TEACHING OUTREACH RESOURCE

Restriction Digest of Plasmid DNA and Cloning

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GO BEYOND WITH GENETICS...

__Explore__ the origins and mechanics of life. __Discover__ causes of disease and their cures. __Solve__ the problems facing our agriculture and natural heritage. __Understand__ the past. __Create__ a better future. __Master__ the world of genetics...

WELCOME...

...to this Genetics teaching resource, created by Genetics Otago and the Genetics Teaching Programme at the University of Otago.

Our aim is to engage young minds with Genetics and to do this we have developed a range of resources that include information, worksheets and activities or experiments that will help you to plan exciting Genetics classes for your students.

Where possible we have endeavoured to align and link the content of the resources to the New Zealand Curriculum.

If you have any questions relating to the content of the resources or would like to organise an onsite teaching session on one of our topics please contact us at go@otago.ac.nz.

Contents

Overview

Plasmids are a powerful tool in genetic research, particularly in genetic engineering and gene therapy. In this resource we provide background information, instructions and worksheets necessary to provide a basic understanding of plasmids, restriction enzymes and digestions and the use of plasmids in cloning. Students will be given the opportunity to work through a series of exercises to understand the concepts before testing for a cloned gene within a plasmid using gel electrophoresis (electrophoresis kits are available on request).

OBJECTIVES

- Successfully follow a scenario through a logical set of steps to reach an informed conclusion.
- Understand the utility of plasmids in cloning and why restriction enzymes are an important part of this process.
- Appreciate the utility of cloning in plasmids and other organisms, with consideration to the implications of such genetic manipulation both biologically and ethically.
- Interpret the results of an electrophoresis gel and apply the results to a cloning model.

SECTIONS

Part A: Plasmids

- Activity One – Application of Plasmids (**Creating a Paper Plasmid**)

Part B: Restriction Enzymes and Digests

- Activity Two – Restriction Digests (**Digesting DNA**)

Part C: Cloning a Gene

- Activity Three – Cloning (**Mini research project**)
- Activity Four – Cloning a Gene (**Cloning a Gene**) including an optional practical component.

CURRICULUM LINKS

This module is designed to feed into the following curriculum areas at Level 6+, but could be adapted to suit younger students:

- **Nature of science**
  - *Understanding about science* **–** working in groups, working with current scientific theories, collecting evidence and developing a logical argument.
  - *Investigating in science* **–** *c*arrying out investigations, using models and working scientifically with multiple variables.
  - *Communicating in science* **–** Using science vocabulary and relating science understanding to scientific texts.
  - *Participating and contributing* **–** develop an understanding of socio-scientific issues and draw evidence-based conclusions.

- **Living World**
  - Understand the relationship between organisms and their environment.
  - Explore the evolutionary processes that have resulted in the diversity of life on Earth and appreciate the place and impact of humans within these processes.
  - Understand how humans manipulate the transfer of genetic information from one generation to the next and make informed judgments about the social, ethical, and biological implications relating to this manipulation.

PART A

Plasmids

Plasmids

Plasmids are small, circular fragments of DNA that replicate independently to the chromosomal DNA of an organism. In general, they carry very few genes and are typically only a few thousand base pairs in length, their small size makes them stable and easy to manipulate. These factors, along with being cheap and self-replicating make them a key tool in laboratories and in this setting, they are referred to as vectors. Plasmids are common in bacteria, and often provide some sort of competitive advantage to the host cell. These advantages include several mechanisms to survive in harsh environments, the one most often exploited by scientists is antibiotic resistance.

Plasmids can be drawn as maps with all key components clearly identified. Naturally occurring plasmids always contain an Ori and a gene carrying an adaptive advantage (which can be used as a selectable marker). Plasmids engineered in a lab also contain a multiple cloning site, to allow for genetic manipulation and a promotor, which allows replication of a gene once cloned into this site.

During cell division the plasmid (or plasmids) within a cell are replicated and are passed on to daughter cells, this is the simplest method of plasmid inheritance. Whether or not a cell has successfully received a plasmid can be tested by applying the specific selection pressure that the plasmid provides resistance to e.g., an antibiotic.

