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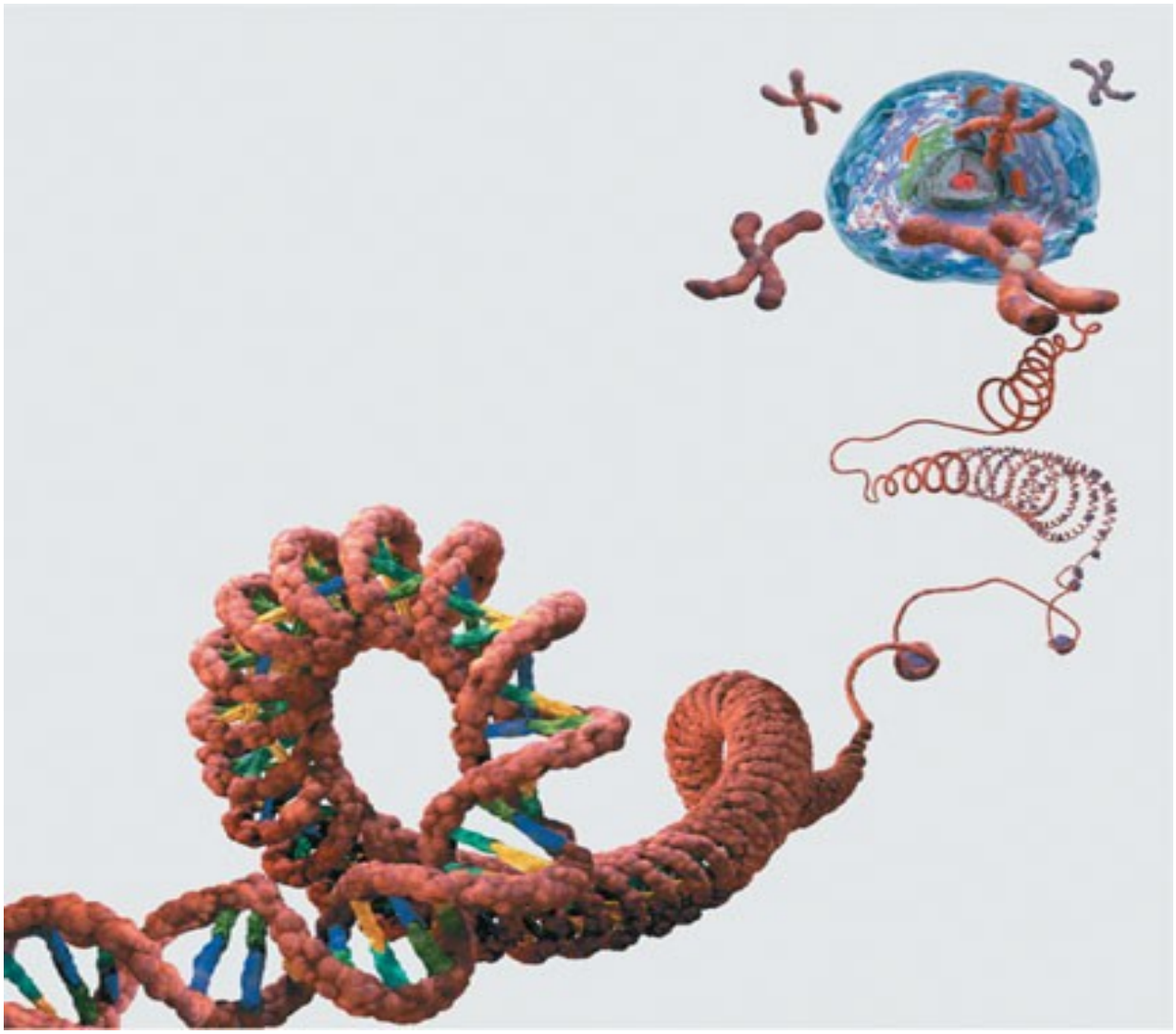
Faculty of Medicine and Surgery

Department of Biochemistry and Molecular biology

Practical Manual of Basic Molecular Biology



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General Laboratory Procedures

Preparation of Solutions

A. Calculation of Molar, % and "X" Solutions .

1. A molar solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight. Ex. To make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g/mol x 5 moles/liter x 0.1 liter = 29.29 g in 100 ml of solution

2. Percent solutions. Percentage (w/v) = weight (g) in 100 ml of solution; Percentage (v/v) = volume (ml) in 100 ml of solution. Ex. To make a 0.7% solution of agarose in TBE buffer, weight 0.7 of agarose and bring up volume to 100 ml with TBE buffer.

3. "X" Solutions. Many enzyme buffers are prepared as concentrated solutions, e.g. 5X or 10X (five or ten times the concentration of the working solution) and are then diluted such that the final concentration of the buffer in the reaction is 1X. Ex. To set up a restriction digestion in 25 µl, one would add 2.5 µl of a 10X buffer, the other reaction components, and water to a final volume of 25 µl.

B. Preparation of Working Solutions from Concentrated Stock Solutions .

Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed. Ex. To make 100ml of TE buffer (10mM Tris, 1mM EDTA), combine 1ml of a 1M Tris solution and 0.2ml of 0.5M EDTA and 98.8ml sterile water. The following is useful for calculating amounts of

stock solution needed: Ci x Vi=Cf x Vf, where Ci = initial concentration, or conc of stock solution; Vi = initial vol, or amount of stock solution needed Cf = final concentration, or conc of desired solution; Vf = final vol, or volume of desired solution

C. **Steps in Solution Preparation:**

1. Refer to a laboratory reference manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical. Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g. Place chemical(s) into appropriate size beaker with a stir bar. Add less than the required amount of water. Prepare all solutions with double distilled water. When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is in preparing solutions containing agar or agarose. Weigh the agar or agarose directly into the final vessel. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using a pH meter. Autoclave, if possible, at 121 deg C for 20 min. Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 μ m or 0.45μm filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it Solid media for bacterial plates can be prepared in advance, autoclaved, and stored in a bottle. When needed, the agar can be melted in a

microwave, any additional components, e.g. antibiotics, can be added and the plates can then be poured.

2. Concentrated solutions, e.g. 1M Tris-HCl pH=8.0, 5M NaCl, can be used to make working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

D. **Glassware and Plastic Ware .**

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid.

Glassware should be rinsed with distilled water and autoclaved or baked at 150 degrees C for 1 hour. For experiments with RNA, glassware and solutions are treated with diethyl-pyrocarbonate to inhibit RNases which can be resistant to autoclaving. Plastic ware such as pipets and culture tubes are often supplied sterile. Tubes made of polypropylene are turbid and are resistant to many chemicals, like phenol and chloroform; polycarbonate or polystyrene tubes are clear and not resistant to many chemicals. Make sure that the tubes you are using are resistant to the chemicals used in your experiment. Micro pipet tips and microfuge tubes should be autoclaved before use.

Using a Micropipette

When scientists need to accurately and precisely deliver smaller volumes of a liquid, they use a pipette–a calibrated glass tube into which the liquid is drawn and then released. Glass and plastic pipettes have been mainstays of chemistry and biology laboratories for decades, and they can be relied upon to dispense volumes down to 0.1mL.

Molecular biologists frequently use much smaller volumes of liquids in their work, even getting down to 0.1µL (that's one ten thousandth of a millilitre, or one ten millionth of a litre). For such small volumes, they need to use a micropipette.

Micropipettes are called a lot of different names, most of which are based on the companies which manufacture. For example, you might hear them called "Gilsons", as a large number of these devices used in laboratories are made by this company. Regardless of the manufacturer, micropipettes operate on the same principle: a plunger is depressed by the thumb and as it is released, liquid is drawn into a disposable plastic tip. When the plunger is pressed again, the liquid is dispensed.

The tips are an important part of the micropipette and allow the same device to be used for different samples (so long as you change your tip between samples) without washing. They come in a number of different sizes and colours, depending on the micropipette they are used with, and the volume to be dispensed.

he most commonly used tips are:

