strain suitable for characterization of the pLacI promoter at different levels of IPTG. Both strains were grown in 10 ml growth tubes containing Luria-Bertani (LB) broth, supplemented with 100 μg/ml ampicillin, at 37 °C in an orbital shaker at 250 rpm. Optical density at 600 nm (OD600) was measured spectrophotometrically using a Varian 50 Bio UV-visible spectrophotometer.

2.2 Routine manipulations

2.2.1 Digestions

EcoRI, XbaI, BcuI and PstI (Fermentas) were used for all BioBrick related digestions. BcuI was used instead of SpeI since both have the same recognition sequence and digestion pattern. Depending on the desired digestion, different reaction mixes and reaction times were used. For insert size inspection, plasmids were digested in 25 μl reactions with XbaI and BcuI (1000 ng plasmid DNA, 1 x Tango buffer (Fermentas), 1 u XbaI and BcuI) in a 37 °C water bath for at least 1 hour. For back vector preparation, plasmids were digested in 25 μl reactions with BcuI and PstI (1000 ng plasmid DNA, 1 x Tango buffer, 1 u BcuI and 2 u PstI) in a 37 °C water bath for at least 1 hour. For back insert preparation, plasmids were digested in 25 μl reactions with PstI and XbaI (1000 ng plasmid DNA, 1 x Tango buffer, 2 u PstI and 1 u XbaI) in a 37 °C water bath for at least 1 hour.

2.2.2 Ligations and transformations

For all assemblies, the Quick Ligation Kit, New England Biolabs, Inc. was used according to protocol. Competent DH5α cells were transformed according to the Quick ligation kit protocol but 200 μl transformed cells were plated instead of 100 μl on LB agar plates supplemented with 100 μg/ml ampicillin, incubated over night at 37 °C. Competent XL1-Blue cells were transformed according to the XL1-Blue competent cells protocol (Stratagene) and 200 μl transformants were plated on LB agar plates supplemented with 100 μg/ml ampicillin, incubated over night at 37 °C.

2.2.3 Agarose gel electrophoresis

Agarose of genetic technology grade (MP Biomedicals, LLC) was mixed with 5 mM sodium tetraborate, pH 7.8, containing 1 μg/ml of the nucleotide binding dye Thiazole Orange (Fluka) and heated until homogenous. Solidified gels were run with 5 mM sodium tetraborate, pH 7.8, as running buffer. Due to the low ion strength of the sodium tetraborate, higher voltages than ordinarily used with i.e. TAE buffer can be applied, effectively speeding up the electrophoresis.

2.3 BioBrick parts from the iGEM 2006 plates

Copies of parts from the Registry of Standard Biological Parts were acquired from DNA plates from the international Genetically Engineered Machine competition 2006 (iGEM 2006) (kindly provided by the Valencia iGEM 2006 team), which is annually arranged by the Massachusetts Institute of Technology. Every well of the iGEM 2006 plates contains a BioBrick part as freeze-dried plasmid DNA at the bottom. Unopened wells were punctured

and plasmids were dissolved in 15 µl dH_2O, of which 1 µl was used to transform and grow DH5α cells according to standard procedures. Empty, opened wells had the remainder of the DNA extracted with 10 µl dH_2O, of which all was used to transform and grow DH5α cells. Plasmids containing each part were prepared from over night cultures using a commercial kit (GenElute Plasmid Miniprep Kit, Sigma). Glycerol stocks (10 % v/v glycerol) of each culture were prepared and stored at –75 °C.

Information about each BioBrick part listed is available from the Registry of Standard Biological Parts (Registry of Standard Biological Parts).

2.3.1 Promoters

The pLacI, the pOmpR and the pOmpRm promoters were obtained from the iGEM 2006 DNA plates (Table 1). Inserts were excised by digestion with XbaI and BcuI and fragment sizes were inspected by agarose gel electrophoresis (2 % w/v agarose, run at 160 V 20 min).

Table 1. Utilized promoter parts and their BioBrick part names.

Promoter	BioBrick part
pLacI	BBa_R0010
pOmpR	BBa_R0082
pOmpRm	BBa_R0083

2.3.2 Fluorescent protein constructs

These BioBrick constructs (Table 2) consist of a ribosome binding site (RBS), a fluorescent protein gene and a double transcriptional terminator (T). These constructs are sometimes referred to by only the fluorescent protein's name. All fluorescent protein constructs were obtained from the iGEM 2006 DNA plates. Inserts were excised by digestion with XbaI and BcuI and fragment sizes were inspected by agarose gel electrophoresis (1 % w/v agarose, run at 160 V for 20 min). The construct's constituent parts are described individually below.

Table 2. Utilized fluorescent protein constructs and their BioBrick names. RFP = Red Fluorescent Protein, GFP = Green Fluorescent Protein, ECFP = Enhanced Cyan Fluorescent Protein, EYFP = Enhanced Yellow Fluorescent Protein.

Fluorescent protein construct	BioBrick part
RBS + RFP + T	BBa_I13507
RBS + GFP + T	BBa_I13504
RBS + ECFP + T	BBa_E0420
RBS + EYFP + T	BBa_E0430

2.3.2.1 Ribosome binding site

Common RBS used as a standard for defining RBS efficiency, BioBrick part BBa_B0034, based on Elowitz Repressilator (Elowitz & Leibler 2000).

2.3.2.2 Transcriptional terminators

Double terminator consisting of terminators BBa_B0010 (from *E. coli rrnB*) and BBa_B0012 (from *E. coli* RNA polymerase), BioBrick part BBa_B0015.

2.3.2.3 Fluorescent proteins

The fluorescent proteins are excited by light of a specific wavelength and re-emits light at a wavelength greater than the excitation wavelength (Table 3).

Table 3. Fluorescent proteins, wavelengths for excitation and emission peaks and their BioBrick names. EYFP can also be excited efficiently at 488 nm (Clontech 2001).

Fluorescent protein	Excitation peak	Emission peak	BioBrick part
RFP	584 nm	607 nm	BBa_E1010
GFP	501 nm	511 nm	BBa_E0040
ECFP	433 nm	475 nm	BBa_E0020
EYFP	513 nm (488)	527 nm	BBa_E0030

2.4 BioBrick vector

All BioBrick parts utilized in this experiment were carried by the pSB1A2 plasmid, which was also used for assembly. This is a high copy number plasmid (100-300 copies per cell) containing the ampicillin resistance gene *ampR* controlled by the pBLA promoter, and a pUC19-derived pMB1 origin of replication. Some read-through transcription, coming into the cloned BioBrick part from both directions, has been reported (Registry of Standard Biological Parts: pSB1A2).

2.5 Standard assembly of BioBrick parts

BioBrick parts can be assembled in either of two ways: by ligating one part upstream of the other part (prefixing it) or ligating it downstream of the other part (postfixing it). These parts may be single BioBrick parts or composite BioBrick assemblies, in either case they will be surrounded by the same standard BioBrick sequence interface. To prefix part 1 with part 2, part 1 is digested with EcoRI and SpeI to create a front insert (FI) and part 2 is digested with EcoRI and XbaI to create a front vector (FV). To postfix part 2 with part 1, part 2 is digested with XbaI and PstI to create a back insert (BI) and part 1 is digested with SpeI and PstI to create a back vector (BV) (Knight 2001). The assembly procedure is illustrated in Appendix A & B. The parts extracted from the iGEM 2006 plates were digested according to this scheme, but using the equivalent enzyme BcuI instead of SpeI, to produce the desired fragments (Table 4).

Table 4. The restriction enzymes required for producing the desired fragments during digestion of each BioBrick part.

Part	Produced fragment	Restriction enzymes
pLacI	Back vector	BcuI and PstI
pOmpR	Back vector	BcuI and PstI
pOmpRm	Back vector	BcuI and PstI
RBS + RFP + T	Back insert	PstI and XbaI
RBS + GFP + T	Back insert	PstI and XbaI
RBS + ECFP + T	Back insert	PstI and XbaI
RBS + EYFP + T	Back insert	PstI and XbaI

Back vector fragments containing promoter parts were purified by agarose gel electrophoresis (1 % w/v agarose, run at 160 V for 45 min) followed by excision and subsequent gel spin extraction using a commercial kit (Illustra DNA and gel band purification kit, GE Healthcare). Back insert fragments were similarly purified but run on a higher density agarose gel at higher voltage (2.5 % w/v agarose, run at 200V for 45 min). An extra high-density agarose gel was used due to special requirements of other samples run simultaneously. Due to low elution volumes, the DNA concentration of each purified fragment could not be measured spectrophotometrically.

Assemblies were constructed by mixing and ligating back vectors with back inserts (Table 5) using a commercial kit (Quick Ligation Kit, New England Biolabs, Inc.) according to protocol. Since the fragment's DNA concentrations were unknown, 3 µl purified back vector was used with 7 µl of purified back insert for each ligation.

Table 5. Constructed BioBrick assemblies are their constituent back vectors and inserts.

