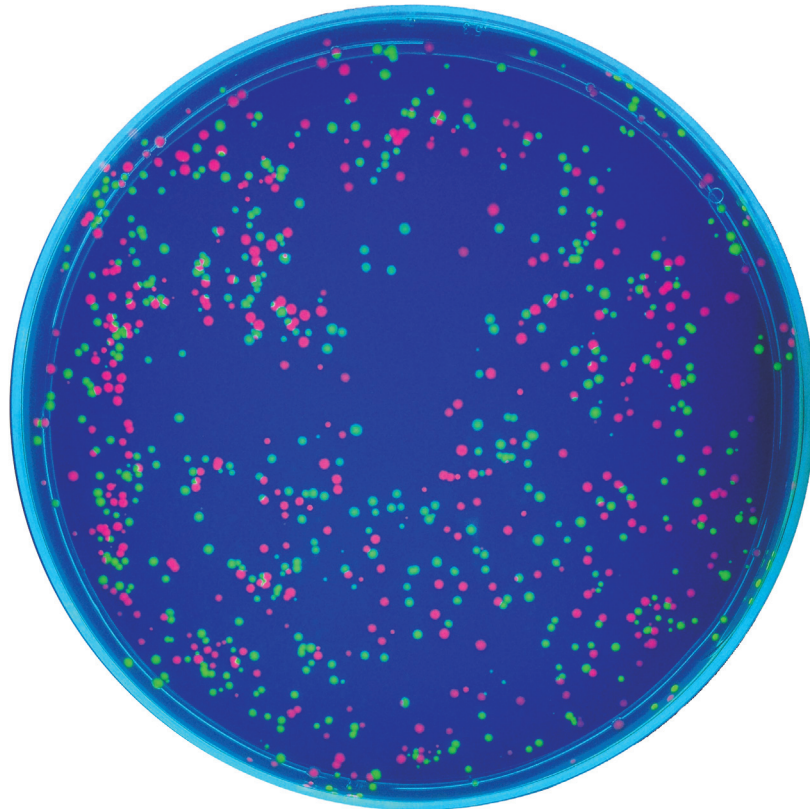


Designer bacteria



Synthetic biology kit

Teacher and
Technician's guide

Contents page

1	Front cover
3	Contents page
4	Introduction to synthetic biology and the practical
5	Equipment and materials
6	Timelines for the practical
7	Practical key and theory task
8-9	Preparation steps and microbiology help
10-11	Digestion, ligation and preparing for transformation
12-13	Transformation and the results
14-15	Good microbiological laboratory practice guidelines
16	Safety and genetic modification

Engineering biological systems

Synthetic means to synthesise something that mimics a natural product. In chemistry, specific reagents can be combined in a controlled environment to produce a whole range of products such as salts, acids and medicines. In the textile industry, synthetic fibres are made such as nylon or acrylic. These can offer certain advantages over natural fibres, for instance being designed to be water or stain resistant.

In biology, synthetic has a similar meaning; scientists can design biological systems for a tailor-made purpose. These pre-designed systems could be individual molecules, cells, or whole tissues or organisms. They can display functions not found naturally in the environment and so the construction of such systems must be tightly regulated.

Synthetic biology is a rapidly growing area of research, systems are often modelled using computer software before they are synthesised. Bacteria and yeast are widely used since it is easy to incorporate new genetic information into their cells, giving them the blueprint for a new protein or compound. An

example of this is the production of artemisinin, which is an antimalarial drug normally isolated from *Artemisia annua*, a herb commonly called sweet wormwood. This is an expensive process and there is an insufficient supply due to the short growing season of the herb. Scientists have come up with two routes to biosynthetically produce this antimalarial drug, one uses yeast and the other uses moss. In both cases the scientists introduce the genes needed for the yeast or moss cells to first produce the precursor molecule to artemisinin, which is then converted into the antimalarial drug. The production of this drug using moss looks very promising since it can be done quickly and on a very large scale, meaning the costs would be relatively low and the efficiency high.

Scientists are only just scratching the surface of what will be possible in the future through synthetic biology, from bacteria or enzymes that could break down plastics and waste to those that could produce renewable energy sources. It is an exciting time to be a scientist!

The practical

The fundamental idea underlying synthetic biology is that scientists can design and make a choice about what genetic blueprint they want to introduce into the host organism they are using. In the case of this practical, the students will have the choice as to whether they would like to make red or green fluorescent bacteria. They will do this by introducing either a red fluorescent gene or a green fluorescent gene (originally isolated from the anemone *Discosoma striata* or the jellyfish *Aequorea victoria*, respectively), which the bacteria will transcribe and translate using its own genetic machinery. This will result in the production of either green or red fluorescent protein. This protein will make the bacteria fluoresce under UV light. In addition to the fluorescent gene, it is necessary to have an antibiotic resistance gene, so we can select which bacteria have taken in the plasmid of interest. If the fluorescent gene is coupled with a kanamycin resistance gene on the same plasmid, when the bacteria are plated out on media containing kanamycin only those with the plasmid can survive.

The students will start by choosing which colour bacteria they would like to make, they will then be given a plasmid with either the red or green gene. In addition, they will get a plasmid containing the gene for kanamycin resistance. The students will cut out the red or green gene using restriction enzymes and cut the plasmid containing the kanamycin resistance gene to allow the addition of the new gene. They will use the same two enzymes to cut both plasmids since each restriction enzyme has a signature of bases they leave after cutting, meaning

that if two plasmids are cut with the same enzymes they should slot seamlessly together with their bases annealing by complementary base pairing (see theory task). The students will use ligase to stick the fluorescent gene into the plasmid containing the kanamycin resistance gene. For more detail see the student guide.

The new plasmid, which contains the red or green gene and kanamycin resistance, will then be transformed into bacteria. These bacteria will be grown overnight on agar plates containing kanamycin and only those containing the plasmid will be able to grow. The next day the colonies can be looked at with a UV torch, to see how many have been successfully transformed with the red or green plasmid. It is of course entirely possible for the kanamycin resistance plasmid to re-ligate with itself, giving bacteria with kanamycin resistance. In this case the result would be white colonies on the plate instead of red or green colonies. There will always be some background colonies containing the re-ligated kanamycin resistance plasmid.

In this guide, there is information regarding the preparation needed to undertake the practical and a comprehensive protocol. In the student's guide, key terms and definitions are highlighted that students should be familiar with following the practical. There is also more detail regarding restriction enzymes and ligase and a quick glance illustrative guide to the practical.

