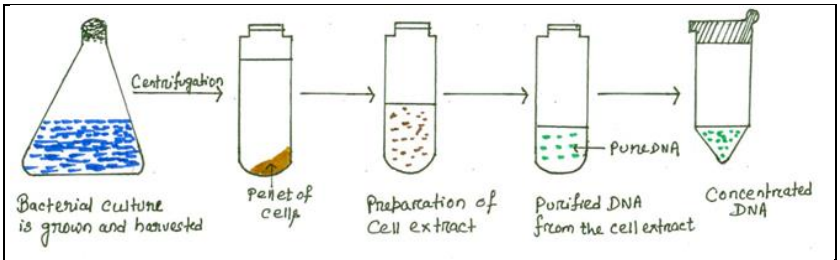
DNA Preparation and Purification

For cloning, there is a requirement of three distinct kinds of DNA i.e, total cell DNA, pure plasmid DNA and phage DNA.

Total cell DNA preparation

Total cell DNA will often be required as a source of material from which to obtain genes to be cloned. Total cell DNA may be DNA from a culture of bacteria, from a plant, from animal cells, or from any other type of organism that is being studied. The steps in the preparation of total cell DNA are: the growth and harvesting of a bacterial culture, breaking of cells to release their contents, purification of the DNA from the cell extract and finally concentration of the DNA.



Preparation of total cell DNA from a bacterial culture

Growing and harvesting a bacterial culture

Most bacteria can be grown without much difficulty in a liquid medium otherwise known as broth culture. M9 is an example of a known defined medium in which a mixture of inorganic nutrients (nitrogen,magnesium and calcium) and glucose(source of carbon and energy) are present. LB is a complex undefined medium in which two of the ingredients, tryptone and yeast extract are complicated mixtures of unknown chemical compounds.

In order to prepare a cell extract, the bacteria must be obtained in as small a volume as possible. Harvesting is therefore performed by spinning the culture in a centrifuge. Low centrifugation

speeds will pellet the bacteria at the bottom of the centrifuge tube, allowing the culture medium to be poured off.

Cell extract preparation

Cell extract preparation is the second step of total cell DNA preparation. Breaking open of bacteral cells can be done by physical methods, and chemical methods. Chemical methods are most commonly used with bacterial cells during DNA preparation. Cell lysis by chemical method is brought about by one chemical that attacks the cell wall while the other disrupts the cell membrane. Cell wall weakening is brought about by lysozyme, **ethylenediamine tetraacetate(EDTA)** or a combination of both. Lysozyme digests the polymeric compounds that give the cell wall its rigidity. Magnesium ions are essential for preserving the overall structure of the cell envelope. EDTA removes magnesium ions and also inhibits cellular enzymes that could degrade DNA. Sometimes a detergent such as sodium dodecyl sulphate(SDS) is added along with the chemicals, because detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membranes.

The insoluble cell debris can be pelleted by centrifugation, leaving the cell extract as a reasonably clear supernatant.

DNA purification from a cell extract

For obtaining pure DNA, Bacterial cell extract is purified from significant quantities of Protein and RNA. DNA associated proteins, as well as other cellular proteins, may be degraded with the addition of a protease. Precipitation of the protein is aided by the addition of a salt such as ammonium or sodium acetate. When the sample is vortexed with phenol-chloroform(1:1 ratio) and centrifuged, the precipitated proteins left as white coagulated mass will remain at the interface between the aqueous and organic layers and can be drawn off carefully. The aqueous solution of nucleic acids (DNA and RNA) can then be removed with a pipette. The only effective way to remove the RNA is with the enzyme ribonuclease, which rapidly degrades these molecules into ribonucleotide subunits.

Concentration of DNA samples and measurement of concentration

Ethanol precipitation is the most frequently used method of concentration. In the presence of a salt (only monovalent cations), absolute ethanol efficiently precipitates polymeric nucleic acids at around a temperature of -20ºC or may be less than that. With a thick solution of DNA the ethanol can be layered on top of the sample, causing molecules to precipitate at the interface. If ethanol is mixed with a dilute solution, the precipitate can be collected by centrifugation, and then redissolved in an appropriate volume of water.

DNA concentrations can be accurately measured by ultraviolet absorbance spectrophotometry. The amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Absorbance is measured usually at 260nm, at which wavelength an absorbance(A_{260}) of 1.0 corresponds to 50µg of double stranded DNA/ml. A pure sample of DNA indicates the ratio of absorbances at 260 and 280nm is 1.8 i.e.,A_{260}/A_{280} is 1.8, for a pure sample of DNA. Ratio less than 1.8 indicates contamination of protein or phenol.

DNA preparation from animal and plant cells

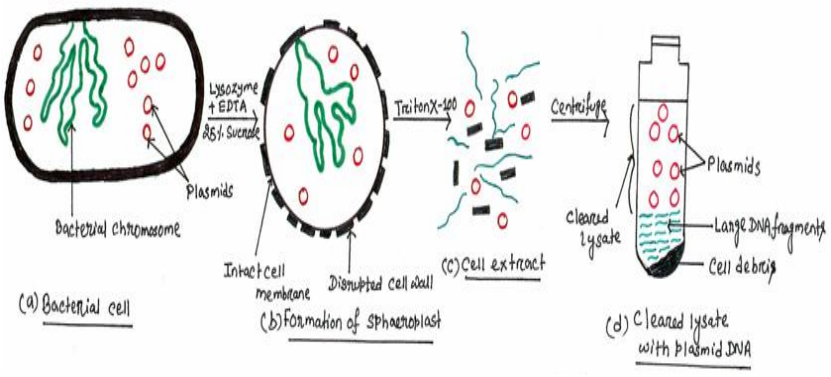
Preparation of DNA from plant and animal cells is different from bacterial cell. Bacterial cell wall degradating enzyme lysozyme has no effect on plant cell wall. whereas most animal cells have no cell wall at all, and can be lysed simply by treating with detergent. Plant tissues consist of large amount of carbohydrates which are not removed by phenol extraction. In this case a detergent called **cetyltrimethylammonium bromide(CTAB)** is used which forms an insoluble complex with nucleic acids. When CTAB is added to a plant cell extract the nucleic acid-CTAB complex precipitates, leaving carbohydrate, protein and other contaminants in the supernatant. The precipitate is then collected by centrifugation and resuspended in 1M Nacl, which causes the complex to breakdown and the RNA removed by ribonuclease treatment.

Plasmid DNA preparation is same as total cell DNA preparation but importantly distinct in one aspect that in plasmid DNA preparation it is always necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA that is also present in the cells.

Plasmids and bacterial DNA differ in conformation(overall spatial configuration of the molecule).Plasmids and bacterial chromosome are circular, but during preparation of the cell extract the chromosome will always be broken to give linear fragments. A method of separating circular from linear molecules will therefore result in pure plasmids.

