IDHAYA COLLEGE FOR WOMEN, KUMBAKONAM

DEPARTMENT OF MICROBIOLOGY

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ISOLATION AND QUANTIFICATION OF DNA

Introduction

DNA isolation of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits used for extraction, and selecting the correct one will save time on kit optimization and extraction procedures

DNA extraction is required for a variety of molecular biology applications. Figure 1 lists the basic steps involved in all DNA extraction methods. Many commercial kits are available to isolate DNA from a variety of biological materials .The sensitivity of polymerase chain reaction (PCR) detection has been shown to be different for various DNA kits. Therefore, selecting the best methodology for your application is crucial.

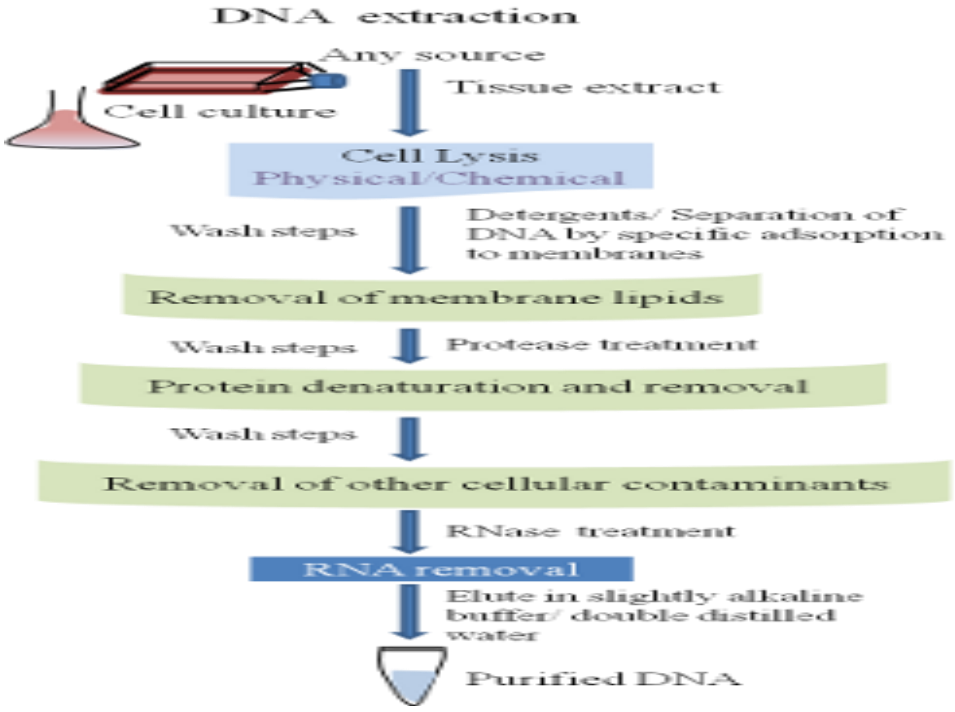

Figure 1. Basic steps involved in all DNA extraction methods.

1. **Sample origin**:

Different kits are used to extract material from specific sources, including human tissues, blood, hair, rodent tissues, leaf tissue, bacteria, yeast, fungi, insect, stool, body fluids, spores, soil, clinical samples (e.g., biopsy samples, fine needle aspirates), forensic samples (e.g., dried blood spots, buccal swabs), and fingerprints .

2. Preparation method:

Sample preparations can be: fresh or previously frozen cell pellets, paraffin-embedded or formalin-fixed tissue sections, frozen tissue sections, ethanol-fixed cells, Oragene®-preserved samples, and samples from forensic sources which might contain very limited material

3. Intended use:

The quality and purity of the DNA provided by the kit should be suitable for the intended downstream application, which could be sequencing, fingerprinting, PCR, quantitative PCR (qPCR), Southern blotting, random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) applications, restriction endonuclease digestion, or the preparation of shotgun libraries.

4. Humic content

If the sample mic content such as compost, sediment and manure, a kit/method that removes humic substances should be used, as they can inhibit downstream applications like PCR.

5. Sample quantity

: The kit to be used depends on the size of the sample being analysed. For example, the number of cultured mammalian cells (10^5-10^7) and bacterial cells (10^6-10^{11}), the weight of human tissue, plant tissue or soil,, the volume of blood, or even trace DNA samples from a crime scene.

6. Yield:

The desired or expected amount of DNA to be purified from the sample. This is dependent upon the sample as well as the downstream applications

7. Simplicity:

kit operation depends on the experience of the user, and the degree of control desired over each stage of the sample processing.

The basic criteria that any method of DNA isolation from any sample type should meet include: (1) efficient extraction of DNA from the sample , (2) production of a sufficient amount of DNA for use in downstream processes, (3) successful removal of contaminants, (4) isolation of high quality and high purity DNA.

Assays to assess sample purity and quality control

Ultraviolet absorbance can be used to assess the purity of the extracted DNA. For a pure DNA sample, the ratio of absorbance at 260 nm and absorbance at 280 nm (A260/A280) is 1.8. A ratio of < 1.8 indicates the sample is contaminated with protein or an organic solvent such as phenol, often used during extraction processes. The quantification of double-stranded DNA can also be assessed by the Qubit assay, which relies on the principle of DNA-selective fluorescent dyes although it may underestimate in DNA extracted after RNA extraction with Trizol DNA quality can be assessed by visualization on agarose gels.

Common DNA extraction methods

Different extraction methods result in different yields and purity of DNA. Some of the extraction methods have been systematically evaluated for specific applications such as soil and sediment samples human microbiome , and fecal samples

Organic Extraction

In this conventional, widely used method, cells are lysed and cell debris is usually removed by centrifugation. Then, proteins are denatured/digested using a protease, and precipitated with organic solvents such as phenol, or 1:1 mixture of phenol and chloroform. The protein precipitate is removed following separation by centrifugation. Purified DNA is usually recovered by precipitation using ethanol or isopropanol.

Silica-based technology

Silica-based technologies are widely employed in current kits. DNA adsorbs specifically to silica membranes/beads/particles in the presence of certain salts and at a defined pH .The cellular contaminants are removed by wash steps. DNA is eluted in a low salt buffer or elution buffer. Chaotropic salts are included in the kit buffers to aid in protein denaturation and extraction of DNA.

Magnetic separation

Magnetic separation is based on DNA reversibly binding to a magnetic solid surface/bead/particles that have been coated with a DNA binding antibody, or a functional group that interacts specifically with DNA After DNA binding, beads are separated from other contaminating cellular components, washed, and the purified DNA is eluted using ethanol extraction .

Anion exchange technology

DNA extraction by anion exchange chromatography is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using a low or medium salt buffer, and purified DNA is eluted using a high salt buffer [This technology is most commonly employed in plasmid isolation kits.

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RNA ISOLATION AND QUNATIFICATION

Introduction

DNA extraction methods cannot be directly applied to RNA as RNA is structurally very different from DNA. RNA is single-stranded, while DNA is mostly double-stranded. It is often difficult to isolate intact RNA. RNases, a group of enzymes that degrade RNA molecules, are abundant in the environment, including on hands and on surfaces and it is difficult to remove/destroy RNases completely. RNA isolation therefore requires cautious handling of samples and good aseptic

techniques. It is important to use only RNase-free solutions during the extraction, as well as RNase-free pipet tips and glassware.

RNA storage and stability

RNAlater solutions from Thermo Fisher and QIAGEN are used by many researchers during RNA isolation, both for stabilizing cellular RNA in tissue samples, and for stabilizing final purified RNA. Ambient storage of tissue samples in RNAlater preserves RNA integrity similarly as storage at low temperature RNAlater is based on the inhibition of RNAses by sulfate salts such as ammonium sulfate at specific pH .RNA quality can be checked using agarose gel electrophoresis. For long-term storage, RNA should be kept at -80°C in single-use aliquots, while the ethanol precipitate of RNA can be stored at -20°C.

Determining RNA quality and quantity

- RNA quality can be determined by examining the ratio of absorption at 260 nm and 280 nm with UV spectrophotometry.
- . RNA can be quantified by measuring the absorption at 260 nm, where 1 absorbance unit is equal to 40 μg/ml, at a pH of about 7.5.
- In addition, the quality of total RNA preparations should be examined through electrophoresis, where both 18S and 28S RNA bands should be very prominent, with the 28S RNA band about twice as intense as the 18S band.

RNA Extraction Methods

The major categories of RNA extraction methods are listed in Table 1. Table 2 lists the basic steps involved in the RNA extraction using organic solvents/chaotropic agents. Other methods utilize some of the steps or similar steps.

Method	**Typical kit**
Guanidinium-acid-phenol Extraction	TRIzol and TRI reagent
Silica technology, glass fiber filters	RNeasy and its variations
Density gradient centrifugation using cesium chloride or cesium trifluoroacetate	
Magnetic bead technology	Dynabeads mRNA DIRECT Micro

Lithium chloride and urea isolation	
Oligo(dt)-cellulose column chromatography	
Non-column poly (A)+ purification/isolation	

Table 1. RNA extraction methods and typical kits.

Table 2. Basic steps involved in the RNA extraction using organic solvents/chaotropic agents

Steps	Characteristic feature
cell lysis and dissolution	Cell lysis can be achieved using buffers or reagents containing chaotropic agents such as guanidinium isothiocyanate, guanidinium chloride, sodium dodecyl sulphate (SDS), sarcosyl, urea, phenol or chloroform. TRIzol or RNAlater or Qiazol [10] can be used to maintain RNA integrity during lysis.
Denaturation of DNA and proteins	DNase can be used to degrade DNA, while proteinase K can be added to digest proteins. Alternatively, repeated organic extraction using phenol and chloroform, or dissolving the sample in buffers containing guanidinium salts, can also be used to remove proteins.
Denaturation and inactivation of RNases	This can be achieved using any of the chaotropic agents mentioned above, such as phenol and chloroform.
Removal/separation of cellular components	RNA can be separated from other cellular components by adding chloroform and centrifuging the solution. This separates the solution into two phases: organic and aqueous phases. The aqueous phase contains RNA.

Precipitation	RNA is often recovered from the aqueous phase using isopropyl alcohol. RNA can also be selectively precipitated from DNA through the use of ammonium acetate. Alternatively, lithium chloride can be used to selectively precipitate RNA from DNA as well as proteins

RNA Isolation and Kits

Two most commonly used kits are RNeasy kits from QIAGEN and TRIzol from Thermo Fisher. RNase-free DNase is used specifically for RNA purification by removing all contaminating genomic DNA. Although commercial kits are most often used, a few studies still use basic organic solvent extraction methods. For example, Meertens L et al purified viral RNA produced through the mMESSAGE mMACHINE SP6 or T7 Transcription Kit from Thermo Fischer Scientific with phenol:chloroform extraction and isopropanol precipitation . .

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Plasmid DNA Purification

Introduction to plasmid purification

Extraction of macromolecules such as DNA, RNA, and protein is common in molecular biology research. The process of extraction and purification of nucleic acids has evolved from being a complex and labor-intensive procedure. Current nucleic acid purification methods provide high sample outputs, purity, and scalability of biomolecules with minimum cross-contamination. Automated systems designed for medium-to-large laboratories are also commonly used.

