

BIO 203

Methods in Molecular Biology

LABORATORY MANUAL

DEPARTEMENT OF BIOLOGY

VIRTUAL UNIVERSITY OF

PAKISTAN

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Sources and Recovery of DNA

Sources of DNA

Purified DNA is required for a variety of molecular biology applications. DNA can be purified from any living organism and its living parts

Origin of Samples:

1. Human tissues i.e histological samples, prenatal samples, postmortem harvesting.
2. Blood, (EDTA).
3. Hair, (follicle part of the hair to be specific).
4. Rodent tissues, as rats are the most common lab mammals used in labs.
5. Leaf.
6. Bacteria, Bacterial cultures.
7. Yeast, yeast cultures.
8. Fungi.
9. Insect, i.e *Drosophila melanogaster*
10. Stool.
11. Body fluids, i.e semen.
12. Spores.
13. Soil.
14. Clinical samples (e.g. biopsy samples, fine needle aspirates).
15. Forensic samples (e.g. dried blood spots, buccal swabs finger prints).

PREPARATION OF REAGENTS

The following general instructions are applicable in the preparation of all reagents.

Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents.

Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared, initials of scientist that prepared reagent, lot number, and expiration date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl [Tris(Hydroxymethyl)aminomethan] pH8

Tris base	121.1g
H ₂ O	to 800ml

Adjust to desired pH with concentrated HCl.
Mix and add H₂O to 1 Liter.

Store at room temperature

0.5 M EDTA (Ethylenediamine Tetraacetic Acid) pH 8.0

Na ₂ EDTA.2H ₂ O	186.1g
H ₂ O	to 700ml

Adjust pH to 8.0 with 10M NaOH (almost 50ml)
Mix and add H₂O to 1 Liter.

Store at room temperature

10M NaOH

NaOH	400 g
H ₂ O	to 1 Liter

Store at room temperature

10 mg/ml Ethidium Bromide

Ethidium Bromide	0.2 g
H ₂ O	to 20ml

Mix well and store at 4°C in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0 (Lysis Buffer)

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	4 ml
H ₂ O	to 1 Liter

Store at room temperature

Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0 for DNA storage

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	0.4 ml
H ₂ O	to 1 Lite

Store at room temperature

Proteinase K (10mg/ml)

Proteinase K	100 mg lyophilized powder
Ultra-pure H ₂ O	to 10 ml

Aliquot and store at approximately -20°C.

CAUTION: Powder and solutions of Proteinase K can be irritating to mucous membranes.

SDS 10% w/v

Sodium dodecyl sulfate 100g H₂O
to 700ml Heat to approximately 65°C to
dissolve.

Bring to a final volume of 1.0 L with ultra pure water.

Store at room temperature.

CAUTION: SDS can be irritating to mucous membranes. Wear safety
glasses, mask and gloves when handling

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl ph 8.0	10 ml
5M NaCl	80 ml
0.5M EDTA	4 ml
H ₂ O	to 1 Liter

Store at room temperature.

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base	242g
Glaciall acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100ml
H ₂ O	to 1 Liter

Store at room temperature

1x TAE (Tris 40mM, Acetate 20mM, EDTA 2mM) Electrophoresis working buffer

50x TAE 10 ml H₂O
to 500 ml The pH of diluted buffer is 8.3.

Store at room temperature.

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base	108g
Boric Acid	55g
0.5M EDTA pH 8.0	40 ml H ₂ O to 1 Liter

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue	0.25 ml
2% Xylene cyanol	0.25 ml
Glycol	7ml
H ₂ O	10ml

Store at room temperature

5M Sodium Chloride

Sodium Chloride	292.2 g
H ₂ O	to 1 Liter

Store at room temperature.

6M Sodium Chloride

Sodium Chloride 351g

H₂O to 1 Liter

Store at room temperature.

Recovery of the DNA

Recovery of the DNA is called DNA extraction. DNA extraction involves many different methods. We will be discussing all those different methods in the next practical. Here in this practical we discuss only the basic steps involved in all DNA extraction methods.

Cell lysis:

Cell lysis is breakdown of the cellular structure so that long strands of DNA can come out from the entanglements of the cellular structures. It can be chemical or physical or combination of the both depending upon the origin of the sample. Cell lysis is achieved by using detergent which destabilized the plasma membrane. To break the cell wall of plants and bacterial samples, the physical force is utilized which may be in the form of sonication. The chemical reagents such as lysozyme, EDTA, lysozyme and EDTA combined, and other detergents are used for the lysis.

Removal of membrane lipids:

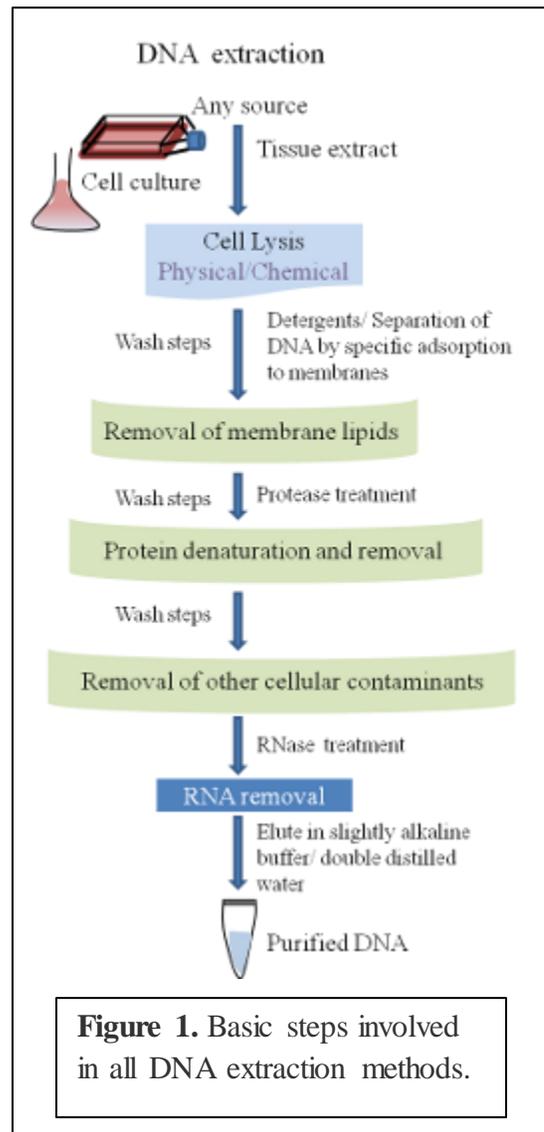
After lysis the membrane lipids are removed and DNA is proceeded to remove other impurities. Once membrane is solubilized by the detergents, it is removed when DNA is washed.

Protein denaturation and removal:

Protease is an enzyme which is used commonly to denature proteins in molecular biology experiments. Once proteins are denatured the soluble components of the proteins will be washed out in the subsequent washings of the DNA.

