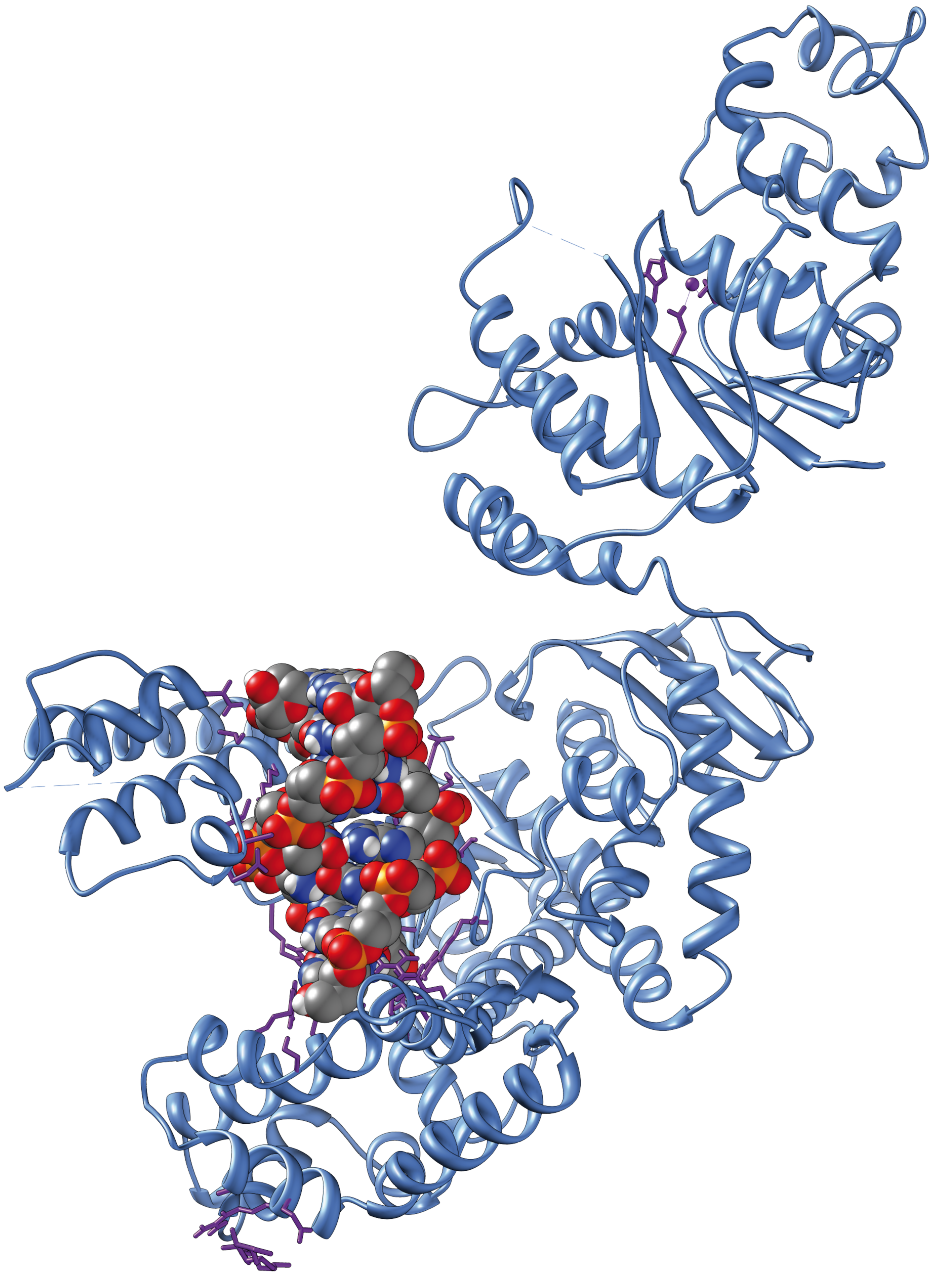
Synthetic Biology Teaching Resources

University of Reading



The PCR, Gibson and DNA assembly

1.0 BETA Instructions and background notes

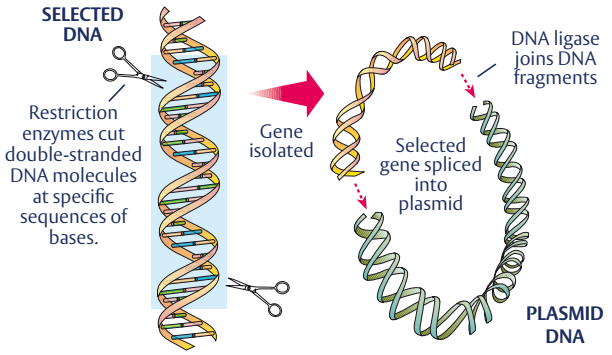
Beyond cut-and-paste

The classical method of constructing novel (recombinant) DNA molecules was developed in the late 1970s. This involves cutting DNA with restriction enzymes (endonucleases) that recognise specific sequences of bases, then pasting the fragments together using another enzyme — DNA ligase.

Although this sounds simple in theory, in practice it can be difficult, error-prone and time-consuming, especially if several different fragments of DNA are to be combined.

This is exactly what synthetic biologists usually want to do — that is, build complex genetic circuits from many components: genes, promoters, terminators and other genetic control elements. Even if several components are successfully joined, the DNA circuit may not work as predicted because it may contain residual sequences or 'scars' that were needed during the assembly process but which interfere with the control and expression of the genes. Even a few nucleotides in the wrong place can have a major effect: therefore the construction often has to be perfect, down to the very last nucleotide.

Many different strategies have been adopted and commercialised over the last few decades in an attempt to overcome these difficulties. One of the simplest and easiest to perform was developed comparatively recently by Daniel Gibson and his colleagues in the Synthetic Biology Group at the J. Craig Venter Institute in the USA. *Gibson Assembly®*, coupled with the ability to design appropriate DNA sequences *in silico* and have them synthesised to order has become the latest method of choice of many synthetic biologists.



Recombinant DNA molecules, like the plasmid shown here, are made by cutting and pasting selected sequences of DNA with restriction enzymes and DNA ligase, respectively. Such recombinant molecules can then be introduced into microbes, plants or animals by a variety of methods.

What you will learn

Educational aims

In the last activity you learnt how to transform bacteria using a ready-made plasmid that carried a gene encoding green fluorescent protein (GFP).

In the following practical procedure you will swap the gene encoding GFP for one encoding red fluorescent protein (RFP). The novel plasmid will then be used to transform bacterial cells. This procedure introduces two important techniques that are used in synthetic biology:

- the polymerase chain reaction (PCR), which is used to prepare numerous copies of the RFP gene and the plasmid 'backbone' for assembly;
- the *Gibson Assembly®* method, which is used to produce the novel plasmid from the two PCR products in a single step.

We also suggest a selection of interesting ready-made genes that you can instert in the place of the GFP if you wish.

Summary of the practical task

Initially, you will have two plasmids: one with a gene encoding GFP and one with a gene encoding RFP. From these you will produce, using the PCR, copies of the RFP gene and a plasmid 'backbone' that carries an antibiotic resistance gene and an origin of replication, but no fluorescent protein gene.

After the components have been amplified, 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®*.

Next, you will use the Gibson method to assemble these two parts into a novel plasmid. Finally, you will use the plasmid you have made to transform bacteria so that they express the RFP. [You can use either the method of transformation used in Kit 1, or a more 'conventional' method that uses calcium chloride and a heat shock to allow the plasmids to enter the cells.]

Making a haystack from a needle

Duplicating DNA — the polymerase chain reaction

A genetic 'photocopier'

The polymerase chain reaction (PCR) has been likened to a genetic 'photocopier'. From a small amount of biological material millions of copies of a chosen section of its DNA can be made quickly, accurately and automatically.

Although it was first devised for carrying out medical diagnostic tests, today the PCR has a wide range of uses ranging from forensic genetic fingerprinting and archæological investigations to tracing the mating and feeding habits of animal species.

A method called 'sexual PCR' or 'DNA shuffling' enables protein engineers to develop better enzymes for industrial use by mimicking the processes of evolution.

Without a clever variant of the PCR called 'cycle sequencing' the Human Genome Project would have been almost impossible — as would much of the molecular biology that is carried out in laboratories around the world. It is hardly surprising therefore that the PCR won for its inventor, Kary Mullis, a Nobel Prize for Chemistry in 1993. What is more surprising is the PCR's unusual origin.

A drive through the woods

The road to the PCR began on a moonlit evening in April 1983. In Kary Mullis's account of his invention, he describes how its two key features occurred to him while driving to his weekend retreat in California. Twice Mullis had to pull over and stop the car: once to calculate the number of copies of DNA that the process might generate; and a second time to sketch a diagram, showing how it might duplicate a specific stretch of DNA.

The idea was so simple that at first people refused to believe that it would work. No one could believe that it hadn't been tried before and found to be impractical for some reason. After Mullis's initial work, his colleagues at the Cetus Corporation, one of the first modern biotechnology companies, carried out extensive research to refine the method and eventually the process was patented.