Equipment and materials

Supplied in the kit

- 8 students' guides
- 2 teacher/ technician's guides
- 16 theory task sheets: 8 red and 8 green
- 25 µl fixed volume pipette
- 1 sachet of kanamycin/LB/IPTG agar
- 1 sachet of LB agar
- 4 foam tube holders
- 2 ultraviolet light key rings
- 20 sterile Petri dishes
- 38 sterile single use 1 ml pipettes
- 16 sterile single use spreaders
- 2 x 1 ml syringes (for aliquoting calcium chloride)
- 4 single use sterile loops
- Bag of 1.5 ml tubes: 8 red, 8 green, 16 purple and 18 clear (for aliquoting plasmids and calcium chloride)
- Bag of 20 clear screw top 1.5 ml tubes (for bacteria when doing the transformation)
- *E. coli* DH10B slope culture
- 4 x 50 ml tubes with 10 ml of LB media in each
- 2 tubes with red plasmid (red lids) should be frozen on arrival
- 2 tubes with green plasmid (green lids) should be frozen on arrival
- 2 tubes with kanamycin resistance plasmid (purple lids) should be frozen on arrival
- 2 tubes of calcium chloride (for transformation)
- 2 foil bags with 0.5 ml tubes: 4 red, 4 green and 8 purple in each, containing the dried restriction enzymes *Eco*R1 and *Spe*I (one bag for each class)
- 2 bags with 8 x 1.5 yellow tubes containing ligase and ligation buffer. These should be frozen on arrival.
- 8 NCBE micro-syringing units
- White graduated tips for micro-syringes
- Yellow tips for aliquoting out plasmids

Provided by you (not supplied in the kit)

- 8 small cups of crushed ice (one for each group of students)
- Waste pots and a suitable disinfectant, such as 1 % (w/v) Virkon® or 5 % (v/v) Biocleanse® (ideally more than one waste pot to be positioned around the classroom)
- Permanent marker pens
- Adhesive tape for sealing the Petri dishes
- Access to at least 2 water baths
- Access to an incubator set at 37 °C
- Access to a benchtop centrifuge
- An autoclave or pressure cooker
- Bunsen burners
- Lab coats
- Access to antibacterial soap and paper towels

NOTES

Obtain ice cubes from a supermarket, wrap them in several thick plastic bags and smash the ice up with a hammer.

CLEAPSS and SSERC (the school safety organisations in the United Kingdom) advise that where necessary, incubation at 37 °C is permitted, provided that good microbiology laboratory practice is followed.

Timelines and practical help

Preparation timeline

To be done before the day of the practical:

Aliquot plasmids and calcium chloride

The day of the practical:

Thaw the plasmids and calcium chloride previously aliquoted

Switch on water baths and check temperatures are correct for steps being done that day

Transformation preparation

To be done not more than 3 weeks before the transformation step:

Make up media and pour LB plates for DH10B *E. coli* and kanamycin plates for practical

Up to a week before the transformation:

Streak DH10B plate and leave to grow at 37 °C overnight. Leave at room temperature until needed for the overnight broths.

The day before the transformation:

Set up overnight broth cultures, leave to grow in 37 °C incubator without shaking.

The day of the transformation:

Check the bacterial cultures, they should appear cloudy if they have grown.

Aliquot the overnight broths into 1.5 ml screw top tubes (1 ml in each) ready to be pelleted in the practical. Try not to aliquot them more than an hour before the practical.

Timeline of the experiment

(Total time for practical if doing in one go: approx. 1 hour 40 mins- 2 hours)

Digestion

Allow 10-15 minutes for setting up and resuspending restriction enzymes, 15 minute incubation at 37 °C with optional 10 minute enzyme denaturation step at 80 °C.

= **approx. 30 minutes** (without denaturation step, if including this then **40 minutes**)



Freeze point: practical can be paused here

Ligation

Allow 10 minutes for spinning ligase tubes in centrifuge before starting and for transferring plasmids into the ligase tube.

10 minute incubation at room temperature and 10 minute ligase denaturation step at 65 °C.

= **approx. 30 minutes**

Digestion and ligation could be done together in a lesson that is an hour or more in length, or they could be done separately in two shorter lessons and the plasmids could be frozen after each stage.



Freeze point: practical can be paused here

Transformation

Allow 10 minutes for pelleting the bacteria in the centrifuge, resuspending them in calcium chloride and adding the ligation

15 minute incubation on ice

Allow 15 minutes for heat shock step (35 seconds in water bath and 2 minutes on ice) and plating out the bacteria (plus taping and labelling the plates)

= **approx. 40 minutes**

The plates then need to dry in the incubator for 30 minutes before being inverted overnight (so the base is uppermost)



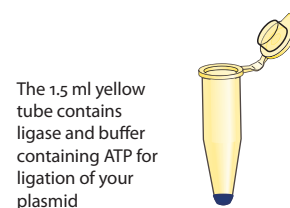
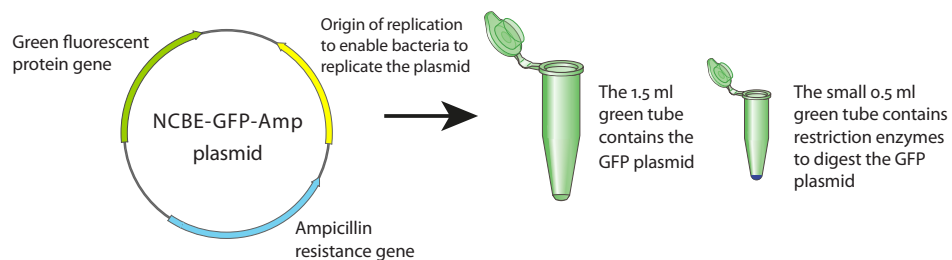
Results

The plates need to be incubated at 37 °C for **24-48 hours**.