Size based separation

Bacterial cell disruption is carried out very gently to prevent wholesale breakage. Treatment with EDTA and lysozyme is carried out in the presence of sucrose, which prevents the cell from bursting. Sphaeroplasts(partially wall less cells) are formed that retain an intact cytoplasmic membrane (Figure-5). Cell lysis is induced by adding a non-ionic detergent Triton X-100 which causes minimal breakage of the bacterial DNA, therefore centrifugation will leave a cleared lysate, consisting almost entirely of plasmid DNA. A clear lysate will however, invariably retain some chromosomal DNA. Size fractionation does not sufficiently help to remove contaminants, and therefore alternative ways for it must be considered.



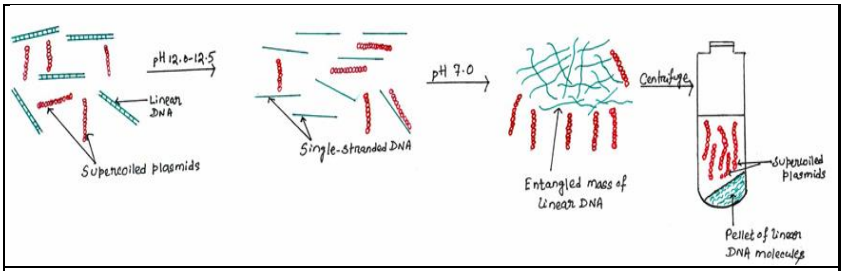
Preparation of a cleared lysate

Conformation based separation

Most plasmids exist in the cell as supercoiled molecules. Supercoiled molecules can be easily separated from non supercoiled DNA. Two different types of conformation based separation are alkaline denaturation and EtBr-CsCl density gradient centrifugation.

Alkaline denaturation

Non-supercoiled DNA is denatured at a narrow pH range. If pH of a cell extract or cleared lysate is increased(12.0-12.5) by addition of NaOH, then the hydrogen bonding in non supercoiled DNA molecules is broken, causing the unwinding of double helix and finally separation of two polypeptide chains . These denatured DNA strands will re-aggregate into a tangle mass by the addition of acid. With the help of centrifugation, the insoluble network can be pelleted, leaving pure plasmid DNA in the supernatant. Under some circumstances (cell lysis by SDS and neutralization with sodium acetate) , most of the proteins and RNA also becomes insoluble and can be removed by centrifugation.

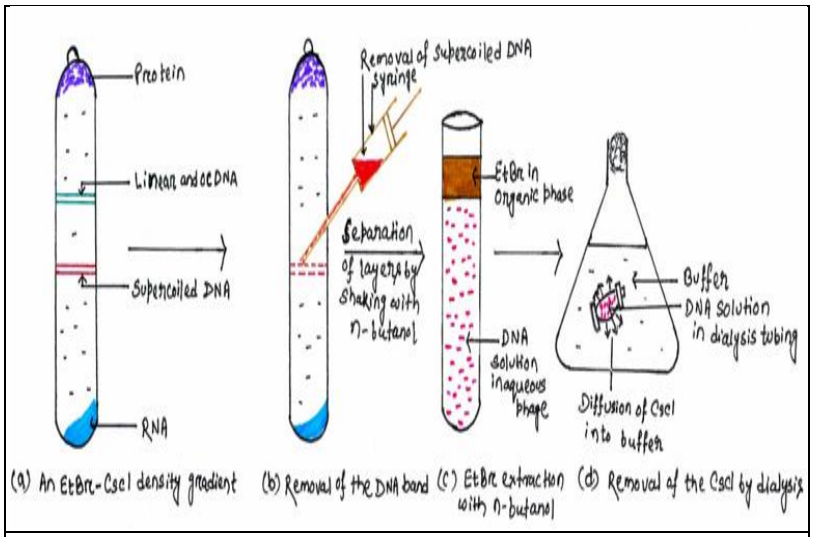


Process of alkaline denaturation

Ethidium bromide-caesium chloride(EtBr-CsCl) density gradient centrifugation

Under high centrifugal force, a solution of cesium chloride (CsCl) molecules will dissociate, and the heavy Cs^+ atoms will be forced towards the outer end of the tube, thus forming a shallow density gradient . DNA molecules placed in this gradient will migrate to the point where they have the same density as the gradient (the **isopycnic point**). Macromolecules present in the CsCl solution when it is centrifuged will form bands at distinct points in the gradient. The gradient is sufficient to separate types of DNA with slight differences in density due to differing (G+C) content, or physical form (e.g., linear versus circular molecules). Density gradient centrifugation in the presence of **ethidium bromide (EtBr)**can be used to separate supercoiled DNA from non-supercoiled molecules . EtBr binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix. Density gradient centrifugation can separate DNA, RNA and protein and is an alternative to phenol extraction and ribonuclease treatment for DNA purification.

EtBr-Cscl density gradient centrifugation is a very efficient method for obtaining pure plasmid DNA. When a cleared lysate is subjected to this procedure, plasmids band at a distinct point, separated from the linear bacterial DNA, with the protein floating at the top of the gradient and RNA pelleted at the bottom. The position of the DNA bands can be seen by shining ultraviolet radiation on the tube, which causes the bound EtBr to fluoresce. The EtBr bound to the plasmid DNA is extracted with n-butanol (Figure-7(c)) and the CsCl removed by dialysis (Figure-7(d)). The resulting plasmid preparation is pure and can be used in cloning.



EtBr-CsCl density gradient centrifugation

Bacteriophage DNA preparation

In bacteriophage DNA preparation, a cell extract is not the starting material, because bacteriophage particles can be obtained in large numbers from the extracellular medium of an infected bacterial culture. When such a culture is centrifuged, the bacteria are pelleted, leaving the phage particles in suspension (Figure-8). The phage particles are then collected from the suspension and their DNA extracted by a single deproteinization step to remove the phage capsid.

DNA Manipulation

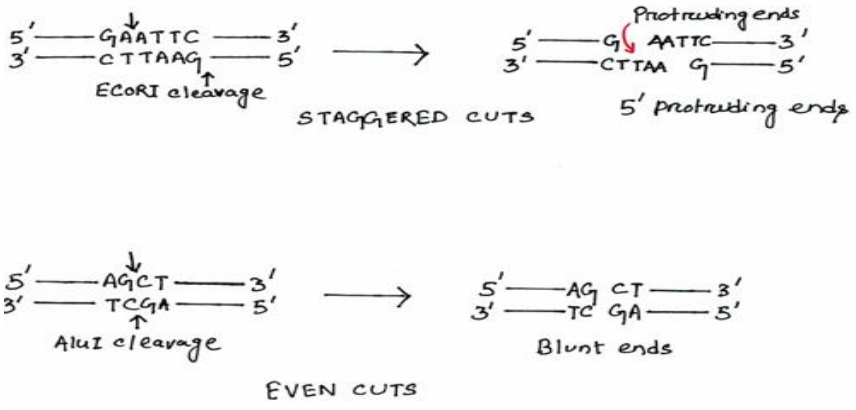
After obtaining pure DNA sample preparation, the next step in gene cloning experiment is construction of the recombinant DNA molecule. Construction of a recombinant DNA molecule is done by cloning the vector as well as the DNA molecule, cutting both at specific points and then joining them together in a controlled manner respectively. Cutting and joining are two examples of DNA manipulative techniques, that underline gene cloning, are carried out by enzymes called restriction endonucleases and ligases respectively.