Mullis was paid a bonus of US$ 10,000 for his invention. The PCR has become so important however, that Cetus was later able to sell the rights associated with the process to Hoffman-La Roche for US$ 300 million.

The breakthrough that made the PCR into a convenient laboratory technique was the isolation of DNA polymerase from Thermus aquaticus, a bacterium found in hot water springs. This enzyme (known as Taq polymerase) is stable at high temperatures and can therefore withstand the rigours of the reaction. In the computer-generated structure shown here, β-pleated sheets are coloured yellow, while the α-helices are magenta. A small fragment of the DNA that is being made is shown as a space-filling model.

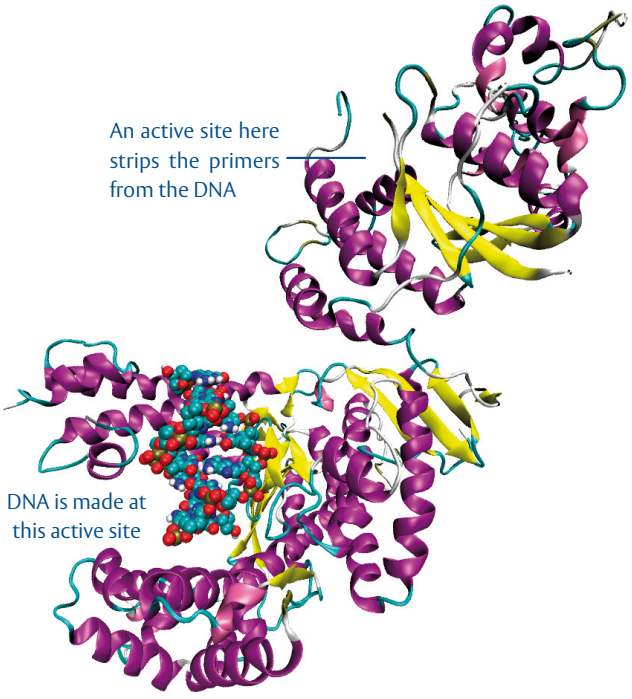
How the PCR works

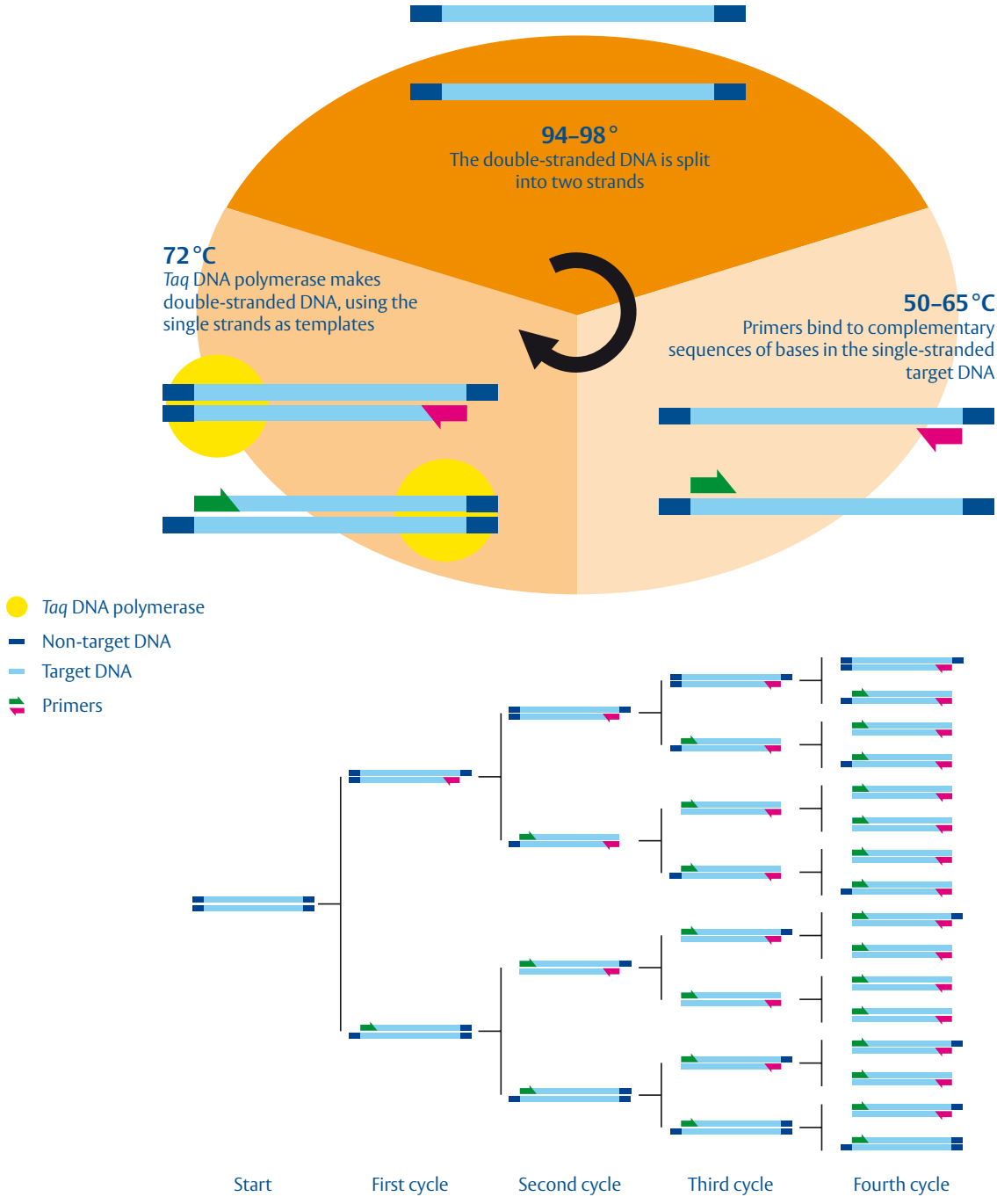
The modern method for PCR (a slightly modified version of Mullis's original idea) operates as follows:

The components

- Double-stranded DNA (the 'template') is extracted from the biological sample under study.
- Two single-stranded 'primers' are made that flank the specific stretch of DNA to be copied. These are generally 15–30 bases long, and are complementary to (in other words, they pair up with) the 5' end of each target DNA strand.
- The template DNA is mixed with the primers.
- Equal proportions of the four different deoxyribonucleoside triphosphates (dNTPs) are added. dNTPs are the units from which DNA is made.
- Heat-stable *Taq* DNA polymerase (obtained from the hot-water bacterium *Thermus aquaticus*) is added to the reaction mix, together with Mg^{2+} ions that are required as a co-factor by the enzyme. This enzyme strings together dNTPs to make new DNA.

All of the components needed for the PCR (apart from the primers and the template DNA) can be dried into a small pellet in the correct proportions. Then, all that needs to be done is to add the primers and template DNA to a tube containing the pellet. This removes the need to dispense very small volumes of numerous reagents and improves the reliability of the process.





The process

Once the chemicals necessary for making DNA have been mixed, the PCR has three steps which are repeated 20–40 times:

Denaturation

The double-stranded DNA is split into two single-stranded templates by heating it to 94–98 °C.

Annealing

The mixture is cooled to 50–65 °C. The primers bind to the complementary strands of DNA by base-pairing.

Extension

Heating to 72 °C (the optimum temperature for *Taq* polymerase) encourages the synthesis of new DNA strands alongside the templates. This step doubles the number of DNA molecules present.

The process of heating and cooling is now repeated. With each cycle (lasting about 2 minutes) the number of copies of the DNA is doubled. After 30 cycles, 1,073,741,764 copies of the target DNA will have been made.

The first attempts at PCR were made using water baths held at three different temperatures. Soon the process was automated — the first PCR machine, made in 1985 with washing machine parts, was known affectionately as 'Mr Cycle'. This machine was only semi-automatic however — before the discovery of temperature-stable *Taq* DNA polymerase, fresh enzyme had to be added with each cycle.

The PCR is a very sensitive method and in theory it can amplify a single DNA molecule. Scrupulous precautions are therefore often necessary to ensure that stray DNA molecules stay out of the reaction mixture.

The Gibson method

Seamless contruction of large DNA molecules

The ability to assemble long sequences of DNA in a predetermined order is an essential technique in synthetic biology. The classical method of 'cutting and pasting' DNA using restriction enzymes and DNA ligase is usually a complex multi-step process. For example, care has to be taken to ensure that the DNA is cut only where required, which means that restriction sites often have to be deleted from sequences.

In 2009, Daniel Gibson and his colleagues described an alternative, novel, method that allows the assembly of DNA molecules using three different enzymes in a single tube at one temperature, using ready-prepared enzymes and reagents. This method can be used to construct multiple DNA molecules, even entire plasmids, up to many hundreds of kilobases in length.

