illuminating

dna

An NCBE / Unilever educational guide

to the reader

Work with DNA is central to many, if not most, developments in modern biotechnology. There is growing public awareness of DNA technologies, their possible applications and wider implications. However, much of the essential debate about current genetics has generated more heat than light.

Fortunately, the basic science upon which DNA technologies are founded features in nearly every school examination syllabus in biology or science. This booklet is intended primarily for post-16 students of biology and their teachers.

The practical exercises described here provide an introduction to some of the classical techniques of molecular biology in a form suitable for the school laboratory. For reasons of safety and expense, some the work in this booklet is not particularly suited to open-ended practical investigations, but some ideas that may provide starting points for additional work of your own are given on pages 36–37. More ideas are provided in other NCBE publications such as: *The Lambda Protocol*; *Investigating Plant DNA* and *The Transformer Protocol*. The latter publication also provides an introduction to ethical, social and other issues raised by DNA technology. All of these publications, the other practical guides in this series, and supplementary resources are available from the NCBE's Web site:

http://www.ncbe.reading.ac.uk

An adequate treatment of the wider issues raised by DNA technology (particularly those associated with human genetics, environmental concerns or food biotechnology) cannot be given in this slim booklet, nor can it take the place of the many excellent school textbooks covering basic biochemistry and molecular biology. It aims merely to provide sufficient information for you to understand the practical exercises. References to other materials are provided throughout and on the back cover. The NCBE's Web site offers a more comprehensive list.

equipment and materials

In this booklet we have tried to describe practical protocols that are relatively inexpensive and require little specialist equipment.

The NCBE endeavours to develop and supply at low cost several of the items required, such as electrophoresis apparatus, restriction enzymes, plasmids and so on.

Because the details of suppliers and their products can change, we have decided not to list them in this booklet. Instead, a supplement giving up-to-date information is available from the NCBE's Web site, or it can be obtained on request from the NCBE. Our address is given on the back page.

Some of the investigations described here require a high-speed microcentrifuge. An inexpensive, award-winning microcentrifuge for school use has been developed by our friend Professor John Cave of Middlesex University and the *Technology Enhancement Programme (TEP®)*, with help from *Science and Plants for Schools* (SAPS) and the NCBE.

acknowledgements

The production of this booklet was very kindly supported by *Unilever plc*. The contents are entirely the responsibility of the NCBE however. *Unilever's* only conditions in sponsoring this booklet were that we made the publication freely available *via* the Internet, and that we consulted our colleague, Dr Malcolm Thomas, in the Department of Education at the University of Aberystwyth. Malcolm made many helpful comments, for which we are grateful. Marjorie Smith of Dollar Academy was very supportive too, giving us tips for making this booklet more palatable to those in Scotland. The molecular structure data for several of the figures came from the *Protein Data Bank* and the *Nucleic Acid Database*; accession numbers are given by the figures. Any errors in this booklet are the author's, of course.

COPYRIGHT

The material in this booklet is copyright.
The author and illustrator, Dean Madden, has asserted his moral right to be identified as the copyright holder under Section 77 of the Copyright, Designs and Patents Act, UK (1988).

PHOTOCOPYING

Photocopies of this booklet, or selections from it may be made for educational use, provided that the copies are distributed free-of-charge or at the cost of reproduction, and the author of the materials is credited and identified as the copyright holder.

contents

dna: molecule of the century

If you were to nominate a 'molecule of the 20th century', one of the best candidates would surely be DNA. Although the science of genetics began around 1900, when the work of Mendel was rediscovered, it wasn't until the 1950s that techniques were developed allowing the structure and function of genes to be probed. The last quarter century has seen a revolution in molecular biology, bringing new knowledge that casts light on the essential unity of life on Earth.

DNA, genes and chromosomes

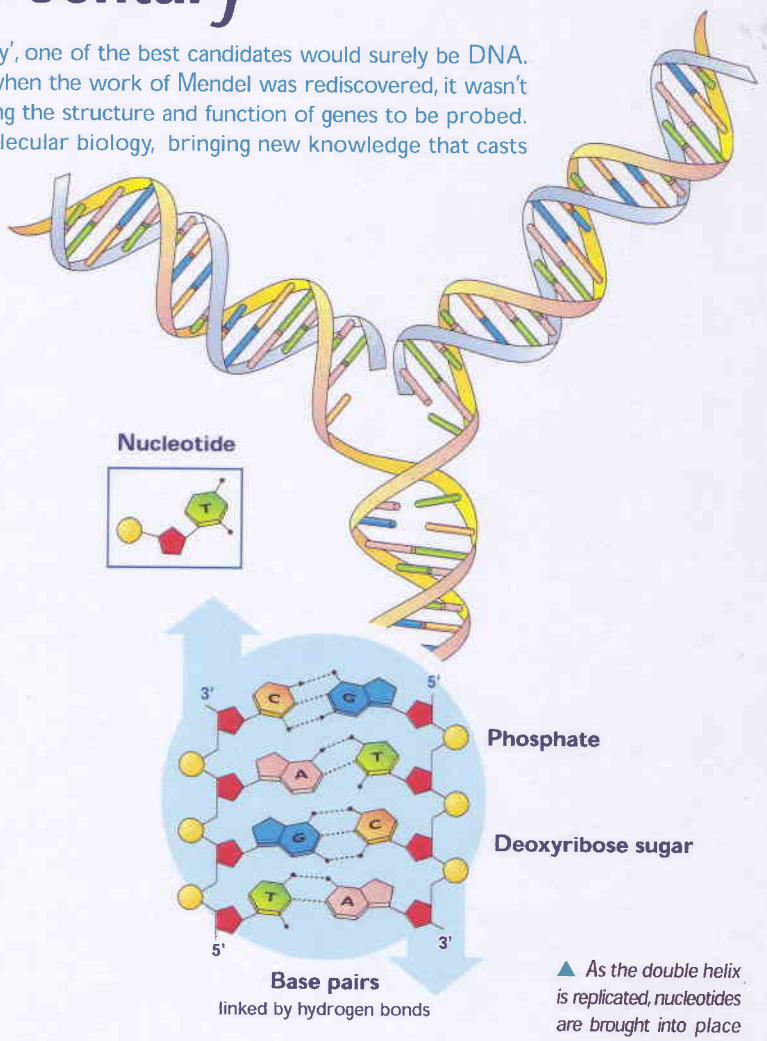
DNA (*deoxyribonucleic acid*), faithfully copied from one *cell* division to the next and passed on through the generations, forms a living link back to some of the first life on our planet. The DNA in our cells is both a record of where we've come from and a predictor of how we might develop, both as individuals and in subsequent generations. Although our environment plays a vital rôle in shaping development, DNA determines all inherited characteristics and is therefore of fundamental importance in understanding biology.

Most DNA is packaged, in fungal (including yeast), plant and animal cells, into *chromosomes* within the nucleus of each cell. Some DNA is also found outside the nucleus: in the *mitochondria* (which release energy for cellular activities); and within the *chloroplasts* (sites of photosynthesis) of plant cells. In bacteria, most of the DNA occurs in a single circular chromosome, although small rings of DNA, called *plasmids*, may also be present.

The *double helix* of DNA can be likened to a twisted rope ladder. The two intertwined helices are chains made from sugar and phosphate molecules linked together alternately. Attached to each sugar molecule is a nitrogenous '*base*'. There are four different bases in DNA: adenine (A); thymine (T); cytosine (C) and guanine (G). Relatively strong *hydrogen bonds* between the bases join the two strands of the double helix together like the rungs of a ladder. A always pairs with T, and C always pairs with G. This '*base pairing*' mechanism ensures identical replication of DNA strands during cell division.

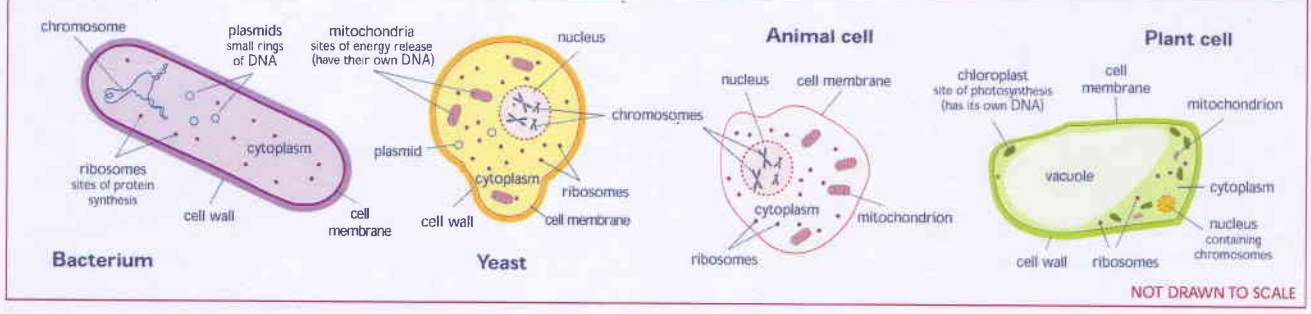
A *gene* is a length of DNA that determines the structure of all or part of a specific *protein*. How does this happen? Proteins are comprised of chains of *amino acids*. The sequence of bases on one DNA strand indirectly spells out the amino acids that are needed to make these chains. A short chain is called a *polypeptide*. A typical protein might be made up of several polypeptide chains linked together and folded into a precise three-dimensional structure.

Each amino acid in a polypeptide is encoded by three consecutive bases in the DNA. The code determining which amino acid each triplet of bases *translates* into — *the Genetic Code* — is virtually the same in all living things.



▲ *As the double helix is replicated, nucleotides are brought into place alongside those of the existing DNA strands. In this way each new double helix consists of a strand from the old molecule (shown here in yellow) and a completely new one (mauve). This type of replication is called 'semi-conservative'.*

cellular components



Also encoded within the DNA are sequences that regulate protein production. These are needed because although most cells of a particular organism contain the same DNA, only certain proteins are made at any one time or in any particular type of cell: that is, only certain genes are *expressed*.

Because the genetic code is universal, it is possible to transfer working genes between organisms — even between completely different species. The process of directly and deliberately transferring, removing or altering genetic information is commonly called *genetic modification* (or *genetic engineering*).

Protein synthesis

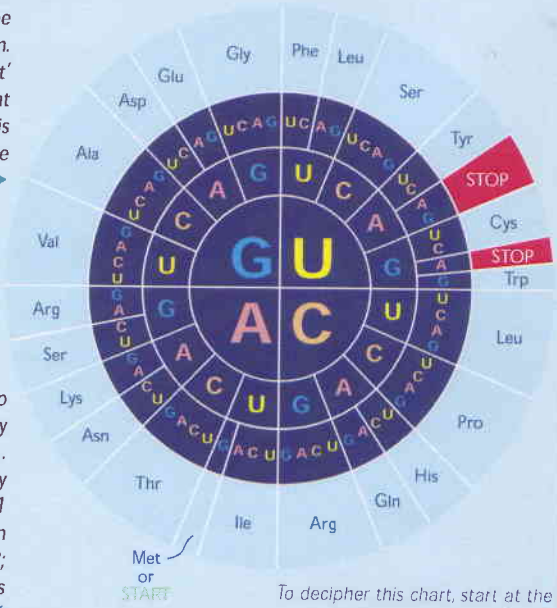
RNA — the genetic go-between

Most of a cell's DNA lies within its one or more chromosomes. However, proteins are not made there, but at special structures called *ribosomes*. Therefore before a particular protein can be made, a copy of the appropriate genetic instructions must first be *transcribed* from the DNA then ferried to the ribosomes. The copied instructions are made from *mRNA* (*messenger ribonucleic acid*), and assembled by an enzyme called *RNA polymerase*. This mRNA is virtually a 'mirror image' of the sequence of bases on one DNA strand, according to the base pairing rules.

On arrival at the ribosomes, the base sequence within the mRNA directs the construction of proteins from amino acids. A sequence of three adjacent bases (a *codon*) in the mRNA molecule determines each amino acid in a polypeptide chain. The amino acids are brought into place by *tRNA* (*transfer RNA*) molecules, which have *anticodons* mirroring the codons in the mRNA.

The Genetic Code

The Genetic Code indicates the amino acids that are encoded by each group of three mRNA bases or codon. There are also 'start' and 'stop' codons that control the synthesis of each polypeptide chain. ▶



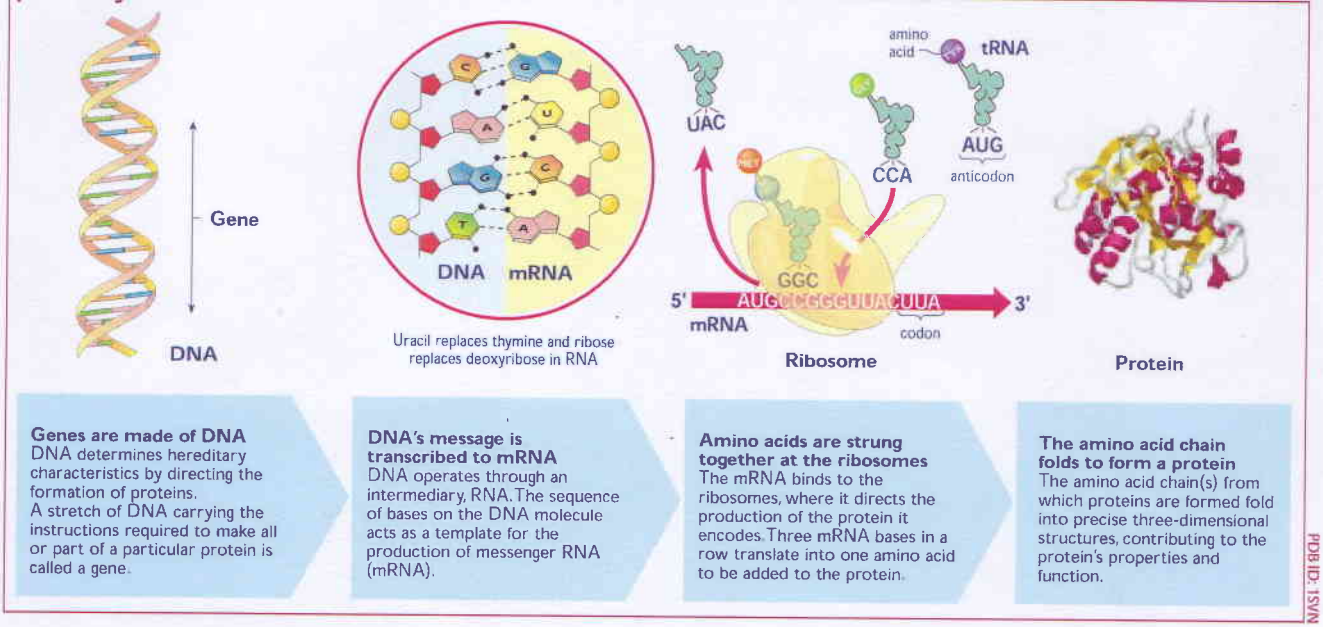
To decipher this chart, start at the centre and read outwards (5'→3').

There are 20 amino acids that commonly occur in proteins. Proteins vary greatly in size: insulin has 51 amino acids; human hæmoglobin has 578; urease (an enzyme) has 4 500. ▼

Alanine	Ala	Glycine	Gly	Proline	Pro
Arginine	Arg	Histidine	His	Serine	Ser
Asparagine	Asn	Isoleucine	Ile	Threonine	Thr
Aspartic acid	Asp	Leucine	Leu	Tryptophan	Trp
Cysteine	Cys	Lysine	Lys	Tyrosine	Tyr
Glutamine	Gln	Methionine	Met	Valine	Val
Glutamic acid	Glu	Phenylalanine	Phe		

The sequence of amino acids in a protein is called its ***primary structure****. The way in which each amino acid chain is twisted (e.g., to form coiled α-helices and flat β-pleated sheets) is its* ***secondary structure****. To show this here, the α-helices are coloured magenta; the β-sheets are yellow, with the arrows pointing to the C-terminus of the polypeptide chain. The amino acid chain(s) fold in on themselves to give a precise shape to the protein — its* ***tertiary structure****.* ▶

protein synthesis



Genes are made of DNA
DNA determines hereditary characteristics by directing the formation of proteins.
A stretch of DNA carrying the instructions required to make all or part of a particular protein is called a gene.

DNA's message is transcribed to mRNA
DNA operates through an intermediary, RNA. The sequence of bases on the DNA molecule acts as a template for the production of messenger RNA (mRNA).

Amino acids are strung together at the ribosomes
The mRNA binds to the ribosomes, where it directs the production of the protein it encodes. Three mRNA bases in a row translate into one amino acid to be added to the protein.

The amino acid chain folds to form a protein
The amino acid chain(s) from which proteins are formed fold into precise three-dimensional structures, contributing to the protein's properties and function.

PDB ID: 1SWN

Enzymes — precise molecular tools

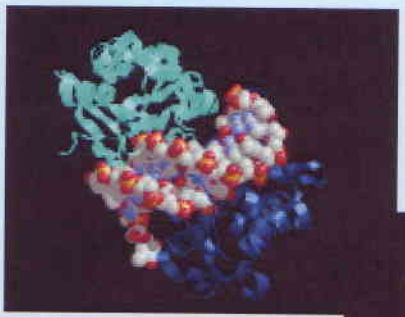
Restricting DNA

Special *enzymes* obtained from bacteria are an essential tool of the molecular biologist. In nature, these enzymes help bacteria to fend off viral attack by precisely dissecting the 'foreign' DNA of invading viruses. In this way, the proliferation of the *viruses* is restricted. *Restriction enzymes* (as they are known) 'recognise' and cut DNA molecules at specific locations. Many hundreds of different restriction enzymes have been isolated from microbes and are available commercially. These restriction enzymes allow almost any section of DNA, and so any given gene, to be isolated.

Sticky ends

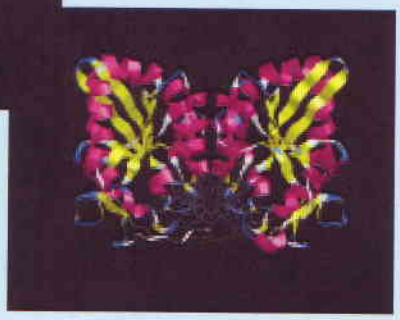
Restriction enzymes leave blunt or staggered ('sticky') ends on the DNA sequences they cut. The end of one DNA molecule with a sticky end will readily link (by hydrogen bonding) to that of another cut with the same enzyme. To join two DNA molecules permanently, however, it is necessary to form stronger chemical bonds along the DNA's sugar-phosphate backbone. An enzyme called *DNA ligase* can do this job.

The function of restriction enzymes and ligase in cutting and pasting DNA molecules is obvious, but the genetic engineer's tool kit would be incomplete without one or two other enzymes.



▲ *Restriction enzyme BamHI, attached to a short length of DNA. Here, the DNA is shown as a space-filling model, while the two polypeptide chains of the restriction enzyme are depicted as blue ribbons.*
NDB ID: PDE020

▼ *The restriction enzyme BamHI, attached to a DNA molecule. This view, down the axis of the DNA, shows the symmetry of the enzyme. For clarity, the DNA is shown as a wireframe structure.*



Source microorganism STRAIN Name	Recognition site (5'→3')
Bacillus amyloliquefaciens H	
BamHI	G↓GATCC
Escherichia coli RY13	
EcoRI	G↓AATTC
Bacillus globigii	
BglI	A↓GATCT
Haemophilus influenzae Rd	
HindIII	A↓AGCTT
Arthrobacter luteus	
AluI	AGC↓T
Thermus aquaticus	
TaqI	T↓CGA
Haemophilus aegyptius	
HaeIII	GG↓CC

◀ *Restriction enzymes bind to specific sequences of bases in double-stranded DNA molecules. They cut the DNA at or near these recognition sites, leaving fragments with blunt or staggered ends. The enzymes take their names from the various microbes from which they are obtained.*

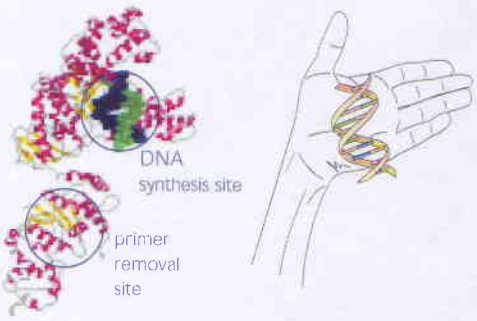
◀ *DNA ligase. ATP provides the energy for this enzyme to work.*
PDB ID: 1A01

DNA from RNA

The enzyme *reverse transcriptase* can be used to assemble a single strand of *complementary* DNA alongside a corresponding piece of mRNA. This is the reverse of the usual situation where DNA dictates the formation of RNA molecules.

Reverse transcriptase can be useful because cells that are making a particular protein will have many more copies of that protein's mRNA in them than the number of copies of the gene itself. It is therefore often easier to search for genetic information amongst these small mRNA molecules rather than along the entire length of the cell's chromosomal DNA.

the most accurate enzyme



PDB ID: 1TAU

The computer image above represents DNA polymerase from the hot-water bacterium *Thermus aquaticus* (*Taq*).

The enzyme is shaped like a hand, with the DNA under replication being held in the 'palm'. A small fragment of DNA is shown here with the newly-formed strand in green and the complementary template strand (against which it is being assembled) in blue.

Unusually, this enzyme has *two* active sites; the one in the lower part of the molecule removes the short 'primers', which are needed to start replication from the finished DNA molecule. DNA polymerases from other organisms go a step further, however, and have a *third* active site, which 'proof reads' the new DNA for errors as it is formed. Human DNA polymerase copies DNA so accurately that less than one error occurs in every billion bases.

Taq DNA polymerase is stable at high temperatures and is used in the polymerase chain reaction (see page 10).

DNA polymerase

Once a desired length of mRNA has been isolated, and the corresponding DNA constructed, a second enzyme — *DNA polymerase* — can be used to assemble a double-stranded helix, using the first DNA strand as a template. All living things make DNA polymerases, and a cell often has several different varieties of the enzyme: some for helping to replicate DNA during cell division and others that conduct day-to-day repair and maintenance.

In humans, several complex proteins act together to ensure that DNA is faithfully copied down the generations; in many other organisms, one multi-functional enzyme does it all.

Gene machines

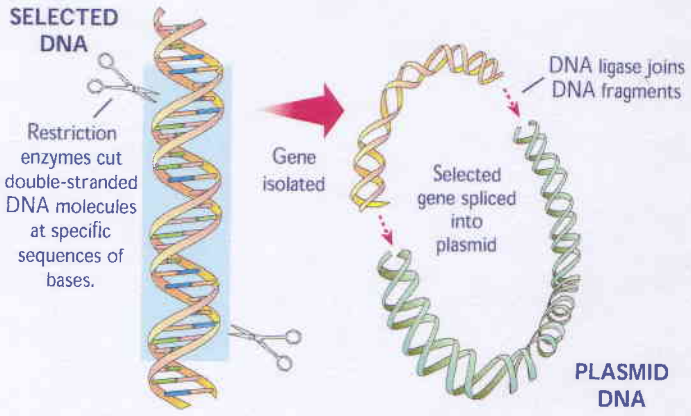
By the judicious use of restriction and other enzymes, molecular biologists are able to assemble DNA molecules that contain one or more genes of interest. Where a particular piece of DNA is difficult to isolate, it is sometimes possible to make it artificially using a *DNA synthesizer*. Under computer control, these machines string together the biochemical building blocks needed to make short stretches of DNA. Of course, to programme the synthesizer it is necessary to know the sequence of bases present in the desired gene; this too can be determined automatically using a *DNA sequencer* (see *Cycle sequencing*, page 13).

Plasmids as vectors

The construction of a suitable DNA molecule is only part of the story. Once this has been done it is necessary to move that molecule into a cell in which it may be expressed and duplicated so that it passes from one cell division to the next. For microorganisms, one of the most successful methods involves the use of plasmids as a vehicle (a *vector*) for transferring genes.

These small rings of DNA carry a limited set of genes and normally constitute only a tiny fraction of the cell's total DNA. Like the DNA of chromosomes, that of plasmids can be cut with restriction enzymes and additional DNA pasted into it. The result is a ring of *recombinant DNA*, which can be put into a bacterium. Specialised plasmids can be used to ferry genes from bacteria into yeast cells or even into plants.

recombinant dna



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.

Changing an organism's genetic make-up by adding 'naked' DNA such as plasmids is called *transformation*. Transformation is a relatively rare event in nature — most cells cannot take up DNA from their environment without first being made 'competent'. This can be done in several ways.

A popular method is to treat the cells with chemicals that produce temporary pores through the cell membrane, then to heat shock the bacteria in the presence of the DNA that is to be introduced. Because both the DNA and cell membrane are electrically uncharged in the circumstances used, the DNA can pass through the pores.

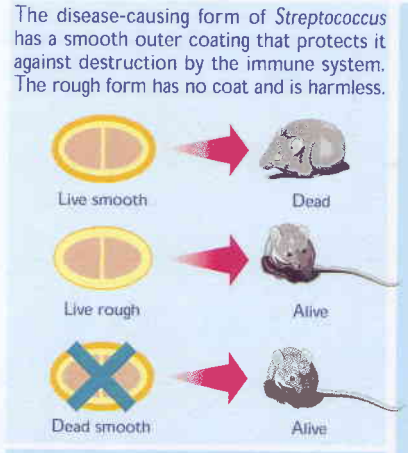
the search for the genetic material

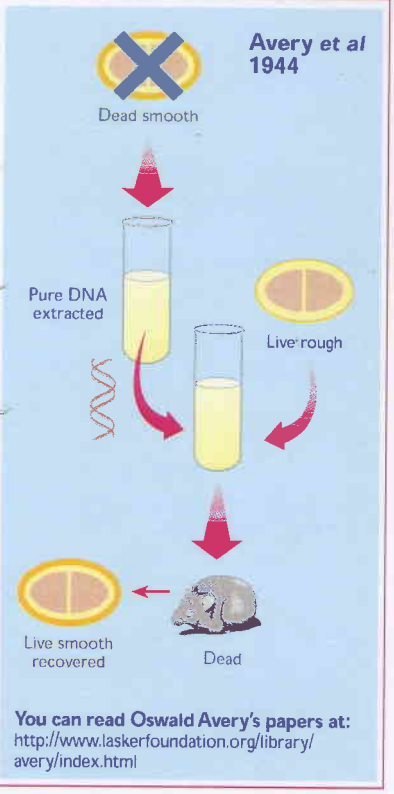
Fred Griffith. Photo courtesy Society for General Microbiology

In 1928, a British medical officer at the Ministry of Health made a remarkable discovery. Fred Griffith found that one strain of *Streptococcus pneumoniae* (then known as *Pneumococcus*) could be converted into another by an unknown, non-living material. The nature of this 'transforming principle' remained a mystery for the next 15 years.

Oswald Avery and his colleagues in the USA carried out meticulous experiments for more than a decade to reveal the identity of the mystery material. Unfortunately, even though their findings (published in 1944) look conclusive to a modern observer, some scientists at the time argued against the Avery team's conclusion that DNA was the molecule of inheritance. Its molecular structure was thought to be too simple to carry the genetic message.

Not until 1952 — just a year before the publication of Watson and Crick's famous 'double helix' letter to *Nature* — were the majority of scientists convinced that DNA was the primary genetic material. It was then that Alfred Hershey and Martha Chase (then an undergraduate) proved, using radiolabelled DNA and protein, that the infective component of a bacteriophage was nucleic acid, and not the structurally more complex proteins.





Genetically modified plants

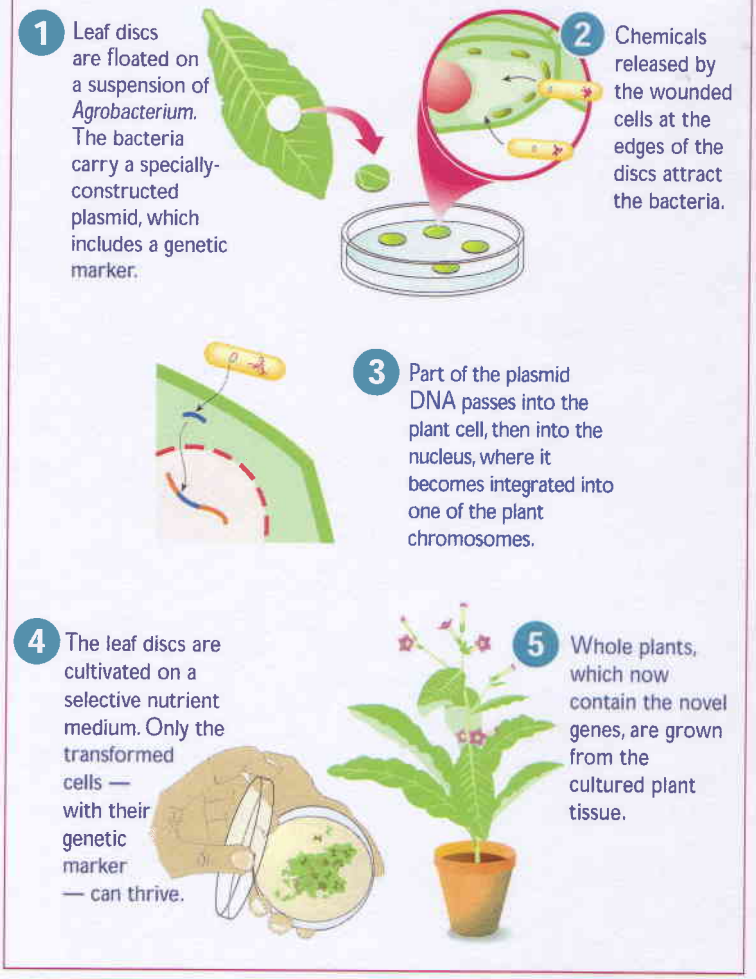
Nature's engineer

A genetic transfer system that is used for a wide variety of plants is the plant tumour-inducing plasmid (*Ti-plasmid*), found in the soil bacterium *Agrobacterium tumefaciens*. Through its plasmid, *Agrobacterium* has the ability to naturally engineer plant cells so that they grow into galls, the cells of which produce special compounds that the bacteria need to thrive.