Preparing nucleic acids for plasmid purification

Preparation of nucleic acids begins with the process of sample collection (bacteria, animal tissue and cells, or plant tissue). Samples must be collected and handled properly to achieve high-quality nucleic acid regardless of the method used for DNA preparation. The path from sample collection

to nucleic acid purification may involve sample collection, transport, archiving, storage, and purification of nucleic acids as a workflow (Figure 1).

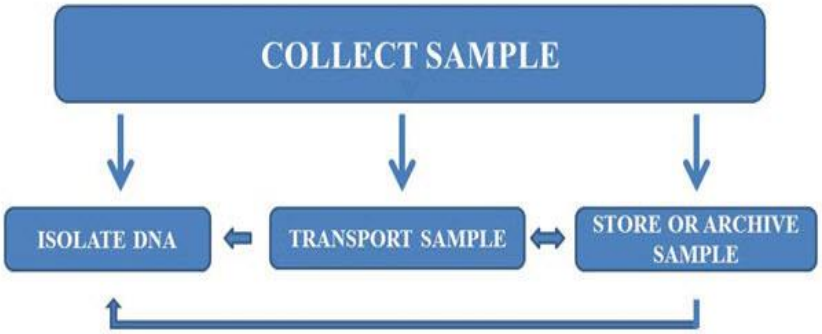

Figure 1. Workflow for sample collection, transport, archiving, and DNA purification.

Nucleic acid isolation

Isolation of DNA involves the lysis of cell membranes, removal of histone proteins, RNA, and lipids, and purification. Various methods available for extraction and purification are detailed in Table 1.

Table 1. Overview of methods for nucleic acid isolation.

Methods	Description of Methods
Conventional Method	
Alkaline Extraction Method (PLED35)	• Alkaline denaturation of high molecular weight chromosomal DNA • Used to isolate plasmid DNA • Recovery process post centrifugation
Cesium Chloride (CsCl) Density Gradient Centrifugation	• Preparative density-gradient ultracentrifugation of DNA • Used to isolate plasmid DNA

	• Purified nucleic acid must be re-precipitated with alcohol
Oligp(dT)-Cellulose Chromatography	• Purification of RNA • Large quantities of RNA from mammalian cells
Guanidinium Thiocyanate-Phenol-Chloroform Extraction (30911)	• A single step liquid – liquid extraction used for the extraction of RNA from cultured cells and most animal tissues • Generates high yield in four hours • Follow-up process done by precipitation with isopropanol
Solid-phase Nucleic Acid Extraction	
Silica Matrices	• For selective DNA binding and DNA purification • Purified DNA molecules can be eluted under low ionic strength (pH ≥7) later by using Tris-EDTA buffer or distilled water
Glass Particles	• Used to separate nucleic acid from other substances in the presence of chaotropic salts solution.
Diatomaceous earth (kieselguhr or diatomite)	• Useful for the purification of plasmid and other DNA by immobilizing DNA onto particles in the presence of a chaotropic agent

	• DNA is eluted with a low salt buffer or in distilled water
Anion-Exchange Material	• Interaction between positively charged diethylaminoethyl cellulose (DEAE) groups on the resin's surface and negatively charged phosphates of the DNA backbone

Plasmid DNA

Plasmids are small, circular, double-stranded DNA used in molecular biology for manipulating and decoding genetic information. They have evolved as key components in any cloning and biotechnological techniques as they are easier to manipulate.

Various methods have been developed for plasmid DNA purification. We are committed to bringing you Greener Alternative Products, which adhere to one or more of The 12 Principles of Greener Chemistry. The GenElute products have Inherently Safer Chemistry, compared to the standard use of phenol and chloroform to perform DNA extractions. Plasmid Miniprep Kit is a simple, rapid, and cost-effective method for isolating plasmid DNA from *E. coli* cultures. The kit combines silica-based membrane technology and the convenience of a spin column format, and recovers up to 20 mg of high copy plasmid DNA per ml of overnight culture. The following are the main steps (Figure 2) in the isolation and purification of plasmid DNA using Plasmid Miniprep Kit.

1. Bacterial cells are harvested via centrifugation, subjected to a modified alkaline-SDS lysis procedure, and the DNA adsorbed onto silica in the presence of high salts.
2. Contaminants are then removed by a simple wash step.
3. The bound DNA is eluted in water or Tris-EDTA buffer. The recovered plasmid DNA is predominately in its supercoiled form.
4. The DNA is ready for use in applications such as restriction enzyme digestion, cloning, PCR, transformation, transcription, conventional and automated sequencing

Some of our plasmid DNA purification kits are tabulated in table 2. Detailed explanation of the different plasmid DNA purification methods (PDF).

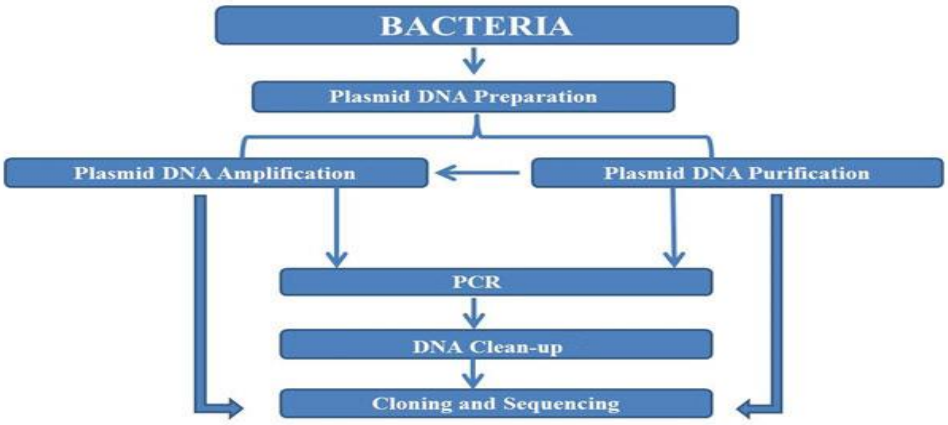

Figure 2. Workflow for nucleic acid preparation and purification from plasmids. PCR DNA clean-up: product numbers NA1020 and PCR9604

Table 2. Plasmid DNA purification kits

Product No.	Product Name	Features
PLN10 PLN70 PLN350	GenElute™ Plasmid Miniprep Kit	• purify up to 20 µg of plasmid DNA per ml of culture • purified plasmid DNA in less than 30 minutes for up to 24 preps • faster than gravity-flow anion exchange columns • no detectable genomic DNA or RNA contamination

		• no phenol/chloroform extraction or alcohol precipitation required contains additional wash buffer for use with enda+ *E. coli* bacterial strains (e.g., hb101 jm101, bl21)
NA0500	GenElute™ HP Plasmid Megaprep Kit	• sophisticated – newest technology ensures improved performance • plentiful – yields 5 mg of high-quality, endotoxin-free (≤0.1eu/µg) plasmid DNA in 90 minutes or less convenient – vacuum format with no ethanol precipitation required
NA0500	GenElute™ HP Plasmid Megaprep Kit	• sophisticated – newest technology ensures improved performance • plentiful – yields 5 mg of high-quality, endotoxin-free (≤0.1eu/µg) plasmid

		DNA in 90 minutes or less convenient – vacuum format with no ethanol precipitation required

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Polymerase chain reaction (PCR)

Introduction

- Polymerase chain reaction (PCR) is a method widely used in molecular biology to rapidly make millions to billions of copies of a specific DNA sample.
- PCR was invented in 1983 by the American biochemist Kary Mullis
- It is fundamental to much of genetic testing including analysis of ancient samples of DNA and identification of infectious agents.
- PCR is now a common and often indispensable technique used in medical laboratory and clinical laboratory research for a broad variety of applications including biomedical research and criminal forensics.

Basic steps

A basic PCR set-up requires several components and reagents including:

- A *DNA template* that contains the DNA target region to amplify

- A *DNA polymerase*; an enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process
- Two DNA *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers there is no double-stranded initiation site at which the polymerase can bind); specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers
- *Deoxynucleoside triphosphates*, or dNTPs (sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase synthesizes a new DNA strand

  A *buffer solution* providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

  Bivalent cations, typically magnesium (Mg) or manganese (Mn) ions; Mg^{2+} is the most common, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis[10]; and *monovalent cations*, typically potassium (K) ions.

Procedure

PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps . The cycling is often preceded by a single temperature step at a very high temperature (>90 °C (194 °F)), and followed by one hold at the end for final product .

PCR methods are as follows:

- *Initialization*: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–

205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

- *Denaturation*: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

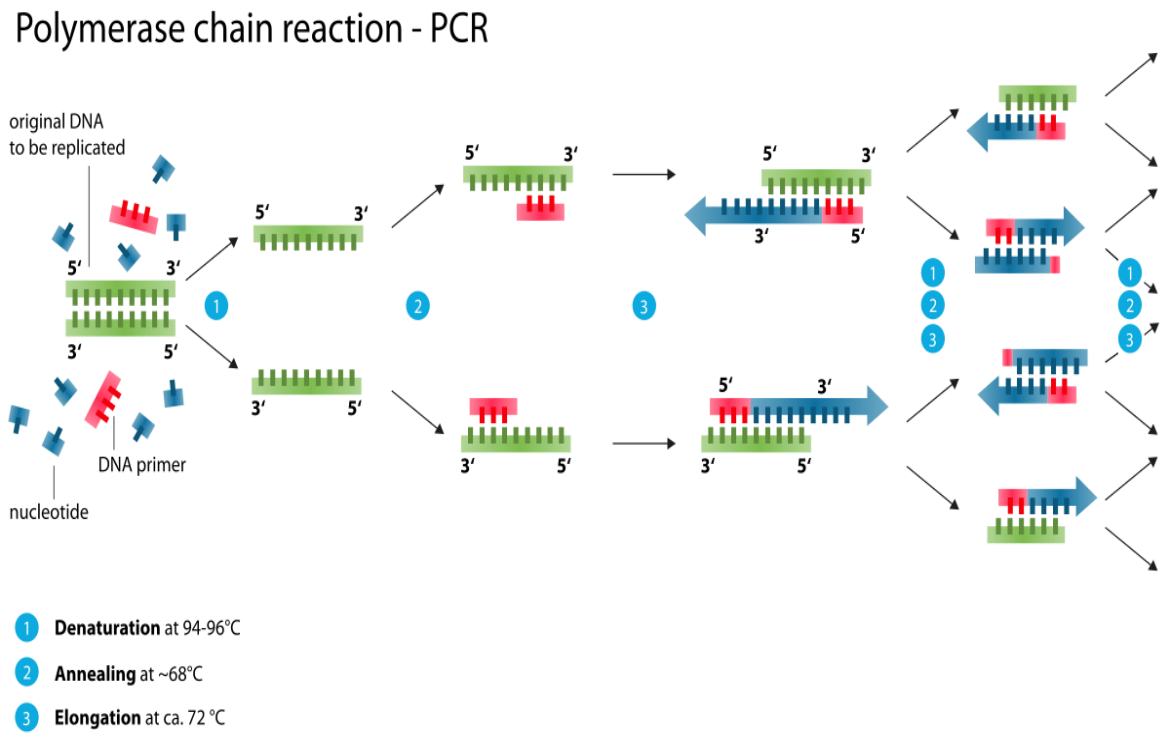
- *Annealing*: In the next step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

    - *Extension/elongation*: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of Taq (Thermus aquaticus) polymerase is approximately 75–80 °C (167–176 °F), though a temperature of 72 °C (162 °F) is commonly used with this enzyme.
    - In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.
    - The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where *n* is the number of cycles. Thus, a reaction set for 30 cycles results in 2^{30}, or 1,073,741,824, copies of the original double-stranded DNA target region.

