BIO 3306: Molecular Biology

TECHNIQUESOF MOLECULARGENETICS

8.1: Prelude to Molecular Genetics

Genetics is the study of the inheritance and variation of biological traits. We have previously noted that it is possible to conduct genetic research without directly studying DNA. Indeed some of the greatest geneticists had no special knowledge of DNA at all, but relied instead on analysis of phenotypes, inheritance patterns, and their ratios in carefully designed crosses. Today, **classical genetics** is often complemented by **molecular biology**, to give **molecular genetics**, which involves the study of DNA and other **macromolecules** that have been isolated from an organism. Usually, molecular genetics experiments involve some combination of techniques to isolate and analyze the DNA or RNA transcribed from a particular gene. In some cases, the DNA may be subsequently manipulated by mutation or by recombination with other DNA fragments. Techniques of molecular genetics have wide application in many fields of biology, as well as forensics, biotechnology, and medicine.

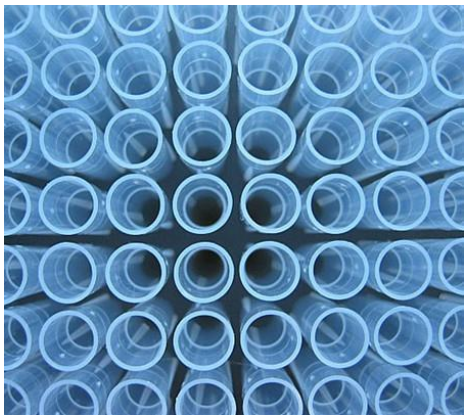


Figure 8.1.1***:*** *Disposable tips for a pipette are used to distribute microliter volumes of liquid in molecular biology. (Flickr- estherase-CC:ANS)*

8.2: Isolating Genomic DNA

DNA purification strategies rely on the chemical properties of DNA that distinguish it from other molecules in the cell, namely that it is a very long, negatively charged molecule. To extract purified DNA from a tissue sample, cells are broken open by grinding or **lysing** in a solution that contains chemicals that protect the DNA while disrupting other components of the cell (Figure 8.2.2). These chemicals may include **detergents**, which dissolve lipid membranes and denature proteins. A cation such as Na^+ helps to stabilize the negatively charged DNA and separate it from proteins such as histones. A chelating agent, such as EDTA, is added to protect DNA by sequestering Mg^{2+} ions, which can otherwise serve as a necessary co-factor for **nucleases** (enzymes that digest DNA). As a result, free, double-stranded DNA molecules are released from the chromatin into the extraction buffer, which also contains proteins and all other cellular components. (The basics of this procedure can be done with household chemicals and are presented on YouTube.)

The free DNA molecules are subsequently isolated by one of several methods. Commonly, proteins are

removed by adjusting the salt concentration so they precipitate. The **supernatant**, which contains DNA and other, smaller metabolites, is then mixed with ethanol, which causes the DNA to precipitate. A small **pellet** of DNA can be collected by centrifugation, and after removal of the ethanol, the DNA pellet can be dissolved in water (usually with a small amount of EDTA and a pH buffer) for the use in other reactions. Note that this process has purified all of the DNA from a tissue sample; if we want to further isolate a specific gene or DNA fragment, we must use additional techniques, such as PCR.

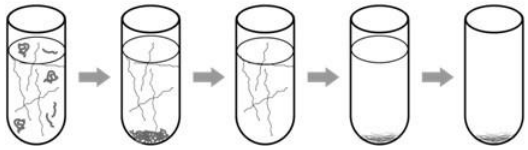


Figure 8.2.2: Extraction of DNA from a mixture of solubilized cellular components by successive precipitations. Proteins are precipitated, then DNA (in the supernatant) is precipitated in ethanol, leaving a pellet of DNA. (Original-Deyholos-CC:AN)

8.3: Isolating or Detecting a Specific Sequence by PCR

Components of the PCR Reaction

The **Polymerase Chain Reaction (PCR)** is a method of DNA replication that is performed in a test tube (i.e. *in vitro*). Here "polymerase" refers to a DNA polymerase enzyme extracted and purified from bacteria, and "chain reaction" refers to the ability of this technique produce millions of copies of a DNA molecule, by using each newly replicated double helix as a template to synthesize two new DNA double helices. PCR is therefore a very efficient method of amplifying DNA.

Besides its ability to make large amounts of DNA, there is a second characteristic of PCR that makes it extremely useful. Recall that most DNA polymerases can only add nucleotides to the end of an existing strand of DNA, and therefore require a **primer** to initiate the process of replication. For PCR, chemically synthesized primers of about 20 nucleotides are used. In an ideal PCR, primers only hybridize to their exact complementary sequence on the template strand (Figure 8.3.3).

Figure 8.3.3: The primer-template duplex at the top part of the figure is perfectly matched, and will be stable at a higher temperature than the duplex in the bottom part of the figure, which contains many mismatches and therefore fewer hydrogen bonds. If the annealing temperature is sufficiently high, only the perfectly matched primer will be able to initiate extension (grey arrow) from this site on the template. (Original-Deyholos-CC:AN)

The experimenter can therefore control exactly what region of a DNA template is amplified by controlling the sequence of the primers used in the reaction.