KEY TERMS

- **Plasmid** – A small extrachromosomal piece of DNA, from ‘cytoplasm’ and ’id’ (Latin for it).
- **Chromosomal DNA** – the primary DNA of an organism that is packaged as chromosome(s).
- **Ori** – Origin of replication, piece of DNA that specifies where replication should begin
- **Vector** – a plasmid created in a lab to hold a specific gene or piece of DNA (sometimes also called a construct)
- **Multiple Cloning Site (MCS)** – an engineered section of DNA containing the restriction sequences for many restriction enzymes.
- **Selectable Marker** – a gene that allows identification of a cell containing the plasmid.

Applications of Plasmids

Plasmids have been of great interest to scientists since their discovery in the 1950's, helping to advance knowledge in a number of areas of bacterial genetics. They are now ubiquitously used in a diverse range of applications based on the introduction of foreign DNA into another cell. Some examples of plasmid use include:

- *The production of drugs or molecules used a therapeutics.* These are generally referred to as recombinant DNA and the most common example given is that of insulin production. The gene for insulin can be engineered into a plasmid which is then placed inside a bacteria which is allowed to grow and divide. This quickly generates millions of copies of the gene which will all express the insulin protein. This protein can then be purified from the bacterial cultures and used to treat insulin dependant diabetes.

- *The production of proteins that glow or fluoresce under certain conditions.* Plasmids can be engineered to contain a gene of interest that will produce a protein fused to a fluorescent tag. These proteins can then be expressed in eukaryotic cells or tissues to track the locations and quantity of protein expression.

- *The production of enzymes and RNA sequences designed to make specific and controlled changes to an organism's genome.* These components are both required for genome engineering using CRISPR technology. A plasmid is used to introduce a guide RNA (gRNA) as well as the the endonuclease protein (i.e., Cas9) into the cells.

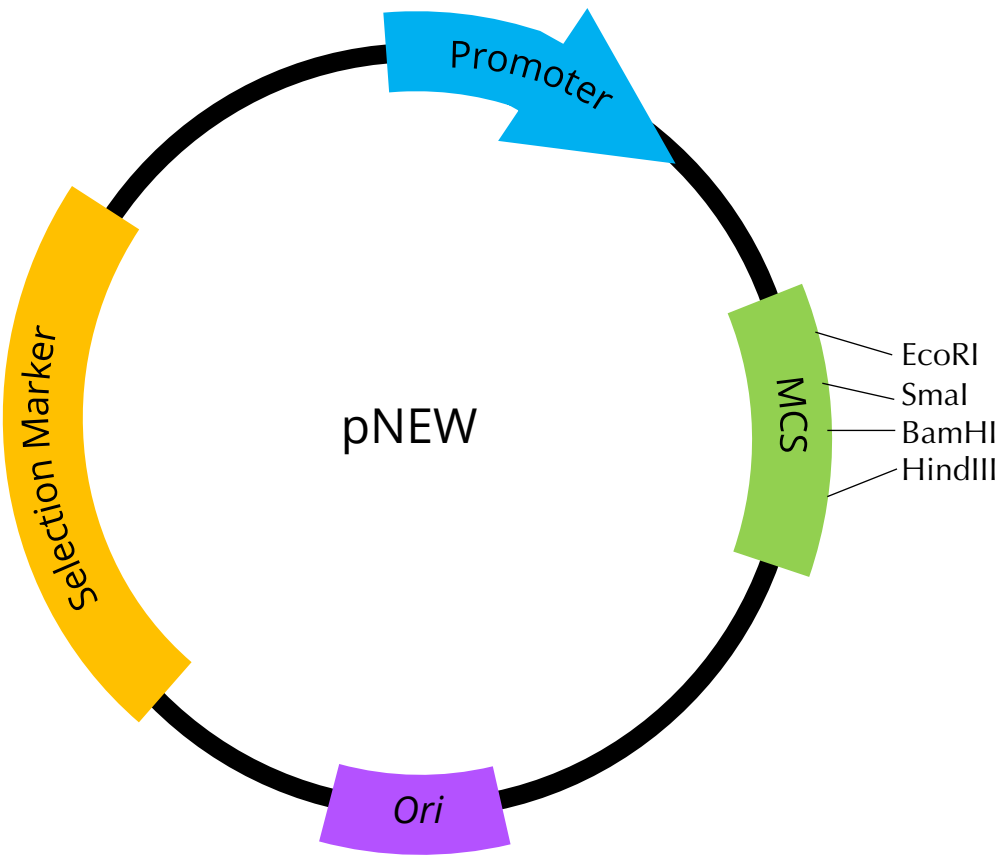
- *To reproduce sections of virus DNA (or RNA) that can be used in research.* Such synthetic viral segments can be used in the production of mRNA-based vaccines such as the Pfizer and BioNTech COVID-19 vaccines. Here the mRNA sequence for the viral antigen (the part our immune system recognises) is mass produced by insertion into a plasmid which then rapidly self replicates. The mRNA can then be purified and used to produce large quantities of vaccine rapidly and at a relatively low cost.

