BIO 301

ESSENTIALS OF GENETICS

LABORATORY MANUAL

DEPARTEMENT OF BIOLOGY

VIRTUAL UNIVERSITY OF PAKISTAN

Contents

Practical 1:
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).

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.

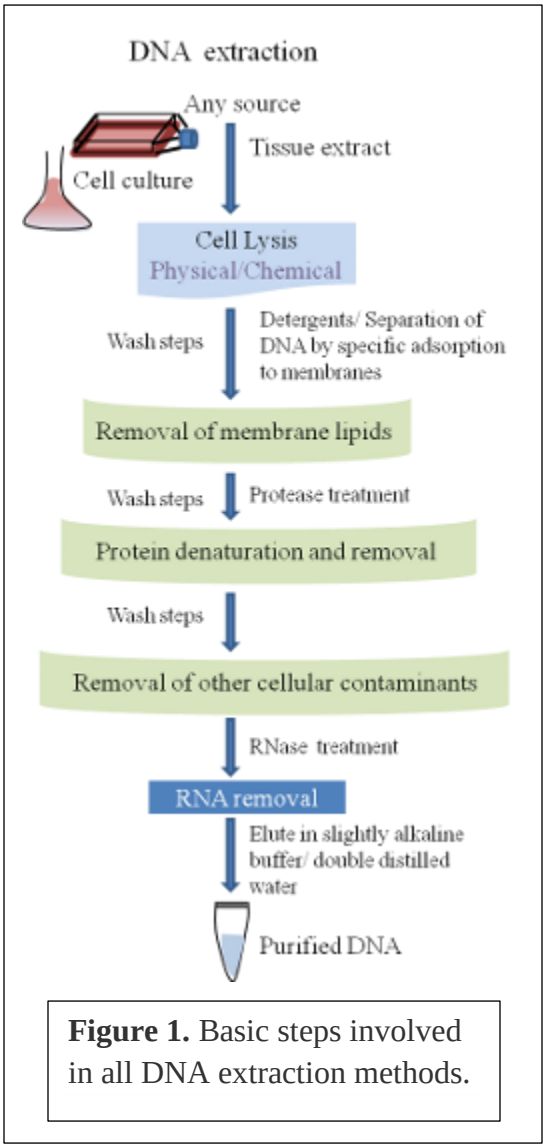


Figure 1. Basic steps involved in all DNA extraction methods.

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.

Practical: 2
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 to 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 (<200µL), 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

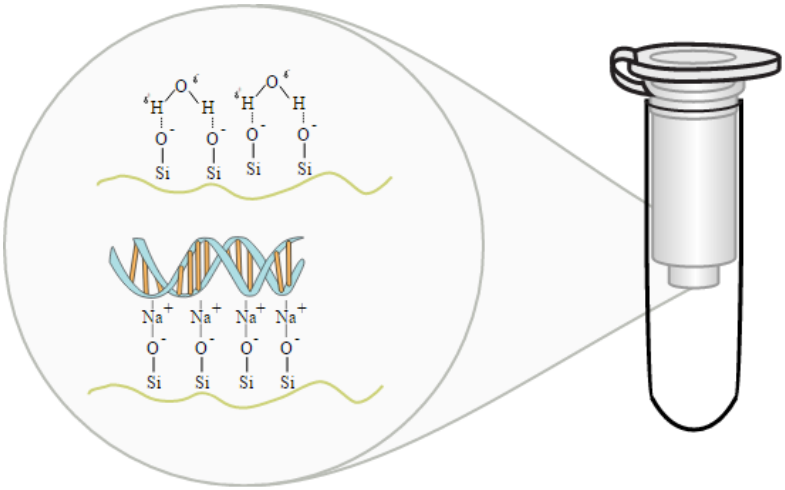
Silica Adsorption Method:

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.

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 µl 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:

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.

READINGS:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3792701/

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.

MATARIALS
- 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, it's 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

Fast Technology Analysis 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

MATARIALS

- 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 µl of Solution 1 to a 6 mm punch washed as above.
2. Incubate 5 minutes at 65^{o}C (deviation from FTA company protocol which incubates at room temperature).
3. Add 260 µl 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, 0.1nm EDTA). Use 0.5µl for a 25µl 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.