        - *Final elongation*: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for

5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

- *Final hold*: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.



Application of PCR:

1. Selective DNA isolation
2. Amplification and quantification of DNA
3. Medical and diagnostic applications
4. Infectious disease applications
5. Forensic applications

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Real-time polymerase chain reaction

Introduction

A real-time polymerase chain reaction (real-time PCR), also known as quantitative polymerase chain reaction (qPCR), is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules) (semi-quantitative real-time PCR).

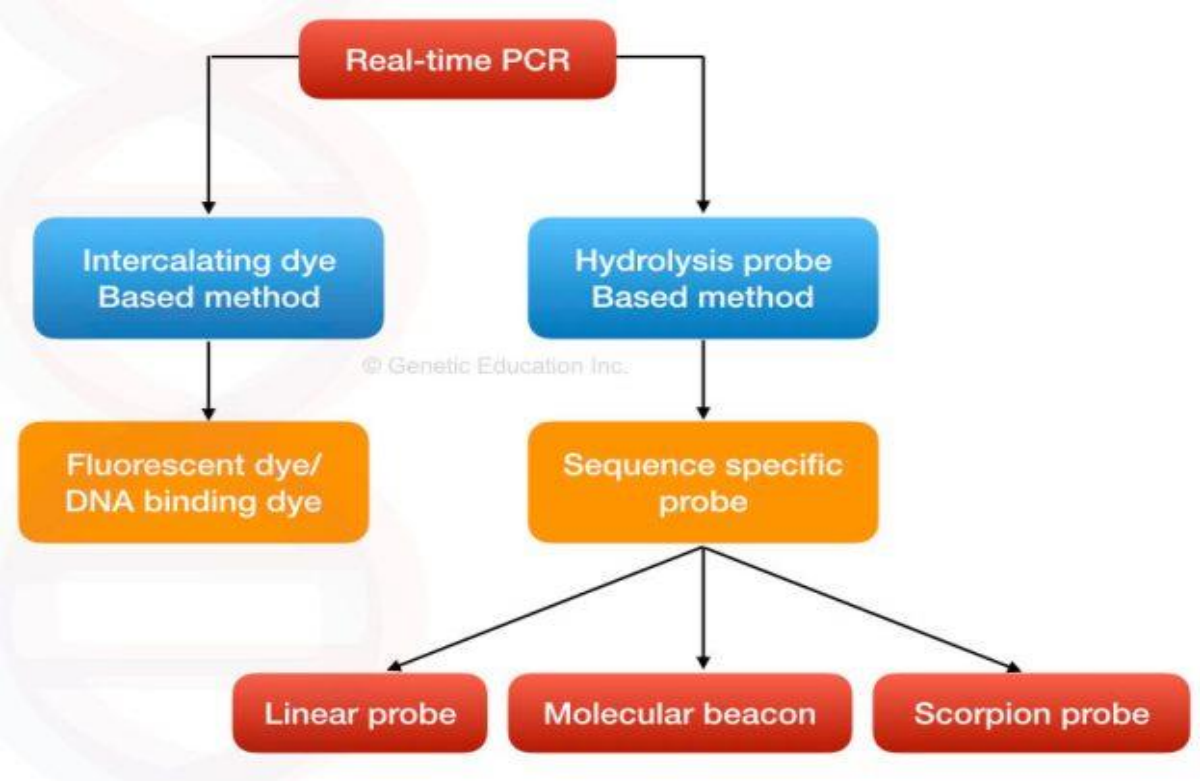
PRINCIPLE

Real-time PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase.

The PCR process generally consists of a series of temperature changes that are repeated 25–50 times. These cycles normally consist of three stages: the first, at around 95 °C, allows the separation of the nucleic acid's double chain; the second, at a temperature of around 50–60 °C, allows the binding of the primers with the DNA template; the third, at between 68–72 °C, facilitates the polymerization carried out by the DNA polymerase. Due to the small size of the fragments the last step is usually omitted in this type of PCR as the enzyme is able to increase their number during the change between the alignment stage and the denaturing stage.

In addition, in four-step PCR the fluorescence is measured during short temperature phases lasting only a few seconds in each cycle, with a temperature of, for example, 80 °C, in order to reduce the signal caused by the presence of primer dimers when a non-specific dye is used. The temperatures and the timings used for each cycle depend on a wide variety of parameters, such as:

the enzyme used to synthesize the DNA, the concentration of divalent ions and deoxyribonucleotides (dNTPs) in the reaction and the bonding temperature of the primers.



Real-time PCR steps

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

1. Denaturation:

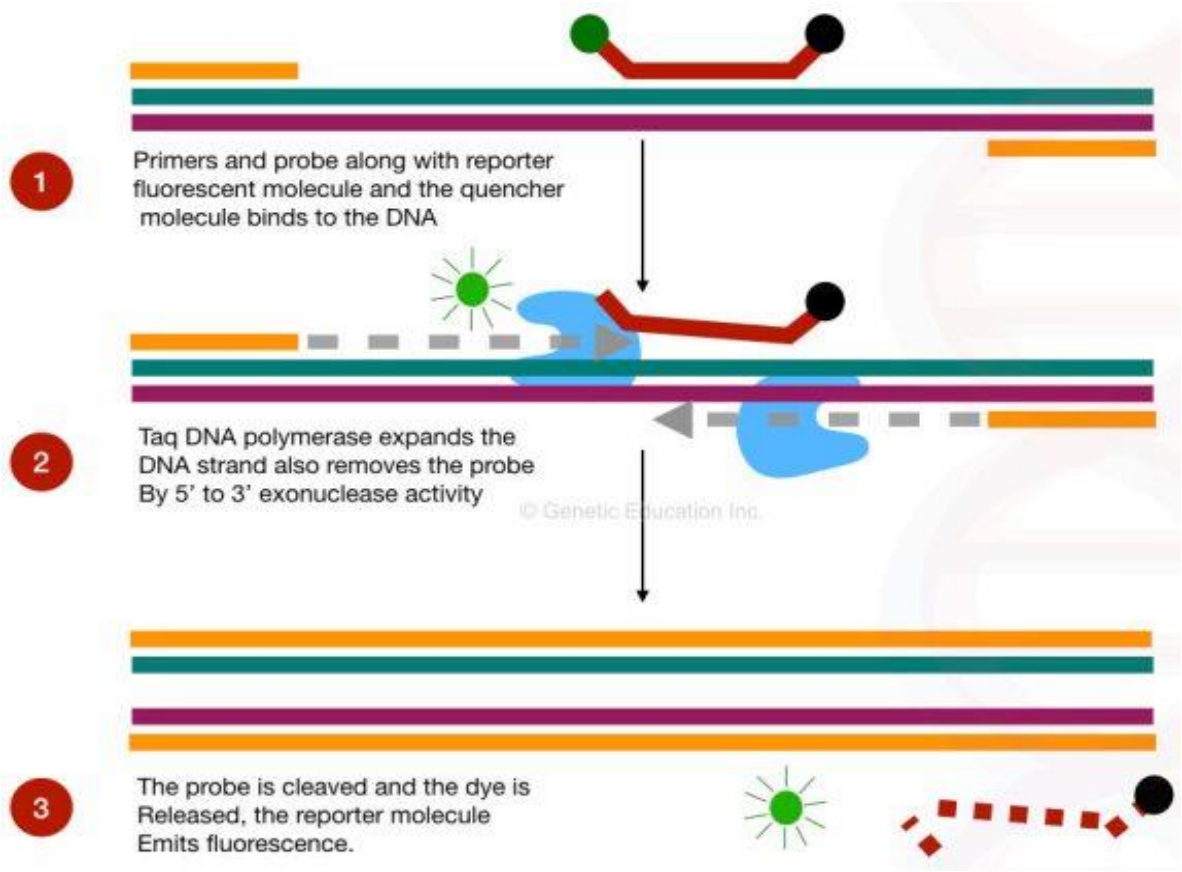
High temperature incubation is used to “melt” double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

2. Annealing:

During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

3. Extension:

At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the



Application of RT-PCR

- ❖ Diagnostic uses
- ❖ Microbiological uses
- ❖ Detection of phytopathogens
- ❖ Detection of genetically modified organisms
- ❖ Clinical quantification and genotyping

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Reverse transcriptase polymerase chain reaction (RT-PCR)

Introduction

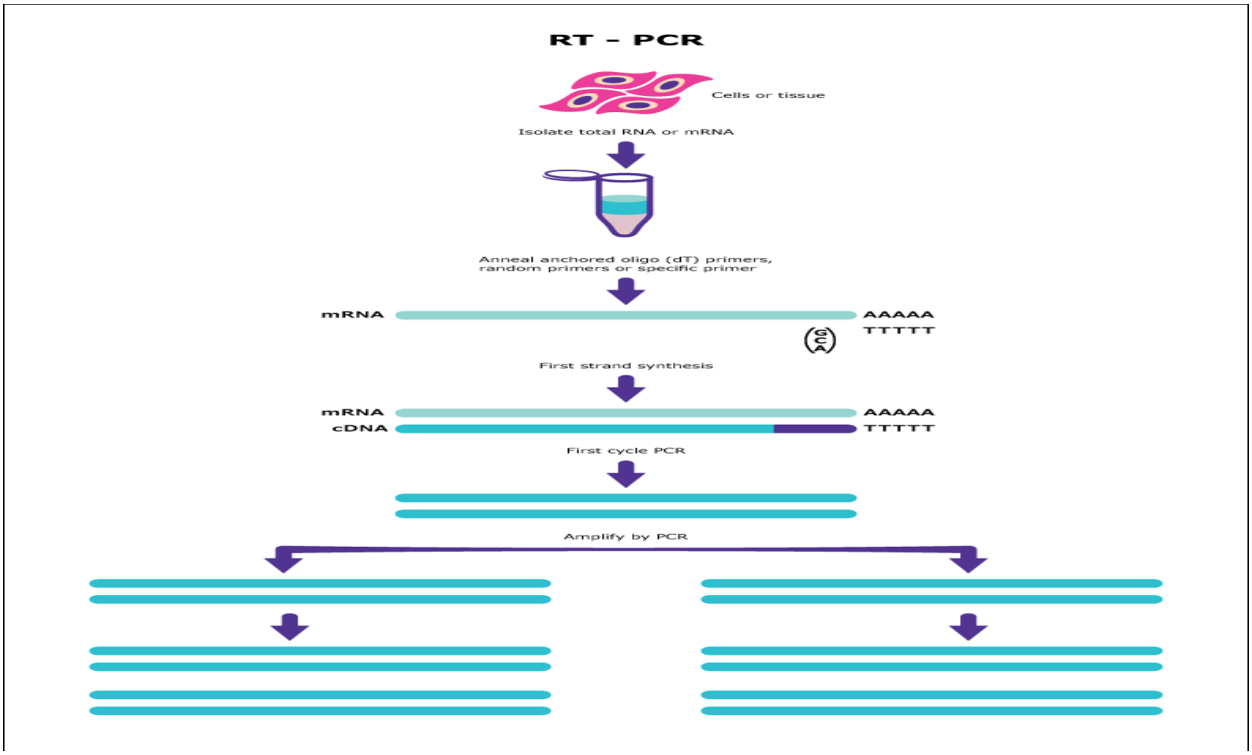
Reverse transcription polymerase chain reaction (RT-PCR) is a variation of standard PCR that involves the amplification of specific mRNA obtained from small samples. It eliminates the need for the tedious mRNA purification process required for conventional cloning techniques.