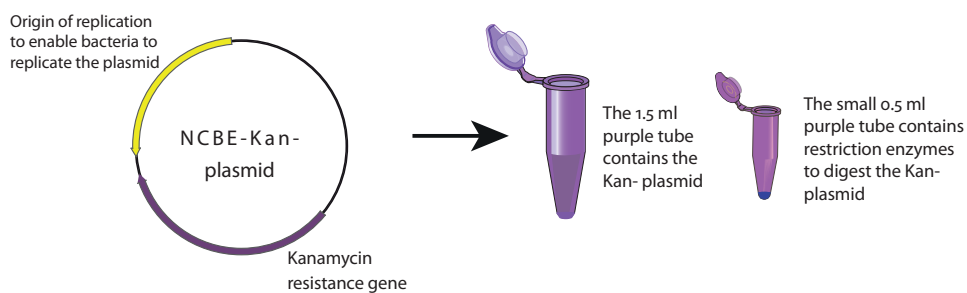
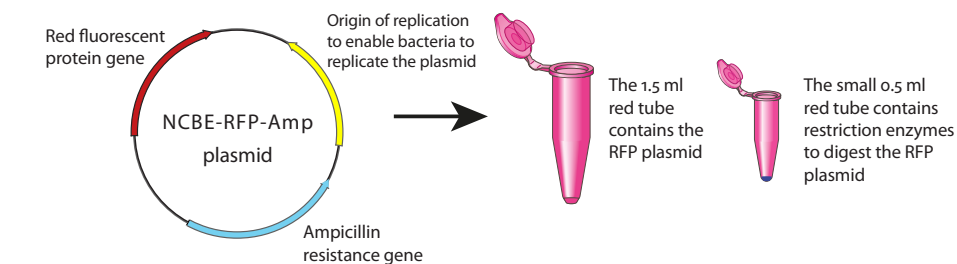
If the students are going to look at the plates and record their results at a later date, then store the plates in the fridge until then.

Allow **20 minutes** to record results, answer questions etc.

Practical Key



The 1.5 ml screw top tube will be given to you for the transformation. It contains 1 ml of DH10B *E. coli*



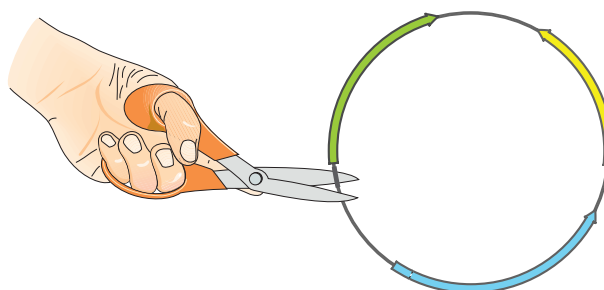
The 1.5 ml clear tube will be given to you for the transformation. It contains calcium chloride solution, which you will use as your transformation buffer



The theory task

Each group will either make red fluorescent bacteria or green. The students will each be given the corresponding theory task.

Plasmids are small circular pieces of DNA. This task shows what the starting plasmids look like, and how when both plasmids are cut with the same two restriction enzymes, the green or red gene can only fit into the second plasmid containing the kanamycin resistance gene in the desired orientation. Refer to the instructions shown on the theory task.



Note: Restriction enzymes have a particular signature of DNA bases they leave after cutting. The enzymes used in this practical leave one strand overhanging the other. We call these "sticky ends". If the same enzyme is used to cut two plasmids then these "sticky ends" should line up and slot seamlessly together. This is how the red or green gene will fit into the plasmid containing kanamycin resistance. Ligase, which is an enzyme that acts like glue, will then stick the fluorescent gene into the kanamycin resistance plasmid.

Preparation

Preparation: to be carried out up to 3 weeks before the practical

- The plasmids will need to be aliquoted into the tubes provided, use the fixed volume pipette to do this. They should have been frozen on arrival so will need to be thawed and spun in a centrifuge to ensure all the liquid is at the bottom of the tube. You have 2 tubes of each plasmid (6 in total), each tube contains enough for a class of 8 groups. Once the plasmids have been aliquoted they need to be stored at -20 °C until the day of the experiment.
 - Clearly label 16 purple tubes with "kan plasmid" and then aliquot 25 µl from the kan resistance plasmid stock tubes (purple lids) into each. You will need 8 of these for each class. There is 240 µl of the kan plasmid in each of the stock tubes .
 - Clearly label 8 red tubes with "red plasmid" and then aliquot 25 µl from the red plasmid stock tubes into each. You will need 4 of these for each class. There is 130 µl included in each of the red plasmid stock tubes.
 - Clearly label 8 green tubes with "green plasmid" and then aliquot 25 µl from the green plasmid stock tube into each. You will need 4 of these for each class. There is 130 µl included in each of the green plasmid stock tubes.
 - Aliquot the calcium chloride, which is supplied as 2 tubes of 2 ml. Each pair will need approximately 120 µl for their transformation. Aliquot the calcium chloride into 16 clear tubes of 150 µl. This can be done using a 1 ml syringe. You will need 8 tubes for each class.

All steps within this practical that involve bacteria or materials to be used with bacteria, such as pouring plates, should be performed using aseptic technique.

- You will need to pour 2 LB agar plates and 18 kanamycin plates for the transformation. Follow the instructions on the LB agar sachets provided; make up 200 ml of LB agar and 200 ml of kanamycin containing agar. First pour 2 x 15-20 ml LB agar plates and then mix the remaining 160-170 ml of LB agar with the 200 ml of kanamycin containing LB agar to dilute the kanamycin concentration. Pour approximately 18 kanamycin plates, which once set, can be stored inverted (base uppermost) in the fridge for 3-4 weeks.

Preparation: to be carried out not more than a week before the transformation

Streak out DH10B *E.coli* onto one of the LB agar plates (not containing kanamycin) and incubate at 37 °C overnight with the plate inverted (agar uppermost). If you wish to streak the plate on a Friday and leave it to grow over the weekend then let it grow at 30 °C. The other LB agar plate can be stored in the fridge to be used for another group at a later date. Once the streak plate has grown, leave it at room temperature until you are ready to grow your overnight broths (see page 9 for more information).

Preparation step: to be carried out the day before the transformation step

Pick a single colony from the DH10B *E.coli* plate with a sterile loop and add to one of the 10 ml LB broths included in the kit. Make sure you swirl the loop rigorously in the broth to dislodge the bacteria. For a class with 8 groups you should set up a minimum of 2 x 10 ml LB broths, as you need 1 ml of culture per group but it is advisable to always set up at least one extra culture in case one doesn't grow. There are enough 10 ml broths included to set up 2 for each class. Once a colony has been added to each broth, make sure the lid is securely closed and then give the tube a good shake to make sure the bacteria is dispersed into the broth. Put the broth cultures into a 37 °C incubator and allow to grow **without shaking** for 16-24 hours (see page 9 for more information).

Preparation steps: to be carried out the day of the experiment

- Thaw the plasmids aliquoted previously, ideally spin them for a few seconds in the centrifuge to ensure all liquid is at the bottom.
- Switch on the water baths and set to the temperatures needed for the sections of the practical being done that day. Make sure to test the temperature with a thermometer since water bath dials tend to not be very accurate.
- Decide which students will make the green or red fluorescent bacteria. Each group will get a large and small purple tube and either a large and small red tube for making the red bacteria or a large and small green tube for making the green bacteria (the small enzyme tubes are found in the foil bags).