Large Blue – 200-1000µL

Small Yellow – 2-200µL

Small White - <2µL

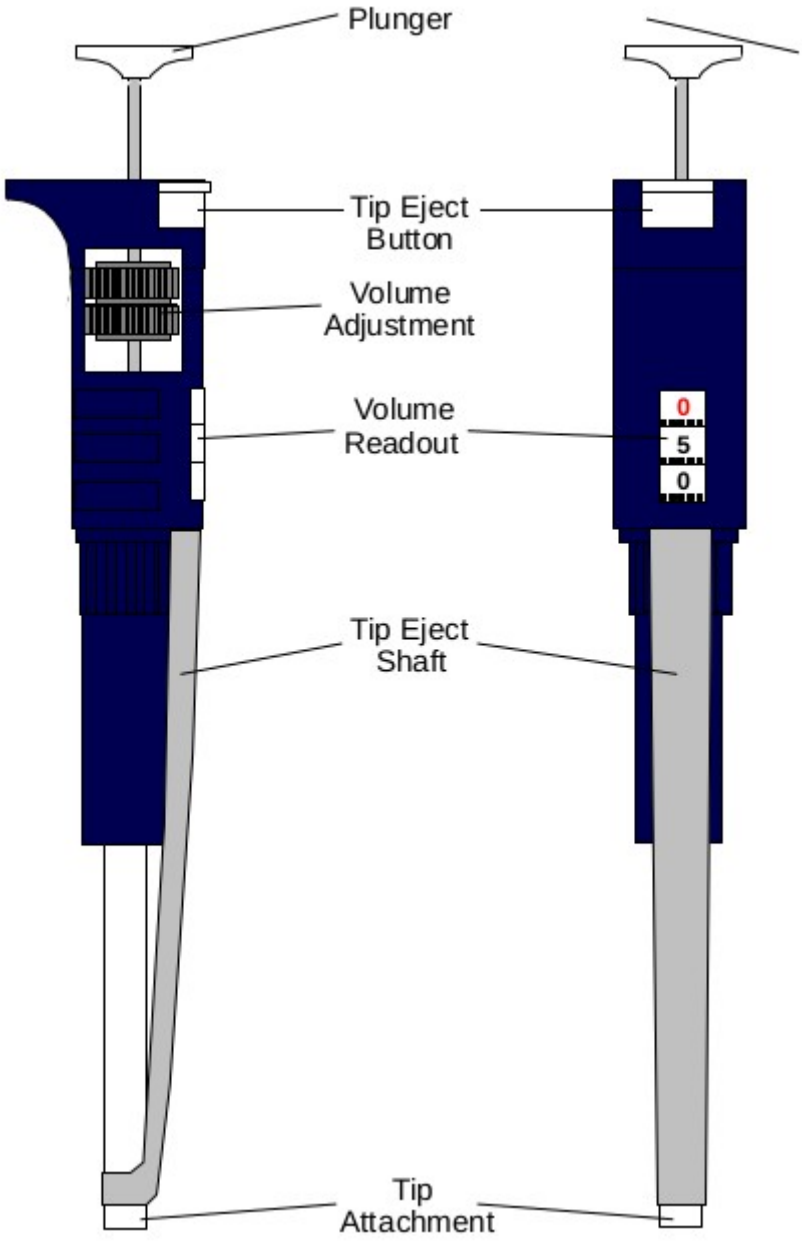

Figure 1: The main parts of the micropipette

Figure 2: Different types of micropepitte

DNA Extraction methods from different tissues

General principle

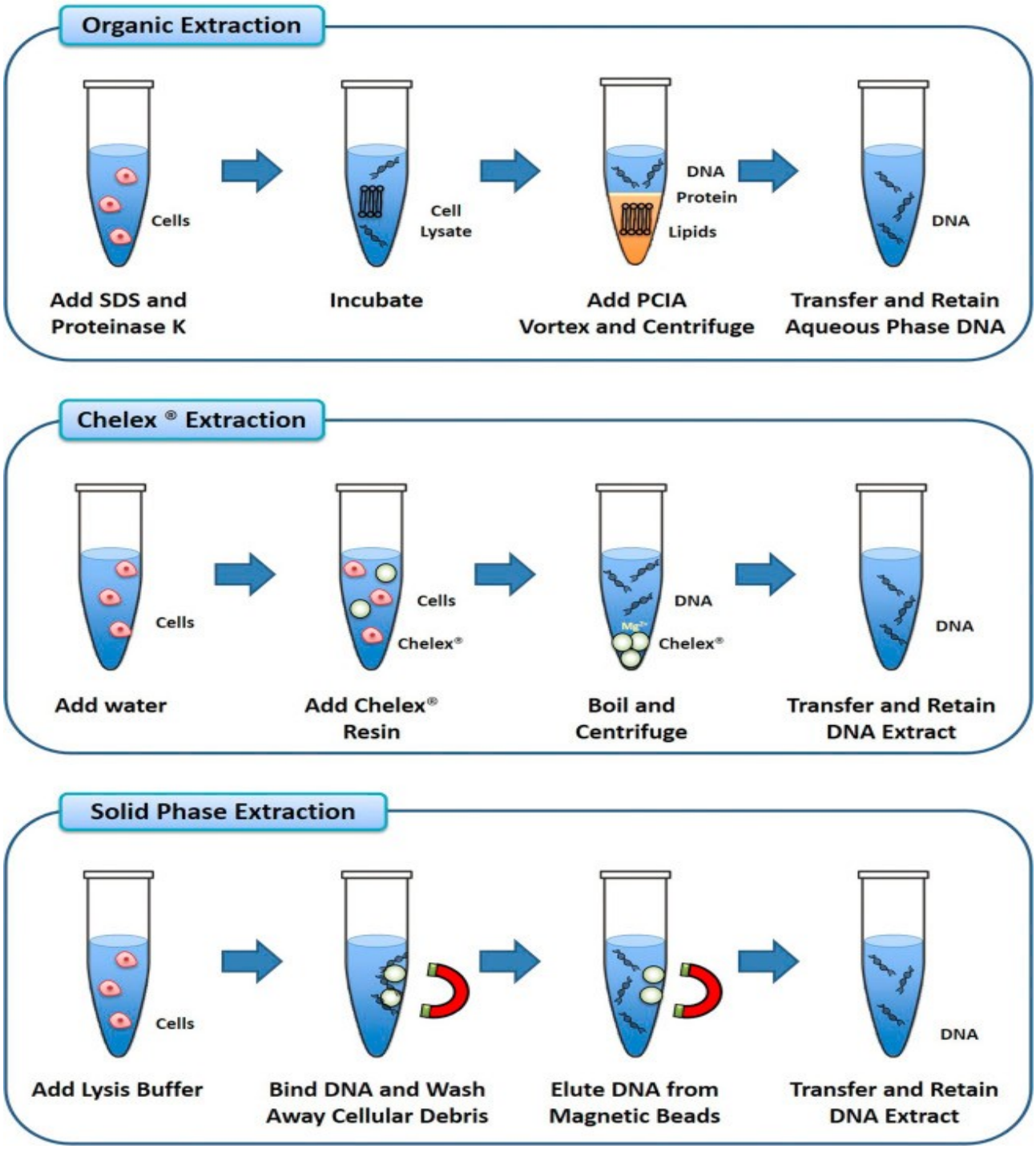

Figure 3: Common steps of the DNA extraction

1- DNA extraction fom Blood Sample using RCLB and WCLB

1. Collect 3 - 5 ml blood in EDTA tubes.
2. Add 10 ml of Red Cell Lysis Buffer (RCLB) Centrifuge for 5min at 6000 rpm.
3. Repeat the previous step till a clear pellet of white blood cells is formed.
4. Discard the supernatant and add 800µl of white Cell lysis Buffer (WCLB) and 10µl proteinase k, 1ml Guanidine chloride and 300µl NH4 acetate. Incubate at 37°C over-night.
5. Add an equal volume of chloroform, mix and centrifuge at 6000 rpm for 5minutes.
6. Transfer the upper layer to a clean tube, add 2 volumes of absolute cold ethanol and incubate over night at -20°C.
7. Centrifuge for 15 min at 10000 rpm, discard the supernatant.
8. Add 2ml of 70% ethanol and centrifuge for 10min at 10000 rpm discard the supernatant.
9. Repeat: the previous step.
10. Discard the supernatant and allow the pellet to dry at air drift for 30 min.
11. Resuspend in 100-200 ml of TE buffer or ddH2O and store at -4°C for short term usage and at -20°C for long term usage.

2- DNA extraction from Buccal cavity using Guanidine chloride and NH4 acetate.

1. Collect the epithelial cell from buccal cavity using brush or swab.
2. Add 2ml lysis buffer.
3. Add 10µl proteinase k, 1ml Guanidine chloride and 300µl NH4 acetate. Incubate at 37°C over- night.
4. Cool to room temperature.
5. Add an equal volume of chloroform, mix and centrifuge at 6000 rpm for 5minutes.
6. Transfer the upper layer to a clean tube, add 2volume of absolute cold ethanol and incubate over night at -20°C.
7. Centrifuge for 15min at 10000 rpm, discard the supernatant.
8. Add 2ml of 70% ethanol and centrifuge for 10 min at 10000 rpm discard the supernatant.
9. Repeat: the previous step.
10. Discard the supernatant and allow the pellet to dry at air drift for 30 min.
11. Add 100-200 ml of TE buffer or ddH2O and store at -4°C for short term usage and at -20°C for long term usage.

DNA extraction using Phenol/chloroform methods

Materials:

phenol:chloroform (1:1)

1. Add an equal volume of buffer-saturated phenol:chloroform (1:1) to the DNA solution.
2. Mix well. Most DNA solutions can be vortexed for 10 sec except for high molecular weight DNA which should be gently rocked.
3. Spin in a microfuge for 3 min.
4. Carefully remove the aqueous layer to a new tube, being careful to avoid the interface. (Steps 1-4 can be repeated until an interface is no longer visible).
5. To remove traces of phenol, add an equal volume of chloroform to the aqueous layer.
6. Spin in a microfuge for 3 min.
7. Remove aqueous layer to new tube.
8. Ethanol precipitate the DNA

Ethanol Precipitation of DNA

Materials:

* 3M Sodium acetate pH 5.2 or 5M ammonium acetate
* DNA
* 100% Ethanol

1. Measure the volume of the DNA sample. Adjust the salt concentration by adding 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M) or an equal volume of 5 M

ammonium acetate (final concentration of 2.0-2.5 M). These amounts assume that the DNA is in TE only; if DNA is in a solution containing salt, adjust salt accordingly to achieve the correct final concentration. Mix well. Add 2 to 2.5 volumes of cold 100% ethanol (calculated after salt addition). Mix well.

2. Place on ice or at -20 degrees C for >20 minutes.
3. Spin a maximum speed in a microfuge 10-15 min.Carefully decant supernatant. Add 1 ml 70% ethanol. Mix. Spin briefly. Carefully decant supernatant. Air dry or briefly vacuum dry pellet.
4. Resuspend pellet in the appropriate volume of TE or water.