To conduct a PCR amplification, an experimenter combines in a small, thin-walled tube (Figure 8.3.4),

all of the necessary components for DNA replication, including DNA polymerase and solutions containing nucleotides (dATP, dCTP, dGTP, dTTP), a DNA template, DNA primers, a pH buffer, and ions (e.g. Mg^{2+}) required by the polymerase. Successful PCR reactions have been conducted using only a single DNA molecule as a template, but in practice, most PCR reactions contain many thousands of template molecules. The template DNA (e.g. total genomic DNA) has usually already been purified from cells or tissues using the techniques described above. However, in some situations it is possible to put whole cells directly in a PCR reaction for use as a template.

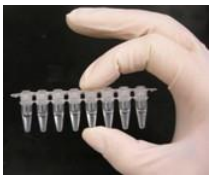


Figure 8.3.4: A strip of *PCR tubes (Wikipedia-madprime-GFDL)*

An essential aspect of PCR is **thermal-cycling**, meaning the exposure of the reaction to a series of precisely defined temperatures (Figure 8.3.5). The reaction mixture is first heated to 95°C. This causes the hydrogen bonds between the strands of the template DNA molecules to melt, or **denature**. This produces two single-stranded DNA molecules from each double helix (Figure 8.3.6). In the next step (**annealing**), the mixture is cooled to 45-65°C. The exact temperature depends on the primer sequence used and the objectives of the experiment. This allows the formation of double stranded helices between complementary DNA molecules, including the annealing of primers to the template. In the final step (**extension**) the mixture is heated to 72°C. This is the temperature at which the particular DNA polymerase used in PCR is most active. During extension, the new DNA strand is synthesized, starting from the 3' end of the primer, along the length of the template strand. The entire PCR process is very quick, with each temperature phase usually lasting 30 seconds or less. Each cycle of three temperatures (denaturation, annealing, extension) is usually repeated about 30 times, amplifying the target region approximately 2^{30}-fold. Notice from the figure that most of the newly synthesized strands in PCR begin and end with sequences either identical to or complementary to the primer sequences; although a few strands are longer than this, they are in such a small minority that they can almost always be ignored.

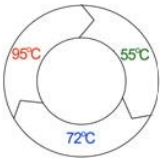


Figure 8.3.5: Example of a thermalcycle, in which the annealing temperature is 55°C. (Original-Deyholos-CC:AN)

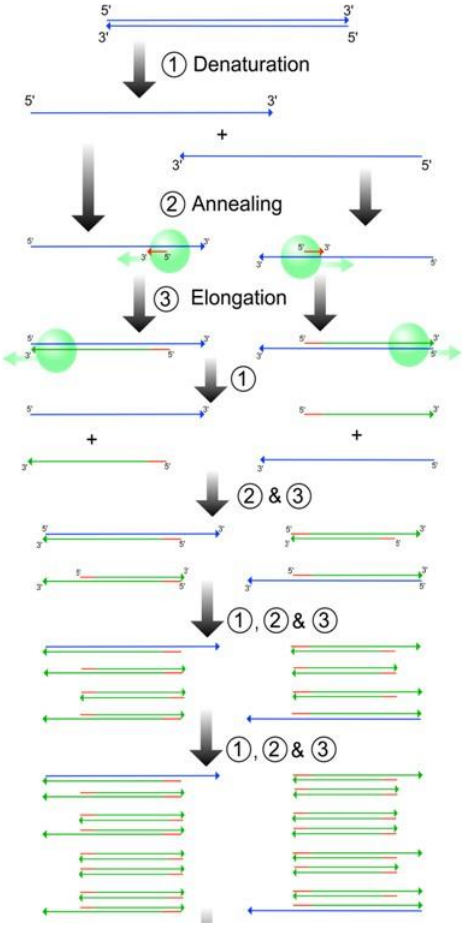


Figure* 8.3.6*: *PCR with the three phases of the thermalcycle numbered. The template strand (blue) is replicated from primers (red), with newly synthesized strands in green. The green strands flanked by two primer binding sites will increase in abundance exponentially through successive PCR cycles. (Wikipedia-madprime-GFDL)*

The earliest PCR reactions used a polymerase from *E. coli*. Because the high temperature of the denaturation step destroyed the enzyme, new polymerase had to be added after each cycle. To overcome this, researchers identified **thermostable** DNA polymerases such as **Taq DNA pol**, from *Thermus acquaticus*, a thermophilic bacterium that lives in hot springs. Taq, and similar thermostable polymerases from other hot environments, are able to remain functional in the repeated cycles of amplification. Taq polymerase cannot usually amplify fragments longer than about 3kbp, but under some specialized conditions, PCR can amplify fragments up to approximately 10kbp. Other polymerases, either by themselves or in combination with Taq, are used to increase the length of amplified fragments or to increase the fidelity of the replication.