Molecular biologists use 'disarmed' (non-tumour-inducing) versions of this plasmid to introduce genes of their choice into plants. As every cell carries a complete copy of all the plant's genes in its chromosomes, it is possible to re-grow an entire plant from a single modified cell. Special plasmids have now been constructed to help transfer fairly large genes into plants.

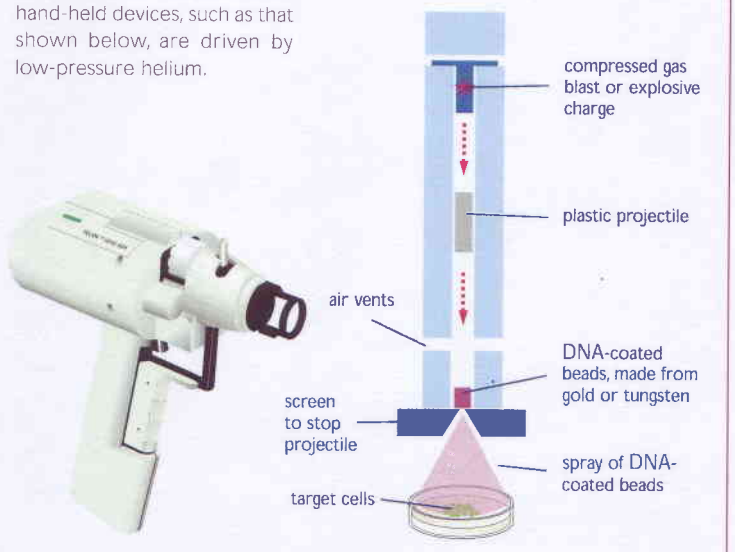
The first species to be modified in this way was tobacco, chosen because it and related plants (*e.g., tomatoes and potatoes*) are easy to propagate by tissue culture. *Agrobacterium* has been especially useful when working with trees which, because they are slow-growing and large, are difficult to improve by conventional plant breeding. Apricot, plum, apple and walnut trees have all been genetically modified with the help of *Agrobacterium*. Unfortunately, most mono-cotyledons (including several important cereal crops) are resistant to infection by *Agrobacterium*. Alternative methods have to be found for these sorts of plants.

transformation with agrobacterium



particle bombardment

Particle bombardment is used to genetically modify a wide variety of species. The first 'gene guns' were housed in heavy metal boxes and used explosive cartridges. Safer, hand-held devices, such as that shown below, are driven by low-pressure helium.



Blasted DNA

A procedure called *particle bombardment* or *ballistic impregnation* has achieved success with several important crops, including rice, wheat and soya.

With this method, the DNA to be introduced into the plant cells is first stuck onto minute tungsten or gold beads. Using a 'gene gun', the DNA-coated particles are fired into soft plant tissue *e.g., callus cells from tissue culture*. This introduces novel DNA into the plant cells, some of which, by chance, integrates into the plant chromosomes.

The cells are grown into miniature plantlets by cultivating them on a special medium that contains appropriate plant growth substances and a selective agent (see *Marker genes*, page 10) that permits only the modified cells to grow. These plantlets are eventually grown up by conventional means in soil.

Screening is necessary to select those plants in which the introduced genes are expressed. Careful checks are also made to ensure that the expression of other genes in the host plant is not affected by the novel introduction.

Efficient electroporation

DNA can also be introduced into the thin-walled tubes that develop from pollen grains by subjecting them to micro- or millisecond pulses of a strong electric field. This technique, known as *electroporation*, causes pores to appear momentarily in the pollen tubes, through which DNA from a surrounding solution can enter. Seeds that develop from ovules fertilised with such pollen carry the introduced genes.

Electroporation also works with plant cells from which the cell wall has been removed by enzyme treatment. From these naked plant cells (called *protoplasts*), whole plants can be regenerated by tissue culture. Entire plant cells can also be treated in this way, albeit with less success than with protoplasts. Electroporation is also used routinely in research to transfer recombinant DNA molecules into a range of microorganisms. It is the most efficient genetic transformation method so far for bacteria.

Genetically modified animals

The DNA of animals can also be modified, but it is necessary to introduce genes at an early stage of development if they are to be present in all the cells of a mature animal and be passed on to its offspring. DNA can be injected into newly-fertilised egg cells through a very fine glass pipette. Only a small proportion of the injected eggs take up the new genes; those that do are transferred into the uterus of a suitable foster mother.

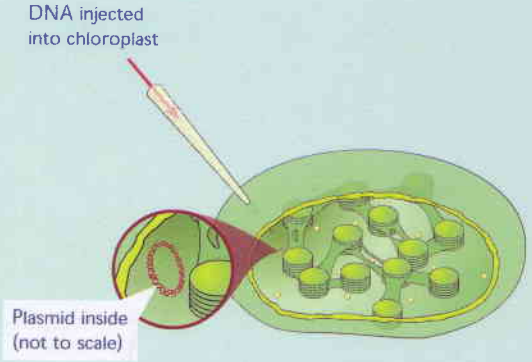
This crude method of genetic modification works for mice, cows, pigs, sheep and goats. Micro-injection can also be used to introduce new genes into fish eggs, but, for obvious reasons, it is not suitable for birds' eggs. However, specially-modified viruses have been used to introduce, for example, disease-resistance into chickens. The viruses are inserted through the shell of the egg. Similar viral vectors can also introduce genes into mammalian cells.

Chloroplast transformation

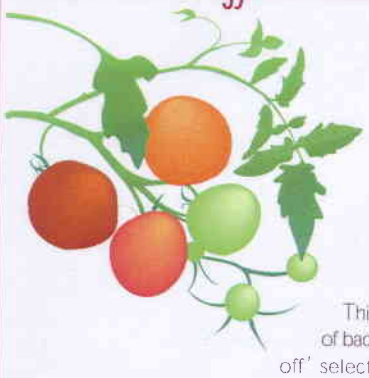
One of the major public concerns about genetically-modified organisms is that they might transfer their introduced genes into other, non-modified organisms with unpredictable and possibly undesirable consequences. Scientists are aware of this problem and have proposed several methods of preventing it. A promising development in plants is the genetic modification of chloroplasts.

Chloroplasts carry their own DNA, which is passed on (in most species) *via* the ovules alone; chloroplast DNA does not end up in pollen grains. If the DNA of chloroplasts could be modified, therefore, it might be possible to prevent the unwanted 'spread' of novel genes *via* pollen.

For the first time in 1999, hollow glass needles with tips a mere 0.1 µm in diameter were used, under microscope guidance, to inject DNA directly into chloroplasts. Such fine tips do not damage the chloroplast membrane, but very high pressures are required to push the DNA from the needle. Controlling the flow of DNA under high pressure is a significant practical problem. It was overcome by sealing a liquid metal alloy into the needle, behind the DNA. When the alloy was heated, it expanded, forcing the DNA out in a controllable manner. Researchers have also used same technique to inject DNA directly into individual bacteria and the nuclei of eukaryotic cells. It remains to be seen how useful this experimental method will become.

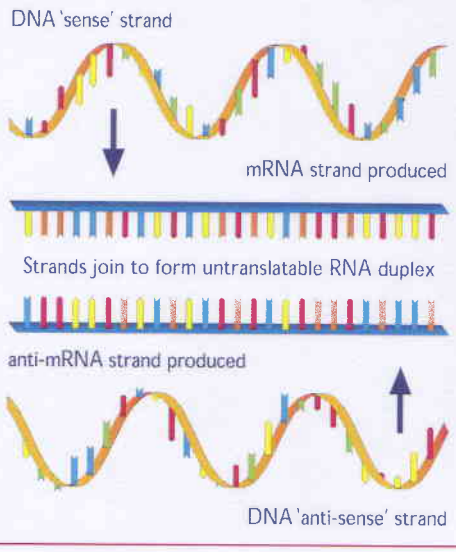


antisense technology

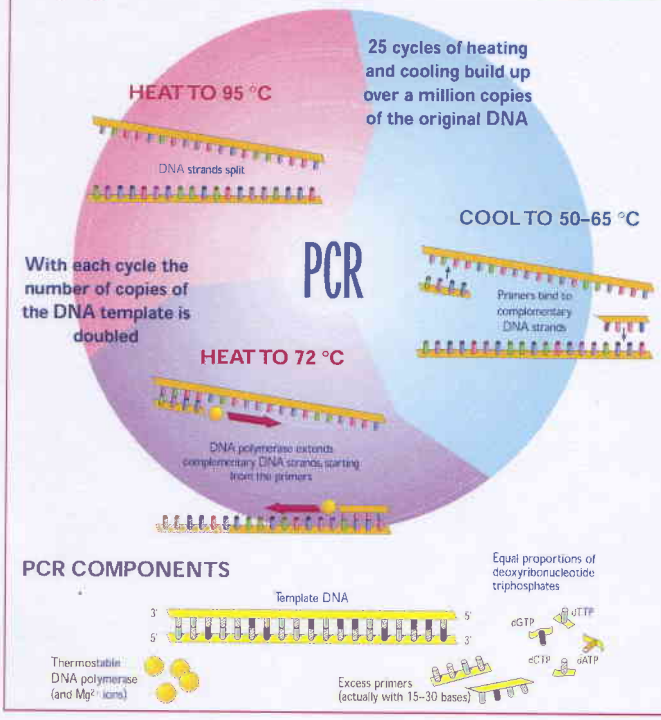


The sequence of bases along only one strand of the DNA double helix directs the production of proteins. This strand is called the 'sense' strand. Antisense RNA is an RNA sequence transcribed from the 'wrong' (antisense) strand of the DNA. When antisense and sense RNA molecules meet, they combine, preventing the production of proteins encoded by their sequences of bases.

This process, which occurs naturally in the control of bacterial genes, can be used by biologists to 'switch off' selected genes. This is done by inserting an appropriate piece of inverted DNA into the sense strand of the cell's DNA. Antisense RNA molecules are then formed from the inverted section when the DNA is transcribed. A diverse range of experimental crops has been produced using this technology, including for a short time in the USA, slow-ripening tomatoes (in which a pectinase gene was 'silenced') although these were never sold in Europe.



the polymerase chain reaction



The components

- Two primers are made that flank the specific stretch of DNA to be copied. These are generally 15–30 bases long, and are complementary to the 5' end of each DNA strand.
- Total (double-stranded) DNA is extracted from the sample under study.
- The DNA and excess primers are mixed.
- Equal proportions of very pure deoxyribonucleoside triphosphates (dCTP, dATP, dGTP, dTTP) are added.
- Heat-stable *Taq* DNA polymerase (originally obtained from the hot-water bacterium *Thermus aquaticus*, but now produced by genetically-modified organisms) is added to the reaction mix, together with Mg^{2+} ions that are required as a co-factor by the enzyme.

The process

- The double-stranded DNA is split into two single-stranded templates by heating it to 95–98 °C.
- The mixture is cooled to 50–65 °C. The primers bind (hybridise) to the complementary strands of DNA.
- Heating to 72 °C (the optimum temperature for *Taq* DNA polymerase) encourages the synthesis of new DNA strands alongside the templates.
- The mixture is heated again to separate the newly-formed DNA strands from their partners.

The cycle of cooling and heating is repeated. With each cycle (lasting about 2 minutes) the number of copies of the template DNA is doubled. After 25 cycles more than a million copies of the DNA will have been made.

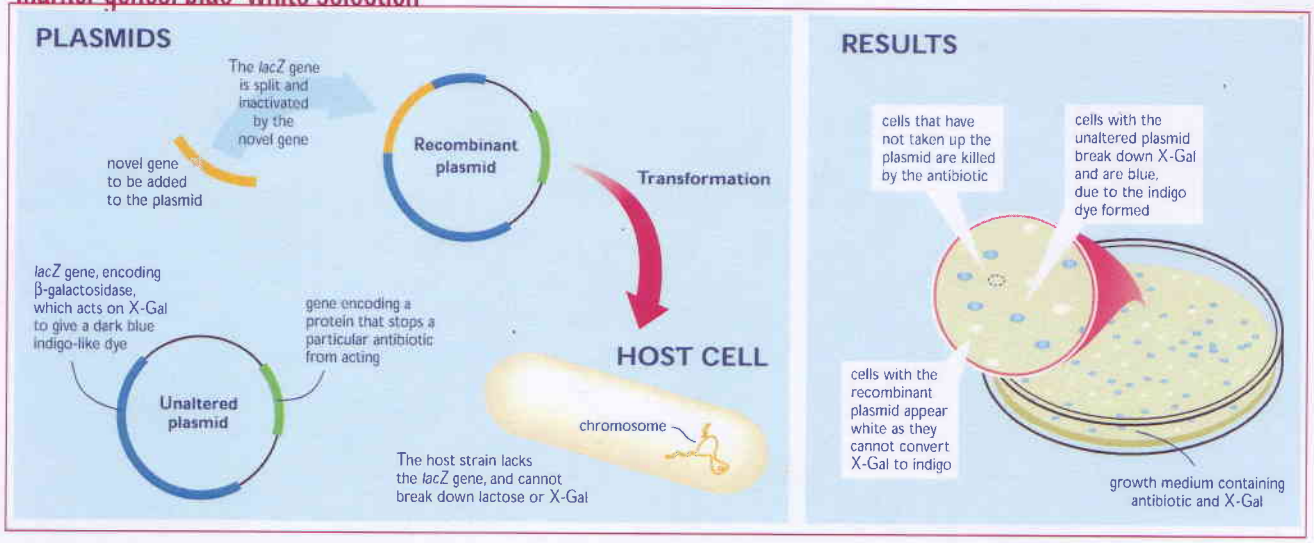
Marker genes

Whatever method is used for genetic modification, only a small proportion of treated cells will take up DNA in a stable and heritable manner. Screening is therefore necessary to discover which cells have done so. Some genes confer upon their hosts the ability to overcome the harmful effects of specific heavy metals, herbicides or antibiotics. Such genes can be used as '*markers*' to identify those cells that have taken up DNA. This is done by coupling the marker gene to the other genes that are to be introduced. When the transformed cells are cultivated on a growth medium which contains the toxic substance (*e.g., antibiotic or herbicide*), only those that have taken up DNA (and can, as a consequence, degrade or otherwise avoid the effects of the toxin) will thrive.

PCR — the genetic photocopier

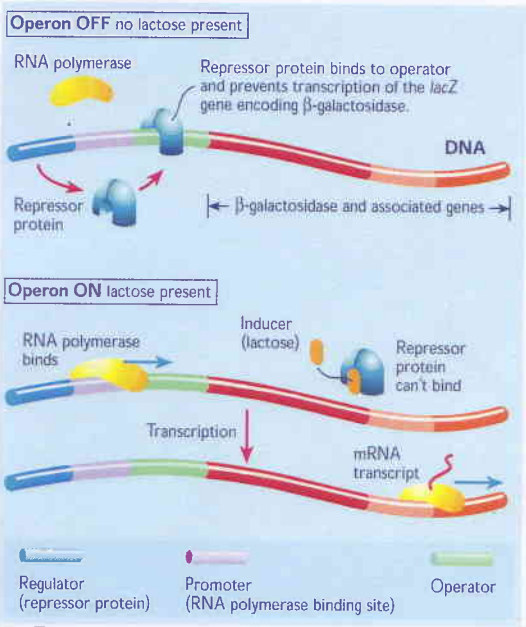
Other methods for identifying transferred genes include the PCR (*polymerase chain reaction*, see box). The PCR has been likened to a 'genetic photocopier'. From a very small amount of biological material, millions of copies of a specific section of its DNA can be made quickly. The PCR lies behind many of the spectacular successes of forensic genetic fingerprinting, where criminals have been identified from the DNA in just a few drops of blood, a couple of cells on a cigarette butt or even on the back of a licked postage stamp. This extremely sensitive method is also used, for example, to test food products for the presence of ingredients that have been derived from genetically-modified sources.

marker genes: blue-white selection



Switched-on genes

The ways in which the expression of genes is regulated have been studied for many years. One controversial method of controlling the expression and 'spread' of novel genes is to regulate them by an external stimulus. For example, it is possible to produce plants that require the application of a specific chemical to grow, or for their introduced genes to be expressed. If the chemical is no longer applied, the plant dies or its novel genes are 'switched off'. Such mechanisms might be used to ensure that, say, insecticides were only produced by modified plants when required, or that modified plants could not stray beyond the limits of sprayed fields. However, fears that similar techniques might be used to increase dependency upon sprays have forced the major agrochemical producers to agree that they will not develop such technologies.

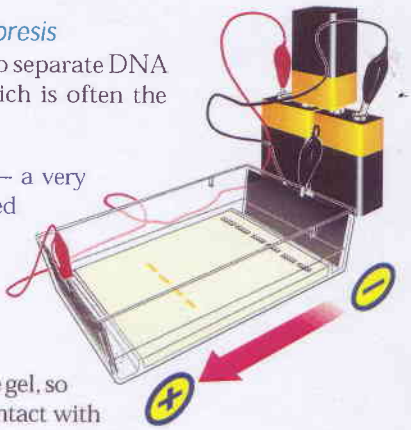


▲ *The lac operon, which was proposed in the late 1950s, explains how E. coli produces the enzyme β-galactosidase only in the presence of lactose or an inducer such as IPTG.*

Genetic analysis

Sorting by size — gel electrophoresis

Gel electrophoresis can be used to separate DNA fragments of different sizes, which is often the first step in their analysis.



First, a gel is cast from *agarose* — a very pure form of agar, which is obtained from seaweed. At one end of the slab of gel are several small wells, made by the teeth of a comb that was placed in the gel before it set.

A buffer solution is poured over the gel, so that it fills the wells and makes contact with electrodes at each end of the gel. Ions in the buffer solution conduct electricity. The buffer also stops the gel from drying out. The invisible DNA fragments are mixed with a small volume of *loading dye*. This dye is dissolved in a dense sugar solution, so that when it is added to the wells, it sinks to the bottom, taking the DNA with it.

▲ *Gel electrophoresis is used to separate DNA fragments of different sizes. This is often the first step in isolating and characterising genes, or mapping their relative positions on chromosomes.*

A current is applied to the electrodes, setting up an electrical field across the gel. Phosphate groups give the DNA fragments a negative electrical charge, so that the DNA migrates through the gel towards the positive electrode. Small fragments move quickly through the porous gel — larger fragments travel more slowly. In this way the pieces of DNA are separated by size. The loading dye also moves through the gel, so that the progress of the electrophoresis can be seen.

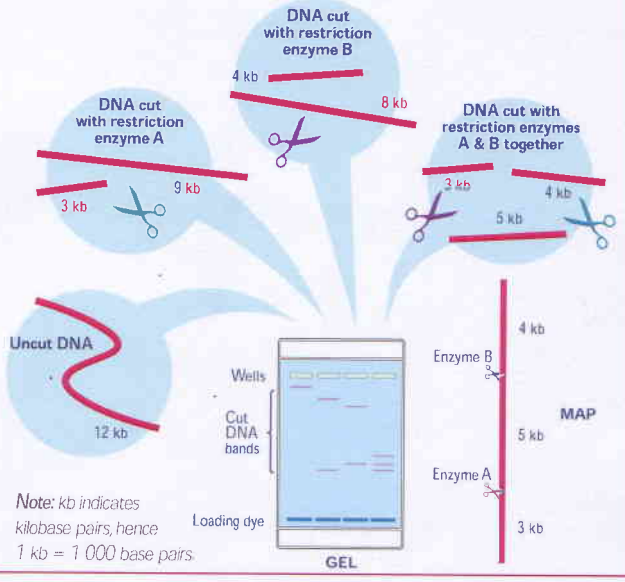
After electrophoresis, the gel is stained to reveal the DNA, either as a smear (many fragments of a wide range of sizes) or bands (each band is comprised of numerous DNA fragments of a similar size). Within a smear, specific bands can by highlighted using *probes* which bind to particular sequences of DNA (or RNA).

restriction site mapping

Construction of a restriction map

This diagram illustrates the principle of restriction site mapping. A 12 kb length of DNA can be cut with two restriction enzymes, A and B. Where do the restriction sites lie, relative to one another? Fragments of DNA produced by cutting with the individual enzymes and both enzymes together are run on a gel.

- *Enzyme A* cuts at one end of the DNA molecule while *Enzyme B* cuts towards the other end.
- The fragments could not be arranged in the sequence 3→4→5 kb because *Enzyme B* produces two pieces of 4 kb and 8 kb, and it is not possible to produce an 8 kb fragment unless the 3 kb piece is next to the 5 kb piece.
- For this reason, the fragments must be arranged in the sequence 3→5→4 kb.



Note: kb indicates kilobase pairs, hence 1 kb = 1 000 base pairs.

linkage maps

In 1913, the very first genetic map was published. It had been devised by Alfred Sturtevant, an undergraduate student at Colombia University (USA).

Alfred realised that the frequency with which characteristics tended to be inherited together might indicate the relative positions of their genes on a chromosome.

Working overnight with data from numerous fruit fly crosses, Sturtevant devised a 'linkage map' showing the relative positions of six fruit fly genes on a chromosome.

You can read Alfred Sturtevant's original paper at:
www.esp.org

1869
J. Friedrich Miescher discovers DNA in the nuclei of cells

1928
Fred Griffith observes bacterial transformation

1938
William Astbury takes X-ray photos of DNA

1944
Oswald Avery and his colleagues show that DNA is the genetic material

1950
Erwin Chargaff finds that A : T and G : C bases in DNA occur in ratios of 1:1

Maurice Wilkins and Ray Gosling obtain X-ray photographs of crystalline DNA

1951
Rosalind Franklin takes X-ray photos of hydrated DNA, revealing a helical structure

Esther Lederberg discovers phage λ

1952
Alfred Hershey and Martha Chase confirm that DNA is the genetic material

1953
James Watson and Francis Crick propose a double helical structure for DNA

1958
Matthew Meselson and Frank Stahl show that DNA replication is semi-conservative

1960
Sydney Brenner and François Jacob discover and explain the rôle of mRNA

Early 1960s
Plasmids carrying antibiotic resistance are discovered by Japanese researchers

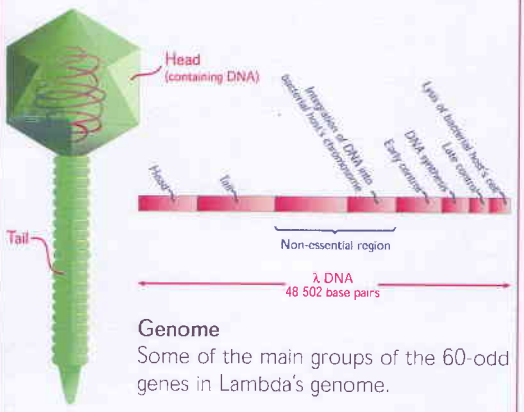
1966
Marshall Nirenberg, H. Gobind Khorana and their co-workers 'crack' the genetic code

Bacteriophage lambda

Bacteriophages (literally: 'bacteria-eaters') are viruses that invade bacteria. To reproduce, bacteriophages (or phages) must take over the molecular machinery of their bacterial hosts. The phage lambda (λ) preys upon *Escherichia coli*. Lambda enjoys a choice of life styles — it can either multiply within its host and destroy it (the lytic cycle), or the λ DNA can be inserted into the bacterial chromosome and remain dormant there for several generations (the lysogenic cycle). An environmental trigger *e.g., ultraviolet light* activates the lytic cycle.

Bacteriophage λ was the first large virus to have its entire genetic make-up (*genome*) mapped and sequenced. It is 48 502 base pairs long. Within this genome are genes that code for the virus's protein coat, bursting (lysis) of the bacterial cell, integration of λ DNA into the host's chromosome and so on. The order in which these genes are activated is important. For example, it would be of little benefit to the virus if the host bacterial cell was broken open (lysed) before new virus particles had been assembled. Consequently, λ has evolved an elaborate system of gene regulation that has been studied in great detail.

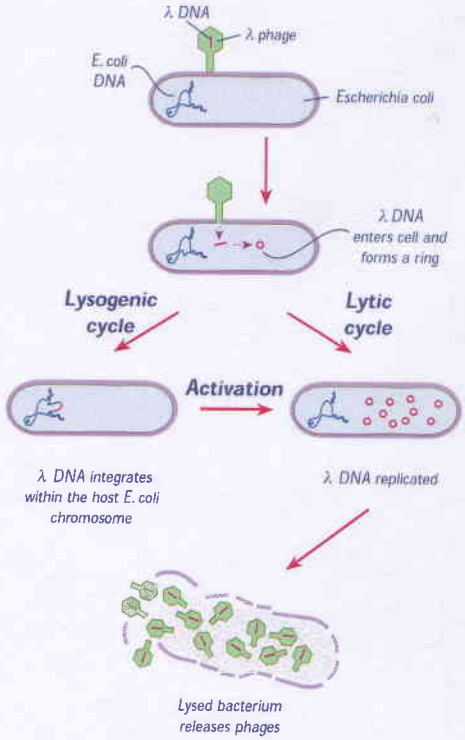
bacteriophage lambda



Genome
Some of the main groups of the 60-odd genes in Lambda's genome.

Structure
Phage λ is a relatively simple organism. It consists of a double-stranded length of DNA coiled around a core of protein and wrapped in a protein coat.

Relatively little of the λ genome is required to package DNA and deliver it into bacterial cells. About 20 000 base pairs can be deleted from its central region and replaced with a similarly-sized fragment of DNA from another organism, without affecting the phage's viability. Several specially-constructed forms of λ, with differing amounts of DNA removed and replaced, are used by molecular biologists to ferry new genes into bacteria. Such vectors are far more efficient at introducing DNA into bacteria than plasmids are. This is hardly surprising because, after all, bacteriophages have evolved to do precisely this job.



▲ *Infection of E. coli by bacteriophage lambda (λ) showing the lytic and lysogenic cycles.*

Mapping and sequencing genomes

The ultimate aim of the *Human Genome Project*, a massive international effort, is to map the positions of the 30–100 000 genes (the exact figure is unknown) and determine the sequence of bases in the entire human genome. This should lead to the ability to diagnose human disease, and in the longer term to devise effective treatments and possibly cures for a wide range of conditions.

Much of this work is now highly automated, with a third of the human genome being sequenced in the UK at the Sanger Centre, near Cambridge. A draft of the genome is to be published in 2000 and the project should be completed by 2002–3.

▲ *Automated sequencing machines are able to determine 450000 bases per day. The interpretation of the vast amount of sequence data produced means that bioinformatics is of increasing importance.*

estimated genome sizes

Organism	Megabases	Made of
Viruses		
• HIV	0.0092–0.0103	ssRNA
• Adenovirus	0.0359	dsDNA
• Herpes Simplex	0.150	dsDNA
• Bacteriophage λ	0.48502	dsDNA
Bacteria		
• *Escherichia coli*	4.7	dsDNA
• *Bacillus subtilis*	4.2	dsDNA
Fungi		
• Baker's yeast	13.5	dsDNA
Nematodes	**75–620**	
• *Caenorhabditis elegans*	80	dsDNA
Insects	**47–12 000**	
• Fruit fly	120	dsDNA
• House fly	840	dsDNA
Birds	**1 100–1 900**	
• Chicken	1 125	dsDNA
Mammals	**2 800–5 200**	
• Human	3 300	dsDNA
• House mouse	3 300	dsDNA
Flowering plants	**95–120 000**	
• *Arabidopsis thaliana*	70	dsDNA
• Tomato	700	dsDNA
• Tobacco	3 500	dsDNA
• Maize	15 000	dsDNA

Except for HIV, these are all figures for haploid genomes

How long is a genome?

There is, roughly, one DNA base per Angstrom of the double helix. An Angstrom is 10^{-10} metres, thus it is easy to estimate the physical size of an organism's DNA. For example:

Organism	Bases	Length
E. coli	4.7 Mb	0.47 mm
Baker's yeast	13.5 Mb	1.35 mm
Human	3 300 Mb	330 mm

Other genomes are also being sequenced, particularly those of economically important plants and animals, organisms that are used in research (such as the fruit fly and the nematode worm *Caenorhabditis elegans*), and many pathogens.