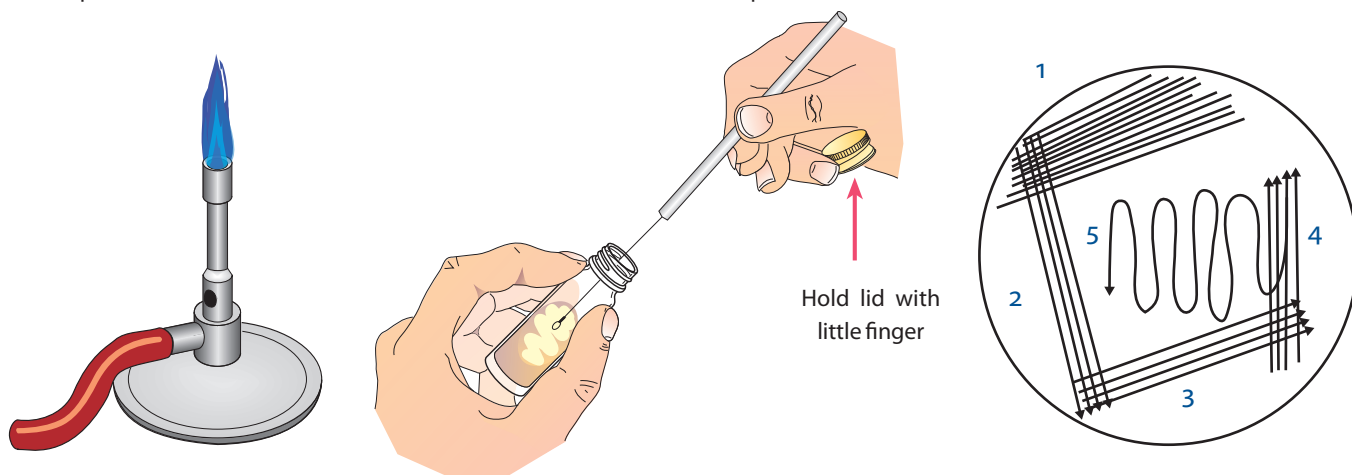
For the transformation:

- The 10 ml bacterial cultures should appear cloudy if they have grown. They can be left at 37 °C until the transformation or they can be kept at room temperature. Aliquot the bacterial cultures using a 1 ml pipette into the 1.5 ml screw top tubes (1 ml in each tube), ideally not more than an hour before the transformation.
- Take the kanamycin LB agar plates out of the fridge and either leave out at room temperature to warm up or ideally put at 37 °C. You will need one plate for each group of students.

Streaking a plate

It is important to streak out the DH10B *E. coli* not more than a week before doing the transformation. Work within 30 cm of a hot blue flame to keep everything sterile. You will need one of the LB agar plates you poured out first, before you made the kanamycin plates. The protocol shown here is for a metal loop and is just one method for streaking a plate, there are several others. If you are using a plastic loop, do not put it into the flame.

1. Flame your metal loop and allow it to cool down before putting it in contact with the bacteria. You can test it is cool enough by touching the agar on the plate.
2. Open the lid of the *E. coli* slope, hold the bottle in your non-dominant hand and screw off the lid using the little finger of your dominant hand which should be holding the loop with thumb and 2 first fingers. Flame the top of the bottle and put your loop in, gently scratch the surface of the agar slope with the loop. Remove the loop from the bottle and flame the top again, replace the lid. It is important not to put the lid down on the workbench to avoid contamination of the slope culture.



3. Pick up the base of the plate and angle it towards the flame. Drag the loop back and forth as can be seen at point 1 in the plate diagram, this creates a well of bacteria. Use the loop to drag down 4-5 times away from the initial well of bacteria (point 2). Place your plate back on the lid and flame your metal loop for a few seconds. This will reduce the amount of bacteria on the loop; increasing the chances of getting single colonies. Once the loop is cool, pass the loop back through the streaks made at point 2 and make another 4-5 straight lines with the loop (point 3) repeat this for point 4. Now flame the loop again, wait for it to cool and then do a wiggly line with the loop from point 4 into the middle of the plate (point 5).

Incubate the plate at 37 °C overnight or at 30 °C if leaving over the weekend. Once grown the plate should be kept at room temperature and be used for the overnight broths within a week of being streaked.

Setting up overnight broths

The overnight broths must be set up the night before the transformation. Again, work on a clean bench around a Bunsen burner. In the kit you will have 10 ml LB broths that you can inoculate with the DH10B from the streak plate.

1. Take one of the sterile loops provided in the kit, unwrap it from the opposite end to the loop.
2. Take your streak plate with the lid facing down, partially lift the base and gently scrape a single colony from the plate using the loop, replace the lid on the plate. Try not to wave the loop around and keep it within 30 cm of the flame.
3. Now pick up the 10 ml broth and working closely to the flame, open the lid. To avoid contamination do not put the lid down on the bench. Put the loop into the broth and mix it quite vigorously to dislodge the bacteria. Remove the loop and replace the lid. Give it a shake to disperse the bacteria since they will be grown without shaking.
4. Repeat this process if you are setting up more than one broth, which is recommended in case one doesn't grow.
5. Grow the broths at 37 °C for 16-24 hours without shaking. If the broths are shaken it causes the bacteria to grow too quickly and be either in stationary phase or dying by the time they are used for the transformation, meaning there are not many viable bacteria to transform.

1 Preparing the plasmids for digestion

Groups making **green** bacteria should have a small **green** enzyme tube and a large **green** tube containing the green plasmid.

Groups making **red** bacteria should have a small **red** enzyme tube and a large **red** tube containing the red plasmid.

