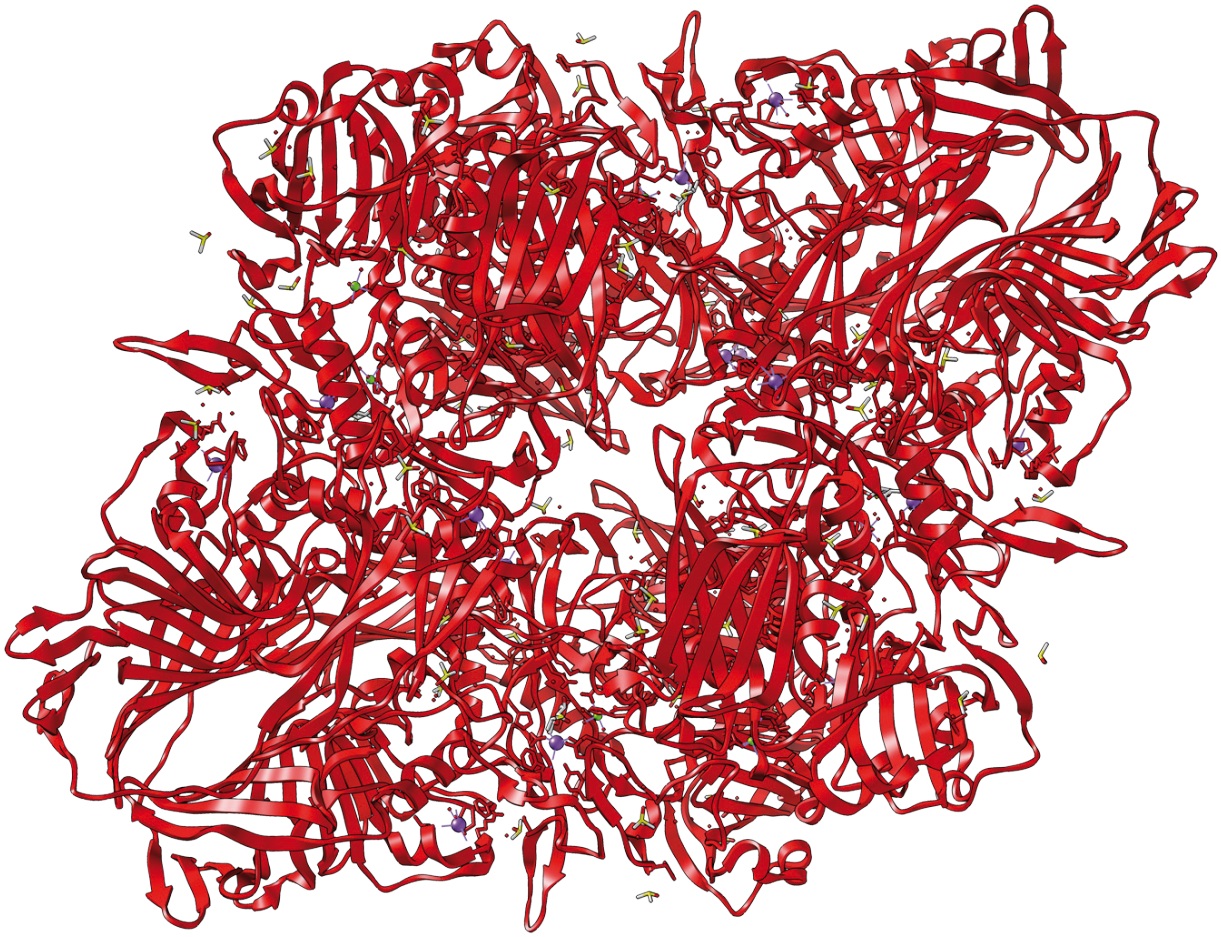
Synthetic Biology Teaching Resources

University of Reading



Engineering
Gene expression

1.0 BETA

Instructions and background notes

A genetic switch

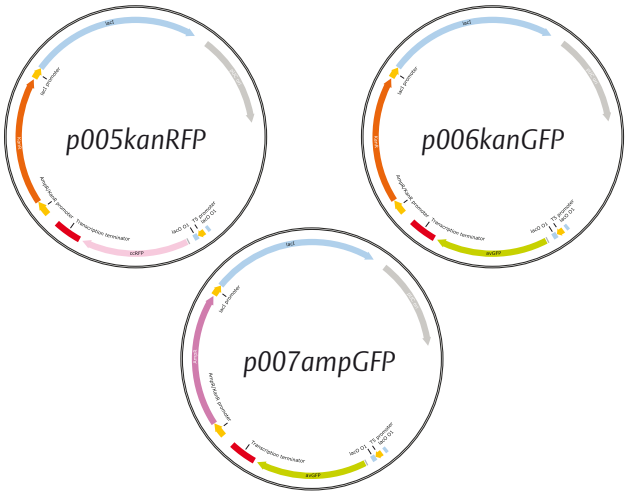
Almost every cell of any living organism contains the complete set of genes needed to make that organism. This is a fact that we tend to take for granted, mainly because it's what we were taught in school, but it was not always clear that this was so.

In a large multi-cellular organism, such as a human, there are many different types of cell and their appearence can differ markedly. Cells can look so different, you might be forgiven for thinking that a human nerve cell, for example, came from a completely different organism to a human sperm cell. At one time it was thought that genes were progressively lost as a multicellular organism developed from a fertilized egg, so that a kidney cell would have a different set of genes to a muscle cell, for example.

We now know that this isn't the case — most cells contain the complete complement of genetic instructions, but at any one time only some of them are active. For instance, a human has about 25 000 genes, but on average, only 5 000 to 15 000 of these are active in a mature, differentiated cell.

Understanding gene regulation — how genes are switched on and off, and how to control the level of gene expression (the 'volume') — is crucial to synthetic biology, because it permits you to design an organism that behaves in the way you wish. This might mean programming it to make the substances you want it to in response to a stimulus or incorporating mechanisms to ensure that it cannot pose an environmental or safety hazard.

Although gene regulation is complex and poorly-understood in eukaryotes, the mechanisms of gene regulation in bacteria are simpler, more predictable and well documented.



What you will learn

Educational aims

In the previous activity you learnt how to use the PCR to generate overlapping DNA parts and combine them using Gibson method to assemble plasmids with novel properties. You also tried another method of bacterial transformation using calcium chloride to introduce plasmids into the cells.