Assembly	Back vector	Back insert
pLacI + RBS + ECFP + T	pLacI	RBS + ECFP + T
pLacI + RBS + EYFP + T	pLacI	RBS + EYFP + T
pLacI + RBS + RFP + T	pLacI	RBS + RFP + T
pLacI + RBS + GFP + T	pLacI	RBS + GFP + T
pOmpR + RBS + ECFP + T	pOmpR	RBS + ECFP + T
pOmpR + RBS + EYFP + T	pOmpR	RBS + EYFP + T
pOmpR + RBS + RFP + T	pOmpR	RBS + RFP + T
pOmpR + RBS + GFP + T	pOmpR	RBS + GFP + T
pOmpRm + RBS + ECFP + T	pOmpRm	RBS + ECFP + T
pOmpRm + RBS + EYFP + T	pOmpRm	RBS + EYFP + T
pOmpRm + RBS + RFP + T	pOmpRm	RBS + RFP + T
pOmpRm + RBS + GFP + T	pOmpRm	RBS + GFP + T

Each assembly is transformed into competent XL1-Blue cells according to protocol (XL1-Blue competent cells, Stratagene). The transformants are plated on LB agar plates containing 100 µg/ml ampicillin and incubated at 37 °C over night. Colonies were counted and two colonies from each assembly were incubated in 5 ml LB supplemented with 100 µg/ml ampicillin at 37 °C over night. Plasmids were extracted from each culture using a commercial kit (GenElute Plasmid Miniprep Kit, Sigma) and sent for sequencing (Macrogen Inc., Seoul, Korea) together with the sequencing primers BBSeq02f (5'TTGTCTCATGAGCGGATACA) and BBSeq02r (5'ATTACCGCCTTTGAGTGAGC), which are identical to previously used sequencing primers (Knight 2001). The acquired sequence chromatogram data was analyzed with SeqMan Pro, Lasergene software suite, DNASTAR Inc. Glycerol stocks (10 % v/v glycerol) of each culture were prepared and stored at –75 °C.

2.6 Promoter characterization

To characterize the promoters, three methods were used: fluorescent imaging, fluorescent microscopy imaging and Northern blotting. Fluorescent imaging measures the fluorescent protein gene transcription indirectly through the fluorescence intensity of expressed fluorescent proteins of a bacterial culture. Fluorescent microscopy imaging measures the fluorescent protein gene transcription indirectly through the fluorescence intensity of expressed fluorescent proteins of individual cells. Northern blotting measures the fluorescent protein gene transcription directly by quantitating the mRNA levels of a bacterial culture.

The promoter-RFP reporter assemblies pLacI-RFP, pOmpR-RFP and pOmpRm-RFP were chosen for promoter characterization since the RFP protein is clearly visible in the visible light spectra. As a negative sample, the RFP construct without inserted promoter (BBa_I13507) was used. Since the pSB1A2 plasmid has some read-through transcription, this negative sample will represent the background transcription not caused by the inserted promoters themselves. These three assemblies plus the negative sample assembly were cultivated according to a standard protocol before quantitating the promoter activity to ensure identical growth and environmental conditions for all samples.

2.6.1 Standard growth conditions and induction

A pipette tip glycerol stock (XL1-Blue cells) from each RFP assembly was incubated in 5 ml LB supplemented with 100 μg/ml ampicillin at 37 °C shaking at 250 rpm over night. The next morning 100 μg/ml ampicillin supplemented LB was pre-warmed to 37 °C in a water-bath. OD600 of the over night cultures were measured and new 5 ml OD600 of 0.1 cultures were inoculated using the pre-warmed broth and the over night cultures. These new cultures were incubated at 37 °C shaking for 2 hours. OD600 was measured and new 5 ml OD600 of 0.1 cultures were inoculated using the 2-hour cultures and the pre-warmed broth; one culture each for the pOmpR-RFP, the pOmpRm-RFP and the negative RFP constructs, and 4 cultures of the pLacI-RFP construct, one supplemented with 100 μM IPTG, one with 10 μM IPTG, one with 1 μM IPTG and one without IPTG. These 7 cultures were incubated for 4 hours shaking at 37 °C and their final OD600 was measured before down-stream applications and measurements.

2.6.2 Fluorescence imaging

Fluorescence quantification was done on the cultured bacteria in a 96-well flat-bottomed round-welled plastic plate using a Pharos FX Plus Molecular Imager (Bio-Rad Laboratories, Inc.). For calibration of each experiment, a RFP protein extract was used as a standard. This experiment was repeated four times with new cells grown according to the standard growth protocol.

2.6.2.1 Standard RFP protein extract

The standard was produced by inoculating the XL1-Blue pLacI-RFP construct in 400 ml LB supplemented with 100 μg/ml ampicillin and 1 mM IPTG in 1l growth flasks in an orbital shaker at 250 rpm at 37 °C for 20 hours. Cells were harvested by centrifugation at 5000 rpm at 4 °C using a Beckman Coulter Avanti J-25 centrifuge with the JLA-16.250 fixed angle rotor (Beckman Coulter). Cell pellets were dissolved in 4 ml native conditions lysis buffer (50 mM monobasic sodium phosphate, 300 mM sodium chloride, pH 8.0) and cells were lysed using an ultrasonic processor (Sonics Vibra cell). Lysates were spun down and the supernatant containing the RFP protein extract was extracted, aliquoted and stored at –20 °C.

2.6.2.2 Pharos FX Plus imaging

Each of the seven culture samples and a 1:10000 dilution of the standard RFP protein extract were loaded in 200 μl tetrads onto the 96-well plate. The samples were analyzed with the Pharos FX Plus Molecular Imager at a 100 μm resolution using a 532 nm excitation laser and a 605 nm bandpass filter.

2.6.2.3 Data processing

Total pixel intensities were calculated from the image for each sample's well using the Quantity One software (Bio-Rad Laboratories, Inc.). The intensity data was processed using Microsoft Excel; measurement background was subtracted from all intensities, biological background (negative RFP construct intensity) was subtracted from all sample intensities and all sample intensity data were calibrated with the diluted standard RFP protein extract intensity. The processed standardized intensity data for different IPTG inducer concentrations of pLacI was used for exponential regression with SigmaPlot 2002 (SPSS Inc.).

2.6.3 Fluorescence microscopy imaging

Samples (10 μl) from each one of the seven cultures were fixated on glass slides by dehydration and new cells were prepared according to the standard growth protocol for each fixation. The fixation was done once by letting the samples dry over night in room

temperature and twice by drying them for 10 min at 65 °C. The glass slide samples were immersed in oil prior to microscopy imaging.

2.6.3.1 Nikon Eclipse 90i imaging

Using a Hamatsu Orca digital camera mounted on a Nikon Eclipse 90i microscope and a 100x Plan Apo objective, RFP fluorescence images were taken of each sample.

2.6.3.2 Data processing

The NIS-Elements Advanced Research software (Nikon) was used for object and object mean intensity quantification. The intensity data was processed using Microsoft Excel.

2.6.4 Northern blotting

All seven cultures were grown according to the standard growth protocol and their RNA was used for Northern blotting. As a standard for calibration, 16S rRNA was used.

2.6.4.1 PCR probe generation

Probe templates for the Northern blotting were made using PCR. Primers f mRFP1 (5'TGTCCCCGCAGTTCCAGTA) and r mRFP1 (5'CCAGTTTGATGTCGGTTTTGTAAG) were used with the plasmid containing the RFP assembly (BBa_I13507) as template in the PCR mix (0.72 ng/µl template, 0.2 mM dNTP mix, 0.2 mM f mRFP1 primer, 0.2 mM r mRFP1 primer, 1 x Taq polymerase buffer (New England Biolabs), 0.02 u/µl Taq polymerase (New England Biolabs)). PCR was run on a MJ Mini PCR machine (Bio-Rad) with an optimized PCR program (5 min at 94 °C, 45 cycles of 15 s at 94 °C and 15 s at 48 °C and 30 s at 72 °C, 5 min at 72 °C, hold at 4 °C). PCR products of size 417 bp were purified by means of agarose gel electrophoresis (1 % w/v agarose, run at 160 V for 20 min) followed by excision and subsequent gel spin extraction using a commercial kit (Illustra DNA and gel band purification kit, GE Healthcare). Probe templates were generated similarly for 16S rRNA but with primers f rrsH 16SrRNA (5'GCGGCCCCCTGGACGAAGAC) and r rrsH 16SrRNA (5'CGGACCGCTGGCAACAAAGGATAA) using *E. coli* strain XL1-Blue gene *rrsH* as template in the otherwise identical PCR mix (0.5 µl XL1-Blue culture used as template). The same PCR program was run but with an primer annealing temperature of 58 °C. The *rrsH* PCR product of size 408 bp was purified in the same manner as the RFP PCR product.

2.6.4.2 Total RNA extraction

The ChargeSwitch Total RNA Cell kit (Invitrogen) was used to extract total RNA from 1 ml of each of the seven cultures.

2.6.4.3 Denaturing gel electrophoresis

A 25 ml RNA denaturing agarose gel (1 % w/v agarose, 0.7 M formaldehyde, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA) containing 1 µg/ml Thiazole Orange (Fluka) was made. Denatured RNA gel samples of 25 µl each were prepared with the maximum volume of total RNA extract (6.7 µl RNA total extract, 2.2 M formaldehyde, 50 % v/v formamide, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA) by heating the mixture to 55 °C for 15 min and adding 2.5 µl 10 x loading buffer. The denatured RNA samples were loaded onto the gel that was run at 90 V for 1 hour using 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA as running buffer. After the run, the gel was UV photographed and washed once in distilled water for 15 min and twice in sterile 10 x SSC (0.3 M sodium chloride, 30 mM sodium citrate, pH ~7-8).

2.6.4.4 Blotting

The gel was put on top of twenty 3 mm Whatman papers in a box filled with 20 x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH ~7-8) halfway up the Whatman papers. The area between the gel and the box was covered with parafilm and a nylon membrane (Hybond-N+, GE Healthcare) cut to the size of the gel and wet in 2 x SSC (60 mM sodium chloride, 6 mM sodium citrate, pH ~7-8) was put on top of the gel. Three 3 mm Whatman papers wet in 2 x SSC was put on top of the membrane. Approximately 5 cm compressed paper towels were put on top of the blot, which was finally covered by a plastic plate carrying a ~0.5 kg weight. The blotting apparatus was left at room temperature over night. After blotting, the position of the wells and the orientation of the gel were marked on the membrane, which was cross-linked with the blotted RNA using an UV cross-linker (UV Stratalinker 1800, Stratagene).

