Lecture – 3

Agarose Gel Electrophoresis

Dr. M. Tariq Javed
Professor
Department of Pathology,
Faculty of Veterinary Science,
University of Agriculture, Faisalabad, Pakistan.

Agarose Gel Electrophoresis

- Gel electrophoresis is a widely used technique for the analysis of **nucleic acids** and **proteins**.
- Agarose gel electrophoresis is routinely used for the preparation and analysis of **DNA**.
- Gel electrophoresis is a procedure that separates molecules on the basis of their rate of movement through a gel under the influence of an electrical field.
- Agarose gel electrophoresis is used to determine the presence and size of PCR products.

Agarose Gel Electrophoresis

- it is usually necessary to separate and visualize the PCR products.
- In most cases, where the products are between 200 and 30,000 bp long, this is achieved by agarose gel electrophoresis.
- Agarose is a natural polysaccharide separated from agar, which is obtained from various species of marine red algae (see wead)
- powder is melted in buffer and allowed to cool -- the agarose forms a gel by hydrogen bonding.
- The hardened matrix contains **pores**, the size of which depends on the concentration of agarose.
- The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.3-3%.

Agarose Gel Electrophoresis

- Electrophoresis is defined as the **movement of ions and charged macromolecules through a medium when an electric current is applied.**
- **Agarose** and **polyacrylamide** are the primary stabilizing media used in the electrophoresis of macro-molecules.
- Macromolecules are separated through the matrix based on size, charge distribution and structure.
- In general, **nucleic acids** migrate through a gel based on **size**, with little influence from base composition or sequence,
- whereas **proteins** separate through the matrix based on **size, structure** and **charge,** because their charge density is not directly proportional to size

Agarose Gel Electrophoresis

- agarose gel is formed on a supporting plate, and then the plate is submerged into a tank containing a suitable electrophoresis buffer.
- **Wells** are preformed in the agarose gel with the aid of a "**comb**" that is inserted into the cooling agarose before it has gelled.
- Into these wells is loaded the sample to be analyzed, which has been mixed with a dense solution (a loading buffer) to ensure that the sample sinks into the wells.
- Gel should be **5-7 mm thick**
- Cover the gel with buffer at least **2 mm depth** in the buffer

Agarose Gel Electrophoresis

- Electrophoresis apparatus is arguably one of the most vital pieces of equipment in the laboratory.
- It consists of four main parts:
  - a power supply (capable of at least 100 V and currents of up to 100 mA),
  - an electrophoresis tank,
  - a casting plate, and
  - a well-forming comb.

Agarose Gel Electrophoresis

- When DNA molecules within an agarose gel matrix are subjected to a steady electric field, they migrate through the gel at **rates that are inversely proportional to the length of the number of base pairs**.
- This is because **larger molecules migrate more slowly** than smaller molecules
- This relationship only applies to linear molecules.

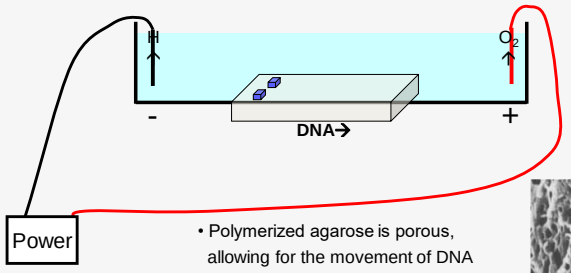
Agarose Gel Electrophoresis

- **Circular molecules**, such as **plasmids**, migrate much more **quickly** than their molecular weight would imply
- The migration rate also depends on other factors, such as the **composition and ionic strength of the electrophoresis buffer** as well as the **percentage of agarose** in the gel.
- The gel percentage presents the best way to control the resolution of agarose gel electrophoresis.

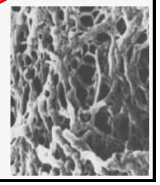
Agarose Gel Electrophoresis

- **Molecular-biology grade agarose** (**high melting point**).
- Running buffer at **1X** concentration
- Sterile distilled water.
- A heating plate or microwave oven.
- Suitable gel apparatus and power pack:
- Ethidium bromide: Dissolve in water at **10 mg/mL**.
- UV light transilluminator (long wave, **365 nm**).
- 5X loading buffer
  - glycerol,
  - EDTA,
  - bromophenol blue
  - xylene cyanol.
- A size marker

- DNA is negatively charged.
- When placed in an electrical field, DNA will migrate toward the positive pole (anode).