Removal of other cellular components:

The other cellular components are removed from the DNA by frequent washing steps.



RNA denaturation and removal:

RNA is important contaminant of the DNA and to have a controlled experiment DNA must be free of any impurity including RNA. RNA is denatured using RNase, which is an RNA digesting enzyme.

DNA elution and storage:

DNA is eluted in an alkaline buffer solution or in double distilled water. Purified DNA is stored at -20°C preferably.

Methods of DNA extraction and purification

The basic criteria a method of DNA isolation from any sample type should meet include

1. Efficient extraction
2. Sufficient amount of DNA extracted for downstream processes
3. Removal of contaminants
4. Quality and purity of DNA

Based on these criteria following different methods are used for the extraction of the DNA.

Organic method

INTRODUCTION

Of all the methods of DNA extraction, the organic method (also known as the phenol-chloroform method) has been the longest in use. This is because it is the most effective at extracting the large amounts of high molecular weight DNA that were required for the RFLPs that created the first DNA fingerprints in the 1980s. This protocol describes the standard method for nucleic acid purification by extraction first with phenol: chloroform and then with chloroform to remove any remaining phenol.

PRINCIPLE:

Organic extractions or Phenol–chloroform extraction is a liquid-liquid extraction technique in biochemistry and molecular biology for purifying nucleic acids and eliminating proteins. In brief, aqueous samples are mixed with equal volumes of a phenol: chloroform mixture. After mixing, the mixture is centrifuged and two distinct phases are formed, because the phenol: chloroform mixture is immiscible with water. The aqueous phase is on top because it is less dense than the organic phase (phenol: chloroform). The proteins will partition into the lower organic phase while the nucleic acids (as well as other contaminants such as salts, sugars, etc.) remain in the upper aqueous phase. The upper aqueous phase is pipetted off and care is taken to avoid pipetting any of the organic phase or material at the interface. This procedure is often performed multiple times to increase the purity of the DNA.

MATERIALS

Reagents

Chloroform

- Ethanol
- Ether (optional)

- Nucleic acid solution to be purified
- Phenol: Chloroform (1:1)
- Tris EDTA (pH 7.8) (optional)
- 3 M sodium acetate pH 5.2 or 5 M ammonium acetate
- 100% ethanol

Equipment

- Automatic pipette fitted with a disposable tip
- Pipettes, large-bore (optional)
- Polypropylene tube
- Rotating wheel (optional)

METHOD

1. Transfer the nucleic acid sample to a polypropylene tube and add an equal volume of phenol: chloroform.

(After mixing and centrifugation this will result in two phases, lower organic phase and upper aqueous phase. The DNA is in the aqueous phase and will be extracted from it later. The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8-8.0.)

2. Mix the contents of the tube until an emulsion forms.
3. Centrifuge the mixture at 80% of the maximum speed that the tubes can bear for 1 minute at room temperature. If the organic and aqueous phases are not well separated, centrifuge again for a longer time.
4. Use a pipette to transfer the aqueous phase to a fresh tube. For small volumes (<200 μ L), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase.
5. Repeat Steps 1-4 until no protein is visible at the interface of the organic and aqueous phases.
6. Add an equal volume of chloroform and repeat Steps 2-4.
7. To recover DNA measure the volume of the aqueous phase.

8. Add 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M).
9. Mix well and add 2 to 2.5 volumes of cold 100% ethanol (calculated after salt addition).
10. Mix well.
11. Place on ice or at -20 degrees C for >20 minutes.
12. Spin a maximum speed in a microfuge 10-15 min.
13. Carefully decant supernatant.
14. Add 1 ml 70% ethanol. Mix. Spin briefly. Carefully decant supernatant.
15. Air dry or briefly vacuum dry pellet.
16. Resuspended pellet in the appropriate volume of Tris EDTA buffer or double distilled water.
17. Proceed with quantification and intended use after storage.

ADVANTAGES AND DISADVANTAGES:

Its other main advantage is the fact that it can be used on a wide range of samples. However, this method does also have some disadvantages including being very labor intensive, being easily contaminated and exposing the scientist carrying out the extraction to dangerous chemicals.

READINGS:

<http://www.fastbleep.com/biology-notes/41/122/1216>

<http://cshprotocols.cshlp.org/content/2006/1/pdb.prot4455>

<http://www.med.upenn.edu/lamitinalab/documents/EthanolPrecipitationofDNA>.

<http://bitesizebio.com/384/the-basics-how-phenol-extraction-works/>

<http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/PHENOL-CHLOROFORM.pdf>

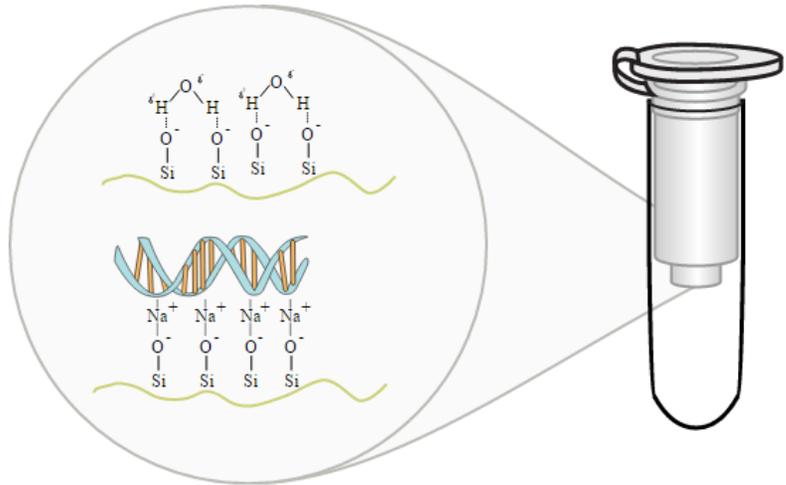
Silica Adsorption Method:

INTRODUCTION:

Silica DNA extraction methods have become the staple for many labs as it is quick reliable and produces very high quality DNA.

PRINCIPLE:

DNA separation by silica adsorption is a method of DNA separation that is based on DNA molecules binding to silica surfaces in the presence of certain salts and under certain pH conditions. Although the mechanism is not fully understood, one possible explanation involves reduction of the silica's surface's negative charge due to the high ionic strength of the buffer. This decrease in surface charge leads to a decrease in the electrostatic repulsion between the negatively charged DNA and the negatively charged silica. Meanwhile, the buffer also reduces the activity of water by formatting hydrated ions. This leads to the silica surface and DNA becoming dehydrated. These conditions lead to an energetically favorable situation for DNA to adsorb to the silica surface.



Figure, 2: Silica in a spin column with water and with DNA sample in specific buffer

MATERIALS:

Kits are available which work with the said principle.

METHODS:

There are two basic steps:

Binding and washing:

The sample is run through a micro-channel, DNA binds to the channel, and all other molecules remain in the buffer solution. The channel is washed free of impurities.