2.6.4.5 Probe hybridisation and washing

The membrane was pre-hybridized with 20 ml Modified Church and Gilbert buffer (7 % w/v SDS, 6.5 M phosphate buffer, 10 mM EDTA) in a 200 ml cylinder glass tube at 65 °C rotating for ~1 hour. The purified RFP PCR product for use as probe template (25 ng) was used with radiolabelled dCTP (Redivue [α-^{32}P] dCTP, GE Healthcare) in the Rediprime II Random prime labelling system (GE Healthcare), according to protocol, to produce radiolabelled DNA probes for RFP mRNA. Unincorporated ^{32}P-dCTP was removed from the probe using the Probe Quant G-50 Micro Columns (GE Healthcare), according to protocol. Probes were denatured at 95 °C for 5 min, cooled on ice and briefly centrifuged. The Modified Church and Gilbert buffer in the glass tube was discarded and exchanged with 20 ml new buffer. The probe was added directly to the buffer and hybridization was performed over night at 65 °C rotating. Low stringency wash solution (0.1 % w/v SDS, 2 x SSC), medium stringency wash solution (0.1 % w/v SDS, 1 x SSC (30 mM sodium chloride, 3 mM sodium citrate, pH ~7-8)) and high stringency wash solution (0.1 % w/v SDS, 0.1 x SSC (3 mM sodium chloride, 0.3 mM sodium citrate, pH ~7-8)) were preheated to 65 °C. The membrane was washed once in the glass tube with low stringency wash solution for 5 min at 65 °C, and then transferred from the glass tube to a plastic box and incubated at 65 °C once with low stringency wash solution for 10 min, wash solution was discarded between the washes. Low stringency wash was removed and replaced with medium stringency wash solution and incubated at 65 °C for 8 min, then discarded and replaced with high stringency wash solution, incubated at 65 °C for 8 min and then discarded. Finally the washed membrane was embedded in Saran Wrap and put into a plastic sheet.

2.6.4.6 Pharos FX Plus Imaging

For exposure, the RFP mRNA probed membrane was put with a K-Screen (Kodak) for 30 min. The K-Screen was analyzed using the Pharos FX Plus Molecular Imager (Bio-Rad Laboratories, Inc.) using the 532 nm laser with the 390 nm bandpass filter. The same membrane, stripped and re-probed with the 16S rRNA probe, was exposed for 15 s on a K-Screen and imaged using the same settings.

2.6.4.7 Membrane stripping and re-probing

After imaging of the RFP mRNA, the membrane was stripped using the hot SDS procedure. The membrane was put in a box where 40 ml boiling 0.1 % w/v SDS solution was poured. After cooling, the SDS solution was discarded and the membrane was washed briefly in 2 x SSC. Re-hybridization was performed as in 2.6.4.5 with the 16S rRNA PCR product as probe template. The washed membrane probed with 16S rRNA probe was exposed and imaged as in section 2.6.4.6.

2.6.4.8 Data processing

Total pixel intensities were calculated from the membrane images for each sample's band using the Quantity One software (Bio-Rad Laboratories, Inc.). The intensity data was processed using Microsoft Excel. All samples' intensity data were calibrated with its corresponding 16S rRNA intensity.

3. Results

3.1 iGEM 2006 plate parts

Plasmids carrying BioBrick DNA parts were extracted from the iGEM 2006 DNA plates and transformed into competent DH5α cells (Invitrogen). Plasmids were prepared from each culture and subsequently digested to extract the parts and inspect the sizes (Figs. 8 & 9).

3.1.1 Promoter parts

Only the excised pLacI fragment was visible on the gel, the pOmpR and pOmpRm fragments were probably too short and few to be visible (Fig. 8).

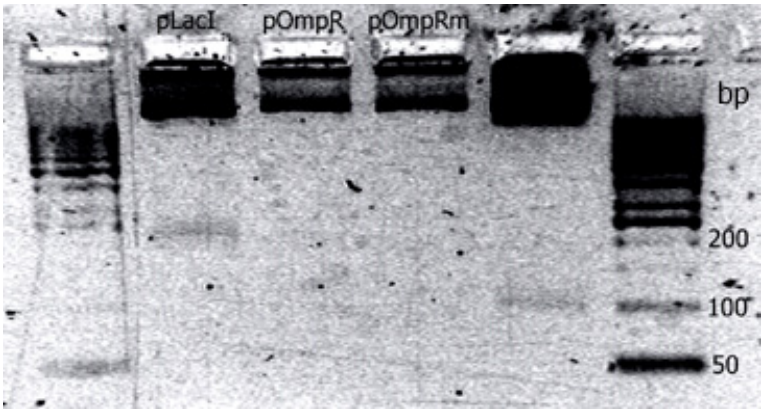

Fig. 8. Excised BioBrick part promoters pLacI, pOmpR and pOmpRm fragment size inspection on an agarose gel. Approximate sizes: pLacI 210 bp, pOmpR 120 bp, pOmpRm 86 bp.

3.1.2 Fluorescent protein construct parts

All excised constructs were of expected size and were clearly visible on the gel (Fig. 9).

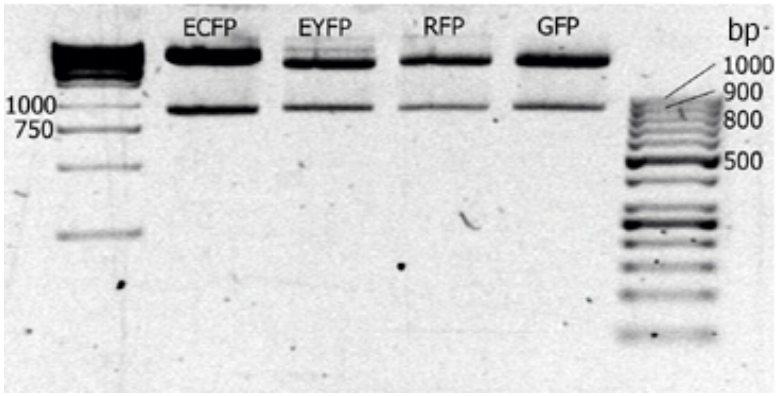

Fig. 9. Excised BioBrick part fluorescent protein constructs ECFP, EYFP, RFP and GFP size inspection on an agarose gel. Approximate construct sizes: ECFP 890 bp, EYFP 890 bp, RFP 870 bp, GFP 890 bp.

3.2 Assemblies

All cultures containing the promoter and fluorescent protein construct BioBricks were grown over night and the plasmids were extracted. The plasmids were digested and the digests were mixed and ligated, all according to the standard BioBrick assembly scheme (section 2.5), to acquire assemblies where each promoter is ligated upstream of each fluorescent protein construct. These reporter constructs were transformed into competent XL1-Blue cells (Stratagene) and plated to obtain colonies. All constructs yielded colonies, but the number of colonies between the constructs differed (Table 6). The colonies containing the pOmpR-RFP

construct were coloured red and a weak pink colour could be discerned from colonies containing the pOmpRm-RFP and pLacI-RFP constructs. This implies that the pLacI promoter is not totally silent.

Table 6. Number of colonies from plated transformations of BioBrick assemblies combining the fluorescent protein constructs ECFP, EYFP, RFP and GFP with the promoters pLacI, pOmpR and pOmpRm.

Reporter construct	pLacI	pOmpR	pOmpRm
ECFP	22	6	7
EYFP	19	4	7
RFP	10	3	21
GFP	5	7	27

Two colonies of each construct were picked and cultivated over night in standard conditions for subsequent plasmid preparation. These 24 plasmid samples were sent for sequencing immediately and the sequencing results confirmed that all promoter-reporter constructs were successfully assembled and cloned except the pOmpR-ECFP construct (Table 7).

Table 7. Sequencing results of selected clones of promoter-reporter assemblies of pLacI, pOmpR and pOmpRm with ECFP, EYFP, RFP and GFP.

Assembly name	Status	Comment
pLacI-ECFP	OK	
pLacI-ECFP	OK	
pLacI-EYFP	OK	
pLacI-EYFP	OK	
pLacI-RFP	OK	
pLacI-RFP	OK	
pLacI-GFP	OK	
pLacI-GFP	OK	
pOmpR-ECFP	-	No promoter
pOmpR-ECFP	-	<55-61% match
pOmpR-EYFP	OK	
pOmpR-EYFP	OK	
pOmpR-RFP	OK	
pOmpR-RFP	OK	
pOmpR-GFP	OK	
pOmpR-GFP	OK	
pOmpRm-ECFP	OK	
pOmpRm-ECFP	OK	
pOmpRm-EYFP	-	No promoter
pOmpRm-EYFP	OK	
pOmpRm-RFP	OK	
pOmpRm-RFP	OK	
pOmpRm-GFP	OK	
POmpRm-GFP	-	99% correct, bad data

3.3 Promoter characterization

The three different promoter-RFP assemblies pLacI-RFP, pOmpR-RFP and pOmpRm-RFP were chosen for promoter characterization studies using the three different methods fluorescence imaging, fluorescence microscopy imaging and Northern blotting. All culture samples to be used for measurement were grown strictly according to the standard growth protocol, which gave seven samples ready for method applications: pLacI-RFP uninduced and induced with 100, 10 and 1 μM of IPTG, pOmpR-RFP, pOmpRm-RFP and a negative sample containing the RFP construct but without inserted promoter.

3.3.1 Fluorescence imaging

For fluorescence quantification, each prepared sample was loaded in tetrads onto a 96-well plate together with the diluted standard RFP protein extract. This was used for imaging using the Pharos FX Plus as described above.