MATARIALS & 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

Practical: 3
Methods 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 ddH_2O 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 ddH_2O. 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.

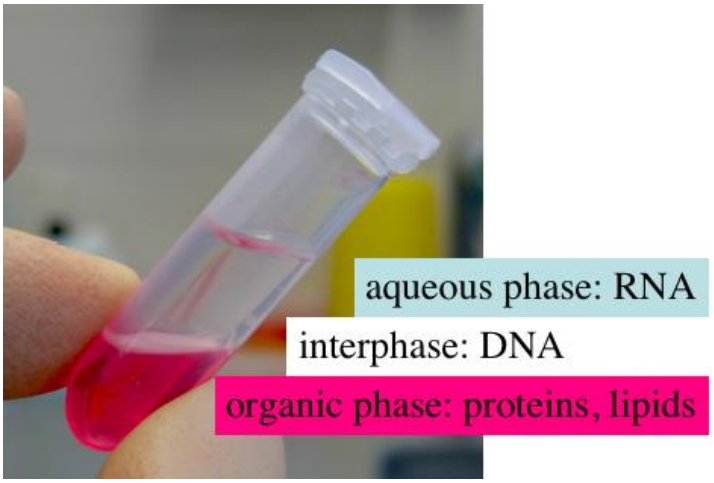


Figure 3: Phases of TRIzol

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

Practical: 4
Quantification of Nucleic acids:

Quantitation of nucleic acids is commonly performed to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity. Reactions that use nucleic acids often require particular amounts and purity for optimum performance. There are several methods to establish the concentration of a solution of nucleic acids, including spectrophotometric quantification and UV fluorescence in presence of a DNA dye.

Spectrophotometry

INTRODUCTION:

Nucleic acids absorb ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample.

PRINCIPLE:

Using the Beer-Lambert Law it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the average extinction coefficient for double-stranded DNA is 0.020 (μg/ml) −1 cm−1, for single-stranded DNA it is 0.027 (μg/ml) −1 cm−1, for single-stranded RNA it is 0.025 (μg/ml) −1 cm−1 and for short single-stranded oligonucleotides it is dependent on the length and base composition. Thus, an Absorbance (A) of 1 corresponds to a concentration of 50μg/ml for double-stranded DNA. This method of calculation is valid for up to an A of at least 2. A more accurate extinction coefficient may be needed for oligonucleotides; these can be predicted using the nearest-neighbor model.

MATERIALS:

- UV/VIS Spectrophotometer
- 1 mL quartz cuvette
- DNA sample(s)
- TE Buffer
- Disposable 1 mL polyethylene Transfer Pipettes (Berol) [2 per group]
- Eppendorf tubes (1.5 mL) [2 per group]
- Ruler
- Kimwipes

METHODS:

I. Setting up the Spectrophotometer (Beckman DU64)

1. Turn on the spectrophotometer at the power strip. Check that the printer is on line and ready.
2. Turn on the UV lamp source and allow it to warm up for 5 minutes.
3. Select the absorbance reading mode (ABS key).
4. Press the SCAN key "Edit" will be displayed.
5. Enter the starting wavelength as 280 nanometers (nm) and press enter.
6. Enter the ending wavelength as 260 nm and press enter.
7. The speed for the scan of the sample will be displayed. It should read 750 nm/min. If it does not, press the STEP key and scroll through the options until 750 nm/min is displayed. Press enter.
8. Upper limit will be displayed. Set the upper limit at 2,000 absorbance. Press enter.
9. Lower limit will be displayed. Set the lower limit at 0.000 absorbance. Press enter. The starting wavelength will reappear.
10. The instrument is now ready to be calibrated against a control solution. The purpose of the calibration is to measure and then subtract from the samples absorbance any absorbance from the buffer solution.
11. Place 200 microliters (μL) of the TE Buffer into the quartz cuvette. This is the solution you will use to calibrate the instrument.
12. Open the sample compartment lid on the instrument.
13. Carefully wipe the cuvette with a Kimwipe and be careful not to get fingerprints on the quartz panels. Place the cuvette into the cuvette holder so that the quartz sides are in the path of the light source (left to right).
14. Close the sample compartment lid.
15. Press CALB. The absorbance of the TE Buffer solution will now be recorded in memory as the "background" and "Bkg" will be displayed.
16. Press READ, Calibration is complete when "Scan" is displayed. The instrument is now ready to measure DNA samples.
17. Open the sample compartment lid and remove cuvette.
18. Discard the 200 μL of TE Buffer.
19. Rinse the cuvette twice with the TE Buffer solution and drain the cuvette onto a Kimwipe.