Recognition sequences

Each type II restriction endonuclease has a specific recognition sequence at which it cuts a DNA molecule. A particular enzyme will cleave DNA at the recognition sequence and nowhere else. Many restriction endonucleases recognize hexanucleotide target sites, but others cut at four, five or even eight nucleotide sequences. Sau3a from (*Staphylococcus aureus* strain 3A) recognizes GATC, and AluI(*Arthrobacter luteus*) cuts at AGCT. Some enzymes with degenerate recognition sequences, cut DNA at any one of a family of related sites. Hinf1(*Haemophilus influenzae* strain Rf), for instance, recognizes GANTC, so cuts at GAATC, GATTC, GAGTC and GACTC.

Blunt ends

The exact nature of the cut produced by a restriction endonuclease is of considerable importance in the design of a gene cloning experiment. The simplest DNA end of a double stranded molecule is called a blunt end. In a blunt-ended molecule both strands terminate in a base pair. Blunt ends are not always desired in biotechnology since when using a DNA ligase to join two molecules into one, the yield is significantly lower with blunt ends. When performing subcloning, it also has the disadvantage of potentially inserting the insert DNA in the opposite orientation desired. On the other hand, blunt ends are always compatible with each other. Many restriction endonucleases make a simple double stranded cut in the middle of the recognition sequence, resulting in a blunt or flush end. *Puv*II and *Alu*II are blunt end cutters .



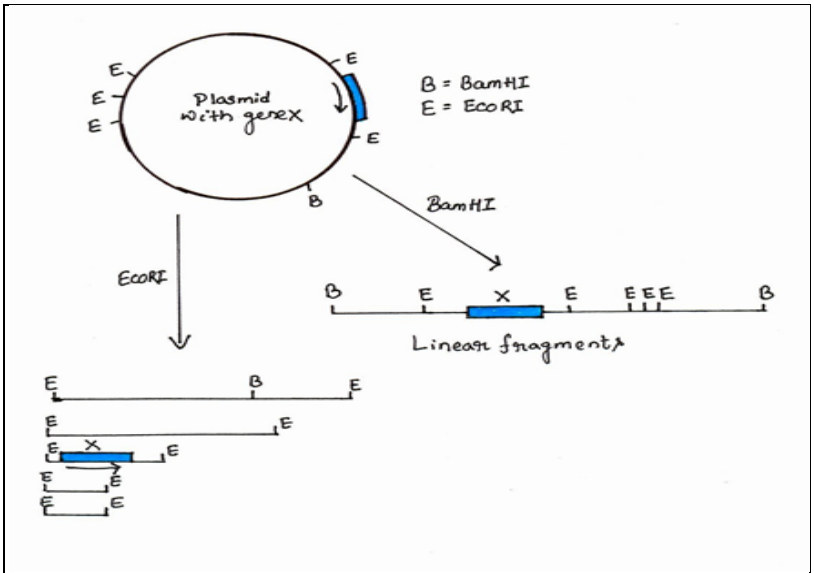
Staggered cuts produced by *Eco*RI cleavage and even cuts produced by *Alu*I cleavage

Sticky ends

Non-blunt ends are created by various overhangs. An overhang is a stretch of unpaired nucleotides in the end of a DNA molecule. These unpaired nucleotides can be in either strand, creating either 3' or 5' overhangs. These overhangs are in most cases palindromic. Longer overhangs are called cohesive ends or sticky ends. Quite a large number of restriction endonucleases cut DNA in a different way. Very often they cut the two DNA strands four base pairs from each other, creating a four-base 5' overhang in one molecule and a complementary 5' overhang in the other. These ends are called cohesive since they are easily joined back together by a ligase. Also, since different restriction endonucleases usually create different overhangs, it is possible to cut a piece of DNA with two different enzymes and then join it with another DNA molecule with ends created by the same enzymes. Since the overhangs have to be complementary in order for the ligase to work, the two molecules can only join in one orientation. Restriction endonucleases with different recognition sequences may produce the same sticky ends. For e.g.,*Bam*HI(recognition sequence GGATCC) and *Bgl*II(recognition sequence AGATCT) both produce GATC sticky ends. The same sticky end is also produced by *Sau*3A, which recognizes only the tetranucleotide GATC .

Restriction digests

Digestion of plasmid or genomic DNA is carried out with restriction enzymes for analytical or preparative purposes, using commercial enzymes and buffer solutions. All restriction enzymes require Mg^{2+} , at a concentration of up to 10 mM, but different enzymes require different pHs, Nacl concentrations or other solution constituents for optimum activity. The buffer solution required for a particular enzyme is supplied with it as a concentrate.



Plasmid digestion by two different restriction enzymes, *Bam*HI and *Eco*RI

A restriction digest will result in a number of DNA fragments, the sizes of which depend on the exact positions of the recognition sequences for the endonuclease in the original molecule. For determining the number and sizes of the DNA fragments after restriction digestion the technique of gel electrophoresis was developed.

Modifications of Cut Ends in DNA

The 3′-ends of DNA strands generated by cleavage always carry a free hydroxyl (—OH) group, while their 5′-ends always bear a phosphate group. Often the ends produced by restriction enzymes have to be modified for further manipulation of the fragments; some of the modifications are summarised below.

1. Removal of the 5'phosphate group of vector DNA by alkaline phosphatase treatment in order to prevent vector circularization during DNA inserts integration.

2. Addition of a phosphate group to a free 5'hydroxyl group by T4 polynucleotide kinase.

3. Removal of the protruding ends by digestion with, say, S1 nuclease; this enzyme digests both 3′- and 5′- protruding ends.

4. Making the single-stranded protruding ends double-stranded by extending the recessed (shorter) strand with, say, Klenow fragment of E. coli DNA polymerase I. This strategy is preferred to SI nuclease digestion for various reasons. (Both the strategies 3 and 4 generate blunt ends, which can be ligated by T_4 polynucleotide ligase.)