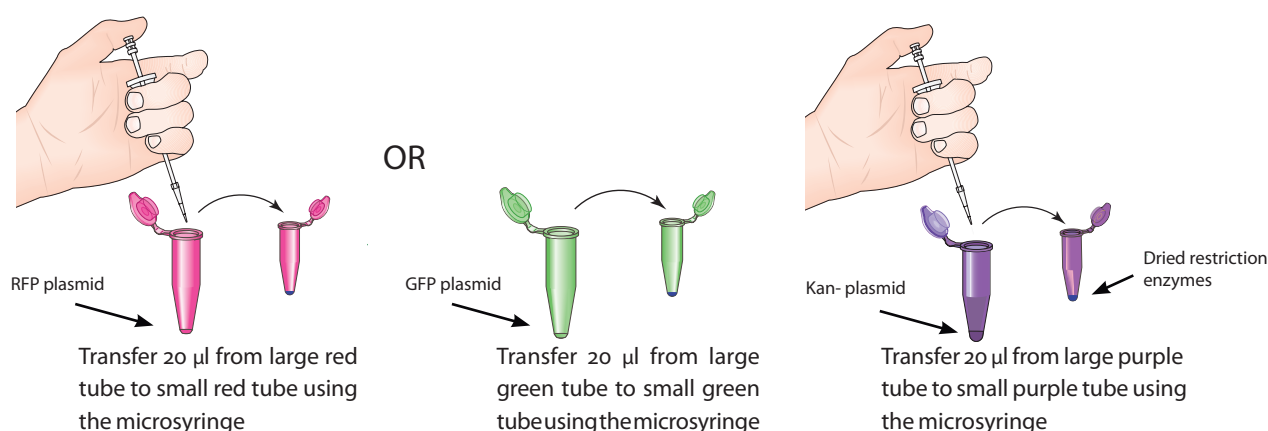
All groups will need a small **purple** enzyme tube and a large **purple** tube containing the kanamycin plasmid.

Students will need to label the enzyme tubes with their initials.

20 μ l of either **red** or **green** plasmid DNA should be transferred into the **red** or **green** enzyme tube using a microsyringe (large **red** tube into small **red** tube or large **green** tube into small **green** tube).

The clear plastic tips for use with the microsyringe are graduated and have 4 marks, the top mark is for 20 μ l (refer to microsyringes in the student guide). They will need to draw the plasmid solution into the tip to the upper mark to give 20 μ l and dispense this into their enzyme tube. They will then need a fresh tip.

The same should be done for the kanamycin plasmid; 20 μ l of the kanamycin plasmid from the large **purple** tube should be transferred into the small **purple** enzyme tube.



2 Digestion of the plasmids

The lids of the enzyme tubes should be shut and left at room temperature for 5 minutes. The dried restriction enzymes need to be resuspended, to do this, students will need to grasp the top of the tube firmly between their thumb and fingers and then vigorously flick the tube with a finger from the other hand until the enzyme mix has been completely resuspended.

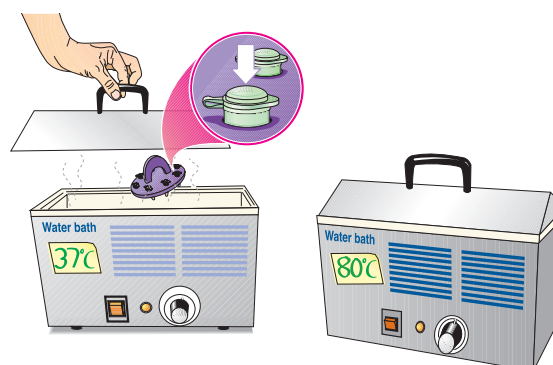
The solution should have a blue hue but there should be no blue spots at the bottom of the tube where there is a concentrated area of dried enzyme.

The plasmid needs to be at the bottom of the tube, so students need to tap the base of the tube firmly on the bench to make sure all the liquid has returned to the bottom.

All small enzyme tubes need to be inserted into the foam tube holders and incubated in the 37 $^{\circ}$ C water bath for 15 minutes. This step allows the enzymes to digest the plasmids; cutting out the green or red genes and cutting the kanamycin plasmid.

Optional step

After digestion, the tubes can be incubated in the 80 $^{\circ}$ C water bath for 10 minutes to denature the restriction enzymes. This step increases the efficiency of the practical by 10-20 % but isn't necessary if you are short of time.



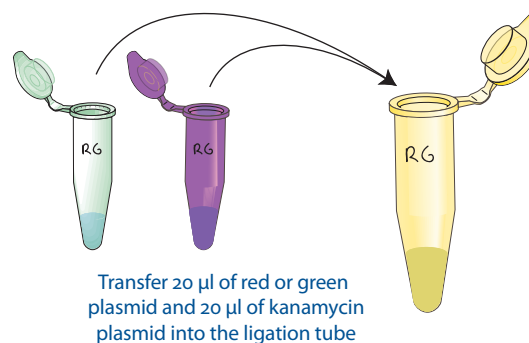
Freeze point: If you wish to stop the practical and continue later, then freeze the digested plasmids in the enzyme tubes. When you wish to continue, thaw the plasmids and start the next step.

3 Ligation

1. Each group now needs a **yellow** ligation tube, which contains ligase; this acts as the glue that will stick the red or green gene into the kanamycin plasmid.
2. The **yellow** tubes need to be spun for a few seconds in a microcentrifuge to make sure all of the ligase is at the bottom of the tube. The students should label the **yellow** tubes with their initials.
3. Using the microsyringes, 20 μ l of the kanamycin plasmid should be transferred from the **purple** tube into the **yellow** ligation tube.
4. 20 μ l of the **red** or **green** plasmid should also be transferred from the **red** or **green** tube into the **yellow** ligation tube.

If there is slightly less than 20 μ l of plasmid when transferring to the ligation tube, this is fine, just transfer as much as possible.

5. The lids of the **yellow** tubes should be closed and the tubes should be flicked to ensure they are properly mixed. The base of the tubes should be tapped on the bench to return all the liquid to the bottom. The ligation reaction should now be incubated at room temperature for 10 minutes and then transferred to a foam tube holder.
6. All the yellow tubes should then be put in the 65 °C water bath for 10 minutes to denature the ligase. Once the ligase has been denatured the tubes should be kept on ice until the students are ready to do the transformation.



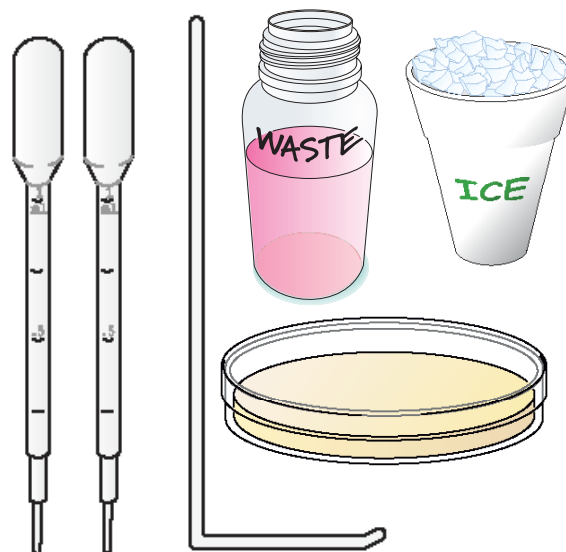
Note: The DNA ligase is mixed with a buffer containing ATP. The buffer will ensure that the pH is correct for the ligase to work, while the ATP will provide the energy necessary for the ligation reaction.