In the following practical procedure you will use ready-made DNA 'parts' to modify the activity of genes in the plasmids you assembled in the previous activity. For example, you will be able to increase or decrease production of GFP, or make GFP active only upon induction by a chemical switch. As before, you will use PCR and *Gibson Assembly®* procedures and transform bacteria with the modified plasmids. You will then measure the performance of your modified organisms.

This procedures introduces an important concept in molecular and synthetic biology: regulation of gene expression and ability to predictably switch genes on and off. Depending on the chosen gene, this procedure also introduces various techniques to quantitatively measure gene activity.

Summary of the practical task

The starting point of all activities in Kit 3 are the plasmids generated in Kit 2: GFP and RFP-expressing plasmids, as well as plasmids encoding the banana smell generator or β-galactosidase.

Initially, you will use the PCR and primers to turn these four plasmids into plasmid 'backbones' that will be able to accept various promoter sequences that will alter the expression of the genes in the plasmids.

After the 'backbones' have been produced, the PCR products must be purified by passing them over a resin column. The concentration of the PCR products must then be measured to ensure that they are combined in the correct proportions in the *Gibson Assembly®*.

You will incorporate the promoters of your choice into the vector backbone using *Gibson Assembly®*, transform bacteria and grow them to compare if and how the gene activity has been modified.

Lastly, you will be able to isolate the newly-constructed plasmids from bacteria and sequence the inserted fragment to confirm correct assembly.

Gene regulation in bacteria

The genome of *E. coli* is about 4.6 million base pairs in length, encoding about 4300 proteins. At any one time, only a fraction of these proteins are being made. How is the expression of these genes regulated?

Promoters and terminators

In a bacterium such as *E. coli*, each gene is flanked by a *promoter* and a *terminator*. The promoter is simply the site where the enzyme RNA polymerase binds to the DNA and starts making complementary messenger RNA (mRNA). This mRNA carries the instructions required to make a protein and it is later 'read' by the bacterium's ribosome, where the protein is strung together from amino acids.

The terminator is a similar instruction in the DNA which tells the RNA polymerase to stop transcribing mRNA and to dissociate from the DNA. You came across these two components — promoters and terminators — in the last practical activity, where the fluorescent protein genes in the plasmids you made were preceded by a T5 promoter and followed by a transcription terminator.

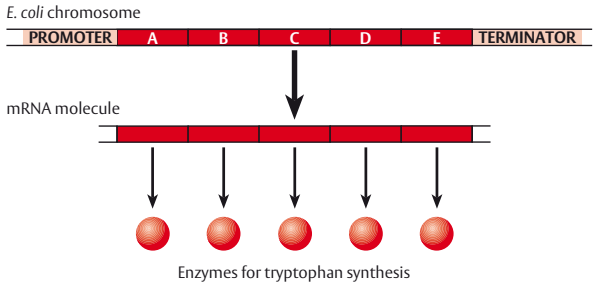
It might be tempting to think of the promoter as the 'on' switch and the terminator as the 'off' switch, but this would be wrong. The promoter and terminator just mark the start and end of the DNA sequence that encodes one or more proteins — these two sequences don't tell the cell when to start and stop making those proteins.

Operons

Sometimes, in bacteria, clusters of two or more related genes are under the control of a single promoter and terminator. A single stretch of mRNA is transcribed from them, allowing the different proteins encoded by the genes to be produced at the same time. For example, there is a group of five genes in *E. coli* encoding enzymes that are needed for synthesising the amino acid tryptophan. Similarly, there are three enzymes needed to break down the sugar arabinose and another three that are needed to absorb and metabolise lactose.

A grouping like this is called an 'operon': hence the groups of genes above are called the 'tryptophan operon', the 'arabinose operon' and the 'lactose (or *Lac*) operon'.

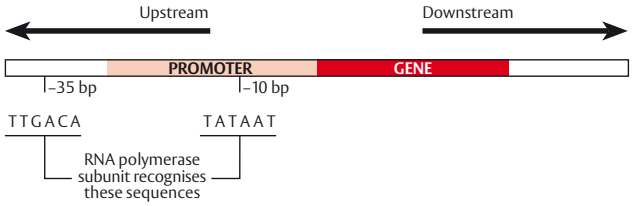
In eukaryotes, in contrast, there are no 'operons' — every gene has its own unique promoter and terminator.



Constitutive promoters

Some proteins are made continuously by the cell. These are usually essential 'housekeeping' proteins that the cell needs to stay alive. Their genes are switched on all of the time, and they are said to be expressed 'constitutively'. A constitutive promoter is therefore one which keeps the gene (or genes) downstream of it switched on all the time and is under no higher level of control.

In technical terms, constitutive promoters have two sites in the DNA (at 10 and 35 bases before the start of the protein-encoding sequence) that a subunit of the RNA polymerase always recognises and binds to. The sequence of bases there hardly ever varies: at −10 bases it is **TATAAT** and at −35 bases it is **TTGACA**. The *strength* of a promoter depends upon how closely the DNA matches these two sequences. A strong promoter will have a perfect, or almost perfect, match, and the RNA polymerase will bind to it every time.



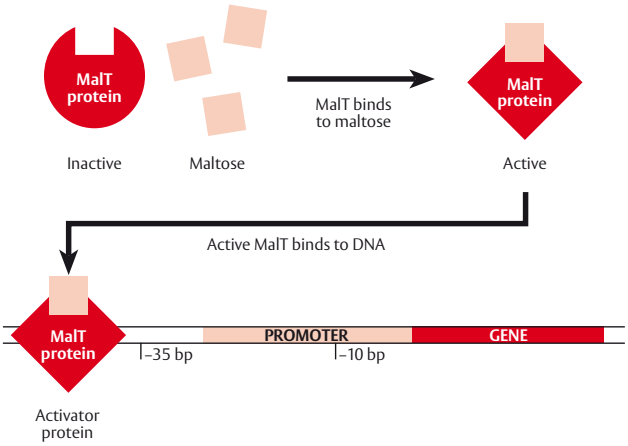
Positive regulation — gene activator proteins

Some proteins are only required at certain times. For instance, it would be wasteful if a bacterium made enzymes for metabolising maltose if this sugar was not present in its surroundings. Gene activator proteins can switch on protein production in such circumstances. How do these proteins work?

The DNA sequences in promoters before genes that are not continuously active are generally more variable than they are in constituative promoters, so they are less easily recognised by the RNA polymerase subunit. A gene activator protein helps the RNA polymerase bind to the DNA. The gene activator protein itself first binds to the DNA, then it guides the RNA polymerase into place.