Elution:

The silica is then dried and DNA is eluted using water or a low salt buffer. An elution buffer removes the DNA from channel walls, and the DNA is collected at the end of the channel.

ADVANTAGES & DISADVANTGES:

It is a quick, reliable method which produces high quality DNA. It is an expensive method and if chewing gum is the source of DNA, the process can be interfered by chewing gum.

READINGS:

<http://www.ncbi.nlm.nih.gov/pubmed/9986822>

<http://bitesizebio.com/13516/how-dna-extraction-rna-miniprep-kits-work/>

<http://herpesvirus.tripod.com/research/protoDNA.htm>

Non-organic method:

INTRODUCTION:

In this method no organic solvents are used that's why it is termed as non-organic method.

PRINCIPLE:

Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification. It is highly suited to this application since the enzyme is active in the presence of chemicals that denature proteins, such as SDS and urea, chelating agents such as EDTA, sulfhydryl reagents, as well as trypsin or chymotrypsin inhibitors. Proteinase K is used for the destruction of proteins in cell lysates (tissue, cell culture cells) and for the release of nucleic acids, since it very effectively inactivates DNases and RNases.

MATERIALS

Solutions/reagents:

- Digestion Buffer (10mM NaCl, 10mM TRIS (pH 8.0), 10mM EDTA (pH 8.0), 0.5% SDS)
- Proteinase K (20mg/ml)
- Sodium Acetate pH 5.2 (3M)
- Ice-cold 98% ethanol
- Ice-cold 70% ethanol
- 1X TE
- Water
- Tissue

Equipment:

- Incubator
- Centrifuge
- Sterile 1.5-ml micro-centrifuge tubes

METHODS:

Tissue Digestion

- Measure out Digestion Buffer into sterile 1.5-ml microcentrifuge tube.
- Add 0.005 volumes Proteinase K.
- That is, for each ml of Digestion Buffer, add 5 μ l of ProteinaseK.
- Homogenize tissue in solution.

- Incubate at 55°C for 1 - 12 hours (overnight).
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 2 minutes at 4°C and aspirate out the top layer.
- Transfer top aqueous layer into sterile 1.5-ml microcentrifuge tube.
- Discard bottom layer.

Precipitation of Protein and Cell Debris

- Add 0.1 volume Sodium Acetate pH 5.2 to sterile 1.5-ml microcentrifuge tube.
- Close the tube tightly and gently mix the contents by inverting the tube.
- Incubate at -20°C for 15 minutes.
- Centrifuge at maximum speed for 20 minutes at 4°C and aspirate out the top layer.
- Transfer top aqueous layer into sterile 1.5-ml microcentrifuge tube.
- Discard bottom layer.
- Be careful not to transfer any of the white solid (cell debris and SDS) into the fresh tube.

Precipitation of Nucleic Acids

- Add 2 volumes ice-cold 98% ethanol to sterile 1.5-ml microcentrifuge tube.
- Close the tube tightly and gently mix the contents by inverting the tube.
- Incubate at -20°C for 15 minutes.
- Centrifuge at maximum speed for 20 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Add 1 ml of ice-cold 98% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Add 1 ml of ice-cold 70% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- (Optional) Add 1 ml of ice-cold 70% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Dry the pellet in air.
- Option 1: Add 10 µl of 1X TE (or) Option 2: Add 10 µl of water.
- Resuspend the pellet by vortexing/by shaking vigorously.
- Ensure to dry the pelleted DNA completely before attempting to resuspend.

ADVANTAGES & DISADVANTGES:

READINGS:

Chelex™ method

INTRODUCTION:

Chelex is one of the oldest methods of DNA extraction and utilizes a chelating resin.

PRINCIPLE

Chelex resin is often used for DNA extraction in preparation for PCR. The Chelex™ protects the sample from DNAases that might remain active after the boiling and could subsequently destroy the DNA. DNAases are enzymes, which naturally occur in all body tissues; they cut DNA into small fragments, rendering it unsuitable for PCR. Magnesium ions are essential cofactors for DNases. Chelex™ resin binds with cations including Mg²⁺. By binding with magnesium ions, the Chelex™ resin renders DNases inoperable, thus protecting DNA from their action.

MATERIALS

- Chelex 300 µL
- Heating Block
- ddH₂O
- Sterile Forceps
- Vortex
- Centrifuge tubes

METHODS

1. Remove premade tubes filled with 300µL 10% Chelex from refrigerator. You will need for each sample, plus 1 extra for a control. Handle the container with gloves and shake out the number you need into your gloved hand. Do not put extra tubes back into jar.
2. Label each Chelex tube to correspond to your sample listed on your Chelex worksheet. Label the tubes on the cap. Put as much information as you can manage, but keep labels legible. Adding initials and a date is always a good idea. Avoid labeling on the side of tube as this writing can be washed off during the incubation stage.
3. Turn on heating block. Set to 95°C. Fill holes with ddH₂O water.
4. Dip forceps into ethanol, then wave forceps through flame of an alcohol burner to ignite. When you are certain that the flame on the forceps has extinguished, repeat 2 times.
5. Using the sterile forceps, remove a small piece of tissue from your sample; uncap the tube of Chelex, place sample in the appropriately labeled tube and close lid. The piece of tissue should be big enough to be visible, but not so big as to be easily visible. Imagine cutting a 0.2mm section of a standard staple. This is plenty big. Too much tissue may inhibit your reactions. The piece of tissue should be about as big as a period.

6. Repeat (step 5) with each sample in a new Chelex tube, being sure to sterilize forceps 3 times between samples (step 4). When finished, make a negative Chelex control by dipping your sterilized forceps into a tube of Chelex slurry. (It may be necessary to wipe excess tissue from forceps with a Kim wipe prior to flame sterilization)
7. When finished with all tubes, vortex samples in Chelex slurry for 10-15 seconds. Be sure lids are snapped on tightly before beginning
8. Spin samples briefly (10-15 sec) at high speed in a microcentrifuge. This step is to ensure that the sample is inside the slurry of Chelex.
9. Incubate samples for 20 minutes at 95°C. The block temperature may drop slightly when doing this step. This drop is normal. Check tubes while incubating to ensure that lids have not popped off.
10. Vortex samples again for 10-15 seconds (Be careful as steam may pop lid off of centrifuge tube. Hold lids down).
11. Spin tubes again at high speed in microcentrifuge to ensure that all contents are in the bottom of the microcentrifuge tube.
12. Samples are ready to use (or not, see below). **ONLY USE SUPERNATE FOR PCR REACTIONS. CHELEX BEAD WILL INACTIVATE TAQ!**

ADVANTAGES & DISADVANTAGES

This is an effective method of DNA extraction. Its advantages are that it is cheap, quick, has a low contamination rate and does not use any dangerous chemicals. However, its disadvantages include being inefficient for use on blood samples, producing low purity DNA samples and being unsuitable for restriction fragment length polymorphism DNA profiling.