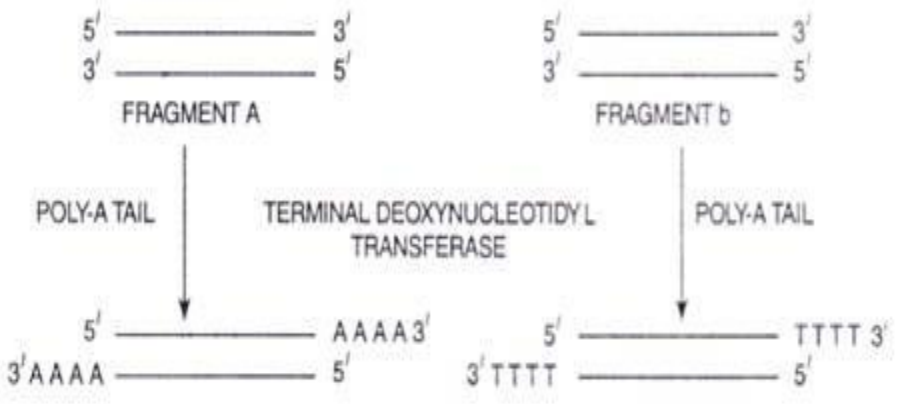
5. Removal of nucleotides from the 5′-ends using A exonuclease.

6. Removal of nucleotides from the 3′-ends using E. coli exonuclease III.

(Both the strategies 5 and 6 convert blunt ends into protruding single-stranded ends of undefined base sequence).

7. Treatment of double-stranded DNAs with exonuclease Ball 1, which simultaneously digests both the strands (from both the ends) of a DNA molecule; this treatment produces shortened DNA fragments with blunt ends.

8. Synthesis of single-stranded tails (protruding ends) at the 3′-ends of blunt-ended fragments by the enzyme terminal deoxynucleotidy1 transferase; this is called tailing. This reaction can be used to generate protruding ends of defined sequence, e.g., poly-A tails on the 3′-ends of the DNA insert and poly-T tails on the 3′-ends of the vector; the protruding ends of the DNA insert and the vector will, therefore, base pair under annealing conditions.

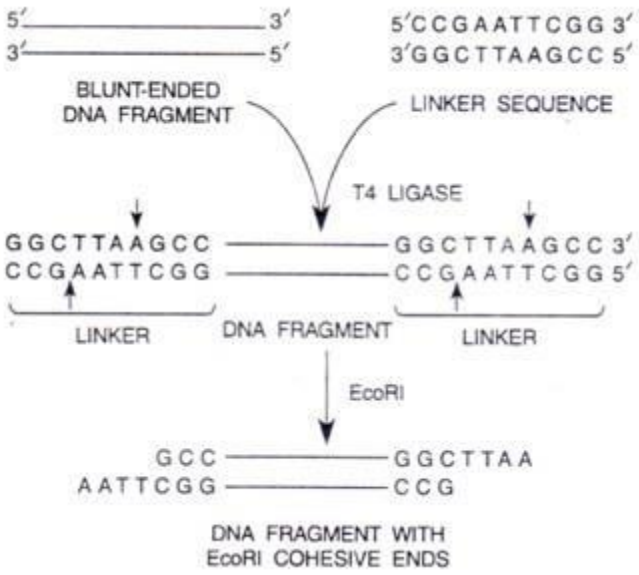


Tailing of blunt-ended DNA fragments A and B using terminal nucleotidyl transferase. Poly-A tail is added to the 3′ ends of fragment A, while poly-T tail is added to the 3′ ends of fragment B so that the protruding ends of fragment A are complementary to those of B.

In practice, the tails on vector and DNA inserts differ in length. As a result, short gaps remain when the ends of DNA insert pair with those of vector. Therefore, Klenow fragment is first used to fill in this gap before DNA ligase is used to ligate them together.

9. Linker and/or adapter molecules can be joined to the cut ends. Linkers are short, chemically synthesized, double-stranded oligonucleotides, which contain within them one or more restriction endonuclease sites, e.g., linker 5'CCGAATTCGG (only one strand of the linker is shown here) contains one EcoRl site. Linkers are fused with blunt-ended DNA fragments; cleavage of the linker with the appropriate restriction enzyme creates suitable cohesive protruding ends .

Linkers have the following two applications: creation of cohesive ends (1) on blunt-ended DNA fragments, and (2) on fragments having unmatched or undefined sequences in their protruding ends. In the latter situation, the DNA fragments are first made blunt-ended using either strategy 3 or 4, following which the selected linkers are ligated to them by T4 ligase.

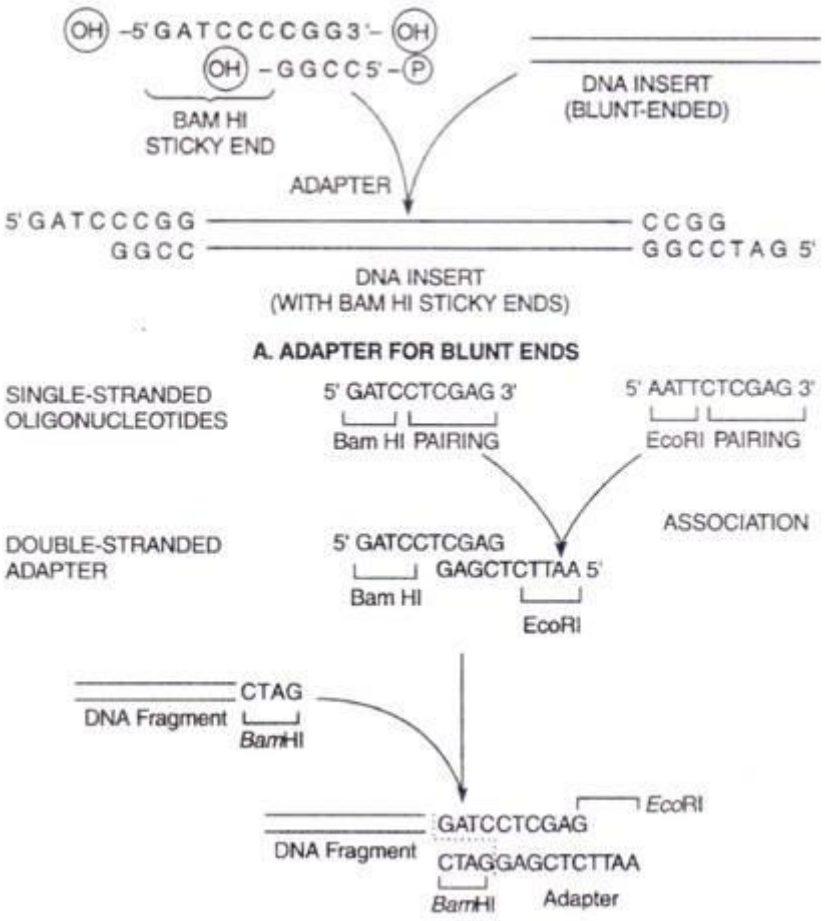


Creation of cohesive ends on blunt-ended DNA fragments. Suitable linkers are ligated to the blunt ends by T4 DNA ligase. The linker is then cleaved with the appropriate restriction enzyme to generate sticky ends.