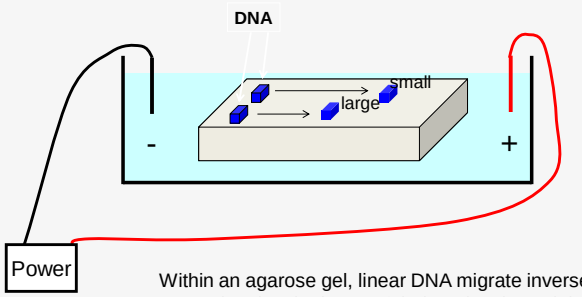
- Polymerized agarose is porous, allowing for the movement of DNA



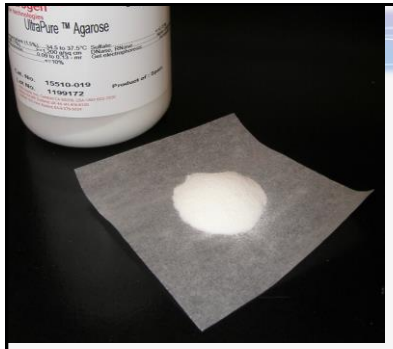
Scanning Electron Micrograph of Agarose Gel (1×1 µm) →

How fast will the DNA migrate?

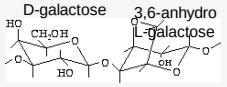
strength of the electrical field, buffer, density of agarose gel…
Size of the DNA!
*Small DNA move faster than large DNA
…gel electrophoresis separates DNA according to size



Within an agarose gel, linear DNA migrate inversely proportional to the log10 of their molecular weight.



Agarose



•Sweetened agarose gels have been eaten in the Far East since the 17th century.

***Lina Hesse**, technician and illustrator for a colleague of Koch was the first to suggest agar for use in culturing bacteria

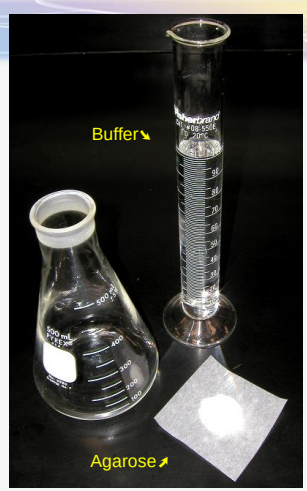
•Agarose was first used in biology when Robert Koch* used it as a culture medium for Tuberculosis bacteria in 1882

Agarose is a linear polymer extracted from seaweed.

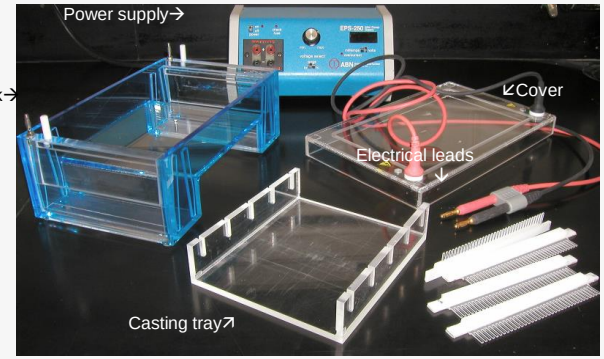
Making an Agarose Gel

An agarose gel is prepared by combining agarose powder and a buffer solution.

Flask for boiling→

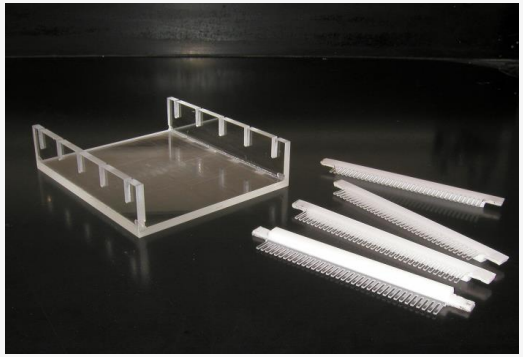


Electrophoresis Equipment



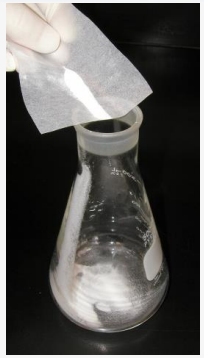
Gel combs↗

Gel casting tray & combs



Preparing the Casting Tray

Seal the edges of the casting tray and put in the combs. Place the casting tray on a level surface. None of the gel combs should be touching the surface of the casting tray.