Freeze point: If you wish to stop the practical and continue later, then freeze the ligated plasmids in the yellow ligation tubes. When you wish to continue, thaw the plasmids and start the next step.

4 Preparing for the bacterial transformation

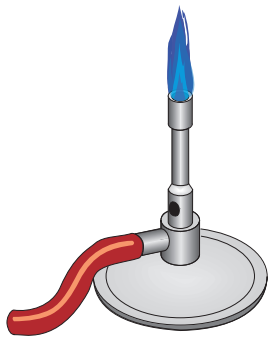
The students should clear their work area and wipe the work surface with disinfectant solution. Each group will need:

- a container of disinfectant for contaminated waste;
- one of the kanamycin containing LB agar plates poured earlier
- a sterile plastic spreader (in its sterile wrapper);
- 2 sterile plastic 1 ml pipettes (in their sterile wrappers).
- a small cup of crushed ice
- a Bunsen burner
- a tube of calcium chloride, which should be put on ice
- a 1.5 ml tube of DH10B *E. coli* recently aliquoted
- their yellow ligation tube



Transforming the bacteria

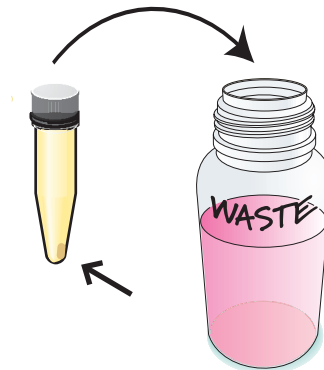
1. The bacteria need to be spun in a microcentrifuge, so the tubes should be balanced and then spun at $2,250 \times g$ for 2 minutes to pellet the bacteria.
2. Following centrifugation, there should be a small pellet of bacteria at the bottom of the tube and the supernatant should have gone from cloudy to clear.
3. The supernatant should be poured off into the waste disinfectant container and the students should add 4 drops of the cold calcium chloride to the bacteria using a sterile 1 ml pipette, the cap of the tube should now be replaced.
4. The pelleted bacteria should be resuspended by flicking the tube (**the bacteria are fragile so do this gently**). No clumps of bacteria should be left and no bacteria should be stuck to the side of the tube. The resuspended bacteria can be kept on ice for a few minutes until the ligations are ready.



Working around a flame helps keep things sterile, use a blue flame so there is a strong upward draught



Calcium chloride on ice



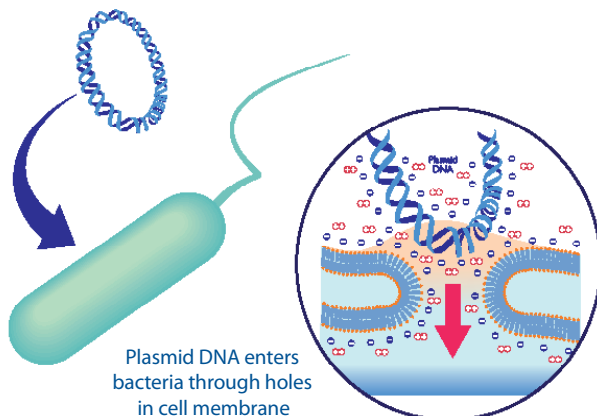
Visible bacterial pellet after centrifugation



Add 4 drops of cold calcium chloride and flick the tube gently to resuspend the bacteria

Note: The bacteria are fragile during the transformation phase so be gentle when handling them

5. Each group now needs to transfer 20 μl of the ligation reaction from the yellow tube into the tube containing the resuspended bacteria. The cap should be replaced and the tube should be tapped a few times to gently mix the contents. The tube should be left on ice for 10-15 minutes.
6. Check the 42 °C water bath is at exactly 42 °C, do this with a thermometer. The students are now going to heat shock the bacteria to encourage pores in the bacterial cell membrane to open; allowing the plasmid DNA to enter.
7. The tubes of bacteria need to be placed into foam tube holders. The bacterial tubes should be put into the 42 °C water bath for **35 seconds** and then transferred straight back to the ice for **2 minutes**. This works best if the bacteria are transferred quickly and carefully from the ice to the water bath and back again to the ice.



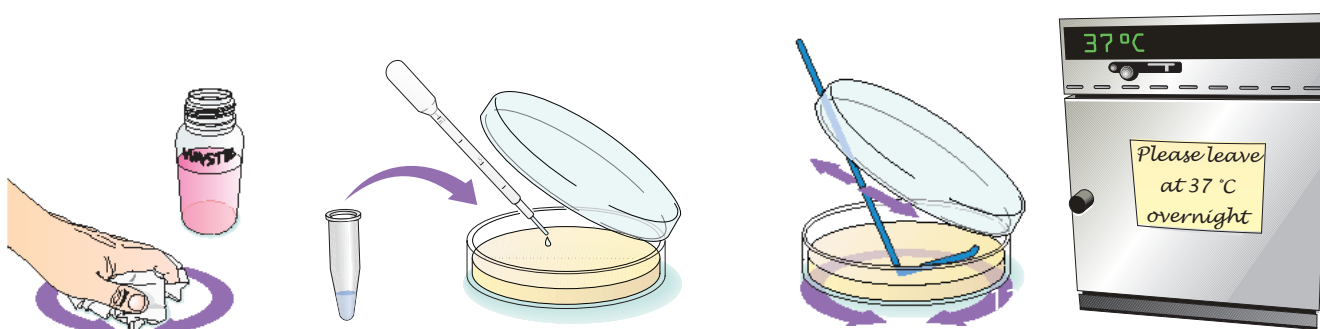
Plasmid DNA enters bacteria through holes in cell membrane



Set two timers, one for 35 seconds and one for 2 minutes

The students need to:

1. Clean their workbench both before and after plating the transformation and work within 30 cm of a flame to help keep things sterile.
2. Take a kanamycin LB agar plate (one for each group) and with one hand lift the lid so it still partially covers the plate, then using a 1 ml pipette, transfer all of the bacteria into the centre of the plate. Put the pipette in the waste container.
3. Use a sterile spreader to gently spread the bacteria out over the surface of the plate and then replace the lid. Put the spreader in the waste container. Tape the plate and clearly label with their name, the date and the experiment.
4. The plates should be moved to the 37 °C incubator with the lid uppermost for at least 30 minutes to allow the transformation to soak into the agar. **The plates shouldn't be left at room temperature for more than a few minutes after the transformation has been plated out.** The plates should then be inverted overnight so the base is uppermost.