Description of RT-PCR:

In RT-PCR, reverse transcriptase and an RNA sample are used in addition to the standard PCR reagents. The reaction mixture is heated to 37 ˚C, which enables the production of cDNA from the RNA sample by reverse transcription. This cDNA anneals to one of the primers leading to first-strand synthesis. Standard PCR proceeds and dsDNA is produced.

The primers used must match the project. If amplification of all of a cell's mRNA is required, oligo (dT) primers are sufficient because they anneal to the poly(A) tails. If a specific mRNA is to be amplified, a coding region-specific primer can be used.

RT-PCR is a common virology diagnostic method and is frequently combined with quantitative real-time PCR (qPCR), which is widely used to quantify RNA transcript levels in cells and tissues.[1] The combination of real-time PCR (qPCR) and reverse transcription PCR is known as quantitative RT-PCR or qRT-PCR.



RT-PCR Kits

All the reagents needed to perform reverse transcription followed by PCR analysis of the synthesized cDNA are included in our RT-PCR kits. Choose the Enhanced AMV kit for difficult experiments with transcripts in low abundance or with complex secondary structures. The Titan One-Tube options offer convenient, high-fidelity reverse transcription and amplification.

Product No.	Product Name	Features
HSRT100	Enhanced Avian HS RT-PCR Kit	• Flexible kit for one or two step RT-PCR • Highly purified eAMV-RT — transcribe difficult

		secondary structure at high temperature
11939823001	Titan One-Tube RT-PCR Kit	• Reduces the error rate in PCR due to the proofreading capability • Threefold higher fidelity than standard Taq
11855476001	Titan One-Tube RT-PCR System	• Reverse Transcriptase AMV for first strand cDNA synthesis • Expand High Fidelity Enzyme with proofreading for cDNA amplification
TOSRTRO	Transcriptor One-Step RT-PCR Kit	• Uses hot-start one-step RT-PCR technology to ensure sensitive and robust reverse transcription. • Provides all the components required for one-step RT-PCR.
KK4752	KAPA PROBE FAST One-Step	• Designed for high-throughput, fast-cycling, one-step RNA quantification. • Suitable for use with all fluorogenic probe-based technologies, including hybridization probes (e.g., FRET), hydrolysis probes (e.g., TaqMan®) and

		displacement probes (e.g., molecular beacons).

cDNA Synthesis Kits & Mixes

To meet your reverse transcription needs, choose from various types of enzymes and several packaging options. All of these products include the reagents necessary for first-strand synthesis and are available as kits with separate reagents for optimization or as complete mixes for quick and convenient First-Strand synthesis.

Product No.	Product Name	Features
STR1	Enhanced Avian First-Strand Synthesis Kit	• Highly purified eAMV-RT — transcribe difficult secondary structure at high temperature • Detects low abundance mRNA.
GE27-9261-01	First-strand cDNA Synthesis Kit	• Pre-assembled bulk reaction mixes to save time and preven contamination • Includes two primers for mRNA with or without Poly(A) tails
11483188001	First Strand cDNA Synthesis Kit for RT-PCR	• Includes AMV RT for higher thermostability vs. M-MuLV RT • Includes Rnase Inhibitor and dNTP Mix for PCR

RDRT	ReadyScript™ cDNA Synthesis Mix	• Sensitive and easy-to-use solution for two-step RT-PCR • ReadyScript Enzyme is an RNAse H(+) modified M-MuLV RT
GE27-9263-01	Ready-to-go™ T-Primed First-Strand Kit	• 50 single-dose, ambient-temperature-stable, first-strand synthesis reactions • Contains NotI-(dT)18 Primer, Cloned FPLCpure M-MuLV RT, RNase Inhibitor, and dNTPs
11117831001	cDNA Synthesis Kit	• Optimized one-tube procedure for the synthesis of double-stranded cDNA up to 3 kb from total RNA or mRNA.
THIFICDNARO	Transcriptor High Fidelity cDNA Synthesis Kit	• Designed to reverse transcribe RNA with increased fidelity compared to other reverse transcriptases. • Optimized for two-step RT-PCR
05893151001	Transcriptor Universal cDNA Master	• Convenient fast solution for cDNA synthesis and real-time PCR analysis. Whichever PCR instrument you are using, this master mix, supplied in two vials, realizes the full

		potential of your qRT-PCR assays.

RT Enzymes — Reverse Transcriptases

Choose from our collection of RT enzymes and find one that suits the complexity of your RNA transcript. Our products include standard Avian Myeloblastosis Virus and Moloney Murine Leukemia Virus Reverse Transcriptases and enhanced versions of these for higher sensitivity

Product No.	Product Name	Features
A4464	Enhanced Avian Reverse Transcriptase	• Greater sensitivity for low abundance mRNA • Best enzyme for transcribing through difficult secondary structure
ERT-RO	Expand™ Reverse Transcriptase	• Transcribe cDNA fragments up to 13.5 kb • Can use cDNA or single-stranded DNA
M1302	M-MLV Reverse Transcriptase	• Uses single-stranded RNA, DNA or RNA-DNA hybrid • Generate first strand cDNA up to 7 kb
10109118001	Reverse Transcriptase AMV	• Includes 5X First-strand cDNA Synthesis Buffer
11062603001	Reverse Transcriptase M-MuLV	• Lacks endonuclease activity and has much lower RNase H activity vs AMV RT

TRANSRTRO	Transcriptor Reverse Trascriptase	• Achieve high sensitivity in two-step RT-PCR. Transcriptor Reverse Transcriptase is used in conventional thermal cyclers and real-time PCR instruments (e.g., the LightCycler® Instruments). • Reverse transcribe difficult templates. The enzyme works well at elevated temperatures, thereby overcoming RNA secondary structure (e.g., GC-rich RNA templates) and facilitating optimal reaction conditions.

Application of RT-PCR:

RT-PCR, also known as Reverse Transcriptase PCR, is a variation of the polymerase chain reaction that typically measures RNA expression levels. In RT-PCR, complementary DNA (cDNA) is made by reverse transcribing of the RNA templates with the enzyme reverse transcriptase.

- This technique is used to qualitatively study gene expression, and can be combined with real time PCR (qPCR) to quantify RNA levels.
- RT-PCR is used in research laboratories to study gene expression, for example in experiments to distinguish exons from introns, and can be used clinically to diagnose genetic diseases and monitor drug therapy.

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GENE CLONING TECHNIQUE

Introduction

The production of exact copies of a particular gene or **DNA** sequence using genetic engineering techniques is called gene cloning.

- The term "gene cloning," "DNA cloning," "molecular cloning," and "recombinant DNA technology" all refer to same technique.
- When DNA is extracted from an organism, all its genes are obtained. In gene (DNA) cloning a particular gene is copied forming "clones".
- Cloning is one method used for isolation and amplification of gene of interest.

DNA cloning can be achieved by two different methods:

1. Cell based DNA cloning
2. Cell-free DNA cloning (PCR)

Requirements for gene cloning :

1. **DNA fragment** containing the desired genes to be cloned.
2. **Restriction enzymes** and **ligase enzymes**.
3. **Vectors** – to carry, maintain and replicate cloned gene in host cell.
4. **Host cell**– in which recombinant DNA can replicate.

Principle of gene cloning

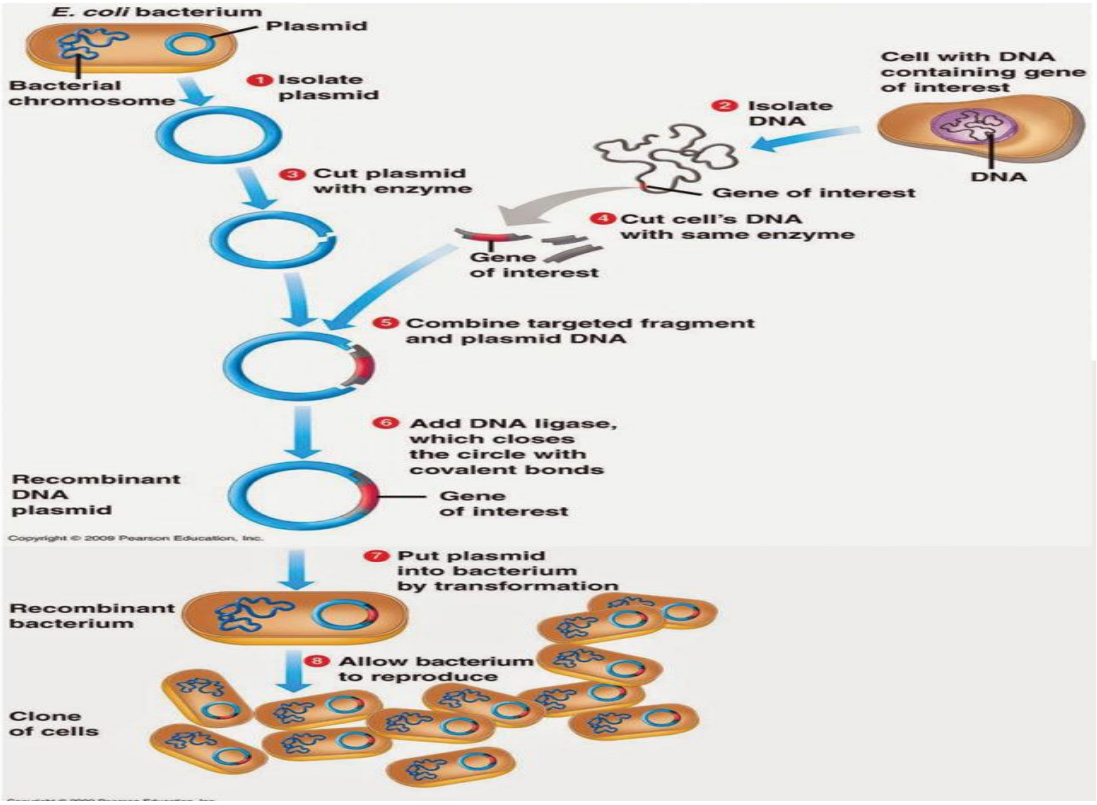
A fragment of DNA, containing the gene to be cloned, is inserted into a suitable vector, to produce a recombinant DNA molecule. The vector acts as a vehicle that transports the gene into a host cell usually a bacterium, although other types of living cell can be used. Within the host cell the vector multiplies, producing numerous identical copies not only of

itself but also of the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

Steps in gene cloning

The basic 7 steps involved in gene cloning are:

1. Isolation of DNA [gene of interest] fragments to be cloned.
2. Insertion of isolated DNA into a suitable vector to form recombinant DNA.
3. Introduction of recombinant DNA into a suitable organism known as host.
4. Selection of transformed host cells and identification of the clone containing the gene of interest.
5. Multiplication/Expression of the introduced Gene in the host.
6. Isolation of multiple gene copies/Protein expressed by the gene.
7. Purification of the isolated gene copy/protein



A.Isolation of the DNA fragment or gene

- The target DNA or gene to be cloned must be first isolated. A gene of interest is a fragment of gene whose product (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.
- The desired gene may be isolated by using restriction endonuclease (RE) enzyme, which cut DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease) producing blunt or sticky ends.
- Sometimes, reverse transcriptase enzyme may also be used which synthesizes complementary DNA strand of the desired gene using its mRNA.