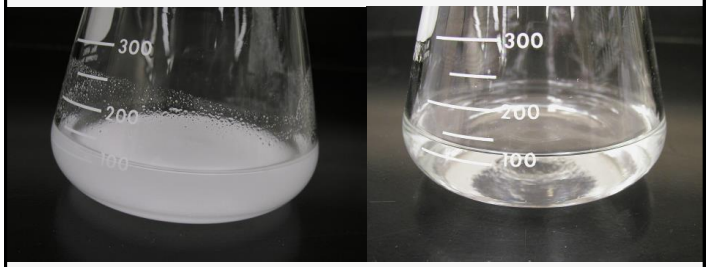


Agarose

Buffer Solution

Combine the agarose powder and buffer solution. Use a flask that is several times larger than the volume of buffer.

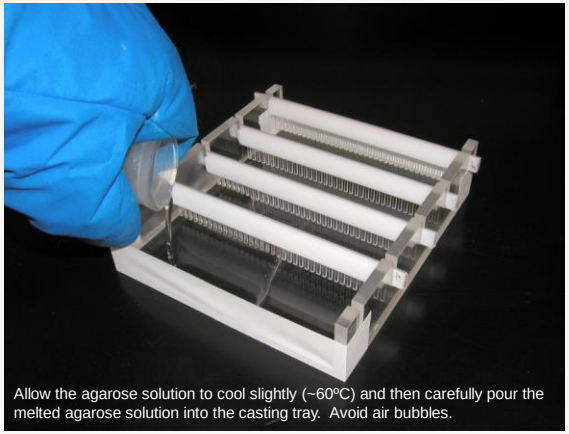
Melting the Agarose



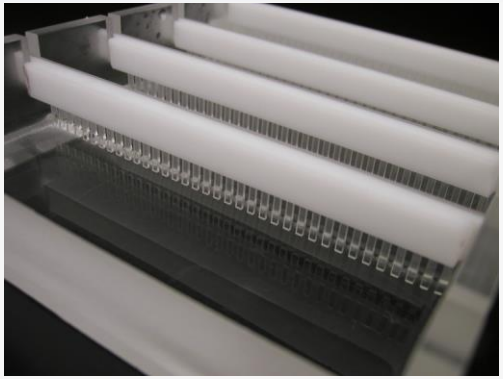
Agarose is insoluble at room temperature (left).
The agarose solution is heated until clear (right).

Gently swirl the solution periodically when heating to allow all the grains of agarose to dissolve.
***Be careful when heating - the agarose solution may become superheated and may boil violently if it has been heated too long in a microwave oven.

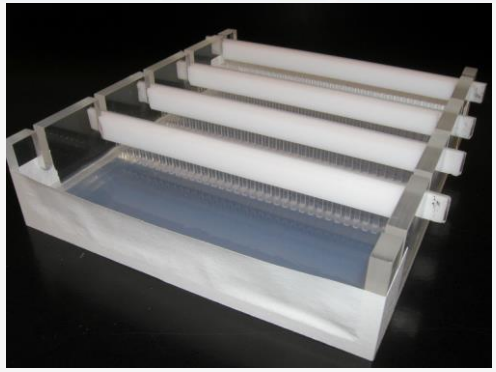
Pouring the gel



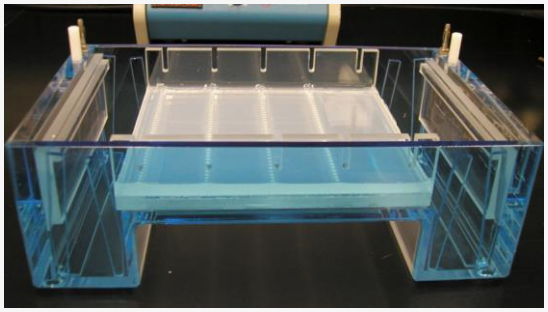
Allow the agarose solution to cool slightly (~60ºC) and then carefully pour the melted agarose solution into the casting tray. Avoid air bubbles.



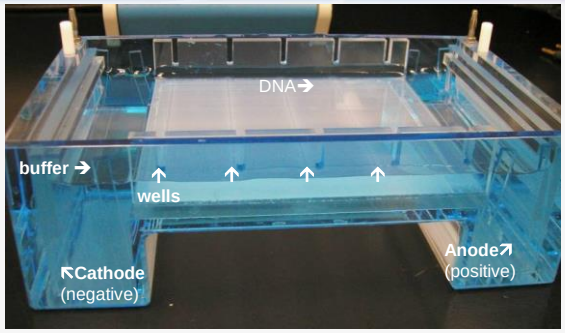
Each of the gel combs should be submerged in the melted agarose solution.