Making use of genetic data

While crop biotechnology or medical applications frequently make the headlines, molecular genetics has led to important discoveries in almost all the biological sciences. For example, in the last few years, the evolutionary relationships of the flowering plants have been completely re-thought based on genetic data. An early application of genetic fingerprinting revealed the hitherto unsuspected mating habits of birds, which proved of great interest to zoologists and conservationists. Archæologists and forensic scientists now make routine use of DNA evidence. Perhaps the most far-reaching insights will emerge from the human genome project, which has already cast light on our origins, our nature and the fundamental unity of all life on Earth.

It is notable that while the early 20th century geneticists (who had almost no knowledge of the genotype) frequently sought to explain the differences between people, often to reinforce their own prejudices, those of the late 20th century have shown how similar we all are. The genes of humans differ by less than 0.1%.

If this new knowledge is to be applied wisely, it is essential that more people understand the basic science and technology of modern genetics and think carefully about the wider implications of such work.

1967
Weiss and Richardson isolate DNA ligase

1970
Hamilton Smith and colleagues isolate and characterise the first sequence-specific restriction enzyme (*HindIII*)

Morton Mandel and A. Higa demonstrate artificial transformation

1972
Janet Mertz and Ron Davis cut and paste DNA molecules

1973
Stanley Cohen and Herbert Boyer put recombinant DNA into bacteria

1977
Fred Sanger sequences ΦX174 virus DNA

1982
The first genetically-modified plants are produced (tobacco)

1983
Kary Mullis conceives of the PCR

Fred Sanger and his colleagues publish the DNA sequence of bacteriophage λ

1986
DNA sequencing is automated

1987
Start of the Human Genome Project

1995
Haemophilus influenzae's genome is sequenced

1996
Saccharomyces cerevisiae's genome is sequenced

1998
Caenorhabditis elegans is sequenced

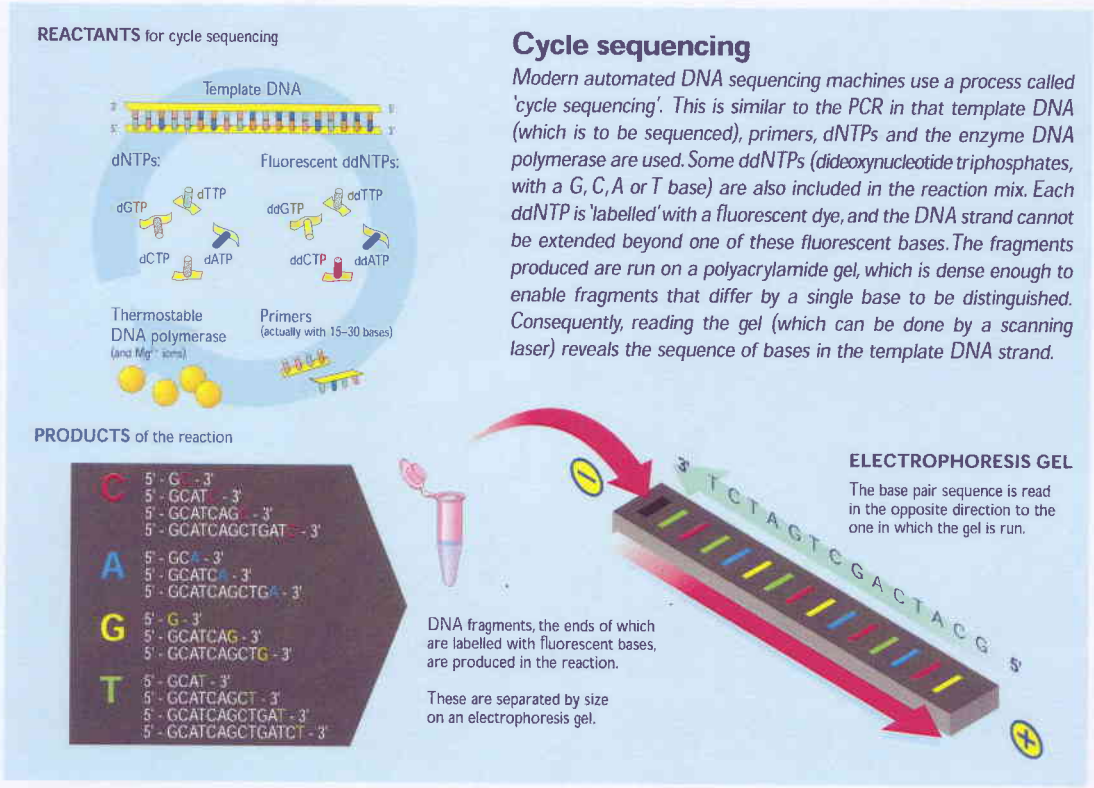
1999
Human chromosome 22 is sequenced

2000
The fruit fly, *Drosophila melanogaster*, is sequenced

A draft sequence of the entire human genome is published

Cycle sequencing

Modern automated DNA sequencing machines use a process called 'cycle sequencing'. This is similar to the PCR in that template DNA (which is to be sequenced), primers, dNTPs and the enzyme DNA polymerase are used. Some ddNTPs (dideoxynucleotide triphosphates, with a G, C, A or T base) are also included in the reaction mix. Each ddNTP is 'labelled' with a fluorescent dye, and the DNA strand cannot be extended beyond one of these fluorescent bases. The fragments produced are run on a polyacrylamide gel, which is dense enough to enable fragments that differ by a single base to be distinguished. Consequently, reading the gel (which can be done by a scanning laser) reveals the sequence of bases in the template DNA strand.



safety guidelines

All of the hazards and risks associated with the investigations in this booklet may be adequately controlled by following normal good laboratory practice. Guidelines for good microbiological practice are described here in detail. In addition, readers will need to refer to any regulations and safety guidelines issued by their local education authority and/or school or college governing body and carry out their own risk assessment.

Sources of microbes

All micro-organisms should be regarded as potentially harmful. However, the severely-debilitated strains of the bacterium *Escherichia coli* used in the investigations in this booklet present minimum risk given good practice. They should be obtained only from recognised educational suppliers.

These laboratory strains should not be confused with the highly-pathogenic *E. coli* Ø157:H7, other pathogenic strains or the normal 'wild type' *E. coli* that is found in the human gut. Unlike these forms, the K12 strains of *E. coli* recommended for use here are unable to inhabit the mammalian gut. K12's origins can be traced back to work in the USA in 1922. Biochemical and genetic studies by Edward Tatum in the 1940s made the strain popular with researchers, and after many millions of generations of laboratory cultivation, it is now known to have undergone significant changes. These have altered the lipopolysaccharides that compose the outer membrane of the bacterial cell, so that it can no longer infect mammals.

The K12 strains suggested for use in this booklet are particularly suitable for transformation by plasmids and grow well on broth and agar plates which are supplemented with amino acids (the LB agar and broth described in the *'Recipes'* section on pages 40–41 contain all the necessary amino acids).

Compared to the wild type *E. coli*, K12 strains such as DH5α are severely weakened and would find it difficult to thrive outside the laboratory. They often have unusual nutritional requirements, and, for example, although DH5α can grow on glucose it is unable to use lactose as an energy source.

Several organisations (see *'Contacts'* box) maintain lists of microbes that are suitable for school use.

Maintenance and storage of cultures

In schools, microbial cultures are often maintained on agar slopes. These should be transferred onto fresh medium every 8–12 weeks or so, and incubated until the organisms have grown. They should then be stored in a cool (10–15 °C), dark place, *not in a refrigerator*. New cultures should be obtained regularly, as repeated subculturing can lead to contamination or genetic changes in the cells.

Good microbiological practice

General precautions

- Any exposed cuts or abrasions should be protected with waterproof dressings before the practical work starts.
- Everyone involved — teachers, technicians and students — should always wash their hands before and after practical work.
- The laboratory door and windows should be closed when work is in progress. This will reduce air movements and consequently the risk of accidental contamination of plates, *etc.*
- It is strongly recommended that laboratory coats are worn, and where necessary (*e.g., when heating liquids*), eye protection.
- High standards of cleanliness must be maintained. Non-porous work surfaces should be used and they should be swabbed with an appropriate laboratory disinfectant before and after each practical session.
- To reduce the risk of ingesting microbes, no hand-to-mouth operations should occur (*e.g., chewing pencils, licking labels, mouth pipetting*). For the same reason, eating, drinking and smoking must not be allowed in the laboratory.

Spills and breakages

Accidents involving cultures should be dealt with as follows:

- Disposable gloves should be worn;
- The broken container and/or spilled culture should be covered with a cloth 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 placed in an infected waste container or disposal bag. This must be autoclaved before disposal;
- The dustpan should also be autoclaved or placed in a suitable solution of disinfectant for 24 hours.

Contamination of skin or clothing

As soon as possible, anyone who has been splashed should wash. Severely contaminated clothing should be placed in disinfectant before it is laundered. Contaminated cleaning cloths should be autoclaved or soaked in disinfectant.

contacts

Association for Science Education
College Lane,
HATFIELD,
AL10 9AA.
T 01707 283000
W www.ase.org.uk

Consortium of Local Education Authorities for the Provision of Science Services at Brunel University,
UXBRIDGE,
UB8 3PH.
T 01895 251496
W www.cleapss.org.uk

Scottish Schools' Equipment Research Centre
St Mary's Building,
23 Holyrood Road,
EDINBURGH,
EH8 8AE.
T 0131 558 8180
W www.sserc.org.uk

Society for General Microbiology
Marlborough House, Basingstoke Road, Spencer's Wood, READING,
RG7 1AE.
T 0118 988 1800
W www.sgm.ac.uk

Microbiology in Schools Advisory Committee
May be contacted via the SGM, above.

Note: ASE, CLEAPSS and SSERC can help their members only.

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 work surfaces and equipment. To obtain a pure culture, sterile growth media and equipment must therefore be used and contaminants must be excluded. These are the main principles of aseptic techniques.

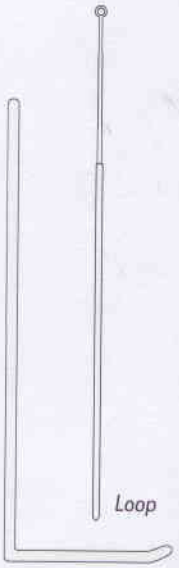
Growth media must be sterilised before use — usually by autoclaving. Sterile containers (flasks, Petri dishes, etc.) should be used. Lids must be kept on these containers to prevent contamination.

It is essential to prepare the work area carefully before you start. All necessary equipment and materials should be arranged so that they are readily-at-hand. Work should be done near a lighted Bunsen burner. Rising air currents from the flame will help to carry away any microbes that could contaminate growth media and pure cultures.

When cultures are transferred between containers, tops and lids should not be removed for longer than necessary. After a lid has been taken from a bottle, it should be kept in your hand until it is put back on the bottle (see picture). This stops contamination of the bench and the culture.

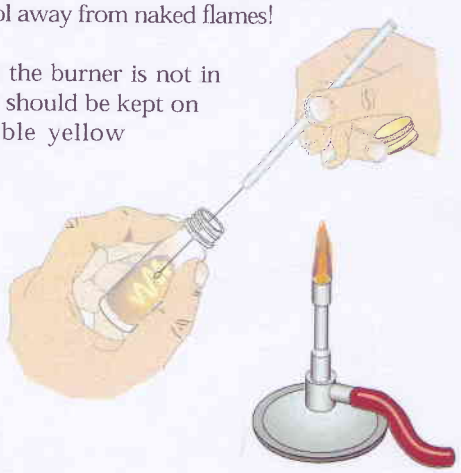
A blue flame about 5 cm high should be used for sterilising loops or wires and flaming the necks of bottles. After removal of the top, the neck of the culture bottle should be flamed for a few seconds. This will kill any microbes present there and cause convection currents which will help to prevent accidental contamination of the culture from the atmosphere. Bottles should not be heated until they become hot and dangerous to handle.

Wire loops, however, *must* be heated until they glow red hot along the entire length of the wire part. This should be done both before and after cultures are transferred. Heat the stem of the loop first as it is brought into the Bunsen burner flame, to reduce sputtering and aerosol formation. Allow the loop to cool before you use it to transfer a culture (some people like to cool the loop after flaming it, by touching it briefly onto the agar at the edge of a culture plate).



Before and after use, glass spreaders should be dipped in alcohol, then passed quickly through the flame and the alcohol allowed to burn off. Great care must be taken to keep the alcohol away from naked flames!

When the burner is not in use, it should be kept on a visible yellow flame.

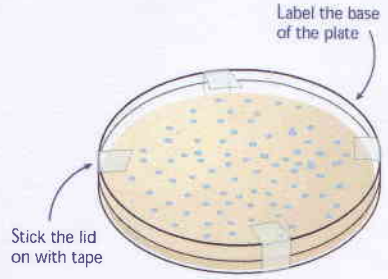


◀ *With practice, it is possible to hold a culture bottle in one hand and the wire loop in the other so that the little finger is free to grip the bottle top against the lower part of the hand.*

incubation of cultures

Label the Petri dish around the edge of the base before inoculation. Your name, date and the name and/or source of the organism used will allow the plate and its contents to be identified.

Where appropriate, use self-adhesive tape to seal Petri dishes as shown here:



The seal will ensure that the plates are not accidentally opened or tampered with. *Do not seal plates completely round their edges as this could create anaerobic growth conditions within the dish.*

Bacterial cultures in Petri dishes should usually be incubated with the base uppermost, so that any condensation that forms falls into the lid and not on the colonies. (If there is heavy condensation in the sterile Petri dish before inoculation, it should be allowed to dry before use.)

Although school texts sometimes warn against doing so, the delicate strains of *E. coli* used in this booklet should be incubated at 37 °C for speedy growth. Good microbiological practice will ensure that human pathogens are not inadvertently cultivated at this temperature.

Transformation preparation

The transformation protocol on pages 26–27 suggests that the host strain of *E. coli* should be grown for several days in advance at 18–25 °C. This gives an extended growth period, increasing the chance that you will be able to take colonies from the plate at the best stage of growth for efficient transformation. Alternatively, cultures for transformation can be inoculated about 24 hours in advance and incubated at 37 °C. Once they have been transformed, it is best to incubate the bacteria at 37 °C.

autoclaving

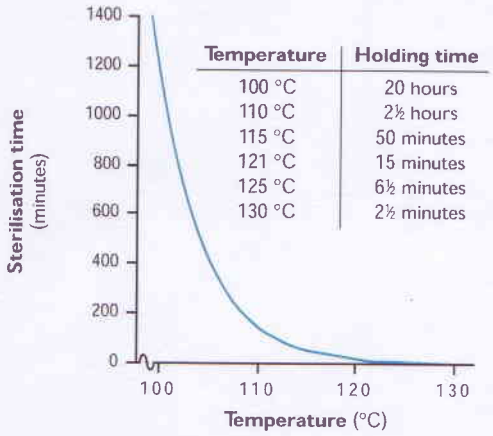
Sterilisation is the complete destruction of all microorganisms, including their spores. All equipment should be sterilised before starting practical work so that there are no contaminants. Cultures and any contaminated material should also be sterilised after use for safe disposal.

Autoclaving is the preferred method of sterilisation for culture media, aqueous solutions and discarded cultures. 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. A domestic pressure cooker or a purpose-built autoclave can be used. Domestic pressure cookers can be used in school laboratories but their small capacity can be a disadvantage when dealing with class sets of material.

Principles of autoclaving

Two factors are critical to the effectiveness of the process. Firstly, all air must be driven from the autoclave. This ensures that high temperature steam comes into contact with the surfaces to be sterilised: if air is present the temperature at the same steam pressure is lower. The materials to be sterilised should be packed loosely so that the air can be driven off. Screw-capped bottles and jars should have their lids loosened slightly to allow air to escape and to prevent a dangerous build-up of pressure inside them.

Secondly, sufficient time must be given for heat to penetrate (by conduction) to the centre of media in flasks or other containers. The times for which media or apparatus must be held at various temperatures for sterilisation are shown below:



Temperature	Holding time
100 °C	20 hours
110 °C	2½ hours
115 °C	50 minutes
121 °C	15 minutes
125 °C	6½ minutes
130 °C	2½ minutes

Notice that just a small difference in temperature can result in a great difference in the time required for sterilisation. It is also important that these temperatures are reached by all materials to be sterilised for the specified time *e.g., the broth in the very centre of a flask.* Three factors determine the duration of the autoclaving process:

- **penetration time**
  the time taken for the innermost part of the autoclave's contents to reach the required temperature (say, 5 minutes);
- **holding time**
  the minimum time in which, at a given temperature, all living organisms will be killed (say, 15 minutes);
- **safety margin**
  roughly half the holding time (about 5 minutes).

Most domestic pressure cookers and autoclaves operate at 121 °C. This gives a total autoclaving period of around 25 minutes. For larger volumes of liquid, such as 1 dm^3 of growth medium in a flask or small fermenter vessel, the holding time should be increased to 20–25 minutes.

The effectiveness of an autoclave can be checked by using autoclave test strips which change colour if the process has worked properly (autoclave tape does not show this).

Disposal and sterilisation

It is very important to dispose of all the materials used in a practical class properly. All containers used for storing and growing cultures must be autoclaved, then washed and rinsed as necessary, before re-use.

Two autoclave bags should be available in the laboratory: one for reusable glassware and another for disposable materials. There should be a discard jar near each work area for items such as microcentrifuge tubes, waste liquid cultures and pipettes. A bucket should be available for disposal of any broken glassware.

After use, disinfectant in the discard jar should be poured away and the items in it should be autoclaved and disposed of. Contaminated paper towels, cloths and plastic Petri dishes should be put into the autoclave bag reserved for disposable items. Any contaminated glassware should be put into the autoclave bag for glassware.

Glassware that is not contaminated can be washed normally. Broken glassware should be put in a waste bin reserved exclusively for that purpose. If the glassware is contaminated it must be autoclaved before disposal. Uncontaminated broken glassware can, of course, be disposed of immediately.

Use and routine care of autoclaves

Different autoclaves and pressure cookers will have different operating instructions and it is important that the manufacturer's instructions are always followed. Care should be taken to ensure that there is enough water in the autoclave so that it does not boil dry during operation. A domestic pressure cooker requires at least 250 cm^3 of water — larger autoclaves may need much greater volumes. The use of distilled or deionised water in the autoclave will prevent the build-up of limescale or corrosion of the metal pressure vessel. Autoclaves should be dried carefully before storage to prevent damage to the vessel.

When the autoclave is used, steam should be allowed to flow freely from it for about one minute to drive off all the air inside. Only then should the exit valve be tightened. After the autoclave cycle is complete, sufficient time must be allowed for the contents to cool and return to normal atmospheric pressure. Premature release of the lid and the subsequent reduction in pressure will cause any liquid inside the autoclave to boil. Therefore the vessel or valve must not be opened whilst under pressure as this may cause scalding and the agar or broth will froth up and may boil over the outside of the containers within.

Chemical sterilisation

Eye protection should be worn when dispensing concentrated disinfectant solutions.

Many different chemicals are used for sterilisation of used equipment and work surfaces. Some disinfectants, such as *MicroSol*[3] (previously known as *VirKon*) can be safely used for most laboratory purposes. Others have specialist uses. **The manufacturer's and supplier's instructions should always be followed with care.**

Clear phenolics are effective against bacteria and fungi but inactive against spores and some types of virus. They are inactivated to some extent by contact with rubber, wood and plastic. Laboratory uses include discard jars and disinfection of surfaces.

Hypochlorites (such as bleaches) are not ideal for sterilisation of used Petri dishes *etc.* as they can be inactivated by protein and plastic materials. However, a freshly made-up solution of sodium chlorate [I] (containing 2 500 ppm available chlorine) is suitable for use in discard jars, into which pipettes, microcentrifuge tubes, *etc.* should be placed after use. Provided they are tall enough to take pipettes and so on, old plastic chemical containers with screw tops make ideal discard jars.

Disposable plastic items

Molecular biologists make extensive use of disposable plastic items, such as Petri dishes, microcentrifuge tubes and single-use pipettes. Although they are not commonly used in the school laboratory, we strongly recommend their adoption as they are inexpensive, save on preparation time and enhance safety by reducing the risk of contamination.

After use, such items can be sterilised and disposed of in the normal waste. Depending upon the use to which they were put, many of these plastics, like polypropylene (from which microcentrifuge tubes are made) can be recycled — but not re-used.

microbial transformation & work with dna

One of the investigations in this booklet involves the 'transformation' of a microorganism by placing plasmid DNA into it. Although this particular protocol uses DNA that could occur naturally within that species (in other words, the 'species barrier' is not crossed), it is still technically defined as genetic modification and is therefore subject to legal control. *Note: The information given here applies only to the United Kingdom.*

EU Directives

Throughout most of the world the use of all live genetically-modified organisms is controlled by law. There are two relevant sets of regulations (Directives) governing genetic modification throughout the European Union. Laws in the United Kingdom have been enacted to comply with these Directives. One covers 'Contained Use' *e.g., work in a laboratory*; the other covers 'Deliberate Releases' of modified organisms into the environment *e.g., field trials of genetically-modified crops.*

'Naked' DNA

'Naked' DNA (including plasmid DNA) is not covered by these regulations as DNA is not an organism. Therefore the use of restriction enzymes, ligases, electrophoresis, PCR and similar procedures do not fall under the 'Contained Use' regulations even if new nucleic acid molecules are formed.

'Self-cloning'

The bacterial transformation suggested in this booklet (on pages 26–27) is known technically (and rather confusingly) as 'self-cloning'. In this context 'cloning' means making copies of plasmid DNA within an organism. Because the plasmids used are made entirely from DNA that could occur naturally within the bacteria involved, the work is *self*-cloning.

Self-cloning using non-pathogenic species and strains, like the severely debilitated *E. coli* strains that have been developed for such work, is *exempt* from the 'Contained Use' regulations. Schools and others may undertake such work without licensing their premises with the Health and Safety Executive. However, somewhat unusually (since these bacteria could be found in nature) the bacteria produced *are* covered by the 'Deliberate Release' regulations. It is therefore essential that the organisms are adequately contained and that a 'release' does not occur.

Containment can be ensured by following good microbiological practice and by the careful selection of suitable host organisms and plasmids *(e.g., host strains that are weakened and 'non-mobilisable' plasmids that cannot transfer their genes into the host's chromosome, or move into other organisms by natural means such as bacterial conjugation).*

IMPORTANT Some kits and practical investigations obtained from the USA, even those that are designed for school use, may not be permitted within the European Union.

A sixth-form student at Chiselhurst and Sidcup Grammar School carrying out a microbial transformation on an NCBE course ▶

Photograph kindly supplied by Marianne Rüffer-Turner.

resources

Richardson, J. (1995) Practical work with DNA. *Education in Science.* **162**, 16–18 (April 1995).

Microbial culture by Susan Issac and David Jennings (1995) Bios Scientific Publishers. ISBN: 1 872748 92 9.

Living biology in schools by Michael Reiss [Ed.] (1996) Institute of Biology. ISBN: 0 900 04903 2.

Safety in science education Department for Education and Employment (1996) Her Majesty's Stationery Office. ISBN: 011 270915 X.

A guide to the genetically-modified organisms (Contained Use) Regulations 2000 Health and Safety Executive (2000) Her Majesty's Stationery Office. ISBN: 0 7176 1186 8.
www.hse.gov.uk

Topics in safety Association for Science Education (2001) [Third Edtn.] Association for Science Education ISBN: 0 86357 316 9.

modelling dna

Model-making can help scientists to understand the structure and function of DNA and proteins. Physical models, such as those which helped James Watson and Francis Crick to reveal the structure of DNA, have been largely replaced by computer-generated images. Much of the software and necessary structure data is available free-of-charge from scientific and educational World Wide Web sites.

PDB ID: 1D66

Computer modelling

RASMOL is a 3-D molecular modelling programme for *Macintosh*, *Windows*, *UNIX* and *VMS* operating systems. It was written by Roger Sayle and can be downloaded free-of-charge from the *RASMOL* Web site (http://www.umass.edu/microbio/rasmol).

The programme is small enough to fit on a single floppy disc. It is a good idea to print out *RASMOL's* 40-page manual (which comes with the programme), as although *RASMOL* is easy-to-use, to make the most of it you will need to use the commands described in the manual.

Molecules can be depicted in seven different forms (wireframe, spacefill, ball-and-stick, ribbon, cartoon, sticks, stereoscopic) and coloured, highlighted and labelled in many ways (for example, to show the general motifs that comprise a protein). It is easy to rotate molecules and zoom in on parts to examine their structures in detail.

Structures

Once you have installed *RASMOL*, you will need to obtain some molecular structure data. An excellent source is the Protein Data Bank (http://www.rcsb.org/pdb/) and its mirror sites around the world.

This database of thousands of molecular structures can be searched in various ways *e.g., by entering the name of a molecule as a keyword.* Selecting the name of a molecule from the list of structures retrieved will enable you to display the selected file within your Web browser or to download it to your computer. Save the structure data as a text file, then launch *RASMOL*.

To view the molecule you have downloaded, open it from within *RASMOL's* 'FILE' menu. *Note: Protein Data Bank and Nucleic Acid Database IDs are given alongside many of the molecular figures in this booklet.*

useful web sites

RASMOL's own Web site (with simple instructions, a discussion list and other resources) can be found at: http://www.umass.edu/microbio/rasmol. This site also has details of RASMOL's stable-mate, CHIME, which enables 3-D models to be incorporated into Web pages.

There are several tutorials on molecular structure at this site, plus instructions for creating your own Web pages that incorporate CHIME models.

The Nucleic Acid Database is held at Rutgers University, New Jersey (USA): http://ndbserver.rutgers.edu/NDB/ndb.html. You can also find the amazing Musical Nucleic Acid Atlas here!

Structures for modern/cell biology—the CHIME plug-in is needed to view these interactive structures: http://info.bio.cmu.edu/courses/biochemMols/BuildBlocks/Molecules.html.

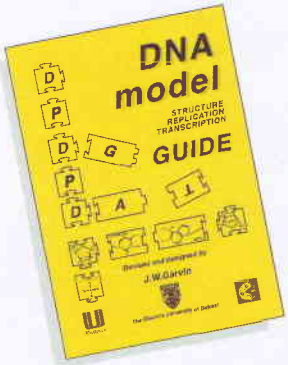
Physical models

Wilbert Garvin, formerly of the Queen's University of Belfast, has designed a 2-dimensional jigsaw-like DNA model kit for use in schools. The model can be used to learn about the structure and function of DNA. The component parts, which are made of robust coloured plastic, are reversible, providing both a simple and an 'advanced' model.

The kit comprises sufficient parts for use by a whole class and comes with a comprehensive guide with instructions for teachers and students. This includes some overhead projector masters.

An *Adobe Acrobat*® (PDF) file of the instruction manual that accompanies the model can be downloaded from the EIBE web site: http://www.reading.ac.uk/EIBE

This manual is available in several languages.



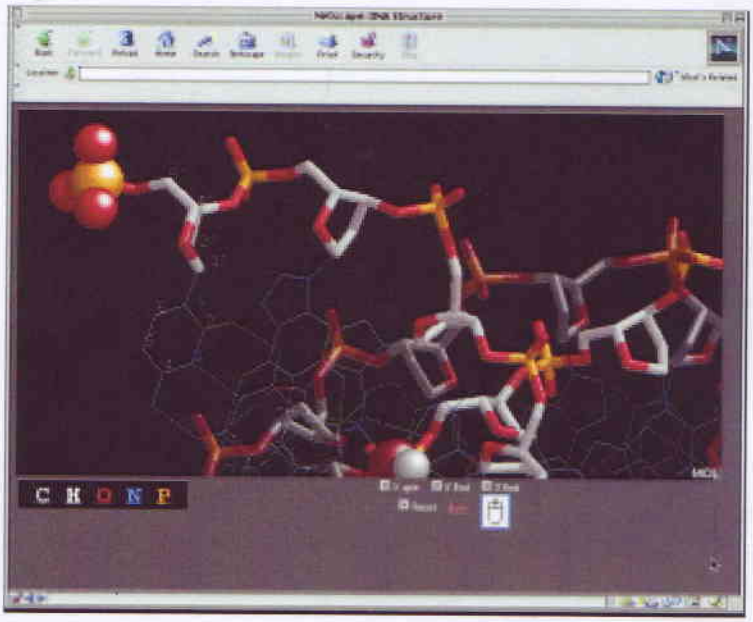
The production of Wilbert Garvin's model kit was jointly supported by Unilever and EIBE, the European Initiative for Biotechnology Education.

resources

Nicholl, L. and Nicholl, D. (1987) Modelling the eukaryotic chromosome: a stepped approach. *Journal of Biological Education* 21 (2) 99–104.

Millar, N. (1996) Computer modelling of biological molecules: free resources on the internet. *School Science Review* 78 (282) 55–60.