B. Selection of suitable cloning vector

- The vector is a carrier molecule which can carry the gene of interest (GI) into a host, replicate there along with the GI making its multiple copies.
- The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected.
- The different types of vectors available for cloning **are plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs)** and **mammalian artificial chromosomes (MACs).**
- However, the most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors.

C. Essential Characteristics of Cloning Vectors

All cloning vectors are carrier DNA molecules. These carrier molecules should have few common features in general such as:

- It must be self-replicating inside host cell.
- It must possess a unique restriction site for RE enzymes.
- Introduction of donor DNA fragment must not interfere with replication property of the vector.
- It must possess some marker gene such that it can be used for later identification of recombinant cell (usually an antibiotic resistance gene that is absent in the host cell).
- They should be easily isolated from host cell.

D. Formation of Recombinant DNA

- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment.
- The mixture of donor DNA fragment and plasmid vector are mixed together.
- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs.
- The resulting DNA molecule is a hybrid of two DNA molecules – the GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination.
- Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the **recombinant DNA technology**.

E. Transformation of recombinant vector into suitable host

- The recombinant vector is transformed into suitable host cell mostly, a bacterial cell.
- This is done either for one or both of the following reasons:
- To replicate the recombinant DNA molecule in order to get the multiple copies of the GI.
- To allow the expression of the GI such that it produces its needed protein product.
- Some bacteria are naturally transformable; they take up the recombinant vector automatically.

For example: *Bacillus*, *Haemophillus*, *Helicobacter pylori*, which are naturally competent.

- Some other bacteria, on the other hand require the incorporation by artificial methods such as Ca^{++} ion treatment, electroporation, etc.

F. Isolation of Recombinant Cells

- The transformation process generates a mixed population of transformed and non-trans- formed host cells.
- The selection process involves filtering the transformed host cells only.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.

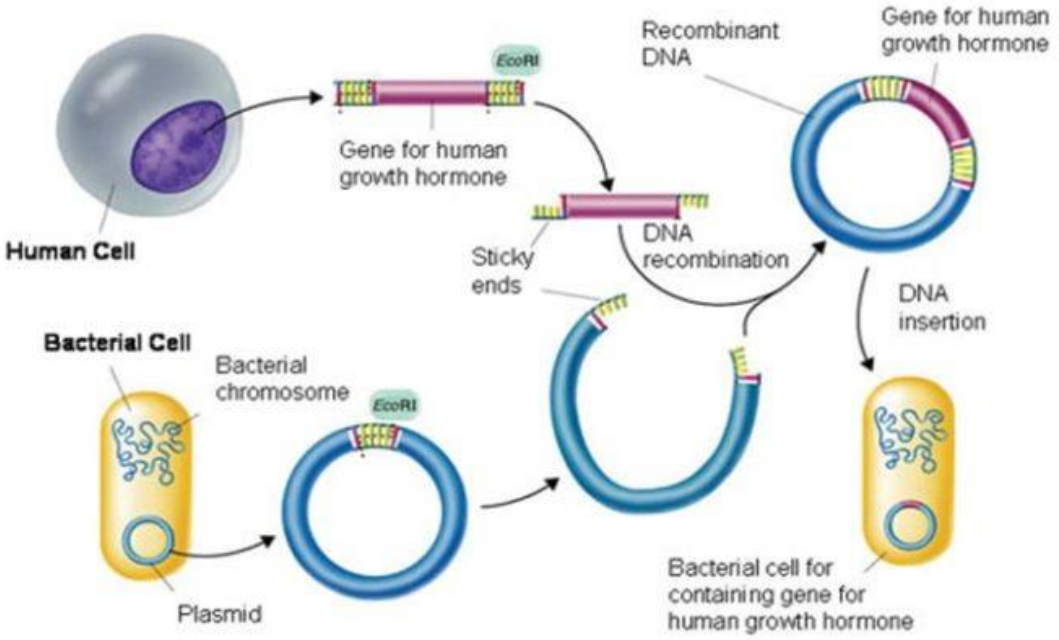
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

G. Multiplication of Selected Host Cells

- Once transformed host cells are separated by the screening process; becomes necessary to provide them optimum parameters to grow and multiply.
- In this step the transformed host cells are introduced into fresh culture media .
- At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them.
- If the aim is obtaining numerous copies of GI, then simply replication of the host cell is allowed. But for obtaining the product of interest, favourable conditions must be provided such that the GI in the vector expresses the product of interest.

H. Isolation and Purification of the Product

- The next step involves isolation of the multiplied GI attached with the vector or of the protein encoded by it.
- This is followed by purification of the isolated gene copy/protein.



Applications of gene cloning

- A particular gene can be isolated and its nucleotide sequence determined
- Control sequences of DNA can be identified & analyzed
- Protein/enzyme/RNA function can be investigated
- Mutations can be identified, e.g. gene defects related to specific diseases

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Restriction digestion Method

Introduction

The process of cleaving DNA at a particular location with the help of the specific type of restriction endonuclease enzyme which helps in mapping, polymorphism study and studying mutation called as restriction digestion.

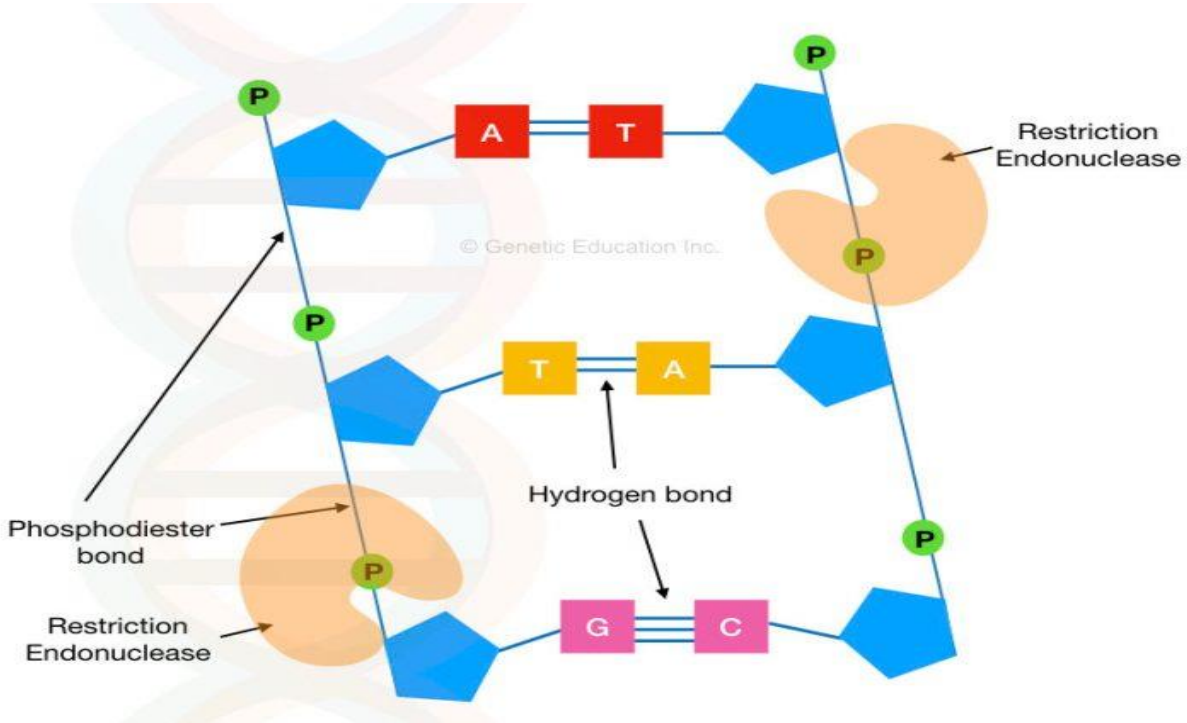
- Restriction endonucleases cut or cleave DNA at a specific location at their specific recognition site.
- The first restriction endonuclease was discovered into the year 1970 (HindII) by *Smith, Thomas Kelly* and *Kent Wilcox*. However, the term was first explained by *W. Arber* in the year 1960.
- *Nathans, Smith* and *W. Arber* were awarded Nobel prize in 1978 for discovering the endonuclease.

Restriction digestion

The restriction endonucleases are involved in the bacterial and prokaryotic defence mechanism and hence the restriction digestion too

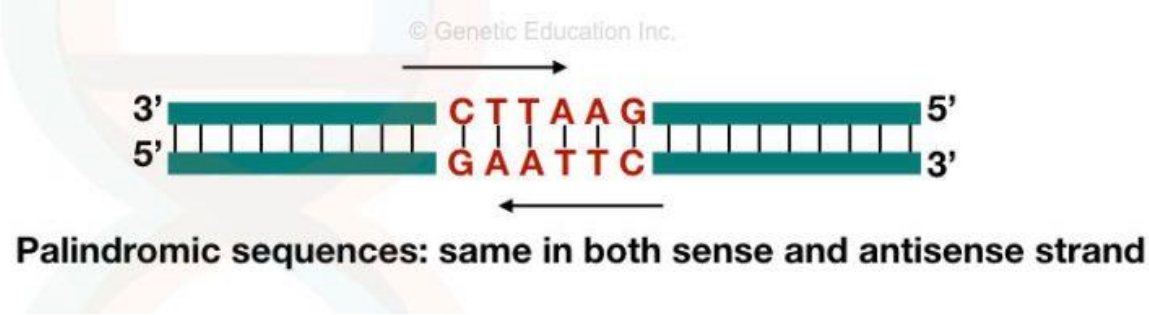
- The virus and phages always target bacterial DNA.