3.3.1.1 Standard RFP protein extract

A total of approximately 3 ml RFP standard protein extract (Fig. 10) was obtained by incubating pLacI-RFP cultures induced with 1 mM IPTG for 20 hours and extracting the proteins as described above.

Fig. 10. Two identical tubes of the RFP protein extract used as standard in fluorescence imaging.

3.3.1.2 Pharos FX Plus imaging

A resulting image from the Pharos FX Plus clearly indicates visible differences in RFP fluorescence levels between the different promoters and the pLacI promoter induced with different concentrations of IPTG (Fig. 11). Four fluorescence-imaging images were taken, one of each one of the four experimental repeats, the same fluorescence pattern is visible in all (images not shown).

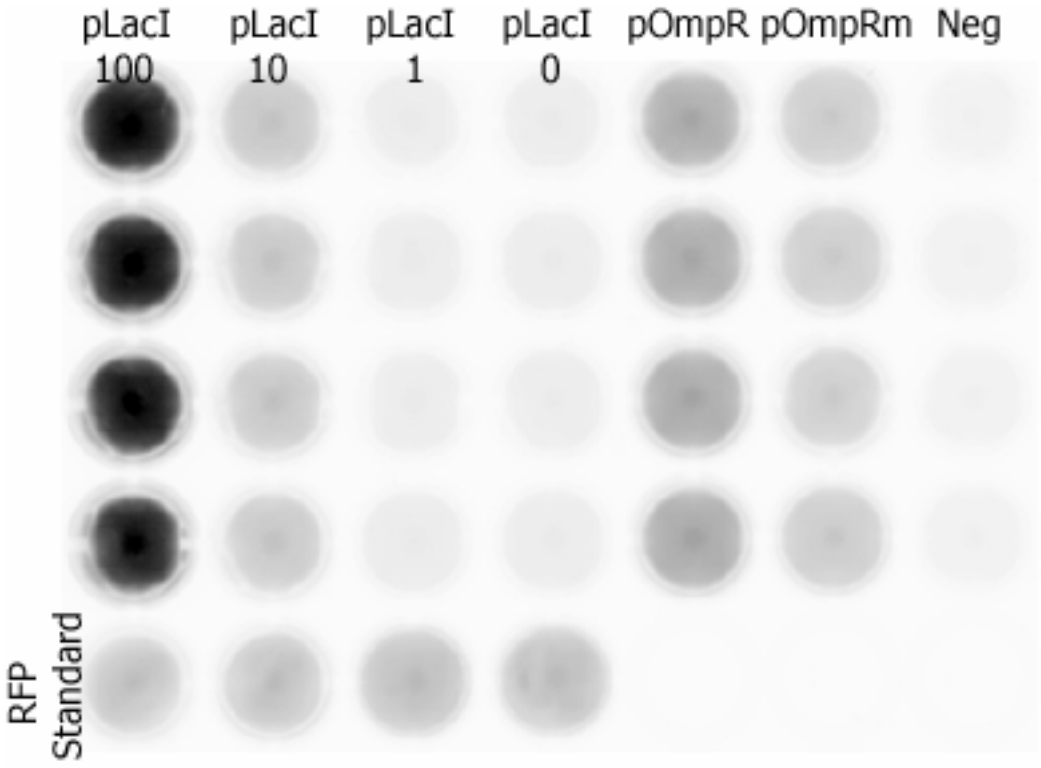

Fig. 11. Fluorescence imaging image of a 96-well plate loaded with tetrads of 200 µl RFP culture samples. Every sample is labeled with its promoter and a number that represents the IPTG concentration used (µM, if relevant).

3.3.1.3 Data processing

The processed and standardized experimental intensity data indicate intensity differences between different samples (Table 8, Fig. 12). Furthermore, standardized intensities normalized to the standardized intensity of pLacI, induced with 100 µM IPTG appear in the same pattern as the non-normalized intensities (Table 8, Fig. 13).

Table 8. Mean values and standard errors (arbitrary units), of the processed and standardized experimental intensity data of all 7 promoter-RFP construct samples, over the four experiments. The first column contains non-normalized data whereas the second contains the intensity data normalized with the intensity of the pLacI 100 µM IPTG sample.

Promoter-RFP construct	**Intensity (n=4) ± SE**	**Normalized intensity (n=4) ± SE**
pLacI 100 µM IPTG	3.66 ± 0.81	1.00 ± 0.00 (by def.)
pLacI 10 µM IPTG	1.12 ± 0.27	0.31 ± 0.06
pLacI 1 µM IPTG	0.45 ± 0.16	0.11 ± 0.03
pLacI 0 µM IPTG	0.31 ± 0.12	0.08 ± 0.02
pOmpR	0.85 ± 0.12	0.26 ± 0.05
pOmpRm	0.33 ± 0.03	0.11 ± 0.03

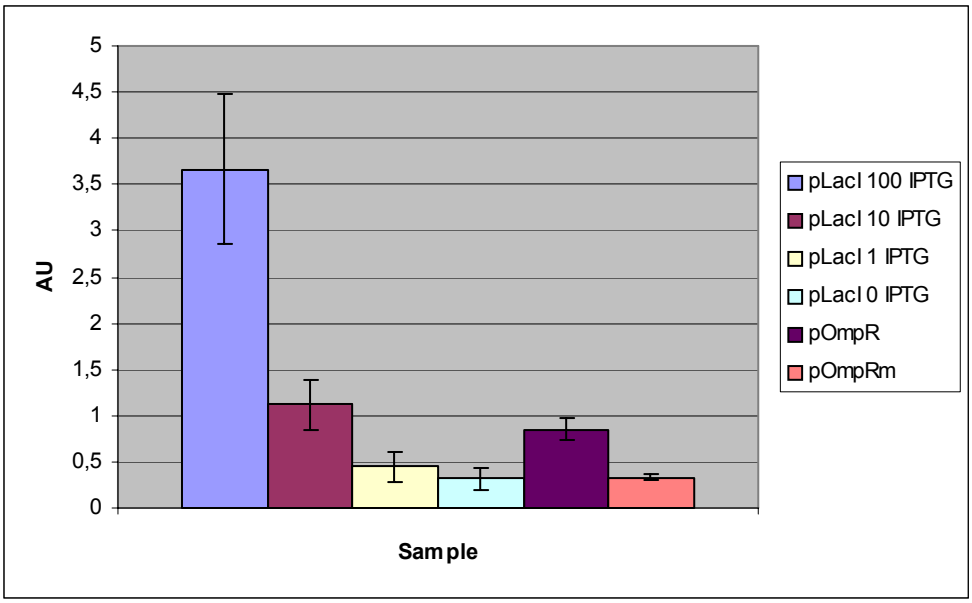

Fig. 12. Promoter-RFP constructs' standardized mean intensities and standard errors (from Table 8) calculated from data from the four fluorescence imaging experiments. Numbers refer to IPTG concentration (μM). Intensity values in arbitrary units.

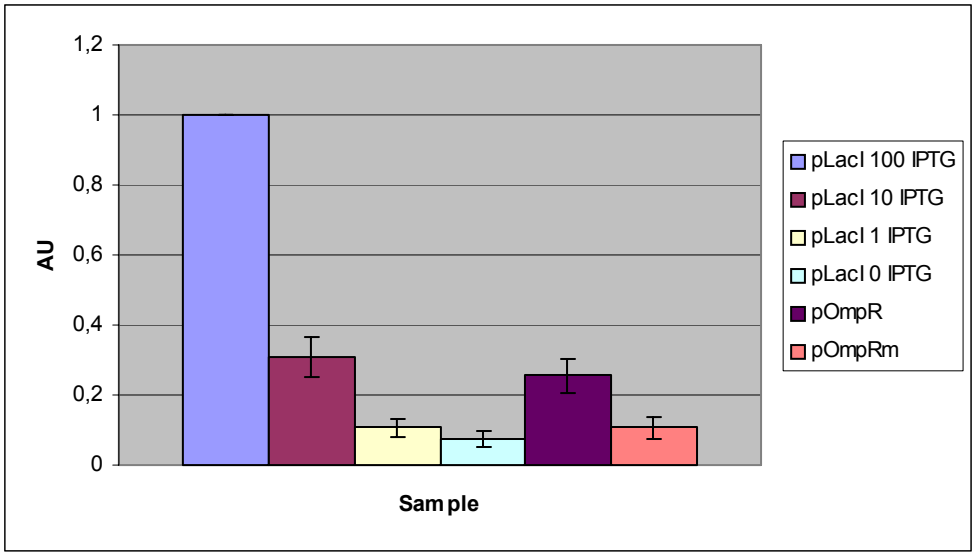

Fig. 13. Promoter-RFP constructs' standardized mean intensities and standard errors calculated from data from the four experiments, normalized to the pLacI (100 μM IPTG) intensity (from Table 8). Numbers refer to IPTG concentration (μM). Intensity values in arbitrary units.

The processed and standardized experimental intensity data for the different IPTG induction levels of pLacI was used for exponential regression (Eq. 1) to show how a possible exponential response of this pLacI-RFP system could look like (Fig. 14).

$f = ae^{bx}$, a = 0.5932 ± 0.2054, b = 0.0182 ± 0.0036. 1.