Ferry, G. (2000) Profile: Protein structure by numbers *HMS Beagle, Issue* 73, March 3. W www.biomednet.com/hmsbeagle

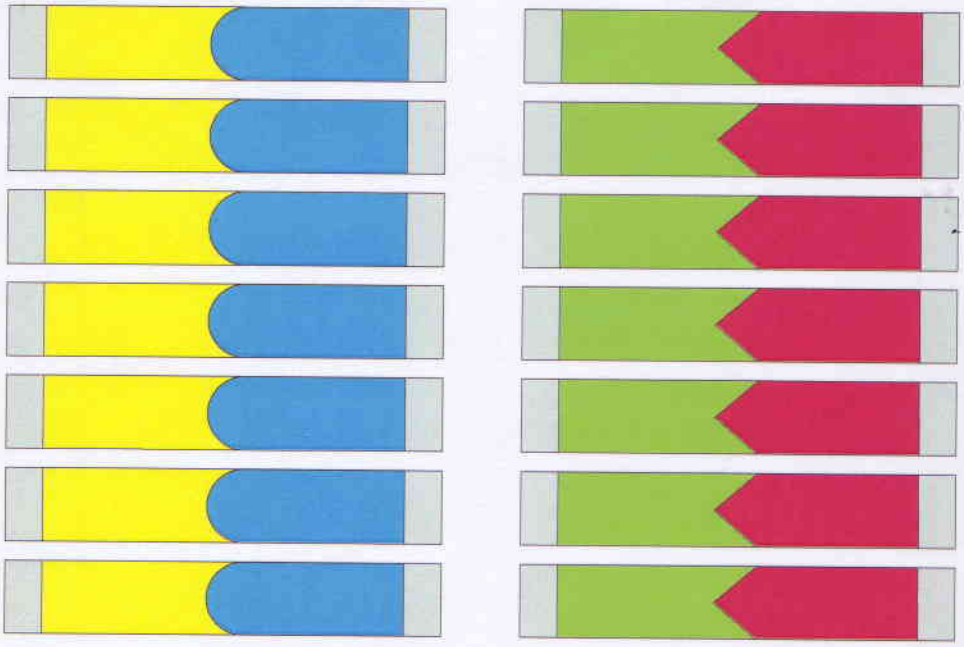


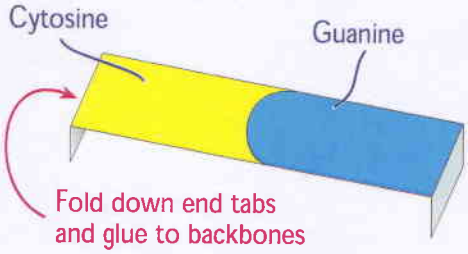
▲ *The CHIME plug-in can be used to view molecular structures within the Netscape Web browser. The frame above comes from a complete suite of animated, interactive CHIME pages designed to help students learn about the structure of DNA. It can be viewed or downloaded entirely (for viewing off-line) from:* http://www.umass.edu/microbio/rasmol

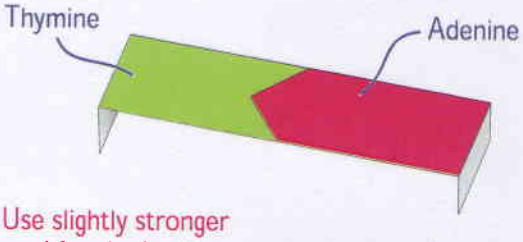
SUGAR-PHOSPHATE BACKBONE

SUGAR-PHOSPHATE BACKBONE

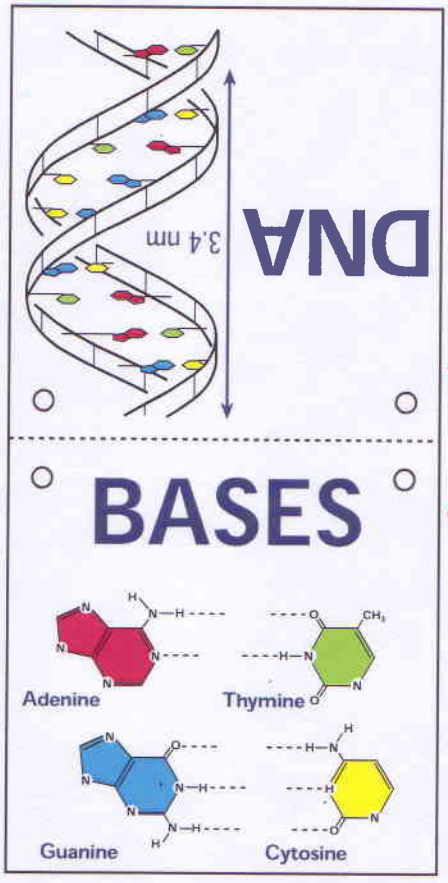
BASE PAIRS



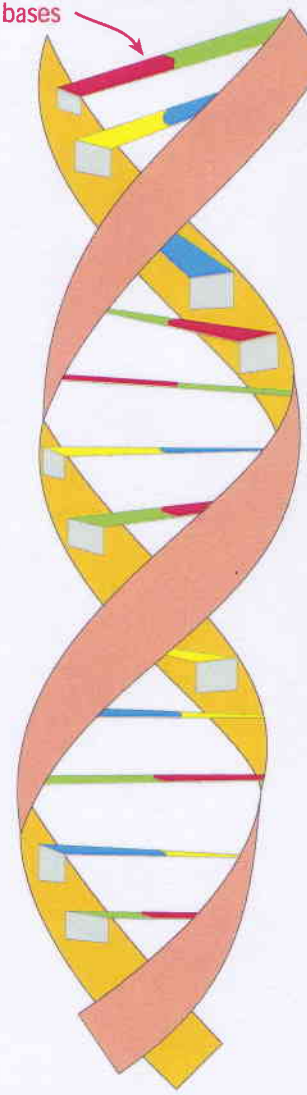




Use slightly stronger card for the bases



LABEL
Fold and glue sides together



Phosphate

Sugar

INSTRUCTIONS

- Print this page onto card
- Cut out the pieces and glue them together
- Hang the label from the bottom of the model

Based on a model devised by CSIRO's 'Double Helix' Science Club

extracting dna

This is a crude but effective method of isolating DNA and RNA from onions. First, the tissue is broken up mechanically. Household detergent is used to degrade both the cell membranes and those surrounding the nuclei. Cell fragments are separated by filtration; the nucleic acids and soluble proteins remain. Protease is used to partly degrade soluble proteins, then the nucleic acids are precipitated into ice cold ethanol. The onion DNA can be run on an electrophoresis gel if it is first dried then dissolved in TE buffer.

Aim

To isolate nucleic acids (DNA and RNA) from onions.

Preparation

The ethanol used must be **ice cold**. Place it in a plastic bottle in a freezer at least 24 hours before you attempt this activity. ***Please read the safety note, below.***

Timing

Isolating the DNA takes about 35 minutes, including an incubation period of 15 minutes.

Materials and equipment

Needed by each person or group

- A small onion
- Washing-up liquid, 10 cm^3 (use a watery type, not the thicker, concentrated variety)
- Table salt, 3 g
- Distilled water, 90 cm^3
- Very cold ethanol, about 6 cm^3, straight from the freezer (industrial methylated spirit, IMS, is suitable, but please see the safety note on this page)
- *Novozymes Neutrase®* (a protease), 2–3 drops
- Ice, in a jug with cold water
- Coffee filter paper (do not use laboratory filter paper, as liquid takes too long to pass through it)
- 1 cm^3 plastic syringe (without a needle)
- Sharp vegetable knife and chopping board
- Large plastic funnel
- 250 cm^3 beakers, 2
- Boiling tube
- Glass rod or spoon for stirring the mixture
- Water bath, maintained at 60 °C

Procedure

1. Dissolve the salt in 90 cm^3 of distilled water. Add the washing-up liquid and mix gently.
2. Chop the onion into small pieces, roughly 5 mm x 5 mm. Add the chopped onion to a beaker with the salty washing-up liquid solution.
3. Stand the beaker in a water bath at 60 °C for *exactly* 15 minutes.
4. Cool the mixture by placing the beaker in an ice water bath for 5 minutes, stirring frequently.
5. Pour the mixture into a blender and blend it for *no more than 5 seconds.*
6. Filter the mixture into a second beaker. Ensure that any foam on top of the liquid does not contaminate the filtrate.
7. Add 2–3 drops of protease to about 10 cm^3 of the onion extract in a boiling tube and mix well.
8. Very carefully pour ice cold ethanol down the side of the boiling tube, to form a layer on top of the onion extract.
9. Leave the tube, undisturbed, for a few minutes. Nucleic acids (DNA and RNA) will precipitate into the upper (ethanol) layer.

Safety

Ethanol in freezers

Most freezers are not spark-proof. There have been instances of explosions when flammable vapours *e.g., from ethanol* have accumulated within a freezer and been ignited by a spark. If you do not have a spark-proof freezer, you MUST ensure that any ethanol is placed in a sealed, vapour-tight plastic container. If you don't have access to a freezer at all, put some ice in an insulated container, then stand the bottle of ethanol in it for several hours before use.

dna from other things

The method described above can be adapted for use with other plant or animal cells, for example, DNA from fish sperm as described here. You can also estimate the amount of DNA produced (see *DNA Quantification*, page 22).

Materials and equipment

- Fish sperm ('milk'), cut from a fresh or frozen fish (*e.g. from cod, salmon or trout*)

- Household detergent
- Saturated solution of table salt (about 13% by mass)
- Cold ethanol (IMS)
- Gauze
- Teaspoon
- Mortar and pestle
- Filter funnel
- 250 cm^3 beaker
- 100 cm^3 measuring cylinder
- Test tube

Procedure

1. Grind a teaspoonful of fish sperm.
2. Add 50 cm^3 of water and stir.
3. Filter through several layers of gauze.
4. Add a few drops of detergent and 50 cm^3 of saturated salt solution. Stir again.
5. Gently pour cold ethanol onto the filtered solution. The DNA precipitates at the border between the water and ethanol.

This method was kindly supplied by Dr Elisabeth Strömberg of the Mathematics and Natural Sciences Resource Centre (M/NfRC), University of Göteborg.

resources

Marmur, J. (1961) A procedure for the isolation of DNA from micro-organisms *Journal of Molecular Biology* **3** (2) 208–218.

This paper describes the original DNA extraction method on which this, and many similar procedures, are based. It is listed here purely for historical interest.

Investigating plant DNA by Dean Madden [Ed.] (1995) National Centre for Biotechnology Education, The University of Reading.

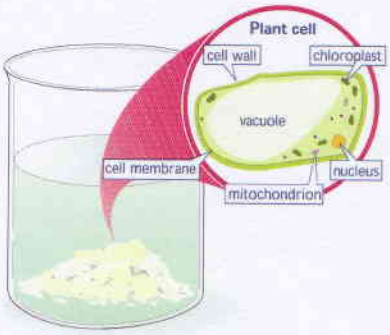
This is a guide from an NCBE practical kit, explaining how to extract DNA from a variety of plant tissues and how to 'run' it on an electrophoresis gel.

Millar, R. (1996) DNA from bacterial cells [Edited by J. Richardson] *SSERC Bulletin* **189** 6–8.

Micro-scale extraction of DNA from bacteria as an alternative to Gram testing.

1 Add 3 g of table salt to 90 cm^3 water. Stir well to dissolve the salt. Gently stir in 10 cm^3 of washing up liquid.

2 Chop the onion into small pieces, and place them in a beaker of the salty washing-up liquid solution.



The washing-up liquid breaks down the cell membranes, releasing DNA from the nucleus, chloroplasts and mitochondria inside each cell.

3 Stand the beaker in hot water at 60 °C for *exactly* 15 minutes.

Hot conditions speed up the process and denature DNases that might degrade the DNA ...

... but leave it too long and the DNA is broken up as well.

4 Cool the mixture by standing the beaker in a jug of icy water for a few minutes.

5 Blend the cooled mixture for *no more than 5 seconds.*

The blender helps to break open the onion cells — but don't blend for too long, or the DNA will be sheared.

6 Filter the chopped onion pieces from the liquid.

This separates the cell wall material from the DNA and proteins, which are now in solution.

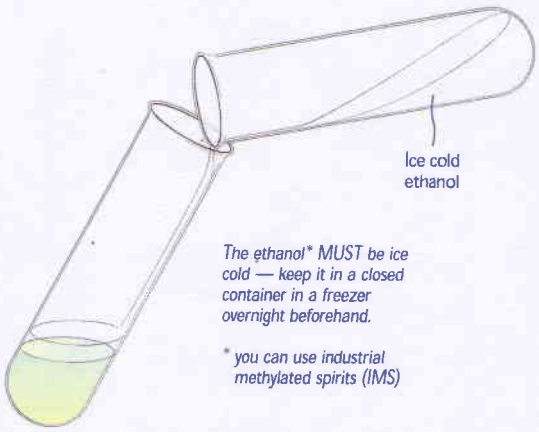
7 Add 2–3 drops of protease enzyme to about 10 cm^3 of the onion extract.

Flick gently to mix the enzyme in

The protease breaks down the protein in the solution. Only a little is needed.

8 Very carefully pour an equal volume of ice cold ethanol onto the surface of the onion extract.

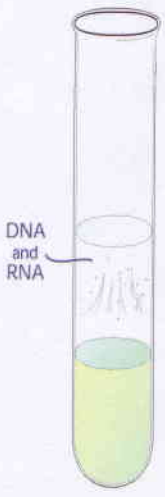
WARNING
Ethanol, if chilled in a freezer, must be stored in a sealed, vapour-tight container.



The ethanol MUST be ice cold — keep it in a closed container in a freezer overnight beforehand.*

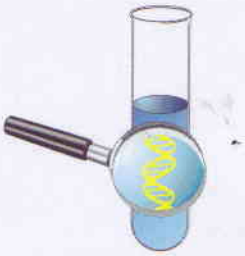
** you can use industrial methylated spirits (IMS)*

9 Bubbles of air carry the DNA and RNA into the upper (ethanol) layer.



DNA doesn't dissolve in ethanol — so it comes out of solution in the upper layer.

dna quantification



A standard method of measuring the amount of DNA in a solution is to measure its absorbance of ultraviolet light. Most schools do not have a UV spectrophotometer however, so the following method, which uses a colorimeter capable of measuring absorbance at 660 nm, can be used instead. For this assay, denatured DNA (strictly, deoxypentose) is reacted with acidified diphenylamine to produce a dark blue-coloured compound.

Aim

To measure the amount of DNA in a solution.

Preparation

The diphenylamine reagent can be prepared in advance, but once diluted it must be used within 24 hours. ***Please see the safety note, below.***

Timing

This takes about 35 minutes (*Quick method*) or 50 minutes plus an overnight incubation (*Quantitative method*).

Materials and equipment

Needed by each person or group

- Standard DNA solution (containing 3 mg per cm^3), *e.g., salmon sperm DNA in saline citrate buffer*
- Diphenylamine reagent, 3 cm^3 per sample (see recipe, below)
- DNA sample in saline citrate buffer
- Saline citrate buffer (used as a 'blank' control)
- Test tubes
- 10 cm^3 plastic syringes (without needles) for dispensing liquids
- Glass marbles, to cover the test tubes
- Anti-bumping granules (*e.g., broken porcelain*)
- Glass rod, for stirring liquids
- Maker pen, fine-tipped, permanent
- Safety spectacles

For the quantitative method, the following items are also needed per class

- Cling film, to cover test tubes
- Spectrophotometer or colorimeter and cuvettes to measure absorbance at 660 nm

Procedure

1. Dispense 3 cm^3 of the DNA solution prepared in the preceding exercise (pages 20–21) into a test tube. Label the tube.
2. Place 3 cm^3 of a standard DNA solution in a second tube and 3 cm^3 of saline citrate buffer in a third. Label all of the tubes appropriately. *These will provide a 'standard' and a 'control' respectively with which you may compare your test sample.*
3. ***Wear eye protection.*** Add 3 cm^3 of diphenylamine reagent to each tube.

Quick qualitative method

4. Place a marble on top of each test tube, then stand the tubes in a beaker of water. Place some anti-bumping granules in the beaker to prevent the water from bubbling too vigorously. Boil the tubes for 10 minutes. *Heating with strong acid converts the deoxyribose of DNA to furfural. Diphenylamine reacts with this to give a deep blue coloured-compound, providing a measure of the amount of DNA present. The exact reaction that occurs is not known. Diphenylamine does not react with ribose derivatives, however, so it is possible to assay DNA in the presence of contaminating RNA.*
5. Compare the colours of the tubes. *Note: Impurities in the DNA preparation can influence the accuracy of the assay.*

Quantitative method

Carry out steps 1–3 as in the *Quick method*, above.

4. Cap the tubes (or cover them with cling film) and leave them overnight (about 17 hours) at 25–35 °C. *This is safer, about three times more sensitive and less prone to 'false positives' (due to diphenylamine reacting with contaminants in the sample) than the 'boiling' method.*
5. Use various dilutions of the DNA standard, in saline citrate buffer, to prepare a standard curve, showing absorbance at 660 nm with known concentrations of DNA.
6. Use the standard curve to determine the concentration of DNA in your sample.

Safety

Diphenylamine reagent

Eye protection should be worn when using diphenylamine reagent and boiling liquids. Diphenylamine is harmful if ingested or inhaled, and may irritate skin or eyes if it comes into contact with them.

diphenylamine reagent

Wear eye protection and use a fume cupboard when preparing this reagent.

Ingredients

- Diphenylamine, 1.5 g
- Glacial acetic acid, 100 cm^3
- Concentrated sulphuric acid, 1.5 cm^3
- Ethanal (acetaldehyde), 1 cm^3

Note: You will need a dark glass bottle to store the diphenylamine solution in.

Procedure

1. Dissolve 1.5 g diphenylamine in 100 cm^3 of glacial acetic acid.
2. Add 1.5 cm^3 of concentrated sulphuric acid.
3. Store the solution in a dark glass bottle.
4. *On the day of use,* prepare a fresh solution of ethanal (1 cm^3) in distilled water (50 cm^3).
5. Add 0.5 cm^3 of this solution to each 100 cm^3 of the diphenylamine solution.

resources

Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid *Biochemical Journal* 62 (2) 314–323.

This paper describes Burton's method of assaying DNA concentration using diphenylamine.

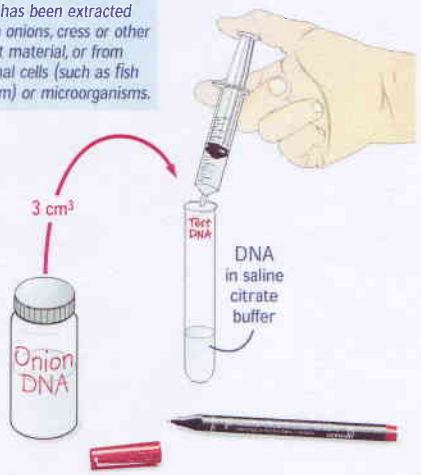
Brown, J. K. (1996) *Agarose gel DNA quantitation [sic]* Access Excellence Activities Exchange www.accessexcellence.org/AE/AEC/AEF/1996/brown_dna.html

An alternative method of quantifying DNA, by comparing samples with known DNA standards on a gel.

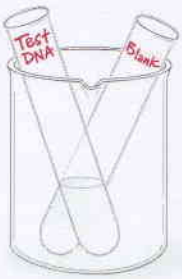
QUALITATIVE METHOD

1 Dispense 3 cm³ of the DNA solution to be tested into a clean test tube. Label the tube appropriately.

Note: *You can test DNA that has been extracted from onions, cress or other plant material, or from animal cells (such as fish sperm) or microorganisms.*



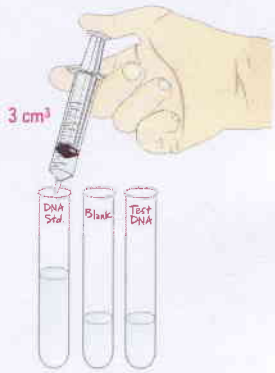
2 Add 3 cm³ of DNA standard solution to a second tube, then place 3 cm³ of saline buffer into a third. Make sure that all three tubes are correctly labelled.



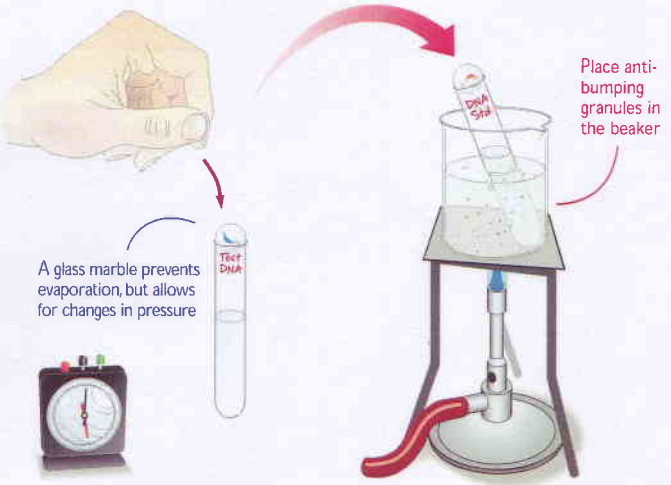
The DNA standard contains 3 mg of dried DNA per cm³

Salmon sperm or calf thymus DNA dissolved in saline citrate buffer is a suitable standard.

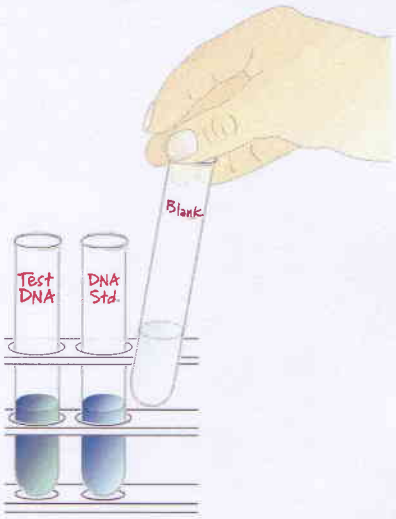
3 Add 3 cm³ of diphenylamine reagent to each tube.



4 Top each tube with a marble, then stand the tubes in a boiling water bath for 10 minutes.



5 Compare the colours of the liquids. Those containing DNA will be blue.



QUANTITATIVE METHOD carry out steps 1–3 as above, then...

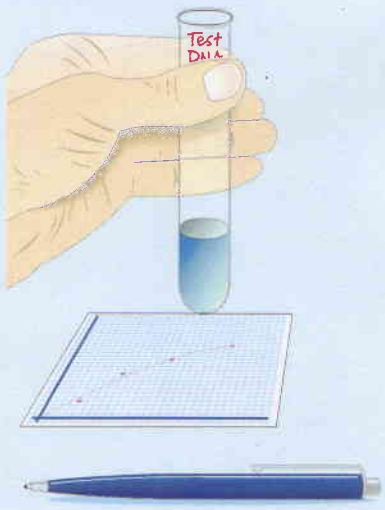
4 Cover the tubes with cling film and incubate them overnight at about 30 °C.

5 Use various dilutions of the standard DNA solution (containing 3 mg per cm³) to prepare a standard curve, showing absorbance at 660 nm.

The exact concentrations needed to prepare a standard curve will vary according to the concentration of DNA in the test sample(s). The following range of dilutions is suggested:

Stock	Undiluted stock solution
1:1	Stock solution : Saline citrate buffer
1:4	Stock solution : Saline citrate buffer
1:8	Stock solution : Saline citrate buffer
Blank	Saline citrate buffer

6 Use the standard curve to determine the concentration of DNA in your sample solution.



Dean Madden

β-galactosidase induction


β-galactosidase PDB ID: 3PBG

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 practical task, 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.

Aim

To induce and measure the production of the enzyme β-galactosidase (lactase) by *E. coli*.

Preparation

You will need cultures of *E. coli* from a strain that possesses the *lacZ* (β-galactosidase) gene. These can be grown on solid agar for the qualitative method or in broth for the quantitative method, 24–48 hours in advance. LB or nutrient broth or agar are suitable. To induce the production of β-galactosidase, lactose must be added to the growth medium (0.1 g per cm^3 of broth). The ONPG solution should be prepared a day before at the earliest. ***Please see the safety note, below.***

Timing

This activity takes about 60 minutes, including an incubation period of 10 minutes.

Materials and equipment

Needed by each person or group

- Cultures of *E. coli* ***See Preparation, above.***
- ONPG (ortho-nitrophenyl-β-D-galactoside) dissolved in Z-buffer, 2 cm^3 per test sample
- Methylbenzene (toluene), 1 drop per test sample
- Test tubes, caps, rack and marker pen
- Inoculation loop
- Pasteur pipettes, attached to 1 cm^3 plastic syringe
- 5 cm^3 syringe, for transferring ONPG solution
- Waste container with disinfectant
- Stopclock
- Safety spectacles

For the quantitative method, the following items are also needed per class

- Water bath maintained at 37 °C
- Access to a fume cupboard
- Hair drier
- Cuvettes for spectrophotometer or colorimeter
- 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).

Procedure

Quick qualitative method

This is used to determine whether a culture is producing β-galactosidase.

1. Using a syringe, transfer 2 cm^3 of ONPG in Z-buffer into each of two test tubes. *One of these tubes will be a 'control'; the other will be for the test culture. Z-buffer is merely a phosphate buffer which maintains the correct pH for the enzyme activity.*
2. Label the tubes appropriately.
3. Use a flamed wire loop to aseptically transfer a large colony from a plate into the test solution. Suspend the microorganisms by agitating the loop, then sterilise it by flaming. ***Take care to introduce the loop into the flame slowly, to avoid sputtering!***
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 membranes, allowing the ONPG to diffuse into the cells.*
5. Let the test tubes stand on the bench, until a strong yellow colour develops. This generally takes 5–20 minutes. *The reaction can be speeded up by incubating the tubes at 37 °C. The colourless ONPG is broken down by β-galactosidase to produce galactose and ortho-nitrophenyl (ONP). ONP is bright yellow in alkaline conditions.*
6. Compare the colour of the tube with the cells to that of the 'control' tube.

Quantitative method

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

1. Using a 1 cm^3 syringe linked to a sterile Pasteur pipette, transfer 0.1 cm^3 of each microbial suspension to be tested into a separate test tube. *Place used pipettes in the waste container.*
2. Transfer 0.1 cm^3 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 cm^3 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).

Safety

Handling microorganisms and methylbenzene

Good microbiological practice must be observed when handling microorganisms. Please refer to the full microbiology *Safety guidelines* on pages 14–17.

Methylbenzene is flammable and produces harmful vapour. Large volumes should therefore be handled in a fume cupboard, although the small amounts used here can safely be handled at the bench (but keep away from flames). Skin and eye contact should be avoided. **Eye protection must be worn.**

resources

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

The lac operon. A short history of a genetic paradigm by Benno Müller-Hill (1996) Walter de Gruyter, Berlin. ISBN: 3 11014830 7.

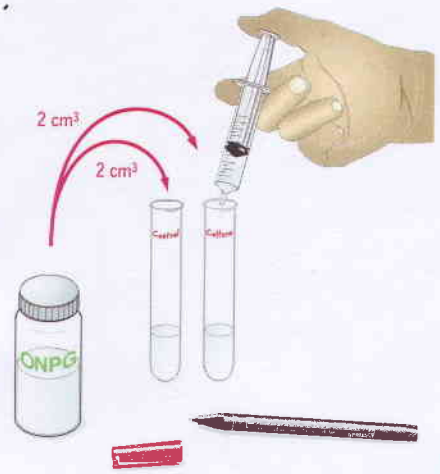
Induction of the lac operon in E. coli. Higher practical 26 in Higher Still Development Unit support materials. Ladywell House, Ladywell Road, EDINBURGH, EH12 7YH.

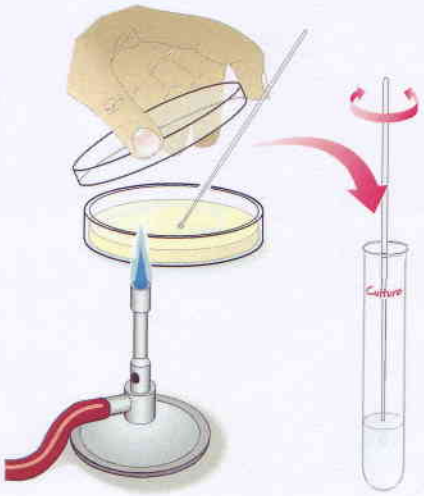
QUALITATIVE METHOD

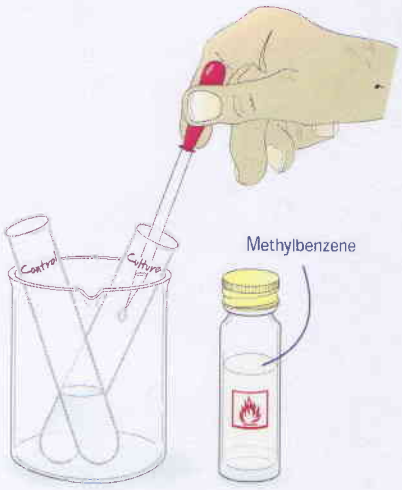
1 2 Transfer 2 cm³ of ONPG in Z buffer into each of two test tubes. Label the tubes appropriately.