How the Gibson assembly works

The basic method of the Gibson assembly is described below It is a relatively complex procedure to understand, and in fact it took Daniel Gibson and his colleagues several steps over a few years to perfect the method. (Unlike Karry Mullis they don't claim to have developed it in one inspired jump!)

The components

- The double-stranded DNA sequences to be assembled. For example, this might be a plasmid 'backbone' with one or more sequences — such as genes encoding fluorescent proteins or control regions — that are to be introduced into the plasmid. These 'parts' have to be carefully designed in advance with overlapping DNA sequences so that they can eventually be joined together. (Computer software is used to do this — then the DNA is synthesised, using the computer-designed sequences.)
- Equal proportions of the four different types of deoxyribonucleoside triphosphates (dNTPs) are needed. dNTPs are the units from which DNA is made. A different dNTP is needed for each of the four different bases (A, C, G and T) that DNA is made from (hence dATP, dCTP, dGTP, dTTP are supplied in the reaction mix).
- A high-fidelity DNA polymerase. This enzyme assembles new DNA molecules from the dNTPs. Although a cheaper DNA polymerase such as that used for the PCR could also be used for this task, a 'high-fidelity' DNA polymerase has the advantage that it proof-reads (checks the accuracy of) the DNA as it assembles it. (It is more than 50 times more accurate than the *Taq* polymerase typically used in the PCR.)
- Magnesium ions, supplied as magnesium chloride. Mg^{2+} ions are needed as a co-factor by the DNA polymerase.
- A heat-labile 5' exonuclease. This enzyme progressively breaks down DNA, starting at the 5' end of any sequence. The exonuclease is used to remove unwanted DNA at the start of the reaction. As it is heat-labile, the enzyme is soon inactivated at 50 °C. Because of this, the DNA which is needed for the later reactions is not destroyed.
- A DNA ligase (to repair any 'nicks' in the newly-assembled DNA molecules).

Both the DNA polymerase and the DNA ligase are capable of withstanding high temperatures. Therefore unlike the exonuclease they are not denatured during the reaction.

The process

All of the ingredients listed above are mixed in a single tube with appropriate buffers and incubated at 50 °C for 15–60 minutes. The sequence of reactions that follows is shown in the diagram overleaf and described below.

The pieces of double-stranded DNA that are to be assembled have already been duplicated by the PCR — there are many millions of each part in the tube. Remember that these parts have been designed with overlapping sequences where they are to be joined (**A** in the diagram).

Initially, the double-stranded sequences are partially broken down by the T5 exonuclease, but in a very specific way. The T5 enzyme removes nucleotides from each double-stranded DNA molecule, starting at the 5' ends of the molecules.

In so doing, the enzyme removes one strand of each overlapping sequence, leaving complementary 'sticky' sequences in each DNA molecule. Because the enzyme keeps on working, at least until it is denatured by heat, it removes slightly more DNA than the overlap and also 'chews up' the non-overlapping 5' end of each molecule — but this damage is repaired later. This step is shown in **B**.

Eventually, the T5 ligase is denatured by the heat and stops working. The complementary ends of the DNA molecules now stick together (anneal) by base-pairing (**C**).

Next, using the complementary DNA strand as a template, the DNA polymerase assembles new DNA strands from dNTPs, filling in the gaps to create complete, double-standed DNA molecules (**D**).

DNA ligase then repairs any 'nicks' in the DNA (**E**) — the 'nicks' are places where, although the base pairs are linked by hydrogen bonds, the DNA's sugar-phosphate 'backbone' is not joined. DNA ligase covalently bonds the stretches of DNA to form a complete molecule (shown as **F** in the diagram). Novel plasmids produced in this way can be used directly for transforming bacteria. The DNA can also be used as a template for PCR or other DNA amplification methods.

Although the diagram shows only two fragments being joined, many fragments (>10) can be joined simultaneously in a single reaction mix.

An outline of DNA assembly using the Gibson method

for simplicity only two stretches of double-stranded DNA to be joined are shown here

A. Two double-stranded lengths of DNA with homologous ends (~20–40 bp overlaps).
B. T5 exonuclease removes nucleotides from the 5' ends of the DNA.
C. T5 nuclease is inactivated. The complementary ends of the two lengths of DNA anneal.
D. DNA polymerase (Phusion® polymerase) fills in the gaps.
E. DNA ligase repairs the nicks.
F. The product. For circular assemblies (such as plasmids), no gaps remain at the extreme 5' ends.

Exonuclease

DNA polymerase

DNA ligase

Equipment and materials

For the PCR

Each person or working group will need

Straight from the kit (refer also to Appendix 2, page 21)

For exchanging *avGFP* for *ccRFP*

To generate the *ccRFP* insert
- template plasmid DNA: 4 µL of *p005kanRFP*, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *1Forward*
- Reverse primer: 4 µL of 1 µM *3Reverse*

To generate the plasmid backbone with *Amp*^{R}
- template plasmid DNA: 4 µL of *p007ampGFP*, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *3Forward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

For exchanging *Kan*^{R} for *Amp*^{R}

To generate the *Amp*^{R} insert
- template plasmid DNA: 4 µL of *p007ampGFP*, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *3Forward*
- Reverse primer: 4 µL of 1 µM *4Reverse*

To generate the plasmid backbone with *ccRFP*
- template plasmid DNA: 4 µL of *p005kanRFP*, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *4Forward*
- Reverse primer: 4 µL of 1 µM *3Reverse*

For inserting 'Banana odour' or β-galactosidase

Insert (supplied ready-made)
- 'Banana odour' insert, containing 10 ng/µL of DNA

Or
- β-galactosidase insert, containing 10 ng/µL of DNA

To generate the plasmid backbone with *Kan*^{R}
- template plasmid DNA: 4 µL of *p006kanGFP*, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *3Forward*
- Reverse primer: 4 µL of 1 µM *1Reverse*

For generating part of either the 'Banana odour' or β-galactosidase asssembly for sequencing

- template plasmid DNA: 4 µL of the construct, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *1Forward*
- Reverse primer: 4 µL of 1 µM *3Reverse*

For generating part of either the *Amp*^{R} or *Kan*^{R} asssembly for sequencing

- template plasmid DNA: 4 µL of the construct, containing 250 pg of DNA per µL
- Forward primer: 4 µL of 1 µM *3Forward*
- Reverse primer: 4 µL of 1 µM *4Reverse*

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 [5]

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.

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

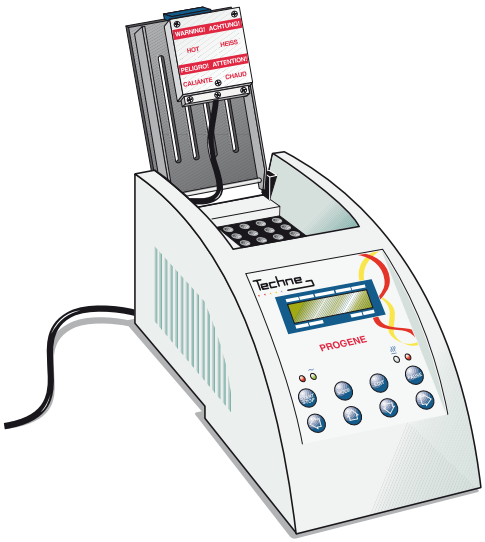
Not in the kit: supplied by you

- clean PCR products in the correct ratios, as prepared by you previously (see pages 6–7)
- heating block or a thermocycler set at 50 °C
- 0.2 mL thin-walled PCR tubes
- micropipette (0–10 µL)
- sterile pipette tips (for the micropipette)
- off-the-shelf *Gibson Assembly*® kit*

  Or

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

* *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)

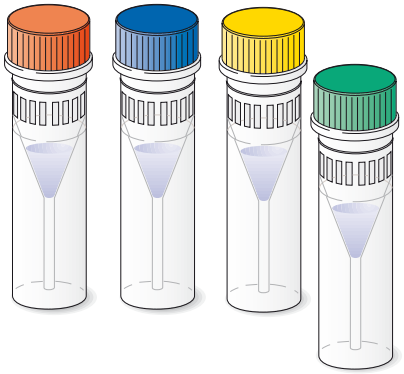
To prepare 6 mL of this buffer

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. Combine all of the ingredients listed above.
2. Add water to make up to 1.2 mL.
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 DNA fragments

PCR, purification and measurement

1. Amplifying the plasmid components

The purpose of this step, using the PCR, is to make copies of the various genetic components which are to be combined later 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 (100 pg/µL)	4 µL

2. Repeat this for the second template and pair of primers.
3. 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.

4. 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 1 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 obtained

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.