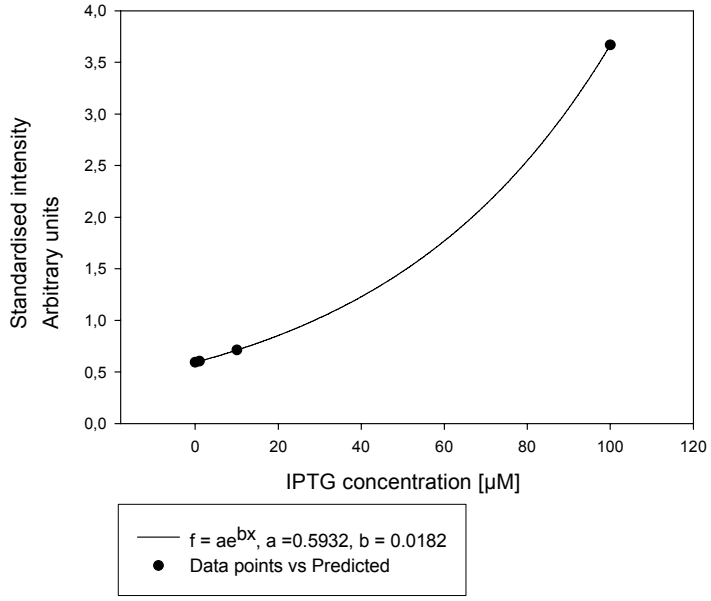

Fig. 14. Exponential regression curve for the standardized intensities of the induced pLacI versus IPTG induction concentration. Standard Error of Estimate = 0.3664. SigmaPlot 2002 (SPSS Inc.).

3.3.2 Fluorescence microscopy imaging

All seven samples were fixated with either one of the two described methods and imaged using the Nikon Eclipse 90i microscope. Fluorescence intensity of bacteria varied depending on method of fixation, area of photography, time-dependent fluorophor bleaching and promoter, with the strongly induced pLacI yielding intense fluorescence (Fig. 15) and the weak pOmpRm yielding low fluorescence.

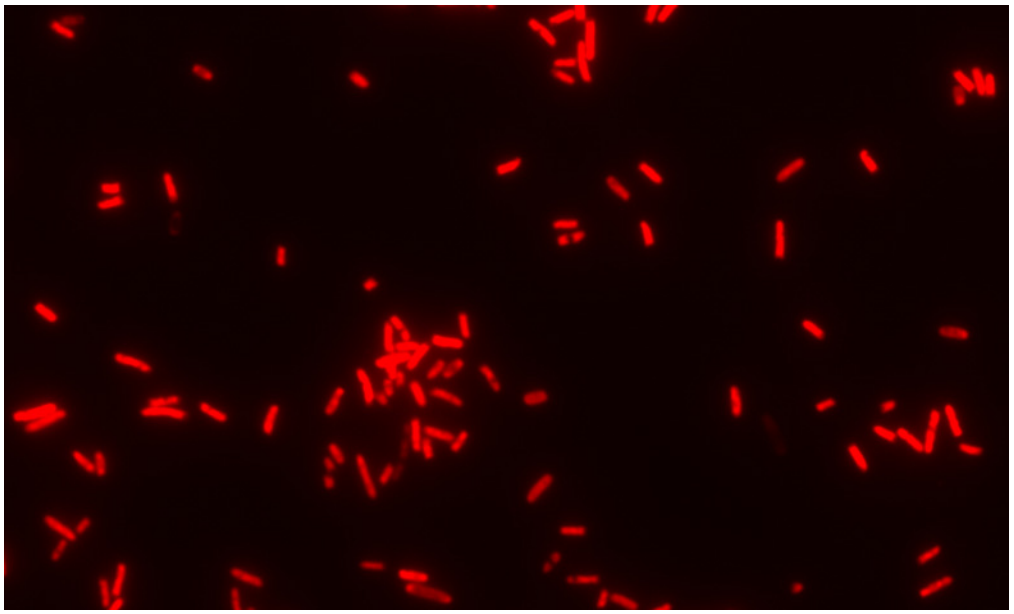

Fig. 15. Fluorescence microscopy image of XL1-Blue cells containing a 100 µM IPTG induced pLacI-RFP construct.

Cells on all samples' images were located using the semi-automated software and each object's mean intensity was calculated. The intensity distribution of the quantitated objects was studied directly (Fig. 16) or as a histogram.

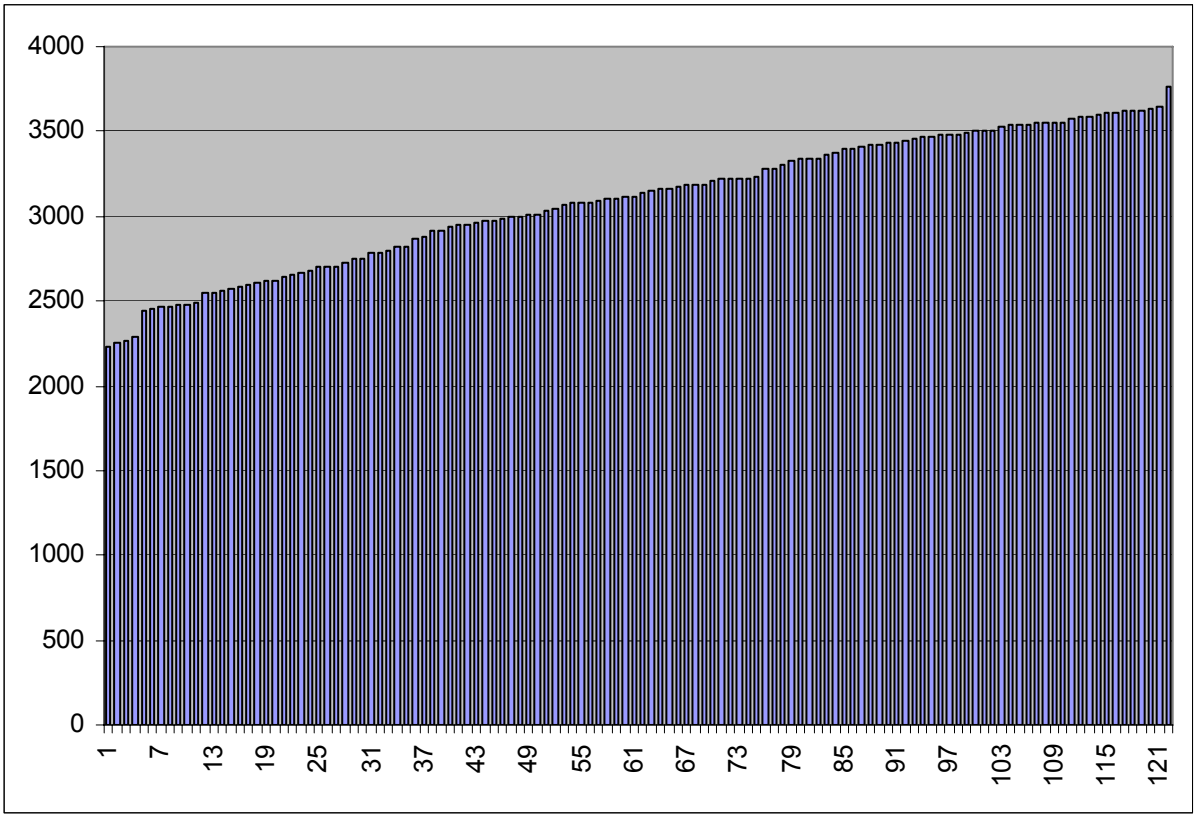

Fig. 16. The object intensity distribution of a fluorescence microscopy image of a pLacI-RFP sample induced with 100 µM IPTG and fixated by dehydration over night at room temperature. Intensity values in arbitrary units.

3.3.2.1 Data processing

The mean intensity was calculated from every sample's counted objects' mean object intensities, both for the samples fixated by dehydration at room temperature over night (Table 9, Fig. 17) and for the samples fixated by dehydration at 65 °C for 10 min (Table 9, Fig. 18).

Table 9. Counted objects' mean object intensities for fluorescence microscopy samples fixated by dehydration at room temperature over night (A) and samples fixated by dehydration at 65 °C for 10 min (B). Intensity values in arbitrary units.

Promoter-RFP construct	Mean intensity A	Mean intensity B
pLacI 100 µM IPTG	3094	466
pLacI 10 µM IPTG	2484	1093
pLacI 1 µM IPTG	604	587
pLacI 0 µM IPTG	461	497
pOmpR	505	421
pOmpRm	339	215

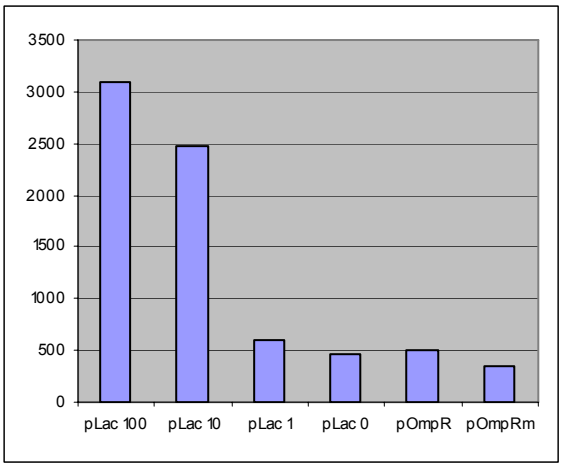

Fig. 17. Counted objects' mean object intensities of fluorescence microscopy samples fixated by dehydration at room temperature over night represented graphically. Intensity values in arbitrary units.