Preparation of Genomic DNA from Bacteria

Materials

TE buffer

10% (w/v) sodium dodecyl sulfate (SDS)

20 mg/ml proteinase K

phenol\chloroform (50:50)

isopropanol

70% ethanol

3M sodium acetate pH 5.2

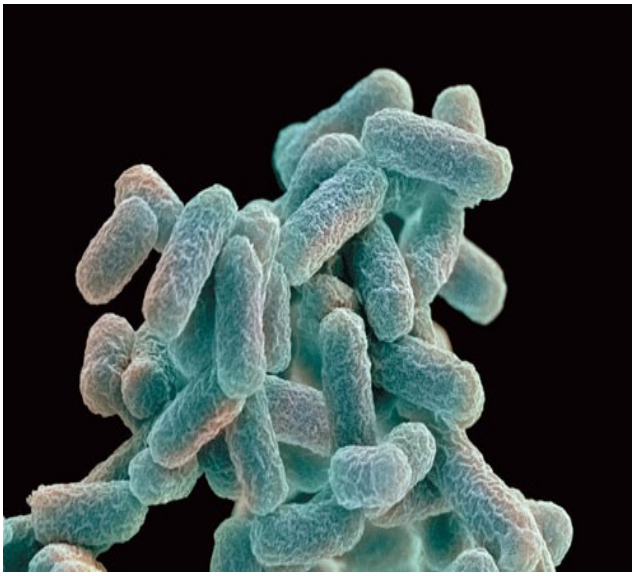

Figure 4: Bacterial culture of E. coli

Preparation of the bacterial culture

1. Grow *E. coli* culture overnight in rich broth.
2. Transfer 2 ml to a 2-ml micro centrifuge tube and spin 2 min.
3. Decant the supernatant.
4. Drain well onto a Kimwipe.
5. Resuspend the pellet in 467 µl TE buffer by repeated pipetting.
6. Add 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K, mix , and incubate 1hr at 37°C.
7. Add an equal volume of phenol/chloroform and mix well but very gently to avoid shearing the DNA by inverting the tube until the phases are completely mixed. CAUTION: PHENOL CAUSES SEVERE BURNS, WEAR GLOVES GOGGLES, AND LAB COAT AND KEEP TUBES CAPPED TIGHTLY.
8. Carefully transfer the DNA/phenol mixture into a Phase Lock Gel™ tube (green) and spin at 12,000 RPM for 10min.

9. Transfer the upper aqueous phase to a new tube and add an equal volume of phenol/chloroform.
10. Again mix well and transfer to a new Phase Lock GelTM tube and spin 10min.
11. Transfer the upper aqueous phase to a new tube.
12. Add 1/10 volume of sodium acetate. Mix.
13. Add 0.6 volumes of isopropanol and mix gently until the DNA precipitates.
14. Spool DNA onto a glass rod (or Pasteur pipet with a heat-sealed end).
15. Wash DNA by dipping end of rod into 1ml of 70% ethanol for 30 sec.
16. Resuspend DNA in at least 200μl TE buffer. Complete resuspension may take several days. Store DNA at 4°C short term, -20 or -80°C long term.
17. After DNA has dissolved, determine the concentration by measuring the absorbance at 260nm.

Working with DNA

A. Storage

The following properties of reagents and conditions are important considerations in processing and storing DNA and RNA. Heavy metals promote phosphodiester breakage. EDTA is an excellent heavy metal chelator. Free radicals are formed from chemical breakdown and radiation and they cause phosphodiester breakage. UV light at 260 nm causes a variety of lesions, including thymine dimers and cross-link. Biological activity is rapidly lost. 320 nm irradiation can also cause cross-link, but less efficiently. Ethidium bromide causes photo oxidation of DNA with visible light and molecular oxygen. Oxidation products can cause phosphodiester breakage. If no heavy metal are present, ethanol does not damage DNA. Nucleases are found on human skin; therefore, avoid direct or indirect contact between nucleic acids and fingers. Most DNases are not very stable; however, many RNases are very stable and can adsorb to glass or plastic and remain active. 5 E C is one of the best and simplest conditions for storing DNA. -20 deg C: this temperature causes extensive single and double strand breaks. -70 E C is probable excellent for long-term storage. For long-term storage of DNA, it is best to store in high salt (>1M) in the presence of high EDTA (>10mM) at pH 8.5. Storage of DNA in buoyant CsCl with ethidium bromide in the dark at 5 E C is excellent. There is about one phosphodiester break per 200 kb of DNA per year. Storage of λ DNA in the phage is better than storing the pure DNA.

B. **Purification**

To remove protein from nucleic acid solutions:

1. Treat with proteolytic enzyme, e.g., pronase, proteinase K
2. Purify on a silica-based column such as a Qiagen PCR Prep Column
3. CsCl/ethidium bromide density gradient
4. Phenol Extract. The simplest method for purifying DNA is to extract with phenol or phenol:chloroform and then chloroform. The phenol denatures proteins and the final extraction with chloroform removes traces of phenol
5. Purify on silica-based column such as Qiagen Brand columns (http://www.qiagen.com)

C. **Quantitation .**

1. Spectrophotometric. For pure solutions of DNA, the simplest method of quantitation is reading the absorbance at 260 nm where an OD of 1 in a 1 cm path length = 50 μ g/ml for double-stranded DNA, 40 μ g/ml for single-stranded DNA and RNA and 20-33 μ g/ml for oligonucleotides. An absorbance ratio of 260 nm and 280 nm gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD 260/OD 280 values of 1.8 and 2.0, respectively. This method is not useful for small quantities of DNA or RNA (<1 μ g/ml).
2. Ethidium bromide fluorescence. The amount of DNA is a solution is proportional to the fluorescence emitted by ethidium bromide in that solution. Dilutions of an unknown DNA in the presence of 2 μ g/ml ethidium bromide are compared to dilutions of a known

amount of a standard DNA solutions spotted on an agarose gel or Saran Wrap or electrophoresed in an agarose gel.

D. **Concentration**

Precipitation with ethanol. DNA and RNA solutions are concentrated with ethanol as follows: The volume of DNA is measured and the monovalent cation concentration is adjusted. The final concentration should be 2-2.5M for ammonium acetate, 0.3M for sodium acetate, 0.2M for sodium chloride and 0.8M for lithium chloride. The ion used often depends on the volume of DNA and on the subsequent manipulations; for example, sodium acetate inhibits Klenow, ammonium ions inhibit T4 polynucleotide kinase, and chloride ions inhibit RNA-dependent DNA polymerases. The addition of MgCl 2 to a final concentration of 10mM assists in the precipitation of small DNA fragments and oligonucleotides. Following addition of the monovalent cations, 2-2.5 volumes of ethanol are added, mixed well, and stored on ice or at -20 E C for 20 min to 1 hour. The DNA is recovered by centrifugation in a microfuge for 10 min (room temperature is okay). The supernatant is carefully decanted making certain that the DNA pellet, if visible, is not discarded (often the pellet is not visible until it is dry). To remove salts, the pellet is washed with 0.5-1.0 ml of 70% ethanol, spun again, the supernatant decanted, and the pellet dried. Ammonium acetate is very soluble in ethanol and is effectively removed by a 70% wash. Sodium acetate and sodium chloride are less effectively removed. For fast drying, the pellet can spun briefly in a Speedvac, although the method is not recommended for many DNA preparations as DNA that has been over dried is difficult to resuspend and also tends to denature small fragments of DNA. Isopropanol is also

used to precipitate DNA but it tends to coprecipitate salts and is harder to evaporate since it is less volatile. However, less isopropanol is required than ethanol to precipitate DNA and it is sometimes used when volumes must be kept to a minimum, e.g., in large scale plasmid preps.

E. Restriction Enzymes

Restriction and DNA modifying enzymes are stored at -20 deg C in a non-frost free freezer, typically in 50% glycerol. The enzymes are stored in an insulated cooler which will keep the enzymes at -20 deg C for some period of time. The tubes should never be allowed to reach room temperature and gloves should be worn when handling as fingers contain nucleases. Always use a new, sterile pipet tip every time you use a restriction enzyme. Also, the volume of the enzyme should be less than 1/10 of the final volume of the reaction mixture.

The Polymerase Chain Reaction (PCR) (Amplification of DNA)

General principle

PCR is a powerful technique for amplifying segments of DNA. PCR is sensitive and quicker technique for DNA amplification. Starting with just one molecule, PCR can produce millions of copies of a DNA segment in just a few hours. PCR requires the use of specific primers and this means that there must be sequence information available for the DNA to be amplified in order for primers to be designed. In fact, amplifications of this size are technically demanding, and, in most cases, investigators use PCR to amplify much smaller fragments of DNA.