When cooled, the agarose polymerizes, forming a flexible gel. It should appear lighter in color when completely cooled (30-45 minutes). Carefully remove the combs and tape.



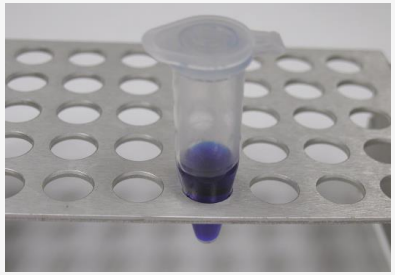
Place the gel in the electrophoresis chamber.



Add enough electrophoresis buffer to cover the gel to a depth of at least 1 mm. Make sure each well is filled with buffer.

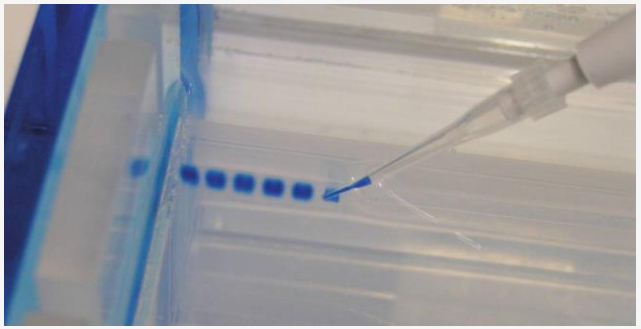
Sample Preparation

Mix the samples of DNA with the **6X sample loading buffer** (w/ tracking dye). This allows the samples to be seen when loading onto the gel, and increases the density of the samples, causing them to sink into the gel wells.



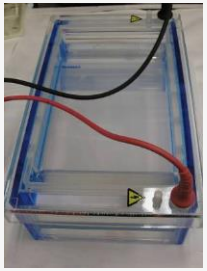
6X Loading Buffer: →
- Bromophenol Blue (for color)
- Glycerol (for weight)

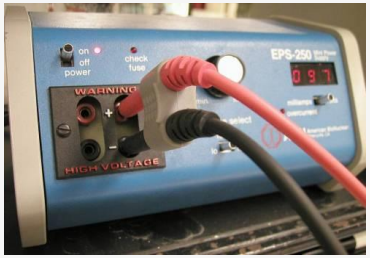
Loading the Gel



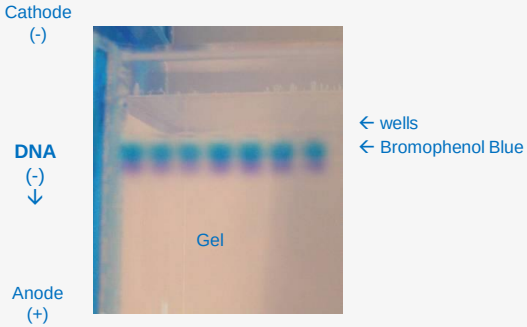
Carefully place the pipette tip over a well and gently expel the sample. The sample should sink into the well. Be careful not to puncture the gel with the pipette tip.

Running the Gel



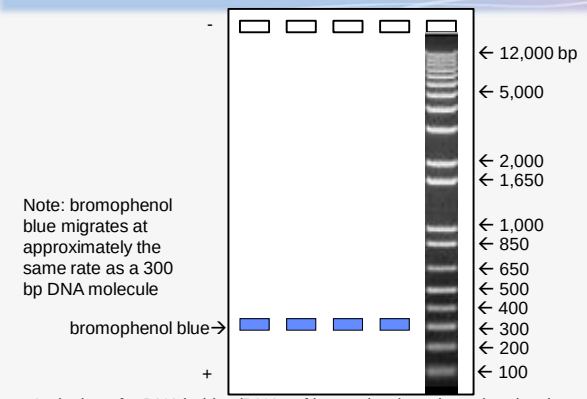


Place the cover on the electrophoresis chamber, connecting the electrical leads. Connect the electrical leads to the power supply. Be sure the leads are attached correctly - DNA migrates toward the anode (red). When the power is turned on, bubbles should form on the electrodes in the electrophoresis chamber.



After the current is applied, make sure the Gel is running in the correct direction. Bromophenol blue will run in the same direction as the DNA.