The activator protein, however, is not normally able to bind to DNA. Only in the presence of a signal molecule can this be done. When the signal molecule binds to the activator protein, it forces the protein to change shape and this new shape is able to bind to the DNA (proteins whose activity is altered by binding to another molecule like this are called *allosteric*).

The signal molecule is often the sugar, for instance, that is broken down by the genes that are encoded downstream of the promoter. The presence of the sugar therefore acts as a switch to trigger the production of the proteins that metabolise it.



In E. coli, an activator protein, MalT, helps RNA polymerase to bind to the promoter upstream of the genes that encode enzymes enabling maltose to be metaolised. The activator protein can only bind to DNA in the presence of maltose, ensuring that the genes are only switched on when necessary.

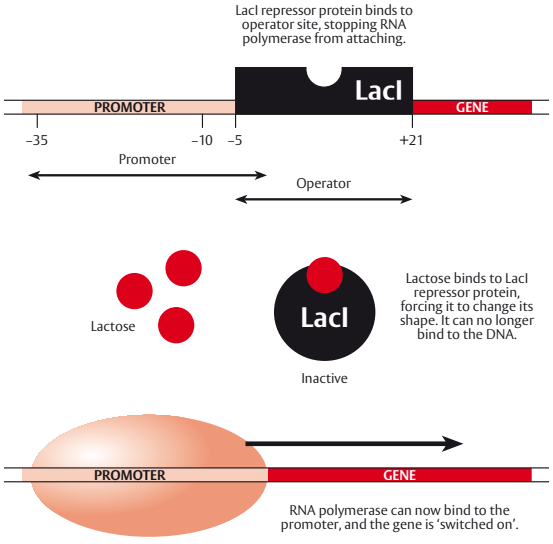
Negative regulation — repressors

In addition to activator proteins which switch genes on, there are also repressor proteins that switch genes off. The most famous of these is the *Lac* repressor protein encoded by the gene *LacI*. It regulates the genes that are needed by *E. coli* to digest lactose. (You came across *LacI* in the three plasmids used in Kit 2.)

When there is no lactose in the growth medium, the *Lac* repressor protein binds to the DNA just before the genes that are involved in digesting lactose. By so doing, it blocks part of the promoter sequence, so that RNA polymerase cannot bind to the DNA. Consequently the genes downstream of the promoter are not transcribed into mRNA.

When lactose is present in the medium, however, it interacts with the *Lac* repressor protein, forcing it to change shape. With its new shape, the protein cannot bind to DNA, so the RNA polymerase binding site is *not* blocked and the lactose-digesting proteins are made.

The tryptophan and L-arabinose operons referred to above are controlled in a similar way by tryptophan and L-arabinose repressor proteins respectively.



Positive and negative regulation — the *Lac* operon — a logic gate

In fact, the *Lac* operon, like many regulatory mechanisms, is slightly more complex than suggested above and the promoter is controlled by *two* different regulatory molecules.

In response to low levels of its preferred energy source (glucose), *E. coli* produces a molecule called cyclic AMP (cAMP). This signal molecule interacts with the *Cyclic AMP Receptor Protein* (CRP) which was formerly known as CAP (the *Catabolite Activator Protein*), forcing that protein to change shape so that it can bind to DNA. When it does so (in several places throughout the *E. coli* genome), it activates genes that would allow *E. coli* to utilise alternative sugars such as lactose, maltose or fructose. CRP is thus a general-purpose activator which operates when glucose is not available.

It would, however, be wasteful of *E. coli* to start producing for example, the three enzymes needed to take up and utilise lactose if there was none of that sugar available either. Switching on the genes that encode the lactose-utilising enzymes therefore requires:

- a general signal is sent by cAMP ('Help! The glucose has run out');
- cAMP binds to the activator protein, CRP;
- CRP guides the RNA polymerase so that it can bind to DNA at the promoter site...
- ...but it can only do so if another signal molecule, lactose, is present and interacts with the *LacI* repressor protein, unblocking the promoter site.

This can be compared to an AND logic gate: only when glucose is absent AND lactose is present are the relevant genes switched on. Many genes are regulated in this way.

CAP protein	Repressor protein	RNA polymerase	Switch ON or OFF
Present	Absent	Binds	On
Absent	Present	Cannot bind	Off
Present	Present	Cannot bind	Off
Absent	Absent	Cannot bind	Off

JARGON BOX

Promoter
Site where RNA polymerase binds to DNA

Operator
Site where repressor protein binds to DNA

Repressor
A protein which prevents RNA polymerase from binding to DNA

Activator
A protein which helps RNA polymerase to bind to DNA

Overview of the procedure

A choice of promoters

If you followed the whole procedure in Kit 2, you now have a set of plasmids with four different proteins whose activity is under control of their original promoters. For *avGFP*, *ccRFP* and β-galactosidase, this promoter is *pLac*. For the banana odour generator, the promoter is *OsmY*. *pLac* is a promoter that only switches the expression of a gene downstream on if there is enough lactose (or IPTG, a lactose analogue) in the medium. In cultures without lactose, the promoter is blocked by LacI (a repressor protein) and the gene downstream is not transcribed. *OsmY* is a promoter that only switches the expression of a gene downstream on when the cells are in the stationary phase of their growth.

If you didn't assemble the four plasmids in Kit 2, you still have the original *p005kanRFP* and *p006kanGFP* plasmids, both with the *pLac* promoter, which you can use in this activity.

For each plasmid, you have an option of exchanging the original promoter for one of two promoters that will switch the gene downstream on all the time, but with different strengths, and another two that will switch the gene on only if arabinose or lactose/IPTG is present in the growth medium. You will then be able to compare their activity with the original promoters. Table 1 shows the names of all the promoter parts available.

Once you select the gene whose expression you want to manipulate — *avGFP*, *ccRFP*, *ATF1* or *LacZ* (β-galactosidate), you must generate the vector into which you will insert the new promoter using *Gibson Assembly®*. All promoters for a given gene are inserted into the same vector, and the PCR product lengths and primers necessary for the generation of the vector backbones are shown in Table 2.

Finally, after you have generated the vector backbone, run it on a gel, purified it and measured its concentration, you must calculate appropriate amount of vector and insert molecules for the *Gibson Assembly®*. Table 3 shows the promoter parts' lengths required for this calculation.

About the promoter parts

Strong constitutive

This genetic part contains a promoter *pFAB4026* from the *BIOFAB* collection. It is one of the strongest promoters tested and characterised by Vivek Muktalik and his colleagues [1]. The strength of the promtoer is given in arbitrary units as 1419, *i.e.* it is about three times stronger than *pFAB4024* (see below). This promoter is constitutive, which means that it is permanently 'on', allowing transcription of the downstream gene.