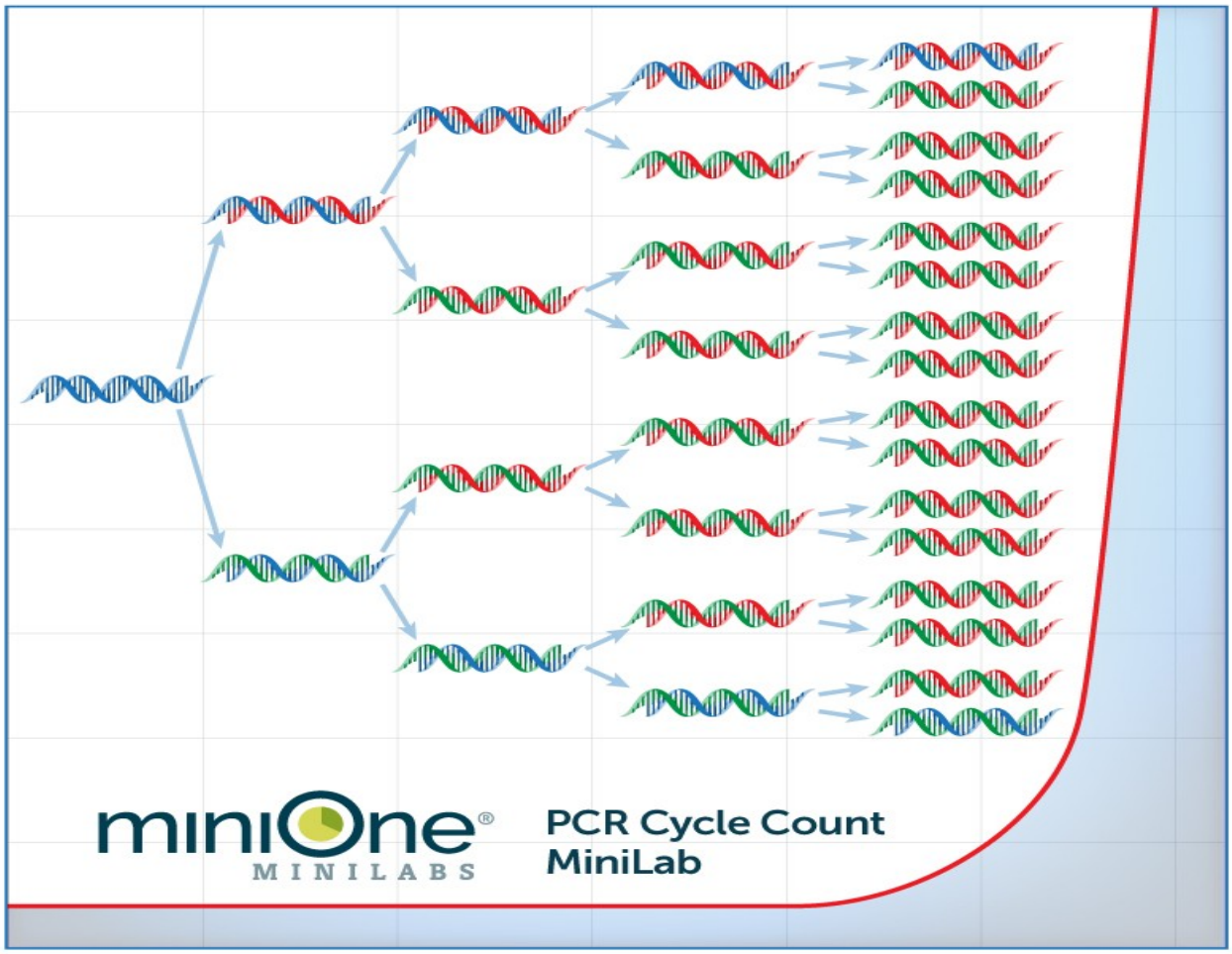

Figure 5: DNA amplification process

Figure 6: The PCR machine

Materials:

sterile water

10X amplification buffer with 15mM MgCl2

10 mM dNTP

50 µM oligonucleotide primer 1

50 µM oligonucleotide primer 2

5 unit/µl Taq Polymerase

template DNA (1 µg genomic DNA, 0.1-1 ng plasmid DNA) in 10 µl mineral oil (for thermocyclers without a heated lid

1. Combine the following for each reaction (on ice) in a 0.2 or 0.5 ml tube:

10X PCR buffer	10µl
Primer 1	1µl
Primer 2	1µl
dNTP	2µl
template DNA and water	85.5µl
Taq Polymerase	0.5µl
Total volume	100µl

2. Prepare a control reaction with no template DNA and an additional 10 µl of sterile water.

3. If the thermocycler does not have a heated lid, add 70-100 µl mineral oil (or 2 drops of silicone oil) to each reaction.

4. Place tubes in a thermal cycler preheated to 94 degrees C.

5. Run the following program:

* 94 degrees C for 3min for the initial denaturation

* 94 degrees C for 30 sec for the denaturation

* 55 degrees C for 1min or annealing temperature appropriate for particular primer pair

* 72 degrees C for 1 min (if product is <500 bp), 3 min (if product is >500 bp) for 30 cycles.

* Program a final extension at 72 degrees C for 7 min.

DNA Agarose Gel Electrophoresis

Principle

Electrophoresis through agarose gels is used to separate, analyze, identify, and purify DNA fragments. DNA contain negative charges because of the back bone PO_4 . This property is exploited to separate DNA based on their size in an electric field with an agarose gel as a stationary phase. The location of bands of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as ethidium bromide; bands containing as little as 20pg of double-stranded DNA can then be detected by direct examination of the gel in ultraviolet (UV) light.

Running Buffer

TRIS Acetate EDTA (TAE) buffer, TRIS Borate EDTA (TBE) buffer both

slightly alkaline (pH 8.00 to 8.5) buffer are used in electrophoresis. Electrophoresis that separates on the basis of charge, ions in the buffer transmit the charge necessary for separation. The buffer, also keeps the pH within a narrow range. This is important because the structure and charge nucleic acid will change if subjected to significant pH changes, thus preventing proper separation. The alkaline pH of the buffer keeps the DNA stable and EDTA chelates divalent cations like Mg^{2+} which is required for DNAase activity.

Gel Loading Dye

Gel-loading buffers are mixed with the samples before loading into the slots of the gel. These buffers serve three purposes: They increase the density of the sample, ensuring that the DNA sinks evenly into the well;

they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates and sued for tracking the sample. Bromophenol blue migrates through agarose gels 2.2-fold faster than xylene cyanol

Staining the Gel

A convenient and commonly used method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide which contains a tricyclic planar group that intercalates between the stacked bases of DNA. Ethidium bromide intercalates into double-stranded DNA in a sequence-independent fashion at a maximum stoichiometry of about two dye molecules per turn of the helix. Ethidium bromide can be used to detect both single-stranded and double-stranded nucleic acids (both DNA and RNA). However, the affinity of the dye for single-stranded nucleic acid is relatively low, and the fluorescent yield is comparatively poor

Materials:

agarose solution in TBE, TAE or SB (generally 0.7-1%)
1X TBE, TAE, or SB (same buffer as in agarose)
gel loading dye
10 mg/ml ethidium bromide

