GENETIC ENGINEERING

Genetic engineering involves the manipulation of genetic information – modifying a single nucleotide in DNA or large sections of chromosomes. Even a single nucleotide change can cause drastic changes in protein structure and function – many changes can lead to new or improved products. The GOAL is to produce organisms with new, improved characteristics – modified organisms are capable of synthesizing new beneficial products.

**Specifics of genetic modification procedure:**

1. The genetic code (DNA) for the desired protein or trait is identified and isolated from a donor cell, confirmed (restriction digestion and/or sequenced) and pasted into a vector (recombinant DNA – rDNA)
2. Recombinant cells are produced when the vector carrying the gene of interest is transferred into new host cells (transformation) grown on agar.
3. Assays are performed to confirm that the inserted DNA is expressed and new proteins are produced.
4. The recombinant cells are grown in culture (cloning), first on a small scale (fermentation) and then on a large scale (manufacturing).
5. The recombinant protein product is isolated and purified from the cell culture. Then the product is analyzed for activity and other qualities before going to market.

Genetic engineered products require significant research and development (R&D). The rationale for investing in these products is based on whether a product can be more successfully produced through genetic engineering than other manufacturing methods.

- Example: Chymosin is the genetically engineered version of the naturally occurring protein, rennin. Rennin (an enzyme purified from calf stomachs) is a protease that cleaves casein, the protein milk into fragments. The casein fragments fall out of solution (precipitation) and appear as curds in milk. Curds are pressed and aged into cheese. Rennin is used in cheese making to speed cheese production. The price and availability of rennin is a problem. Biotechnologist recognized this issue and isolated the rennin gene from calf cells and transferred it into fungal cells. The transformed fungal cells began transcribing the calf DNA and produced rennin. Producing genetically engineered rennin is cheaper and more reliable than obtaining it from cows.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
PLASMIDS

- Joshua Lederberg and William Hayes independently discovered plasmids while studying conjugation – process of transferring DNA by a pilus from one bacterium to another.
- 1952: Lederberg proposed the name plasmid
- 1961: Tsutomu Watanabe and Toshio Fukasawa found that some plasmids carried antibiotic resistance genes.
- 1962: Peter Lobban proposed using restriction enzymes to help recombine DNA.
- 1973: Stanley Cohen, Annie Change, Herbert Boyer and Robert Helling published a paper describing how to construct a functional plasmid

Plasmid structure and function:

- Plasmids are extra chromosomal loops of DNA that can self-replicate in the cytosol of bacteria. They have an origin of replication (ori) and are designated with a “p” in the name.
- Plasmids have genes that code for proteins – they are symbolized by an arrow in the direction of transcription.
- The number of plasmids ranges from 5 to 1,000 per bacterial cell.
- Plasmids can be picked up from the environment and transferred between bacteria. Plasmids are used by their host organism to cope with stress-related conditions. Many plasmids, for example, carry genes that code for the production of enzymes to inactivate antibiotics or poisons. Others contain genes that help a host organism digest unusual substances or kill other types of bacteria. Several characteristics of plasmids make them easy to modify genetically. Firstly, they have relatively small DNA sequences, between 1,000 and 20,000 DNA base pairs. Secondly, they are easy to cut open, without falling apart, and snap back into shape. This makes it easy to insert new DNA into plasmids. Once a new DNA is inserted, the modified plasmid can be grown in bacteria for self-replication to make endless copies.
- Plasmids have 2 main uses: (1) to express recombinant proteins, (2) to house genes that have been cloned
- Plasmids are constructed to make cloning easy. They have an area called multiple cloning site (MCS) that has a series of unique restriction enzyme recognition sites. The MCS is used to open up the plasmid to receive the gene of interest – plasmid with a gene (red) inserted into the MCS (green).

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
GENETIC ENGINEERING STEPS:
If a potential product is to be made through genetic engineering, it must already be produced in some type of cell. Using genetic engineering techniques, scientists can locate and isolate DNA, coax cells to accept the DNA instructions and begin synthesizing the compound. Isolation and purification of DNA depend on the type of cell.

STEP 1: Isolating Genetic Information
Retrieving the DNA – explode or lyse the cell. Some cells have cell walls that must be eliminated. All cells have cell membranes and cytoplasmic constituents that must be removed. When cells burst open, all the contents are released and the DNA must be separated from the other materials. The procedure for extracting bacterial chromosomal DNA requires sufficient quantity of starting cells grown in agar or in broth culture.