A potential drawback of linkers is as follows. The linkers must be cleared by the concerned restriction enzyme to generate the sticky ends. In case, one or more recognition sites for this enzyme are present within the DNA insert, it will also be cleaved into two or more pieces. In such a case, adapters are employed.

10. Adapters are short, chemically synthesized DNA double strands, which already have one or both sticky ends . When a blunt end is converted into a sticky end, the adapter has one blunt end and one sticky end corresponding to the concerned restriction enzyme. The blunt end of the adapter is ligated to the blunt ends of the DNA insert, which are now converted into sticky ends.

In order to prevent ligation of a further adapter molecule to a sticky end so produced, the 5′-terminus at the sticky end of the adapter molecules is occupied by a —OH rather than the normal phosphate group. After the adapters have been attached to the DNA insert, their 5′-ends are phosphorylated by polynucleotide kinase so that the DNA insert can be ligated to the vector.



Examples of adapters. A. An adapter used to create BamHl sticky ends at the blunt ends of a DNA insert. B. A conversion adapter produced by associating two oligonucleotides, each having a different recognition sequence at its 5′-end but a complementary sequence for base pairing at the 3′-end.

GENE TRANSFER METHODS

Chemical methods

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compoundsare introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell. An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

- Calcium phosphate
- DEAE dextran
- Cationic Lipid
- Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

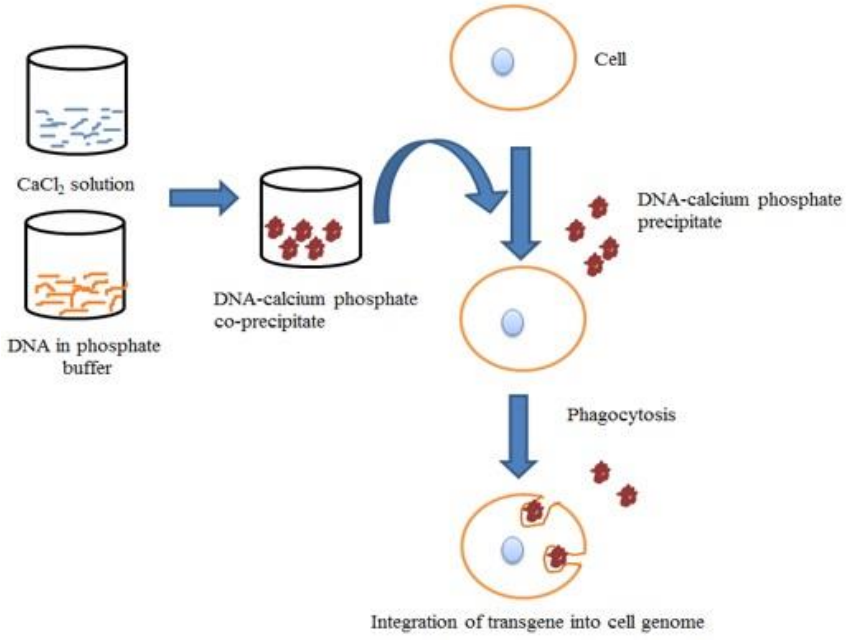
Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction. Inthis method,the precipitates of calcium phosphate and DNAbeing small and insolublecan be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis

and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.



Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable ortransient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.

DEAE-Dextran (Diethylaminoethyl Dextran)mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.
- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.
- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfectionbut not forstable transfection
- Typically produces less than 10% delivery in primary cells.

Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes areartificial phospholipid vesicles used for the deliveryof a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosisthus forming DNA- liposome complex. This complexfuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

- Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

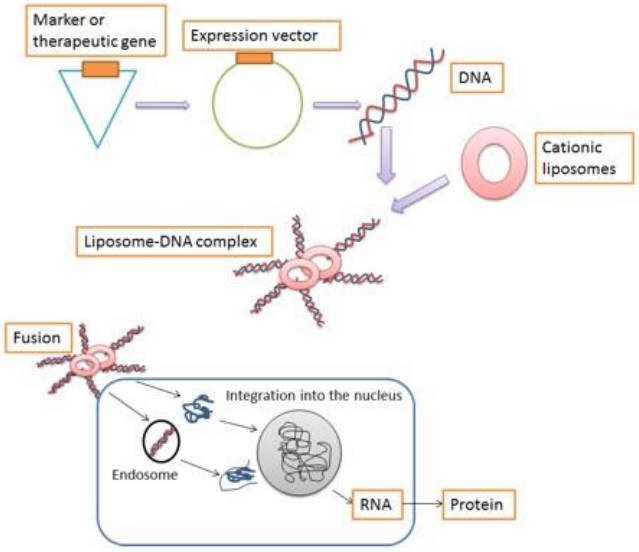
Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum.A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

Negatively charged liposomes

- Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.
- However, formation of lipoplex, a complex between DNA and anionic lipidscan occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which canneutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.
- They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro*gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.



Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.

- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.

- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.
- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bondsin the reducing environment of the cytoplasm.

Chitosan

- Chitosan, a biodegradable polysaccharide is composed of D-glucosamine repeating units and can be used as a non-viral gene carrier.
- It can efficiently bind and protect DNA from nuclease degradation.
- The biocompatibility and low toxicity profile makes it a safe biomedical material for clinical applications.
- Chitosan DNA nanoparticles can transfect several different cell types with relatively low transfection efficiency.
- Modified chitosans such as trimethylated chitosan and chitosan conjugated with deoxycholic acid have been developed to increase the solubility of chitosan at neutral pH which can efficiently transfect COS-1 cells.
- Chitosans with different molecular weights exhibit different DNA binding affinities. The efficiency of transfection is determined by the particle stability which is one of the rate-limiting steps in the overall transfection process.

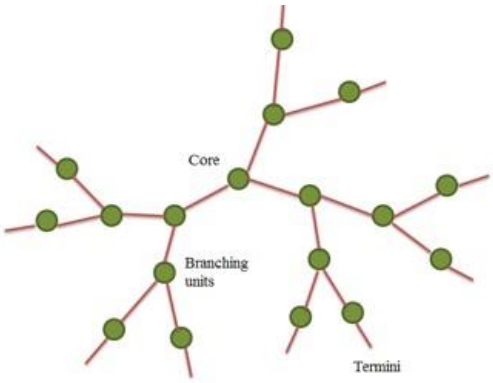
Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.
- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.

- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.

- Once inside the cell, these complexes are then transported to the endosomes where these are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.

- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.



Structure of a dendrimer.