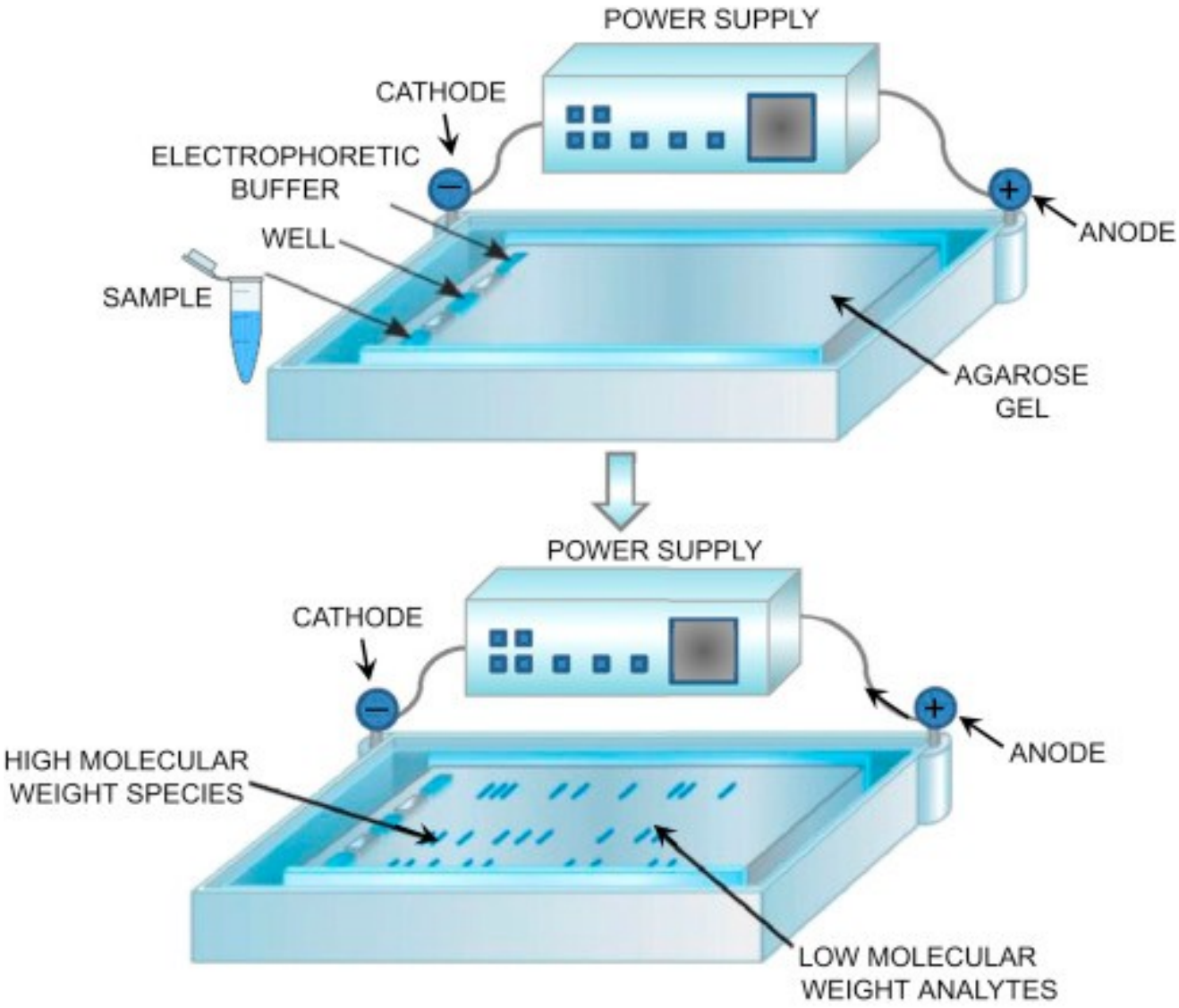

Figure 7: DNA gel electrophoresis device

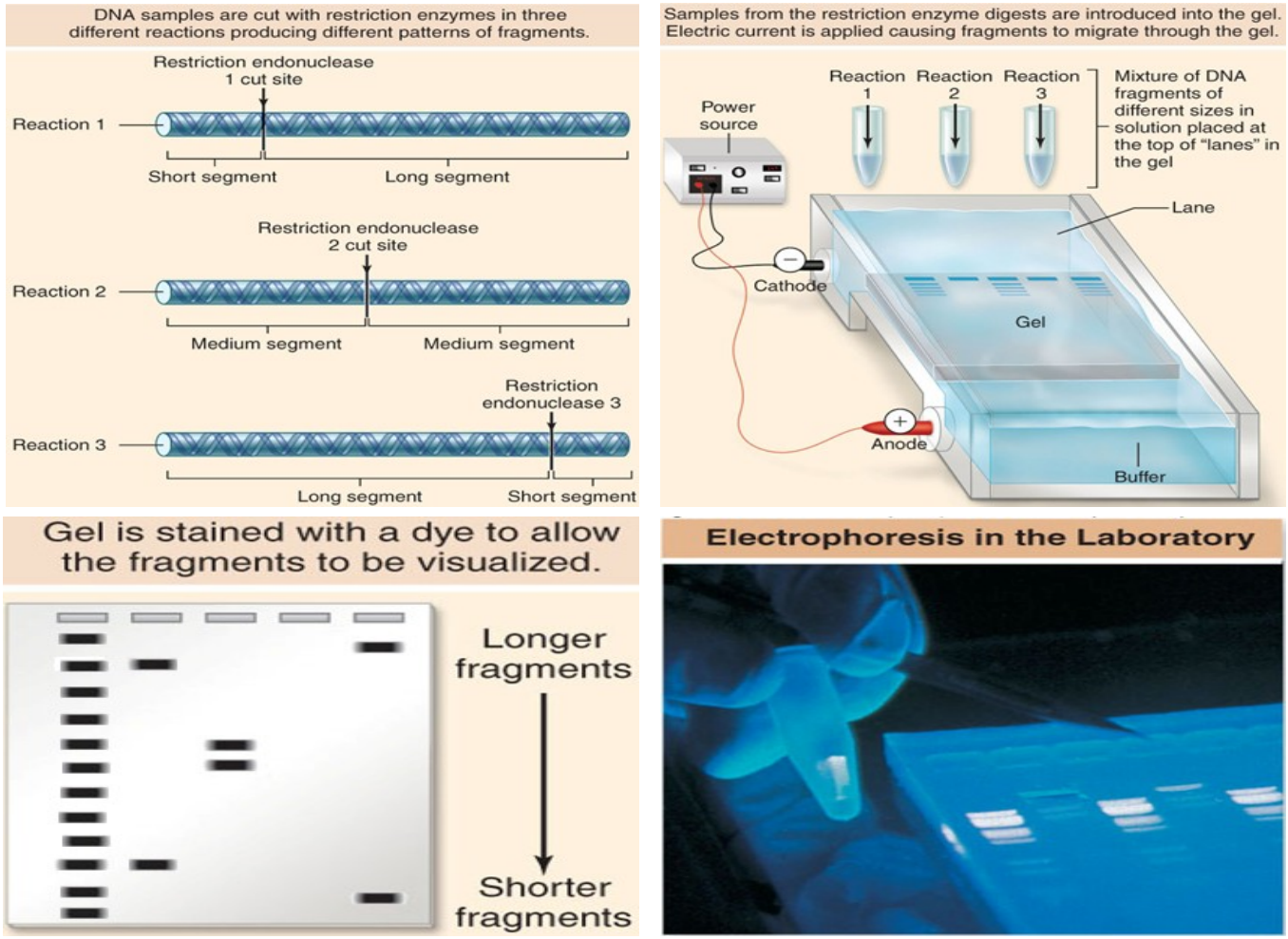

Figure 8: Steps of DNA gel electrophoresis

Procedure_

1. To prepare 100 ml of a 0.7% agarose solution, measure 0.7 g agarose into a glass beaker or flask and add 100 ml 1X buffer. Microwave or stir on a hot plate until agarose is dissolved and solution is clear.
2. Allow solution to cool to about 55 degrees C before pouring. (Ethidium bromide can be added at this point to a concentration of 0.5 µg/ml)

3. Prepare gel tray by sealing ends with tape or other custom-made dam. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray
4. Pour 50 degree C gel solution into tray to a depth of about 5 mm. Allow gel to solidify about 20 minutes at room temperature.
5. To run, gently remove the comb, place tray in electrophoresis chamber, and cover (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose). Excess agarose can be stored at room temperature and remelted in a microwave. To prepare samples for electrophoresis, add 1 µl of 6x gel loading dye for every 5 µl of DNA solution. Mix well. Load 5-12 µl of DNA per well (for minigel). Electrophorese at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.
6. If the gel was not stained with ethidium during the run, stain the gel in 0.5 µg/ml ethidium bromide until the DNA has taken up the dye and is visible under short wave UV light, if the DNA will not be used further, or with a hand-held long-wave UV light if the DNA is to be cut out and purified.

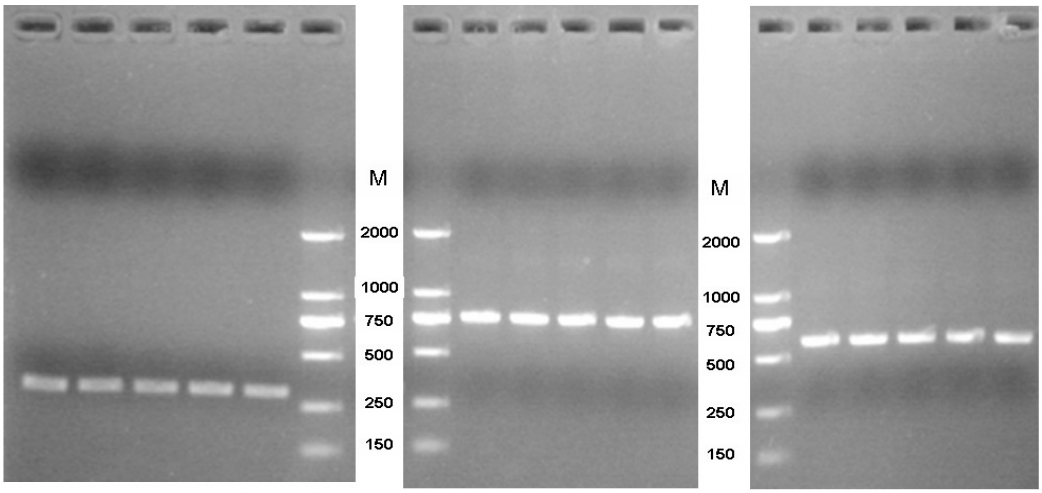

Figure 9: DNA visualized in the gel. Recognize the DNA marker

Purification of DNA Fragment from an Agarose Gel

Background:

DNA can be easily isolated and purified after size selection on an agarose gel. The fragment of interest is simply cut out of the gel with a razor blade and purified by a number of different methods. The easiest is to use a method that involves first dissolving the agarose slice in a solution at 50°C, then binding the DNA from the melted agarose to a silica-gel membrane.

1. Prepare an agarose gel in TAE buffer using the four-well combs. (Preparative agarose gels should be run using 1X TAE electrophoresis and gel buffer as the borate in TBE interferes with some purification resin). Load the DNA. To visualize the DNA after staining, do not expose the DNA to shortwave UV light as this will introduce nicks. Visualize the bands with a hand-held long wave UV light and cut out the band with a clean razor blade (Note: place gel on a glass slide to avoid cutting the surface of the transilluminator).
2. After cutting out the band, follow the procedure for DNA fragment purification using Qiagen QIAquick or Qiaex II purification systems following the manufacturer's procedure. Estimate the approximate concentration of the DNA obtained by running 10% of the eluate on an agarose gel against a DNA mass ladder.

Restriction Enzyme and DNA Digestion

General principle

Restriction enzymes are also called 'molecular scissors' as they cleave DNA at or near specific recognition sequences known as restriction sites. These enzymes make one incision on each of the two strands of DNA and are also called restriction endonucleases. Restriction enzymes present in bacteria and used to degrade the invading viral DNA by cleaving it at specific restriction sites. At the same time, the host cell protects its own DNA from being cleaved by employing other enzymes called methylases enzymes. The restriction enzymes catalyse the hydrolysis of the bond between the 3'-oxygen atom and the phosphorus atom in the phosphodiester backbone of DNA.