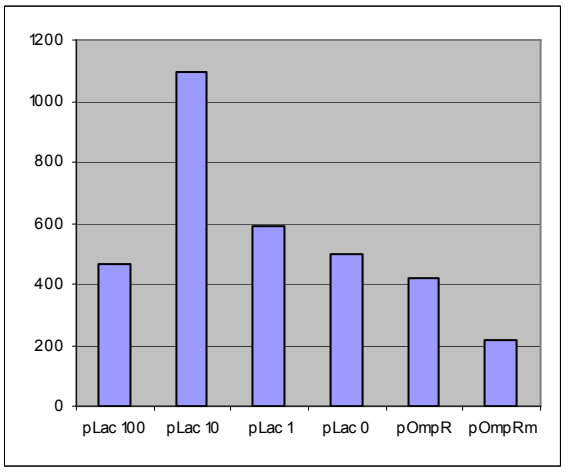

Fig. 18. Counted objects' mean object intensities of fluorescence microscopy samples fixated by dehydration at 65 °C for 10 min represented graphically. Intensity values in arbitrary units.

3.3.3 Northern blotting

The RFP mRNA and 16S rRNA probe templates were prepared by PCR, purified (Fig. 19, Fig. 20) and the gel extracts were used for preparing radioactively labelled nucleotide probes.

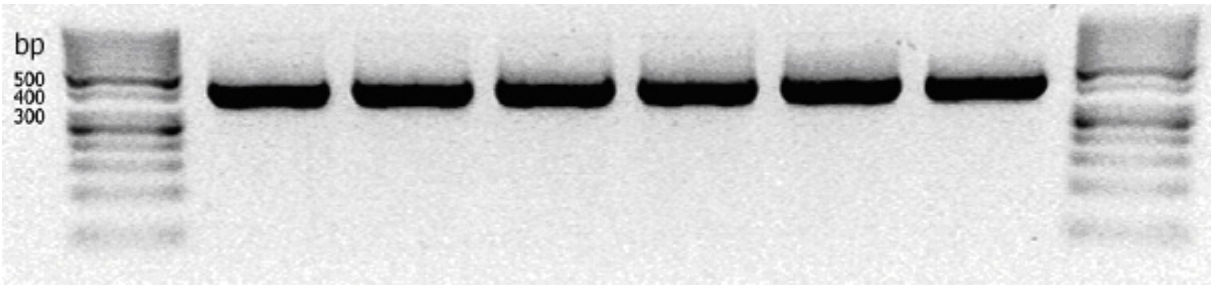

Fig. 19. Agarose gel purification of the RFP probe template PCR product. Object is loaded in all wells and expected size is 417 bp.

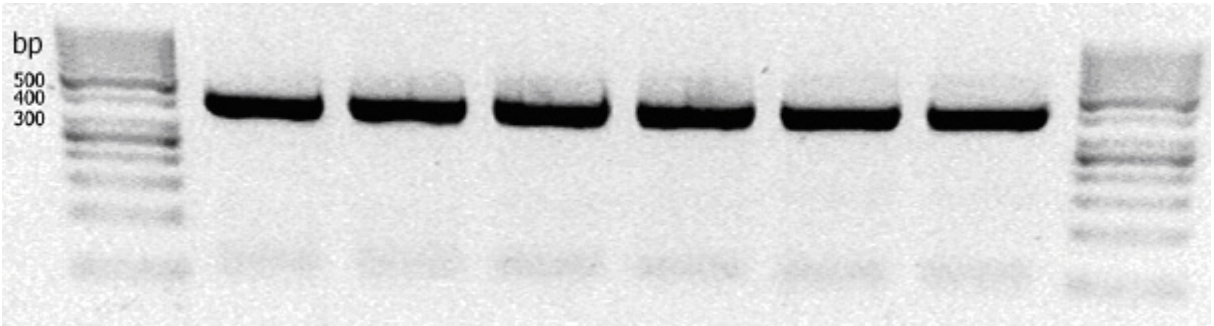

Fig. 20. Agarose gel purification of the 16S rRNA probe template PCR product. Object is loaded in all wells and expected size is 408 bp.

The seven prepared sample cultures were used for RNA extraction using a commercial kit, the extracted RNA was loaded on a denaturing agarose gel and run. The separated nucleotide samples were capillary blotted to a membrane and fixed using an UV-light cross-linking apparatus. After fixing, each probe was hybridised to the membrane (in turn), which was washed, exposed to an image plate, imaged and stripped after each hybridisation.

3.3.3.1 Pharos FX Plus imaging

Images from the blot were obtained for both the RFP probe (Fig. 21) and the 16S rRNA probe (Fig. 22). The bands, which appeared smeared, cannot estimated for their size due to bad RNA ladder, but it is highly unlikely that something else than the correct RFP mRNA and 16S rRNA would give bands corresponding so strongly to the expected bands. An observed intensity tendency is that the strong RFP mRNA signal of the pLacI 100 µM IPTG sample coincides with a strong 16S rRNA signal.

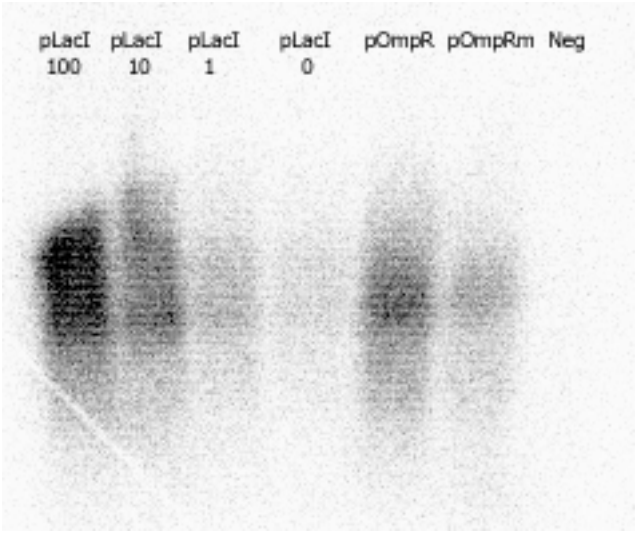

Fig. 21. The exposed RFP Northern blot. RFP constructs are represented by their promoter's names, numbers represent the IPTG concentration (µM, if relevant). Neg represent the negative RFP construct.

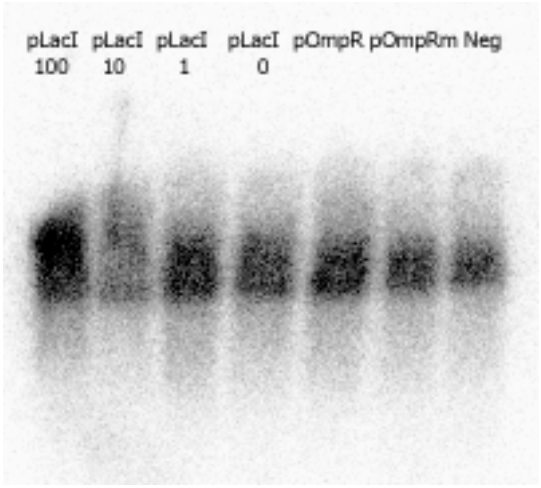

Fig. 22. The exposed 16S rRNA Northern blot. RFP constructs are represented by their promoter's names, numbers represent the IPTG concentration (µM, if relevant). Neg represent the negative RFP construct.

3.3.3.2 Data processing

Identical area volumes were defined to encompass bands as correct as possible for the RFP and the 16S rRNA images. Since it is possible that the 16S rRNA probe is somewhat unspecific, or that those results are inappropriate to use for calibration, intensity values were calculated both without (Table 10, Fig. 23) and with 16S rRNA calibration (Table 10, Fig. 24).

Table 10. RFP mRNA Northern blot intensity values from promoter-RFP construct samples without 16S rRNA calibration (A) and Northern blot intensity values with 16S rRNA calibration (B). Intensity values in arbitrary units.

Promoter-RFP construct	Intensity A	Intensity B
pLacI 100 µM IPTG	7520	0,553
pLacI 10 µM IPTG	4900	0,558
pLacI 1 µM IPTG	2270	0,172
pLacI 0 µM IPTG	1150	0,098
pOmpR	5110	0,396
pOmpRm	2540	0,238

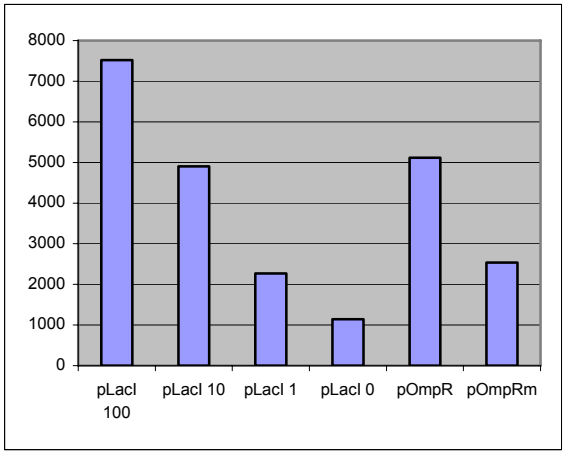

Fig. 23. RFP mRNA Northern blot intensity values from promoter-RFP construct samples without 16S rRNA calibration. Numbers equal IPTG concentration (μM). Intensity values in arbitrary units.

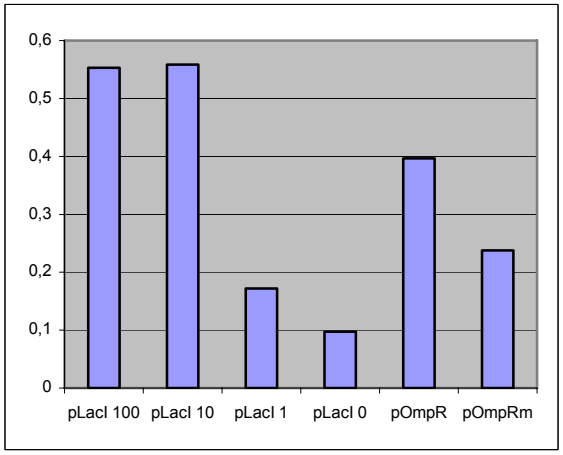

Fig. 24. RFP mRNA Northern blot intensity values from promoter-RFP construct samples calibrated with 16S rRNA. Numbers equal IPTG concentration (μM). Intensity values in arbitrary units.