Activity One: Have the students complete the worksheet '**Creating a Paper Plasmid**'.

Please note all worksheet in this booklet will need to be printed single sided for the students as they will be required to cut certain portions up.

There are plasmid models that can be used alongside the paper models (or instead of, with small classes) included in our electrophoresis kits, details on these kits can be found on page 16.

The plasmid that the students will construct is representative of the plasmid pNEW shown below. For practical reasons, the paper plasmid is much smaller (in terms of Base pairs) than an actual plasmid, but still contains all relevant features.



ANSWERS

1. A plasmid is a small, circular fragment of DNA that replicates independently to the chromosomal DNA of an organism.
2. They are cheap, versatile and easy to manipulate and maintain
3. Any two applications can be accepted – students may do some research to discover applications for themselves. Examples include those detailed on page 5.
4. The selectable marker is important in the laboratory to select for cells which contain a plasmid (often by killing those that do not, though colour differentiation markers also exist).

PART B

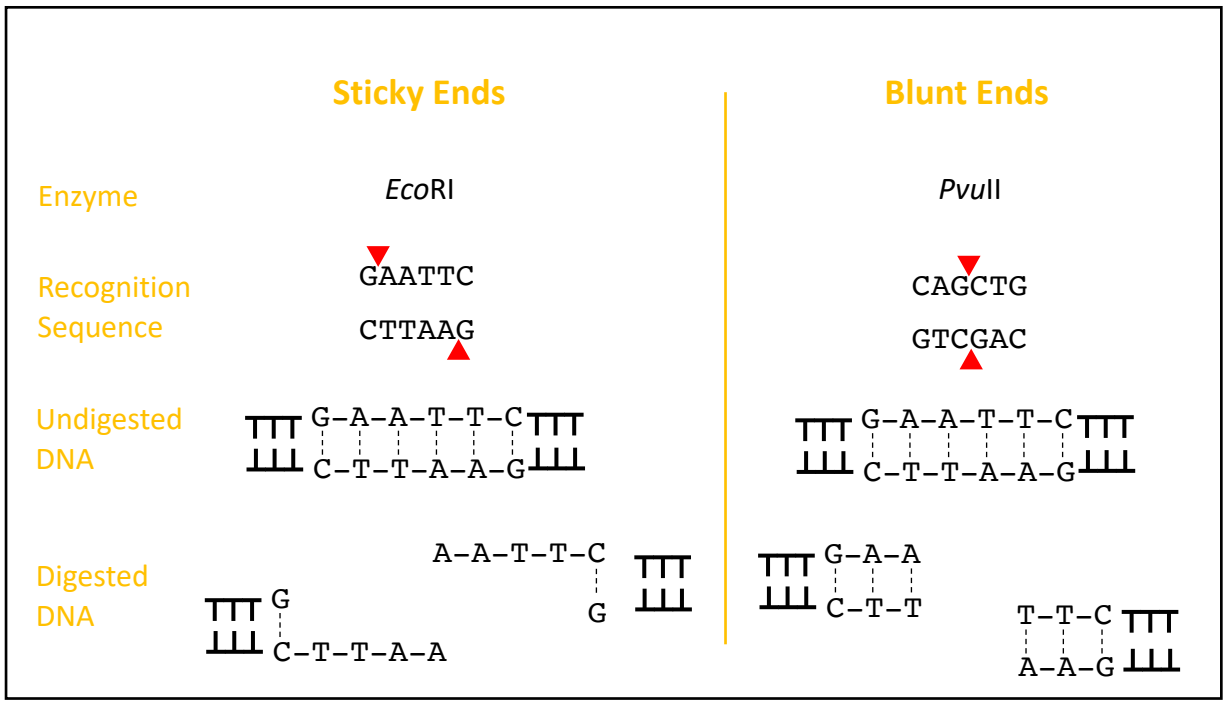
Restriction Enzymes and Digests

Restriction Enzymes

Restrictions enzymes (or endonucleases) cut DNA and are sometimes referred to as molecular scissors. They are found in bacteria and act as an immune system of sorts, targeting and cutting up foreign DNA that may enter the cell ('restricting' the infection of foreign DNA). The DNA of the host cell is protected from its own restriction enzymes by specific modifications.

Restriction enzymes are named for the species they are found in e.g., *Eco*RI is an *E. Coli* specific enzyme, and each has a specific short DNA sequence that it recognises. The enzyme cleaves the DNA at these recognition sequences which are unique to each enzyme and usually palindromic.

Broadly speaking, restriction enzymes cut in two different ways; 1) blunt cuts, where the DNA is cut straight through both strands at the same base position, and 2) sticky end cuts, in which the enzyme cuts at one base on one strand and a different base on the other strand leaving an overhang or 'sticky end' of bases on each side of the cut (see the diagram below).