After completion of the thermalcycling (amplification), an aliquot from the PCR reaction is usually loaded onto an **electrophoretic gel** (described below) to determine whether a DNA fragment of the expected length was successfully amplified or not. Usually, the original template DNA will be so dilute that it will not be visible on the gel, only the amplified PCR product. The presence of a sharp band of the expected length indicates that PCR was able to amplify its target. If the purpose of the PCR was to test for the presence of a particular template sequence, this is the end of the experiment. Otherwise, the remaining PCR product can be used as starting material for a variety of other techniques such as sequencing or cloning.

8.4: Cutting and Pasting DNA- Restriction Digests and DNA Ligation

Restriction Enzymes

Many bacteria have enzymes that recognize specific DNA sequences (usually 4 or 6 nucleotides) and then cut the double stranded DNA helix at this sequence (Figure 8.4.7). These enzymes are called site-specific **restriction endonucleases**, or more simply "**restriction enzymes**", and they naturally function as part of bacterial defenses against viruses and other sources of foreign DNA. To cut DNA at known locations, researchers use restriction enzymes that have been purified from various bacterial species, and which can be purchased from various commercial sources. These enzymes are usually named after the bacterium from which they were first isolated. For example, ***EcoRI*** and *EcoRV* are both enzymes from *E. coli*. *EcoRI* cuts double stranded DNA at the sequence GAATTC, but note that this enzyme, like many others, does not cut in exactly the middle of the restriction sequence (Figure 8.4.8). The ends of a molecule cut by *EcoRI* have an overhanging region of single stranded DNA, and so are sometimes called **sticky-ends**. On the other hand, *EcoRV* is an example of an enzyme that cuts both strands in exactly the middle of its recognition sequence, producing what are called **blunt-ends**, which lack overhangs.

Figure 8.4.7: An EcoRI dimer (blue, purple) sits like a saddle on a double helix of DNA (one strand is green, one is brown). This image is looking down the center of the helix.(NCBI-?-PD)

Figure 8.4.8: The recognition sequence for EcoRI (blue) is cleaved by the enzyme (grey). This particular enzyme cuts DNA at a position offset from the center of the restriction site. This creates an overhanging, sticky-end. (Original-Deyholos-CC:AN)

DNA Ligation

The process of **DNA ligation** occurs when DNA strands are covalently joined, end-to-end through the action of an enzyme called **DNA ligase**. Sticky-ended molecules with complementary overhanging sequences are said to have **compatible ends**, which facilitate their joining to form recombinant DNA. Likewise, two blunt-ended sequences are also considered compatible to join together, although they do not ligate together as efficiently as sticky-ends. Note: sticky-ended molecules with non-complementary sequences will not ligate together with DNA ligase. Ligation is therefore central to the production of recombinant DNA, including the insertion of a double stranded DNA fragment into a plasmid vector.

8.5: Cloning DNA - Plasmid Vectors

Plasmids are Naturally Present in Some Bacteria

Many bacteria contain extra-chromosomal DNA elements called **plasmids**. These are usually small (a few 1000 bp), circular, double stranded molecules that replicate independently of the chromosome and can be present in high copy numbers within a cell. In the wild, plasmids can be transferred between individuals during bacterial mating and are sometimes even transferred between different species. Plasmids are particularly important in medicine because they often carry genes for pathogenicity and drug- resistance. In the lab, plasmids can be inserted into bacteria in a process called transformation.

Using Plasmids as Cloning Vectors

To insert a DNA fragment into a plasmid, both the fragment and the circular plasmid are cut using a restriction enzyme that produces compatible ends (Figure 8.5.1). Given the large number of restriction enzymes that are currently available, it is usually not too difficult to find an enzyme for which corresponding recognition sequences are present in both the plasmid and the DNA fragment, particularly because most plasmid vectors used in molecular biology have been engineered to contain recognition sites for a large number of restriction endonucleases.

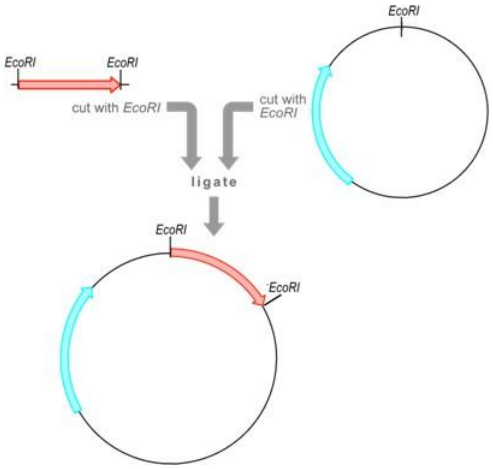


Figure 8.5.1: Cloning of a DNA fragment (red) into a plasmid vector. The vector already contains a selectable marker gene (blue) such as an antibiotic resistance gene. (Original-Deyholos-CC:AN)

After restriction digestion, the desired fragments may be further purified or selected before they are mixed together with ligase to join them together. Following a short incubation, the newly ligated plasmids, containing the gene of interest are **transformed** into *E. coli*. Transformation is accomplished by mixing the ligated DNA with *E. coli* cells that have been specially prepared (i.e. made **competent**) to uptake DNA. Competent cells can be made by exposure to compounds such as $CaCl_2$ or to electrical fields (**electroporation**). Because only a small fraction of cells that are mixed with DNA will actually be transformed, a **selectable marker**, such as a gene for antibiotic resistance, is usually also present on the plasmid. After transformation (combining DNA with competent cells), bacteria are spread on a bacterial agar plate containing an appropriate antibiotic so that only those cells that have actually incorporated the plasmid will be able to grow and form colonies. This can then be picked and used for further study.