READINGS

<https://www.ncbi.nlm.nih.gov/pubmed/1867860>

<https://www.eeb.ucla.edu/Faculty/Barber/Web%20Protocols/Protocol2.pdf>

<http://www.fastbleep.com/biology-notes/41/122/1216>

FTA method

INTRODUCTION:

The FTA is an acronym for fast technology analysis. The FTA paper extraction method was initially used as a method of DNA collection in forensic science but due to the ease of the process has become a popular method of extraction.

Its Basic methodology is that

1. Sample source (usually blood) is dropped onto the paper and as it dries the cells are lysed and the DNA becomes trapped within the matrix of the paper.
2. The paper is punched to create discs, which are washed in a test tube.
3. The discs are then washed with a solvent and added to the PCR mix

PRINCIPLE

Biological samples, such as blood and saliva, adhere to the paper through the mechanism of entanglement, while the mixture of chemicals lyses cells and denatures proteins. Because nucleases are inactivated, the DNA is essentially stable when the sample is properly dried and stored. Nucleic acid damage from nucleases, oxidation, ultraviolet light (UV) damage, microbes, and fungus is reduced when samples are stored on the FTA card

MATERIALS

- FTA Purification Reagent
- TE Buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0)
- Solution 1: 0.1N NaOH, 0.3mM EDTA, pH 13.0
- Solution 2: 0.1M Tris-HCL, pH 7.0

METHODS

Procedure for wash protocol

1. Take a 6 mm punch using a regular single-hole paper punch from a dry spot and place in a 1.5 mL microtube. To ensure that there is no cross-contamination, rinse the cutting end of the punch with ethanol and let it dry between samples.
2. Add 1000 µl of FTA Purification Reagent to microtube and flash vortex 5 times or manually to mix.
3. Incubate for 5 minutes at room temperature (tube may be given moderate manual mixing or vortex if desired).
4. Remove and discard all spent FTA Purification Reagent using a pipette.
5. Repeat steps 2-4 twice, for a total of three (3) washes with FTA Purification Reagent.
6. Add 1000 µl of TE Buffer.

7. Incubate for 5 minutes at room temperature.
8. Remove and discard all spent TE Buffer with a pipette.
9. Repeat steps 6-8 twice for a total of three (3) washes with TE Buffer (punch should look white or pale with most of the blood removed).

Procedure for pH treatment

Time scheme: High pH for 5 minutes, neutralized for 10 minutes

1. Add 140 μ l of Solution 1 to a 6 mm punch washed as above.
2. Incubate 5 minutes at 65°C (deviation from FTA company protocol which incubates at room temperature).
3. Add 260 μ l of Solution 2 and flash vortex 5 times to mix.
4. Incubate 10 minutes at room temperature.
5. Flash vortex 10 times.
6. Remove punch and squeeze to recover maximum volume of elute (can use a clean pipette tip to remove punch).

Elute contains gDNA in TE (66mM Tris-HCL, 0.1mM EDTA). Use 0.5 μ l for a 25 μ l PCR reaction.

ADVANTAGES & DISADVANTAGES

A marketable advantage of the FTA[®] technology is that samples spotted on treated cards may be stored at room temperature. The chemicals on the FTA cards enhance the preservation of the DNA and inactivate many dangerous pathogens that may be found in liquid blood samples or dried biological stains. Because the cards are small in size (approximately 3.5" x 5"), they are easily packaged, shipped, and stored for data basing .its other advantages include being easily repeated, easily automated and the added bonus that there is no need to quantify DNA extracted by FTA before PCR. However, due to the smaller nature of the "discs" of DNA obtained by this method, static electricity often causes them to jump out of their set location, leading to contamination.

READINGS

<http://www.fastbleep.com/biology-notes/41/122/1216>

<https://www.promega.com/~media/files/resources/application%20notes/genetic%20identity/an102%20extraction%20and%20isolation%20of%20dna%20from%20blood%20cards%20and%20buccal%20swabs%20in%20a%2096%20well%20format.pdf?la=en>

https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1392818611307/litdoc28982222_20140311044843.pdf

Differential method

INTRODUCTION:

Differential extraction methods are used to separate spermatozoa from other cell types. Spermatozoa are more difficult to lyse than other cells and conditions can be set so that all cells except spermatozoa are lysed. The supernatant containing the DNA from these cells is removed from the sperm cells, which can then be lysed separately.

PRINCIPLE

Differential extraction (also known as differential lysis) refers to the process by which the DNA from two different types of cells can be extracted without mixing their contents. The most common application of this method is the extraction of DNA from vaginal epithelial cells and sperm cells from sexual assault cases in order to determine the DNA profiles of the victim and the perpetrator. Its success is based on the fact that sperm cells have protein disulfide bonds in their outer membrane which makes them more resilient to extraction than epithelial cells.

MATERIALS & METHODS

The differential extraction steps are:

- Optional wash step
 - Some laboratories have incorporated an optional wash step at the beginning of the procedure to remove cellular debris and contaminants. The sample is gently washed in a buffer and detergent and the supernatant is removed (wash fraction). This can be done under refrigerated conditions or at room temperature.
- Non-sperm cell lysis
 - An extraction buffer containing a buffer, detergent, and Proteinase K is added to the sample and incubated. This step lyses all cells except spermatozoa. The supernatant containing the DNA from the lysed cells (fraction 1) is removed after pelleting the spermatozoa. The sperm pellet is often washed numerous times with a buffer to remove excess DNA from this lysis step. If this wash is not done, it is not unusual to see a low level of fraction 1 DNA in fraction 2. If any of the sperm cells are weak or otherwise compromised, these may lyse in the first step, leaving a low level of fraction 2 DNA in fraction 1.
- Sperm cell lysis
 - The pelleted sperm cells are lysed under more stringent conditions, using a buffer, detergent, DTT, and a higher concentration of Proteinase K (fraction 2), and are subsequently incubated.
 - Both fractions (including the wash fraction, if appropriate) are extracted separately with the phenol/chloroform/isoamyl alcohol combination and purified.

ADVANTAGES & DISADVANTAGES

The success of differential extraction depends on the sperm head resisting the processes that readily lyse epithelial and white blood cells. Separating the sources of DNA from different contributors to a stain, namely a male donor and female victim, lessens the difficulty associated with mixture interpretation during data analysis, source attribution, and/or statistical calculations. The value of differential extraction is demonstrated by the requirement in the Quality Assurance Standards (QAS) for inclusion in the laboratory's recorded procedures. Details of the method are provided in the laboratory manual.

READINGS

http://www.nature.com/app_notes/nmeth/2012/120805/pdf/an8507.pdf

http://www.biotype.de/fileadmin/user/Dokumente/20120821_Biotype_Differential_Lysis_SOP.pdf

http://projects.nfstc.org/pdi/Subject03/pdi_s03_m02_03.htm

Method of RNA purification from blood

INTRODUCTION:

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. Several methods are used in molecular biology to isolate RNA from samples; the most common of these is TRIzol extraction.