- The phage attacks the bacterial cell and destroys it. The endonucleases recognise the foreign DNA, bind on it and attack it, by doing this it cut the foreign DNA into small pieces and destroys vital genes of the phages.
- The endonuclease attacks the foreign DNA and cleaves the phosphodiester bond between the adjacent nucleotide on both the strands.
- Numerous REase attacks numbers of different sequences present into the foreign DNA and protect the bacterial DNA from the attacks of the phages and other viruses
- Here the process of methylation protects the bacterial own DNA from the nucleophilic attack. By transferring methyl groups to adenine or cytosine residues of the DNA of its own it produces N6-methyladenine or 5-methylcytosine.
- Once the methyl groups added to the DNA sequence the enzyme cannot identify their recognition sequence and hence it escapes the digestion process.



Palindromic sequences

The sequences of DNA in a sense strand from 5’ to 3’ and antisense strand from 3’ to 5’, are same, these types of DNA sequences are the palindromic sequences.

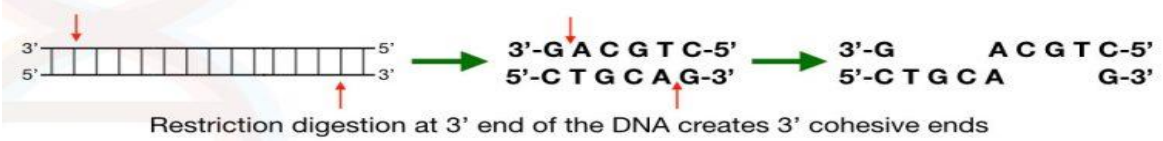


Palindromic sequences: same in both sense and antisense strand

The restriction endonuclease digestion system helps the bacterial own DNA from the phage invasion and from the other virus.

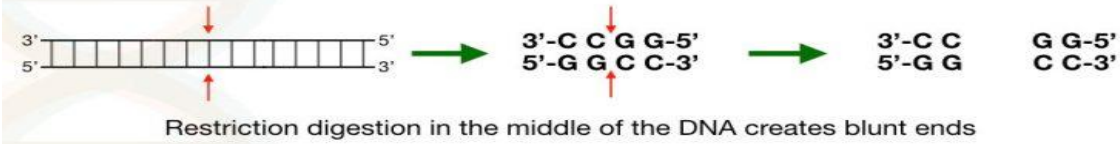
In vitro process of Restriction digestion

- From the discovery of the REase, different REase are utilised for different types of applications. Due to different recognition sites, different REase cuts different sequences of the DNA.
- Humans genome is very big, millions of recognition site are present for one particular type of REase. Hence cutting one specific sequence in a whole genome is nearly impossible by REase



Restriction digestion at 3’ end of the DNA creates 3’ cohesive ends

Restriction digestion at 5’ end of the DNA creates 5’ cohesive ends



Restriction digestion in the middle of the DNA creates blunt ends

The process of RE starts with DNA extraction. We have to extract the high quality of DNA from the source. The best method for DNA extraction for the restriction digestion is phenol-chloroform DNA extraction method, an enzymatic method of DNA extraction or you can use the ready to use DNA extraction kits.

Methods of restriction digestion

1. RFLP, restriction fragment length polymorphism. The method is the combination of polymerisation and restriction digestion followed by the agarose gel electrophoresis.
2. The RFLP, AFLP and STRP are the most common types of methods used into the research as well as in diagnostic which contains the restriction digestion.
3. In recent time, the Restriction fragment length polymorphism is most popular in disease diagnosis of single gene disorders.
4. The AFLP, amplified fragment length polymorphism is also of the important tool used in the plant research. Also, the short tandem repeat polymorphism is best applicable along with restriction digestion.
5. The restriction digestion is further, involved in the method of gene editing, especially, in the zinc finger nuclease method of gene transfer.
6. Direct digestion is also one of the best methods for the digestion of the whole genome, it is used to creates the library for microarray and DNA sequencing.

Application of restriction digestion

- **Population-wide polymorphism and mutation study:** The population wise study is very important for identification of the harmful alleles, the restriction digestion method is one of the powerful tools in studying the single gene mutation in different diseases such as sickle cell anaemia and Huntington’s disease. Also, new variations or polymorphism can be identified but can not be characterised
- Linkage mapping is possible by the restriction digestion method.
- Used in DNA probe preparation
- DNA fingerprinting and paternity verification is possible by the RFLP method of restriction digestion. Restriction digestion was commonly used in the criminal verification and paternity test since long.

- Cloning and expression vector preparation.

Alkaline phosphatases treatment of cloning vector

Introduction

Alkaline phosphatases are routinely used to reduce the background from empty, religated vectors during cloning of DNA fragments, since dephosphorylated DNA termini cannot be ligated by DNA ligase. The phosphatase treatment will effectively reduce the background of “empty” clones by >95%.

Dephosphorylation is a common step in traditional cloning workflows to ensure that the vector does not re-circularize during ligation. If a vector is linearized by a single restriction enzyme, or has been cut with two enzymes with compatible ends, use of a phosphatase, such asto remove the 5´ phosphate reduces the occurrence of vector re-closure by intramolecular ligation. Decreased re-circularization reduces the background during subsequent transformation. If the vector is dephosphorylated, it is essential to ensure that the insert contain a 5' phosphate to allow ligation to proceed. Each double-strand break requires that one intact phosphodiester bond be created before transformation (and in vivo repair).

cloning

In cloning protocols, a DNA fragment is ligated into a plasmid vector. Especially if the vector is cut with a single restriction enzyme, chances are much higher that the vector religates back on itself rather than on an added DNA fragment. This results in a high fraction of “empty clones”, or background.

Alkaline phosphatases are routinely used to reduce the background from empty, religated vectors during cloning of DNA fragments, since dephosphorylated DNA termini cannot be ligated by DNA ligase. The phosphatase treatment will effectively reduce the background of “empty” clones by >95%. However, cloning procedures and also phosphatase treatment may be cumbersome and error prone.

SAP offers greater convenience to this procedure, since the enzyme may be completely removed by a simple heat inactivation step. SAP is active in all buffers used for restriction enzymes, so SAP can be added either during restriction digestion, or directly after. With SAP, the user can forget elaborate calculations and multi-step incubations, because the enzyme completely dephosphorylates DNA during one, simple, incubation.

Protocol including restriction cutting

In this protocol, SAP (Shrimp alkaline phosphatase)is present during restriction cutting, so the termini are dephosphorylated as soon as they are formed. In this protocol, the minimum effective amount of SAP is proportional to the amount of restriction enzyme added (i.e. the rate of terminus formation). In the simple sense, use at least 0.1 U SAP per Unit restriction enzyme, and proceed to complete cutting. The amount of restriction enzyme may differ from the protocol below, please use amounts recommended by the supplier.

- 1 µg plasmid
- 5 Unit restriction enzyme
- 5 µl 10x restriction enzyme buffer
- 1 Unit SAP
- dH2O to 50 µl

Incubate at 37°C for 1 hour, inactivate as recommended for the restriction enzyme used. Proceed to ligation protocol.

Quick dephosphorylation of cut plasmid

Efficient dephosphorylation can be achieved in short time using high amounts of SAP. When restriction cutting is complete, simply add 5 U SAP per µg vector to your restriction mix and incubate for further 5 min at 37°C. Inactivate as recommended for the restriction enzyme. Proceed to ligation protocol.

Gene transfer techniques: chemical methods

Introduction

- 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.
- 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.

chemical methods

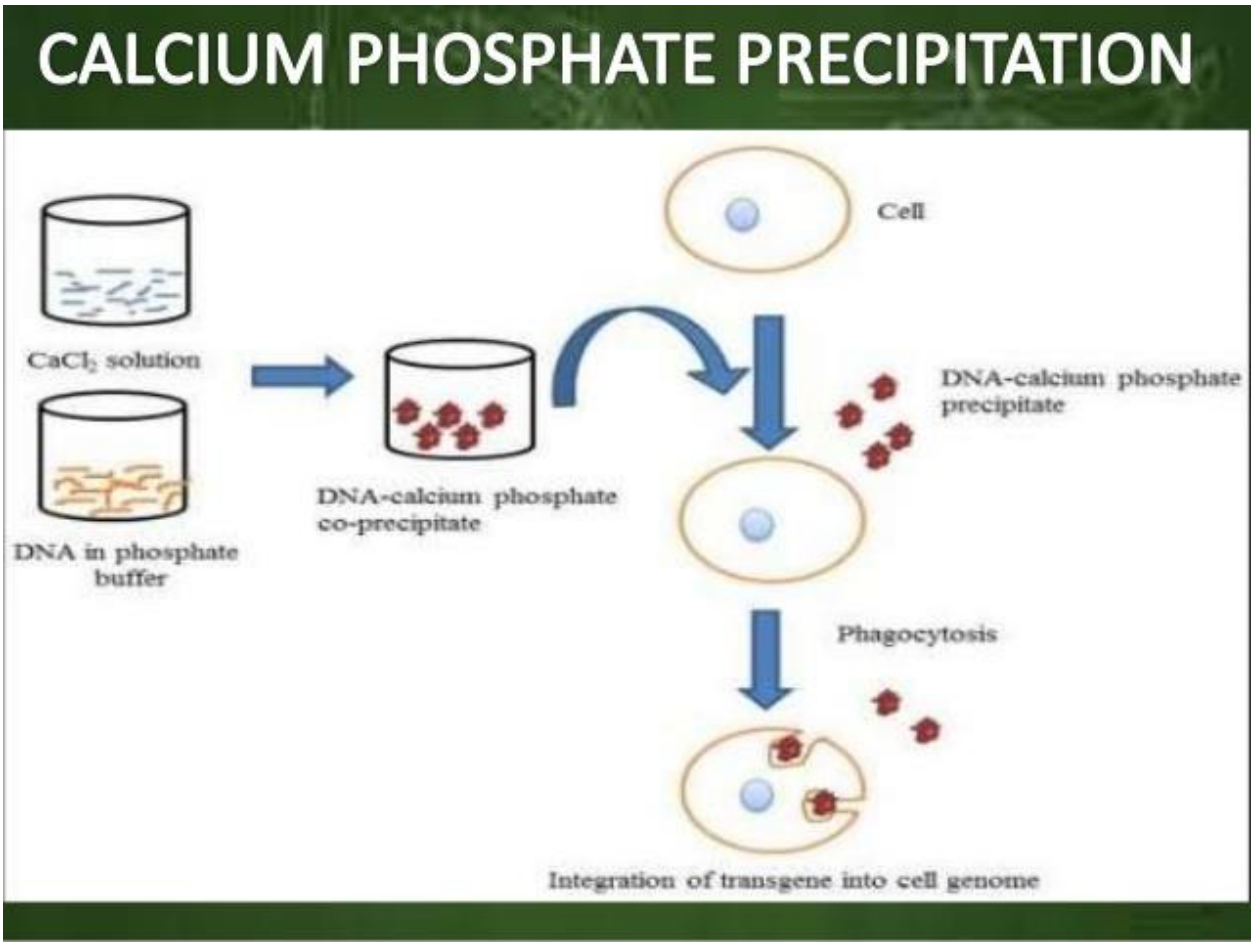
The commonly used methods of chemical transfection use the following,

1. Calcium phosphate 2. DEAE dextran

3. Cationic Lipid 4. Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

Calcium phosphate mediated DNA transfer

- This method is based on the precipitation of plasmid DNA and calcium ions by their interaction.
- In this method, the precipitates of calcium phosphate and DNA being small and insoluble can 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 or transient 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.