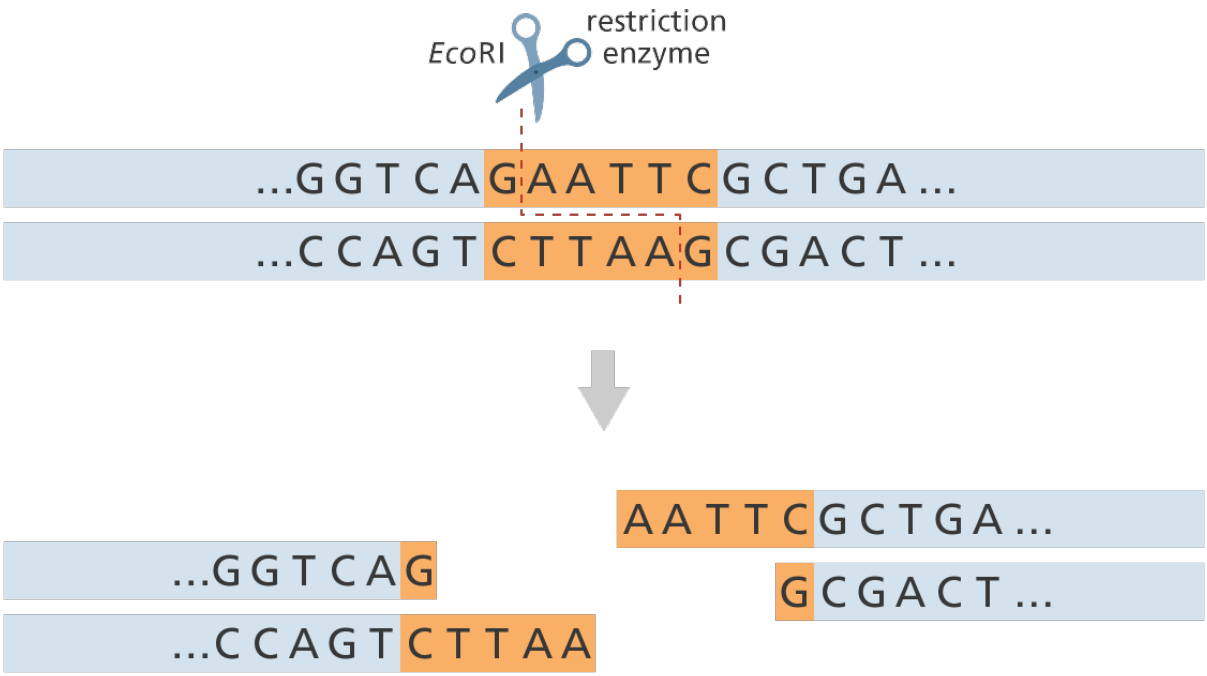

Figure 10: The principal action of the restriction enzymes

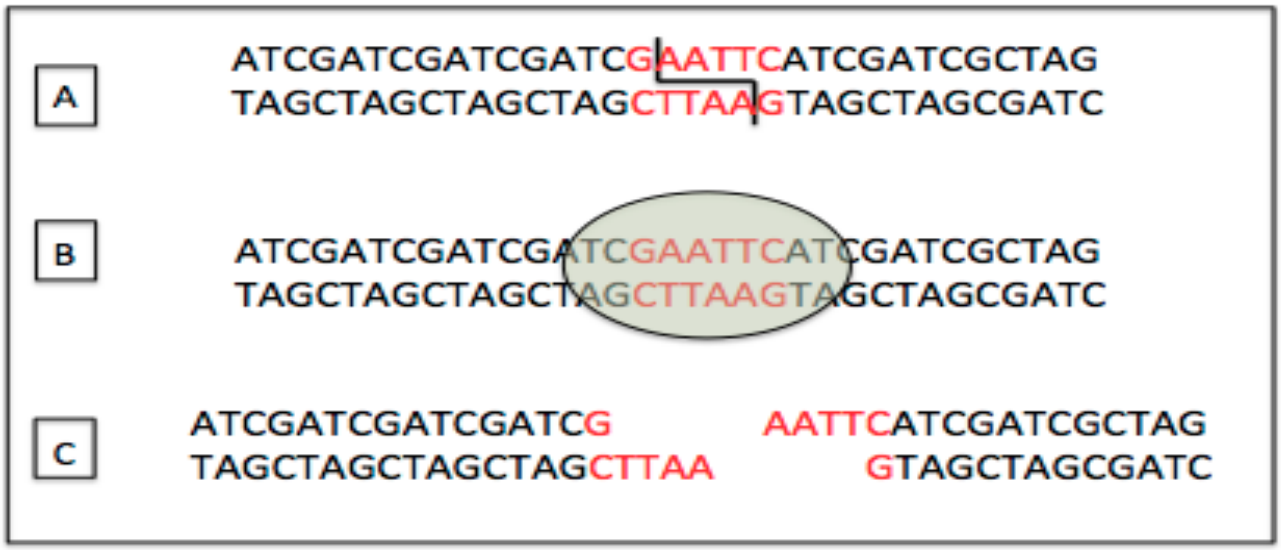

Figure 11: Principle of restriction enzymes cutting site_

Materials:

10X restriction enzyme buffer (according to manufacturer's recommendation)

* DNA
* sterile water
* restriction enzyme
* phenol:chloroform (1:1) (optional)

1- Add the following to a microfuge tube:

* 2 µl of appropriate 10X restriction enzyme buffer
* 0.1 to 5 µg DNA
* sterile water to a final volume of 19 µl (Note: These volumes are for analytical digests only. Larger volumes may be necessary for preparative digests or for chromosomal DNA digests.

2- Add 1 to 2µl (3 to 20 units) enzyme and mix gently. Spin for a few seconds in microfuge. Incubate at the appropriate temperature (usually 37 degrees C) for 1 to 2 hours. Run a small aliquot on a gel to check for digestion.

1. If the DNA is to be used for another manipulation, heat inactivate the enzyme (if it is heat labile) at 70 degrees C for 15 min, phenol/ chloroform extract and ethanol precipitate, or purify on Qiagen DNA purification column (http://www.qiagen.com).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

General principle

The separation of macromolecules in an electric field is called electrophoresis. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called SDS-PAGE. SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides.

In a gel of uniform density the relative migration distance of a protein (Rf, the f as a subscript) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between Rf and mass can be plotted, and the masses of unknown proteins estimated.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions.

The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

Figure 12: The SDSPAGE device

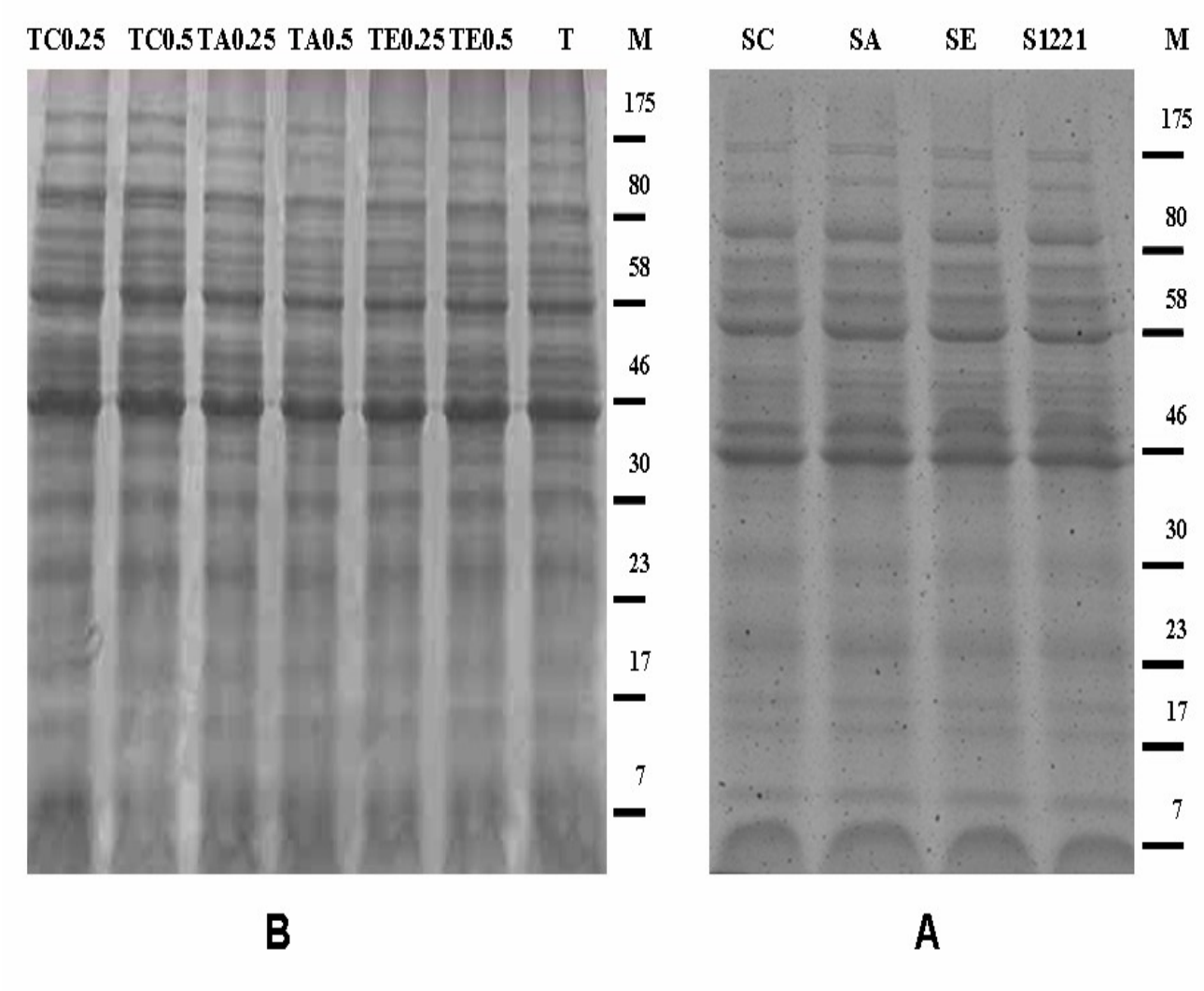

Figure 13: Separation of two protein samples (A, B) in the gel of the SDSPAGE

Materials

Vertical Electrophoresis apparatus

Gel plates, Spacers, Comb

Ethanol

Vaseline

Distilled water

Buffers and reagents

Acrylamide (30%)

Dissolve 290g of acrylamide and 10g of N,N'-methylbisacrylamide in 600ml of H2O. Adjust the volume to 1L with H2O. Sterilize the solution by filtration (0.45 micron pore size). Check the pH (should be 7.0 or less). Store in dark bottles at room temperature.