Table 1

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** (kb)	**Time** using *NEB Phusion*® DNA polymerase	**Time** using *NEB Vent*$_R$® DNA polymerase	**Insert length** (kb)	**Time** using *NEB Phusion*® DNA polymerase	**Time** using *NEB Vent*$_R$® DNA polymerase
Gibson Assembly® to swap *avGFP* (*p007ampGFP*) for *ccRFP* (*p005kanRFP*)	3 592	60 sec	3 min 40 sec	1 129	20 sec	1 min 15 sec
Gibson Assembly® to swap *Kan*^{R} (*p005kanRFP*) for *Amp*^{R} (*p007ampGFP*)	3 525	60 sec	3 min 40 sec	1 206	20 sec	1 min 15 sec
PCR to generate backbone for introduction of β-galactosidase	3 541	60 sec	3 min 40 sec	*Not applicable, as this 'part' is provided ready-made*	—	—
PCR to generate backbone for introduction of banana odour generator	3 541	60 sec	3 min 40 sec	*Not applicable, as this 'part' is provided ready-made*	—	—

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 be far 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

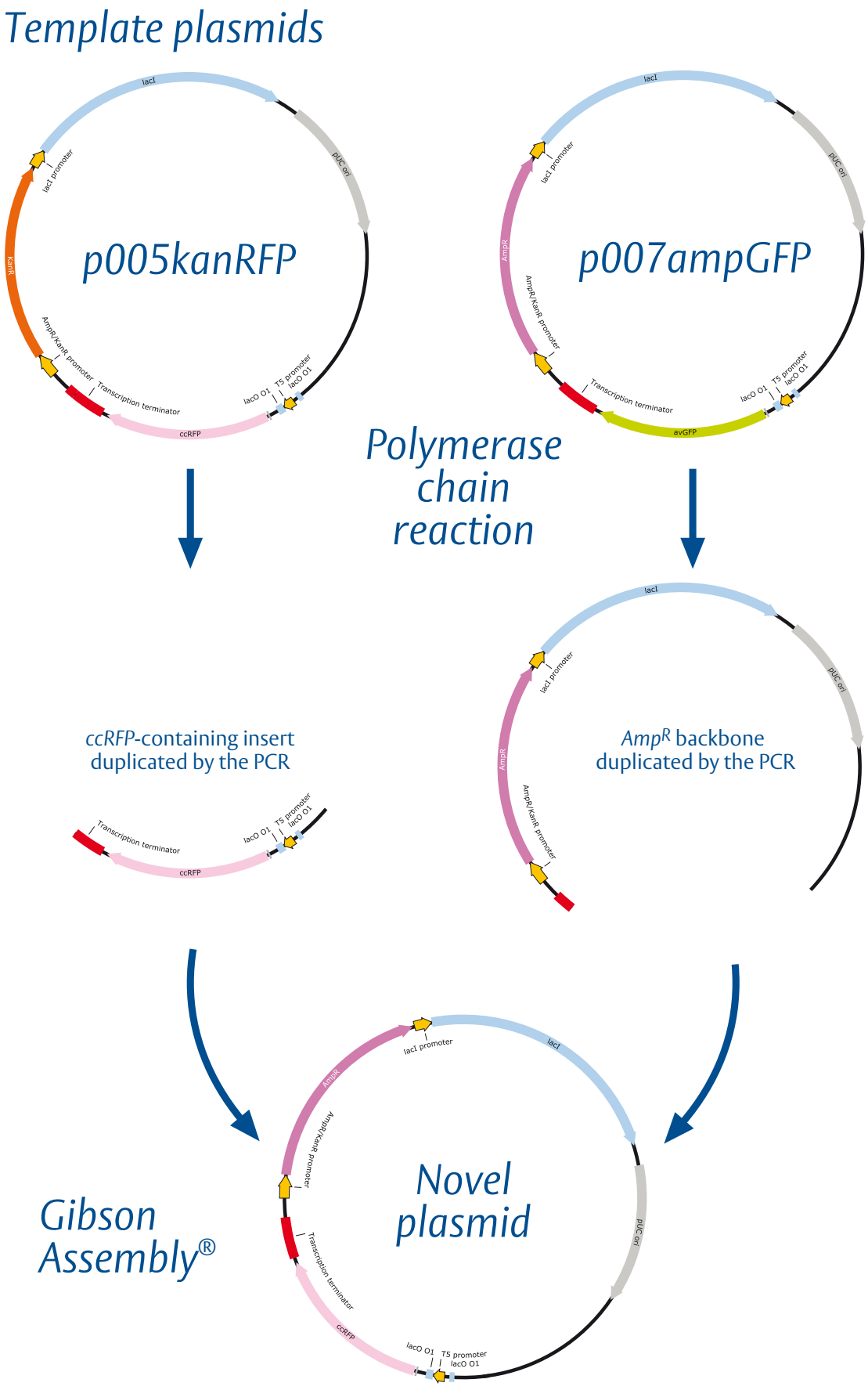
1. Add 5 µL of DNA to be assembled (the PCR products mixed in the correct proportions) to 15 µL of *Gibson Assembly*® master mixture. [Note that you can use up to 10 µL of DNA if using off-the-shelf NEB *Gibson Assembly*® master mix.]
2. The DNA should generally be in equimolar amounts. Use 10–100 ng 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 this kit (pages 14–17), will require more DNA.

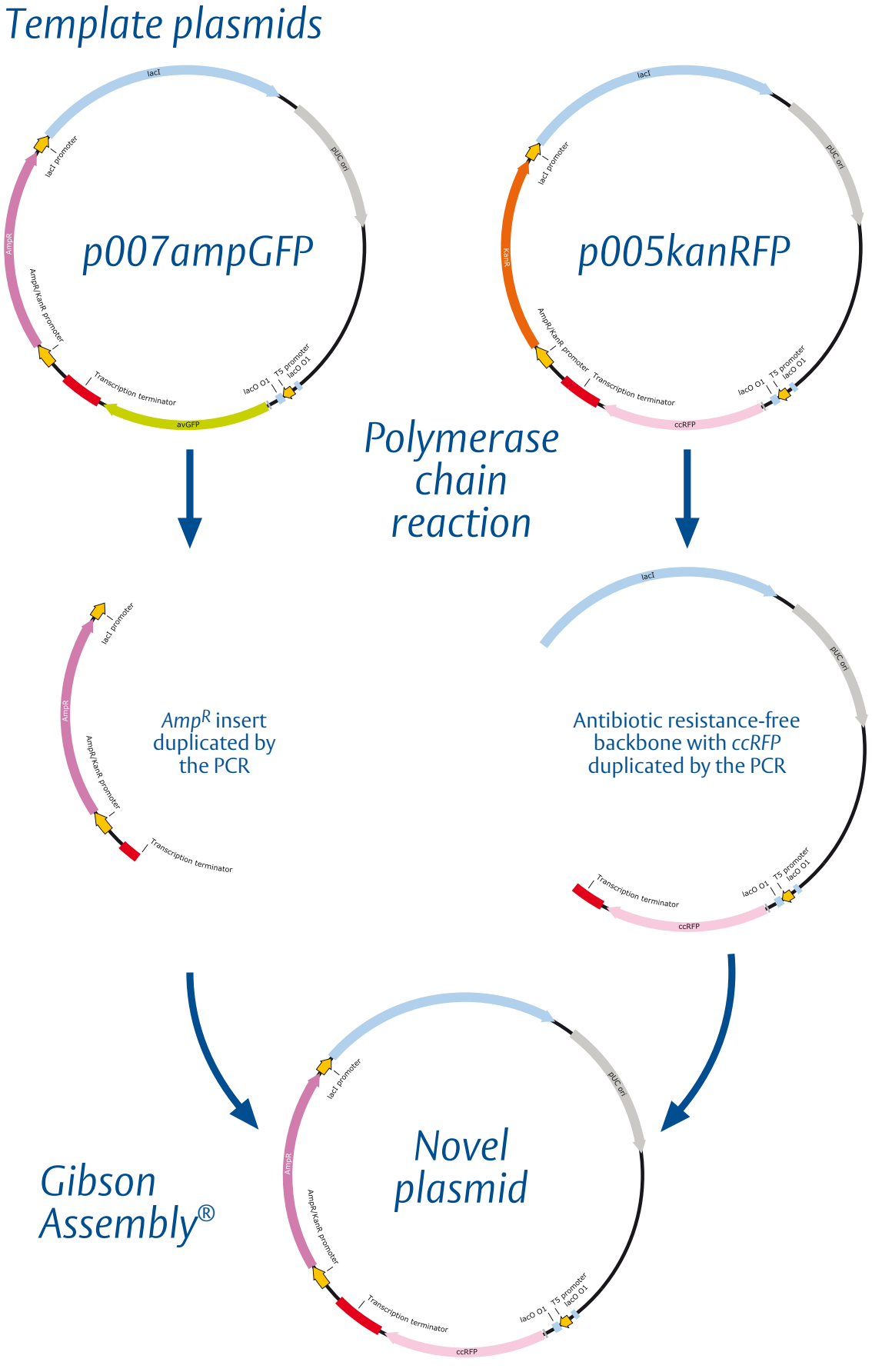
   Typically, a *Gibson Assembly*® reaction *as described here* will produce 180–370 ng of DNA in total, so you will need to use *all* of this DNA to transform each scraping of bacterial cells.