Weak constitutive

This part contains a promoter *pFAB4024*, again from the *BIOFAB* collection. It is a moderately weak promoter tested and characterised by Vivek Muktalik and his colleagues [1]. The strength of the promtoer is given in arbitrary units as 515, *i.e.* it is about three times weaker than the *pFAB4026*. This promoter is also constitutive, which means that it is permanently switched on, allowing transcription of the downstream gene.

Inducible by L-arabinose

This part contains a *pBAD* promoter that is inducible by arabinose, *i.e.* the promoter allows for transcription of the downstream gene only if there is L-arabinose in the environment [3].

In addition, this part contains an *AraC* gene under control of its endogenous *pC* promoter. *AraC* encodes a repressor of the *pBAD* promoter (similar in action to the LacI repressor in the *pLac* promoter) [3]. The relationship between arabinose concentration and rate of induction of this promoter is described in [4].

Inducible by lactose or IPTG

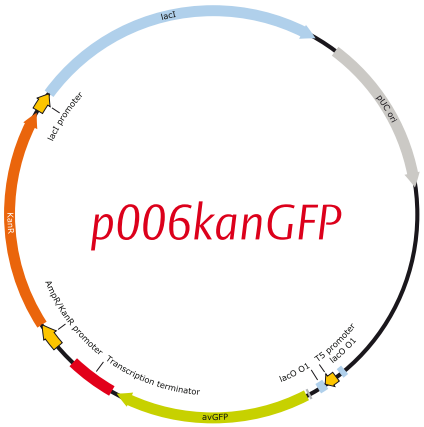
This genetic part contains a hybrid promoter comprising of operator sites (sequences bound by the LacI protein in the absence of lactose or IPTG) from the classic *pLac* promoter. These flank a promoter from the bacteriophage T5. In the absence of lactose or IPTG this promoter is repressed by Lac repressor protein, LacI, which is constituatively produced from the plasmid. The original plasmids *p005kanRFP* and *p006kanGFP* contain this promoter. A detailed description of the *Lac* promoter, repressor and operator system — one of the best known bacterial gene regulation systems — is given in the references listed below [5, 6].

References

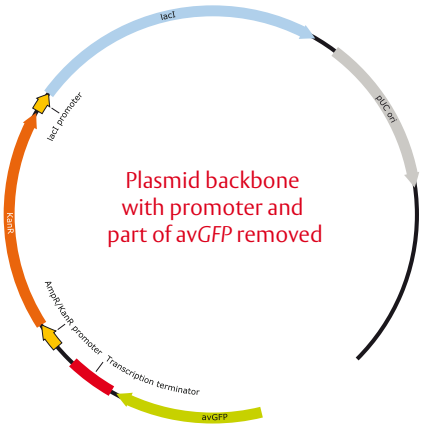
1. Mutalik, V.K. *et al.* (2013) Precise and reliable gene expression *via* standard transcription and translation initiation elements. *Nature Methods* **10**: 354–602.
2. Schleif, R. (2000) Regulation of the L-arabinose operon of *Escherichia coli*. *Trends in Genetics* 16: 559–565.
3. An animation showing the operation of the L-arabinose operon: **http://www.youtube.com/watch?v=EVmnIDc0K70**
4. iGEM registry. Description of the pBAD series of promoters. **http://parts.igem.org/PBAD_Promoter_Family**
5. *The lac operon. A short history of a genetic paradigm* by Benno Müller-Hill (1996) Walter de Gruyter, Berlin. ISBN: 3 11014830 7.
6. Sandwalk. Laurence A. Moran's blog: **http://sandwalk.blogspot.co.uk/2008/02/repression-of-lac-operon.html**

Example: Engineering *GFP* expression

Template plasmid

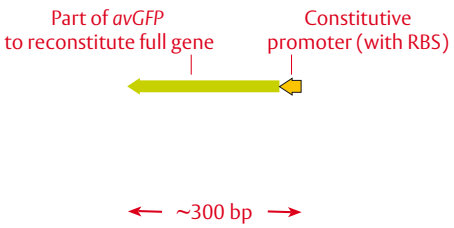


Polymerase chain reaction

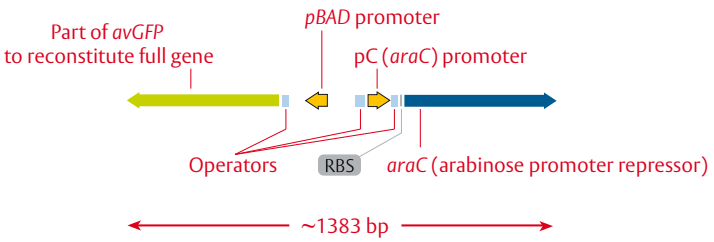


Gibson Assembly®

Inserts with constitutive promoters of different strengths



Insert with promoter inducible by arabinose



Promoters and primers

Table 1

Promoter part names to modify the expression level of each of the four proteins (avGFP, ccRFP, ATF1 and β-galactosidase). These promoters are already present in the plasmids p005kanRFP and p006kanGFP.

Product	Promoter part name for avGFP	Promoter part name for ccRFP	Promoter part name for ATF1	Promoter part name for β-galactosidase
Strong constitutive	P1	P5	P9	P13
Weak constitutive	P2	P6	P10	P14
Inducible by L-arabinose	P3	P7	P11	P15
Inducible by lactose or IPTG	P4	P8	P12	P16

Table 2

PCR product lengths for *Gibson Assembly*®. Note that the elongation time during PCR depends on the type of DNA polymerase used. For example, NEB *Phusion*® polymerase synthesises ~4kb of DNA in 1 minute, whereas NEB *Vent*$_R$® polymerase is approximately four times slower.

Product	Vector length (bp)	Time using *NEB Phusion*® DNA polymerase	Time using *NEB Vent*$_R$® DNA polymerase	Primers required
avGFP plasmid backbone	4288	1 min 15 sec	5 min	2AForward 1Reverse
ccRFP plasmid backbone	4238	1 min 15 sec	5 min	2BForward 1Reverse
ATF1 plasmid backbone	5081	1min 30 sec	6 min	2CForward 1Reverse
Beta-galactosidase plasmid backbone	6376	1min 50 sec	7 min 30 sec	2DForward 1Reverse

Table 3

Promoter parts' lengths required to calculate correct ratio of plasmid backbone to promoter part in each *Gibson Assembly*® reaction.