II. Sample Preparation

20. Place 200 μL of the DNA sample in the cuvette and place the cuvette in the sample holder.

21. Press READ. The absorbance of the sample between 260 and 280 nm will be measured and plotted as a graph on the printer.

22. Repeat steps 20 and 21 for any other DNA samples that you have been assigned.

ADVANTGES & DISADVATAGES

It is an easier and cost effective method with reliable results if performed adequately. The protein contamination cannot be reliably assessed with a 260:280ratio; this also means that it contributes little error to DNA quantity estimation.

READINGS

http://www2.le.ac.uk/departments/emfpu/genetics/explained/quantification
https://www.ncbi.nlm.nih.gov/pubmed/18201813
https://www.ncbi.nlm.nih.gov/pubmed/7702855

Agarose Gel Electrophoresis

INTRODUCTION:
Agarose gel electrophoresis is another way to quickly estimate DNA concentration.

PRINCIPLE
The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, the DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity. Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA will be distinct.

MATERIALS
To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are required.

METHODS:
A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field. Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/µl (100ng divided by 2µl). Standards used for quantitation should be labeled as such and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal.

ADVANTGES & DISADVATAGES

- Easiest and quick method
- Ethidium bromide is a known mutagen; precautions need to be taken for its proper use and disposal.

READINGS
https://worldwide.promega.com/resources/pubhub/enotes/how-do-i-determine-the-concentration-yield-and-purity-of-a-dna-sample/

http://www.ncbi.nlm.nih.gov/pubmed/21638536

Practical: 5
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.

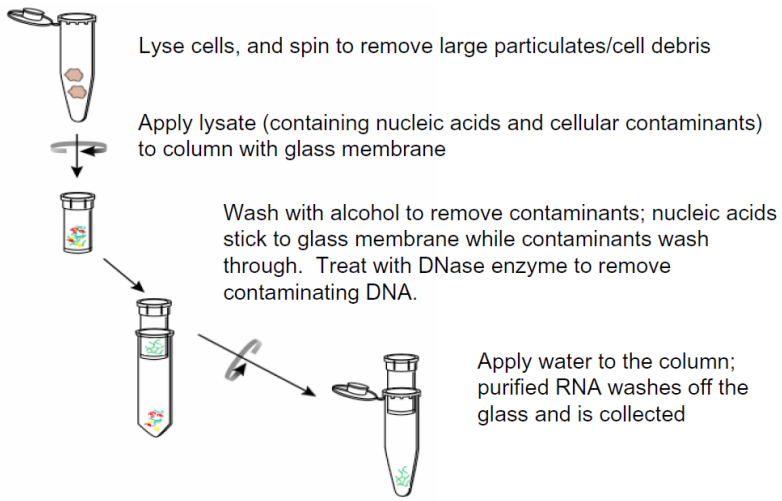


Figure 4: **Affinity purification of total RNA.**

PRINCIPLE:

The affinity selection of polyadenylated mRNA using oligodeoxthymidylate (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.
- Oligodeoxthymidylate-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.
- 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

Practical: 6
Chromosome staining, banding of chromosomes (C, Q, R, T).

INTRODUCTION:

Chromosomes display a banded pattern when treated with some stains. Bands are alternating light and dark stripes that appear along the lengths of chromosomes.

Unique banding patterns are used to identify chromosomes and to diagnose chromosomal aberrations, including chromosome breakage, loss, duplication, translocation or inverted segments.