3 Aseptically transfer a colony from the plate to one of the tubes of ONPG solution. Twiddle to disperse the cells, then flame the loop to kill any remaining cells.

4 6 Add a drop of methylbenzene to each tube, cap the tubes and shake well to mix. Stand the tubes for 5–20 minutes until a yellow colour develops.

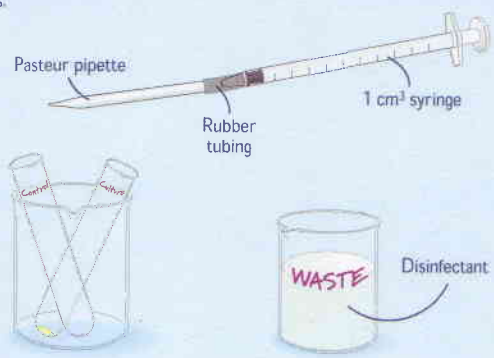






QUANTITATIVE METHOD

1 3 Transfer 0.1 cm³ of each culture to be tested into a separate test tube. Discard used pipettes into disinfectant. Use a similar volume of LB broth as a 'control'. Label the tubes.

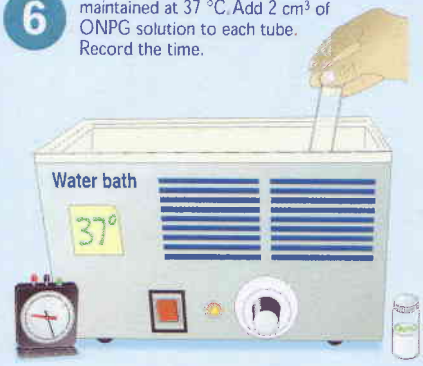


4 Add a drop of methylbenzene to each tube. Cap the tubes firmly and shake them well.

5 FUME CUPBOARD

Use a hair drier to evaporate the methylbenzene from each tube.

6 Transfer the tubes to a water bath maintained at 37 °C. Add 2 cm³ of ONPG solution to each tube. Record the time.



7 Record the optical density of the samples at 420 nm at 10 minute intervals until no further colour change is seen.

8 Plot a graph of the results (OD_{420} vs. Time)

CALCULATION OF ENZYME ACTIVITY

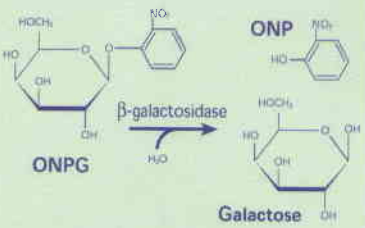
The enzyme units in a given sample can be calculated as follows:

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

The optical density (OD) at 420 and 550 nm is read from the sample.

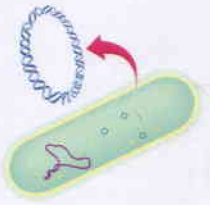
The 1.75 x OD_{550} is an optional correction factor which allows for light-scattering by *E. coli* cell debris, *etc.*

OD_{600} reflects the cell density at the start of the reaction
t = time of the reaction in minutes
v = the volume of the culture in cm³



Note: *A fully-induced culture grown on glucose-containing medium has an activity of about 1 000 units, whereas an uninduced culture has an activity of 1 unit.*

extraction of plasmid dna



This is the 'classical' method of isolating plasmid DNA; with it you can see the purpose of each step. Quicker, easier methods, using small resin filters that nest within a microcentrifuge tube, are also available as kits from suppliers of materials for molecular biology, but these tend to be too costly for everyday teaching purposes.

Aim

To isolate plasmid DNA from bacteria. The plasmids produced in this way can be cut and ligated *in vitro* and 'run' on an electrophoresis gel. However, they should not be used for transformations as their identity cannot easily be verified.

Preparation

An overnight culture of *E. coli* containing a suitable (*i.e., self-cloned*) plasmid is required. Sterile tubes and tips are not needed, however. ***Please see the safety note, below.***

Timing

This activity takes about 60 minutes, including a period of 30 minutes when the materials are being chilled.

Materials and equipment

Needed by each person or group

- 1 cm^3 liquid culture of *E. coli* containing plasmid DNA, grown overnight at 37 °C. It is best to use a plasmid with a high copy number (*i.e., where numerous copies exist within each bacterial cell*) such as pUC18 or pUC19. *Antibiotic is needed in the medium to maintain the plasmid (e.g., ampicillin for pUC18, pUC19 or pBLU).*
- Glucose / EDTA / Tris (GET) buffer, 100 µL
- SDS / Sodium hydroxide (SDS / NaOH), 200 µL
- Potassium acetate / Ethanoic acid (KOAc), 150 µL
- Tris / EDTA (TE) buffer, 15 µL
- Ice cold ethanol (95%), 400 µL
- 1.5 cm^3 microcentrifuge tubes, 2
- Micropipettes or similar dispensing devices
- Tips for micropipette, 8
- Crushed ice, in an expanded polystyrene cup
- Microcentrifuge for 1.5 cm^3 microcentrifuge tubes, capable of producing at least 8 000 x g. With a slower centrifuge (*e.g., 2 000–3 000 x g*) the times stated here should be doubled.
- Hair drier

Procedure

1. Spin down 1 cm^3 of bacterial culture in a 1.5 cm^3 microcentrifuge tube for 1 minute or until a mass of cells some 2–3 mm in diameter is visible.
2. Decant the supernatant (liquid) into disinfectant in a waste container.
3. Ensure that *all* the liquid is removed, using a micropipette to draw off any that remains in the tube.
4. Add 100 µL of GET buffer to the tube. Resuspend the cells by closing the tube, then flicking the side repeatedly with a finger. *EDTA in the buffer chelates Mg^{2+} and Ca^{2+} ions, which are necessary for DNase action and cell membrane stability.*
5. Add 200 µL of SDS/NaOH solution. Mix by gently inverting the closed microcentrifuge tube. Leave to stand for 5 minutes at room temperature. *SDS is an ionic detergent which dissolves the phospholipids and proteins of the cell membrane. This lyses the cells, releasing the DNA. The sodium hydroxide splits both the plasmid and chromosomal DNA into single-stranded molecules, but each denatured plasmid remains stuck as two intertwined rings.*
6. Add 150 µL of KOAc and mix gently by inverting the closed microcentrifuge tube. Leave to stand on ice for 5 minutes. *Treatment with KOAc restores the pH of the solution to neutral — the DNA consequently renatures. KOAc also precipitates the SDS, phospholipids, proteins and large DNA molecules.*
7. Centrifuge the tube for 5 minutes to spin down the cell debris and chromosomal DNA.
8. Very carefully transfer 400 µL of the supernatant (liquid) to a clean microcentrifuge tube. Ensure that none of the cell debris is carried over. *The supernatant contains the plasmid DNA.*
9. Add 400 µL of ice cold ethanol. Mix gently.
10. Leave the tube in a deep freeze (-18–20 °C) for 10–30 minutes, then centrifuge for 10 minutes at high speed. You should now have a *very small* but *just* visible pellet.
11. Decant the supernatant into a waste container, taking care not to discard the pellet of plasmid DNA.
12. With a micropipette, remove the last drops of liquid. If convenient, leave the uncapped tube to stand at room temperature for 15 minutes or use a hair drier so that all the alcohol evaporates.
13. Dissolve the pellet in 15 µL of TE buffer; vigorous mixing is needed to ensure that this happens. Store the plasmid solution in a freezer, or run it on a gel.

Safety

Handling microorganisms and ethanol in freezers

Good microbiological practice should be observed when handling microorganisms. These procedures are explained on pages 14–17. Please refer to the safety note about ethanol in freezers on page 20 also.

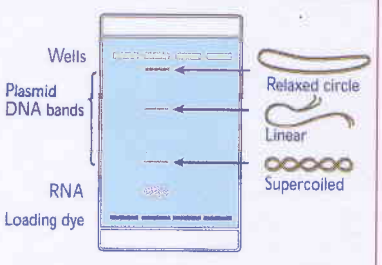
resources

Laboratory DNA Science. An introduction to recombinant DNA techniques and methods of genome analysis by Mark Bloom, Greg Freyer and David Micklos (1996) The Benjamin/Cummings Publishing Company, Menlo Park. ISBN: 0 8053 3040 2.

running plasmid dna on a gel

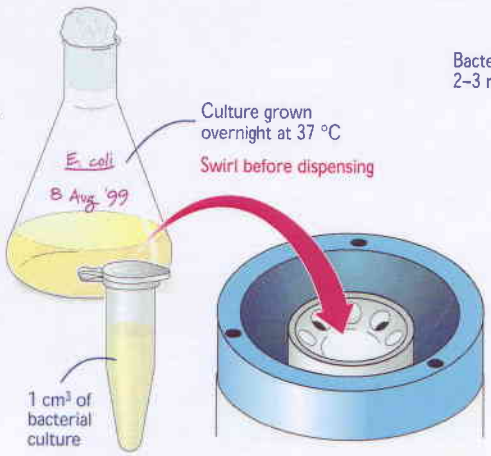
Procedure

1. Mix 15 µL of plasmid DNA solution with 4 µL of loading dye.
2. Very carefully load the sample into a well in a 0.8% agarose gel.
3. Run the gel until the loading dye has moved 30–40 mm.
4. Pour off the buffer, leave the gel in the tank and pour on 0.04% Azure A solution for *4 minutes only.*
5. Wash the gel surface with water.
6. The plasmid bands will appear as the stain moves down through the gel. Three bands, corresponding to three physical forms of the plasmid, should be seen.



Relaxed: only one strand of the plasmid is broken, hence the plasmid loses some of its coiling.
Linear: both strands of the DNA helix are broken.
Supercoiled: plasmid is tightly twisted.

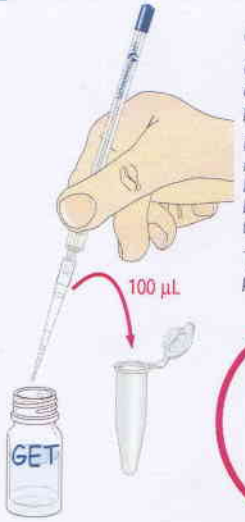
1 Spin 1 cm³ of bacterial culture for 1 minute at 8 000 x g, or until a pellet is formed.



Remember to balance the centrifuge!

2 **3** Pour the supernatant into disinfectant. Use a micropipette to remove as much liquid as possible from the pellet.

4 Add 100 µL of *ice-cold* GET buffer to the pellet. Cap the tube and resuspend the cells well by tapping the tube vigorously until no lumps remain.

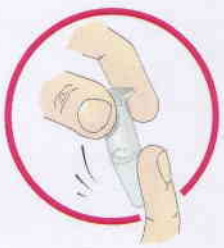


Glucose/EDTA/Tris

Glucose maintains the osmostic pressure and prevents lysis of the cells.

EDTA weakens the cell membrane and chelates Mg^{2+} ions that are needed by DNases, protecting the DNA from breakdown.

Tris buffers the solution at pH8.

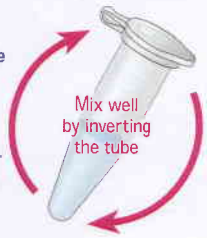


5 Add 200 µL of SDS + NaOH solution. Mix well by inverting the capped tube. Leave for 5 minutes on ice.

Sodium Dodecyl Sulphate + Sodium hydroxide

The SDS dissolves the cell membrane lipids and degrades the cellular proteins.

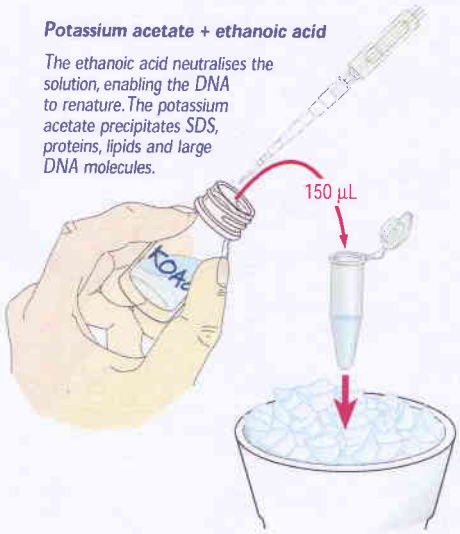
The NaOH splits the double-stranded DNA into single strands.



6 Add 150 µL of *ice-cold* KOAc solution. Mix well. *A white precipitate should appear.* Stand the tube on ice for 5 minutes.

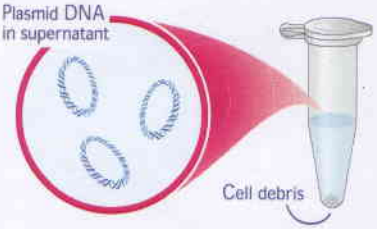
Potassium acetate + ethanoic acid

The ethanoic acid neutralises the solution, enabling the DNA to renature. The potassium acetate precipitates SDS, proteins, lipids and large DNA molecules.

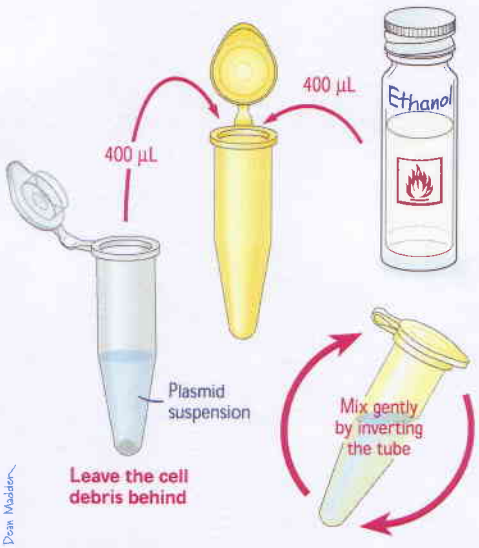


7 Spin down the cell debris for 5 minutes. *The plasmid DNA remains in the supernatant.*

Remember to balance the centrifuge!

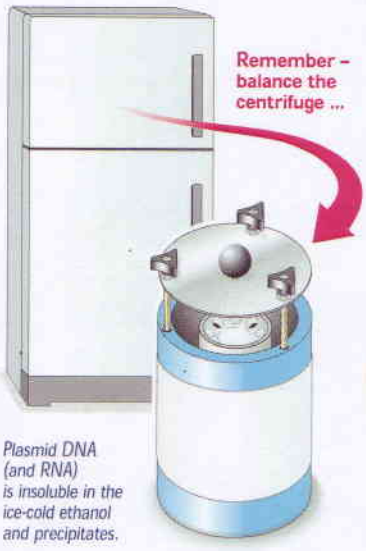


8 **9** Transfer 400 µL of the supernatant to a new tube. Add 400 µL of *ice-cold* ethanol.



Leave the cell debris behind

10 Leave the tube at -20 °C for 10–30 minutes. Centrifuge for 10 minutes at ≥ 8 000 x g.



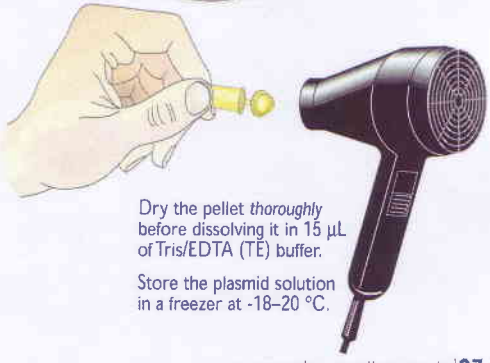
Remember – balance the centrifuge ...

Plasmid DNA (and RNA) is insoluble in the ice-cold ethanol and precipitates.

11 **13** Decant the liquid from the tube. Remove any remaining liquid using a micropipette.

Take care not to pour away or dislodge the *very* small DNA pellet

Note: The pellet may almost be invisible.

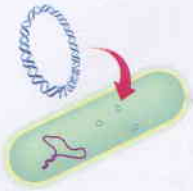


Dry the pellet *thoroughly* before dissolving it in 15 µL of Tris/EDTA (TE) buffer.

Store the plasmid solution in a freezer at -18–20 °C.

Dean Madden

bacterial transformation



This is a 'generic' method of transforming cells; it will work with most 'cloning' strains of bacteria and plasmids. Because colonies are taken directly from Petri dish cultures (rather than liquid cultures at the optimum phase of growth), the protocol uses a specially-devised transformation buffer to improve the transformation efficiency. This protocol is for plasmids that have the *lacZ* gene (and hosts that lack it) so that X-Gal can be used as a marker.

Aim

To transform an artificially-competent K12 strain of *Escherichia coli* bacteria with plasmid DNA.

Preparation

Petri dish cultures of a suitable laboratory strain (see below) of *E. coli* should be prepared 3–4 days before this work is undertaken. These cultures should be grown at room temperature (18–25 °C). Petri dishes of media should be prepared no more than one week in advance. Plasmid DNA can be dispensed in advance and frozen at -18–20 °C until it is needed.

Timing

This activity takes about 50 minutes, plus overnight incubation of the transformed cells.

Materials and equipment

Needed by each person or group

- 10 µL of plasmid DNA solution in TE buffer, at a concentration of 2 ng per µL. Any plasmid constructed from DNA that can occur naturally within the host *E. coli*, that contains a selective antibiotic marker and the *lacZ* gene can be used *e.g., p2k (from the NCBE), pBLU or pUC18.*
- Access to a stock culture plate of *E. coli* K12. Suitable strains are non-pathogenic, enfeebled and harbour no plasmids of their own. They must also lack the ability to metabolise lactose. Suggested strains are DH5α or JM101.
- Petri dishes, 2 containing a suitable selective medium *e.g., LB agar with X-Gal and antibiotic (kanamycin for p2k, ampicillin for pBLU and pUC18). pUC18 also requires 79 mg of isopropyl-β-galactoside (IPTG) per dm^3 of medium to induce the production of β-galactosidase. p2k and pBLU do not need this as their lacZ (β-galactosidase) gene is unregulated.*
- Sterile microcentrifuge tubes, each containing 250 µL of sterile transformation buffer, 2
- Sterile microcentrifuge tube containing 500 µL of sterile LB broth, warmed to 37 °C
- Crushed ice, in an expanded polystyrene cup
- Sterile disposable Pasteur pipettes, 4
- Inoculation loop
- Spreader (2, if using the disposable type)
- Screw-topped waste container, *e.g., an old plastic chemical jar*, containing disinfectant solution
- Marker pen, fine tipped, permanent
- Floating holder for tubes, *e.g., cut from polystyrene*
- Water bath set at 40–42 °C
- Incubator set at 37 °C

Procedure

1. Place two tubes of sterile transformation buffer on ice and leave them to cool for at least 5 minutes.
2. With a wire loop, aseptically remove 2–3 colonies from the stock plate. Place the loop in the transformation buffer and agitate it vigorously to dislodge the bacteria. Cap the tube. Flame the used loop. Repeat this step with the second tube.
3. Tap the side of each capped tube to resuspend the cells. Keep both tubes on ice. *This step prepares the cells to take up plasmid DNA. Positively-charged ions in the transformation buffer neutralise the cell surface (and later, the plasmid DNA) so that the plasmid DNA can approach it.*
4. Use a sterile pipette to add all the bacterial suspension from one of the tubes to that containing 10 µL of plasmid DNA solution. Place the used pipette in disinfectant. Cap the tube and flick it gently to mix the plasmid into the cell suspension.
5. Place both tubes in a foam holder. Heat shock the bacteria by floating the tubes in a water bath at 40–42 °C for *exactly* 30 seconds. *The heat shock is thought to help drive the plasmids into the cells.*
6. Remove the tubes from the water bath and using another sterile pipette, place 250 µL of warmed (37 °C) LB broth to each tube. Cap the tubes and mix their contents by tapping the tubes again. Leave the tubes at 37 °C *for at least 20 minutes. This 'recovery period' is necessary to allow expression of the plasmid's antibiotic resistance gene.*
7. Use separate sterile pipettes to place about 250 µL of each culture onto separate Petri dishes containing LB agar with antibiotic and X-Gal. Place the used pipettes in disinfectant solution.
8. Use a sterile spreader to evenly distribute the culture over the surface of one plate. Reflame the spreader, then spread culture over the second plate. Flame the spreader after use. Seal and label both plates, let the liquid soak in, then incubate them, inverted, overnight at 37 °C.
9. Cells transformed with plasmid DNA are able to metabolise the colourless compound X-Gal, producing blue-coloured indigo dye.

Safety

Handling microorganisms

Good microbiological practice must be observed when handling microorganisms. ***Transformed cells MUST be destroyed by autoclaving after use.*** Refer to the full microbiology *Safety guidelines* on pages 14–17.

◀ *Biology students at St. Katherine's School, near Bristol, trying to transform bacteria.*

Photograph kindly supplied by Sue Morgan.

resources

The transforming principle. Discovering that genes are made of DNA by Maclyn McCarty (1986) W.W. Norton & Company, New York. ISBN: 0 393 30450 7.

Laboratory DNA Science. An introduction to recombinant DNA techniques and methods of genome analysis by Mark Bloom, Greg Freyer and David Micklos (1996) The Benjamin/Cummings Publishing Company, Menlo Park. ISBN: 0 8053 3040 2.

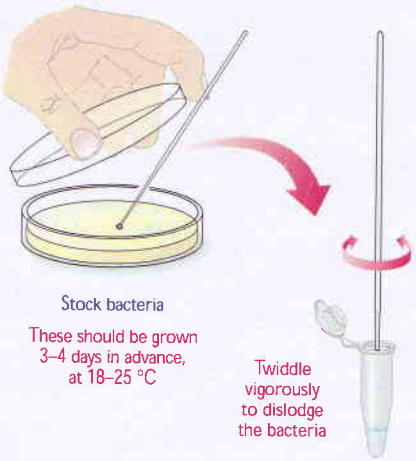
The transformer protocol Students' and Technical Guides by Dean Madden (2000) National Centre for Biotechnology Education, The University of Reading.

This is a guide from an NCBE practical kit, enabling 'self-cloning' bacterial transformation.

1 Chill the transmission buffer on ice for **at least 5 minutes** before you start.

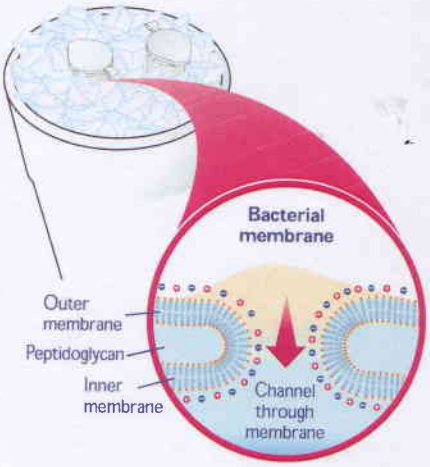
The transformation buffer contains positively-charged ions e.g. Ca^{2+}. These ions bind to the negatively-charged phosphate groups of the DNA, and the phospholipids of the cell membranes, shielding their negative charges. This allows the DNA to approach the cell membrane and to pass through channels in it that are formed where the inner and outer cell membranes meet.

2 Scrape 2–3 colonies from the stock plate. **Do not dig into the agar.** Mix the cells into the cold buffer. **Repeat with the second tube.**

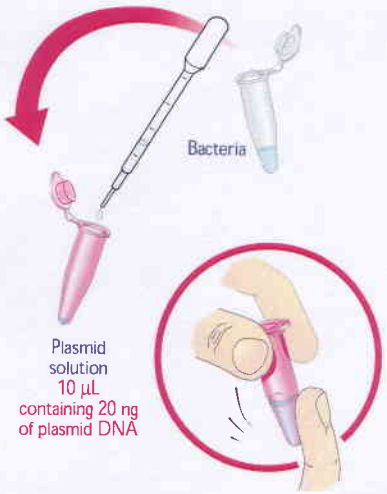


3 Chill both tubes on ice for **10 minutes**.

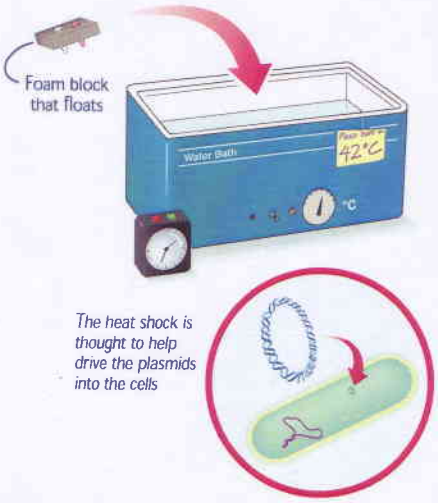
This prepares the cells to take up plasmids, that is, it makes them 'competent'.



4 Add all the bacteria from one tube to the plasmid DNA. Close the tube and mix gently by tapping the side of the tube.



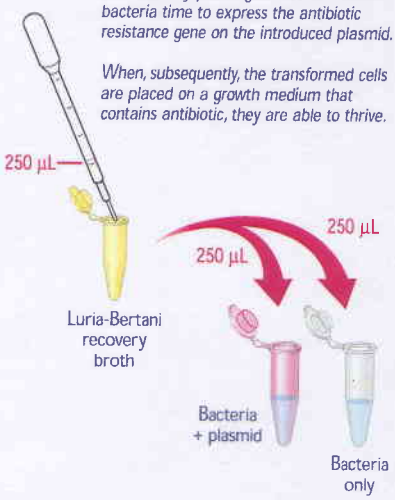
5 Heat shock the bacteria in both tubes for **exactly 30 seconds** at 40–42 °C.



6 Add 250 µL of LB recovery broth, warmed to 37 °C, to each tube. Mix gently by tapping. Incubate for **at least 20 minutes** at 37 °C.

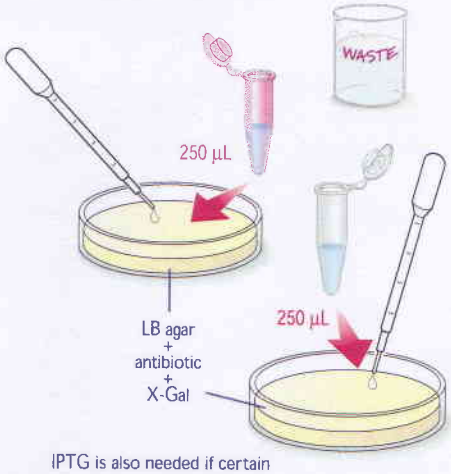
This recovery period gives the transformed bacteria time to express the antibiotic resistance gene on the introduced plasmid.

When, subsequently, the transformed cells are placed on a growth medium that contains antibiotic, they are able to thrive.



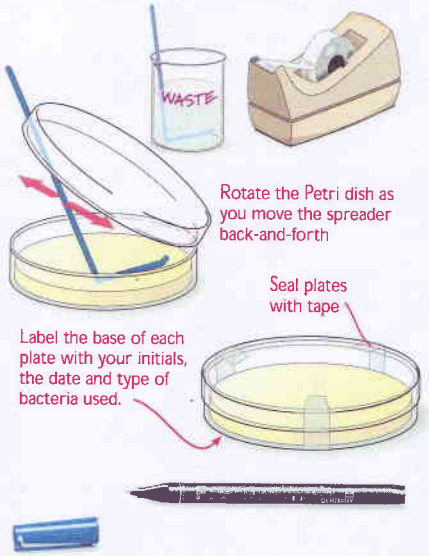
7 Add 250 µL of bacterial cell suspension to each plate. Use a new pipette each time.

The bacteria survive better if the plates are pre-warmed to 37 °C.

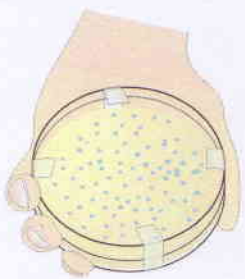


IPTG is also needed if certain plasmids are used, to induce the formation of β-galactosidase

8 Spread the culture all over the plates, using a new sterile spreader for each one. Let the culture soak in if necessary. Incubate overnight, inverted, at 37 °C.

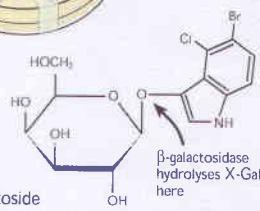


9 Colonies transformed with plasmid DNA are blue. This colour is indigo, which is made by linking two molecules that result from hydrolysis of the X-Gal.



BIOHAZARD

TRANSFORMED CELLS MUST BE DESTROYED AFTER USE



Dean Madden

restriction and ligation

T4 DNA ligase can be used to join double-stranded DNA fragments that have staggered or blunt ends, produced by the action of a restriction enzyme. Here the enzyme *Eco*RI is used to sever plasmid DNA which is then re-joined using DNA ligase. The resulting fragments are separated by gel electrophoresis.

Aim

To demonstrate the action of T4 DNA ligase on plasmid DNA. Any plasmid with an *Eco*RI restriction site can be used *e.g., pUC18, pBR322.*

Preparation

The T4 DNA ligase and buffer should be kept in a freezer at -18–20 °C until within 5–10 minutes of use and returned to the freezer IMMEDIATELY after use.

Timing

This activity takes about 50 minutes, plus running and staining time for the gel.