1. Cultured cells are transferred to a buffered solution that may contain the enzyme lysozyme. Lysozyme gently degrades the bacterial cell walls by breaking down the structural carbohydrate, peptidoglycan. When the cell walls are gone, osmotic pressure causes the cells to rupture, dumping their cell contents.
2. A detergent is added (sarkosyl or sodium dodecyl sulfate-SDS) to dissolve membrane lipids and precipitate proteins. Often proteases and salts are added to degrade the remaining proteins and polypeptides. To remove the RNA, RNase is added.
3. The sample is spun in a centrifuge to pellet (precipitate) the molecular debris. The bacterial DNA stays in solution in the supernatant. The supernatant can be poured off and the genomic DNA used for gene isolation. The DNA in a supernatant can also be pulled all the way out of solution by alcohol extraction – alcohol is layered on top of the DNA solution and the DNA strands are either spooled out with a glass rod or precipitated into a pellet with a centrifuge.

The steps in bacterial plasmid DNA isolation are similar the above procedure with a few exceptions. The cell lysis solution for plasmid isolation needs an additional base (NaOH) and SDS.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
STEP 2: Probing DNA for Genes of Interest

Once the DNA is removed from the cells and purified from contaminant molecules, the search for genes of interest begins. **Probes** are used to identify the gene of interest. Probes are DNA or RNA molecule that is complementary to the DNA sequence being sought.

- Probes can be made from RNA molecules. RNA is extracted from cells – use of RNA as a probe is called mRNA/DNA hybridization. mRNA probes degrade at high temperatures so scientists often produce copy DNA (cDNA) probes. Using reverse transcriptase and DNA polymerase, copies of RNA, complementary to gene of interest, can be synthesized.

**Methods:**

- Using **Restriction Enzymes** and **gel electrophoresis** – Restriction enzymes digest the DNA isolated for the source cells. The restriction fragments are then split into single-stranded pieces of DNA and run on an agarose gel. Once the DNA pieces are separated, a solution containing probes is flushed over the gel. The probe molecules are “tagged” with either radioactive marker or a fluorescent label. The probes bounce around the gel. If they bump into a complementary sequence, they bind to it. This process is called **hybridization.** The hybridization “tag” shows the location of the DNA sequence of interest. If the tag is a radioactive label, then an x-ray film can be exposed, resulting in a permanent record – autoradiogram. Other methods of visualizing process include using chemiluminescent (color) or fluorescent markers.

- Using **Southern Blotting** – a gel with the DNA fragments is transferred to a positively charged membrane (a blot). The blot is then probed. Once the DNA sequence of interest is identified on a gel, it can be removed for further processing.

- Using **Polymerase Chain Reaction** (PCR) - Primers (similar to probes) recognize a certain DNA sequence and make possible the replication of millions of copies of it within a few hours. Samples with primers are loaded into a thermal cycler for replication. The PCR product can be confirmed by gel electrophoresis and used in genetic engineering.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
STEP 3: Transforming Cells
Once the gene is isolated, it can be transferred into a new host cell. If the recipient cells express the genes and produce a nonnative protein, the cells will exhibit new traits. These cells are transformed. Transformation is the uptake and expression of foreign DNA by a cell. Transformations do occur in nature – this is how bacteria acquire new traits.

- Bacteria naturally have the ability to take up DNA from dead cells. The cells taking up DNA showed new characteristics --- they are transformed. When scientist first tried this process, very few cells were transformed --- the transformation efficiency was low. Scientist observed that plasmid DNA, small rings of extrachromosomal bacteria DNA, was taken up easily and better expressed by certain bacteria cells.
- Scientist proposed using plasmid DNA as vectors to carry genes of interest into cells – introduce genes of interest into plasmids (rDNA). The rDNA plasmids were actually doing the transformation. Since the 1st transformation in 1970s, scientists now use viral DNA as vectors of genetic information. Viruses are used to transform cells – called transduction.
- Transfection is used to describe mammalian transformations. Viruses are often used as vectors to carry genes of interest into mammalian host cells. Cosmids are also used as vectors – cosmids are very large plasmids. It’s also common to inject DNA directly into the nucleus of mammalian cell using a microinjection syringe.