Restriction Digests

Using a restriction enzyme to cut DNA is called a restriction digest. Each enzyme has its own optimal cutting conditions and so the reaction must be done at a specific temperature and salt concentration.

The restriction enzyme is placed into a small tube with the DNA and buffer (a typical reaction is 10 – 50 ul). The mixture is then incubated at the appropriate temperature for 1 – 16 hours after which it is heat shocked with a high temperature to stop the enzyme activity.

Because each enzyme cuts at only its specific recognition site restriction enzymes can be used in the laboratory to manipulate DNA in other organisms, allowing for recombination of DNA and genes.

When you are using a restriction digest as a precursor to cloning, you must digest your plasmid vector and your PCR product, containing the gene you would like to clone, with the same restriction enzyme so that they have the same sticky ends.

KEY TERMS

- **Restriction Enzyme** – (Molecular scissors) an enzyme derived from a bacteria with the ability to cut DNA (or RNA) in a very specific manner.
- **Recognition Sequence** – the short DNA sequence recognized by a restriction enzyme
- **Recognition Site –** the site within the recognition sequence where an enzyme makes its cut
- **Blunt end** – where DNA is cut through both strands at the same location leaving no overhanging bases
- **Sticky end** – where DNA is cut at a different location on each strand, leaving an overhang of 4 or 5 bases on each strand
- **Restriction Digest** –the act of using a restriction enzyme in a reaction to cut DNA
- **Ligation**– joining two pieces of DNA cut by the same restriction enzyme using ligase (molecular glue)

Activity Two: Have the students complete worksheet '**Digesting DNA**'.

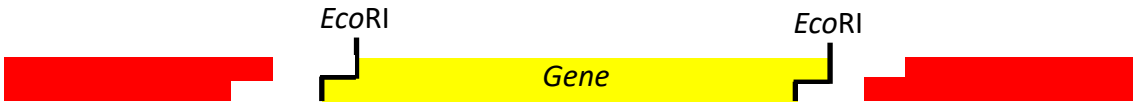
ANSWERS

1. *Bam*HI = 2x in plasmid and 2x in genomic DNA, *Eco*RI = 1x in plasmid and 2x in genomic DNA, *Hind*III = 2x in plasmid only, *Sma*I = 1x in plasmid and 1x in genomic DNA
2. The students should choose EcoR1 it cuts only once in the plasmid DNA, within the multiple cloning site so does not disrupt any plasmid genes. It also cuts twice in the genomic DNA closely flanking the target region but not disrupting it. EcoRI also creates the sticky ends we require for cloning.