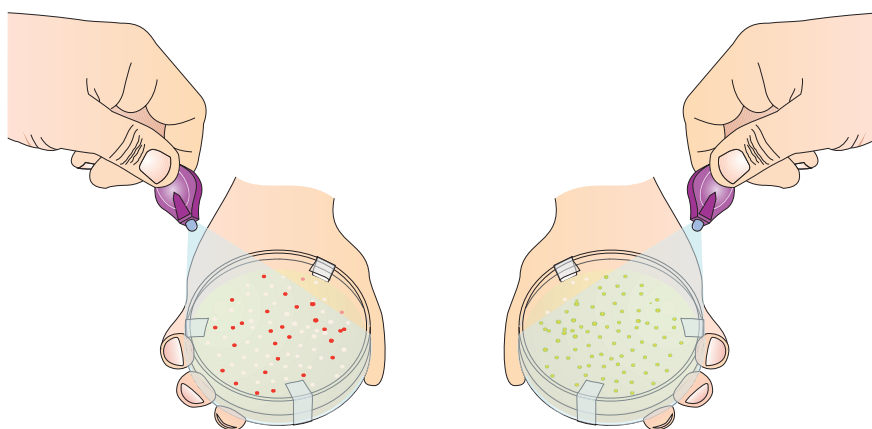
5 The Results

24 to 48 hours later...

The plates should be left to grow at 37 °C for a minimum of 24 hours. Sometimes if the transformation efficiency is low it may take longer for red or green colonies to appear. The red colonies will appear red even without UV light.

The green colonies appear white in daylight and will only appear green under UV light, so the bacterial colonies will need to be checked carefully with the UV torch.

If the students are going to look at the plates and record their results at a later date, then store the plates in the fridge until then.



Students should count how many fluorescent colonies they have and how many white colonies. They can calculate the percentage of fluorescent colonies to see how efficient the reaction was.

There is a set of questions on the back of the student guide to make sure the students have understood the practical. Testing that the students know the key terms listed on page 7 of the student guide could also test their understanding.

Good microbiological laboratory practice

General precautions

- Any exposed cuts and abrasions should be protected with waterproof dressings before the practical work starts.
- There is no need to wear disposable gloves, except if a person has skin condition such as eczma or abrasions or cuts to the skin that cannot be covered with waterproof dressings (either because they are too large or awkward to cover or the person concerned is allergic to plasters).
- Everyone involved — teachers, technicians and students should wash their hands before and after practical work.
- Laboratory doors and windows should be closed while practical work is in progress. This will reduce air movements and consequently the risk of accidental contamination of plates, etc.
- High standards of cleanliness must be maintained. Non-porous work surfaces should be used and they must be swabbed with an appropriate laboratory disinfectant before and after each practical session. (Virkon® or Biocleanse® are the disinfectants of choice for school microbiology).
- No hand-to-mouth operations should occur (e.g., chewing pencils, licking labels, mouth pipetting). Eating, drinking and smoking must not be allowed in the laboratory.
- Those carrying out the work should wear laboratory coats and, where necessary, eye protection.

Spills and breakages

- Accidents involving cultures should be dealt with as follows:
- Disposable gloves should be worn.
- The broken container and/or spilt culture should be covered with paper towels soaked in disinfectant.
- After not less than 10 minutes, it must be cleared away using paper towels and a dustpan.
- The contaminated material must be submerged in a suitable disinfectant for 24 hours or placed in a microbiological disposal bag.
- The contaminated material must then be autoclaved before disposal. The dustpan should also be autoclaved or placed in a bucket of suitable disinfectant solution (e.g., Virkon®) for 24 hours.
- Contaminated paper towels should be autoclaved.

Contamination of skin or clothing

As soon as possible, anyone affected should wash with antibacterial soap. Severely contaminated clothing should be placed in disinfectant before it is laundered.

Sources of microbes

All micro-organisms should be regarded as potentially harmful. However, the strain of *E. coli* used in this kit presents minimum risk given good microbiology laboratory practice. Other species of bacteria must not be used for this work, as this might contravene the regulations governing genetic modification.

In general, stock slope cultures of bacteria should be kept in the dark at room temperature, not in a fridge. Slope cultures should be subcultured onto fresh nutrient agar every six weeks or so. You should not attempt to maintain the culture for an extended period, however, as mutations can occur in the storage conditions that are found in schools, and these may lead to the failure of the practical work. Cultures may also become contaminated with repeated sub-culturing. If in doubt, obtain a fresh culture.

Aseptic techniques

The aims of aseptic techniques are:

- To obtain and maintain pure cultures of microorganisms;
- To make working with microorganisms safer.

A 'pure culture' contains only one species of microorganism, whereas a 'mixed culture' contains two or more species.

Contamination of cultures is always a threat because microbes are found everywhere; on the skin, in the air, and on inanimate objects. To obtain a pure culture, sterile growth media and equipment must be used and contaminants must be excluded. These are the main principles of aseptic techniques.

It is unrealistic to expect inexperienced school students to be fully accomplished at aseptic techniques. Sterile, disposable items are therefore provided in this kit, so that the necessary procedures can be carried out as easily and safely as possible.

Sterile Petri dishes should be used. Lids must be kept on containers to prevent contamination.

Practical work should be carried out near a Bunsen burner flame. Rising air currents from the flame will carry away any microbes that could contaminate growth media and pure cultures.

When cultures are transferred, tops and lids of containers should not be removed for longer than necessary. After a lid has been taken from a bottle, it should be kept in the hand until it is put back on the bottle. This prevents contamination of the bench and the culture. After removal of the top, the neck of the culture bottle, if glass, should be flamed briefly for 1–2 seconds. This will kill any microbes present there and produce convection currents which will help to prevent accidental contamination of the culture. With practice, it is possible to hold a bottle containing the microbes in one hand and the loop or pipette in the other in such a way that the little finger is free to grip the bottle top against the lower part of the hand. (In this case, it is important that the bottle top should be loosened slightly before the inoculation loop is picked up.)

Obviously, unlike glassware, the sterile plastic 50 ml tubes, spreaders, loops, microcentrifuge tubes and pipettes that are provided in this kit must not be flamed.

When the Bunsen is not in use it should be kept on the yellow flame, so that it can be seen. A blue flame about 5 cm high should be used for flaming the necks of glass bottles.

Avoid contaminating the work area. Any non-disposable instruments should be sterilised immediately after use and used plastic pipettes, tubes and other plastic items should be placed directly into a jar of fresh disinfectant (Virkon®) solution, so that they are completely immersed.

Incubation of cultures

Label the Petri dish around the edge of the base. A student's name, date and the name of the organism used will allow the plate and its contents to be identified.