4. Discussion

4.1 Characterization methodology

All methods are dependent on the raw material for the measurements, in this case, the cultured bacteria. Therefore it is of outmost importance that the sample bacteria are grown under identical conditions, during the same time-intervals, and prepared identically for measurements. In this study, all cultures prepared for experimental measurements were grown according to the standard growth protocol (section 2.6.1). Still, minute differences in temperature, pH, nutrient accessibility, osmolarity, incubation times and cell densities may add up to substantial measurement uncertainties and variations. All these environmental factors can elicit stress responses that affect lots of genes' expression, a good example being the alternative sigma factor RpoS whose large regulon generally is expressed in nutrient deprived cells or cells affected by external stress (Vijayakumar *et al.* 2004). Cell density is of special importance since it has been found that global expression patterns differ greatly between active growing high-density cultures and low-density cultures (Liu *et al.* 2000). These factors and others unknown most probably have contributed to parts of the data variance observed in this study. To deal with this experimental culture noise, standard routines have to be implemented and as many culture environmental factors as possible have to be monitored and compensated for. Here, culture differences due to cell densities were partly compensated for by normalization with OD600 values. Ideally, automation would be desired since it lowers the number of experimental steps and human involvement while increasing the control of the environment.

When comparing the difference in signal between the samples pLacI-RFP induced with 100 μM IPTG and pLacI-RFP induced with 10 μM IPTG, the results from fluorescence imaging, fluorescence microscopy imaging and Northern blotting are quite different. Fluorescence imaging gives a signal difference of 3.5 times between pLacI-RFP induced with 100 μM and 10 μM of IPTG (Fig. 13), the same difference is 1.2 times for fluorescence microscopy imaging (Fig. 17) and 1.5 times for Northern blotting (Fig. 23). The 3.5 fold difference measured by fluorescence imaging is likely to be the most correct since the amount of saturated pixels is controlled during the imaging process and set to a low, fixed value; the amount of saturated pixels were not controlled in this manner in the other two methods. Hence, it is probable that the lower signal measurements of pLacI samples induced with 100 μM of IPTG obtained from fluorescence microscopy imaging and Northern blotting are misleading.

In the sub-field of promoter characterization, a lot of work has been done prior to this study. Fluorescence quantification is common, i.e. by using confocal laser scanning microscopy to quantify the copper induction of a GFP-coupled promoter (Granger & Cyr 2001) or by using fluorescence-activated cell sorting (FACS), which was used for characterizing a whole promoter library coupled to GFP (Alper *et al.* 2005). As a measure of promoter strength, Alper *et al.* calculated the relative fluorescence units per absorbance units per hour, using a previously defined dynamical model (Leveau & Lindow 2001) that compensates for cell-growth effects plus GFP maturation and synthesis factors. Even though Alper *et al.* had to use constant approximations of the GFP degradation rate and the GFP maturation rate, this model should provide a more accurate metric of the promoter strength than the standardized absolute intensity value obtained through fluorescence imaging in this study. However, if only the relative differences in promoter efficiencies are desired, the standardized absolute intensities obtained here are probably enough. Further, if a common standard is used to standardize the intensities, data from different studies will also be comparable.

4.1.1 Fluorescence imaging

This method measures fluorescence on a macroscopic level; this means that the observed fluorescence intensity for every sample is in fact a vast collection of individual bacteria's mean fluorescence. Because of this, stochastic effects due to low numbers of fluorescent objects are of no concern, which renders this method robust. Live cells are taken directly from the culture and measurement is quick, this means that the cells are in good shape during measurement, and that fluorophor bleaching is of small importance. One of the major advantages is also a large drawback - the method measures fluorescence on a macroscopic level, this means per definition that it is impossible to measure fluorescence on an individual cell scale. When it comes to promoter characterization, one major flaw is that fluorescence imaging only measures the transcription indirectly. Many factors are important for the translation of mRNA into protein; the added variation from the translation step may make small variations in mRNA levels unclear and conclusions drawn from such data may be more or less erroneous. However, the results from the Northern blotting (Fig. 21, Fig. 23) indicate that these macroscopic fluorescence-imaging measurements (Fig. 12) are quite accurate in serving as means of transcriptional quantification, at least for the big picture. More data is needed to draw further conclusions about the accuracy of fluorescence imaging to quantitate promoter activity on a finer scale.

4.1.2 Fluorescence microscopy imaging

With this method individual cells are studied, for large bacteria or eukaryotes even parts of cells may be scrutinized. This is the major perk of this method; by measuring individual cell's fluorescence on a microscopical level a lot more information may be obtained than from macroscopic measurement methods like fluorescence imaging. Further, it is possible to quantitate a lot of objects on a microscopic image using semi-automated software, rendering it more statistically robust than otherwise. But even though the experimental procedure is straightforward and no hazardous chemicals are involved, fixating the cells while preserving a correct representation of their fluorescence may be hard. Bleaching is another problem of major concern; while searching for a good image, and whilst exposing it, the fluorescence intensity drops noticeably fast. This is hard to counter, but fast imaging and exposure using set times may partly reduce the problem. Also, cells with low intensity levels may not be detected, this will bias the data toward greater than real intensities since the missed low intensity cells will be left out of the statistics; the detected cells with higher intensities will give a too high mean intensity value. Increasing the exposure time in order to detect low intensity cells will increase the signal from high intensity cells and their pixels might become saturated. This will lead to underestimation of the fluorescence from high intensity cells, something that is likely to have happened for the pLacI-RFP sample induced with 100 μM IPTG (Fig. 17) in this study. Because of this, microscopy imaging is most suited for measuring cell intensities of relative medium strength or higher, with intensity differences between samples low enough to avoid intensity data bias in either direction. Additionally, this method also measures promoter activity indirectly, with the translation step in between, giving the same drawbacks as for fluorescence imaging. The Northern blotting results (Fig. 21, Fig. 23) do, at least largely, support also the fluorescent microscopy results (Fig. 17, Fig. 18). In this study though, there are large variations in the intensity results because of different fixation methods, different bleaching times and the population of cells chosen for sampling. Therefore it is strongly recommended that the methodology is further evaluated and that standard methods for choosing sample populations, imaging and fixation are devised. When this is done, fluorescence microscopy imaging should prove useful in single-cell promoter characterization, because it has previously been used successfully to characterize promoters (Sentchilo *et al.* 2003).

4.1.3 Northern blotting

Even though this method is macroscopic in the sense that it measures RNA levels of bacterial cultures, it is the only method evaluated in this study that measures the mRNA levels directly. Hence, additional effects upon the promoter characterization data caused by translation or the fact that fluorescence based techniques measures on the protein level are avoided. This is also the big advantage of this otherwise time consuming method which involves several toxic or radioactive chemicals. Extracting the RNA from the sample cultures, running it on a denaturing gel, blotting it and probing for the correct target are four steps that together add up to experimental noise and systematical errors, even provided that the RNA extractions and gel runs of the different samples are unbiased and equal. This problem can be partly overcome by calibrating the measured data with the transcription level of one or several reference genes, in this study 16S rRNA was used, which also compensates for discrepancies in loaded amounts of RNA and pipetting errors. Nevertheless, reference genes with a true constant transcription level, so called housekeeping genes, are hard to find. In fact the transcriptional level of many reference genes has been found to vary significantly. Instead, normalization using a molecule, e.g. a unique RNA molecule, externally added as early as possible in the experimental procedure may be a better choice (Huggett *et al.* 2005). The RFP probe used here seems to be specific since there is no binding in the lane where the negative RFP construct is (Fig. 21). However, it is possible that the 16S rRNA probe binds also the RFP mRNA since a strong 16S rRNA signal coincides with a strong RFP mRNA signal at the pLacI sample induced with 100 μM IPTG (Fig. 22). But this could also be due to variations in the sample amount of rRNA. The specificity of the 16S rRNA probe is testable by a hybridization experiment where it is applied to RNA samples that do not contain rRNA; probe-binding would then implicate unspecificity. This was not done during this study. Generally, unspecific binding is alleviated with higher stringency washing procedures, however in this case this was possibly not performed successfully. If the 16S rRNA probe does have a high degree of unspecific binding and even higher stringency washes fail to remove unspecific binding, the 16S rRNA calibration probe has to be re-designed or another probe must be used. Because of the unknown specificity of the 16S rRNA probe, mainly the uncalibrated data (Fig. 21) is used for comparison with other promoter activity data in this study.

4.2 Promoter characterization

The different experiment's results support each other, which strengthens the conclusions that can be drawn about each promoter. As results, relative differences between the samples were obtained from the three methods, which are what can be compared and used for drawing conclusions. The measured strengths of a certain promoter cannot be directly compared between the methods since a common standard is missing. It is certain that the 100 μM IPTG induced pLacI sample has by far the most active transcription of RFP mRNA, one can also conclude that pOmpR is considerably stronger than pOmpRm and that pLacI induced with 1 and 0 μM IPTG and pOmpRm have the weakest RFP transcription. Since these results agree with what was expected if one considers the knowledge about the different promoters (section 1.4), conclusions drawn from these results are further substantiated.