The plasmid and genomic DNA that the students have 'digested' will now have this structure

pNEW

Genomic DNA



PART C

Cloning a Gene

Cloning

Cloning is a technique used in a laboratory setting to make exact copies of DNA. This is most often a single gene, but it can extend to whole organisms. Cloning occurs naturally in a number of settings; bacteria replicate by cloning themselves, meaning that all bacteria in a single colony are genetically identical, but cloning also occurs in eukaryotes including humans. All cells that undergo mitosis rather than meiosis are clones of their mother cell, this means that all of your skin cells, for example, are exact clones of one another.

There are a number of different methods of cloning available, all with different benefits and techniques. While most rely, in some way, on a linear vector joining to a linear PCR product, there are also methods that rely on recombination of DNA. Restriction digest and subsequent ligation (the original cloning technique) remains the most commonly used, due to its versatility, ease and low cost.

There are currently three main applications of cloning:

1. *Gene cloning:* this is the most common type of cloning and creates copies of single genes or segments of DNA to be used in a laboratory setting. This type of cloning is often a first step in experiments to discover the function of genes or the effects of damage to a gene.
2. *Reproductive cloning:* this creates copies of whole organisms. The first successful cloning of an entire animal was with Dolly the sheep in 2007, since then scientists have cloned a number of other mammals including pigs, cats, horses and dogs. Some scientists have hope that cloning may be able to be used in the de-extinction of animals.
3. *Therapeutic cloning:* this form of cloning creates embryonic stem cells (ESC). Researchers hope that because ESC are pluripotent (meaning they have the ability to differentiate into any type of cell), they will be able to use such cells to grow tissue to replace injured or diseased tissues in humans.

Activity Three *:* Have the students work in small groups or individually to investigate a cloning application and/or a cloning technique in more detail. They should include the technical details of the experiment as well as the biological and ethical implications of conducting such research. They could make short presentations to the class on their findings or write a short essay on the topic.