A range of different chromosome treatments produce a range of banding patterns: Q-bands, R-bands, C-bands and T-bands.

In recent years a number of chromosome banding techniques have been developed that employ molecular cytogenetic techniques, for example fluorescence in situ hybridization (FISH).

Chromosome structure and bands

The chromosomes of eukaryotes are composed of a combination of nuclear DNA and proteins. During mitosis chromosomes replicate and condensed through coiling forming chromosomes consisting of two chromatids joined at the centromere. Each chromatid condenses approximately ten-thousand fold reaching maximal condensation at metaphase – DNA of roughly 5 cm in length is condensed to 5 micrometers in a metaphase chromosome. These condensed chromosomes are visible under the light microscope.

During the condensation process DNA is looped around protein complexes called nucleosomes. This primary structure then undergoes more cycles of coiling producing the well-known metaphase chromosome structure. Some of the looped segments of DNA are close together and condense more than others, forming regions known as domains. These closely condensed domains tend to stain more darkly than the areas where the loops are more loosely arranged.

In the order Diptera, which includes Drosophila, salivary gland chromosomes undergo repeated rounds of replication without cell division forming highly replicated chromosomes – polytene chromosomes. A polytene chromosome in a Drosophila salivary gland cell can contain as many as five thousand alternating dark and light bands. In these chromosomes the dark bands correspond to highly condensed domains and the lighter bands to less condensed DNA. It is in the less condensed areas where active genes can be identified. A gene becomes active by

unravelling to permit transcription into messenger RNA. These unravelled regions are observed as "puffs" under the microscope. The gene becomes inactive by resolving the "puff" through condensation.

C-Banding

C-banding stains areas of heterochromatin, which is tightly packed and repetitive DNA. C-banding is specifically useful in humans to stain the centromeric chromosome regions and other regions containing constitutive heterochromatin - secondary constrictions of human chromosomes 1, 9, 16, and the distal segment of the Y chromosome long arm.

Q-Banding

Quinacrine mustard, an alkylating agent, was the first chemical to band chromosomes viewed under a fluorescence microscope. Quinacrine dihydrochloride has subsequently been substituted by quinacrine mustard. The alternating bands of bright and dull fluorescence are called Q bands. The bright bands are primary composed of DNA rich in adenine and thymine, while the dull bands are rich in guanine and cytosine.

Q bands are especially useful for distinguishing the human Y chromosome and various chromosome polymorphisms involving satellites and centromeres of specific chromosomes.

R-banding

Reverse banding (R-banding) involves the incubation of slides containing metaphase chromosomes in hot phosphate buffer and stained with Giemsa. The banding pattern that results is essentially the reverse of G bands. R bands are GC-rich. The AT-rich regions are selectively denatured by heat leaving the GC-rich regions intact. Fluorochromes that are GC specific also produce a reverse chromosome banding pattern. R-banding is helpful for analyzing the structure of chromosome ends, since these areas usually stain light with G-banding.

T-Banding

T-banding involves the staining of telomeric regions of chromosomes using either Giemsa or acridine orange after controlled thermal denaturation. T bands apparently represent a subset of the R bands because they are smaller than the corresponding R bands and are more strictly telomeric.

MATERIALS & METHODS

Harvesting of culture

- Spindle inhibitors – Colchicine/colcemed (0.1μg/ml)
- Hypotonic treatment – 0.075M KCl
- Fixation (3:1 methanol : acetic acid)
- Preparation of slides
- Slides stained with 4% Giemsa for 20-25min
- Screening of slides to study the morphology of chromosome

- Construction of karyotype

C-banding

- Treat the slides in 0.2 N HCI for one hr at room temperature.
- Rinse in de-ionized water.
- Immerse in 1% barium hydroxide at 50°C for 5-15 min.
- Rinse in deionized water.
- Incubate at 60°C in 2XSSC buffer for one hr.
- Rinse in de-ionized water and stain in 4% Giemsa stain for 90 min.
- Rinse in de-ionized water, dry and examine under oil immersion.