Note:

This protocol assumes the investigator is beginning this with one full Yellow-Top (type A) BD Vacutainer tube of human blood (equals roughly 8 ml) to yield approximately 30 µg of RNA.

Additional Note:

RNA is very easily degraded by ever-present RNAses. Therefore, all of the tubes and solutions in this protocol must be RNase-free (autoclaving does NOT inactivate RNAses). One cannot overemphasize the need for a clean work environment when working with RNA.

PRINCIPLE:

Guanidinium isothiocyanate (powerful protein denaturant)

Inactivation of RNases

Acidic phenol/chloroform

Partitioning of RNA into aqueous supernatant for separation

Note: low pH is crucial since at neutral pH DNA not RNA partitions into the aqueous phase.

MATERIALS:

10x RBC Lysis Buffer

- 10.0 g KHCO_3
- 2.0 ml 0.5 M EDTA
- 89.9 g NH_4Cl

Dissolve the above in approximately 800 ml ddH₂O and adjust pH to 7.3. QS To 1 liter and mix thoroughly. This solution is stable for 6 months at 2 – 8° C in a tightly closed bottle.

1x RBC Lysis Buffer

Simply dilute the 10x stock solution 1:10 with ddH₂O. This is Stable for 1 week at room temperature.

TRIzol Reagent OR RNA STAT-60 Reagent

TRIzol Reagent Invitrogen Life Technologies: Cat No. 15596018

or

RNA STAT-60 Reagent Tel-Test: Cat No. CS-111

Other Reagents Needed

- Phosphate Buffered Saline (PBS)
- Isopropanol (2-propanol)
- Ethanol
- RNase-free water
- RNase-Away (a cleaning solution that neutralizes RNases on bench tops, pipettor, centrifuges, and other equipment).

METHODS:

- 1) Transfer contents of tube into a 50 ml polypropylene conical centrifuge tube.
- 2) Bring volume to 45 ml with RBC Lysis Buffer (recipe follows protocol).
- 3) Let stand at room temperature for 10 minutes.
- 4) Pellet cells at 600 x g (approx. 1,400 rpm) for 10 minutes in a room temp centrifuge (program#3).
- 5) Carefully decant supernatant.
- 6) Gently resuspend the pellet in 1 ml of RBC Lysis Buffer and transfer to a 1.5 ml microcentrifuge tube. – Let stand for 5 minutes.
- 7) Pellet cells for 2 minutes by centrifuging in a microfuge at room temperature at 3000 rpm.
- 8) Carefully aspirate the supernatant.
- 9) Resuspend the pellet in 1 ml of sterile DPBS.
- 10) Pellet cells as in step 7.
- 11) Carefully aspirate the supernatant.
- 12) Add 1200 µl of TRIzol solution to each tube and resuspend the cells. Note: for a full 8 ml blood tube, the 1200 µl TRIzol solution can be split into 2, 600 µl aliquots and frozen at -80 C until further processing.
- 13) Add 0.2 ml of Chloroform (CHCl₃) and vortex each tube for 15 seconds, ONE AT A TIME.
- 14) Centrifuge the samples at 13,000 rpm for 10 minutes at 4°C.
- 15) Remove the upper phase and transfer to a clean microcentrifuge tube. Be careful not to remove any of the white interface when collecting the upper phase of the extraction
- 16) For the future collection of micro RNA (miRNA), carefully remove ~20% of the volume of the upper phase from step 16 and place into another clean, labeled, 1.5ml microfuge tube. Store this aliquot at -80 C until further processing.
- 17) To the remaining upper phase from step 16, add an equal volume of cold isopropanol and invert to mix.
- 18) The samples can be placed in a -20°C freezer to precipitate.
- 19) Samples are centrifuged at 13,000 rpm for 10 minutes at 4°C.

Note: you may be able to see a small white pellet of RNA at the bottom of the tube after this step.

20) Carefully decant the supernatant, and rinse the pellet with 0.5 ml of ice-cold 75% ethanol. The 75% EtOH should be prepared RNase-free and stored at -20°C.

21) Centrifuge the samples at 13,000 rpm for 10 minutes at 4°C.

22) Decant the supernatant.

23) Using a pipettor, carefully remove all of the remaining liquid in the bottom of the tube.

24) Allow the pellet to dry for 5 to 10 minutes to remove any remaining ethanol.

25) Dissolve the RNA pellet by adding 20 µl of RNase-free H₂O to each sample.

26) RNA should be quantitated within 2 hours of elution. It can be kept at 4°C until that time; it can also be held temporarily at -20°C until permanent storage at -80°C. Repeated freeze-thaws are to be avoided, so RNA should be aliquoted for transfer as soon as possible after quantitation.

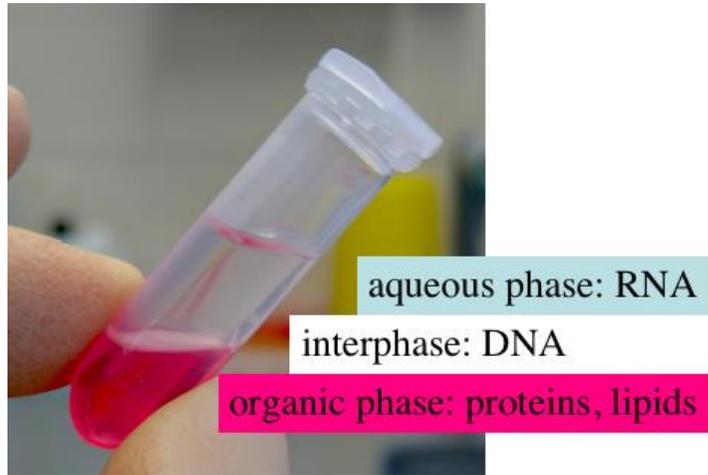


Figure 3: Phases of TRIzol

ADVANTAGES & DISADVANTAGES:

- TRIzol or tri (name depends on manufacturer) combines phenol and guanidine isothiocyanate and thereby some of the advantages of the above two
- Removes protein and DNA but depends on pipetting skills (disturbing the phases leads to contamination)
- RNA is protected by the reagent during the extraction procedure
- Phenol and chloroform are potentially harmful reagents (handle under the hood)

READINGS:

<https://www.thermofisher.com/pk/en/home/references/ambion-tech-support/rna-isolation/tech-notes/total-rna-from-whole-blood-for-expression-profiling.html>

<https://www.lerner.ccf.org/gmi/gmb/documents/RNA%20Isolation.pdf>

<http://www3.appliedbiosystems.com/sup/URLRedirect/index.htm?xDoD=4332809>

<https://www.ncbi.nlm.nih.gov/pubmed/17417019>

<http://www.nature.com/nprot/journal/v1/n2/full/nprot.2006.83.html>

<https://www.ncbi.nlm.nih.gov/pubmed/16028681>

http://openwetware.org/wiki/RNA_extraction_using_trizol/tri

http://openwetware.org/wiki/RNA_extraction#TRIzol_or_tri_followed_by_chloroform_and_precipitation

Affinity purification of total RNA.