Molecular biologists use plasmids as **vectors** to contain, amplify, transfer, and sometimes express genes of interest that are present in the cloned DNA. Often, the first step in a molecular biology experiment is to **clone** (i.e. copy) a gene into a plasmid, then transform this recombinant plasmid back into bacteria so that essentially unlimited copies of the gene (and the plasmid that carries it) can be made as the bacteria reproduce. This is a practical necessity for further manipulations of the DNA, since most techniques of molecular biology are not sensitive enough to work with just a single molecule at a time. Many molecular cloning and recombination experiments are therefore iterative processes in which:

1. a DNA fragment (usually isolated by PCR and/or restriction digestion) is cloned into a plasmid cut with a compatible restriction enzyme
2. the recombinant plasmid is transformed into bacteria
3. the bacteria are allowed to multiply, usually in liquid culture
4. a large quantity of the recombinant plasmid DNA is isolated from the bacterial culture

5. further manipulations (such as site directed mutagenesis or the introduction of another piece of DNA) are conducted on the recombinant plasmid
6. the modified plasmid is again transformed into bacteria, prior to further manipulations, or for expression

8.6: DNA Analysis - Gel Electrophoresis

A solution of DNA is colorless, and except for being viscous at high concentrations, is visually indistinguishable from water. Therefore, techniques such as **gel electrophoresis** have been developed to detect and analyze DNA (Figure 8.6.11).

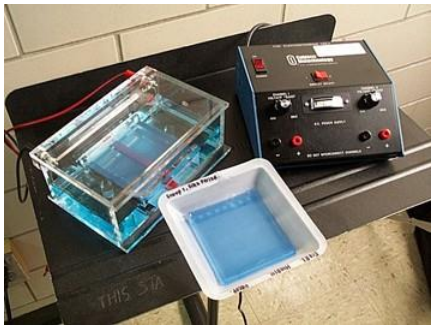


Figure 8.6.11: Apparatus for agarose gel electrophoresis. A waterproof tank is used to pass current through a slab gel, which is submerged in a buffer in the tank. The current is supplied by an adjustable power supply. A gel (stained blue by a dye sometimes used when loading DNA on the gel) sits in a tray, awaiting further analysis, such as photography under a UV light source. (Flickr- 457088634_585df11af5_o-pending)

This analysis starts when a solution of DNA is deposited at one end of a gel slab. This gel is made from polymers such as **agarose**, which is a polysaccharide isolated from seaweed. The DNA is then forced through the gel by an electrical current, with DNA molecules moving toward the positive electrode (Figure 8.6.12).

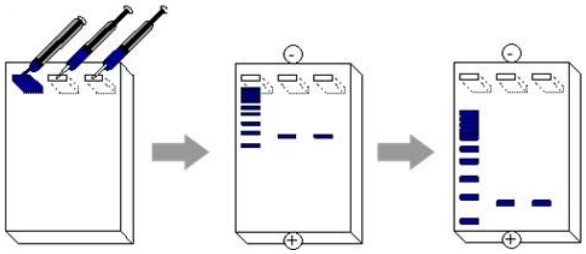


Figure 8.6.12: Agarose gel electrophoresis. DNA is loaded into wells at the top of a gel. A current is passed through the gel, pulling DNA towards the positively charged electrode. The DNA fragments are separated by size, with smaller fragments moving fastest towards the electrode. (Wikipedia-Magnus Manske_PD)

As it migrates, each piece of DNA threads its way through the pores, which form between the polymers in the gel. Because shorter pieces can move through these pores faster than longer pieces, gel electrophoresis separates molecules based on their size (length), with smaller DNA pieces moving faster than long ones. DNA molecules of a similar size migrate to a similar location in each gel, called a **band**. This feature makes it easy to see DNA after staining the DNA with a fluorescent dye such as **ethidium**

bromide (Figure 8.6.13). By separating a mixture of DNA molecules of known size (**size markers**) in adjacent lanes on the same gel, the length of an uncharacterized DNA fragment can be estimated. Gel segments containing the DNA bands can also be cut out of the gel, and the size-selected DNA extracted and used in other types of reactions, such as sequencing and cloning.