Q-banding

- Dehydrate the slides by dipping in alcohol with decreasing concentration 90%, 70% and 50% one min each.
- Rinse in distilled water. .
- Wash the slide in phosphate buffer at pH 6.8.
- Stain the slide in quinacrine mustard (5 mg in 100 mI) or in quinacrine dihydrochloride 5% for 20 min.
- Rinse in phosphate buffer and mount in the same buffer.
- Examine under fluorescent microscope.

R-banding

- Age the slides for 7 -10 days .
- Place the slides in a Coplinjar containing phosphate buffer ofpH 6.5 at 85°C and incubate for 20-25 min.
- Stain the slides in 0.01% acridine orange in the phosphate buffer pH 6.5 for 4-6 min. Rinse in phosphate buffer and mount in the same buffer.
- Examine under fluorescent microscope.

T -banding

- Age the slide for 7 days.
- Place the slides in PBS pH 5.0 for 20-60 min at 87°C.
- Rinse in PBS.
- Stain in 3% Giemsa in phosphate buffer pH 6.8 at 87°C, leave for 5-30 min and rinse.
- Slides are stained in Hoechst 33258 stain for 10 min (Hoechst stain 0.5 pg/m1 of phosphate buffer).Rinse in phosphate buffer and examine in fluorescent microscope.
- Alternatively, the stained slides are covered with a cover slip and placed in a wet chamber under UV lamp for 2 to 3 hrs or under direct sunlight for 2 hrs.
- Remove the cover slip and stain in Giemsa stain for 10 min.
- Rinse in buffer, dry and mount in DPX.

READINGS:

http://geneticssuite.net/node/25

https://www.researchgate.net/file.PostFileLoader.html?id...assetKey...

www.pathology.washington.edu/.../main.php?file=banding%20patterns

www.pitt.edu/~super7/31011-32001/31081.ppt

Practical: 7
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 °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 (Tm) of the primers.

Initialization step:

This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °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 94–98 °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 50–65 °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 °C below the Tm 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.

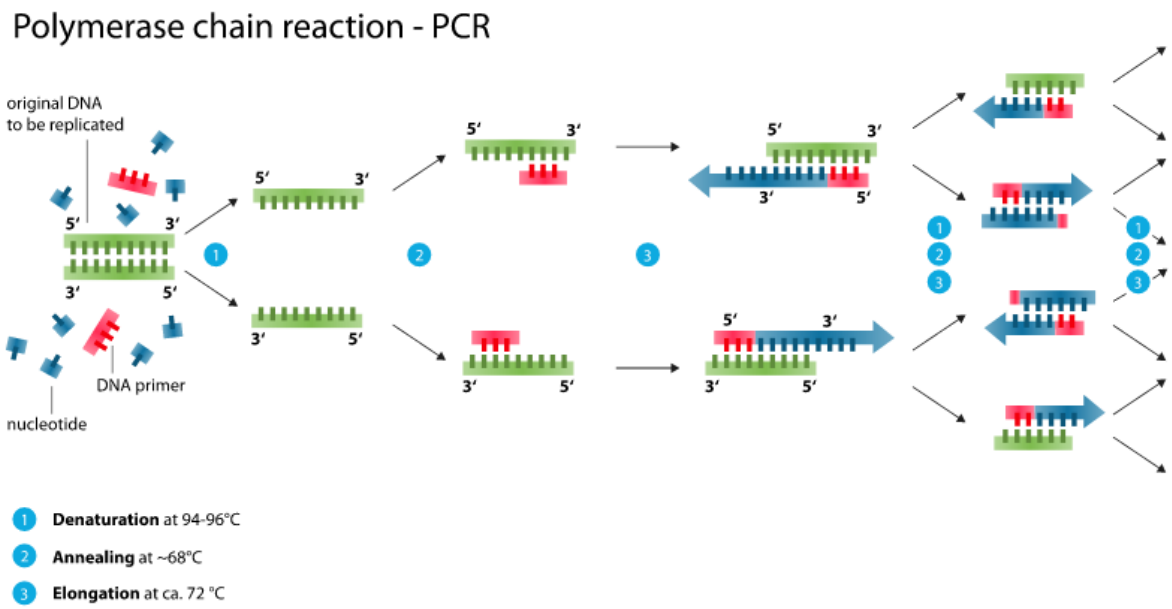


Figure 5: Polymerase Chain Reaction

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 contractions 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 (Tm) 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 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/

Practical: 8
Southern blotting

INTRODUCTION:

Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples.