Promoter part name	Promoter part length (bp)
P1	302
P2	301
P3	1383
P4	403
P5	311
P6	310
P7	1396
P8	414
P9	418
P10	417
P11	1494
P12	556
P13	667
P14	666
P15	1748
P16	810

PCR primers to generate various backbone plasmids

PCR primers to generate backbone from *avGFP* plasmid:
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
2AForward: TGTCAGTGGAGAGGGAGAAGG

PCR primers to generate backbone from *ccRFP* plasmid:
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
2BForward: GTACAGGCAAACCGTATGAG

PCR primers to generate backbone from *ATF1* plasmid:
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
2CForward: AGCTTTGAGGGAAATCTGCC

PCR primers to generate backbone from β-galactosidase plasmid:
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
2DForward: GTTTCATCTGTGGTGCAACG

Equipment and materials

For the PCR and *Gibson Assembly*®
Each person or working group will need

Straight from the kit:

For manipulating expression level of *avGFP*

To generate the plasmid backbone
- template plasmid DNA: 4 µL of *avGFP*-carrying plasmid constructed in Kit 2, or the original *p006kanGFP* plasmid, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *2AForward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

Ready-made insert, one for each construct
P1, P2, P3, P4 (see page 7)

For manipulating expression level of *ccRFP*

To generate the plasmid backbone
- template plasmid DNA: 4 µL of *ccGFP*-carrying plasmid constructed in Kit 2, or the original *p005kanRFP* plasmid, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *2BForward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

Ready-made insert, one for each construct
P5, P6, P7, P8 (see page 7)

For manipulating expression level of *ATF1*

To generate the plasmid backbone
- template plasmid DNA: 4 µL of banana-odour-generator-carrying plasmid constructed in Kit 2, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *2CForward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

Ready-made insert, one for each construct
P9, P10, P11, P12 (see page 7)

For manipulating the expression level of β-galactosidase

To generate the plasmid backbone
- template plasmid DNA: 4 µL of β-galactosidase-carrying plasmid constructed in Kit 2, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *2DForward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

Ready-made insert, one for each construct
P13, P14, P15, P16 (see page 7)

Not in the kit: supplied by you

- PCR 'Mastermix' (2×): 25 µL per pair of reactions [1]
- thermal cycler
- microcentrifuge
- micropipettes (*e.g.*, 0–10 µL and 40–200 µL)
- sterile pipette tips (for the micropipettes)
- 0.2 mL thin-walled PCR tubes
- equipment and materials for agarose gel electrophoresis *e.g.*, gel tank and power supply, 1.5% (w/v) agarose, TAE or TBE buffer, dye to detect DNA in the gel [2]
- suitable DNA ladder [3]
- kit for cleaning up PCR products (one column for each PCR product) [4]
- apparatus for DNA quantification (if not quantifying by gel electrophoresis) [5]

- clean PCR products in the correct ratios, as prepared by you previously (see pages 5–7)
- off-the-shelf *Gibson Assembly*® kit [6]

  Or

  15 µL home-made *Gibson Assembly*® master mixture

Notes

1 We recommend using *Phusion*® DNA polymerase master mix (*NEB* Cat. No. M0531S) or *ThermoScientific* (Cat. No. F-548S). Note that conventional *Taq* polymerase is unsuitable for this task as it generates unwanted adenine overhangs which interfere with the *Gibson Assembly*®.

2 We recommend using the *Lonza FlashGel*™ system to run electrophoresis in under 10 minutes.

3 For example, *FastRuler* Middle Range DNA Ladder, Ready-to-Use, 100–5 000 bp, from *Thermo Scientific* (Cat. No. SM1113).

4 For example, *GeneJET (Thermo Scientific)* or *Qiagen* kits.

5 For example, *NanoDrop*® *(Thermo Scientific)* or *Qubit*® (*Life Technologies*) apparatus.

6 *New England Biolabs* (Cat. No. E2611S). Alternatively, you could use the *NEB Gibson Assembly*® Cloning Kit (Cat. No. E5510S) that combines assembly and transformation protocols.

Home-made *Gibson Assembly*® master mix

ISO buffer (5x)

To prepare 6 mL of this buffer

Ingredients

- 3 mL 1 M Tris-HCl, pH 7.5
- 150 µL 2 M $MgCl_2$
- 60 µL 100 mM dGTP
- 60 µL 100 mM dATP
- 60 µL 100 mM dTTP
- 60 µL 100 mM dCTP
- 300 µL 1 M DTT
- 1.5 g PEG-8000
- 300 µL 100 mM NAD
- Sterile ultrapure water

Method

1. Combine all of the ingredients listed above.
2. Add water to make up to 6 mL.
3. Aliquot in 100 µL volumes and store frozen at -20 °C.

Assembly master mixture

To prepare 1.5 mL of this mixture

Ingredients

- 320 µL 5× ISO buffer (recipe above)
- 0.64 µL 10 U/µL T5 exonuclease
- 20 µL of 2 U/µL *Phusion*® polymerase
- 160 µL of 40 U/µL *Taq* ligase
- Sterile ultrapure water

Method

1. 1. Combine all of the ingredients listed above.
2. 2. Add water to make up to 1.2 mL.
3. 3. Aliquot in 15 µL volumes and store frozen at -20 °C.

This assembly mixture can be stored at -20 °C for at least a year. The enzymes remain active following 10 freeze-thaw cycles, and possibly many more.

Note
This mixture is ideal for the assembly of DNA molecules with 20–150 bp overlaps. For DNA molecules overlapping by more than 150 bp, prepare the assembly mixture using 3.2 µL 10 U/µL T5 exonuclease instead.

Reference

Gibson, D. (2009) One-step enzymatic assembly of DNA molecules up to several hundred kilobases in size. *Nature Protocol Exchange*. **www.nature.com/protocolexchange/protocols/554**

Preparing the plasmid backbone

PCR, purification and measurement

1. Amplifying the plasmid backbone

The purpose of this step, using the PCR, is to make copies of a selected plasmid backbone which will later be combined with ready-made parts using the *Gibson Assembly®* method.

1. Using a 0–10 µL micropipette, and a new sterile pipette tip for each item, place the following components in a 0.2 mL thin-walled PCR tube:

– PCR 'Master mix' (2×)	12.5 µL
– Forward primer (1 µM)	4 µL
– Reverse primer (1 µM)	4 µL
– Template plasmid DNA (< 1 ng total)	4 µL

Note: If you are using the plasmids you constructed in the Kit 2, the concentration of your template DNA will vary. As a rule of thumb, the total amount of template should be kept below 1 ng.