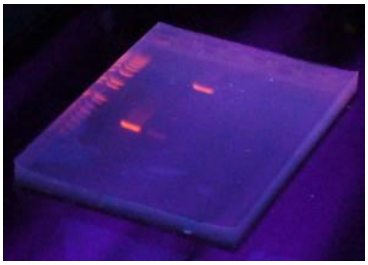


Figure 8.6.13: An agarose gel stained with ethidium bromide and illuminated by UV light. The stain associated with DNA is fluorescent. (Wikipedia-Transcontrol-GFDL)

8.7: DNA Analysis- Blotting and Hybridization

Bands of DNA in an electrophoretic gel form only if most of the DNA molecules are of the same size, such as following a PCR reaction, or restriction digestion of a plasmid. In other situations, such as after restriction digestion of chromosomal (genomic) DNA, there will be a large number of variable size fragments in the digest and it will appear as a continuous smear of DNA, rather than distinct bands. In this case, it is necessary to use additional techniques to detect the presence of a specific DNA sequence within the smear of DNA separated on an electrophoretic gel. This can be done using a "Southern Blot".

Southern Blots

A **Southern blot** (also called a **Southern Transfer**) is named after Ed Southern, its inventor. In the first step, DNA is digested with restriction enzymes and separated by gel electrophoresis (as discussed above). Then a sheet or **membrane** of nylon or similar material is laid under the gel and the DNA, in its separated position (bands or smear), is transferred to the membrane by drawing the liquid out of the gel, in a process called **blotting** (Figure 8.7.1). The blotted DNA is usually covalently attached to the nylon membrane by briefly exposing the blot to UV light. Transferring the DNA to the sturdy membrane is necessary because the fragile gel would fall apart during the next two steps in the process. Next, the membrane is bathed in a solution to **denature** (double stranded made single stranded) the attached DNA. Then a **hybridization** solution containing a small amount of single-stranded **probe** DNA that is complementary in sequence to a target molecule on the membrane. This probe DNA is labeled using fluorescent or radioactive molecules, and if the hybridization is performed properly, the probe DNA will form a stable duplex only with those DNA molecules on the membrane that are exactly complementary to it. Then, the unhybridized probe is washed off and remaining radioactive or fluorescent signal will appear in a distinct band when appropriately detected. The band represents the presence of a particular DNA sequence within the mixture of DNA fragments.

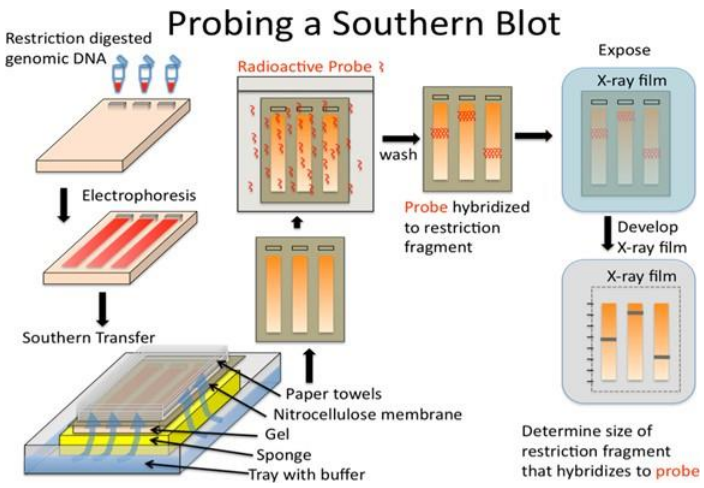


Figure 8.7.1: A diagram of Southern blotting. Genomic DNA that has been digested with a restriction enzyme is separated on an agarose gel, then the DNA is transferred from the gel to a nylon membrane (grey sheet) by blotting. The DNA is immobilized on the membrane, then probed with a radioactively labeled DNA fragment that is complementary to a target sequence. After stringent washing, the blot is exposed to X-ray film to detect what size frament the probe is bound. In this case, the probe bound to different- sized fragments in lanes 1, 2, and 3. In the last image the orange represent the position of the digested DNA, but it is not actually present on the X-ray film. (Original-J. Locke-CC:AN)

The probe is sequence specific (requires complementarity). However, variation in hybridization temperature and washing solutions can alter the **stringency** of the probe. At maximum stringency (higher temperature) hybridization conditions, probes will only hybridize with the exact target sequences that are perfectly complementary (maximum number of hydrogen bonds). At lower temperatures, probes will be able to hybridize to targets to which they do not match exactly, but only are roughly complementary for part of the sequence.

Southern blotting is useful not only for detecting the presence of a DNA sequence within a mixture of DNA molecules, but also for determining the size of a restriction fragment in a DNA sample. Southern blots are useful for detecting fragments larger than those normally amplified by PCR, and when trying to detect fragments that may be only distantly related to a known sequence. Applications of Southern blotting will be discussed further in the context of molecular markers in a subsequent chapter. Southern blotting was invented before PCR, but PCR has replaced blotting in many applications because of its simplicity, speed, and convenience. Following the development of the Southern blot, other types of blotting techniques were invented.

Northern Blots

The **Northern blot** involves the size separation of RNA in gels like that of DNA. Because we wish to determine the native size of the RNA transcript (and because RNA is single stranded) no restriction enzymes are ever used. Because most RNA is single stranded and can fold into various conformations thorough intra-molecular base pairing, the electrophoresis separation is more haphazard and the bands are often less sharp, compared to that of double stranded DNA.