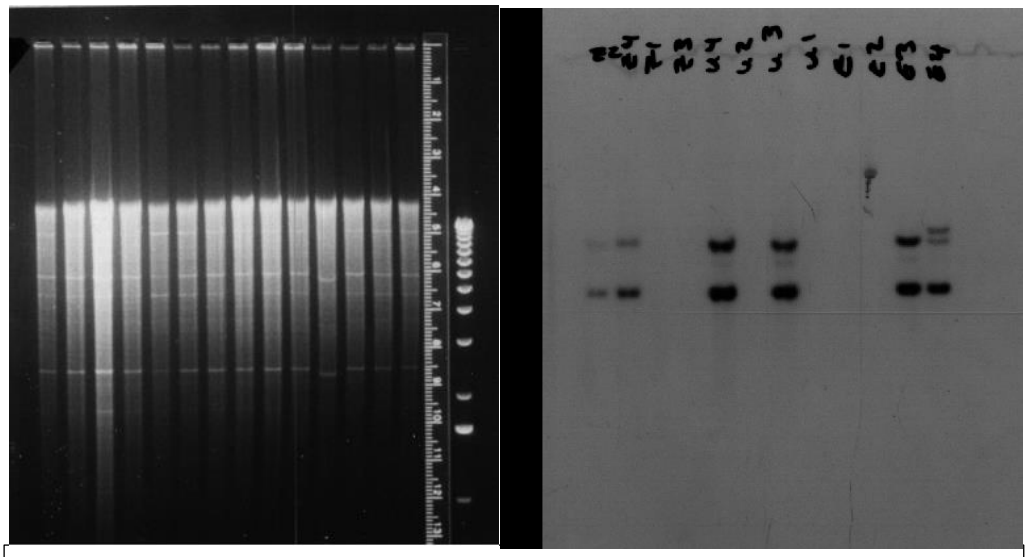


Figure 6. The figure on the left shows a photograph of a 0.7% agarose gel that has 14 different samples loaded on it (plus molecular weight marker in the far right lane and a glowing ruler used for analysis of the results). Each sample of DNA has been digested with the same restriction enzyme (EcoRI). Notice that the DNA does not appear as a series of discrete bands but rather as a smear. The DNA was transferred to nitrocellulose and then probed with a radioactive fragment of DNA that was derived from the transformed gene. The figure on the right is a copy of the X-ray film and reveals which strains contain the target DNA and which ones do not.

PRINCIPLE:

Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

MATERIALS &METHODS:

1) DNA (genomic or other source) is digested with a restriction enzyme and separated by gel electrophoresis, usually an agarose gel. Because there are so many different restriction fragments on the gel, it usually appears as a smear rather than discrete bands. The DNA is denatured into single strands by incubation with NaOH.

2) The DNA is transferred to a membrane which is a sheet of special blotting paper. The DNA fragments retain the same pattern of separation they had on the gel.

3) The blot is incubated with many copies of a probe which is single-stranded DNA. This probe will form base pairs with its complementary DNA sequence and bind to form a double-stranded DNA molecule. The probe cannot be seen but it is either radioactive or has an enzyme bound to it (e.g. alkaline phosphatase or horseradish peroxidase).

4) The location of the probe is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen or gives off light which will expose X-ray film. If the probe was labeled with radioactivity, it can expose X-ray film directly.

ADVANTGES & DISADVATAGES

Southern blots allow investigators to determine the molecular weight of a restriction fragment and to measure relative amounts in different samples.