4.2.1 The pLacI promoter

All three methods of promoter strength quantification support the theoretically falling ability of pLacI to promote transcription when the IPTG concentration goes down. This should occur exponentially, which is expected for this *lac* operator controlled promoter in the XL1-Blue strain that contains the functional lactose permease gene *lacY*. Lactose permease transports its own gene's inducer IPTG into the cell, which establishes a positive feedback loop that leads to a sharp rise in internal IPTG concentration at externally lower concentrations (Jensen *et al.*

1993). It seems though, that this positive feedback loop is weaker in *E. coli* compared to *Pseudomonas fluorescens*, which lacks the extra means of IPTG import or diffusion that *E. coli* has (Hansen *et al.* 1997). Therefore, the exponential nature of IPTG induced *lac*-type promoters in *E. coli* can be expected to be weaker than in other species more dependent on lactose permease to import IPTG. More data of this specific pLacI system at different IPTG concentrations is needed to conclude whether it responds exponentially to a linear, external increase of the IPTG concentration. The exponential curve (Fig. 14) adapted to obtained fluorescence imaging data by regression is a good example of how the response could look if it is exponential in nature. Moreover, the pLacI promoter sequence only contains two of the wild-type lacI operators described in section 1.4.1, this, and the fact that the expression of the *lac* repressor depends upon the strain and hence is relative, makes it incorrect to exactly compare these results with other studies of the *lac* operon or the *lac* promoter. Yet, a previous study of the *lacUV5* promoter, which has a mutation that renders the promoter insensitive to glucose, in cells expressing the *lac* repressor and the lactose permease, has shown a response to increasing IPTG concentrations that is sigmoidal, close to peaking at 10 μM IPTG and reaching steady state at 100 μM IPTG (Jensen *et al.* 1993). In this study, using this specific pLacI system, an IPTG concentration of 10 μM does not give an induction close to the steady state level since the difference in signal strength between pLacI samples induced with 10 and 100 μM IPTG is 3.5 times (Fig. 12). Nonetheless, the response can be expected to be sigmoidal, as it also has been for another *lac* repressor containing *lac*-type promoter system in *Erwinia herbicola* (Leveau & Lindow 2001).

4.2.2 The pOmpR and pOmpRm promoters

pOmpR should be considerably stronger than pOmpRm (section 1.4.2 and 1.4.3), which it is according to data from all three methods of promoter quantification. As the pOmpR and pOmpRm promoters were included to provide references to the pLacI promoter, and to be quantitated at a constant transcriptional level, not much can be said about their characteristics without further studies where e.g. the medium osmolarity or pH is varied. However, the results show that the pOmpR promoter, using the standard growth conditions used here, has a strength slightly below the pLacI promoter induced with 10 μM IPTG.

4.3 Synthetic biology

As a standard tool for assembling genetic parts, Synthetic biology has proven to be extremely valuable in this study. Previous work in non-Synthetic biological cloning and genetic part assembly was much more time and hence resource consuming (unpublished). Furthermore, as a tool for comparing methods of promoter characterization and actually quantifying the promoter's abilities of promoting transcription, Synthetic biology has proven to be indispensable. A prerequisite for being able to compare the transcription of different promoter-reporter constructs was that the constructs, except from their differences in promoter sequence, must be identical. This demand was extremely well solved by one of the key concepts of Synthetic biology, standardization, which in this case meant that the plasmids and all sequences except the different promoters were identical. When more complex assemblies like whole metabolical pathways are to be characterized, the standardization concept becomes even more important, not only because of the partly identical sequences but because of the easier and faster assembly.

5. Future development

5.1 Characterization methodology

For characterizing promoters and other small genetical elements, fluorescence imaging and fluorescence microscopy imaging seems most promising of the methods used in this study. This is partly due to the robustness of fluorescence imaging, the possibilities of quantifying intensities of individual cells, or parts of cells, in fluorescence microscopy imaging but also because of their ease of use, which is important if the methods are to be used in routine. Another method suitable for routine characterization is flow cytometry or fluorescence-activated cell sorting (FACS), which may be used for quantifying fluorescence intensities for large amounts of cells. FACS has previously been used successfully for quantifying promoter strength by calculating the geometric mean of the fluorescence distribution for each promoter construct (Alper 2005). Northern blotting, or other direct measurements of RNA levels like reverse transcriptase quantitative PCR (RT q-PCR), will probably be of importance in identifying conditions where fluorescent protein levels are accurate representatives for the mRNA transcription. As nowadays complicated procedures like Northern blotting or RT q-PCR become more automatized, these methods may prove to be better methods for routine characterization of genetic elements. For more accurate metric measurements of promoters' abilities to promote transcription, the model mentioned above defined by Leveau & Lindow and utilized by Alper *et al.* may be used (Leveau & Lindow 2001, Alper *et al.* 2005). This model still needs to approximate certain kinetic factors though, and it requires that the dynamics of the fluorescent protein in question is well characterized.

5.2 Promoter characterization

In order to generally elucidate the functions of promoters and characterize them, all environmental factors relevant to their function should be varied in turn and the response in transcriptional activity measured. When it comes to inducers, like IPTG for the pLacI promoter or pOmpR-P for the pOmpR and pOmpRm promoters, the inducer concentrations should be varied until enough data points for accurate characterization have been obtained. This is a simple matter for pLacI where the levels of IPTG can be controlled easily, but more complex for pOmpR and pOmpRm that respond to pOmpR-P levels which in turn are controlled by osmolarity, temperature and pH (Alphen & Lugtenberg 1977). The cell density of cultures containing pOmpR or pOmpRm is another highly relevant factor that needs to be taken in consideration. These promoters are derived from the porin gene *ompC*, which expression is altered by the cell density; high cell densities up-regulate the expression whereas low densities down-regulate it (Liu *et al.* 2000). The variation of environment should also include different host species, or chassis, of relevance. Finally, regression models should be used to mathematically describe the promoters so they can be used in modeling and future design of complex circuits and assemblies in Synthetic biology.

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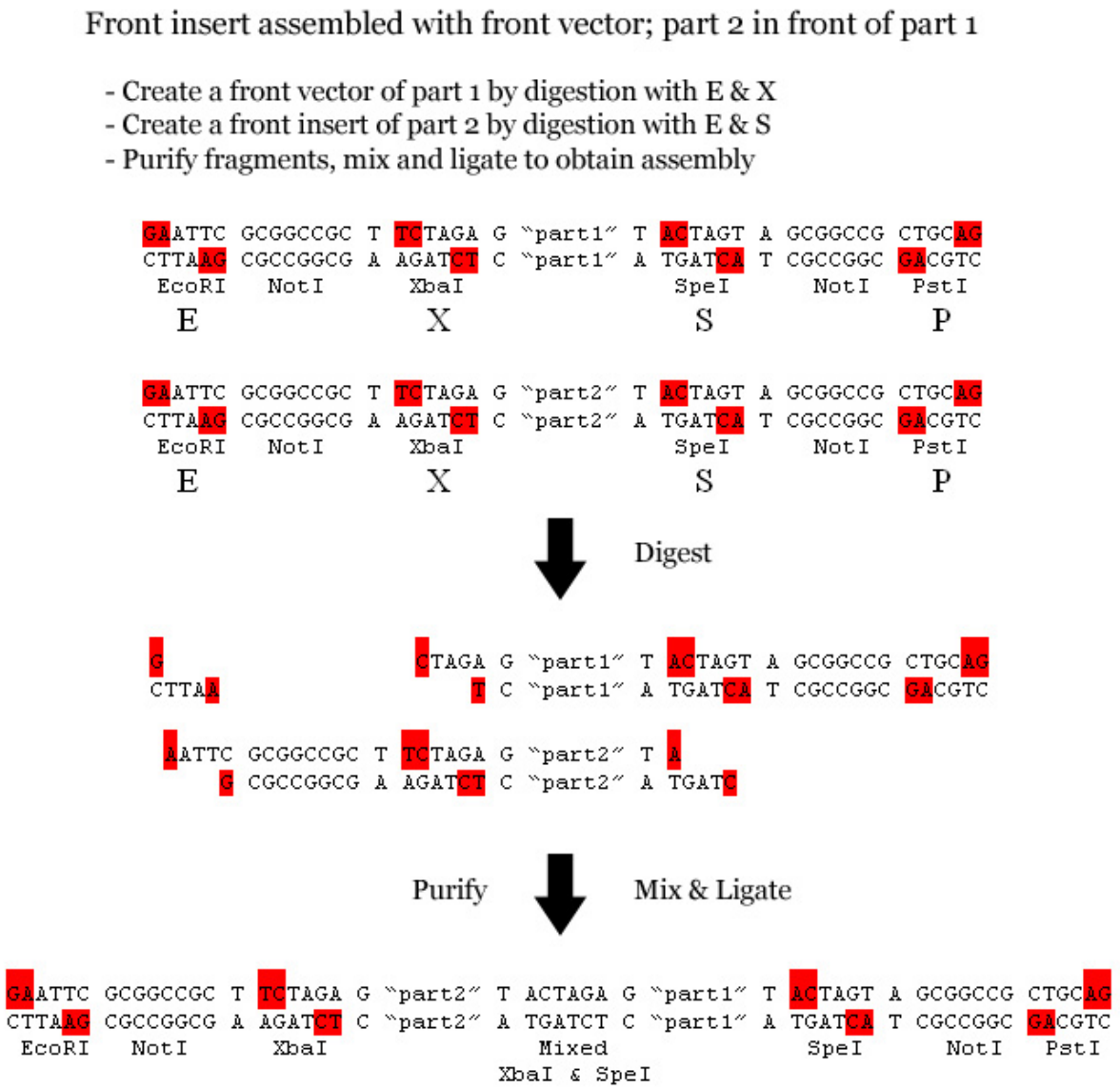
8. Appendices

8.1 Appendix A – BioBrick assembly scheme for front fragments



8.2 Appendix B – BioBrick assembly scheme for back fragments