Gel casting Buffer

1.5 M Tris-HCl, pH8.8 (to prepare resolving gel): Dissolve 18.15g of Tris base in 80mL distilled water. Adjust pH to 8.8 using 6N HCl. Make up the final volume to 100mL with distilled water

0.5 M Tris-HCl, pH6.8 (to prepare stacking gel): Dissolve 6g of Tris base in 80mL distilled water. Adjust pH to 6.8 using 6N HCl. Make up the final volume to 100mL with distilled water

2X Loading Buffer

Bromophenol blue 0.004%

2-mercaptoethanol 10%

Glycerol 20%

SDS 4%

Tris-HCl 0.125 M

10X Running Buffer

Tris-HCl 25 mM

Glycine 200 mM

SDS 0.1% (w/v

SDS (10%)

Dissolve 10 g in 100 ml of distilled water.

APS (10%)

Dissolve 100 mg in 1 ml of distilled water (Need to be prepared fresh for every experiment

Staining solution

Stain: Dissolve 0.4g of Coomassie blue R350 in 200 mL of 40% (v/v) HPLC grade methanol in water with stirring as needed. Filter the solution to remove any insoluble material. Add 200mL of 20% (v/v) acetic acid in water. The final concentration is 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid.

Distaining solution

Add 500mL of HPLC- grade methanol to 300mL of HPLC grade water. Add 100mL of reagent grade acetic acid and, after mixing, adjust the total volume to 1000mL with water. The final concentrations are 50% (v/v) methanol in water with 10% (v/v) acetic acid

TEMED

PROCEDURE

1. Assemble the plates for casting gel

2. Clamp the assembly of plates to fix it in a gel casting apparatus. Ensure the assembly is leak proof by filling water between the plates. Silicon grease can be applied to spacer to make a water-tight seal.

3. Prepare the separation gel. Mix in the following order: (see the table below)

Gel %	Water (mL)	30% acrylamide (mL)	1.5 M Tris-HCl, pH 8.8 (ml)	10% SDS (µL)	10% APS (µL)	TEMED*(µL)
8%	4.6	2.6	2.6	100	100	10
10%	3.8	3.6	2.6	100	100	10
12%	3.2	4.0	2.6	100	100	10
15%	2.2	5.0	2.6	100	100	10

4- Pour gel, leaving ~2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.

5. Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out (In ~30min, the gel should be completely polymerized.

6. Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.

7. Prepare the stacking gel (4%). Mix in the following order:

-H2O 6.1 mL

-Acrylamide/bis (30%, 37.5:1) 1.3 mL

-Tris–HCl (0.5 M, pH 6.8) 2.5 mL

-SDS, 10% 100 µL

-TEMED 10 µL

Ammonium persulfate (APS), 10% 100 µL

8. Pour stacking gel on top of the separation gel.

9. Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized.

10. Add 25µl of sample loading buffer to protein sample. Add 25µl of sample loading buffer to 25µl of protein marker. Place it in a boiling water bath for 5 minutes.

11. After the stacking gel has set, carefully remove the comb and the bottom spacer. Wash the wells immediately with distilled water to remove non-polymerized acrylamide. Fill the bottom reservoir with 1X reservoir buffer and carefully fix the plate to the apparatus without trapping any air bubbles between the buffer and the bottom of the gel. Fix the plates to PAGE apparatus. Fill the top reservoir with 1X reservoir buffer.

12. Load samples and molecular mass protein markers into wells for separation by electrophoresis.

13. Set voltage at 100 V and switch on the power supply.

14. When the dye front comes to 0.5 cm above the bottom of the gel, turn off the power. This will take approximately 1 to 11/2 hours

15. Remove the gel plates and gently pry the plates apart using a spatula or similar tool, not at the notch. Cut off the wells and the stacking gel and place the gen in a water containing Tray

16. Wash the gel Gently and discard the water

17. Add 100 ml of staining solution and keep it in the rocker for overnight.

18. Remove the staining solution and add 100 ml of distaining solution and change the distaining solution for every one hr till the bands are visible.

Western blotting

PRINCIPLE

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) Electrophoresis -separation by size, (2) Blotting-transfer to a solid support, and (3) Immunodetection-marking target protein using a proper primary and secondary antibody to visualize.

Electrophoresis

Mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis

Blotting

Blotting is transfer of resolved proteins from the gel onto a surface of a suitable membrane, done commonly by electrophoresis and referred to as electroblotting. The gel is placed in contact with nitrocellulose membrane which is then sandwiched between filter paper, two porous pads and two plastic supports. The entire set up is then placed in an electrophoretic tank containing blotting buffer. The proteins get transferred to the corresponding position on the membrane as resolved on the polyacrylamide gel, forming a mirror image of the gel.

Immunodetection

Protein of interest on the membrane is further located by immunodetection. The membrane is then incubated with labels antibodies

specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. The electroblotted sample will then be detected using anti-mouse GAPDH IgG (Cell signaling) as primary antibody and corresponding secondary antibody labeled with Horse Radish Peroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and Dimethylamino benzidine (DAB) as a chromogen. HRP acts on hydrogen peroxide to release oxygen, which oxidizes the DAB to DAB oxide. The DAB oxide is deposited wherever enzyme is present and appears as a light brown band on the NC membrane

WESTERN BLOT ANALYSIS

STEP 2: Set up

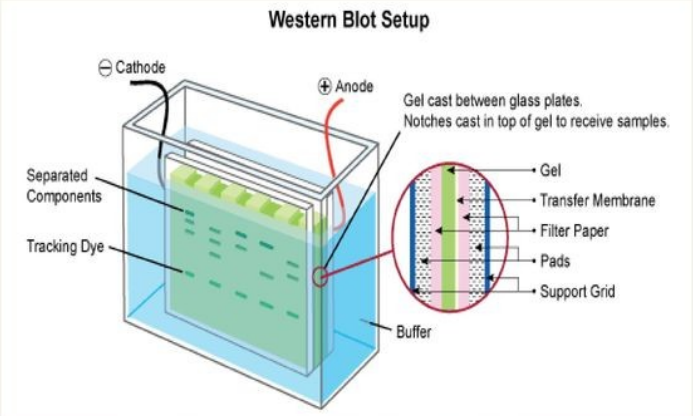

Figure 3 Western Blot Setup (Leinco Technologies, 2017)

- Protein concentrations in lysate determined
- Protein samples are heated to denature and give them a net negative charge
- Gel electrophoresis
- Electrophoretic transfer