Materials and equipment

Needed by each person or group

- Plasmid DNA in sterile distilled water, 20 µL (at a concentration of 50 ng per cm^3)
- Dried *Eco*RI, 1 tube (with an activity of 10 units)
- T4 DNA ligase solution, 1 µL (with an activity of 1 unit per µL)
- 5x DNA ligase buffer with ATP, 4 µL (*this is usually supplied with the T4 DNA ligase*)
- OPTIONAL: 1 kb DNA ladder solution, 10 µL in TE buffer (containing 1 µg of DNA)
- Bromophenol blue loading dye, 8 µL
- Agarose solution, 12 cm^3 (0.8% in TBE buffer, melted in a microwave oven then kept molten in a water bath at 55–60 °C)
- TBE buffer, 15 cm^3
- Azure A solution (0.04% in water)
- Crushed ice, in an expanded polystyrene cup
- Tips for micropipette, 8
- 1.5 cm^3 microcentrifuge tubes, 2
- NCBE gel electrophoresis equipment with 6-toothed comb and carbon fibre electrode material
- Foam holder for tubes (*this can be made by punching holes from a strip of foam using a suitably-sized cork borer*)
- Marker pen, fine tipped, permanent
- Micropipette or similar device, 1–10 µL
- Water bath or incubator set at 37 °C
- Power supply for electrophoresis equipment (*2–5 x 9 volt batteries*)

Procedure

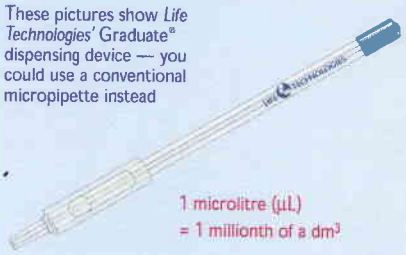
1. Add 20 µL of plasmid DNA solution to the tube containing the dried *Eco*RI.
2. Draw the liquid up and down in the micropipette tip a few times to ensure that the enzyme is properly rehydrated. *The liquid in the tube should have an even, pale blue hue, with no concentration of blue dye at the bottom of the tube.*
3. Cap the tube, label it and place it in a foam holder.
4. Incubate the tube in a water bath or incubator at 37 °C for 30 minutes.
5. While the tube is incubating, prepare the electrophoresis equipment. Cut two carbon fibre electrodes to fit the tank, each 22 mm x 42 mm. Place the comb in the tank and pour in sufficient agarose solution (about 12 cm^3) to fill the central section. Leave the tank undisturbed for about 15 minutes until the gel has set and looks opaque.
6. Remove the plasmid DNA solution (which has now been cut by the enzyme) from the water bath or incubator.
7. Take 5 µL of the plasmid DNA solution and add it to a new microcentrifuge tube.
8. Add 2 µL of bromophenol loading dye to this sample, then store it on ice. *The addition of loading dye stops the reaction, because the dye mixture contains EDTA, which chelates Mg^{2+} ions, which are needed by the restriction enzyme.*
9. To the remaining 15 µL of plasmid DNA solution, add 4 µL of 5x DNA ligase buffer and 1 µL of T4 DNA ligase. *The ligase buffer contains ATP and magnesium ions, which are required by the ligase.* ***The stock DNA ligase is unstable at room temperature, and it should be returned to the freezer immediately after you have dispensed it.***
10. Draw the liquid up and down in the micropipette tip a few times to ensure that the enzyme, buffer and plasmid are mixed. Leave the mixture to stand at room temperature ***for 2 minutes only.***
11. Stop the reaction by adding 2 µL of loading dye to the ligase, buffer and plasmid mixture. Store the mixture on ice alongside the unligated sample.
12. Pour sufficient TBE buffer over the agarose gel to cover it to a depth of 2–3 mm. Gently ease the comb from the gel.
13. Load the first sample of cut plasmid DNA into one of the wells in the gel. Into each of two other wells load about 10 µL of the cut, ligated plasmid DNA. If desired, add 10 µL of 1 kb DNA ladder solution mixed with 2 µL of loading dye to another well. *This will provide DNA fragments of known sizes with which the fragments of plasmid DNA may be compared.*
14. Put the carbon fibre electrodes in either end of the tank. Check that there is still enough buffer in the tank and add a little more if necessary (the buffer soaks into the electrode tissue). Cover the gel with the comb and electrophorese for 2–12 hours (depending upon the voltage used).
15. When the loading dye reaches the end of the gel, disconnect the power supply, remove the electrodes and pour the buffer off the gel. *Leave the gel in the tank for staining.*
16. Flood the gel with Azure A solution and let it stand for ***exactly 4 minutes***.
17. Pour off the stain, and wash the gel surface with water. *Do not leave any water on the gel.*
18. Wrap the gel in a plastic bag. The dye will migrate through the gel, staining the DNA as it does so. This will take 15–30 minutes, and the best results will be visible after about 12 hours.

resources

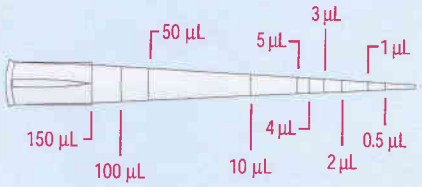
The biology of plasmids by David Summers (1996) Blackwell, Oxford. ISBN: 0 632 03436 X

Russell, G. A. (1997) Practical DNA technology in school — 4: Plasmids and ligase. *Journal of Biological Education* **31** (2) 135–140.

These pictures show *Life Technologies'* Graduate® dispensing device — you could use a conventional micropipette instead

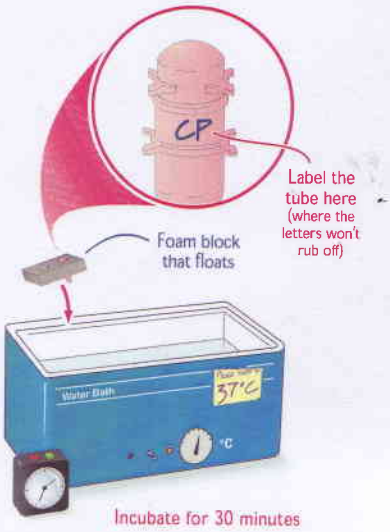


Calibrated Triple Check® tip

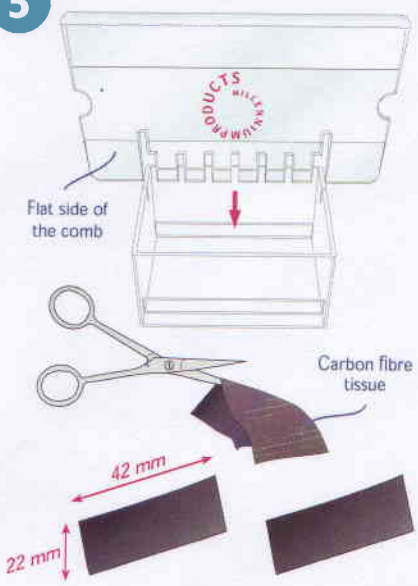


1 3

4

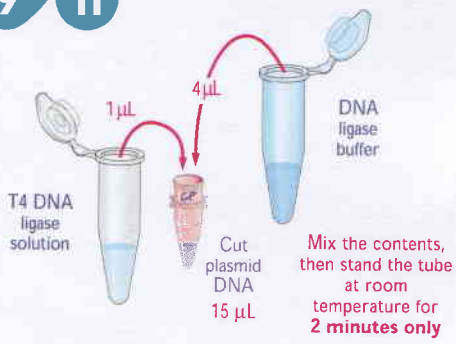


5

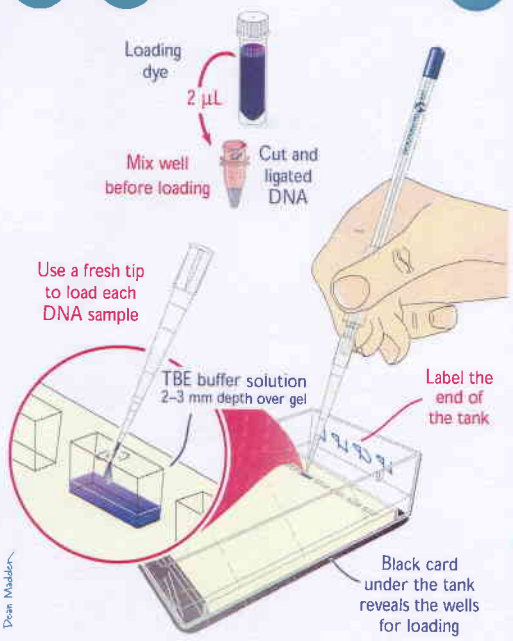


6 8

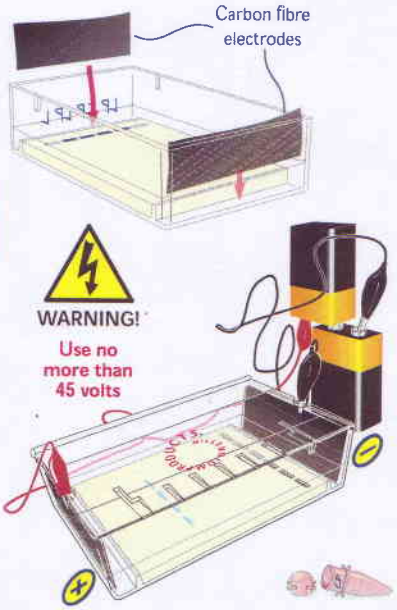
9 11



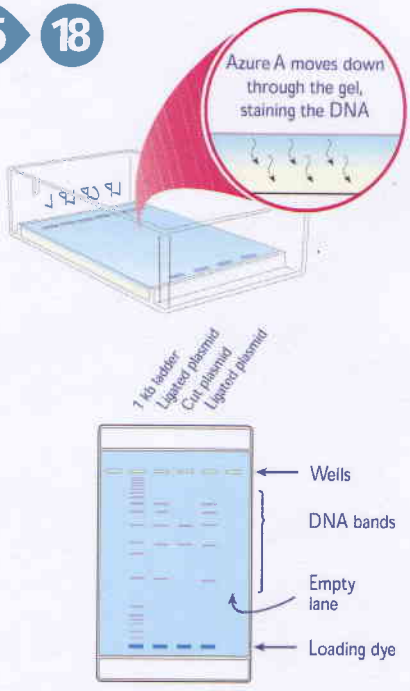
12 13



14



15 18



Dean Madden

restriction site mapping

In addition to their role in cutting DNA molecules, restriction enzymes help in the initial steps of analysing nucleotide sequences. This exercise demonstrates the principle of restriction site mapping, using DNA from the Lambda (λ) bacteriophage. Two or three enzymes can be used to cut the DNA. A 'DNA ladder' provides a 'molecular ruler' with fragments of known sizes against which the λ fragments can be measured.

Aim

To locate the relative positions of restriction sites for *Eco*RI and *Bam*HI (and optionally, *Hin*dIII) on Lambda DNA.

Preparation

Study these instructions carefully, as this task is complex.

Timing

This activity takes about 90 minutes, plus running and staining time for the gel.

Materials and equipment

Needed by each person or group

- Dried *Eco*RI, 3 tubes
- Dried *Bam*HI, 2 tubes
- OPTIONAL: Dried *Hin*dIII, 2 tubes
- Dried λ DNA, 1 tube for one double digest, 2 tubes for two double digests (each containing 10 µg of DNA)
- 1 kb DNA ladder solution, 20 µL in TE buffer (containing 1 µg of DNA)
- Bromophenol blue loading dye, ~16 µL
- Agarose solution, 12 cm^3 (0.8% in TBE buffer, melted in a microwave oven then kept molten in a water bath at 55–60 °C)
- TBE buffer, 15 cm^3
- Azure A solution (0.04% in water)
- Crushed ice, in an expanded polystyrene cup
- Tips for micropipette
- NCBE gel electrophoresis equipment with 4-toothed comb (for 2 enzyme-mapping) or 6-tooth comb (for 3 enzyme-mapping)
- Carbon fibre electrode material
- Foam holder for tubes (*this can be made by punching holes from a strip of foam using a suitably-sized cork borer*)
- Marker pen, fine tipped, permanent
- Micropipette or similar device, 1–10 µL
- Water bath or incubator set at 37 °C
- Hot water bath maintained at 70 °C
- Power supply for electrophoresis equipment (*2–5 x 9 volt batteries*)

Procedure

1. Rehydrate 10 µg of dried λ DNA with 50 µL of distilled water, to give a concentration of 0.2 µg of DNA per µL. *If you are mapping with two enzymes, use one tube of dried DNA; with three enzymes, two tubes will be required.*
2. Add 10 µL of the DNA solution to each of the dried enzyme tubes for single digests. Then add an extra 10 µL of distilled water to each of these tubes. Mix their contents well until the colour from the dried enzyme is uniformly distributed through the liquid. Cap the tubes and label them.
3. For the double digests, label all the tubes carefully. Then add 20 µL of the DNA solution to each *Eco*RI tube. Mix well as before. Next add 20 µL of water to each of the second enzyme tubes, and mix until the enzyme is rehydrated. Combine the contents of each of the second enzyme tubes with an the *Eco*RI tubes (see diagram, opposite). *These 'double digests' will show where the different restriction sites lie relative to one another. As the dried enzyme preparations contain a buffer, it is important to ensure that the volumes of liquid (DNA solution or water) used to rehydrate them are correct.*
4. Incubate all of the restriction digests in a water bath or incubator at 37 °C for 30–45 minutes.
5. While the tubes are incubating, cast a gel from molten agarose. *It is important that the gel is of uniform thickness, so that during electrophoresis the DNA will move evenly through the gel.*
6. Cut two electrodes from carbon fibre tissue, and when the gel has set, pour some TBE buffer over it and ease the comb out gently.
7. Add 2 µL of bromophenol blue loading dye to each single enzyme digest and to the DNA ladder. Add 4 µL of loading dye to each of the double digests. As you add the dye to each tube, stir it into the DNA sample. ***Remember to use a new tip for each sample to avoid cross-contamination.***
8. Cap all of the tubes. Float them in a water bath at 70 °C for exactly 10 minutes, then immediately place them on ice. *The heat treatment breaks bonds that sometimes cause fragments from the two extreme ends of the λ DNA to stick together (this would form a large aberrant band on the gel). Rapid cooling is necessary to stop the unwanted bonds from re-forming.*
9. Place the gel where it is to be run. Ensure that there is sufficient buffer over the gel, then load each DNA sample into a well. Only half of the sample will be needed for each of the double digests. Add 10 µL of 1 kb DNA ladder solution mixed with 2 µL of loading dye to another well. ***Once again, remember to use a clean tip for each sample.***
10. Put an electrode at each end of the tank. Top up the buffer if necessary.
11. Cover the gel *e.g., with the comb* and electrophorese for 2–12 hours (depending upon the voltage used).
12. When the loading dye reaches the far end of the gel, disconnect the batteries, remove the electrodes and pour the buffer away. *Leave the gel in the tank for staining.*
13. Flood the gel with Azure A solution and let it stand for ***exactly 4 minutes***.
14. Pour off the stain, then wash the gel surface with water. *Do not leave any water on the gel.*
15. Wrap the gel in a plastic bag to reduce any evaporation. The dye will migrate through the gel, staining the DNA as it does so. This will take 15–30 minutes, and the best results will be visible after about 12 hours.
16. Analyse the results by measuring the distance moved by each group of fragments in the ladder, then calculating the approximate sizes of the digested DNA samples and the relative positions of the restriction sites in the λ genome.

resources

Ehrman, P. (1990) A user-friendly method for teaching restriction enzyme mapping. *The American Biology Teacher* **52** (7) 429–435.

Russell, G. A. and Miller, M. B. (1996) Practical DNA technology in school — 3: Mapping and methylation. *Journal of Biological Education* **30** (4) 289–294.

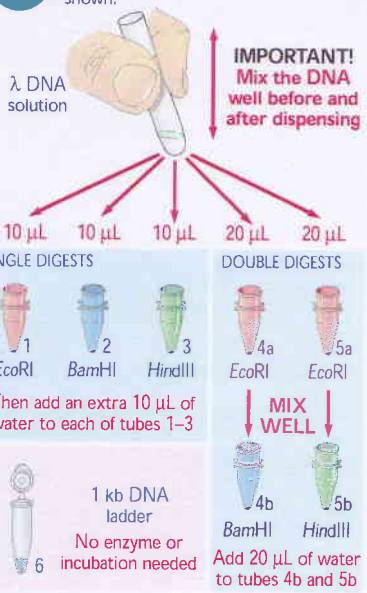
Laboratory DNA Science. An introduction to recombinant DNA techniques and methods of genome analysis by Mark Bloom, Greg Freyer and David Micklos (1996) The Benjamin/Cummings Publishing Company, Menlo Park. ISBN: 0 8053 3040 2.

The Lambda Protocol by Dean Madden (1996) National Centre for Biotechnology Education, The University of Reading.

A guide from an NCBE kit for gel electrophoresis of lambda DNA.

Web Cutter
A free on-line programme to help restriction mapping of DNA sequences.
www.medkem.gu.se/cutter/

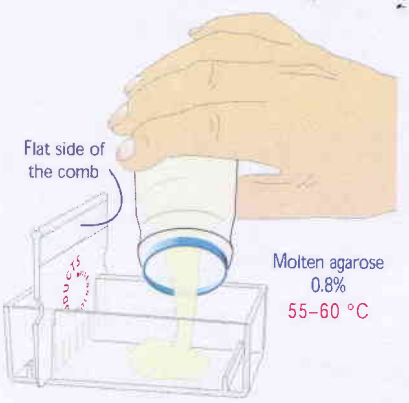
1 3 Rehydrate the λ DNA using 50 µL of water. Label and fill the tubes as shown:



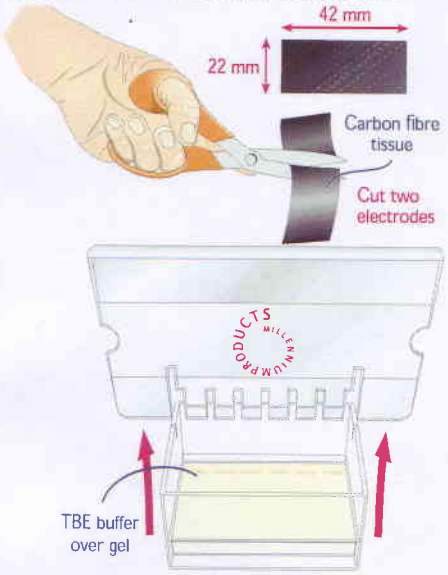
4 Cap all of the tubes, ensure that they are labelled, then incubate tubes 1–5 at 37 °C for 30–45 minutes.

5 Place the gel tank on a level surface. Fit the comb, then pour in the molten agarose. Leave the gel to set for 15–20 minutes.

CAUTION
Hot molten agarose can scald

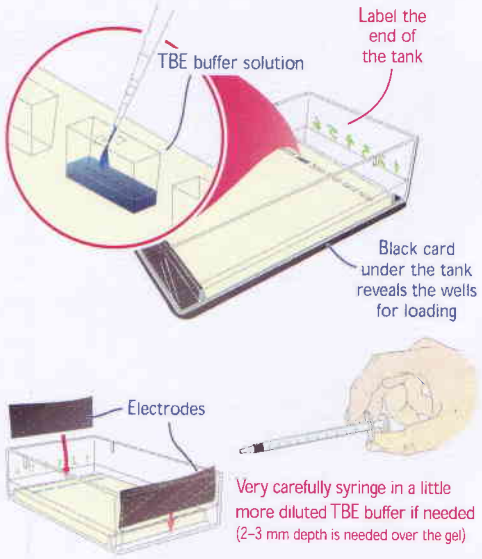


6 While the gel is setting, cut two electrodes from carbon fibre tissue. Once the gel has set, pour on some buffer then gently ease out the comb.

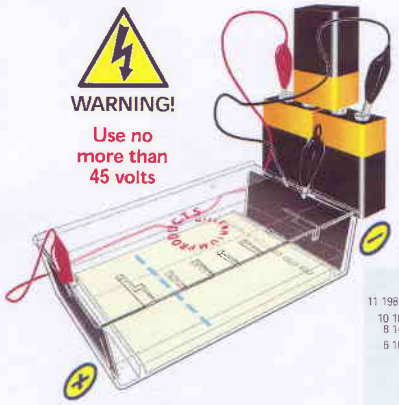


7 8 Add loading dye to each sample. Mix well, then heat to 70 °C for 10 minutes. Cool on ice.

9 10 Place the gel where it is to be run. Load the samples, using a new tip for each. Fit an electrode into each end of the tank.



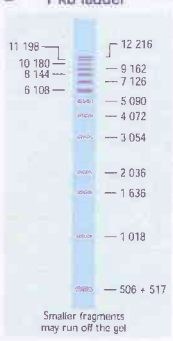
11 Connect batteries to the electrodes. Place the comb over the tank. Run until the loading dye reaches the end of the gel.



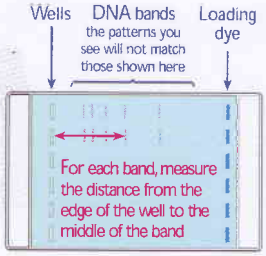
No. of 9 V batteries	Time taken to run gel
2	4 hours
3	3.5 hours
4	2 hours
5	1 hour

NEVER connect directly to a mains electricity supply!
Rechargable batteries may be used with this equipment.

12 13 Pour off the buffer, and throw away the electrodes. Leave the gel in the tank. Stain with Azure A solution for 4 minutes only, then pour away the stain. Wash the gel surface with water.



Millimetre graph paper beneath the gel is a convenient way to measure distances



16

ANALYSIS

1. Record the distances moved by all the bands on the gel. Measure from the leading edge of each well to the mid point of each band.
2. Use $\log_{10}$/normal graph paper (you will need two log cycles to fit the data on the graph). For the 1 kb DNA ladder, plot the distance moved by each band (X axis) against the size of the fragments (Y axis). *This will give you a straight line graph which can be used to find the approximate sizes of fragments from the DNA that has been cut with restriction enzymes.*
3. Use the graph to estimate the sizes of fragments in all the DNA samples that have been 'restricted'.
4. From the single enzyme treatments, work out the approximate location of restriction sites on the DNA for each individual enzyme.
5. From the 'double digests' try to determine the relative positions of the various restriction sites on the lambda DNA.

Dean Madden

amplifying lambda dna

Prepared PCR reagents are available in dried form from several suppliers. This protocol is based on those that require a final reaction volume of 25 µL. For other products simply adjust the amount of DNA and primer solutions used. The amplified sequence is a 500 base pair (bp) length of Lambda's total genome (48 502 bp).

Aim

To amplify a 500 bp fragment of Lambda (λ) DNA.

Preparation

The water baths must be pre-heated. Good organisation (*e.g., with one person acting as a time-keeper while the other transfers the tubes between water baths*) is essential for success.

Timing

This activity takes about 50 minutes, plus preparation, running and staining time for the gel. The PCR product may be stored in a freezer before electrophoresis if this proves more convenient.

Materials and equipment

Needed by each person or group

- λ DNA solution, 2 µL (containing 50 ng of DNA) (if using dried λ DNA from the NCBE, simply resuspend the contents of a tube in 400 µL of water)
- Primers, 2 µL of each (10 µM):
  5'-GATGAGTTCGTGTCCGTACAACTGG-3'
  5'-GGTTATCGAAATCAGCCACAGCGCC-3'
- Ready-prepared PCR mix (containing buffered dNTPs, *Taq* polymerase and $MgCl_2$ *e.g., an Amersham-Pharmacia 'Ready-To-Go' bead*)
- Sterile distilled or deionised water, 19 or 20 µL
- OPTIONAL: 1 kb DNA ladder solution, 20 µL in TE buffer (containing 1 µg of DNA)
- Orange G loading dye, 8 µL
- Agarose solution, 12 cm^3 (0.8% in TBE buffer, melted in a microwave oven then kept molten in a water bath at 55–60 °C)
- TBE buffer, 15 cm^3
- Azure A solution (0.04% in water)
- Tips for micropipette, 6
- NCBE gel electrophoresis equipment with 4- or 6-toothed comb and carbon fibre electrode material
- Floating holder for tubes (*this can be made by punching holes from a strip of foam*)
- Marker pen, fine tipped, permanent
- Micropipette or similar device, 1–10 µL
- Water baths, 3 (maintained at 96, 58 and 72 °C)
- Stopclock
- Power supply for electrophoresis equipment (*2–5 x 9 volt batteries*)

Procedure

1. Take a tube containing a pellet of the PCR reagents. Carefully add 2 µL of Lambda DNA solution. Using a fresh tip each time, add 2 µL of each of the primer solutions. Finally add 19 or 20 µL of sterile distilled water, giving a total of 25 or 26 µL. *Although a total volume of 25 µL is best, 26 µL may be more convenient to dispense if you are using an NCBE microsyringe.*
2. Cap the tube and mix the contents quickly but thoroughly by flicking the end of the tube with your finger. Tap the tube firmly on the table to return any small drops of solution to the bottom of the tube. *Ensure that the cap is closed tightly, as heating in the next step may cause it to pop open. Note: Usually, oil is placed on top of PCR reactants to stop them from evaporating. No oil is needed for this method, as any liquid that condenses on the lid tends to be knocked back into the tube as it is moved between the water baths.*
3. Put the tube in a floating rack. Immerse the rack in the 96 °C water bath for ***exactly 30 seconds***. ***Take care not to scald your fingers!*** *Do not leave the tube in the water bath for too long, or the* Taq *polymerase may be damaged.*
4. Transfer the tube (in its rack) to the 58 °C water bath for ***exactly 1 minute***.
5. Finally, transfer the tube (again in its rack) to the 72 °C water bath for ***exactly 30 seconds***.
6. Repeat steps 3–5 to give 15–20 cycles in total.
7. Hold the tube in the 58 °C water bath for ***exactly 5 minutes***. *This ensures that all of the DNA becomes double-stranded again.*
8. Add 4 µL of Orange G loading dye to the PCR product and mix well. *The sample may be frozen now and stored for electrophoresis at a later time.*
9. Pour sufficient TBE buffer over the agarose gel to cover it to a depth of 2–3 mm. Gently ease the comb from the gel. Cut two carbon fibre electrodes.
10. Load the amplified DNA into one or two of the wells in the gel. If desired, add 20 µL of 1 kb DNA ladder solution mixed with 4 µL of loading dye to another well. *This will provide DNA fragments of known sizes with which the fragments of amplified DNA may be compared.*
11. Put the electrodes in either end of the tank. Check that there is still enough buffer in the tank, and add a little more if necessary (the buffer soaks into the electrode tissue). Cover the gel with the comb and electrophorese until the loading dye reaches half way down the gel or further (this will take 3–12 hours, depending upon the voltage).
12. Disconnect the power supply, remove the electrodes and pour the buffer away. *Leave the gel in the tank for staining.*
13. Flood the gel with Azure A solution and let it stand for ***exactly 4 minutes***.
14. Pour off the stain, and wash the gel surface with water. *Do not leave any water on the gel.*
15. Wrap the gel in a plastic bag to reduce any evaporation. The dye will migrate through the gel, staining the DNA as it does so. This will take 15–30 minutes, and the best results will be visible after about 12 hours.

Safety

Water baths

Take care when using the boiling water bath. It may be necessary to use forceps to lift the tube rack from the water and to wear protective gloves.

resources

Mullis, K.B. (1990) The unusual origin of the polymerase chain reaction. *Scientific American* **262** (4) 36–43.

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

Making PCR *Key documents describing the development of PCR* sunsite.berkeley.edu/pcr/

Willmott, C. (1998) An introduction to the polymerase chain reaction. *School Science Review* **80** (290) 49–54.

Betsch, D. and Berard, J. (1999) D1S80 PCR with the $25 thermal cycler. *Biochemical Education* **27** (1) 45–47.

Cold Spring Harbor Laboratory's DNA Learning Center *Animated simulations of PCR, cycle sequencing etc.* vector.cshl.org

1 Place the primers, template DNA and water on the side of a tube containing the PCR reactants.

Sterile water 19–20 µL

Primer A — 2 µL
Primer B — 2 µL
λ DNA — 2 µL

2 Mix the contents, then cap the tube firmly.

PCR bead, containing:
- buffer
- *Taq* polymerase
- dNTPs
- $MgCl_2$

The bead disintigrates in the liquid

Tap the tube gently on the bench to return the contents to the bottom.