The main gene transfer methods using biological means are as follows:

- Bacterial gene delivery i.e. bactofection.
- Delivery using a viral vector i.e. transduction

Bactofection

It is a method of direct gene transfer using bacteria into the target tissue, organ or organism. The genes located on the plasmids of the transformed bacterial strains are delivered and expressed into the cells. The gene delivery may be intracellular or extracellular. It has a potential to express

various plasmid-encoded heterologous proteins (antigens, toxins, hormones, enzymes etc.) in different cell types.

Uses

- Bactofection can be used for DNA vaccination against various microbial agents such as viruses, fungi, protozoans and other bacteria.
- It can be used in the treatment of several tumours like melanoma, lung carcinoma and colon carcinoma in mice.

Advantages

- Simple, selective and efficient transfection.
- Low synthesis cost and can be administered easily.

Disadvantages

- Unwanted side effects associated with host-bacteria interaction. This can be reduced by using genetically modified bacteria which contain suicide genes that ease the bacterial destruction and thus reduces the risk of clinical infections.

Transduction

This method involves the introduction of genes into host cell's genome using viruses as carriers. The viruses are used in gene transfer due to following features-

- Efficiency of viruses to deliver their nucleic acid into cells
- High level of replication and gene expression.

The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene .

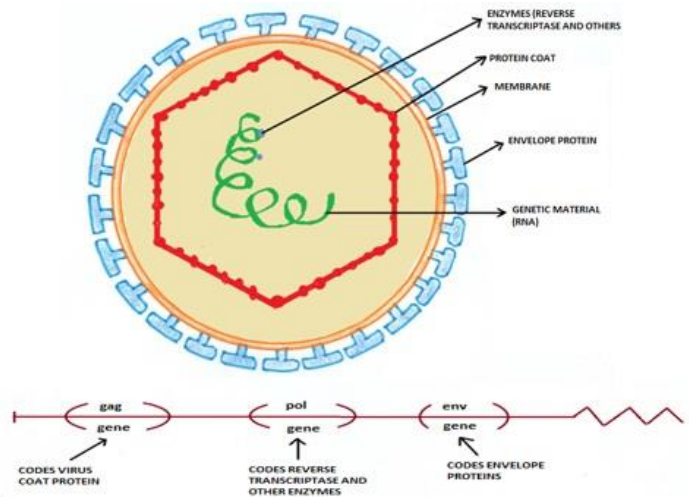
Viral vectors

Various kinds of viruses can be used as viral vectors, viz

1. Adenovirus
2. Adeno- associated virus (AAV)
3. Herpes virus
4. Retrovirus
5. Lentivirus

Vector	**Host cells**	**Entry pathway**	**Vector genome forms**	**Transgene expression**	**Uses**
Retrovirus	Actively dividing cells	Receptor-binding, membrane fusion	Integrated	Long term (years)	SCID, Hyperlipedemia, solid tumors
Lentivirus	Dividing and non-dividing cells	Receptor-binding, membrane fusion	Episomal	Stable	Hematopoetic cells, muscles, neuron, hepatocytes
Adeno virus	Most cells	CAR (Coxsackie and Adenovirus Receptor)-mediated endocytosis endosomal escape	Episomal	Transient (short term for weeks)	CNS, hepatocytes, pancreas
Adeno-associated virus	Most cells	Receptor-mediated endocytosis endosomal escape	Episomal (90%) Integrated (10%)	Medium to long term (year)	lung , muscle, heart, CNS
Herpes virus	Most cells	Endocytotic or membrane fusion	Episomal	Transient	Suitable particularly for nervous system

Retroviruses are RNA viruses that replicate via a ds-DNA intermediate. The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell. The capsid contains two copies of the RNA genome, as well as reverse transcriptase/integrase. After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.



Structure of a Retrovirus vector.

Life cycle of retroviruses

A retrovirus, on binding to a cell surface receptor, enters the cell where it reverse transcribes the RNA into double-stranded DNA. Viral DNA gets integrated into the cell chromosome to form a provirus. Cellular machinery transcribes, processes the RNA and undergoes translation into viral proteins. The viral RNA and proteins are then assembled to form new viruses which are released from the cell by budding (Figure 5-1.4.4(b).).

Retroviral genome

The integrated provirus comprises three genes (*gag*, *pol* and *env*). The *gag* gene encodes a viral structural protein, *pol* encodes the reverse transcriptase and integrase and *env* gene encodes viral envelope proteins. Retrovirus can be classified as oncoviruses, lentiviruses, and spuma-viruses. Oncoviruses are simple whereas lentiviruses and spuma-viruses are complex retroviruses.

Viral genomic RNA is synthesized by transcription from a single promoter located in the left LTR and ends at a poly-A site in the right LTR. Thus, the full-length genomic RNA is shorter than the integrated DNA copy and lacks the duplicated LTR structure. The genomic RNA is capped and polyadenylated, allowing the *gag* gene to be translated. The *pol* gene is also translated by read through, producing a Gag–Pol fusion protein, which is further processed into several distinct polypeptides. Some of the full-length RNA also undergoes splicing, eliminating the *gag* and *pol* genes and allowing the downstream *env* gene to be translated. Two copies of the full-length RNA genome are incorporated into each capsid requiring a specific *cis* -acting packaging site termed ψ. The reverse transcriptase/ integrase are also packaged.

Construction of a retroviral vector and propagation in helper cell

The retroviral construct involved in gene delivery comprises two constructs-

• A vector consisting of all *cis* -acting elements required for gene expression and replication

• A helper cell expressing all the viral proteins (*gag, pol, env*) lacking in vector and support the replication of vector. Helper cell lacks RNA containing packaging signal which is required for formation and release of infectious particles but not for non-infectious viral particles.

When the vector DNA is introduced into a helper cell, helper cell produces the viral proteins which help in the assembly of viral particles containing RNA transcribed from the viral vector. These viral particles on infecting the target cell, reverse transcribe the vector RNA into ds-DNA which gets integrated into the host genome forming a provirus which encodes the gene of interest. Target cells do not express viral proteins and cannot generate infectious viral particles containing the vector RNA and thus cannot infect other target cells .

Advantages

• Insert size up to 8 kb

• Integration into host genome resulting in sustained expression of the vector

• Vector proteins are not expressed in host

Disadvantages

- Infection by retrovirus requires dividing cells
- Low titres (10^6 -10^7)
- Random integration
- Poor *in vivo* delivery

Various physical or mechanical methods areemployed to overcome this and aid ingene transfer as listed below-

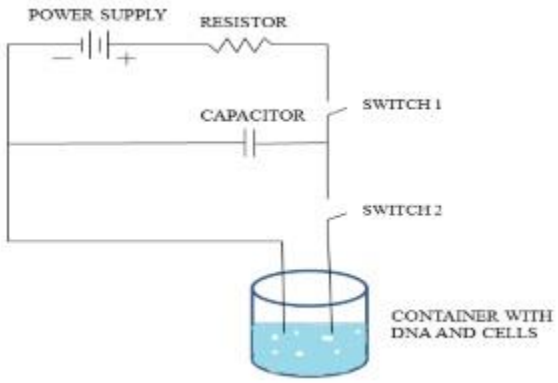
1. Electroporation
2. Microinjection
3. Particle Bombardment
4. Sonoporation
5. Laser induced
6. Bead transfection

Electroporation

- Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane.
- This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells.
- It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells.
- It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.