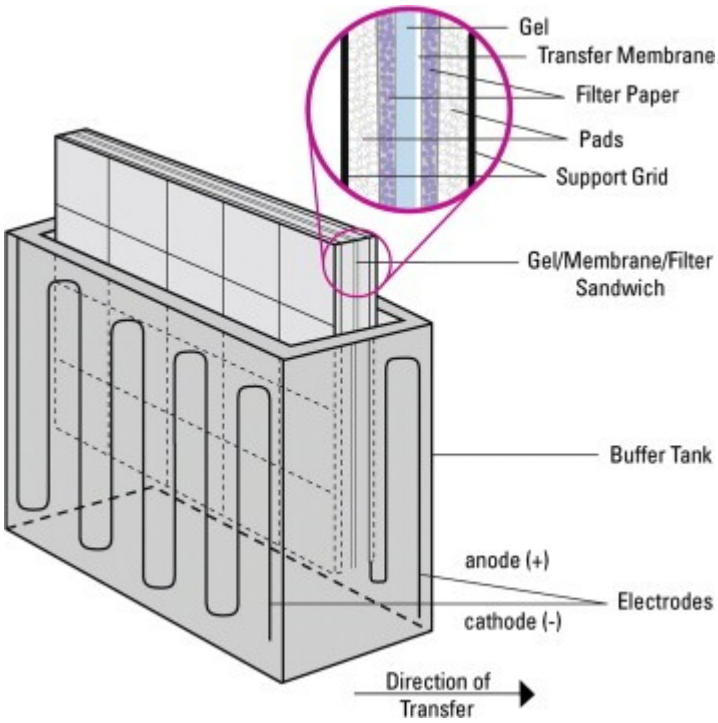

Figure 14: Western blot sandwich device

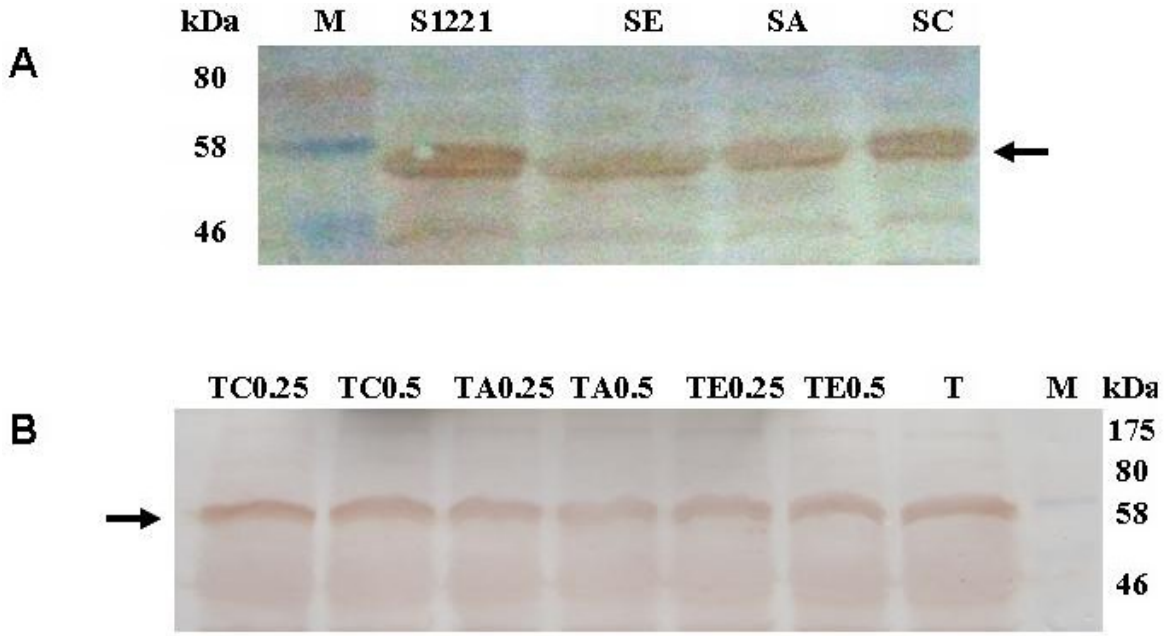

Figure 15: The result of western blot

Procedure

1. Cut PVDF membrane to the appropriate size, activate with absolute methanol for 5 sec, and incubate in distilled water for 5 min.
2. For electroblotting, equilibrate in transfer buffer and follow the standard blotting procedure to transfer the proteins to the membrane. For dot blotting, keep membrane wet until ready to use.
3. After protein has been transferred to the membrane, wash again in absolute methanol for a few seconds and allow to dry at room temperature for 30 min. or more.
4. Block in 30 ml of 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), gently rocking, 1 hr, room temperature.

5. Add appropriate dilution of primary antibody (typically 1:5000 or 1:10,000) prepared in 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), incubate 30 min, room temperature, gently rocking.
6. Wash three times in 20 ml 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block) for 5 min each. Add appropriate dilution of secondary antibody conjugated to alkaline phosphatase prepared in 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), gently rocking, 30 min, room temperature.
7. Wash as in step #6.
8. Then, wash twice with 1X Western buffer without I-block.
9. At the end of the second final wash, leave some buffer in the container to keep the membrane moist. With the membrane facing protein-side up, add 0.5 ml of substrate solution directly into the remaining liquid, mix well, and pipet (with a p1000) the solution over the membrane to ensure the entire surface comes into contact with the substrate. Gently agitate for a few minutes, remove membrane to a paper towel and let dry completely. The substrate solution can be reused immediately for additional membranes.
10. Scan membrane using the Molecular Dynamics Storm or other suitable instrument.

Western Blotting Solutions:

- 1X Transfer buffer: 25 mM Tris, 192 mM Glycine, pH 8.3. Mix 3.03 g Tris and 14.4 g glycine; add water to 1 liter - do not add acid or base to pH - it should be >8.0. Use 0.5X for transfer in 20% methanol.

10X Western Buffer: 200 mM Tris pH = 7.5; 1.5 M NaCl (containing 0.1% Tween-20 and 0.2% I-Block). To prepare 1X Western Buffer, dilute 10X buffer to 1X, adding Tween-20 to 0.1%. Remove 50 ml and set aside for the last two washes. To the remainder, add I-Block to 0.2% (Cat #T2015, Applied Biosystems - formerly Tropix). To dissolve I-Block, heat solution in a beaker briefly in a microwave to about 60°C, then stir until dissolved (solution will be cloudy). Bring to room temperature before using.

- Primary antibody: For his tagged proteins - Anti-His monoclonal antibody - BD Bioscience #631212
- Secondary antibody: Goat anti-mouse alkaline phosphatase conjugated - Biorad #170-6520.
- Substrate: ECF chemifluorescent substrate - Amersham #RPN5785. Mix substrate with accompanying buffer as per manufacturer's recommended instructions, prepare 1 ml aliquots and store at -20°C.

Southern Blotting

Principle

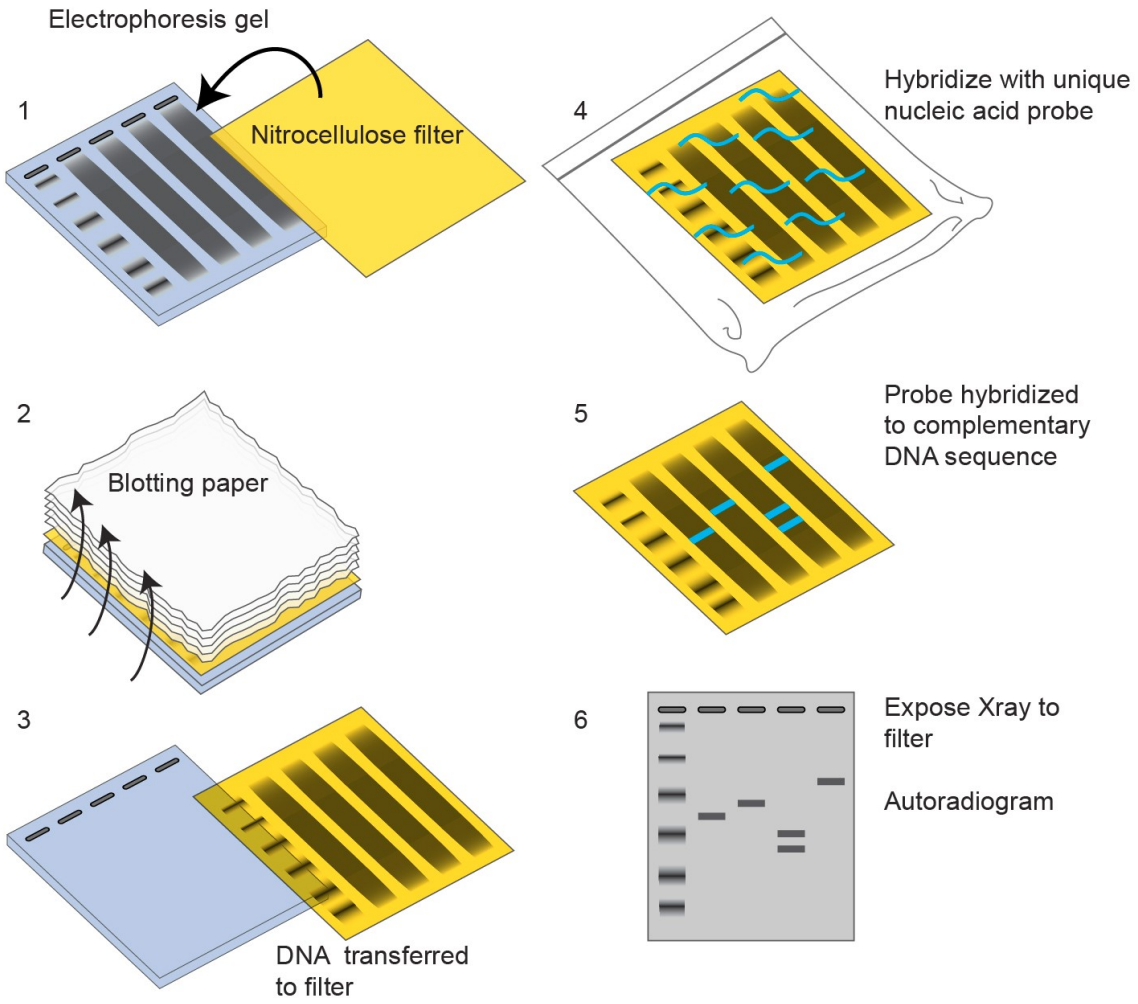

Figure 16: The steps of the southern blot

Steps

1. Electrophoresis of DNA is carried out in a neutral agarose gel system. Prepare a 0.8-1% agarose gel containing 1x TAE buffer. Ethidium bromide can be added to a final concentration of 0.2 µg/ml.
2. Apply the samples to the gel.
3. Run the gel in 1x TAE. buffer at 4V/cm until the bromophenol blue indicates that the sample has run for a sufficient distance.
4. Following electrophoresis, visualize the gel under UV transillumination and photograph with a ruler.
5. i) Depurination, 10 minutes at room temperature with gentle agitation (optional). This step is necessary if target sequences are greater than 10 Kb in size
   ii) Denaturation, 25 minutes at room temperature with gentle agitation.
   iii) Neutralization, 30 minutes at room temperature with gentle agitation. When using nitrocellulose membranes, the neutralization time should be extended to 45 minutes. Include a rinse in distilled water between each step
6. Assemble the capillary blotting apparatus using 10X SSC as the transfer buffer. Allow the DNA to transfer overnight onto Hybond N+.
7. The following day, disassemble the apparatus, mark the membrane appropriately and fix the DNA to the membrane by UV crosslinking or baking (2 hours at 80°C). For nitrocellulose membranes, bake for 2 hrs. at 80°C in a vacuum oven.

solutions

Hybridization buffer

5x SSC

1 in 20 dilution Liquid Block (Amersham) or other blocking reagent

0.1%(w/v) SDS

5%(w/v) Dextran sulphate

EDTA stock

0.5M EDTA pH8.0

SDS stock

10% or 20% (w/v) SDS

Depurination solution

250mM HCl

Denaturation solution

1.5M NaCl

0.5M NaOH

Neutralization solution

1.5M NaCl

0.5M Tris-HCl

pH adjusted to 7.5

20x SSC

0.3M Na(3) citrate

3M NaCl