2. Place the capped tubes in a thermal cycler, and run the following programme:

   To ensure that the template DNA becomes single-stranded
   95 °C for 30 seconds.

   Repeat the following three steps 30 times:

   - *Denaturation of the DNA template strands*
     95 °C for 10 seconds
   - *Annealing of the primers to their target sequences*
     60 °C for 15 seconds
   - *Extension/DNA synthesis*
     72 °C for X seconds (refer to Table 1 for the duration)

   Ensures that the DNA fragments are fully extended
   72 °C for 5 minutes.

3. Once the 30 cycles are complete, hold the product at 10 °C or store the tubes in a fridge at 3–5 °C until you are ready to proceed with Step 2.

2. Running the product on a gel

Take a small 3 µL sample of the PCR product and run it on a gel alongside a suitable DNA ladder (*e.g.*, 100–5 000 bp). This will give you an indication of how well the amplification has worked. You should see a clean, sharp band of the expected size (refer to Table 2 for the sizes of the different PCR products). There may be some smaller primer-dimers present, but these should not interfere with the success of the DNA assembly. If you see don't see the expected results, *do not proceed to the next step*. Check all of the reagents and run the amplification again.

3. Purifying the PCR products

This step removes free nucleotides, ions and buffers from the PCR products, leaving clean DNA sequences. Clean DNA is necessary not only for the DNA assembly method, but also when you measure the amount of DNA before assembly.

You should use a commercial silica-based PCR purification kit (such as *GeneJET*, *Qiagen* and others).

Important note: The following instructions are given simply to provide an idication of the principal steps involved in such purifications. The instructions provided with the particular kit you use must be followed, as products differ from one another and may change over time.

1. Measure the volume of one of the PCR products using a micropipette and clean tip.
2. Return the PCR product to the tube and add an equal volume of Binding Buffer to the tube. Mix thoroughly by drawing the liquid up and drown in the tip a few times.
3. Transfer the mixture to the *GeneJET* purification column. Centrifuge for 1 minute. Discard the flow-through.
4. Add 700 µL of Wash Buffer to the *GeneJET* purification column. Centrifuge for 1 minute. Discard the flow-through and place the purification column back in the collection tube.
5. Centrifuge the empty *GeneJET* purification column for 1 minute to completely remove any residual wash buffer.
6. Transfer the *GeneJET* purification column to a clean 1.5 mL microcentrifuge tube. Add 35 µL of ultrapure water to the centre of the *GeneJET* purification column membrane to wash out the DNA. Take care not to touch the membrane with the pipette tip as you might damage it.
7. Incubate at room temperature for 1 minute then centrifuge for 1 minute.
8. The purified DNA will be in the collection tube.
9. Repeat this procedure for each PCR product.

4. Measuring the amount of DNA

It is essential to meanure the amount of DNA in the PCR products as you will need to combine them in the correct proportions for the *Gibson Assembly®*.

There are several methods of measuring the amount of DNA such as running it alongside a marker of known concentration on a gel or using commercial DNA measurement apparatus *e.g.*, *NanoDrop®* or *Qubit®* equipment.

It is a wise precaution to ensure that the DNA sample is cleaned up before measuring the concentration, as some measurement methods, especially spectrophotometric ones, can give false readings if this is not done.

The *Gibson Assembly*®

Joining the DNA fragments to make a novel plasmid

1. Calculating the amounts of DNA required

A critical requirement of the *Gibson Assembly*® is that there must be similar numbers of the DNA molecules to be joined. Therefore you would normally add equimolar amounts of each DNA component to the reaction mix.

When joining an insert to a vector (as in our case), however, there should be roughly *twice* the amount of the insert as the vector. This is simply to increase the probability of the insert reacting with the plasmid. Note that this proportion is a 'rule of thumb', not a precise ratio.

For a home-made Gibson master mix (see page 8), the total amount of DNA in the assembly reaction should be between 0.02–0.5 pmol and 100–500 ng. In your reaction, however, there will likely be less DNA than this maximum amount.

The combined volume of DNA fragments added should be no more than 5 µL, to avoid diluting the reactants too much. This volume restriction does not apply if you are using a ready-made *Gibson Assembly*® master mix from *New England Biolabs*, however, where up to 10 µL of DNA may be added.

To calculate the amount of DNA present (in picomoles):

$$\text{DNA (pmol)} = \frac{\text{Mass of DNA in ng} \times 1\,000}{\text{Length of DNA fragment in base pairs} \times 650}$$

Note: 650 is the average mass, in daltons, of a single nucleotide.

2. The *Gibson Assembly*® method

1. Add 5 µL of DNA to be assembled (the PCR products mixed in the correct proportions) to 15 µL of *Gibson Assembly*® master mixture. [Note that you can use up to 10 µL of DNA if using off-the-shelf NEB *Gibson Assembly*® master mix.]
2. The DNA should generally be in equimolar amounts. Use 10–100 ng of each ~6 kb DNA fragment. For larger DNA segments, increasingly proportionate amounts of DNA should be added (*e.g.*, 250 ng of each 150 kb DNA segment).
3. Incubate at 50 °C for 15 to 60 minutes (60 minutes is optimal).
4. The finished product of the Gibson method can be used directly for transforming cells. If you are using electrocompetent *E. coli*, you can transform the cells using 1 µL of the assembly product for each 30 µL of bacterial cell suspension.

   Other methods, such as the rapid colony method using TSS buffer described in Kit 1 and the rapid colony method using calcium chloride solution, described in Kit 2, will require more DNA.

   Typically, a *Gibson Assembly*® reaction *as described here* will produce 180–370 ng of DNA in total, so you will need to use *all* of this DNA to transform each scraping of bacterial cells.

   Note that if you wish to calculate the efficiency of your transformation (see page 15 of KIt 2), you will need to run a replicate *Gibson Assembly*® and estimate the amount of DNA in the Gibson product by gel electrophoresis.

After the transformation

Quantifying the change in expression levels

Identification of PCR template carryover contamination

Following the successful assembly and transformation, the cells must be plated on agar plates to enable bacteria carrying the newly-assembled plasmids to be distinguished from bacteria carrying the original plasmid used to generate vector backbone (carryover contaminant).

For example, if you started with *p006kanGFP* and exchanged its lactose–inducible promoter for a constitutive promoter, you should not grow the transformed cells on plates with lactose or IPTG.