INTRODUCTION:

High-quality mRNA is needed for a number of molecular biology techniques, including cDNA library construction. Not surprisingly, numerous mRNA extraction kits are now commercially available.

PRINCIPLE:

The affinity selection of polyadenylated mRNA using oligodeoxythymidylate (Oligo (dT)).

MATERIALS

- All materials used in this procedure should be sterile and of molecular biology grade.
- All Tris-containing solutions are prepared using RNase-free water and autoclaved.
- All other solutions, unless otherwise stated, should be treated directly with diethyl pyro carbonate (DEPC) and autoclaved. DEPC is an efficient, nonspecific inhibitor of RNase activity. It is, however, a carcinogen and should be handled in a fume hood with extreme care. Hands are a major source of RNase activity. Because of this, gloves should be worn for all procedures.
- RNase-free water: Add 0.1% DEPC to water. Allow to stand overnight at 37°C and autoclave to destroy residual DEPC activity. All solutions except Tris, which inactivates DEPC, can be treated in the same way.
- SDS (sodium dodecyl sulphate): SDS is dangerous if inhaled and should be weighed in a fume hood. A 10% stock solution is normally prepared. This solution is unstable if autoclaved, however any residual RNase activity can be destroyed by heating the solution at 65°C for 2 h.
- Oligodeoxythymidylate-cellulose (oligo (dT)): Oligo (dT) cellulose is available commercially.
- Although the binding capacity of oligo(dT) cellulose varies between different suppliers, a general rule is to use 25 mg of oligo(dT) for each 1 mg of total RNA. Suspend oligo (dT) cellulose in loading buffer at a concentration of 5 mg per 1 mL loading buffer.
- Oligo (dT) is insoluble and should be resuspended by gentle tapping or inversion. Do not put it in a vortex. It can be stored either dry at 4°C or in suspended in loading buffer at –20°C.

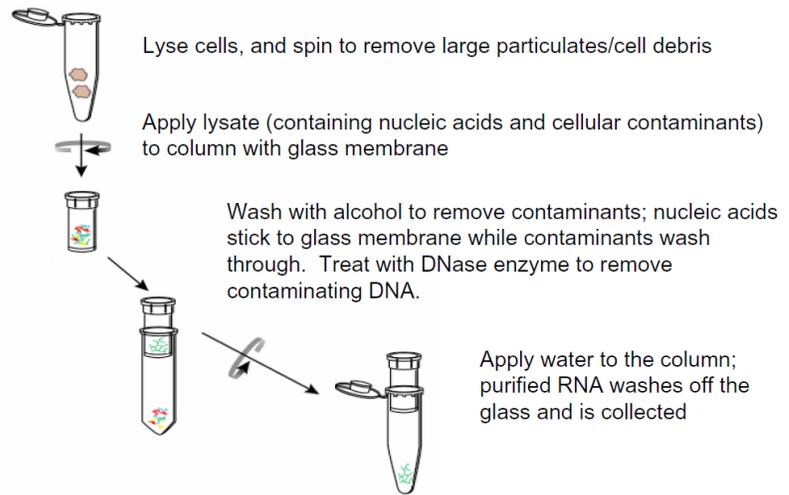


Figure 4: Affinity purification of total RNA.

- RNase-free glass wool and Pasteur pipets: Wrap both the glass wool and pipets in aluminium foil and bake at 200°C for 2–4 h to remove any RNase activity.
- 5 M NaCl: Store at room temperature.
- 3 M Sodium acetate pH6: Store at room temperature.
- Absolute alcohol: Store at –20°C.
- 70% ethanol: Prepare this solution using DEPC-treated water. Store at 4°C.
- Loading buffer: 0.5 M NaCl in 0.5% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 Store at room temperature.
- Elution buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The buffer can be stored at room temperature but should be preheated to 65°C prior to use.
- Recycling buffer: 0.1 M NaOH, which should be prepared immediately before use and used fresh.

METHODS

Preparing an Oligo (dT) Column

Oligo (dT) columns are available commercially or can be prepared by using a 1–3 mL syringe. Preparing your own columns is both easy and cheap.

1. Remove the plunger from the syringe and plug the base with glass wool.
2. Add oligo (dT) cellulose to the syringe using a sterile RNase-free Pasteur pipet. The oligo (dT) cellulose will collect, as a column, above the glass wool. The loading buffer will escape through the glass wool and can be discarded. To ensure that the oligo (dT) cellulose is packed and free from air locks, add 3 volumes of loading buffer using a pipette and allow the solution to run through the column. The column is now ready for immediate use and should not be allowed to run dry.

Isolation of Poly (A+) RNA:

1. Resuspend the RNA pellet in loading buffer or, if the buffer is in solution, add 1/10th volume of 5 M NaCl (see Note 1).
2. Heat denature the RNA and immediately load it onto the column and apply 3 volume of loading buffer.
3. Reapply the eluate to the column.
4. Wash with 3 volume of loading buffer (see Note 4). Discard eluate.
5. Recover the bound poly (A+) mRNA by adding 3 volume elution buffer. Collect the mRNA in a sterile tube on ice (see Note 5).
6. The mRNA is precipitated by adding 1/10th volume of 3 M sodium acetate and 2 volume of ice-cold absolute ethanol. An overnight precipitation at –20°C maximizes the precipitation of RNA.
7. Centrifuge at 15,000g for 15 min to pellet the RNA. Discard the supernatant.
8. Wash the RNA pellet in ice-cold 70% ethanol. Centrifuge at 15,000g for 5 min to repellet the RNA which may have been disturbed by washing. Discard the supernatant.

Isolation of mRNA:

9. Dry the RNA pellet. Once it is dry, resuspend it in DEPC-treated water.

10. Assess the purity and integrity of mRNA.

ADVANTGES & DISADVANTAGES:

- Eliminates need for organic solvents
- Compatible with a variety of sample types (tissue, tissue culture cells, white blood cells, plant cells, bacteria, yeast, etc.)
- DNase treatment eliminates contaminating genomic DNA
- Excellent RNA purity and integrity
- Less amount of RNA obtained.
- Expensive

READINGS

https://lifescience.roche.com/wcsstore/RASCatalogAssetStore/Articles/NAPI_Manual_page_166-169.pdf

<http://www.sabiosciences.com/newsletter/RNA.html>

<http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryId=11763&catalogId=10101&productId=&top=Y&storeId=11765&langId=-1>

<http://www.thermofisher.com/pk/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/mrna-extraction.html>

PCR and its types:

(Nested, Multiplex, Reverse transcriptase, Real time, hot start, Asymmetric, Long, Allele specific, colony, In-situ, Inverse, AFLP PCR).