Making Recombinant DNA – (part A of Step 3)
1. Plasmid digestion: To carry a gene(s) into a cell, a plasmid or other vector must be “spliced” or cut open. Restriction enzymes (endonucleases) recognize specific A, C, G and T sequences within DNA molecules and cut the DNA strands. Some restriction enzymes cut across the DNA strand to produce “sticky ends”. Sticky ends have one side of DNA strands that is longer than the other. These overhangs allow for complementary matches between two DNA pieces cut by the same enzyme. A plasmid and the gene of interest can be cut by the same restriction enzyme – their sticky ends will match.
2. The gene(s) of interest is then pasted into the open plasmid. DNA ligase is used to paste the gene into the open plasmid. This produces recombinant DNA (rDNA) plasmid – contains DNA from two different species.

More than 1200 restriction enzymes have been discovered and isolated. It’s thought that restriction enzymes evolved as a defense against invading viral DNA. Restriction enzymes are named based on their origin. Restriction enzymes sequences are palindromic (it reads the same forward and backwards). Example: EcoRI finds the DNA sequence GAATTC in the 5'-3' strand, it cuts between G and A. It cuts the complementary strand in a 5'-3'.

More information is included in the image that cannot be accurately transcribed.
Restriction enzymes are used to cut DNA into pieces of manageable DNA for analysis – restriction fragments. Studies of the differences in fragment length that result from digestion can reveal information about the difference in DNA sequences --- technique is called restriction fragment length polymorphism (RFLP) analysis. Applications of RFLP analysis:

- DNA fingerprinting – unique patterns result from the DNA analysis of an individual. A section of DNA is subjected to restriction digestion analysis. Restriction digestion fragments are run on a gel creating a banded pattern. Only identical twins or asexually produced clones have the exact DNA fingerprint. Fingerprints are made using a particular restriction enzyme – the different RFLPs are seen on a gel electrophoresis.
- PCR – targets specific sections of genetic information and produces millions of copies.
- Evolutionary studies – banding patterns help scientist understand DNA mutations. Recognizing these differences help to identify related organism and understand how to address needs of threatened or endangered species.

Performing a Transformation (part B of Step 3)
1. Grow the host cells in broth culture.
2. Keeping the cells on ice, make them competent (ready to take up DNA – make the cell membrane porous) with a treatment of calcium chloride (CaCl₂) or magnesium chloride (MgCl₂).
3. Add the rDNA plasmids to the competent cells.
4. Heat shock the cells by rapidly moving them from ice to hot water bath (37°C or 42°C for 20 to 90 seconds, depending on the strain of cells used), and then quickly put them back on ice. This is called a heat shock/cold shock. A heat shock enlarges pores and “sucks” more plasmids into cells. A cold shock traps the plasmids inside.
5. Add a nutrient broth for cell recovery and gene expression at some optimum temperature (such as 37°C for E. coli).
6. Plate out the cells on some kind of selection media/agar that shows that the cells are producing the new protein.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
Tricks to induce bacteria to take in foreign DNA more efficiently –

(1) Create large pores or channels in the outer boundaries of the cell – this is called competency. How does it work? One theory suggests that membrane proteins are arranged to make intramembrane channels or pores. Adding cations covers the membrane channel proteins with a positive charge. The proteins in the channels may repel each other, expanding channels. When cells are treated with ions, they are ‘competent’. Competency increases transformation efficiency by thousands of times. Using a molecule like CaCl$_2$ to induce competency is called chemical competency. Exposing cells to an electric field is called electroporation (use an electroporator).

(2) Give cells heat and cold shocks after they have been treated with rDNA. When cells are transferred from cold to hot, they swell rapidly, pulling in DNA at or near the membrane. When quickly transferred back to cold, the cells shrink rapidly and trap the DNA inside.

Once cells have been transformed, they undergo a recovery period. The cells are given nutrients in a sterile broth and allowed to repair their membranes. The cells that survive grow and divide. They are spread on Petri plates containing selection media and grown in incubation ovens. Selection media has a specific ingredient that makes it easy to tell if transformed cells are growing on the plate – may be an antibiotic, a nutrient or a type of chemical.

Selection media kills off or slows the growth of non-transformed cells. Only the transformed cells grow – this is called selection. Selection screens the cells to see if they are making the new proteins from the newly acquired gene. As transformed cells grow and reproduce, colonies of identical transformed cells arise. The transformed cells begin expressing their new genes. The colonies are clones of transformed cells – all expressing the same gene and producing the same proteins.