The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram (Figure 5-3.1.1(a).).



When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.

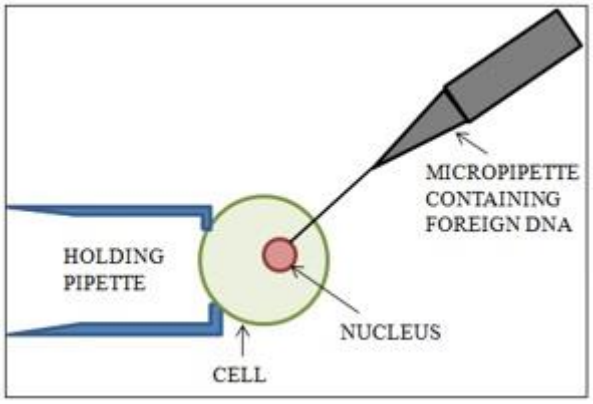
- Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.
- This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
- When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.
- The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

Microinjection

- DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.
- This method is widely used for gene transfection in mammals.
- It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.
- It is used to identify the characteristic function of dominant genes.

Procedure

- The delivery of foreign DNA is done under a powerful microscope using a glass micropipette tip of 0.5 mm diameter.
- Cells to be microinjected are placed in a container. A holding pipette is placed in the field of view of the microscope thatsucks and holds a target cell at the tip. The tip of micropipette is injected through the membrane of the cell to deliver the contents of the needle into the cytoplasm and then the empty needle is taken out.



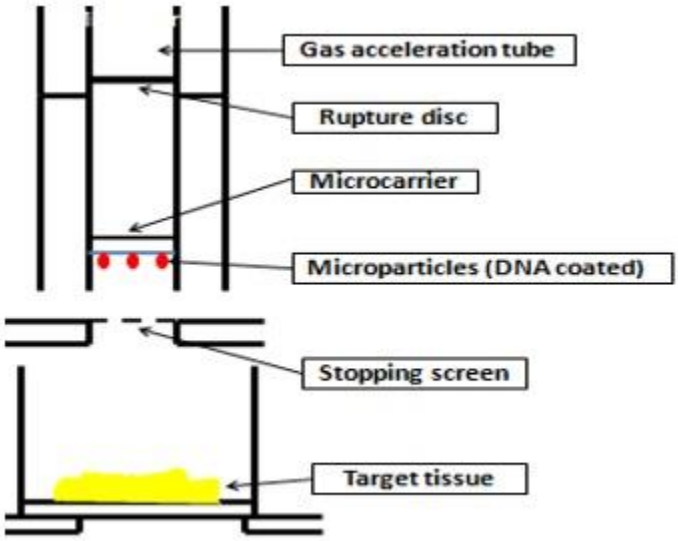
Delivery of DNA into a cell through microinjection.

Particle bombardment

This method is commonly employed for genetic transformation of plants and many organisms.

- This method is applicable for the plants having less regeneration capacity and those which fail to show sufficient response to *Agrobacterium*- mediated gene transfer in rice, corn,wheat, chickpea,sorghum and pigeon-pea.

The biolistic gun employs the principle of conservation of momentumand uses the passage of helium gas through the cylinder with arrange of velocitiesrequired for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.



Sonoporation

- Sonoporation involves the use of ultrasound for temporary permeabilization of the cell membrane allowing the uptake of DNA, drugs or other therapeutic compounds from the extracellular environment.

- This method leaves the compound trapped inside the cell after ultrasound exposure.

- It employs the acoustic cavitation of micro bubbles for enhancing the delivery of large molecules like DNA.The micro bubbles form complex with DNA followed by injection and ultrasound treatment to deliver DNA into the target cells.
- Unlike other methods of transfection, sonoporation combines the capability to enhance gene and drug transfer.

Laser induced transfection

- It involves the use of a brief pulse of focused laser beam.
- In this method, DNA is mixed with the cells present in the culture and then a fine focus of laser beam is passed on the cell surface that forms a small pore sufficient for DNA uptake into the cells. The pore thus formed is transitory and repairs soon.

Bead transfection

- Bead transfection combines the principle of physically producing breaks in the cellular membrane using beads.
- In this method, the adherent cells are incubated for a brief period with glass beads in a solution containing the DNA.

Immunoporation is a recently developed transfection process involving the use of new type of beads, Immunofect^{TM} beads, which can be targeted to make holes in a specific type of cells.

Molecular characterization of transgenes

- **PCR**- Simplest and fastest method. Prone to false positives.
- **Southern Blot**- Confirms insertion of the tDNA into the genomic DNA of the target organism, as well as provides insertion copy number.
- **Northern Blot**- Confirms the presence of RNA transcript accumulation from the transgene of interest.

- **Western Blot**- Confirms presence of the PROTEIN produced from the inserted transgene of interest.
- **qRT-PCR**- Provides a relative expression level for the gene of interest—transcript—like Northern blot.

Gene silencing is a general term used to describe the regulation of gene expression. In particular, this term refers to the ability of a cell to prevent the expression of a certain gene. Gene silencing can occur during either transcription ortranslation and is often used in research. In particular, methods used to silence genes are being increasingly used to produce therapeutics to combat cancer and diseases, such as infectious diseases and neurodegenerative disorders.

Gene silencing is often considered the same as gene knockout. When genes are silenced, their expression is reduced. In contrast, when genes are knocked out, they are completely erased from the organism's genome and, thus, have no expression. Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes, such as RNAi, CRISPR, or siRNA, generally reduce the expression of a gene by at least 70% but do not completely eliminate it. Methods using gene silencing are often considered better than gene knockouts since they allow researchers to study essential genes that are required for the animal models to survive and cannot be removed. In addition, they provide a more complete view on the development of diseases since diseases are generally associated with genes that have a reduced expression.