5DEAE-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 transfection but not for stable transfection • Typically produces less than 10% delivery in primary cells.

Lipofection

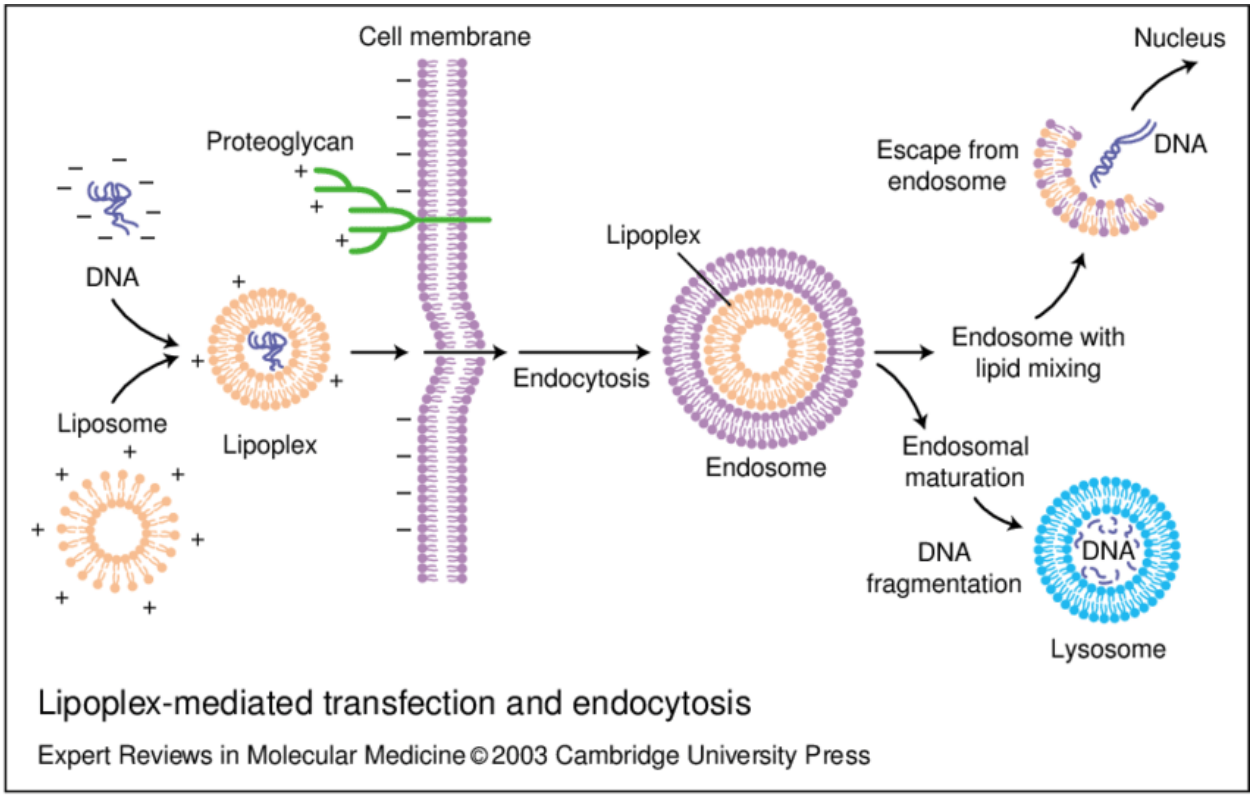
• Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.

• Liposomes are artificial phospholipid vesicles used for the delivery of 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 fusion and endocytosis thus forming DNA- liposome complex. This complex fuses 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.

. Liposome Action



Lipoplex-mediated transfection and endocytosis

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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

Other Methods

Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers..

Polyethylenimine

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.

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.

- 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

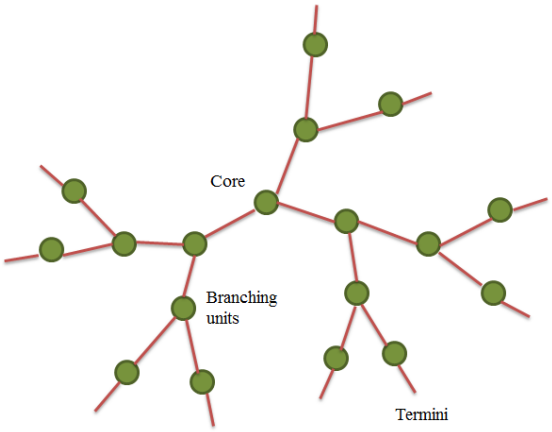
Polyphosphoester

- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications.

- They can efficiently bind and protect DNA from nuclease degradation They exhibit a significantly lower cytotoxicity than Poly-L-Lysine or polyethylenimine both *in vitro* and *in vivo.*

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.



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Gene transfer using Electroporation method

Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving electric field-mediated membrane permeabilization. Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

The basic technique of electroporation for transferring genes into mammalian cells is depicted in Figure ..The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

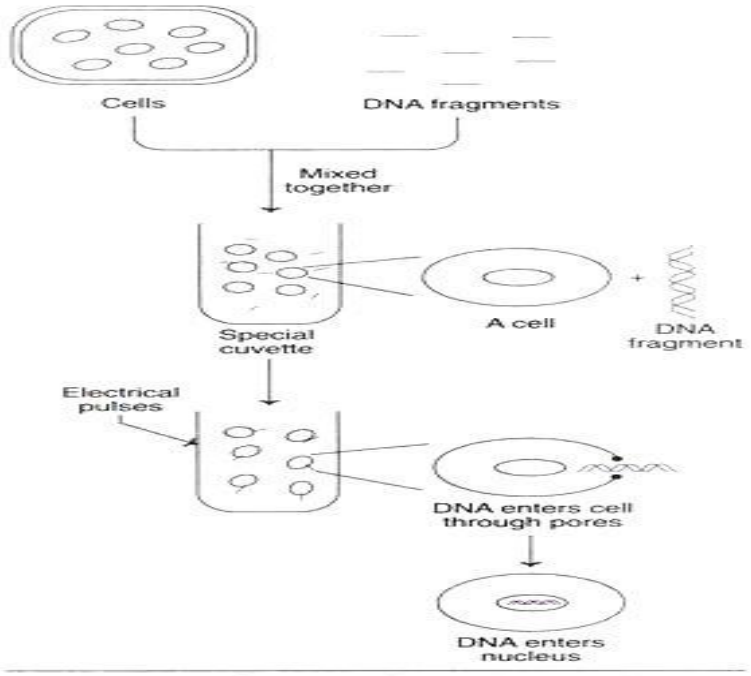


***Fig. 6.11** : Gene transfer by electroporation.*
***(Note** : Magnification depicted on right side)*

Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Advantages:

- Electroporation can transfer foreign material effectively, faster than any other viral or non-viral vector-mediated delivery system.
- In comparison with the viral vectors, It's safer. The chance of infection is negligible. Also, it's non-toxic, non-allergic and non-mutagenic.
- Transfection or transformation can perform on any living cells like mammalian, plant, bacteria, algae and yeast cells.
- It is used for *in vivo* as well as *in vitro* gene therapy.
- It can transfer any sized plasmid DNA into cells.

Broadly, the electroporation method is effective, reproducible, fast, efficient and easy to use. Importantly, the insertion is transient and stable.

Applications:

- It's used in gene transfer and gene therapy experiments.
- Foreign DNA, gene, viral DNA or plasmid DNA can be inserted into target cells using the present method.
- It is effectively employed in knockout mice construction. Gene expression studies are done using the gene knockout or gene knock-in studies.
- The electroporation technique is often used for cancer prevention. Anticancer agents or gene can be inserted in affected cells.
- It's used in vaccine development.it is used in drug discovery and drug research as well.
- It is often used to induce a rapid and robust immune response.
- The electroporation technique practised for generating monoclonal antibodies since long.
- The present method is also useful in RNA interference studies, gene expression and gene silencing studies.
- Broadly, the major applications of it are in farming, plant tissue culture, microbiology, medicines and molecular biology.
- Apart from its major applications, the electroporation method is also widely used for transferring dye, drugs, protein, antibodies, peptides and chemicals into cells for various purposes.

Colony hybridization

Introduction

- Colony hybridization begins with culturing sparsely populated bacterial colonies on a nutrient agar plate.

- These colonies are symmetrically replicated on a nitrocellulose filter by direct contact, after which the cells on the filter membrane are lysed and their DNA is denatured, allowing it to bind to the filter.
- These DNA clusters are then hybridized to a desired radioactively-labelled RNA or DNA probe (chosen specifically beforehand) and screened by autoradiography.
- DNA clusters that exhibit a desired gene are then matched up to the corresponding (living) bacterial colonies, which can be isolated for further growth and experimentation.

Definition of Colony Hybridization:

Colony hybridization is the "Blot analysis technique" where the bacterial cells are transferred from the solid nutrient medium to the absorbent material. Colony hybridization can define as the method for the isolation of the specific DNA sequences or genes from the bacterial cells containing hybrid DNA, by the means of a nitrocellulose membrane filter. The transferring medium then goes through several chemical and physical treatment.

Transferring medium of Colony Hybridization:

- The nitrocellulose filter paper is the transferring medium of the colony hybridization which forms replicas of the master plate. The nitrocellulose acts as a membrane which contains the exact copies of the gene to that of the master plate. Nitrocellulose filter paper acts as the "Blotting pad".

Colony hybridization involves the following steps:

Preparation of Master plate:-

First, inoculate the bacterial cell suspension on the solid agar medium to prepare the master plate. After the inoculation, the number of bacterial colonies will develop with different plasmids which refer as "Master or Reference plate".