INTRODUCTION:

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

PRINCIPLE

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers, the short DNA fragments containing sequences complementary to the target region along with a DNA polymerase, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

MATERIALS

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis.

Following reagents are required for PCR

- DNA template that contains the DNA region (target) to amplify
- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target
- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C
- Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

- Bivalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis
- Monovalent cation potassium ions

METHODS

The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps. In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2–3 discrete temperature steps, usually three (Figure below). The cycling is often preceded by a single temperature step at a high temperature ($>90\text{ }^{\circ}\text{C}$), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers.

Initialization step:

This step consists of heating the reaction to a temperature of $94\text{--}96\text{ }^{\circ}\text{C}$ (or $98\text{ }^{\circ}\text{C}$ if extremely thermostable polymerases are used), which is held for 1–9 minutes.

Denaturation step:

This step is the first regular cycling event and consists of heating the reaction to $94\text{--}98\text{ }^{\circ}\text{C}$ for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step:

The reaction temperature is lowered to $50\text{--}65\text{ }^{\circ}\text{C}$ for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should only bind to a perfectly complementary part of the template. If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically the annealing temperature is about 3–5 $^{\circ}\text{C}$ below the T_m of the primers used.

Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation. It is very vital to determine the annealing temperature in PCR. This is because in PCR, efficiency and specificity are affected by the annealing temperature. An incorrect annealing temperature will cause an error in the test.

Extension/elongation step:

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify. As a rule-of-thumb, at its optimum temperature, the DNA polymerase polymerizes a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation

This single step is occasionally performed at a temperature of 70–74 °C (this is the temperature needed for optimal activity for most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold:

This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Polymerase chain reaction - PCR

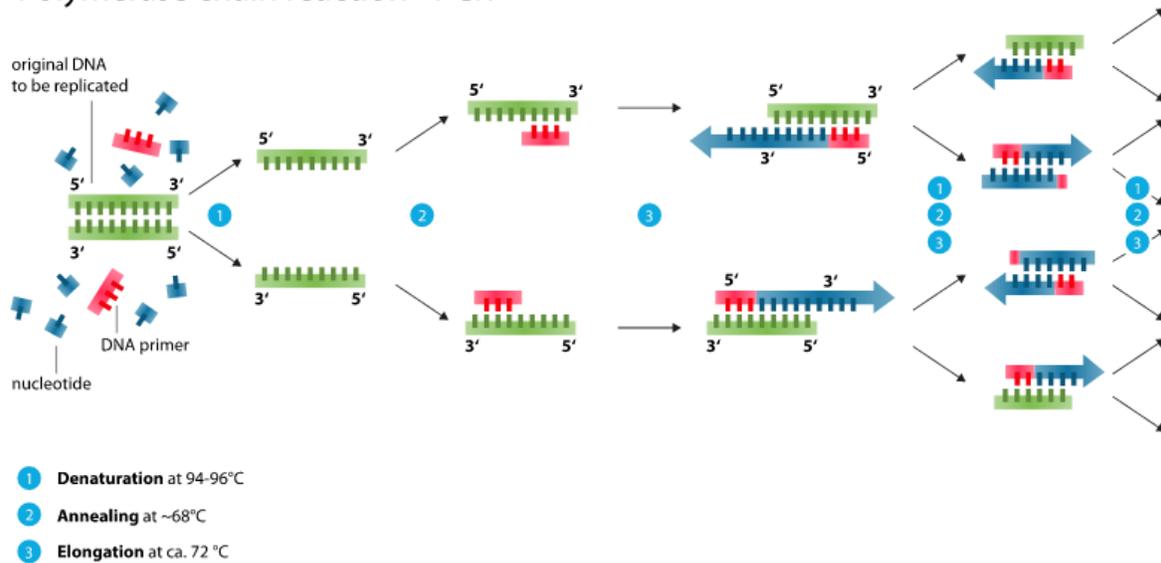


Figure 5: Polymerase Chain Reaction

ADVANTGES & DISADVATAGES

Selective DNA isolation, Amplification and quantification of DNA, Disease diagnosis, DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments that are made. Additionally, the specificity of the PCR fragments can mutate to the template DNA, due to nonspecific binding of primers. Furthermore, prior information on the sequence is necessary in order to generate the primers

PCR VARIATIONS:

Nested PCR:

It Increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

Multiplex-PCR:

It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

Reverse Transcription PCR (RT-PCR):

It is useful for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (Rapid Amplification of cDNA Ends).

Quantitative PCR (qPCR):

It is used to measure the quantity of a target sequence (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Quantitative PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative PCR has a very high degree of precision. Quantitative PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (real-time PCR) but this abbreviation should be used only for reverse transcription PCR. qPCR is the appropriate contraction for quantitative PCR (real-time PCR).

Hot start PCR:

A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95 °C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

Asymmetric PCR:

Preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two

complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (T_m) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Nanoparticle-Assisted PCR (nanoPCR):

In recent years, it has been reported that some nanoparticles (NPs) can enhance the efficiency of PCR (thus being called nanoPCR), and some even perform better than the original PCR enhancers. It was also found that quantum dots (QDs) can improve PCR specificity and efficiency. Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are efficient in enhancing the amplification of long PCR. Carbon nanopowder (CNP) was reported to be able to improve the efficiency of repeated PCR and long PCR. ZnO, TiO₂, and Ag NPs were also found to increase PCR yield. Importantly, already known data has indicated that non-metallic NPs retained acceptable amplification fidelity. Given that many NPs are capable of enhancing PCR efficiency, it is clear that there is likely to be great potential for nanoPCR technology improvements and product development.

Allele-specific PCR:

A diagnostic or cloning technique based on single-nucleotide variations (SNVs not to be confused with SNPs) (single-base differences in a patient). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNV (base pair buffer around SNV usually incorporated). PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

Inverse PCR:

It is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self-ligation, resulting in known sequences at either end of the unknown sequence.

Colony PCR:

Colony PCR is a method used to screen for plasmids containing a desired insert directly from bacterial colonies without the need for culturing or plasmid purification steps.

In situ PCR:

In situ PCR is a method in which the polymerase chain reaction actually takes place in the cell on a slide, and the product can be visualized in the same way as in traditional in situ hybridization.

AFLP-PCR:

AFLP-PCR uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The amplified fragments are separated and visualized on denaturing polyacrylamide gels, either through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments.