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

Change *avGFP* to *ccRFP*

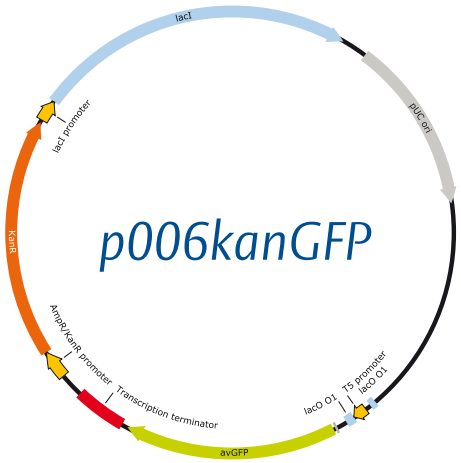


Change *Kan*^{R} to *Amp*^{R}

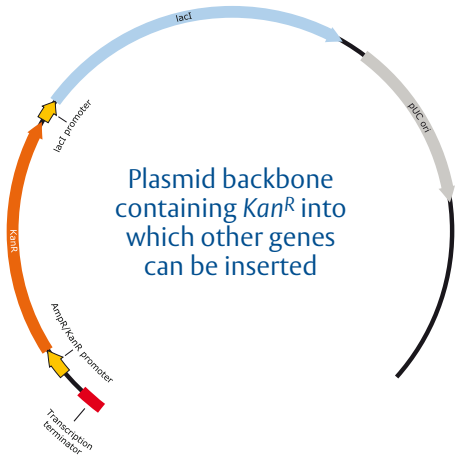


Add β-galactosidase or ‘banana odour’

Template plasmid

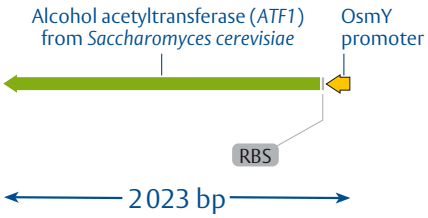


Polymerase chain reaction

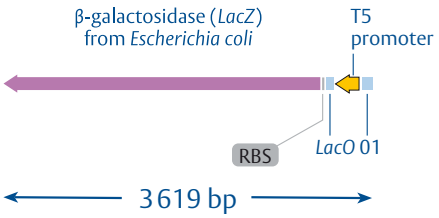


Gibson Assembly®

‘Banana odour’ insert



β-galactosidase insert



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

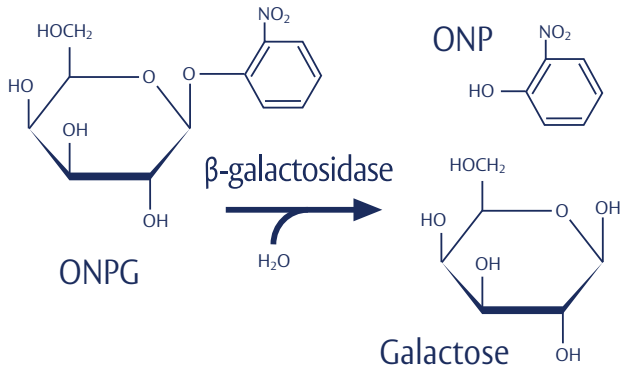
Used for detecting and measuring β-galactosidase activity
Makes 100 mL . Store in a refrigerator, for no more than 24 hours.

- 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.

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.



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

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 (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**

Equipment and materials

For rapid colony transformation using calcium chloride

Each person or working group will need

Straight from the kit

- a copy of the these instructions
- a sterile single use spreader
- a sterile single use 5 µL inoculation loop
- 100 µL of 50 mM sterile calcium chloride solution, dispensed into a 1.5 mL microcentrifuge tube
- 100 µL of sterile LB broth, dispensed into a 1.5 mL microcentrifuge tube

Prepared in advance

(see pages 4 to 6 of Kit 1 for instructions)

- access to a stock culture of *E. coli*, K-12 strain TG2, ***prepared no more than 48 hours in advance*** (to be shared by group)
- plasmid DNA from the *Gibson Assembly*® dispensed into a microcentrifuge tube, on ice.
- a Petri dish containing about 15 mL of LB agar, with 25 mg/mL kanamycin and 50 µg/mL IPTG.

Not in the kit: supplied by you

- a small insulated cup of crushed ice
- a micropipette (*e.g.*, 40–200 µL)
- sterile pipette tips (for the micropipette)
- floating holder for 1.5 mL microcentrifuge tubes
- a permanent marker pen
- access to an incubator set at 37 °C
- a Bunsen burner
- a discard jar of freshly-prepared 1% (w/v) *Virkon*® solution
- paper towels

IMPORTANT

You must wear a lab coat and follow good microbiology laboratory practice while carrying out the practical work (see pages 13–15 of the guide from Kit 1).

For rapid colony transformation using TSS

Each person or working group will need

Straight from the kit

- a copy of the the instructions from Kit 1
- a sterile single use spreader
- a sterile single use 5 µL inoculation loop

Prepared in advance

(see pages 4 to 6 of Kit 1 for instructions)

- access to a stock culture of *E. coli*, K-12 strain TG2, ***prepared no more than 48 hours in advance*** (to be shared by group)
- plasmid DNA from the *Gibson Assembly*® added to 130 µL of transformation buffer (TSS) and dispensed into a 1.5 mL microcentrifuge tube, on ice
- a Petri dish containing about 15 mL of LB agar, with 25 mg/mL kanamycin and 50 µg/mL IPTG.

Not in the kit: supplied by you

- a small insulated cup of crushed ice
- a micropipette (*e.g.*, 40–200 µL)
- sterile pipette tips (for the micropipette)
- floating holder for 1.5 mL microcentrifuge tubes
- a permanent marker pen
- access to an incubator set at 37 °C
- a Bunsen burner
- a discard jar of freshly-prepared 1% (w/v) *Virkon*® solution
- paper towels

IMPORTANT

You must wear a lab coat and follow good microbiology laboratory practice while carrying out the practical work (see pages 13–15 of Kit 1).

Note: For a practical protocol for the TSS method of transformation, please refer to Kit 1, pages 16–18.

Rapid colony transformation

The 'classic' method using calcium chloride and a heat shock

Before you start, put on a lab coat and wash your hands with antibacterial soap.

1. Wipe down the work surface using disinfectant (*Virkon®*) solution and paper towels. Set out the work area with everything you need, including a container of disinfectant solution for contaminated waste and a Bunsen burner with a yellow flame to provide an updraft of warm air, which will carry potential contaiminants away.
2. Place the sterile calcium chloride solution on ice and leave it to chill for approximately 10 minutes.
3. Use a sterile loop to scrape a visible mass of bacteria from the stock plate (but don't dig into the agar). Put the loop of bacteria into the ice-cold calcium chloride solution and agitate it vigorously to dislodge the bacteria and suspend them in the solution. Hold the tube almost horizontally as you do so. Once you have suspended the bacteria, place the contaminated loop in the disinfectant solution.
4. Close the tube, return it to the ice, and leave it for a further 10 minutes. *In this time, the positively-charged calcium ions will neutralise the cell membrane, which will become more rigid as it cools, so that pores in the membrane are exposed.*
5. Using a sterile tip on the micropipette, add *all* of the plasmid DNA preparation from the Gibson reaction to the bacterial suspension. Gently draw the liquid in the tube up and down a few times to ensure that the bacteria and the plasmid are well mixed. Close the tube tightly and dispose of the used tip into the disinfectant.
6. Using a floating microcentrifuge holder, place the tube in a water bath at 42 °C for *exactly* 30 seconds.
7. Return the tube to the ice. *During this time, the plasmid DNA, which will also have been neutralised by the calcium ions, will be taken up by the bacteria.*
8. Using a new sterile tip, add 100 µL of sterile LB broth at room-temperature to the bacterial suspension. Close the tube, dispose of the tip and leave the tube at room temperature for 10 minutes. *This will allow the fragile bacterial cells to recover and start expressing the kanamycin resistance gene on the introduced plasmid.*
9. Put a new sterile tip on a micropipette and use it to drop 150 µL of bacterial suspension onto the centre of a Petri dish of LB/Kanamycin/IPTG agar. Place the contaminated pipette tip and open tube of bacteria in the disinfectant.
10. Use a sterile spreader to coat the surface of the agar with the bacterial suspension. Rotate the plate as you do this, so that the culture is spread evenly over the plate. Once you have done this, place the contaminated spreader in the disinfectant.
11. Allow the liquid to soak into the agar, then invert the plate and write your name, the date and the bacteria used on the edge of the base of the Petri dish. Place the Petri dish, inverted (agar uppermost) in an incubator at 37 °C. Clean the work area with disinfectant and wash your hands with antibacterial soap.
12. Incubate the plates overnight — ideally for 24 hours — then examine the bacteria. There is no need to use an ultraviolet light. Colonies expressing RFP should appear bright pink.

After the plates have been examined, dispose of all contaminated materials and cultures by autoclaving them.

Transformation efficiency

1. Count the number of transformed colonies on your plate.

   Number of colonies = _____
2. Calculate the mass, in micrograms (µg), of plasmid DNA used.