Cells that carry the original plasmid from carryover contamination with PCR template would grow and express the *avGFP* on such plate, just as your newly created cells with *avGFP* under constitutive promoter. To distinguish the two categories of cells, plate the transformed bacteria on a plate without lactose or IPTG.

In some situations, for example in cells expressing *ATF1* (the banana odour generator), distinguishing cells carrying new constructs from PCR template carryover contamination will not be straightforward, especially on solid agar plates. However, in such cases, you can inoculate broth with single colonies picked from the agar plate, and grow them in broth supplemented with the appropriate chemical.

For example, if you started with a plasmid with *ATF1* under control of the *OsmY* promoter and exchanged it to an L-arabinose–inducible promoter, you should grow your colonies in broth supplemented with L-arabinose and the *ATF1* substrate, isoamyl alcohol (see page 15). Cultures with the L-arabinose promoter will begin to smell like bananas much earlier than ones under control of the *OsmY* promoter (after few hours as opposed to after 24–36 hours).

Alternatively, you may simply decide to sequence the insert from the plasmid isolated from the colonies after *Gibson Assembly*® and transformation, to confirm correct assembly, then to identify colonies carrying the desired construct.

Comparing gene activity in generated constructs

Quantification of the activity of different promoters naturally depends on the nature of the gene the promoter regulates. In every case in this kit, however, bacteria carrying the correctly-assembled plasmid must be grown in liquid medium (broth).

While visual, qualitative comparisons of for instance, the amount of GFP fluorescence are possible with bacteria growing on agar plates, quantification of gene expression levels require bacteria grown in liquid medium.

All comparisons between different promoters and controls must be done in cultures of identical optical density.

For plasmids expressing *avGFP* or *ccRFP*, promoter activity is best measured using a fluorimeter, a plate reader or flow cytometry. The manufacturer's instructions for such measurements must be followed in each case, as they will depend on the machine used.

ATF1 and β-galactosidase promoter activity can be measured using the assays described on the following pages.

Measuring promoter inducibility

Another useful excercise to better characterise the inducible promoters in different plasmids is to analyse the relationship between the amount of inducer of the promter and the level of response (fluorescence, enzymatic reaction, odour, *etc.*). This is most straightforwardly done for GFP, RFP and β-galactosidase genes under inducible promoters by preparing a series of broth cultures of the same optical density and growing them after the addition of an inducer in a range of concentrations.

For the T5 lactose-inducible promoter, we recommend trying IPTG concentrations in range of 1 µM–10 mM.

For the L-arabinose-inducible promoter, we recommend trying arabinose concetrations in range of 0.1 µM–1 mM.

Assaying the banana odour

A banana-like odour is produced when the enzyme alcohol acetyl transferase (encoded by *ATF1*, from *Saccharomyces cerevisiae*), made by genetically-modified *E. coli*, acts on isoamyl alcohol converting it to isoamyl acetate.

The transformed bacteria must be grown in LB broth to which kanamycin (25 mg/mL) and isoamyl alcohol (1 µL/mL) has been added. The banana odour is produced in the stationary phase of growth, as the *OsmY* promoter in the insert is activated only at this time.

A comprehensive description of the protocols and challenges in quantifying the smell of this generator has been published by Dixon and Kuldell (2011). Unlike these authors, however, in our activity we insert the banana smell generator into a conventional *E. coli* strain, rather than one that has been altered to eliminate the characteristic smell of the species. In our experience this procedure still works well.

The most accurate way to measure banana smell is to use gas chromatography to separate and quantify different compounds present in the sample.

A simpler alternative would be to prepare a range of standard solutions of known isoamyl acetate concentration or of commercial banana flavouring and to compare these to the smell of growing bacterial cultures (autoclaving the cultures destroys the smell).

It is important that the cultures remain plugged with non-absorbent cotton wool so that they can be smelt safely. Students should be discouraged from smelling the cultures too often since if they are smelt too often, it can give some people a headache.

Commercial food essences are normally dissolved in oil, which is not miscible with water, so we have developed a method of emulsifying the flavouring using gum arabic and a small high-speed motor. This emulsion can then be dispersed in water to produce a range of standards that can be kept for many months (for details, see the project website).

Dixon and Kudell suggest the following arbitrary dilutions of commercial banana flavouring as standards:

Standard	Concentration (%)	Volume in 25 mL water
0	0	0
1	0.1	25 µL
2	0.25	62.5 µL
3	0.5	125 µL
4	1	250 µL
5	2.5	625 µL
6	5	1.25 µL

Reference

James Dixon and Natalie Kuldell (2011) BioBuilding: Using Banana-Scented Bacteria to Teach Synthetic Biology. In Christopher Voigt, editor: *Methods in Enzymology* **497**: 255–271. Burlington: Academic Press. ISBN: 978 0 12 385075 1.

This publication is available here: **http://openwetware.org/images/b/bc/MiE_00012.pdf**

Assaying β-galactosidase activity

The *lac* operon is the classic example of gene regulation, in which the production of β-galactosidase (lactase) is induced by the presence of lactose in the growth medium. In this assay, ONPG, rather than lactose, is used as a substrate for the enzyme. After partial disruption of the cell membrane with methylbenzene, colourless ONPG is added. It moves into the cells where it is broken down to form the yellow-coloured product, ONP.

Preparation

You will need cultures of *E.coli* that possess the *LacZ* (β-galactosidase) gene. The must be grown in LB broth for 24–48 hours in advance. To induce the production of β-galactosidase, lactose must be added to the growth medium (0.1 g per mL of broth). The ONPG solution should be prepared a day before at the earliest.

Materials and equipment

Needed by each person or group

- cultures of *E. coli* in LB broth
- ONPG (ortho-nitrophenyl-β-D-galactoside) dissolved in Z-buffer, 2 mL per test sample
- methylbenzene (toluene), 1 drop per test sample
- test tubes, caps, rack and marker pen
- inoculation loop
- a micropipette (*e.g.*, 40–200 µL)
- sterile pipette tips (for the micropipette)
- 5 mL syringe, for transferring ONPG solution
- waste container with disinfectant
- stopclock or timer
- safety spectacles

The following items are also needed per class

- water bath maintained at 37 °C
- access to a fume cupboard
- hair drier
- spectrophotometer or colorimeter to measure optical density at 420 nm (a 440 nm filter or similar can be used if a 420 nm filter is not available)
- cuvettes for spectrophotometer or colorimeter

Method

This method is used to determine how much β-galactosidase is produced by a culture.