- Sometimes it is difficult to select for the desired gene directly – additional genes can be added to rDNA plasmids for selection.
- Example: The beta-galactosidase (B-gal) gene is inserted into recombinant plasmids as a selection gene. In cells, the B-gal gene produces beta-galactosidase, an enzyme that converts the carbohydrate X-gal to a blue product. If cells that are transformed with a recombinant B-gal plasmid are grown on X-gal agar, the colonies turn blue. Biotechnicians can add other genes of interest to the B-gal plasmid and use blue colonies to determine whether cells are transformed.
- Example: Another selection gene is the green fluorescent protein (GFP) gene which is normally found in a jellyfish (Aequorea victoria). When a recombinant vector plasmid contains the GFP gene, transformed cells in UV light will glow a fluorescent green color.


Example: 50 ng of plasmid DNA is transformed into a final transformation volume of 500 μl & 10 μl of this volume is spread on an agar plate. Assume that 60 CFU (colony forming units) are observed on the agar plate. Note: 1 μg is 1,000 ng, so 50 ng = 0.05 μg of DNA

Steps:
1. First, count the number of colonies growing on the LB/ampicillin (LB/amp) agar plate. In this case, the CFU is 60.
2. Next, determine the amount of plasmid DNA (in μg) spread on the LB/amp agar plate. In this example, only 10 μl of a 500 μl transformation was spread on the plate

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DNA spread = \frac{\text{Volume spread (μl)} \times \text{DNA in transformation (μg)}}{\text{Total volume of transformation (μl)}}

= 10 \mu l \times 0.05 \mu g / 500 \mu l
= 0.001 \mu g

3. Next, calculate transformation efficiency by dividing the CFU by the amount of DNA spread on plate.

Transformation efficiency = \frac{60 \text{ CFU}}{0.001 \mu g}
= 60,000 \text{ CFU/μg} or 60 \times 10^4 \text{ CFU/μg}

**STEP 4: Scale-Up Process**

Huge volumes of transformed cells are needed to manufacture the amounts of proteins required to make a marketable product. To produce the large volumes, the selected transformed cells are grown in increasingly larger containers – this is scale-up. Scale-up process begins with the transfer of a transformed cell colony to liquid media – about a 50 mL of broth. The nutrient broth allows more room and more nutrients. If the culture does well, the culture is scaled-up to 1 or 2 L spinner flasks (contains a propeller blade to keep cells suspended and aerated), then increased up to 10 L, etc...

During each scale-up, the cell growth rate, product concentration and product activity is measured. The requirements for each cell system are unique – optimum temperature, pH, nutrient concentration and oxygen content. In early scale-ups, the progress is monitored “by hand”. Samples are taken at regular intervals and tested for each factor – requirements are added to maintain optimum growing conditions. When batches are large enough, 10 L or more, computers monitor and adjust batches in the fermenters (automated containers used for the growth of microorganism cultures – fermentation). To maintain sterile conditions, the fermentation tanks have pipes going into and out of them for monitoring and adjustment. As the culture moves from scale-up to fermentation to manufacturing, the process becomes more automated.

 Cultures are also monitored for contamination. Ensuring sterile conditions throughout the scale-up requires careful procedures – a strict protocol for cleaning and sterilizing equipment. To ensure sterile conditions, workers “gown up” and scale-up is done in “clean rooms” which have HEPA (high-efficiency particulate air) filters.

During the scale-up process, assays are done to measure protein concentration and activity. Assay service departments in R&D develop, conduct test and verify the products as they move through the pipeline. To support manufacturing, Quality Control (QC) department conducts test and provides verification. The QC department performs additional assays and more extensive testing. Test results from QC are sent to the FDA or other regulatory agencies.

**Fermentation, Manufacturing and GMP**

Many biotechnology products are manufactured by alcoholic or lactic acid fermentation.

- **Alcoholic fermentation** – process by which certain yeast and bacteria cells convert glucose to carbon dioxide and ethanol under anaerobic conditions.
- **Lactic Acid fermentation** – process by which certain bacteria cells convert glucose to lactic acid under anaerobic conditions.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
In biotechnology, fermentation refers to growing cells (bacteria or fungi) under optimum conditions for maximum cell division and product production. Many biotech companies have fermentation departments that grow their cell lines in highly controlled fermentation tanks to ensure the highest productivity.

- Scale-up begins with the transfer of transformed cells to broth. Volumes of broth cultures begin as small quantities in flask. A large colony, growing well on Petri plates and producing product is used as the seed. The seed colony may have several million identical transformed cells in it. With the ideal temperature, pH, aeration and nutrients, the cells will grow and multiply as quickly as every 20 minutes. Exponential growth occurs when a cell culture doubles in cell count with every cell cycle.