Western Blots

In a **Western blot,** protein is size separated on a gel (usually an acrylamide gel) before transferring to a

membrane, which is then probed with an antibody that specifically binds to an antigenic site on the target protein. This antibody is then detected by other antibodies with some fluorescent or color production marker system. It will also give bands proportional to the amount and size of the target protein (Figure 8.7.2).

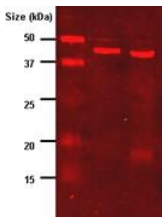
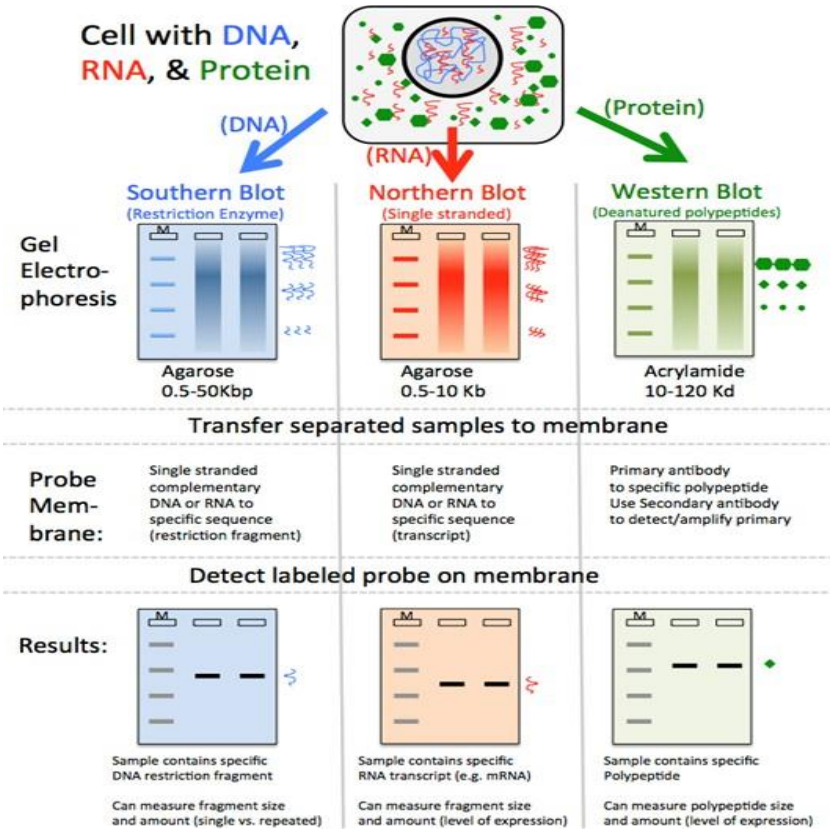


Figure 8.7.2: Western Blot using a anti-lipoic acid primary antibody and an IR-dye labelled secondary antibody in Leishmania major extracts. (Wikipedia-TimVickers–CC:AS)



A comparison of all three blotting methods is shown in Figure 8.7.3.

Figure 8.7.3: Comparison of Southern, Northern, and Western blots. Size and amount of DNA, RNA, and polypeptides can be determined using similar blotting methods. DNA is in blue, RNA in red, and polypeptides in green. A marker lane is shown in the left of each gel to determine size. A eukaryote cell is shown, but the same methods can be applied to prokaryotes, too. (Original- Locke-CC:AN)

8.8: Transgenic organisms

General principles of transgenesis

Transgenic organisms contain foreign DNA that has been introduced using biotechnology. Foreign DNA (the **transgene**) is defined here as DNA from another species, or else recombinant DNA from the same species that has been manipulated in the laboratory then reintroduced. The terms transgenic organism and genetically modified organism (**GMO**) are generally synonymous. The process of creating transgenic organisms or cells to be come whole organisms with a permanent change to their germline has been called either **transformation** or **transfection**. (Unfortunately, both words have alternate meanings. Transformation also refers to the process of mammalian cell becoming cancerous, while transfection also refers to the process of introducing DNA into cells in culture, either bacterial or eukaryote, for a temporary use, not germ line changes.) Transgenic organisms are important research tools, and are often used when exploring a gene's function. Transgenesis is also related to the medical practice of gene therapy, in which DNA is transferred into a patient's cells to treat disease. Transgenic organisms are widespread in agriculture. Approximately 90% of canola, cotton, corn, soybean, and sugar beets grown in North America are transgenic. No other transgenic livestock or crops (except some squash, papaya, and alfalfa) are currently produced in North America.