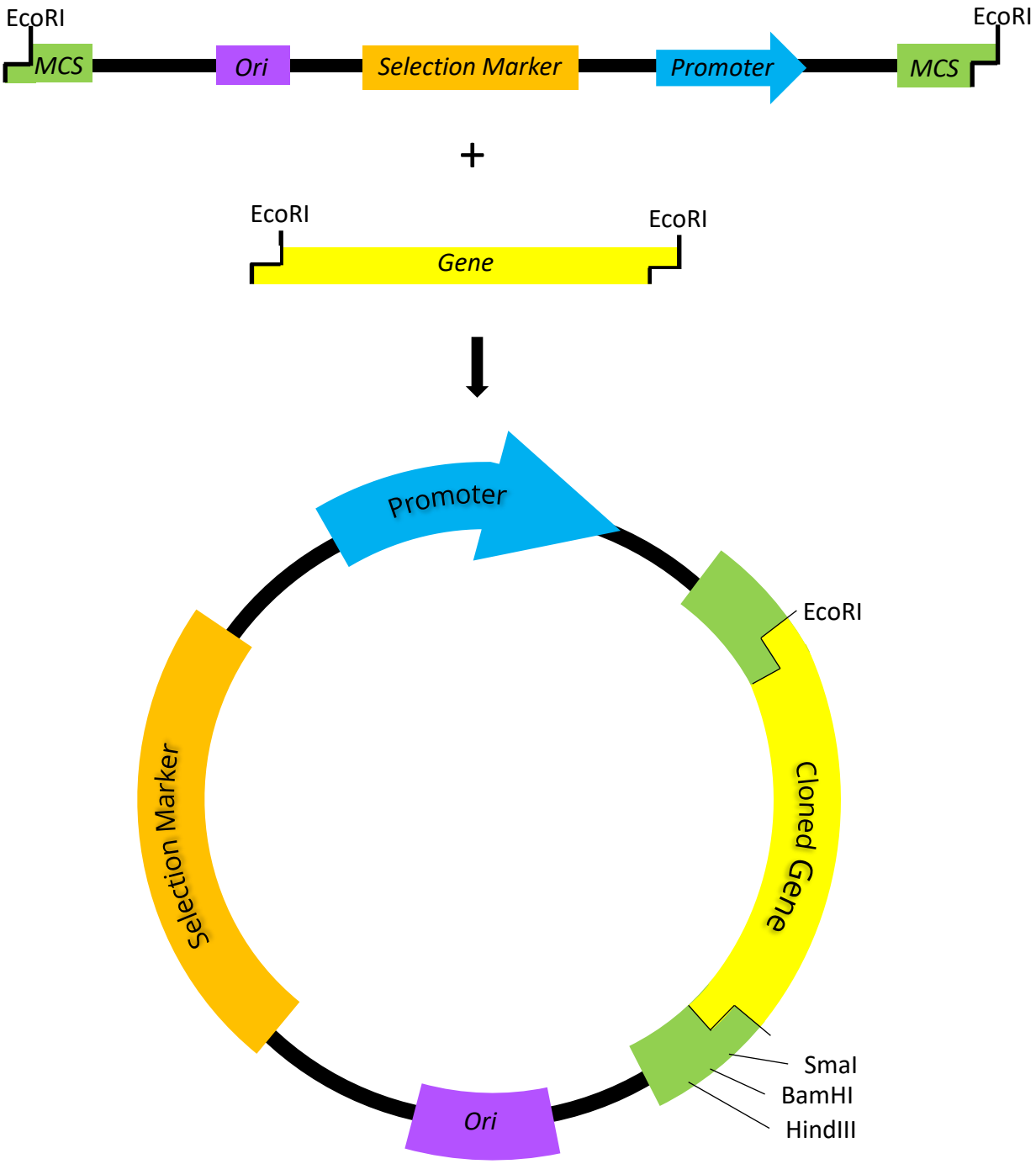
Cloning a Gene

Once the digest has occurred, DNA cut with the same enzyme can be ligated (stuck together) using another enzyme called ligase (think of this as molecular glue). This process is the basis of cloning.

A ligation reaction is also done in a small tube (total reaction volume is usually ~20ul). The reaction contains the digested plasmid vector and digested PCR product (gene of interest), DNA Ligase and a DNA Ligase buffer. This reaction is complete in ~10 mins at room temperature and is again stopped by heat shocking the Ligase enzyme. The ratio of plasmid DNA to your gene of interest is usually about 3:1, this should result in less of the plasmids simply reannealing to themselves rather than joining with a gene of interest.

Once the ligation has been completed the plasmids need to be inserted into bacterial cells in order to replicate. This process is called transformation. The transformed bacteria are then allowed to grow in a medium containing the agent requiring the selectable marker (e.g. an antibiotic), this results in only those cells that were successfully transformed with the plasmid to grow. The plasmid can then be purified from the bacteria and used in future experiments.

Following purification of the plasmid it is wise to check the success of the cloning step. This can be done by simply re-digesting a sample of the plasmid with the same restriction enzyme used previously and running the resulting reaction on an electrophoresis gel.


EcoRI
MCS
Ori
Selection Marker
Promoter
MCS
EcoRI
+
EcoRI
Gene
EcoRI
Promoter
EcoRI
Cloned Gene
SmaI
BamHI
HindIII
Ori
Selection Marker

Activity Four: Have the students complete worksheet **'Cloning a Gene'.**

If you are planning on doing the associated practical experiment (see below), this can be done as part of this activity as a means for the students to check the predictions they have made.