- When a broth culture is first inoculated, it appears clear. When it becomes more concentrated with cells, it turns cloudy. After the initial inoculation, it takes some time (lag phase) before reaching the exponential phase. The concentration of cells in the culture can be monitored using a spectrophotometer. Over time, the cells begin to use up the nutrients, slows their growth and division – this is the stationary phase. The culture must be transferred into a new, larger broth culture.

- The fermentation protocols are different for different cell lines and for different products. Ultimately, the goal is to produce enough product for purification and marketing. The purified product must be formulated or prepared for delivery and storage. During the manufacturing, the company follows good manufacturing practices (cGMP).

**STEP 5: Retrieving Plasmids after Transformation**

After transformation, it is necessary to extract the transforming plasmid from the transformed cells. This extraction process is called preparation. The amounts of plasmid recovered will vary.

- A miniprep is a plasmid isolation that yields about 20 to 30 μg of DNA (usually 20 μg in a 50 μL sample).
- A midiprep isolates plasmids from 15 to 25 mL of culture with the goal of isolating several hundred micrograms of DNA.
- A maxiprep starts with 100 mL of culture, giving yields of over 500 μg of plasmid DNA.
- Megapreps and Gigapreps can be done for larger yields of plasmids.

In any prep procedure, the cells must be exploded and the DNA separated from all the other molecules in the cell. Most laboratories use commercially kits for plasmid preparations.

**Miniprep Procedure** – Transformed cells are spun down, and enzymes and other reagents are added to explode the cells. Salts precipitate the proteins, which can be discarded. Alcohol washes and centrifugation clean to precipitate a pellet of plasmids on the bottom of the tube. The plasmid pellet is resuspended in TE buffer and stored at -20°C until ready to use.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*
Reasons for Performing a Prep – Extracting and analyzing the transforming plasmid verifies that a cell received, and is processing the new genes carried in on the transforming plasmid. Another reason is to collect more plasmids for future transformations – transformed cells may not store well for long periods of time. Plasmid DNA can be stored almost indefinitely at very low temperatures.

Testing for the Presence of DNA – The concentration and purity of the sample must be determined by using a DNA indicator assay test. A common way to determine the concentration of DNA in a sample is to use a UV spectrophotometer. Scientists have determined that DNA molecules absorb a maximum amount of light at 260 nm and can detect DNA in a sample by setting a UV spec to this wavelength.

To calculate the concentration of DNA, use a simple ratio. It is known that 50 μg/mL of pure DNA absorbs approximately 1 au of light at 260 nm.

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\frac{50 \, \mu g/mL}{1 \, \text{au at 260 nm}} = \frac{X \, \mu g/mL}{\text{the absorbance of sample at 260 nm}}
\]

For example, if a plasmid absorbs 1.05 au at 260 nm, then its concentrated value would be approximately __________ μg/mL. This is a good yield for a miniprep, but this concentration is so high that the sample would need to be diluted for use in a transformation.

Assuming the plasmid sample is pure and not contaminated by RNA or protein. Often, RNA and proteins contaminate plasmid preps. By measuring a sample’s absorbance at 280 nm (the wavelength of max light absorbance for a colorless protein) and comparing that value to the absorbance at 260 nm, one can establish a ratio of nucleic acid (DNA or RNA) to protein.

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\frac{\text{Absorbance (au) at 260 nm}}{\text{Absorbance (au) at 280 nm}} = \text{purity value of the sample}
\]

A purity value of at least 1.8 is expected for pure DNA and preferred when conducting plasmid preparations. If the purity value is over 2.0, the sample is probably contaminated with RNA. Depending on what the sample is to be used for, RNA contamination could be a problem – using RNases during and after prep can reduce RNA contamination. If the purity value is below 1.5, the sample has substantial contamination which could be a serious problem. If the purity value reaches 1.0, the sample is worthless. Using proteases and column chromatography during or after prep may reduce protein contamination.

When a sample’s DNA purity and concentration is determined, a plasmid sample can be used for restriction digestion to confirm that the purified plasmid is the one desired. Once the plasmid has been retrieved from transformed cells and its identity is confirmed through restriction digestions, it may be used in subsequent transformations.

*Adapted from “Biotechnology: Science for the New Millennium” by Ellyn Daugherty.*