Use self-adhesive tape to seal Petri dishes at a few places around the rim. The seal will ensure that the plates are not accidentally opened or tampered with. Do not seal plates completely as this could create anaerobic growth conditions within the dish.

Bacterial cultures in Petri dishes should be incubated with the base uppermost, so that any condensation that forms falls into the lid and not onto the colonies.

Incubation at 37 °C

Although in general, 30 °C is regarded as the upper limit for the incubation of microbial cultures in UK schools, the delicate strains of *E. coli* used for cloning work often require incubation at 37 °C. Good microbiological practice, coupled with the use of selective growth media will ensure that contaminating human pathogens are not inadvertently cultivated at this temperature. Official safety guidelines for school microbiology from the Association for Science Education, CLEAPSS, SSERC and others in the UK allow for this possibility.

Disposal

It is very important to dispose of all the materials used in a practical class properly, especially any item that has been in contact with cultures of bacteria. All non-disposable containers used for storing and growing cultures must be autoclaved, then

washed and rinsed as necessary, before re-use.

There should be a discard jar of fresh disinfectant (Virkon®) near each work area. Disposable plastic pipettes, spreaders, tubes and any liquid from cultures should be put into the disinfectant pot immediately after use. After soaking for 24 hours, these materials should be autoclaved then disposed of in the normal waste.

Contaminated paper towels, cloths and plastic Petri dishes should be put into an autoclave bag and sterilised by autoclaving before placing in the normal waste.

Glassware that is not contaminated (e.g., flasks used for making up media) can be washed normally. Broken glassware should be put in a waste bin reserved exclusively for that purpose. If any glassware is contaminated it must be autoclaved before disposal. Uncontaminated broken glassware can be thrown away immediately.

Autoclaving

Sterilisation is the complete destruction of all micro-organisms, including their spores.

All equipment should be sterilised before starting practical work so that there are no contaminants. Cultures and contaminated material should also be sterilised after use for safe disposal.

Autoclaving is the preferred method of sterilisation for culture media, aqueous solutions and discarded cultures. To comply with the regulations governing genetic modification, it is essential that any genetically-modified microorganisms are killed before disposal. Autoclaving is the most reliable method of achieving this.

The process uses high pressure steam, usually at 121 °C. Microbes are more readily killed by moist heat than dry heat as the steam denatures their proteins. Domestic pressure cookers can be used in school laboratories instead of autoclaves, but their small capacity can be a disadvantage when dealing with class sets of material.

Handling disposable sterile items

The plastic pipettes, loops, tubes and spreaders provided in this kit are sterile. Remind students that the packets should be opened with care to ensure that the tips of pipettes, spreaders and so on are not touched.

These items should all be placed in a discard container of disinfectant after use, where they should be left for 24 hours before autoclaving and disposal in the normal waste.

Safety and genetic modification

Contained Use

All practical work that involves the production or use of genetically-modified organisms (GMOs) is strictly regulated by law throughout the European Union. There are two relevant sets of EU regulations (Directives) governing genetic modification. Laws in the United Kingdom and elsewhere within the EU are enacted to comply with these Directives. One Directive covers 'Contained Use' e.g., work in a laboratory; the other covers 'Deliberate Releases' of GMOs into the environment e.g., field trials of genetically-modified crops.

In general, anyone carrying out work with GMOs must do so only on premises that have been registered with the relevant authority. In England and Wales, this is the Health and Safety Executive (HSE) — or its equivalent in Scotland and Northern Ireland. The organisation, such as a university or research facility, under whose auspices the work is to be done must usually set up a local expert safety committee and procedures to oversee and control the work. These stringent requirements would seem to preclude any work with GMOs in the majority of schools. There is, however, a limited amount of practical work that can be done in schools.

'Self-cloning'

The practical procedure described in this kit is known technically as 'self-cloning'. Here, 'cloning' means making copies of DNA within an organism. Originally, the definition of self-cloning was restricted to taking DNA from one species and making copies of it (cloning it) in the same species — hence the term self-cloning. Later, this definition was widened slightly to include 'marker' genes and control sequences, which might come from other species, provided that these elements had a long history of safe use in the organism concerned.

Self-cloning using non-pathogenic microorganisms, such as the strain *E. coli* provided with this kit, is exempt from the Contained Use regulations. The bacteria produced are covered by the Deliberate Release regulations, however, and it is therefore essential to ensure that an accidental 'release' of the organism into the environment does not occur. This is achieved by physical, chemical and biological containment.

Physical and chemical containment

The genetically-modified microorganisms (GMMOs) must be physically and chemically contained by good microbiology laboratory practice, including the destruction of the cultures after use.

Biological containment

The GMMOs are also biologically contained, by the selection of a suitable host strain and the careful construction of the plasmid DNA. So, for example, in the current practical procedure, the strain of *E. coli* lacks the ability to pass on the introduced DNA by the natural bacterial 'mating' process of conjugation, and the plasmid DNA is non-methylated so that if it did enter a wild-type bacterium, it would be degraded by that organism's own restriction enzymes.

Alterations to the procedure

It follows from what has been stated above that no attempt should be made to alter or add to the procedure described in this kit in a way that might bring those following it outside the umbrella of self-cloning and into the realm of Contained Use. If this was to be done without notifying the relevant authorities and following the other procedures that such work legally requires, users could place themselves and others at risk, and could ultimately be subject to legal action.

Further information

Additional guidance on work with GMOs can be found on the HSE's web site: www.hse.gov.uk/biosafety/gmo/ and in the following publication: The Genetically Modified Organisms (Contained Use) Regulations 2014. Health and Safety Executive (2014) The Stationery Office, London.

ISBN: 978 0 7176 6641 6. This official document is aimed principally at research scientists, but the latest also mentions work in schools and can be downloaded from the HSE's web site.

Practical advice on safety in school science is available to schools that are members in England, Wales and Northern Ireland from CLEAPSS (www.cleapss.org.uk) and in Scotland from SSERC (www.sserc.org.uk).

Most schools and colleges in the UK will have a copy of Topics in Safety, which includes chapters on both microbiology and work with DNA: Topics in safety (2001) [Third edition] Association for Science Education. ISBN: 0863573169.

An updated (October 2014) version of Chapter 16, covering work with DNA, can be found on the NCBE's web site: www.ncbe.reading.ac.uk/dnasafety

Video demonstrations of basic microbiology laboratory techniques and other useful information can be found on the Microbiology Society's YouTube channel: www.youtube.com/channel/UCs2_8IXc1SJLvQuGHft_TAA/videos