ELECTROPHORESIS KITS

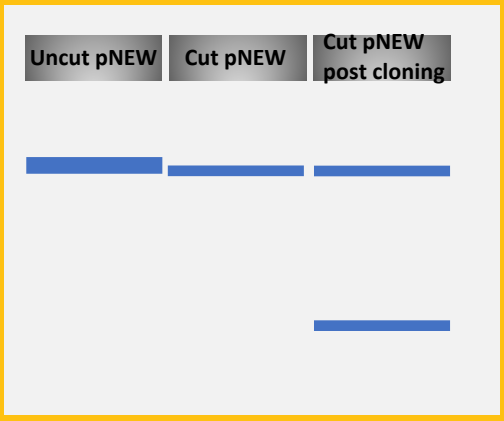
The final confirmation of cloning step can be done as a practical exercise using the Genetics on the GO kits available from us. In this case the students will make and pour an agarose gel that they will then load 'DNA' samples into and analyse the results to determine if the cloning was successful. The kit also contains 6x plasmid models that can be used instead of (or as well as) the paper plasmid exercise. If you would like to borrow one of these kits, please contact us at go@otago.ac.nz.

ANSWERS

1. The plasmid should be put into bacterial cells and allowed to grow on (or in) media containing the agent for selection (e.g., antibiotic). Once the bacteria have grown the plasmid, and the cloned gene from within it, can be purified from the cells.

2. Samples should include the uncut (undigested) original plasmid prior to cloning, the cut (digested) original plasmid and the cut plasmid containing the cloned gene..

3. The bands would appear as shown on the gel



4. The uncut pNEW plasmid will appear as a large single band. There may well be smaller or larger secondary bands in this lane, or a smear of DNA due to the DNA fragment being circular and undergoing supercoiling. The cut pNEW should run more cleanly, producing a single large band as this DNA is now linear. The final lane should show two bands, the larger of which should be the same size as the cut pNEW band and the smaller being the gene you have cloned.

Extra for experts (not in worksheet): **Ask the students what other banding patterns would be possible in the final lane?**

The final lane must contain bands because we have selected for cells that contain a plasmid earlier in the experiment, however the plasmid will not necessarily contain the gene we have cloned, resulting in the final lane looking the same as the 'cut pNEW lane'. This can happen if the digested plasmid ligates back to itself rather than to a copy of the gene (PCR product). In the lab we try to combat this by adding more PCR product than vector to the ligation mixture, this works simply by increasing the probability of the vector finding a PCR product before finding itself. This final lane could also contain a brighter large band if the vector has ligated to a second vector instead of (or as well as) the PCR product.

Creating a Paper Plasmid

The sequence 'Plasmid Sequence' is *representative* of a real plasmid sequence, we will call this plasmid pNEW. In reality, the sequence would be much longer, but all necessary components of the plasmid are represented in this sequence.

Follow the below instructions and answer the questions to create your pNEW Plasmid.

1. Cut out the columns of plasmid sequence P #1 - #5.
2. Tape the strips together in order, i.e., the top of #2 onto the bottom of #1, the top of #3 to the bottom of #2 etc. until all the strips are taped together.
3. Tape the bottom of #5 to the top of #1 to form one continuous loop. This is your plasmid pNEW.
4. Using different colours, identify and mark the following elements on your plasmid:
    - *i.* *Ori* - `TTT TCC ACA`
    - *ii.* *Promotor* — `GCA AAA TTA TTT TGG AAA AGC`
    - *iii.* *Selection Marker (Kanamycin Resistance)* – `GGA TCC GGG TCG`
    - *iv.* *Multiple Cloning Site* — `GAA TTC CCC GGG GGA TCC AAG CTT`

1. What is a plasmid?

_____

2. Why are plasmids useful in a laboratory setting?

_____

_____

3. Briefly describe two applications of a plasmid:

_____

_____

4. What is the purpose of the selection marker?

_____

_____

Plasmid Sequence

P #1	P #2	P #3	P #4	P #5
T A	G C	A T	C G	T A
T A	G C	T A	C G	T A
T A	T A	T A	G C	G C
T A	C G	T A	G C	G C
C G	G C	T A	A T	T A
C G	G C	G C	A T	C G
A T	G C	G C	T A	A T
C G	G C	A T	T A	G C
A T	A T	A T	C G	G C
A T	A T	A T	C G	G C
T A	G C	A T	C G	C G
C G	C G	G C	C G	C G
C G	T A	C G	G C	A T
C G	T A	C G	G C	T A
C G	G C	C G	G C	A T
C G	A T	G C	G C	T A
G C	A T	G C	G C	C G
A T	G C	T A	A T	C G
G C	C G	C G	T A	T A
G C	A T	T A	C G	C G
A T	A T	T A	C G	T A
T A	A T	A T	A T	T A
C G	A T	A T	A T	A T
C G	T A	A T	G C	
G C	T A	C G	C G	