   (You will need to measure the exact amount of plasmid DNA there was in your *Gibson Assembly®* product)

   Mass of plasmid DNA used = _____
3. Note the volume of bacterial cell suspension you spread on your plate.

   Volume of suspension spread on plate = _____
4. Calculate the fraction of the cell suspension you spread on your plate (volume of suspension spread [3] ÷ total volume of suspension (in µL)).

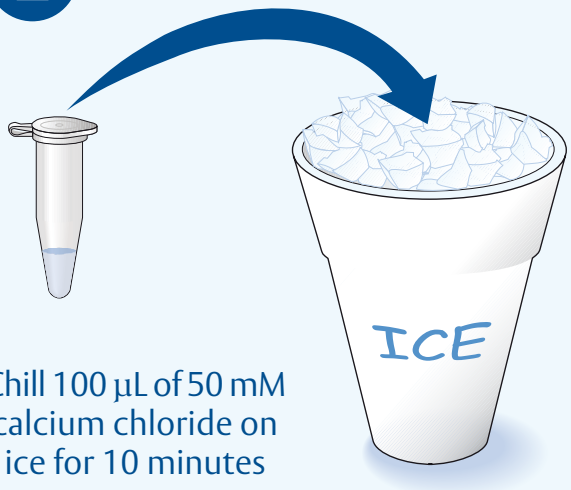
   Fraction of suspension spread on plate = _____
5. Calculate the mass of plasmid DNA (in µg) contained in the cell suspension you spread on the plate (total mass of plasmid [2] × fraction spread [4]).

   Mass of plasmid DNA spread on plate = _____
6. Calculate the transformation efficiency (number of colonies [1] ÷ mass of plasmid spread [5]).

   Transformation efficiency = _____

1

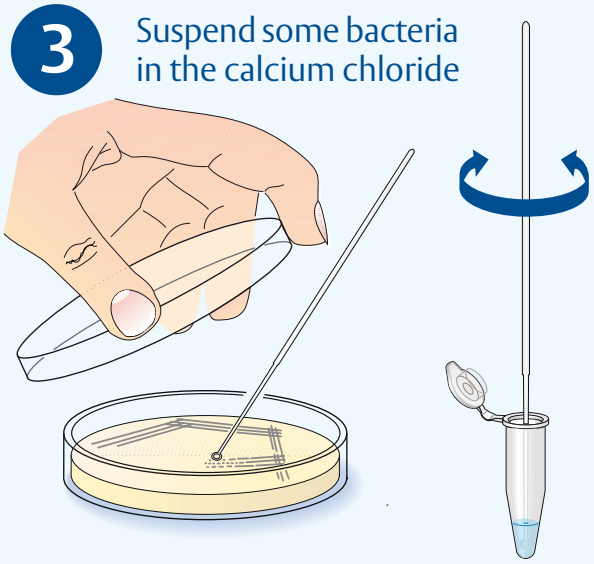
2



Chill 100 µL of 50 mM calcium chloride on ice for 10 minutes

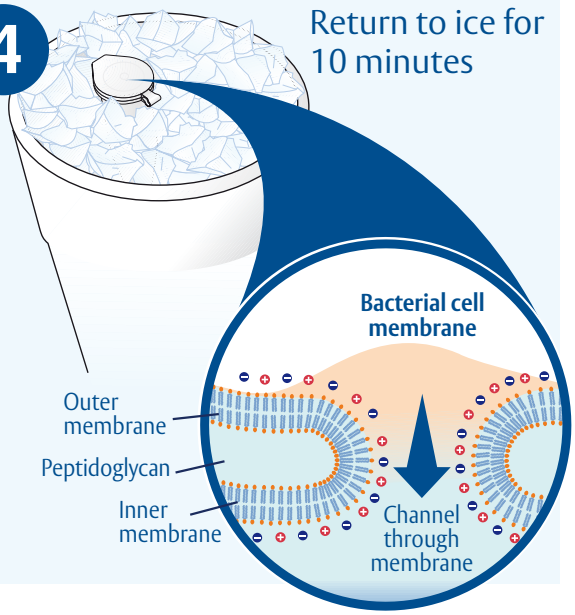
3

Suspend some bacteria in the calcium chloride



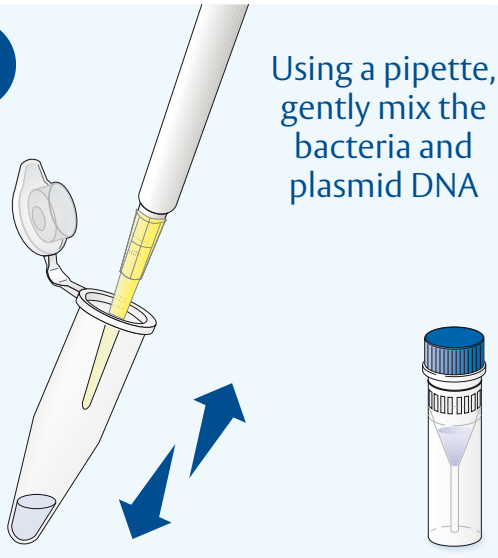
4

Return to ice for 10 minutes



5

Using a pipette, gently mix the bacteria and plasmid DNA



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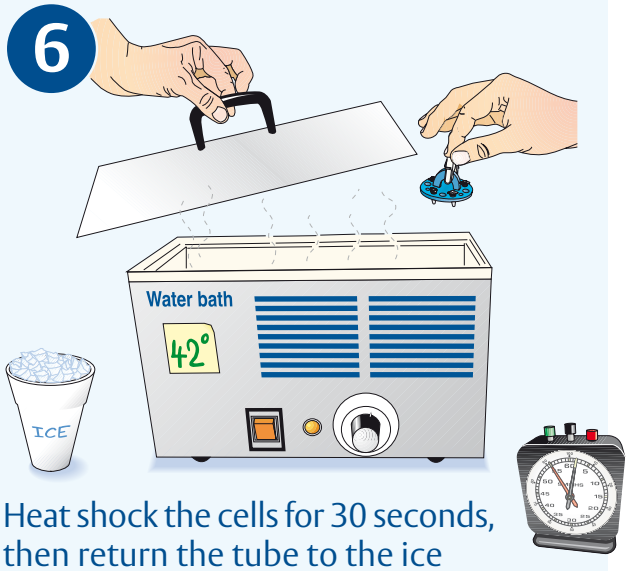
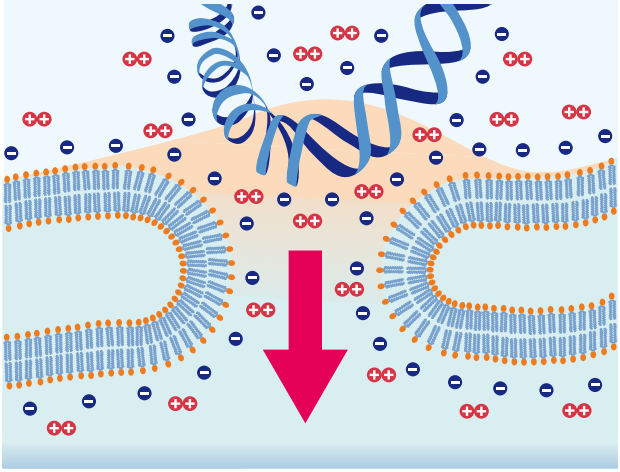
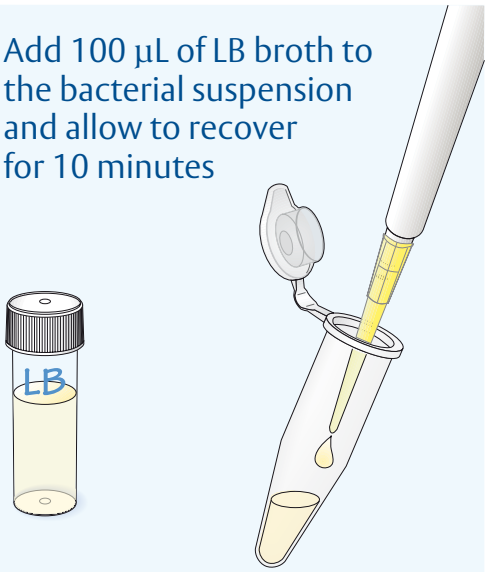


Heat shock the cells for 30 seconds, then return the tube to the ice

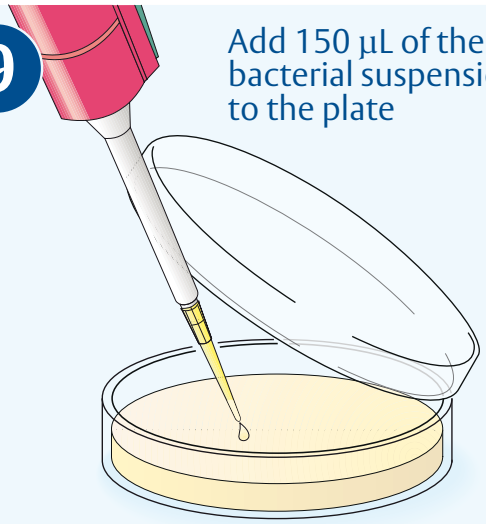
7 Plasmid DNA enters bacteria via pores in the cell membrane