1. Transfer 100 µL of each microbial suspension to be tested into a separate test tube.
2. Transfer 100 µL of LB broth to a 'control' tube.
3. Label the tubes appropriately.
4. Add a drop of methylbenzene to each tube, cap the tubes and shake well to mix. *Methylbenzene kills the cells and partially disrupts the cell membrane, allowing the ONPG to diffuse into the cells.*
5. Perform the next operation in a fume cupboard. Use a hair drier to evaporate the methylbenzene. *Methylbenzene is lighter than water and will appear as a 'greasy' film on the surface. You must wait until all of this solvent has evaporated before proceeding to the next step.*
6. Transfer the samples to a water bath maintained at 35–37 °C. Add 2 mL of ONPG in Z-buffer to each sample. Record the time.
7. Measure the optical density (OD) of the samples at 420 nm (or 440 nm) at 10 minute intervals until there is no further colour change.
8. Plot a graph of the results (OD_{420} *vs*. Time).
9. The enzyme units in a given sample can be calculated as follows:

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}} = \text{Units of } \beta\text{-galactosidase}$$

The optical density (OD) at 420 and 550 nm is read from the sample.
The 1.75 × OD_{550} is an optional correction factor which allows for light-scattering by *E. coli* cell debris, *etc.*

OD_{660} reflects the cell density at the start of the reaction
t = time of the reaction in minutes
v = the volume of the culture in mL

Note: A fully-induced culture has an activity of about 1 000 units, whereas an uninduced culture has an activity of 1 unit.

Recipes

Z-buffer

Used for making up ONPG reagent. Makes 100 mL
Do not autoclave. *Store in a refrigerator, for up to several months.*

- $Na_2HPO_4.7H_2O$, 1.6 g
- $NaH_2PO_4.H_2O$, 0.55 g
- KCl, 0.075 g
- $MgSO_4.7H_2O$, 0.025 g
- β-mercaptoethanol, 0.27 mL

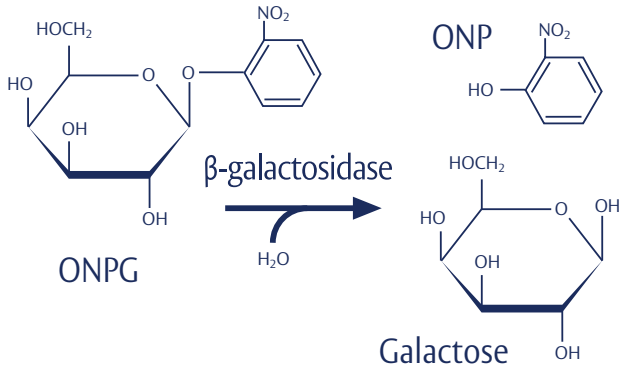
1. Add the ingredients listed above to 80 mL of deionised or distilled water.
2. Stir to dissolve.
3. Adjust the pH to 7.0 with 1 or 2M NaOH.

ONPG reagent

Used for detecting and measuring β-galactosidase activity
Makes 100 mL . Store in a refrigerator, for no more than 24 hours.

- Z-Buffer, 100 mL
- Ortho-nitrophenyl-β-D-galactoside (ONPG), 0.55 g

1. Dissolve the ONPG in the buffer.
2. Store in a bottle, wrapped in foil.



Breakdown by β-galactosidase of (colourless) ONPG to (yellow) ONP and galactose.

Reference

A short course in bacterial genetics: Laboratory manual and handbook by Jeffrey Miller (1992) Cold Spring Harbor Laboratory Press, New York. ISBN: 0 87969 349 5.

Further information

Molecular methods

Mullis, K.B. (1990) The unusual origin of the polymerase chain reaction. *Scientific American* **262**: 36–43.

Making PCR. A story of biotechnology by Paul Rabinow (1996) The University of Chicago Press. ISBN: 0 226 70147 6.

Gibson, D.G. *et al* (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods* **6**: 243–345.

Gibson, D.G. (2011) Enzymatic assembly of overlapping DNA fragments. *Methods in enzymology* **498**: 349–361.

Perkel, J.M. (1914) Seamlessly re-writing the lab cloning manual. *Biotechniques* **56**: 12–14.

Web sites

University of Cambridge: 'Guide to Gibson assembly' **www.synbio.org.uk/dna-assembly/guidetogibsonassembly.html**

University of Cambridge iGEM Team: The Gibson Assembly Song: **www.youtube.com/watch?v=WCWjJFU1be8**

New England Biolabs: Introduction to Gibson assembly (animation): **www.youtube.com/watch?v=tlVbf5fXhp4&list=PLQl3WvQSRR-tsJEzvaVpsfe1tixg9Bzk3&feature=share&index=5**

GFP-like proteins: Molecule of the Month, June 2014 **www.rcsb.org/pdb/101/motm.do?momID=174**

Molecular structure data

The molecular images in this booklet were created using data from the *Protein Data Bank*: **www.rcsb.org/pdb**

The data for β-galactosidase molecule on the cover of this document was published in: Dugdale, M.L. *et al* (2010) Importance of Arg-599 of β-galactosidase (*Escherichia coli*) as an anchor for the open conformations of Phe-601 and the active-site loop. *Biochemistry and Cell Biology* **88**: 969-979 [PDB ID: 3MUY].

The software used to produce this image was *UCSF Chimera*, which can be obtained free-of-charge from: **www.cgl.ucsf.edu/chimera/**

A comprehensive review of b-galactosidase structure and function is available in: Juers, D. H., Matthews, B. W. and Huber, R. E. (2012) *LacZ* β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. *Protein Science* **21**: 1792–1807.

Genetic modification method and microbiology safety

A guide to the genetically modified organisms (contained use) regulations 2000. Health and Safety Executive (2000) The Stationery Office, London. ISBN: 978 0717617586. *This official document, which is aimed principally at academic researchers, can be downloaded from the HSE's web site:* **www.hse.gov.uk/biosafety/gmo/**

C.T. Chung, Niemela, S. and Miller, R.H. (1989) One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA.* **86**, 2172–2175. *This is the method for transforming cells used in this kit.*

Plasmids and DNA sequence data

The sequences of the plasmids and other inserts needed for these practical tasks are available from *Figshare*: **www.figshare.com** [Search for 'Practical synthetic biology']

The plasmids and inserts will be available from the National Centre for Biotechnology Education, University of Reading: **www.ncbe.reading.ac.uk**

For those unable to obtain the plasmids from the NCBE, they have also been deposited in *AddGene*: **https://www.addgene.org** [Search for 'Bryk']

Updated versions of the written and other educational resources may be found on the UNIGEMS project website: **practicalsyntheticbiology.net/resources**

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