READINGS

http://www.bio.davidson.edu/genomics/method/Southernblot.html

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

https://askabiologist.asu.edu/southern-blotting

https://www.mun.ca/biology/scarr/Gr12-18.html

Practical: 9
Northern blotting

INTRODUCTION:

The northern blot is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample

PRINCIPLE:

Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting.

MATERIALS & METHODS

Northern blots allow investigators to determine the molecular weight of an mRNA and to measure relative amounts of the mRNA present in different samples.

1) RNA (either total RNA or just mRNA) is separated by gel electrophoresis, usually an agarose gel. Because there are so many different RNA molecules on the gel, it usually appears as a smear rather than discrete bands.

2) The RNA is transferred to a sheet of special blotting paper called nitrocellulose, though other types of paper, or membranes, can be used. The RNA molecules retain the same pattern of separation they had on the gel.

3) The blot is incubated with a probe which is single-stranded DNA. This probe will form base pairs with its complementary RNA sequence and bind to form a double-stranded RNA-DNA molecule. The probe cannot be seen but it is either radioactive or has an enzyme bound to it (e.g. alkaline phosphatase or horseradish peroxidase).

4) The location of the probe is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen or gives off light which will expose X-ray film. If the probe was labeled with radioactivity, it can expose X-ray film directly.

ADVANTGES & DISADVATAGES

Analysis of gene expression northern blotting is able to detect small changes in gene expression that microarrays cannot. The microarrays have over northern blots is that thousands of genes can be visualized at a time, while northern blotting is usually looking at one or a small number of genes.

A problem in northern blotting is often sample degradation by RNAses. The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material;

ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity, which is important to reduce false positive results.
The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.

READINGS

http://www.bio.davidson.edu/courses/genomics/method/northernblot.html

https://www.thermofisher.com/pk/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/northern-blotting.html

http://www.nature.com/scitable/definition/northern-blot-287

Practical: 10

Western blotting.

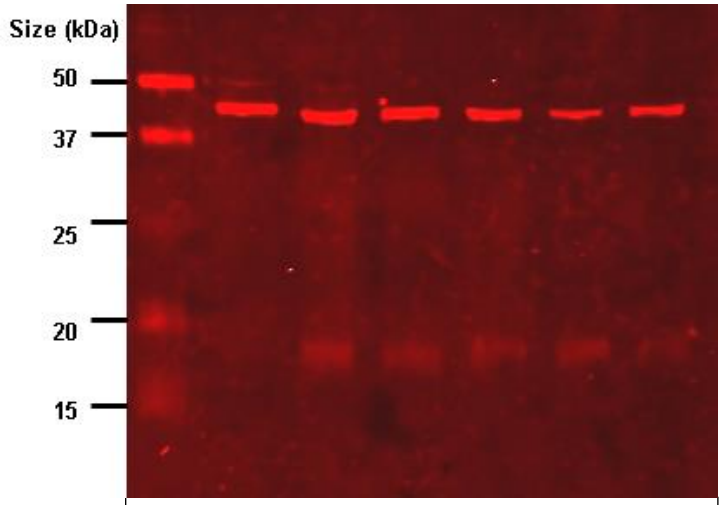


Figure: 7 Western blots using an antibody that recognizes proteins modified with lipoic acid.

INTRODUCTION:

The western blot (sometimes called the protein immunoblotting) is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract.

PRINCIPLE

It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein. The gel electrophoresis step is included in western blot analysis to resolve the issue of the cross-reactivity of antibodies.

MATERIALS & METHODS:

Western blots allow investigators to determine the molecular weight of a protein and to measure relative amounts of the protein present in different samples.

1. Proteins are separated by gel electrophoresis, usually SDS-PAGE.
2. The proteins are transferred to a sheet of special blotting paper called nitrocellulose, though other types of paper, or membranes, can be used. The proteins retain the same pattern of separation they had on the gel.
3. The blot is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the nitrocellulose. An antibody is then added to the solution which is able to bind to its specific protein. The antibody has an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) or dye attached to it which cannot be seen at this time.
4. The location of the antibody is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen and photographed.

ADVANTGES & DISADVATAGES

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted

on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.