8 Add 100 µL of LB broth to the bacterial suspension and allow to recover for 10 minutes



9 Add 150 µL of the bacterial suspension to the plate



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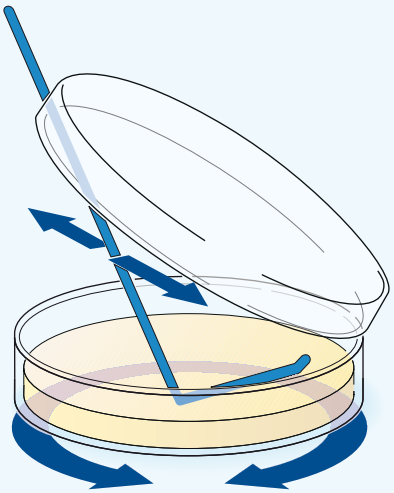
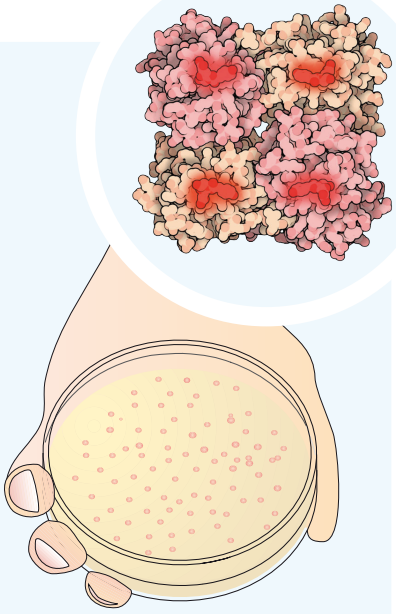


11

12



Red fluorescent protein expressed

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

Cold Spring Harbor DNA Learning Center. PCR animation: **www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html**

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 *Taq* polymerase was published in: Eom, S.H., *et al.* (1996) Structure of *Taq* polymerase with DNA at the polymerase active site. *Nature* **382**, 278–281. [PDB ID: 1TAU].

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

Genetic modification 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/**

The SACGM Compendium of guidance. Available as PDF files from the HSE web site: **www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/**

Detailed guidance from the Scientific Advisory Committee on Genetic Modification (UK). The guidance represents what is considered to be good practice in the UK.

European Union Directive 2009/41/EC on the contained use of genetically modified micro-organisms: **eur-lex.europa.eu/legal-content/en/NOT/?uri=CELEX:32009L0041**

Software

Any standard primer-design software, standalone or online, is suitable for setting up *Gibson Assembly®*, although the addition of the overhangs complementary to the other assembled molecule(s) may have to be done manually.

In contrast, *SnapGene®* (**www.snapgene.com**) is one of several software packages that have recently been updated to include primer design specifically for *Gibson Assembly®*. *SnapGene®* is paid-for software but there is a fully-functional 30-day trial version. *SnapGene®* is the most convenient and visually appealing software currently available, and it has a free 'viewer' application so that those without *SnapGene®* itself may open *SnapGene®* files.

The following alternative software packages are also useful:

NEBuilder for *Gibson Assembly®:* is a free online tool for primer design: **nebuilder.neb.com**

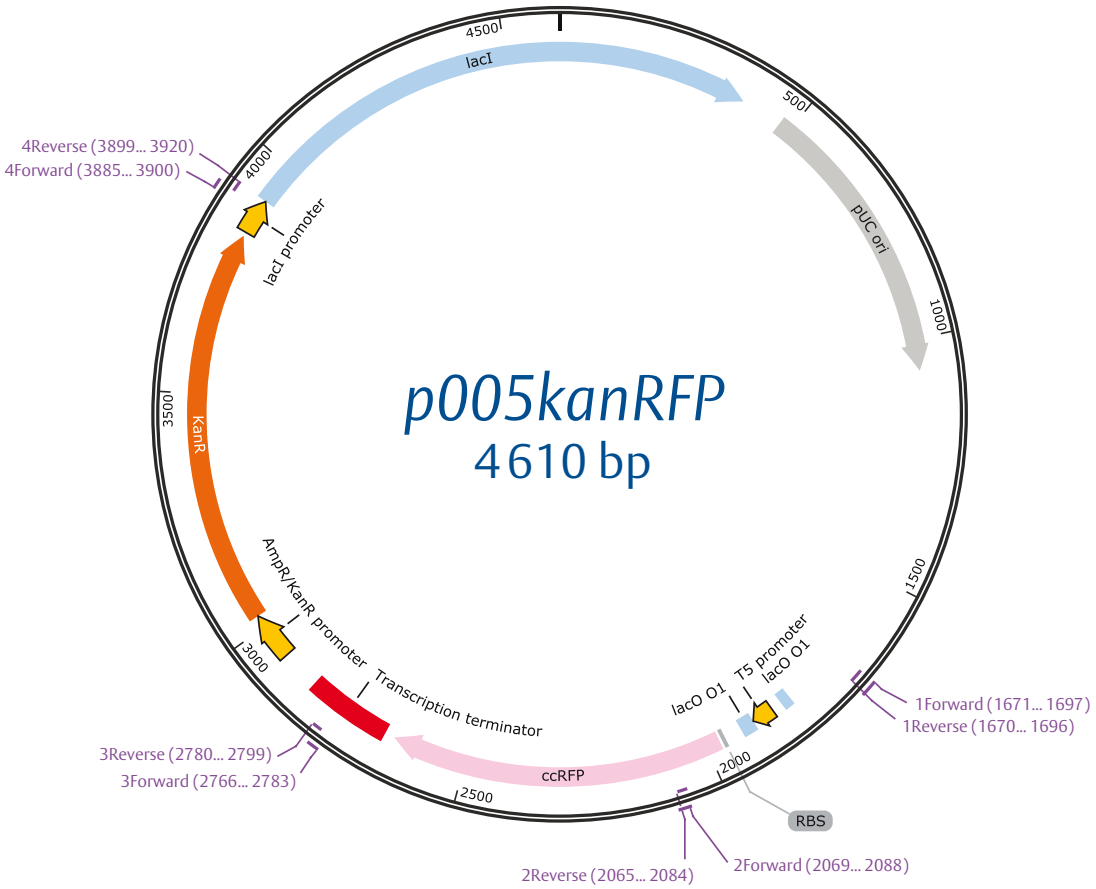
ApE is a donation-based plasmid editor developed by M. Wayne Davis for *Macintosh®* and *Windows®* computers. It has a primer finding option but does not have a dedicated *Gibson Assembly®* feature: **biologylabs.utah.edu/jorgensen/wayned/ape/**

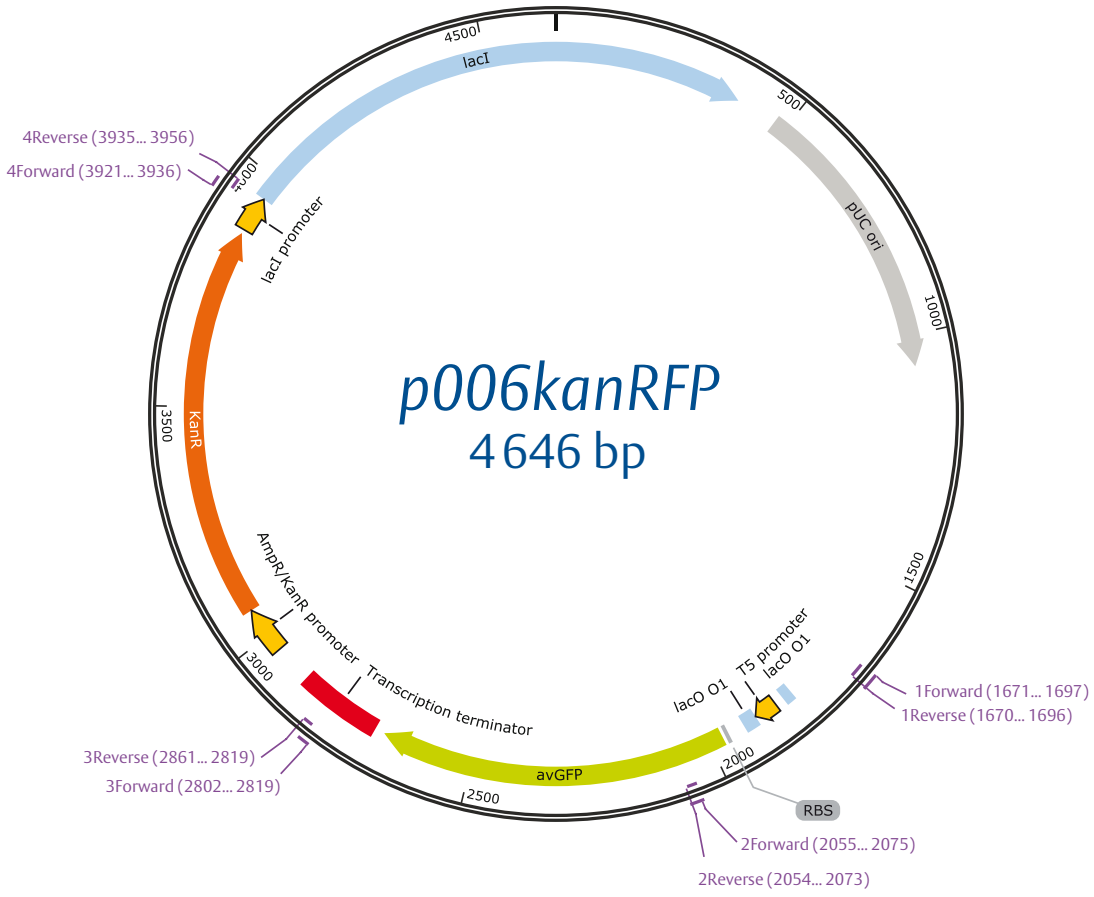
Primer3, a long-standing best-in-class solution for designing PCR primers, can also be used, although it does not have a dedicated *Gibson Assembly®* solution: **primer3.ut.ee**