READINGS

https://en.wikipedia.org/wiki/Polymerase_chain_reaction

file:///C:/Users/User/Desktop/Polymerase_chain_reaction.svg

<http://link.springer.com/protocol/10.1385%2F1-59259-384-4%3A3>

<https://www.ncbi.nlm.nih.gov/pubmed/2999980>

http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1993/mullis-lecture.html

<http://www.ncbi.nlm.nih.gov/tools/epcr/>

Analysis Of proteins by SDS

SDS gel preparation:

To make 12% SDS-PAGE gel, the resolving gel components for 20 ml were added as follows.

a) Resolving Gel:

Component	Amount(mL)
Aqua dest.	8.6
40 % acrylamide mix	6
Tris (1.5 M, pH 8.8)	5

- 200 μ L of 10% SDS solution was added
- 20 μ L of APS (25%) was added
- 20 μ L of TEMED was added.
- This gel mixture was added to the Gel apparatus after careful mixing and avoiding froth. Isopropanol was added on top of the gel to remove the bubbles and even the upper front of the gel.

b) Stacking Gel:

The resolving gel is allowed to polymerize and then stacking gel mixture is added to at the top after removing isopropanol layer.

For 5 ml of stacking gel

Component	Amount(mL)
Aqua dest.	3.645
40 % acrylamide mix	0.625
Tris (1.0, pH 6.8)	0.630

- 50 μ L of 10% SDS solution was added
- 05 μ L of APS (25%) was added
- 05 μ L of TEMED was added.
- This gel mixture was added to the Gel apparatus after careful mixing and avoiding froth.
- Combs were placed avoiding entrapment of any air bubbles.

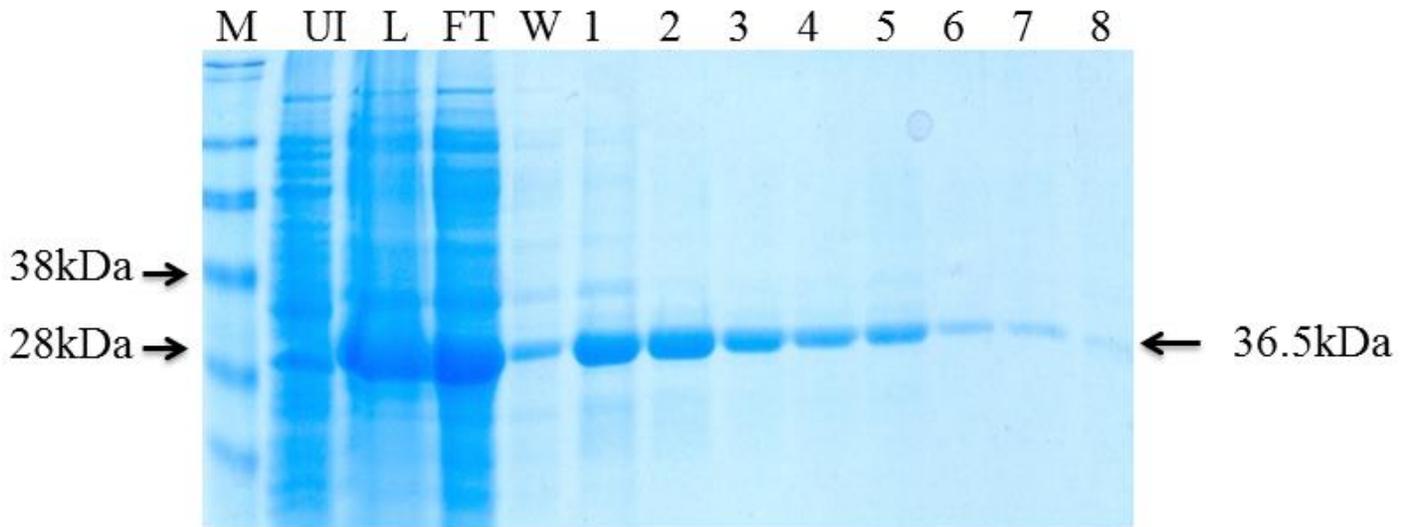


Figure 1: Purification of His-tagged PROTEIN (326 aa) by Ni-NTA affinity purification. Protein expression induced by 0.5 mM IPTG for 3 hours was used for affinity purification using Ni-NTA beads. Uninduced was collected before induction, load was lysate sample before addition of Ni-NTA beads. Flow-through is lysate which passed through the column, Wash is wash buffer passed through the column, while rest are the eluted fractions. All the protein samples were boiled in SDS loading buffer and resolved on SDS-PAGE where expected 36 kDa protein is visible in eluted fractions (1-5). M: protein ladder UI: uninduced; L: Lysate/load; FT: Flow through; W: Wash; 1-4: serial elutions with 100mM imidazole; 5-6: serial elutions with 250mM imidazole and 7-8: serial elutions with 400mM imidazole. Stained with colloidal coomassie stain.

Comparing plasmids of different molecular weights using molecular weights marker

Principle:

Plasmid DNA isolation

- A single white bacterial colony of the transformant will be inoculated into 5 mL of LB medium containing 100 µg/mL ampicillin in a test tube.
- The culture will be incubated overnight at 37°C in shaking incubator.
- Three mL of culture will be poured into new microfuge tube and centrifuge at 12000 rpm for 1 min and supernatant will be discarded.
- Pellet will be resuspended into 100 µL of ice-cold solution I by vigorous shaking
- Then 200 µL of freshly prepared solution II will be added. Contents will be mixed by inverting the tube gently 4-5 times
- Then tube will be incubated on ice for 5 min.
- Finally 150 µL of Ice-cold solution III will be added. The tube will be inverted gently several times to disperse the solution III through the viscous bacterial lysate.
- Tube will be stored on ice for 3 -5 min and centrifuge at 12000 rpm for 5 min at 4°C.
- Supernatant will be transferred to fresh tube and equal volume of phenol:chloroform (1:1) will be added and centrifuge at 12000 rpm for 5 min.
- Supernatant will be transferred to fresh tube.
- DNA will be precipitated with 2 volume of absolute ethanol and store at -20°C for half an hour. Centrifugation will be done at 12000 rpm for 10 min at 4°C.
- Supernatant will be discarded and the tubes will be allowed to stand on paper towel in an inverted position to allow all the solution to dry away.
- Pellet will be washed with 500 µL of 70% ethanol.
- Supernatant will be removed and pellet allow to air dry .
- Eighty µL of water and 1 µL of RNase will be added and left for 30 min at 37°C and then stored at -20°C.
- The isolated plasmid will be analyzed on 1% agarose gel electrophoresis.

Restriction analysis

- The presence of insert in pTZ57R/T will be confirmed by restriction digestion of the recombinant plasmid.
- Initially the plasmid will be restricted with suitable restriction enzyme using 2X Tango buffer.

- The reaction mixtures each of 15 μL will be prepared as mentioned in below table 8.1 and then incubated at 37°C for 4 h.
- Electrophoresis will be done with 1% agarose gel to visualize the required product under UV light.

Table 8.1: Restriction analysis

Reagents	Sample Amylase
10X Yellow-Tango buffer	3 μL
<i>EcoRI</i>	1 μL
<i>HindIII</i>	1 μL
DNA	10 μL
Total	15 μL