- A western blot is also used as the definitive test for bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ western blotting.
- A western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, a western blot is sometimes used to confirm FIV+ status in cats.

READINGS:

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

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3456489/

https://www.thermofisher.com/pk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-western-blotting.html

https://www.abdserotec.com/western-blotting.html

http://www.bio-rad.com/en-us/applications-technologies/introduction-western-blotting

http://www.nature.com/scitable/definition/western-blot-288

http://www.bio.davidson.edu/courses/genomics/method/westernblot.html

Practical: 11
Genetics problems

Simple Genetics Practice Problems

1. For each genotype, indicate whether it is heterozygous (HE) or homozygous (HO)

AA ____ Bb ____ Cc ____ Dd ____	Ee ____ ff ____ GG ____ HH ____	Ii ____ Jj ____ kk ____ Ll ____	Mm ____ nn ____ OO ____ Pp ____

2. For each of the genotypes below, determine the phenotype.

Purple flowers are dominant to white flowers PP _____ Pp _____ pp _____	*Brown eyes are dominant to blue eyes* BB _____ Bb _____ bb _____
Round seeds are dominant to wrinkled RR _____ Rr _____ rr _____	*Bobtails are recessive (long tails dominant)* TT _____ Tt _____ tt _____

For each phenotype, list the genotypes. (Remember to use the letter of the dominant trait)

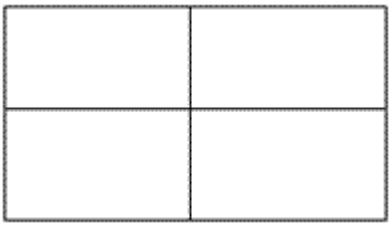
Straight hair is dominant to curly.	*Pointed heads are dominant to round heads.*
_____ straight _____ straight _____ curly	_____ pointed _____ pointed _____ round

4. Set up the square for each of the crosses listed below. The trait being studied is round seeds (dominant) and wrinkled seeds (recessive)

Rr x rr

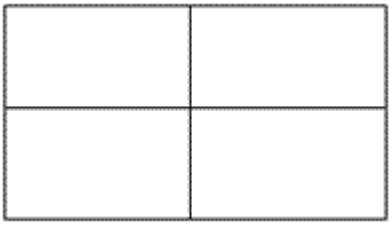
What percentage of the offspring will be round? _____

Rr x R r



What percentage of the offspring will be round? _____

RR x Rr



What percentage of the offspring will be round? _____

Practice with Crosses. Show all work!

5. A TT (tall) plant is crossed with a tt (short plant).
What percentage of the offspring will be tall? _____

6. A Tt plant is crossed with a Tt plant.
What percentage of the offspring will be short? _____

7. A heterozygous round seeded plant (Rr) is crossed with a homozygous round seeded plant (RR). What percentage of the offspring will be homozygous (RR)? _____

8. A homozygous round seeded plant is crossed with a homozygous wrinkled seeded plant. What are the genotypes of the parents? _____ X _____

What percentage of the offspring will also be homozygous? _____

9. In pea plants purple flowers are dominant to white flowers. If two white flowered plants are cross, what percentage of their offspring will be white flowered? _____

10. A white flowered plant is crossed with a plant that is heterozygous for the trait. What percentage of the offspring will have purple flowers? _____

11. Two plants, both heterozygous for the gene that controls flower color are crossed. What percentage of their offspring
will have purple flowers? _____.
What percentage will have white flowers? _____

12. In guinea pigs, the allele for short hair is dominant.
What genotype would a heterozygous short haired guinea pig have? _____
What genotype would a purebreeding short haired guinea pig have? _____
What genotype would a long haired guinea pig have? _____

13. Show the cross for a pure breeding short haired guinea pig and a long haired guinea pig.
What percentage of the offspring will have short hair? _____

14. Show the cross for two heterozygous guinea pigs.
What percentage of the offspring will have short hair? _____.
What percentage of the offspring will have long hair? _____

15. Two short haired guinea pigs are mated several times. Out of 100 offspring, 25 of them have long hair. What are the probable genotypes of the parents?

_____ X _____ Show the cross to prove it!