To make a transgenic cell, DNA must first be transferred across the cell membrane, (and, if present, across the cell wall), without destroying the cell. In some cases, **naked DNA** (meaning plasmid or linear DNA that is not bound to any type of **carrier**) may be transferred into the cell by adding DNA to the medium and temporarily increasing the porosity of the membrane, for example by **electroporation**. When working with larger cells, naked DNA can also be **microinjected** into a cell using a specialized needle. Other methods use **vectors** to transport DNA across the membrane. Note that the word "vector" as used here refers to any type of carrier, and not just plasmid vectors. Vectors for transformation/transfection include **vesicles** made of lipids or other polymers that surround DNA; various types of particles that carry DNA on their surface; and infectious viruses and bacteria that naturally transfer their own DNA into a host cell, but which have been engineered to transfer any DNA molecule of interest. Usually the foreign DNA is a complete expression unit that includes its own cis-regulators (e.g. promoter) as well as the gene that is to be transcribed.

When the objective of an experiment is to produce a **stable** (i.e. heritable) transgenic eukaryote, the foreign DNA must be incorporated into the host's chromosomes. For this to occur, the foreign DNA must enter the host's nucleus, and recombine with one of the host's chromatids. In some species, the foreign DNA is inserted at a random location in a chromatid, probably wherever strand breakage and non-homologous end joining happen to occur. In other species, the foreign DNA can be targeted to a particular locus, by flanking the foreign DNA with DNA that is homologous to the host's DNA at that locus. The foreign DNA is then incorporated into the host's chromosomes through homologous recombination.

Furthermore, to produce multicellular organisms in which all cells are transgenic and the transgene is stably inherited, the cell that was originally transformed must be either a gamete or must develop into tissues that produce gametes. Transgenic gametes can eventually be mated to produce homozygous,

transgenic offspring. The presence of the transgene in the offspring is typically confirmed using PCR or Southern blotting, and the expression of the transgene can be measured using reverse-transcription PCR (RT-PCR), RNA blotting, and Western (protein blotting).

The rate of transcription of a transgene is highly dependent on the state of the chromatin into which it is inserted (i.e. **position effects**), as well as other factors. Therefore, researchers often generate several independently transformed/transfected lines with the same transgene, and then screen for the lines with the highest expression. It is also good practice to clone and sequence the transgenic locus from a newly generated transgenic organism, since errors (truncations, rearrangements, and other mutations) can be introduced during transformation/transfection.

Producing a transgenic plant

The most common method for producing transgenic plants is **Agrobacterium-mediated transformation** (Figure 8.8.1).

Agrobacterium tumifaciens is a soil bacterium that, as part of its natural pathogenesis, injects its own tumor-inducing (**Ti**) plasmid into cells of a host plant. The natural T_i plasmid encodes growth-promoting genes that cause a gall (i.e. tumor) to form on the plant, which also provides an environment for the pathogen to proliferate. Molecular biologists have engineered the T_i plasmid by removing the tumor-inducing genes and adding restriction sites that make it convenient to insert any DNA of interest. This engineered version is called a **T-DNA** (transfer-DNA) plasmid; the bacterium transfers a linear fragment of this plasmid that includes the conserved "left-border (LB)", and right-border (RB)" DNA sequences, and anything in between them (up to about 10 kb). The linear T-DNA fragment is transported into the nucleus, where it recombines with the host-DNA, probably wherever random breakages occur in the host's chromosomes.

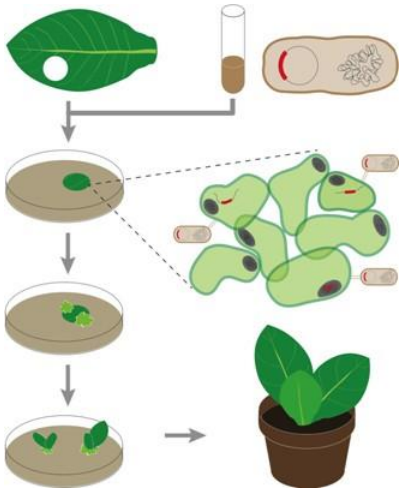


Figure 8.8.1: Production of a transgenic plant using Agrobacterium-mediated transformation. The bacterium has been transformed with a T-DNA plasmid that contains the transgene and a selectable marker that confers resistance to a herbicide or antibiotic. A bacterial culture and plant tissue (e.g. a leaf punch) are co-cultured on growth medium in a Petri dish. Some of the plant cells will become infected by the bacterium, which will transfer the T-DNA into the plant cytoplasm. In some cases the transgene will become integrated into the chromosomal DNA of a plant cell. In the presence of certain combinations of hormones, the plant cells will

dedifferentiate into a mass of cells called callus. The presence of a selective agent (e.g. herbicide or antibiotic) in the growth medium prevents untransformed cells from dividing. Therefore, each callus ideally consists only of transgenic plant cells. The resistant calli are transferred to media with other combinations of hormones that promote organogenesis, i.e. differentiation of callus cells into shoots and then roots. The regenerated transgenic plants are transferred to soil. Their seeds can be harvested and tested to ensure that the transgene is stably inherited. (Original-Deyholos-CC:AN)

In Arabidopsis and a few other species, flowers can simply be dipped in a suspension of Agrobacterium, and ~1% of the resulting seeds will be transformed. In most other plant species, cells are induced by hormones to form a mass of undifferentiated tissues called a callus. The Agrobacterium is applied to a callus and a few cells are transformed, which can then be induced by other hormones to regenerate whole plants (Figure 8.8.2). Some plant species are resistant (i.e. "**recalcitrant**") to transformation by Agrobacterium. In these situations, other techniques must be used such as **particle bombardment**, whereby DNA is non- covalently attached to small metallic particles, which are accelerated by compressed air into callus tissue, from which complete transgenic plants can sometimes be regenerated. In all transformation methods, the presence of a selectable marker (e.g. a gene that confers antibiotic resistance or herbicide resistance) is useful for distinguishing transgenic cells from non-transgenic cells at an early stage of the transformation process.