3 DENATURE DOUBLE-STRANDED DNA

30 seconds

Water bath 96°

4 ANNEAL PRIMERS TO SINGLE-STRANDED DNA

60 seconds

Water bath 58°

5 EXTEND DNA BETWEEN PRIMERS

30 seconds

Water bath 72°

6 REPEAT STEPS 3-5 FOR 15–20 CYCLES

7 AFTER THE LAST CYCLE, INCUBATE THE TUBE AT 58 °C FOR 5 MINUTES

8 Orange G loading dye

4 µL

Mix well before loading

Amplified lambda DNA

9 Flat side of the comb

PCR PRODUCTS

0.8% agarose gel

Carbon fibre tissue

42 mm

22 mm

Cut two electrodes

Dean Madden

10 Label the end of the tank

TBE buffer solution 2–3 mm depth over gel

Black card under the tank reveals the wells for loading

OPTIONAL DNA LADDER:

4 µL

1 kb ladder DNA

Mix well before loading

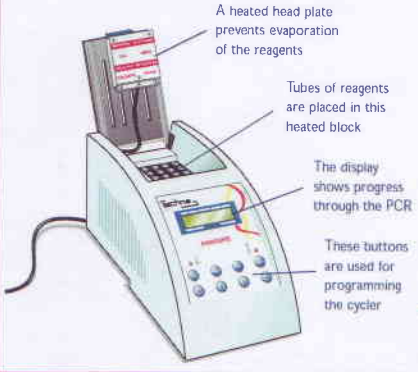
Orange G loading dye

11 Electrodes

12 15 The Orange G diffuses once the gel has been run. After staining, the amplified DNA fragments will appear as a single blue band just behind the loading dye.

DNA Gel electrophoresis
Concentrated
DNA Stain

pcr with a thermal cycler

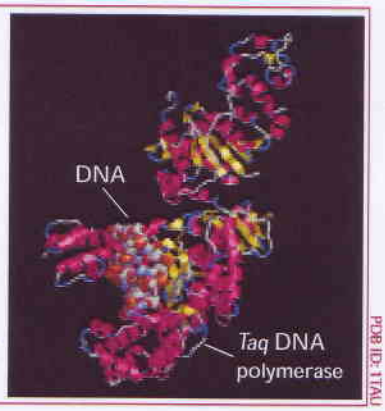


If you have a thermal cycler, use this 3-step programme:

'Hot start' : 1 cycle
96 °C for 2 minutes (denatures all double strands)

Main programme : 30 cycles
96 °C for 30 seconds (DNA denatured)
58 °C for 1 minute (primers anneal to template DNA)
72 °C for 30 seconds (DNA strands elongate)

End programme : 1 cycle
58 °C for 5 minutes (DNA made double-stranded again)



A computer-generated image of Taq DNA polymerase, with a fragment of DNA (space-filling model) at the active site. ▶

ideas for investigations

In a school or college context, for reasons of both safety and expense, the scope for open-ended practical investigations with DNA is limited. Within these constraints, there are some ideas which can very easily form the basis of a worthwhile practical project. Some ideas for such projects are given here.

Modelling DNA pp. 18–19

After experimenting with *RASMOL* and the structure data from the Web sites mentioned in the DNA model-making section, you may wish to try your hand at more advanced techniques. *PovChem* is a molecular illustration package that takes data in PDB format, and outputs *POV* files suitable for 3-D ray-tracing. It is available at low cost from http://www.chemicalgraphics.com/PovChem/ The ray-tracing software, *POV*, is free-of-charge from http://www.povray.org

An introduction to molecular modelling with links to numerous Web sites is provided in Georgina Ferry's *HMS Beagle* article that is referred to on page 18. Computer software such as the *Swiss Pdb Viewer* (plus the necessary tutorial text) is available free-of-charge *via* the internet from: http://www.expasy.ch/spdbv/mainpage.htm

If computers don't appeal, you could try to create your own cut-out model for demonstrating protein synthesis. Wilbert Garvin's DNA model, which is referred to in the text, could provide a useful starting point. The manual that accompanies the DNA model can be downloaded from the EIBE Web site: http://www.reading.ac.uk/EIBE

Extracting DNA pp. 20–21

Using variations on the simple methods described in this section, it is possible to extract DNA from a wide variety of microbial, plant and animal tissues. Additional protocols are given in *Investigating Plant DNA*, which is available from the NCBE's Web site. *Be warned that pectin is abundant in some plant tissues, and precipitates to form a DNA-like gel in cold ethanol!* (How could you test that any material you extract really is DNA?)

With these methods, it is not straightforward to extract DNA that is noticeably different in *quality* when it is electrophoresed. Most crude extracts merely produce a 'smear' of DNA fragments down the gel. However, given a high-speed centrifuge (such as that developed by TEP® — http://www.tep.org.uk), it is possible to separate DNAs of different molecular masses. How could you adapt the method given on pages 20–21 to extract chloroplast (rather than chromosomal) DNA from plant tissue? Materials have been developed by Dr Leighton Dann for *Science and Plants for Schools*, enabling the investigation of chloroplast DNA. See the SAPS Web site for details: http://www-saps.plantsci.cam.ac.uk/

DNA quantification pp. 22–23

It should not be very difficult to devise an investigation which involves the measurement of the amount of DNA extracted from a particular source. For example, do certain storage conditions lead to a change in the amount of DNA in onions? Can you think of other situations in which similar changes might occur? What might their biological significance be?

If you have a DNA sample to test which forms a distinct band on a gel rather than a smear (for example, chloroplast or plasmid DNA), it is possible to determine its concentration by running it on a gel alongside a DNA standard, which its intensity may be compared by eye. λ DNA, restricted with *Hin*dIII, forms a suitable standard.

A tube of the dried λ DNA supplied by the NCBE contains 10 µg of DNA. If this is diluted in 100 µL of water and 20 µL is then restricted with *Hin*dIII and run on a gel, the bands will contain roughly the following amounts of DNA:

Sizes of fragments (kb)	DNA in band (µg)
23.13	0.95
9.42	0.39
6.56	0.27
4.36	0.18
2.32	0.10
2.03	0.08
0.56	<0.01
0.13 runs off gel	~2.00 µg in total

For this type of quantification, you may find it better to use a more sensitive stain like Nile blue rather than Azure A (see page 39).

Induction of β-galactosidase pp. 24–25

With this protocol, you can compare the activity (number of units) of β-galactosidase (lactase) produced by different K12 strains of *E. coli*.

Other chemicals, apart from lactose, can also be used to 'switch on' the production of this enzyme. For example, IPTG (isopropyl-β-galactoside), which cannot be cleaved by β-galactosidase, is a widely-used inducer of lactase production. You can determine the effect of IPTG on the induction of β-galactosidase by adding it to the growth medium instead of lactose. A good starting point would be to add 79 mg of IPTG to each dm^3 of growth medium. You could try to determine the minimum amount required to 'switch on' the gene.

Extraction of plasmid DNA pp. 26–27

Several investigations are possible here, particularly if you couple the extraction method with the quantification procedure from pages 22–23. Can frozen preparations of bacterial cultures be used for this extraction method? How might freezing affect the yield of plasmid and the ease with which it can be extracted from the cells? At what stage of bacterial growth is plasmid yield the greatest? Once you have the plasmid DNA, how should it be stored to prevent deterioration? For example, does exposure to sunlight (or another UV source) damage DNA, so that it runs differently on an electrophoresis gel? If you are making several preparations of plasmid DNA and have access to a microcentrifuge, you may find it more convenient to use the resin filter method of extracting and purifying DNA. Simple kits containing all the items for this technique are available from molecular biology suppliers.

Bacterial transformation pp. 28–29

There are numerous variations on the technique described here which can be attempted to try to improve the efficiency of transformation.

You might try altering:

- the age of the host cells used;
- the amount of plasmid DNA used;
- the duration of the heat shock;
- the intensity of the heat shock (*i.e., its temperature*);
- the duration and/or temperature of the recovery period.

To determine the effect of altering these factors, it is useful to calculate the *transformation efficiency*. This is expressed as the number of transformed colonies produced per µg of plasmid DNA. The transformation efficiency can be calculated as follows:

1. Calculate the mass, in µg, of plasmid DNA used in Step 4 (page 28). Concentration of the plasmid DNA x Volume of plasmid DNA solution used = Mass of plasmid.
2. Determine how much of the cell suspension you spread, in µL, onto the LB/antibiotic/X-Gal plate. Volume of suspension spread / Total volume of suspension = Fraction of cell suspension spread.
3. Calculate the mass of plasmid contained in the cell suspension spread onto the LB/antibiotic/X-Gal plate. Mass of plasmid x Fraction of cell suspension spread = Mass of plasmid spread.
4. Determine the number of colonies per µg of plasmid DNA. Colonies counted / Mass of plasmid spread (µg) = Transformation efficiency.

Restriction and ligation pp. 30–31

Here the ligation reaction is allowed to proceed for two minutes only. What might the effect be of longer (or shorter) ligation reactions? Can you ligate DNA molecules from different sources (*e.g., two different plasmids*) that have been cut with the same enzyme? How well would other restriction enzymes work? ***N.B. DNA cut and pasted in this way must not be re-inserted into living cells, nor must full-length viral (e.g., lambda DNA be combined with DNA from other sources) (see the Safety guidelines on page 17).***

Restriction site mapping pp. 32–33

There are many different natural mutants of λ DNA available from molecular biology suppliers. You may like to try mapping some of these in the same way as the 'standard' (wild-type) λ.

Although a similar exercise with plasmid DNA might appeal, be warned that many modern plasmids have their restriction sites clustered within a small region, and so yield minute DNA fragments that cannot easily be resolved on an agarose gel. Consequently, mapping exercises with such plasmids are likely to be disappointing, at least with the range of inexpensive restriction enzymes that are available to schools (rarer enzymes may cut the plasmids elsewhere, yielding bigger fragments, but these are far more expensive).

Amplifying lambda DNA pp. 34–35

Unfortunately, there is relatively little to be gained from altering the protocol described here. Most variations (*e.g., in water bath temperatures or the duration of the incubations*) are likely to result in no amplification of the λ DNA at all, or non-specific amplification (where the primers bind at the wrong location — revealed by a 'smear' rather than a distinct band on the gel). Since the materials used for this work are relatively expensive, experimentation of this type is likely to be beyond the scope of most secondary schools.

However, there are now numerous inexpensive PCR preparations available (that is, mixtures of dNTPs, *Taq* polymerase and $MgCl_2$). A comparison of these should be within the reach of many schools. Perhaps greater enjoyment would come from trying to design your own PCR machine. The first-ever PCR machine was a home-made device called 'Mr Cycle'. It heated and cooled between just two temperatures. We've seen designs using thermostatically-controlled valves (to let hot and cold water into and out of small tanks), hair driers and light bulbs. Our ingenious friend Eske Brunn built a PCR machine at his school in Denmark. This was a computer-controlled robot arm that transferred tubes between three 'water baths', improvised from coffee jugs!

resources

This PDF file was last updated on 3 October, 2000.

You can find updates and more information on the NCBE's Web site:

www.ncbe.reading.ac.uk

running gels

Effect of voltage on gel electrophoresis

What's the best voltage to use?

At low voltages, migration of linear DNA is proportional to the voltage applied. As the voltage is increased, the mobility of the higher molecular mass fragments is increased differentially (the larger fragments tend to 'catch up' with the smaller ones). Hence the effective range of separation is *decreased* as the voltage is increased. For the best resolution, 0.8% agarose gels should be run at no more than 5 V per cm (as determined by the distance between the electrodes).

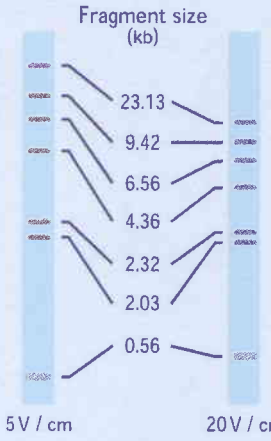
Calculating the resolution of a gel

For λ DNA digested by *Hin*dIII (shown on the right), the resolution can be calculated by dividing the distance between the 23 and 2 kb fragments by the total distance travelled by the 2 kb fragment.

NCBE electrophoresis equipment

This particular equipment MUST NOT be connected directly to a mains electricity supply. It was designed to be used with batteries at low voltages and currents. You can use up to five 9 volt batteries (MN1604, 6LR61, 6LF22 or PP3).

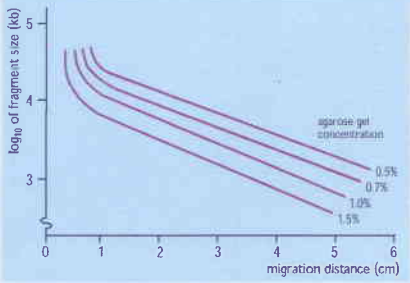
Number of 9 V batteries	Time taken to run gel
2	4 hours
3	3.5 hours
4	2 hours
5	1 hour



*Hin*dIII digest of λ DNA

Fragment size

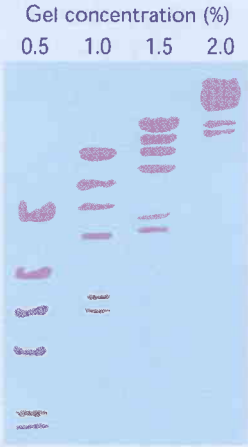
Within limits, there is a linear relationship between the *logarithm* of the molecular mass of linear DNA molecules and their movement through a gel. The molecular mass of DNA fragments is roughly proportional to their size.



Gel concentration

There is a linear relationship between the *logarithm* of the mobility of the DNA and the gel concentration. By altering the agarose concentration it is possible to control the range of sizes of fragments that can be separated by electrophoresis.

Agarose (% w/v)	Separation range (kb)	Gel strength
0.3	60 – 5	very weak
0.6	20 – 1	weak
0.7	10 – 0.8	moderate
0.9	7 – 0.5	moderate
1.2	6 – 0.4	strong
1.5	4 – 0.2	strong
2.0	3 – 0.1	strong



*Hin*dIII digest of λ DNA

resources

The technical guides that accompany the NCBE's gel electrophoresis equipment are available from the Centre's Web site: www.ncbe.reading.ac.uk

DNA Science. A first course in recombinant DNA technology by David Micklos and Greg Freyer (1990) Carolina Biological Supply Company / Cold Spring Harbor Laboratory Press. ISBN: 0 89278 411 3.

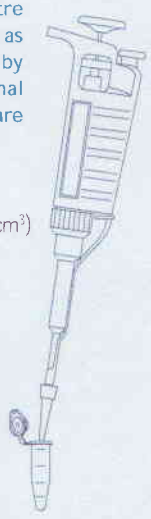
This book includes a useful 'trouble-shooting' guide for diagnosing poor gels.

Biological nomenclature. Standard terms and expressions used in the teaching of biology by Alan Cadogan [Ed.] (2000) Institute of Biology. ISBN: 0 900 49036 5.

This book has details of the recommended units of measurement, terminology etc.

UNITS OF MEASUREMENT

Small volumes and masses, which may be unfamiliar to school-based biologists, are referred to in this booklet. The units dm^3 and cm^3 have been used in preference to litre and millilitre (to which they are almost equivalent) as both cm^3 and dm^3 are often preferred in schools, by some examination boards and in the National Curriculum in England and Wales (although none are true SI units).



Volume

1 cubic decimetre (dm^3) = 1 000 cubic centimetres (cm^3)
1 cubic centimetre (cm^3) = 1 000 microlitres (μL)

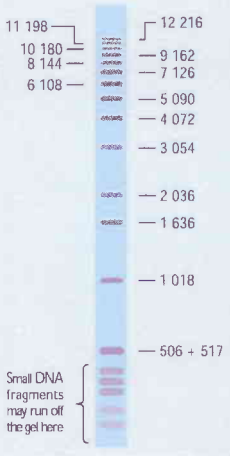
Mass

1 gram (g) = 1 000 milligrams (mg)
1 milligram = 1 000 micrograms (μg)
1 microgram (μg) = 1 000 nanograms (ng)
1 nanogram (ng) = 1 000 picograms (pg)

Note: Various devices for pipetting microlitre volumes are available; the NCBE can advise on suppliers, suitability for school use and approximate costs.

1 kb DNA LADDER

This 'ladder' or 'ruler' provides DNA fragments of known sizes, for comparison with those whose size is not known. The sizes of the fragments are in base pairs, and they are spaced at roughly 1 kb intervals.



How much ladder should I use?

With the NCBE equipment, 1 μg of ladder is needed per well with the 6-toothed comb; with the 4-tooth comb, use 2 μg per well.

Note: Not all 1 kb DNA ladders have this selection of fragment sizes; details will be given by the supplier.

dna revealed

Ethidium bromide, a potent mutagen

In research laboratories, ethidium bromide and similar fluorescent compounds are normally used to visualise DNA on a gel. Unfortunately, ethidium bromide and its breakdown products are potent mutagens and carcinogens and therefore *they should not be used in schools*. Such dyes are often flat molecules with similar dimensions to DNA base pairs. When ethidium bromide binds to DNA, it slips between adjacent base pairs and stretches the double helix. This explains the dye's mutagenic effect — the 'extra bases' cause errors when the DNA replicates. In addition, short-wavelength UV light (which itself is harmful) is required for ethidium bromide to fluoresce and reveal the DNA. For reasons of safety and because UV light of this wavelength causes unwanted mutations in the DNA being studied, several researchers have sought alternative methods of revealing DNA.

Safer alternatives

Crystal violet binds to DNA in a similar way to ethidium bromide and although it is a mutagen, it is not thought to be as harmful as ethidium bromide. Because it can be viewed in normal daylight (avoiding the need for damaging UV light), some researchers have advocated its use where functional DNA is to be recovered from a gel.

Thiazin dyes

The most widely used alternatives to ethidium bromide are methylene blue and its oxidation products, such as Azure A, Azure B, Toluidine blue O and Brilliant cresyl blue. These dyes are used individually or as mixtures (often in proprietary formulations). Although their exact mode of action is unknown, they are thought to bind ionically to the outside of nucleic acids (to the negatively-charged phosphate groups) and can therefore be used to detect both DNA and RNA.

Such dyes are not as sensitive as ethidium bromide, and some of them colour the gel heavily. Consequently, prolonged 'destaining' may be necessary before the DNA bands can easily be seen. Several dyes also fade rapidly after use — methylene blue falls into both categories and is therefore not ideal for staining DNA on a gel.

All of the thiazin dyes may be used in aqueous solution at a concentration of about 0.02–0.04% and applied to the gel after it has been run. They may also be dissolved in mild alkaline solutions (*e.g., running buffer; not over about pH 8*). Destaining with dilute acetic acid may be necessary for alkaline solutions.

The age of the dye may have a considerable effect upon the results achieved. For example, old samples of methylene blue will almost certainly contain a proportion of other dyes (such as Azure A and B) and these breakdown products may be responsible for much of the staining. Dye solutions are best stored in glass bottles (some dyes will stain plastic containers), either wrapped in foil or kept in the dark.

Staining DNA on the move

Recently, several commercial products have emerged that enable the DNA to be seen as it moves across the gel. Suppliers seldom reveal their composition, but several of these stains contain Nile blue sulphate (also known as Nile blue A), a dye which had not previously been noted for its ability to stain DNA. Adkins and Burmeister (1996) give useful guidance as to its use as well as hints for identifying other dyes which may be useful for visualising DNA.

All of the dyes used for staining 'mobile' DNA are cationic — that is, they are positively charged in the gel buffer, at pH 8. They move through the gel in the opposite direction to the DNA, latching onto the DNA molecules as they meet them. So that sufficient dye remains in the gel, it is added to both the gel and the buffer above it. However, a far lower concentration (1–3 μg per cm^3) of dye is necessary for this method than for post-electrophoresis staining. This is because too much dye will neutralise the negatively-charged DNA fragments, slowing their movement and reducing the resolution or even preventing the DNA from moving at all. Consequently, there is a compromise to be struck between visibility and resolution. Better results are usually achieved by staining the DNA *after* the gel has been run, rather than staining during the run.

Drying gels

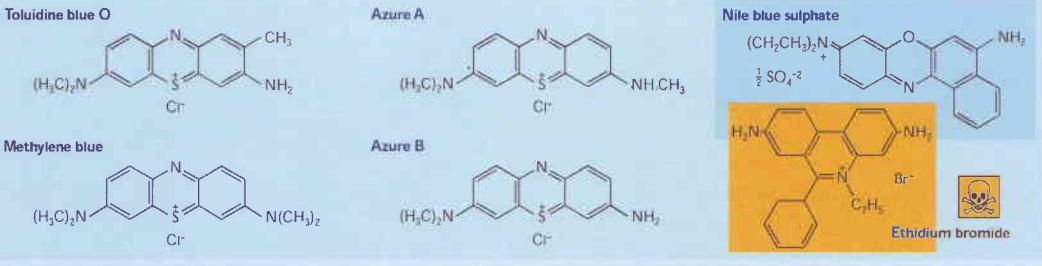
It is also possible to dry a gel after the dye has been applied, and thereby to concentrate the dye in bands which would otherwise be difficult to see. So that the gel dries evenly, it is advisable to place the wet gel on a sheet of good-quality writing paper, and to place this on several sheets of filter paper. Moisture from the gel soaks into the filter paper, while the writing paper layer stops too much of the dye from soaking out of the gel. Gels should be dried at room temperature.

Light boxes

White light boxes (such as those sold for viewing photographic negatives or transparencies) are ideal for observing stained gels; the larger models tend to be more robust and reliable. A yellow-coloured filter may help when photographing gels that have been stained with blue dyes.

Safety

Although several dyes that can be viewed in normal daylight are thought to be relatively safe, they have not been as intensively studied as the fluorescent dyes for long-term toxic effects. Some of these visible dyes, apparently, intercalate DNA like ethidium bromide so they too have a potential for mutagenesis and, depending on absorption and metabolism, a potential for carcinogenesis. As with all laboratory chemicals, suitable safety precautions should be exercised when handling any dyes, particularly when they are in dry, powdered form.



resources

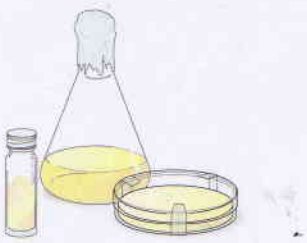
Methylene blue
Yung-Sharp, D. and Kumar, R. (1989) Protocols for the visualisation of DNA in electrophoretic gels by a safe and inexpensive alternative to ethidium bromide. *Technique* **1** (3) 183–187.

Brilliant cresyl blue
Santillán Torres, J. and Ponce-Noyoia, P. (1993) A novel stain for DNA in agarose gels *Trends in Genetics* **9** (2) 40.

Nile blue
Adkins, S. and Burmeister, M. (1996) Visualization of DNA in agarose gels as migrating colored bands: Applications for preparative gels and educational demonstrations *Analytical Biochemistry* **240** (1) 17–23.
W www-personal.umich.edu/~steviema/blueDNA.html

Crystal violet
Rand, N. (1996) Crystal violet can be used to visualise DNA bands during gel electrophoresis and to improve cloning efficiency *Technical Tips Online* W www.biomednet.com/db/tto

recipes



gel electrophoresis

TBE buffer

Used for making up agarose gel and as the electrophoresis buffer

10x concentrate – Makes 1 dm^3

Store at room temperature.

Ingredients

- Sodium hydroxide, 1 g
- TRIS base, 108 g
- Boric acid, 55 g
- Ethylene diamine tetraacetic acid (EDTA, disodium salt), 7.4 g

Procedure

1. Add the ingredients listed above to 700 cm^3 of deionised or distilled water.
2. Stir to dissolve.
3. Make up to 1 dm^3 with deionised or distilled water.

Note: Once made up, 1 volume of this TBE concentrate should be diluted with 9 volumes of distilled water before use.

IMPORTANT

DO NOT use TRIS-HCl to make up this buffer. You must use TRIS base.

TE buffer

DNA is dissolved in this buffer for storage and electrophoresis

Makes 100 cm^3

Store at room temperature.

Ingredients

- 0.5 M Ethylene diamine tetraacetic acid (EDTA, disodium salt, m.m. 372.24), 200 µL *(See recipe, below)*
- 1 M TRIS (pH 8, m.m. 121.10), 1 cm^3

Procedure

1. Mix the EDTA and TRIS solutions.
2. Make up to 100 cm^3 with deionised or distilled water.

Note: TE buffer should not be used to dissolve DNA that is to be cut with restriction enzymes. This is because the EDTA in the buffer will chelate metal ions needed as co-factors by the enzymes. Use sterile distilled water instead.

⚠ CAUTION

Avoid inhaling TRIS or EDTA powders. Wear a mask over your nose and mouth. Both chemicals are irritants, and can cause harm through physical contact and inhalation.

Loading dyes

Used for loading DNA fragments into the electrophoresis gel

Makes 100 cm^3

Store at room temperature.

Ingredients

- Bromophenol blue, 0.25 g
  OR
  Orange G, 0.5 g
- Sucrose, 50 g
- 1 M TRIS (pH 8, m.m. 121.10), 1 cm^3

Procedure

1. Add the ingredients listed above to 60 cm^3 of deionised or distilled water.
2. Stir to dissolve.
3. Make up to 100 cm^3 with deionised or distilled water.

Note: Bromophenol blue loading dye is appropriate for all of the protocols except the PCR, where Orange G should be used instead.

Agarose gel

Used to separate small fragments of nucleic acids in an electric field

0.8% agarose – Makes 100 cm^3

Store at room temperature.

Ingredients

- Agarose (electrophoresis grade), 0.8 g
- TBE buffer (1x, from concentrate, above), 100 cm^3

Procedure

1. Add the agarose powder to the TBE buffer.
2. Heat in a boiling water bath or microwave oven to melt the agarose. Less than a minute at full power in a 940 watt oven is sufficient to melt 100 cm^3 of gel. The container used to hold the molten agarose must not be sealed, but lightly covered with plastic film that has been punctured with one or two small holes.
3. Swirl the gel halfway through the heating cycle to ensure that it is thoroughly mixed.
4. Once molten, the agarose solution can be kept in this state at 55–60 °C in a water bath.
5. Ensure that the agarose solution is mixed well before casting gels.

⚠ CAUTION

Hot, molten agarose can scald and so it must be handled with care. It is advisable to let the molten agarose cool until it is comfortable to handle before pouring the gel.

Azure A

Used to stain nucleic acids on a gel

2x concentrate – Makes 50 cm^3

Store at room temperature.

Ingredients

- Azure A, 0.08 g
- Ethanol (40% aqueous solution), 50 cm^3

Procedure

1. Add the Azure A powder to 50 cm^3 of 40% ethanol.
2. Stir to dissolve.

⚠ CAUTION

The concentrated DNA stain is flammable and must not be used near naked flames or other sources of ignition. The stain bottle must be kept closed to prevent evaporation of the solvent. When diluted to its working concentration (0.04% in 20% ethanol), the stain presents no serious safety hazard, although care should be taken to avoid splashes on the skin or eyes *e.g., wear protective gloves and glasses.* Used stain may be diluted with water and washed down the drain.

Note: Once made up, this Azure A concentrate should be diluted with an equal volume of distilled water before use.

plasmid extraction

The first three of these solutions are used as stocks for preparing the other solutions.

0.5 M EDTA (pH 8.0)

Makes 100 cm^3

Store at room temperature.

Procedure

1. Add 18.6 g of EDTA (disodium salt) to 80 cm^3 of deionised or distilled water.
2. Adjust the solution to pH 8.0 by slowly adding sodium hydroxide (about 2.2 g of NaOH pellets). Monitor with a pH meter.
3. Mix vigorously. EDTA will dissolve only after the pH has reached 8.0 or greater.

5 M Potassium acetate

Makes 200 cm^3

Store at room temperature.

Procedure

1. Add 98.1 g of potassium acetate to 160 cm^3 of distilled or deionised water.
2. Add deionised or distilled water to make 200 cm^3 of solution in total.

Sodium dodecyl sulphate

10% solution — Makes 100 cm^3

Store at room temperature.

Procedure

1. Dissolve 10 g of electrophoresis-grade SDS in 80 cm^3 of distilled or deionised water.
2. Add deionised or distilled water to make 100 cm^3 of solution in total.

⚠ CAUTION

Avoid inhaling SDS powder; wear a mask over your nose and mouth.

Potassium acetate + acetic (ethanoic) acid

Makes 100 cm^3

Store at 4 °C, so that the solution will be precooled and ready for use.

Procedure

1. Add 60 cm^3 of 5 M potassium acetate and 11.5 cm^3 of glacial acetic acid to 28.5 cm^3 of deionised or distilled water.

Note: The strong smell of acetic acid distinguishes the finished KOAc + acetic acid solution from the KOAc stock; the two can easily be confused.

1% SDS / 0.2 N NaOH

Ideally, this should always be prepared immediately before use.

Makes 10 cm^3

Store at room temperature for 1 or 2 days only. A precipitate can form at colder temperatures. Warm the solution and shake it gently to dissolve the precipitate.

Procedure

1. Mix 1 cm^3 of 10% SDS and 0.5 cm^3 of 4 N NaOH into 8.5 cm^3 of distilled water.

transformation

Luria-Bertani (LB) media

For the culture of E. coli

Makes 1 dm^3

Once sterilised, can be stored at 4 °C or room temperature for up to 3 months.

Ingredients
- Yeast extract, 5 g
- NaCl, 10 g
- Tryptone, 10 g

For a solid medium, add:
- Agar, 15 g

Procedure
1. Add the dry ingredients to a clean 2 dm^3 flask that has been rinsed with distilled or deionised water. While stirring, add 1 dm^3 of distilled or deionised water.
2. Adjust the pH to 7.5 using a pH meter or pH paper (typically, you might need to add about 0.5 cm^3 of 4 M NaOH).