Digesting DNA

Using the 'Genomic DNA' sequence and the 'Restriction Enzymes' sequences along with the paper plasmid you constructed previously, follow the instructions below to digest your plasmid and DNA ready for cloning. The part of the sequence in bold is the gene you are going to clone – remember to keep it intact! Answer the questions as you go.

1. Cut out the columns of Genomic DNA sequence G #1 - #4.
2. Tape the strips together in order, i.e., the top of #2 onto the bottom of #1, the top of #3 to the bottom of #2 etc. until all the strips are taped together.
3. Draw a box around each of the restriction enzyme recognition sequences in a different colour.
4. Search through your plasmid and the genomic DNA and outline any instances of the 4 restriction enzyme recognition sequences.
5. Choose the best enzyme and cut your plasmid and genomic DNA at all of its recognition sites (remember to cut at the correct bases on each strand)

Tips:

- The best restriction enzyme will cut the plasmid once and the genomic DNA twice.
- Do not use an enzyme that will cut through important parts of the plasmid.
- Use all the bold text in the genomic DNA, if you cut any of it you may cause a mutation
- Keep as little of the non-bold genomic DNA as possible

1. How many restriction sites did you find for each enzyme?

_____

2. Why did you choose the enzyme you did?

_____

_____

_____

_____

_____

_____

_____

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Genomic DNA

G #1	G #2	G #3	G #4
A T	A T	**T A**	A T
A T	T A	**T A**	G C
A T	C G	**G C**	A T
T A	C G	**C G**	C G
T A	T A	**G C**	C G
G C	G C	**C G**	A T
G C	A T	**G C**	G C
A T	A T	**C G**	A T
T A	T A	**C G**	A T
T A	T A	**C G**	T A
C G	C G	**C G**	T A
G C	A T	**G C**	C G
G C	A T	**G C**	A T
T A	C G	**G C**	A T
A T	**T A**	**A T**	G C
A T	**A T**	**A T**	G C
T A	**C G**	**G C**	G C
G C	**A T**	**C G**	G C
T A	**A T**	**C G**	A T
G C	**A T**	**T A**	T A
T A	**A T**	**A T**	C G
C G	**A T**	**G C**	C G
A T	**A T**	T A	T A
G C	**A T**	A T	G C
G C	**T A**	T A	C G

Restriction Enzymes

BamHI:

```
G | C
G | C
A | T
T | A
C | G
C | G
```

SmaI:

```
C | G
C | G
C | G
G | C
G | C
G | C
```

EcoRI:

```
G | C
A | T
A | T
T | A
T | A
C | G
```

HindIII:

```
A | T
A | T
G | C
C | G
T | A
T | A
```

Cloning a Gene

Now that you have digested your plasmid and genomic DNA you can ligate your gene of interest into your plasmid.

Follow the instructions below and answer the questions to ligate your plasmid and then predict what results you would get if you were to check your cloning in a lab.

1. Line up the sticky ends of your plasmid with those of your gene of interest
2. Use tape to stick the gene into the plasmid, completing the circle.

1. Briefly describe what you would now do with your plasmid to clone your gene.

To ensure that the cloning has been successful, the purified plasmid is re-digested with the same restriction enzyme used to construct it. The resulting product is checked for size by using gel electrophoresis.

2. Label the lanes of the gel below with the 3 samples that should be run. (Hint: think about which controls you should use).

3. Draw bands onto your gel, predicting what you should see for each lane.

4. Explain why each lane is giving the result you predict.

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We hope you have enjoyed this lesson. Feedback is very welcome to:
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