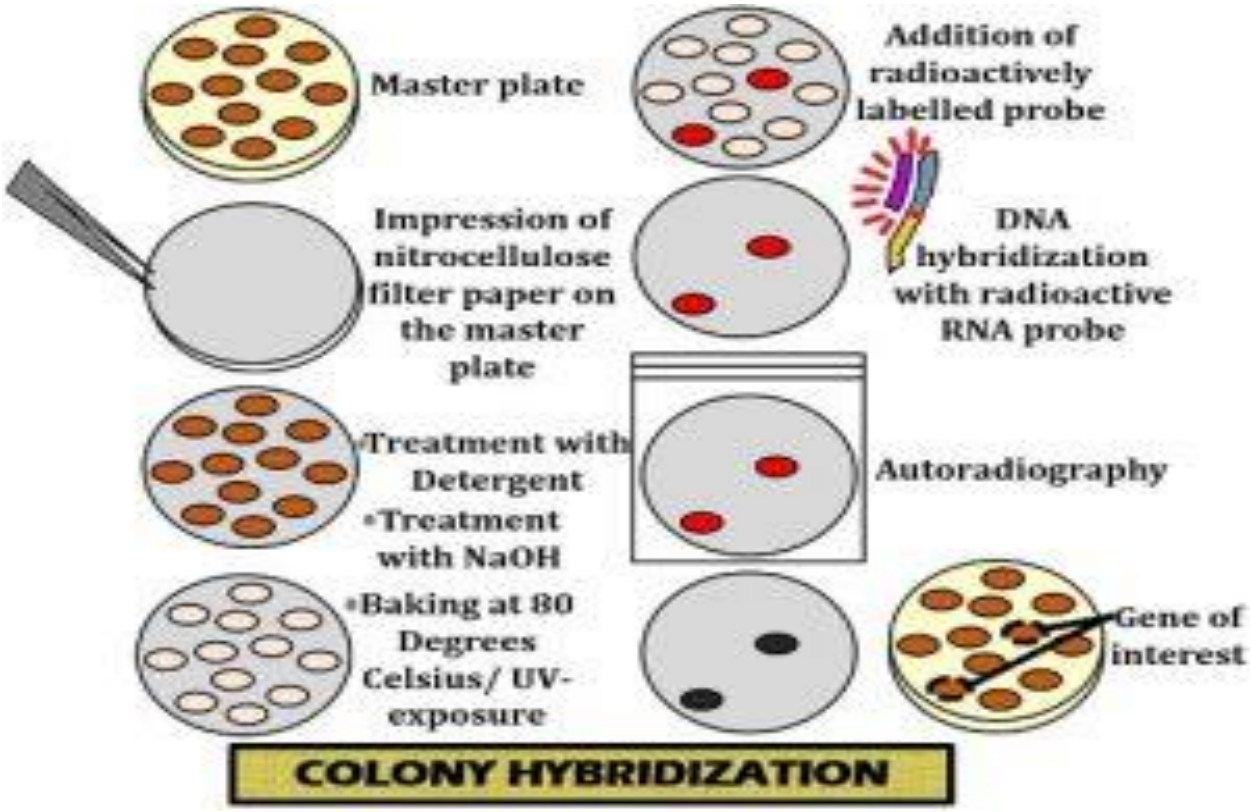
Formation of replicas over a nitrocellulose filter:-

Then transfer the bacterial cells from the master plate on to the membrane or filter by the means of "Nitrocellulose filter". Press the nitrocellulose filter paper over the surface of the master plate.

This compression of the filter membrane will form replicas or copies of the bacterial cells as that of the master plate.

Treatment of filter medium with SDS:-

After that treat the nitrocellulose filter paper with the detergent like SDS (Sodium dodecyl sulfate) to lyse the bacterial cells.



Treatment of filter medium with alkali:-

Treat the filter medium with the alkali like sodium hydroxide in order to separate the DNA into single strands.

Fixation of DNA onto the filter medium:-

To fix the DNA onto the nitrocellulose filter paper, either bake the filter paper at 80 degrees Celsius or expose it to the UV light.

Addition of radioactive probe:-

Hybridize the nitrocellulose filter paper containing imprints of the plasmid DNA by the addition of radioactive RNA probe. This radioactive RNA probe will code the desired gene of sequence from the bacterial cells.

Washing and Autoradiography:-

Wash the filter paper to remove unbound probe particles. After that, expose the nitrocellulose filter paper to the X-ray film by the method refer as "Autoradiography". The colony which will appear after autoradiography will refer as "Autoradiogram" which carry the genes of interest.

Identification of the desired gene:-

Then compare the developed autoradiogram with the master plate to identify the colonies containing a gene of interest.

The cells which contain the desired gene can grow in the liquid medium and can further process for the isolation of recombinant plasmid DNA.

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Blue–white screening Method

Introduction

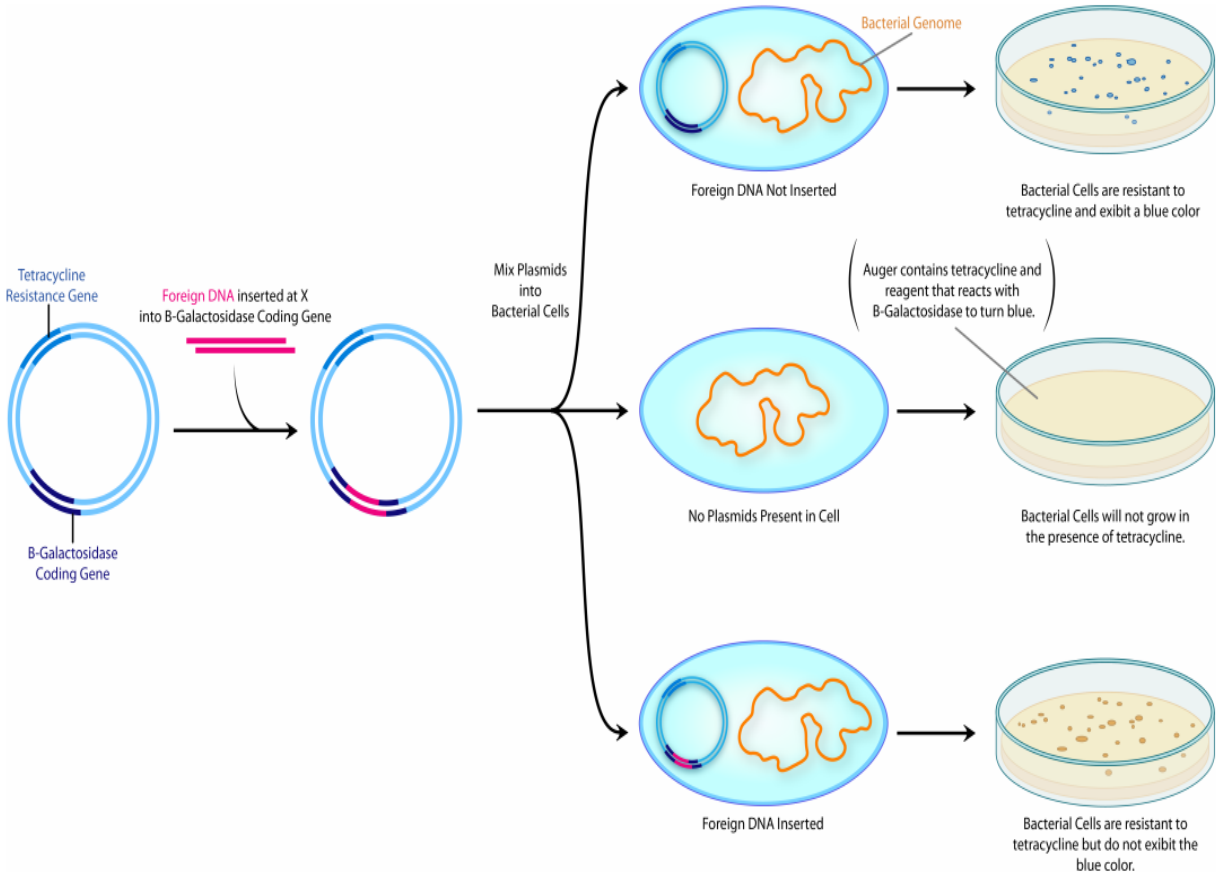
- The **blue–white screen** is a screening technique that allows for the rapid and convenient detection of recombinant bacteria in vector-based molecular cloning experiments.
- DNA of interest is ligated into a vector. The vector is then inserted into a competent host cell viable for transformation, which are then grown in the presence of X-gal.
- Cells transformed with vectors containing recombinant DNA will produce white colonies; cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies. This method of screening is usually performed using a suitable bacterial strain, but other organisms such as yeast may also be used.

Molecular mechanism:

- β-galactosidase is a protein encoded by the *lacZ* gene of the *lac* operon, and it exists as a homotetramer in its active state. However, a mutant β-galactosidase derived from the M15

strain of *E. coli* has its N-terminal residues 11—41 deleted and this mutant, the ω-peptide, is unable to form a tetramer and is inactive.

- In this method of screening, the host *E. coli* strain carries the *lacZ* deletion mutant (*lacZΔM15*) which contains the ω-peptide, while the plasmids used carry the *lacZα* sequence which encodes the first 59 residues of β-galactosidase, the α-peptide.
- Neither is functional by itself. However, when the two peptides are expressed together, as when a plasmid containing the *lacZα* sequence is transformed into a *lacZΔM15* cells, they form a functional β-galactosidase enzyme.
- The blue/white screening method works by disrupting this α-complementation process. The plasmid carries within the *lacZα* sequence an internal multiple cloning site (MCS). This MCS within the *lacZα* sequence can be cut by restriction enzymes so that the foreign DNA may be inserted within the *lacZα* gene, thereby disrupting the gene that produces α-peptide.
- The presence of an active β-galactosidase can be detected by X-gal, a colourless analog of lactose that may be cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo.
- This results in a characteristic blue colour in cells containing a functional β-galactosidase.
- Blue colonies therefore show that they may contain a vector with an uninterrupted *lacZα* (therefore no insert), while white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert in *lacZα* which disrupts the formation of an active β-galactosidase.
- The recombinant clones can be further analyzed by isolating and purifying small amounts of plasmid DNA from the transformed colonies and restriction enzymes can be used to cut the clone and determine if it has the fragment of interest.



Uses of Blue-white screening method

- Blue-white screening provides a convenient and powerful way to distinguish bacterial colonies or phage plaques that contain a cloning vector with a DNA insert, from those containing empty vectors with no insert DNA
- The blue–white screen is a screening technique that allows for the rapid and convenient detection of recombinant bacteria in vector-based molecular cloning experiments. DNA of interest is ligated into a vector
- This method of screening is usually performed using a suitable bacterial strain, but other organisms such as yeast may also be used.
- Blue-white screening provides a convenient and powerful way to distinguish bacterial colonies or phage plaques that contain a cloning vector with a DNA insert, from those containing empty vectors with no insert DNA.

Immunochemical detection Method

Immunochemical detection and isolation of DNA from metabolically active bacteria. ... To identify actively growing bacteria, we adapted a technique from immunocytochemistry to **detect** and selectively isolate DNA from bacteria incorporating bromodeoxyuridine (BrdU), a thymidine analog.

- A simple immunoassay has been developed which can be used in the isolation of particular gene(s) from a clone bank of recombinant plasmids.
- A clone bank of the DNA is constructed with a plasmid vector and maintained in Escherichia coli.
- The recombinant clones were filtered onto a hydrophobic grid membrane and grown up into individual colonies, and a replica was made onto nitrocellulose paper.
- The bacterial cells were then lysed with chloroform and the proteins were immobilized onto the nitrocellulose paper.
- The nitrocellulose paper is then reacted with a rabbit antibody preparation made against the particular antigenic product to detect the recombinant clone which carries the corresponding gene.
- The bound antibodies can be detected easily by a colorimetric assay using goat anti-rabbit antibodies conjugated to horseradish peroxidase.
- Positively reacting clones can be recovered from the master hydrophobic grid membrane filter for further characterization.
- We proposed to call this method "colony ELISA blot" and described the isolation of the genes coding for the soluble antigens of Pasteurella haemolytica using this method.