For solid medium (agar)

a. Cover the flask with aluminium foil and autoclave for 15–20 minutes at 121 °C.
b. While the liquid is being autoclaved, the agar may sink to the bottom of the flask. Swirl to mix the agar evenly after autoclaving.
c. Let the flask cool to 55–60 °C (until the flask can be held comfortably in your hands). *Note: If the agar medium begins to solidify it can be re-melted by autoclaving again for not more than 5 minutes.*
d. While the liquid is cooling, label the bases of the Petri dishes with the type of medium to be added. If you are using sterile polystyrene plates, cut the end of the plastic bag carefully, so that it can be re-used to store the poured plates. Spread the Petri dishes out on the bench ready to pour.
e. When the agar has cooled, lift the lid of each Petri dish just enough to pour the agar in. Don't put the lid down on the bench. Quickly add enough agar to cover the bottom of the plate (12–15 cm^3). Tilt the plate to spread the agar and replace the lid immediately.
f. Continue pouring plates. Occasionally, flame the mouth of the flask to maintain sterility.
g. To remove bubbles from the surface of the poured agar, briefly touch the surface with a burner flame while the agar is still molten.
h. Allow the agar to solidify undisturbed (about 15 minutes).
i. If possible, invert the plates and incubate them for several hours or overnight at 37 °C. This dries the agar, limiting condensation when the plates are stored in a fridge—it also allows any contaminated plates to be detected.
j. Stack the plates in their original plastic sleeves for storage in a fridge at 4 °C.

For liquid medium (broth)

Use the smallest practical bottle size, since once you open a bottle the chances of it becoming contaminated are high. Always check bottles of media for microbial contamination by swirling the bottle and looking for cloudiness; autoclave any cloudy medium and discard it. LB broth that looks clear can be regarded as sterile.

a. Dispense the liquid in aliquots *e.g. 10 cm^3 in 15–25 cm^3 bottles for small volume cultures.*
b. Loosen the bottle caps and autoclave for 15–20 minutes at 121 °C.

Note: For broth to which an antibiotic or other chemicals are to be added, autoclave the medium first, then add the antibiotic etc., then dispense into Petri dishes.

For media with added antibiotic and/or other components

It is very important *not* to add antibiotics to hot broth or agar, as the heat will denature them. The best way to avoid this problem and ensure good results is to stand the flasks of media, after autoclaving, but before dispensing, in a water bath at 55 °C.

Once the media have cooled to this temperature, add the required amount of antibiotic (and/or other ingredient), swirl to mix, and pour the plates or dispense the medium as above.

X-Gal solution

For addition to LB agar (use 1 cm^3 of this stock solution for every 300 cm^3 of agar)

Makes 100 cm^3

Store in the dark, for no more than a year, frozen at -18–20 °C.

Ingredients
- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 0.5 g
- N,N-dimethyl formamide (DMF), 25 cm^3

Procedure
1. *Wear protective gloves and a mask.*
2. Add the X-Gal and DMF to a 100 cm^3 flask.
3. Stir to dissolve.
4. Dispense in 1 cm^3 aliquots into 1.5 cm^3 microcentrifuge tubes. Freeze at -18–20 °C. It is not necessary to sterilise this solution.

WARNING!
DMF is toxic and mutagenic. Handle it with care. Always wear gloves and a dust mask.

Antibiotic solutions

For addition to LB agar or broth (add 1 cm^3 of either stock solution to 500 cm^3 of medium). Use ampicillin for pUC18 or pBLU; kanamycin for p2k.

Makes 25 cm^3

Once sterilised, can be stored for one year, frozen at -18–20 °C, or at 4 °C for 3 months.

Ingredients
- Kanamycin sulphate, 0.156 g
  OR
- Ampicillin, 1.25 g

Procedure
1. Add either antibiotic powder to 25 cm^3 of distilled water in a 100 cm^3 flask, and stir to dissolve.
2. Prewash a 0.45 or 0.22 µm filter by drawing through 50–100 cm^3 sterile distilled or deionised water.
3. Pass the solution through the washed filter.
4. Dispense 1 cm^3 aliquots into sterile 1.5 cm^3 microcentrifuge tubes. Store frozen at -18–20 °C.

Transformation buffer

For production of competent cells

Makes 200 cm^3

Store at room temperature.

Ingredients
- PIPES, 0.61 g
- $MnCl_2.4H_2O$, 2.18 g
- $CaCl_2$, 0.44 g
- KCl, 3.73 g

Procedure
1. Add all the components except the $MnCl_2$ to 180 cm^3 of distilled or deionised water.
2. Stir to dissolve.
3. Adjust the pH to 6.7 with 5N KOH, then add the $MnCl_2$ (the pH must be below 7 to prevent the precipitation of manganese salts).
4. Make up to 200 cm^3 with distilled or deionised water.
5. Prewash a 0.45 or 0.22 µm filter by drawing through 50–100 cm^3 distilled or deionised water.
6. Pass the transformation buffer through the washed filter.
7. Dispense into sterile containers.

Note: Sterile filtered, 50 mM calcium chloride solution can be used instead of this buffer, but will not give such good results.

GET buffer

For extraction of plasmid DNA

Makes 100 cm^3.

Store at room temperature.

Ingredients
- Glucose, 0.9 g
- 1 M TRIS (pH 8.0), 2.5 cm^3
- 0.5 M Ethylene diamine tetraacetic acid (EDTA, disodium salt, m.m. 372.24), 2 cm^3
- Distilled or deionised water, 94.5 cm^3

Procedure
1. Add the dry ingredients to a clean beaker.
2. While stirring, add the distilled or deionised water to the dry ingredients.

ß-galactosidase production

Z buffer

Used for making up ONPG reagent

Makes 100 cm^3

Do not autoclave. Store in a refrigerator, for several months.

Ingredients
- $NaH_2PO_4.7H_2O$, 1.6 g
- $NaH_2PO_4.H_2O$, 0.55 g
- KCl, 0.075 g
- $MgSO_4$, 0.012 g
  OR
  $MgSO_4.7H_2O$, 0.025 g

Procedure
1. Add the ingredients listed above to 80 cm^3 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 cm^3

Store in a refrigerator, for no more than 24 hours.

Ingredients
- Z-Buffer, 100 cm^3
- Ortho-nitrophenyl-β-D-galactoside (ONPG), 0.55 g

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

⚠ CAUTION
ONPG is harmful. Avoid inhaling the powder; wear a mask over your nose and mouth.

dna quantification

Saline citrate buffer

This buffer should be used for DNA samples to be quantified using the diphenylamine method

Makes 1 dm^3

Store in a refrigerator.

Ingredients
- NaCl, 0.878 g
- Sodium citrate, 0.294 g

Procedure
1. Dissolve the ingredients in 900 cm^3 of deionised or distilled water.
2. Bring the pH to 7.0 with 1 M hydrochloric acid.
3. Make up to 1 dm^3 with deionised or distilled water.

glossary

Agarose gel — a firm gel made from agarose, a very pure derivative of agar (from seaweed). Used in the **electrophoresis** of relatively large pieces of **DNA** (100–6 000 bp).

Agrobacterium tumefaciens — a **bacterium** that can naturally transfer **genetic** information into plant **cells**, thereby causing crown gall disease. (See **Ti plasmid**.)

Allele — one of several alternative forms of a **gene** or **DNA sequence** occurring at the same position (**locus**) on **homologous chromosomes**.

Amino acid — the units from which all **proteins** are made. There are twenty different sorts of **amino acid** that occur commonly in **proteins**.

Antibiotic — a substance that kills or prevents the growth of **bacteria**, which is usually produced by other **bacteria** or fungi *e.g., Penicillium*. **Antibiotics** are also made synthetically or by chemical modification of the natural product.

Anticodon — found in **tRNA**, the **complementary sequence** to a **codon**, which specifies an **amino acid** during **protein** synthesis. (See **Codon**.)

Antisense (RNA) — a **sequence** of **mRNA** (dubbed **antisense mRNA**) which has been **transcribed** from the wrong (**antisense**) strand of a **DNA** molecule. The **antisense mRNA** combines with the true ('sense') **mRNA**, effectively 'switching off' the **gene**.

Bacteriophage (or phage) — a **virus** that infects **bacteria** *e.g., bacteriophage λ*.

Bacterium (plural bacteria) — a relatively simple organism consisting of one **cell**, in which there is no **nucleus**, but the **chromosomal DNA** is free within the **cytoplasm** as a ring.

Base (of nucleic acid) — one of the chemical sub-units found in **nucleic acid** molecules which in **DNA** (in groups of three) carry the code for particular **amino acids**. In **DNA**, the bases are adenine (A), thymine (T), cytosine (C) and guanine (G). Uracil (U) is found in place of thymine in **RNA** molecules.

Base pair (bp) — two nitrogenous **bases** in a **nucleic acid** which link reversibly by hydrogen bonds. In **DNA**, cytosine always pairs with guanine and adenine always links to thymine. (In **RNA** molecules, adenine joins to uracil.)

Callus (tissue) — undifferentiated (non-specialised) plant **cells**, often produced in tissue culture.

Carcinogen — a chemical that causes **genetic** changes leading to cancer.

cDNA (complementary DNA) — a **DNA** strand synthesised on an **RNA** template by the action of the **enzyme reverse transcriptase**. *Note: This is the reverse of the usual situation where the DNA sequence determines that of the RNA molecule.*

Cell — the smallest sub-unit, capable of independent division, from which living things are composed.

Chloroplast — the sites of photosynthesis in plant **cells**. Chloroplasts contain **DNA** which is passed down the female line *i.e., in ovules*.

Chromatid — one of the two identical filaments found in **chromosomes** as they are copied during **cell** division.

Chromosome — the structure containing **DNA** that carries **genetic** information. Humans have 23 pairs of **chromosomes** in their body (**somatic**) **cells**, one of each pair from either parent. The **gametes** (eggs and sperm) contain only 23 (unpaired) **chromosomes**. In **eukaryotes**, the DNA is associated with **proteins** (histones). In **prokaryotes** (mainly **bacteria**) the **chromosome** is a ring with no associated **proteins**.

Clone (of an organism) — an organism which is **genetically** identical to another *e.g., identical twins; plants from cuttings; cloned sheep*.

Clone (of cells) — a group of **genetically** identical cells *e.g., bacteria or plant cells in culture* arising from asexual division.

Clone (of DNA) (verb) — to make identical copies of a particular piece of **DNA**.

Codon — a group of three adjacent **bases** in **mRNA** coding for an **amino acid** or acting as a signal to stop or start the synthesis of a particular **protein** (See **Anticodon**.)

Competent (of bacteria) — **bacteria** that are capable of absorbing **DNA** from their surroundings.

Complementarity (of bases in nucleic acids) — the pairing of specific **bases** in **nucleic acid** molecules, between adenine and thymine (or uracil in **RNA**) and cytosine and guanine.

Conjugation — the transfer of **DNA** between different 'mating strains' of **bacteria**. This process requires an F ('fertility') **plasmid**, encoding specialised **proteins** that help the transfer to occur.

Crossing-over — a natural process occurring during **meiosis** in sexually-reproducing organisms, during which sections of similar (**homologous**) **chromosomes** are exchanged. Also called **recombination**. *Note: Do not confuse with recombinant DNA used in the narrow sense of genetic engineering or genetic modification.*

Cyto — of, or referring to, a **cell**.

Cytoplasm — the material inside a **cell** membrane that surrounds the sub-cellular components such as the **nucleus**.

DNA (deoxyribonucleic acid) — in most organisms, **DNA** carries the primary genetic information. **DNA** is a polymer consisting of long chains of **nucleotides**. Each **nucleotide** consists of a nitrogenous **base** linked to a sugar (deoxyribose) and a phosphate molecule.

DNA ligase — an **enzyme** used to couple double-stranded **DNA** molecules together.

DNA polymerase — an **enzyme** that joins deoxyribonucleoside triphosphates (dNTPs) to form a **DNA** strand alongside a **complementary DNA** template strand.

DNA sequencer — an automated machine that can determine the **sequence** of **bases** in a **DNA** molecule.

DNA synthesizer — a programmable machine that can assemble short stretches of **DNA** from their constituent **nucleotides**.

Diploid — a **cell** or organism with two complete sets of **chromosomes** *e.g., most human cells (except for the sex cells) have two sets*.

Dominant — one of a pair of **alleles** that is **expressed** in the **phenotype**, regardless of the nature of the other **allele** (*cf.* **Recessive**).

Double helix — the three-dimensional structure of **DNA**, in which two phosphate-deoxyribose strands are (almost always) arranged as a right-handed helix. Between the two strands, holding them together, are **base pairs**; there are normally 10 **base pairs** per turn of the helix.

Electrophoresis — a technique for separating differently-sized fragments of **DNA**, based on their mobility in an electric field. As **DNA** fragments generally carry a negative electrical charge, they move towards the positive electrode when a current is applied. Large fragments move relatively slowly, whereas small pieces move quite fast, thereby achieving separation.

Electroporation — a method of **genetic modification** in which holes are produced in **cells** by subjecting them to a brief, high-voltage pulse of electricity. This permits **DNA** from the surrounding solution to enter the **cells**.

Enzyme — a **protein** catalyst that speeds up a specific chemical reaction.

Eukaryote — an organism that has a **nucleus**.

Exon — *Expressed sequence*. **Sequences** of **DNA** in a **gene** that code for a **protein** product after the intervening, non-coding sequences (**introns**) have been spliced out. [In practice, the whole **gene** is transcribed into **mRNA**, which is then processed to remove the introns, forming mature **mRNA** coding for the **protein** alone.] *Note: This applies only to eukaryotes — bacteria do not have introns.*

Expression — the manifestation of a particular characteristic specified by a **gene**. At any given time or in any particular **cell**, most characteristics are not **expressed**, but are masked by other, **dominant**, **genes** or are regulated in other ways.

Gamete — a **haploid** sex **cell**, such as a sperm or egg.

Gene — a unit of inheritance consisting of a sequence of **DNA** to which a specific function can be assigned. At least three types of **genes** are recognised: **structural genes** which code for particular **proteins**; **regulatory genes** which control the **expression** of the other **genes**; and **genes** which yield **transfer** or **ribosomal RNA**.

Gene therapy — the attempt to treat human disease by genetic intervention.

Genetic code — strictly speaking, a cipher. The groups of three **bases** that encode specific **amino acids**.

Genetic engineering — also called **genetic manipulation** or **genetic modification**. The production of new combinations of **genetic** material and transferring that **DNA** into an organism in which it *may* not naturally occur.

Genetic fingerprinting — a technique that enables **genetic** relationships between close relatives or the identity of an individual to be established — usually beyond reasonable doubt.

Genetic map (relating to chromosomes) — a diagram showing the relative positions of **genes** on **chromosomes**. Much of the effort of the **Human Genome Project** is directed towards mapping **chromosomes**.

Genome — the complete (**haploid**) **genetic** content of a **cell**, **plasmid** or **virus**.

Genotype — the **genetic** make-up of an organism.

Haploid — a **cell**, tissue or organism having one **chromosome** set *e.g., human sperm cells*.

Heredity — the transfer of **genetic** information from parents to their offspring.

Heterozygote, Heterozygous (adj.) — a **diploid** organism or **cell** with two different forms (**alleles**) of a particular **gene** or **chromosome**.

Homologous (of chromosomes or DNA) — Similarity due to relationship by common evolutionary descent. A **gene** and its mutant derivative, or two **chromosomes** with the same array of **genes** but possibly differing in exact detail by **mutation**, are said to be **homologous**.

Homozygote, Homozygous (adj.) — a **diploid** organism or **cell** with two identical forms (**alleles**) of a **gene**.

Human Genome Project — the international effort to map the positions of all 100 000 human **genes**, and to determine their **DNA sequences**. This is expected to be completed by 2002–3.

Intron — *Intervening sequence*. A stretch of **DNA** or **mRNA** that is not **expressed** as a **protein** or as mature **mRNA** (*cf.* **Exon**).

'Junk' DNA — **DNA** that does not encode **proteins**. 90% of human **DNA** is thought to fall into this category.

Linkage — the tendency of pairs or groups of **genes** to be inherited together because they are found on the same **chromosome**. **Genes** can be assigned to linkage groups according to the **chromosomes** on which they are found.

Locus (plural loci) — the location of a particular **gene** or **DNA sequence** on a **chromosome**.

Marker (genetic) — a distinguishing **genetic** trait that can be used to detect the presence of a particular **gene** or **DNA sequence**.

Meiosis, Meiotic (adj.) — a type of 'double' **cell** division which leads to the production of (**haploid**) **cells** with only one set of **chromosomes**. Human **gametes** (eggs and sperm) are formed by **meiosis**.

Mitochondrion (plural mitochondria) — a **sub-cellular** organelle in **eukaryotic** cells concerned with the release of energy for metabolic activities. **Mitochondria** have their own **DNA** that is passed down the female line alone.

Mitosis, Mitotic (adj.) — **cell** division in **eukaryotes** leading to a pair of daughter **cells** with the same number of **chromosomes** as the **cell** they originated from.

mRNA — *messenger RNA*. A section of **RNA** **transcribed** from a **DNA** molecule that carries the code for the **amino acid** sequence of a **protein** or **polypeptide**.

Mutagen — a chemical or other agent (such as ultraviolet radiation) which causes **mutations** in a **cell's DNA**.

Mutation — the process by which a (random) change occurs in the **DNA** or **chromosomes**.

Nucleic acid — a large biological polymer (**DNA** or **RNA**) made up of **nucleotides**.

Nucleotide — the 'building blocks' from which **nucleic acids** are composed. Each **nucleotide** consists of a **base**, a sugar and a phosphate group.

Nucleus (plural nuclei) — the membrane-bound structure within **eukaryotic cells**, that contains the **chromosomes**.

Operon — Two or more adjacent **structural genes** together with the regions of **DNA** that control the **expression** of the **gene(s)**. The *lac* **operon** is a classic example of such a mechanism, where the **gene** for β-galactosidase (lactase) is 'switched on' only in the presence of its substrate, lactose.

Phenotype — all the characteristics of an organism that can be determined without **genetic** tests *e.g., the visible characteristics that arise from the interaction of both genetic and environmental influences*.

Plasmid — a small ring of **DNA** found in many **bacteria** and some yeasts. **Plasmids** are able to replicate independently of the **chromosome(s)**, and may pass from one **cell** to another. They often carry **genes** of use to the host **cell** *e.g., antibiotic resistance*. They are one of the principal **vectors** used in **genetic modification**.

Polyacrylamide — a type of gel used for **electrophoresis**, generally to separate small **DNA** fragments (6–1 000 bp) and **proteins**.

Polygenic — a **trait** controlled by or associated with more than one **gene**.

Polymerase (DNA or RNA) — an **enzyme** which catalyses the production of **nucleic acid** molecules from subunits such as dNTPs. (See **DNA polymerase**.)

Polymerase chain reaction (PCR) — a laboratory process by which a specific **DNA** sequence is duplicated ('amplified') several millions of times in only a few hours.

Polymorphism — the presence of several forms of a **genetic** characteristic in a population.

Polypeptide — a molecule formed from a chain of **amino acids** condensed together. **Proteins** often consist of several **polypeptide** chains, each made up of several hundred **amino acid** residues. Each kind of **polypeptide** is encoded by a single **gene**.

Primer — in **DNA** synthesis, a short piece of single-stranded **DNA** that initiates the formation of a **complementary** strand.

Probe — a specific single-stranded **DNA** **sequence**, often labelled with radioactive phosphorus (^{32}P) or fluorescent dyes. This aids the detection of a **gene** with a **complementary sequence** of single-stranded **DNA** or **RNA** to which the **probe** can bind.

Prokaryote — an organism that lacks a **nucleus** and does not undergo **mitosis** or **meiosis** *e.g., a bacterium*.

Protein — a molecule composed of many **amino acids**, folded into a particular shape so that it may form a specific function. There are many types of **proteins** *e.g., most enzymes are proteins*.

Protoplast — a **cell** (**bacterial** or plant) from which the **cell** wall has been removed.

Recessive — **trait expressed** in organisms that are **homozygous** for a particular **allele**, but not in those who are **heterozygous** for the **allele**.

Recombinant DNA — **DNA** formed by joining DNA from two different sources (usually different **species**).

Regulatory gene — a gene that controls the **transcription** of a **structural gene**.

Replication (of DNA) — the synthesis of a new double-stranded **DNA** molecule alongside an existing single strand according to the **base-pairing** rules.

Restriction enzyme or restriction endonuclease — an **enzyme** which will cut **DNA** molecules at precise sequences of **base pairs**. Such **enzymes** are produced by microorganisms as a defence against 'foreign' **nucleic acids** *e.g., from invading bacteriophages*.

Reverse transcriptase — an **enzyme** that enables the synthesis of **DNA** from **RNA**. (See **Retrovirus**.)

Ribosome — the site of **protein** synthesis in the **cell**, composed of **rRNA** and **protein**.

RNA (ribonucleic acid) — a similar biological polymer to **DNA**, but with the sugar ribose instead of deoxyribose in its structure, and with the **base** uracil in place of thymine. Various forms of **RNA** are found: these include **mRNA** (*messenger RNA*); **tRNA** (*transfer RNA*); and **rRNA** (*ribosomal RNA*). Some types of **RNA** can perform a catalytic function similar to that of **enzymes**. Most **RNA** molecules are single-stranded, although they can form double-stranded hydrogen-bonded units.

RNA polymerase — the **enzyme** that **transcribes** the information encoded in **DNA** into **RNA**.

rRNA — *ribosomal RNA*. **RNA** molecules that, with certain polypeptides, form the **ribosomes**.

Sequence — the precise order of **bases** in a **nucleic acid** or **amino acids** in a **polypeptide**.

Southern blotting — a technique developed by Ed Southern. **DNA** is transferred from an **agarose** gel after **electrophoresis** to a membrane, where the **DNA** fragments may be detected using **probes**. [Northern blotting is a similar technique for detecting **RNA** fragments.]

Somatic cells — all body cells other than sex **cells** or the **cells** that give rise to the sex **cells**.

Species — organisms that are capable of interbreeding to produce viable offspring.

Structural gene — a **gene** that codes for a **polypeptide** or **protein**, such as an **enzyme**.

Ti (tumour-inducing) plasmid — a **plasmid**, carried by the microbe *Agrobacterium tumefaciens*, part of which naturally integrates into plant **chromosomes**, where it triggers the growth of galls ('Crown gall disease'). 'Disarmed' versions of this **plasmid**, with their tumour-inducing ability removed, are used as **vectors** to **genetically modify** some plant **species** (usually dicotyledons).

Trait — a **phenotypic** characteristic associated with the **expression** of one or more **genes**.

Transcription — the synthesis of a strand of **RNA**, using a single strand of a **DNA** molecule as a template. This is done by **enzymes**, according to the sequence of **bases** present in the **DNA** molecule.

Transduction — this involves the transfer of **DNA** between **bacteria**, mediated by **bacteriophage** infection. The **bacteriophage** picks up some of the host **cell's DNA**, which is then passed on to **bacteria** that are subsequently infected.

Transformation — a change in the **genetic** nature of a **cell** or organism brought about by the uptake of **DNA**.

Transgenic (adj.) — an organism or stretch of DNA containing **genetic** material artificially placed there from another **species**.

Translation — the production of a **polypeptide**, using the information encoded in **mRNA**.

Transposon — a piece of **DNA** that can insert at random into **plasmid** or **chromosomal DNA**.

tRNA — *transfer RNA*. A variety of **RNA** molecules that carry specific **amino acids** to the sites on the **ribosomes** where **proteins** are made.

Vector — an autonomously-replicating and specially-constructed **DNA** molecule, derived from a **plasmid** or **virus** used to carry a **cloned DNA sequence** into a cell.

Virus — a submicroscopic agent which contains **genetic** material but must invade another **cell** to replicate.

Zygote — a **diploid cell** formed by the fusion of egg and sperm **nuclei** within the **cell**.

resources

Radford, A. and Baumberg, S. (1986) A glossary of terms for teaching genetics. *Journal of Biological Education* **21** (2) 127–135.

Primer on molecular genetics
www.bis.med.jhmi.edu/Dan/DOE/intro.html

other sources of information

These resources are aimed specifically at school students or provide an introduction for general readers. Where possible, paperback editions are referred to. Additional references accompany each of the practical exercises inside. All of the materials are reviewed on the NCBE's Web site, which gives links to many more Web sites.

DNA TECHNOLOGY PRACTICAL TEXTS

DNA Science. A first course in recombinant DNA technology by David Micklos and Greg Freyer (1990) Carolina Biological Supply Company / Cold Spring Harbor Laboratory Press. ISBN: 0 89278 411 3.

Laboratory DNA Science. An introduction to recombinant DNA techniques and methods of genome analysis by Mark Bloom, Greg Freyer and David Micklos (1996) The Benjamin / Cummings Publishing Company. ISBN: 0 8053 3040 2.

DNA GEL ELECTROPHORESIS

The Lambda Protocol by Dean Madden (1996) [Second edtn.] National Centre for Biotechnology Education, The University of Reading.

Brown, N. (1995) Electrophoresis for the visually impaired: the modification of the lambda protocol and its use with visually impaired A-Level students. *Journal of Biological Education* **29** (3) 166–169.

Note: Both of the resources mentioned above are available from the NCBE's Web site.

BIOCHEMISTRY & MOLECULAR BIOLOGY

Life chemistry and molecular biology. An introductory text by E. J. Wood, C. A. Smith and W. R. Pickering (1996) Portland Press. ISBN: 1 85578 064 X.

Essential cell biology. An introduction to the molecular biology of the cell by Bruce Alberts, *et al.* (1997) Garland Publishing. ISBN: 0 8153 2971 7.

Essential Cell Biology is a lavishly-illustrated introductory version of an undergraduate text, and is suitable for 'A' Level students. It includes a CD-ROM.

Understanding gene cloning. A guide for the curious by Karl Drlica (1997) [Third edtn.] Wiley & Sons. ISBN: 0 471 13774 X.

Route maps in gene technology by Matthew R. Walker with Ralph Rapley (1997) Blackwell Science. ISBN: 0 632 03792 X.

Biotechnology from A to Z by William Bains (1998) Oxford University Press. ISBN: 0 19 963693 1.

Molecular biology notebook PC CD-ROM for schools (1999) Details and a Web site with linked resources can be found at: http://www.iacr.bbsrc.ac.uk/notebook/index.html

ETHICAL & MORAL ISSUES

Ethics, Morality and Crop Biotechnology by Roger Straughan and Michael Reiss (1996) Biotechnology and Biological Sciences Research Council. ISBN: 070 840 570 3.

Engineering genesis. The ethics of genetic engineering in non-human species by Donald and Ann Bruce (1998) Earthscan Publications. ISBN: 1 85383 570 6.

Ethics, Morality and Animal Biotechnology by Roger Straughan (1999) Biotechnology and Biological Sciences Research Council. ISBN: 070 840 6157.

LabNotes. New Biology and Society. Available from The Wellcome Trust's Web site: http://www.wellcome.ac.uk/education

Bioethics Net http://www.bioethics.net

FUN

The cartoon guide to genetics by Larry Gonick and Mark Wheelis (1991) HarperCollins. ISBN: 780062 730992.

Get a grip on genetics by Martin Brookes (1999) Weidenfeld & Nicolson. ISBN: 0297 82699 9.

The complete idiot's guide to decoding your genes by Linda Tagliaferro and Mark V. Bloom (1999) Alpha Books. ISBN: 0 02 863586 8.

HISTORY & BIOGRAPHY

Rosalind Franklin and DNA by Anne Sayre (1978) W. W. Norton & Company. ISBN: 0 393 00868 1.

The double helix. A personal account of the discovery of the structure of DNA by James D. Watson [Gunther Stent, Ed.] (1980) W. W. Norton & Company. ISBN: 0 393 95075 1.

The Transforming Principle. Discovering that genes are made of DNA by Maclyn McCarty (1990) W.W. Norton & Company. ISBN: 0 393 30450 7.

What mad pursuit by Francis Crick (1990) Penguin Books. ISBN: 0 14 011973 6.

The eighth day of creation by Horace Freeland Judson (1996) [Second edtn.] Cold Spring Harbor Laboratory Press. ISBN: 0 87969 478 5.

A Passion for DNA by James D. Watson [Walter Gratzer, Ed.] (2000) Oxford University Press. ISBN: 0 19 850697 X.

Genome: the autobiography of a species in 23 chapters by Matt Ridley (2000) Fourth Estate. ISBN: 1 85702 835 X.

Classical papers in genetics http://www.esp.org

National Centre for Biotechnology Education www.ncbe.reading.ac.uk
European Initiative for Biotechnology Education www.reading.ac.uk/EIBE
Cold Spring Harbor's DNA Learning Center vector.cshl.org
Access Excellence (National Health Museum, USA) www.accessexcellence.org

National Centre for Biotechnology Education, The University of Reading, Whiteknights, PO Box 228, Reading, RG6 6AJ.
Telephone: + 44 (0) 118 987 3743. Facsimile: + 44 (0) 118 975 0140. eMail: NCBE@reading.ac.uk
Copyright © Dean Madden, 2000 *ISBN: 0 7049 1370 4*