Antisense oligonucleotides

Antisense oligonucleotides were discovered in 1978 by Paul Zamecnik and Mary Stephenson. Oligonucleotides, which are short nucleic acid fragments, bind to complementary target mRNA molecules when added to the cell. These molecules can be composed of single-stranded DNA or RNA and are generally 13-25 nucleotides long. The antisense oligonucleotides can affect gene expression in two ways: by using an RNase H-dependent mechanism or by using a steric blocking mechanism. RNase H-dependent oligonucleotides cause the target mRNA molecules to be degraded, while steric-blocker oligonucleotides prevent translation of the mRNA molecule. The majority of antisense drugs function through the RNase H-dependent mechanism, in which RNase H hydrolyzes the RNA strand of the

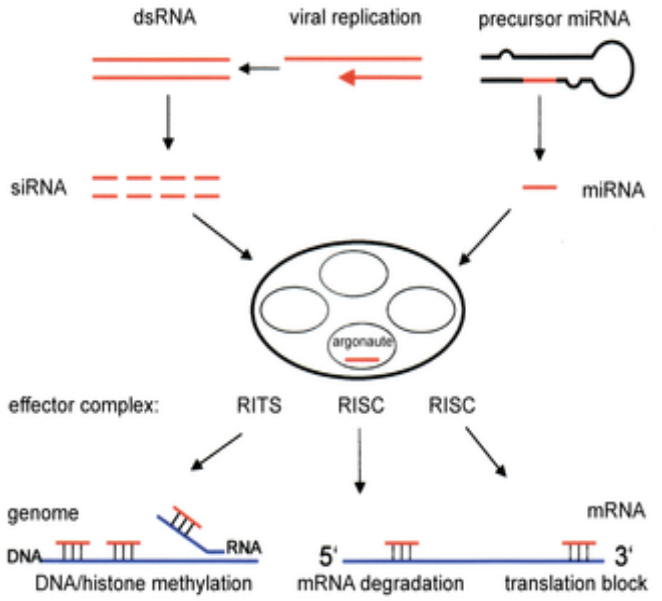
DNA/RNAheteroduplex. This mechanism is thought to be more efficient, resulting in an approximately 80% to 95% decrease in the protein and mRNA expression.

Ribozymes

Ribozymes are catalytic RNA molecules used to inhibit gene expression. These molecules work by cleaving mRNA molecules, essentially silencing the genes that produced them. Sidney Altman and Thomas Cech first discovered catalytic RNA molecules, RNase P and group II intron ribozymes, in 1989 and won the Nobel Prize for their discovery. Several types of ribozyme motifs exist, includinghammerhead, hairpin, hepatitis delta virus, group I, group II, and RNase Pribozymes. Hammerhead, hairpin, and hepatitis delta virus (HDV) ribozyme motifs are generally found in viruses or viroid RNAs. These motifs are able to self-cleave a specific phosphodiester bond on an mRNA molecule. Lower eukaryotes and a few bacteria contain group I and group II ribozymes. These motifs can self-splice by cleaving and joining together phosphodiester bonds. The last ribozyme motif, the RNase P ribozyme, is found in *Escherichia coli* and is known for its ability to cleave the phosphodiester bonds of several tRNA precursors when joined to a protein cofactor.

The general catalytic mechanism used by ribozymes is similar to the mechanism used by protein ribonucleases. These catalytic RNA molecules bind to a specific site and attack the neighboring phosphate in the RNA backbone with their 2’ oxygen, which acts as a nucleophile, resulting in the formation of cleaved products with a 2’3’-cyclic phosphate and a 5’ hydroxyl terminal end. This catalytic mechanism has been increasingly used by scientists to perform sequence-specific cleavage of target mRNA molecules. In addition, attempts are being made to use ribozymes to produce gene silencing therapeutics, which would silence genes that are responsible for causing diseases.

RNA interference



RNA interference (RNAi) is a natural process used by cells to regulate gene expression. It was discovered in 1998 by Andrew Fire and Craig Mello, who won the Nobel Prize for their discovery in 2006. The process to silence genes first begins with the entrance of a double-stranded RNA (dsRNA) molecule into the cell, which triggers the RNAi pathway The double-stranded molecule is then cut into small double-stranded fragments by an enzyme called Dicer. These small fragments, which includesmall interfering RNAs (siRNA) and microRNA (miRNA), are approximately 21-23 nucleotides in length. The fragments integrate into a multi-subunit protein called the RNA-induced silencing complex, which contains Argonaute proteins that are essential components of the RNAi pathway. One strand of the molecule, called the "guide" strand, binds to RISC, while the other strand, known as the "passenger" strand is degraded. The guide or antisense strand of the fragment that remains bound to RISC directs the sequence-specific silencing of the target mRNA molecule. The genes can be silenced by siRNA molecules that cause the endonucleatic cleavage of the target mRNA molecules or by miRNA molecules that suppress translation of the mRNA molecule. With the cleavage or translational repression of the mRNA molecules, the genes that form them are essentially inactive. RNAi is thought to have evolved as a cellular defense mechanism against invaders, such as RNA viruses, or to combat the proliferation of transposons within a cell's DNA. Both RNA viruses and transposons can exist as

double-stranded RNA and lead to the activation of RNAi. Currently, siRNAs are being widely used to suppress specific gene expression and to assess the function of genes

Construction of siRNA vectors

There are several methods for preparing siRNA, such as chemical synthesis, in vitro transcription, siRNA expression vectors, and PCR expression cassettes. Irrespective of which method one uses, the first step in designing a siRNA is to choose the siRNA target site.

General Design Guidelines

If you prefer to design your own siRNAs, you can choose siRNA target sites in a variety of different organisms based on the following guidelines. Corresponding siRNAs can then be chemically synthesized, created by in vitro transcription, or expressed from a vector or PCR product.

1. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide.

Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (1) that siRNAs with 3' overhanging UU dinucleotides are the most effective. This is also compatible with using RNA pol III to transcribe hairpin siRNAs because RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail.

In Elbashir's and subsequent publications, siRNAs with other 3' terminal dinucleotide overhangs have been shown to effectively induce RNAi. If desired, you may modify this target site selection strategy to design siRNAs with other dinucleotide overhangs, but it is recommended that you avoid G residues in the overhang because of the potential for the siRNA to be cleaved by RNase at single-stranded G residues.

2. Select 2-4 target sequences.

Research has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75-95% reduction. Choose target sites from among the sequences identified in Step 1 based on the following guidelines:

- Researchers find that siRNAs with 30-50% GC content are more active than those with a higher G/C content.
- Since a 4-6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of > 4 T's or A's in the target sequence when designing sequences to be expressed from an RNA pol III promoter.
- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16-17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST.

3. Design appropriate controls.

A complete siRNA experiment should include a number of controls to ensure the validity of the data. The editors of Nature Cell Biology have recommended several controls (2). Two of these controls are:

- **A negative control siRNA with the same nucleotide composition as your siRNA but which lacks significant sequence homology to the genome.** To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.
- **Additional siRNA sequences targeting the same mRNA.** Perhaps the best way to ensure confidence in RNAi data is to perform experiments, using a single siRNA at a time, with two

or more different siRNAs targeting the same gene. Prior to these experiments, each siRNA should be tested to ensure that it reduces target gene expression by comparable levels.