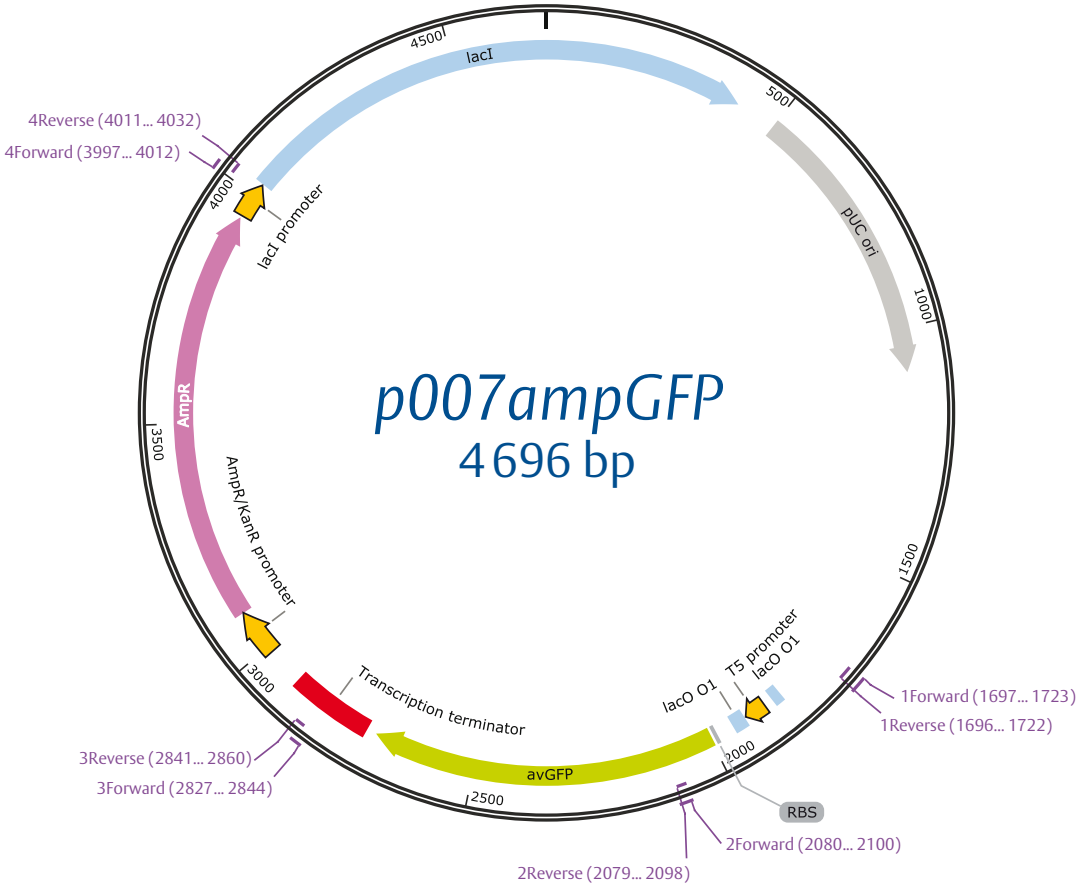
Geneious, a comprehensive molecular biology software suite, enables *Gibson Assembly®* helper in its paid option (although this is disabled in the trial version): **geneious.com**

A large and relatively expensive software package for molecular biology from *Life Technologies*, *Vector NTI*, also has a *Gibson Assembly®*–dedicated primer design facilities: **www.lifetechnologies.com/uk/en/home/life-science/cloning/vector-nti-software.html**

Appendix 1: Plasmid maps







Origin of replication

This is necessary for the plasmid to be reproduced within the bacterium. This particular one ensures that between 50 and 700 copies of the plasmid are made in each cell.

Prometers (various)

Before a stretch of mRNA (and subsequently protein) is produced, RNA polymerase has to attach to the DNA in front (upstream) of the gene. Promoters are DNA sequences that provide a binding site for RNA polymerase (in fact, part of the RNA polymerase molecule binds at *two* locations, 10 and 35 bases before the start of the gene).

Ribosome binding site (RBS)

This site is necessary to initiate efficient and accurate production of proteins. The mRNA it generates consists of a site that the ribosome binds to plus a spacer (together known as the Shine-Dalgarno sequence).

Terminator

Just as there is a site in front of the gene that allows production of the mRNA, there is also a site after (downstream of) the gene that forces the RNA polymerase to stop making mRNA and to dissociate from the DNA.

Red fluorescent protein (*ccRFP*)

A gene from the coral *Corynactis californica*. It encodes a bright pink protein that fluoresces in light of 570 nm.

Green fluorescent protein (*avGFP*)

A gene from the jellyfish *Aequorea victoria*. The protein it encodes glows bright green in light of ~385 nm.

Ampicillin resistance (*Amp*^{R})

The gene *Amp*^{R} encodes the enzyme β-lactamase, which breaks down the β-lactam ring in ampicillin and similar antibiotics, preventing them from acting.

Kanamycin resistance (*Kan*^{R})

The gene *Kan*^{R} encodes aminoglycoside 3'-phospho-transferase (APH). This enzyme alters the antibiotic kanamycin, preventing it from working.

LacI (and *LacO*)

LacI encodes a repressor protein, which, in the absence of lactose or IPTG in the growth medium, binds to the *LacO* sites, preventing the transcription of the genes ahead of them.

Appendix 2: Primer sequences

PCR primers for exchanging *avGFP* with *ccRFP*

To generate *ccRFP* insert from plasmid *p005kanRFP*
1Forward: CTCGAAAATAATAAAGGGAAAATCAGT
3Reverse: CTCAGAAGTGAAACGCCGTA
PCR product size: 1129 bp

To generate backbone from plasmid *p007ampGFP*
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
3Forward: ACTACCATCGGCGCTACG
PCR product size: 3592 bp

PCR primers for exchanging *Kan*^{R} with *Amp*^{R}

To generate backbone from plasmid *p005kanRFP*
4Forward: CGCCCGGAAGAGAGTC
3Reverse: CTCAGAAGTGAAACGCCGTA
PCR product size: 3525 bp

To generate *Amp*^{R} insert from plasmid *p007ampGFP*
4Reverse: CATATTCACCACCCTGAATTGA
3Forward: ACTACCATCGGCGCTACG
PCR product size: 1206 bp

For the Further investigations (introducing various parts in place of *avGFP*)

DNA parts (provided ready-made in the kit)
β-galactosidase gene (based on *BioBrick* part BBa_K909006)
Banana odour generator (based on *BioBrick* part BBa_J45250)

To generate *p006kanGFP* plasmid backbone
1Reverse: CTGATTTTCCCTTTATTATTTTCGAGA
3Forward: ACTACCATCGGCGCTACG
PCR product size: 3541 bp

To generate part of either the banana or lactase assembly in *p006kanGFP* for confirmation by sequencing
1Forward: CTCGAAAATAATAAAGGGAAAATCAGT
3Reverse: CTCAGAAGTGAAACGCCGTA
PCR product sizes (depending on insert chosen):
~1100 bp for *avGFP* and *ccRFP*;
2023 bp for banana;
3619 bp for β-galactosidase.

To generate part of either the *Amp*^{R} or *Kan*^{R} assembly in *p006kanGFP* for confirmation by sequencing
4Reverse: CATATTCACCACCCTGAATTGA
3Forward: ACTACCATCGGCGCTACG
PCR product sizes (depending on insert chosen):
1206 bp for *Amp*^{R}
1155 bp for *Kan*^{R}

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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National Centre for Biotechnology Education, University of Reading, 2 Earley Gate, Reading RG6 6AU. United Kingdom
Tel: + 44 (0) 118 9873743. Fax: + 44 (0) 118 9750140. eMail: NCBE@reading.ac.uk Web: www.ncbe.reading.ac.uk