Figure 8.8.2: Organogenesis of flax shoots from calli. (Original-J. McDill-CC:AN)

Producing a transgenic mouse

In a commonly used method for producing a transgenic mouse, **stem cells** are removed from a mouse embryo, and a transgenic DNA construct is transferred into the stem cells using electroporation, and some of this transgenic DNA enters the nucleus, where it may undergo homologous recombination (Figure 8.8.3). The transgenic DNA construct contains DNA homologous to either side of a locus that is to be targeted for replacement. If the objective of the experiment is simply to delete ("**knock-out**") the targeted locus, the host's DNA can simply be replaced by selectable marker, as shown. It is also possible to replace the host's DNA at this locus with a different version of the same gene, or a completely different gene, depending on how the transgenic construct is made. Cells that have been transfected and express the selectable marker (i.e. resistance to the antibiotic neomycin resistance, neoR, in this example) are distinguished from unsuccessfully transfected cells by their ability to survive in the presence of the selective agent (e.g. an antibiotic). Transfected cells are then injected into early-stage embryos, and then are transferred to a foster mother. The resulting pups are chimeras, meaning that only some of their cells are transgenic. Some of the chimeras will produce gametes that are transgenic, which when mated with a wild-type gamete, will produce mice that are hemizygous for the transgene. Unlike the chimeras, these hemizygotes carry the transgene in all of their cells. Through further breeding,

mice that are homozygous for the transgene can be obtained.

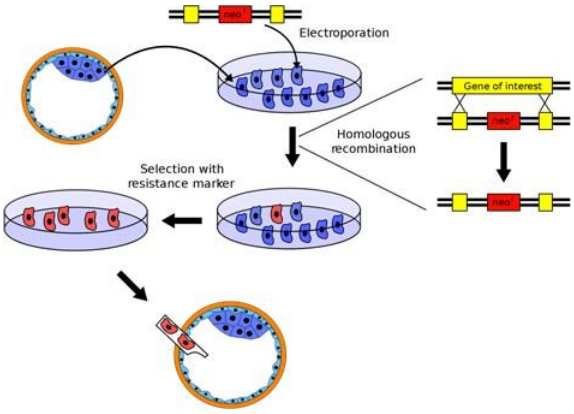


Figure 8.8.3: Production of a transgenic mouse. Stem cells are removed from an embryo, and are transfected (using electroporation) with a transgenic construct that bears a neomycin resistance gene (neor) flanked by two segments of DNA homologous to a gene of interest. In the nucleus of a transgenic cell, some of the foreign DNA will recombine with the targeted gene, disrupting the targeted gene and introducing the selectable marker. Only cells in which neor has been incorporated will survive selection. These neomycin resistant cells are then transplanted into another embryo, which will grow into a chimera within a foster mother. (Wikipedia-Kiaergaard-CC:AN)

Human gene therapy

Many different strategies for human gene therapy are under development. In theory, either the **germline** or **somatic** cells may be targeted for transfection, but most research has focused on somatic cell transfection, because of risks and ethical issues associated with germline transformation. Gene therapy approaches may be further classified as either ***ex vivo*** or ***in vivo***, with the former meaning that cells (e.g. stem cells) are transfected in isolation before being introduced to the body, where they replace defective cells. *Ex vivo* gene therapies for several blood disorders (e.g. immunodeficiencies, thalassemias) are undergoing clinical trials. For *in vivo* therapies, the transfection occurs within the patient. The objective may be either stable integration, or **non-integrative** transfection. As described above, stable transfection involves integration into the host genome. In the clinical context, stable integration may not be necessary, and carries with it higher risk of inducing mutations in either the transgene or host genome). In contrast, transient transfection does not involve integration into the host genome and the transgene may therefore be delivered to the cell as either RNA or DNA. Advantages of RNA delivery include that no promoter is needed to drive expression of the transgene. Besides mRNA transgenes, which could provide a functional version of a mutant protein, there is great interest in delivery of **siRNA** (small-inhibitory RNAs), which can be used to silence specific genes in the host cell's genome.

Vectors for *in vivo* gene therapy must be capable of delivering DNA or RNA to a large proportion of the targeted cells, without inducing a significant immune response, or having any toxic effects. Ideally, the vectors should also have high specificity for the targeted cell type. Vectors based on viruses (e.g. **lentiviruses**) are being developed for in both *in vivo* and *ex vivo* gene therapies. Other, non-viral vectors (e.g. vesicles and nanoparticles) are also being developed for gene therapy as well.