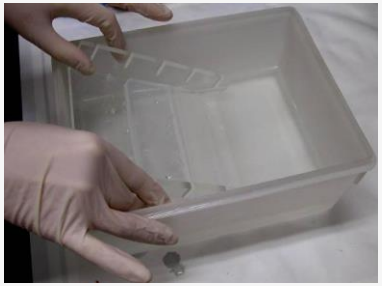
DNA Ladder Standard



Note: bromophenol blue migrates at approximately the same rate as a 300 bp DNA molecule

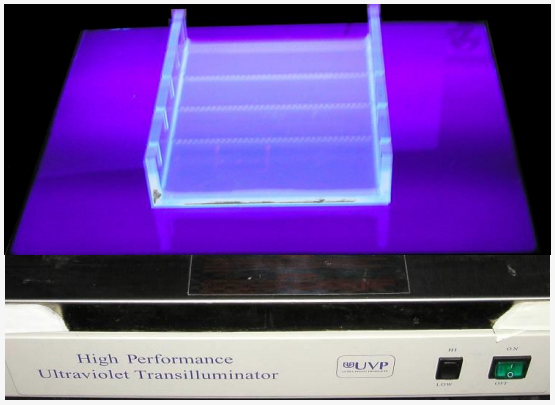
Inclusion of a DNA ladder (DNAs of know sizes) on the gel makes it easy to determine the sizes of unknown DNAs.

Staining the Gel

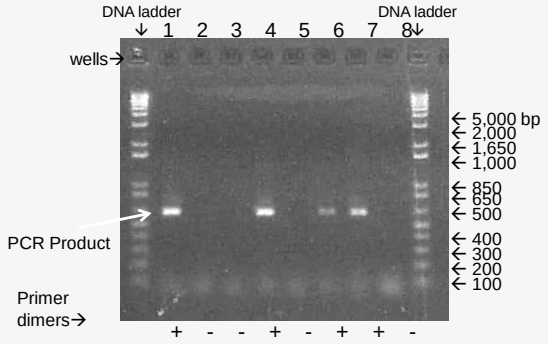


- Place the gel in the staining tray containing warm diluted stain.
- Allow the gel to stain for 25-30 minutes.
- To remove excess stain, allow the gel to destain in water.
- Replace water several times for efficient destain.

Ethidium Bromide requires an ultraviolet light source to visualize



Visualizing the DNA (ethidium bromide)



Samples # 1, 4, 6 & 7 were positive for Wolbachia DNA

Agarose Gel Electrophoresis

- Concentration of agarose also affects migration
- Higher concentration of agarose, the more it retards the movement of all DNA fragments
- Small DNA fragments require higher concentrations of agarose

Agarose Gel Electrophoresis

- Agarose gels must be prepared and run in a buffer containing ions.
- Ions are charged particles (like those found in salt) and are necessary to carry a charge
- A buffer is a substance that resists changes in pH.
  - It will neutralize a base---make it into water
  - It will neutralize an acid---make it into water

Agarose Gel Electrophoresis

- During electrophoresis water undergoes hydrolysis : H_2O → H+ and OH-
- The anode (+ /red) pole becomes alkaline because OH- will accumulate at this pole
- The cathode (-/black) pole becomes acidic because H+ will accumulate at this pole

Agarose Gel Electrophoresis

- The buffer is either TBE or TAE
  - TBE is made with Tris/Boric Acid/EDTA
  - TAE is made with Tris/Acetic Acid/ EDTA

Agarose Gel Electrophoresis

- EDTA is the metal chelator (ethylenediamine tetraacetic acid)
- Used in many buffers to protect molecules because molecule destroying enzymes often use metals
- Used in most **shampoos**, **detergents** and **shower shine** products

Agarose Gel Electrophoresis

- The voltage applied to the gel affects how quickly the gel runs
- The higher the voltage, the more quickly the gel runs……But that often reduces the quality of the DNA separation
- >>>>>>>>>>It also generates heat which reduces the quality of the DNA separation

Agarose Gel Electrophoresis

- When running an analytical gel, the optimal resolution is obtained at about 10 V/cm of gel.
- When fragments of **5 kb and above** are to be analyzed, better resolution is obtained at about 5 V/cm.
- Fragments smaller than 1 kb are normally resolved better at higher V/cm.
- For **larger DNA**, the best choice is TAE in combination with a low field strength (1-2 V/cm).
- A 0.5 x TBE buffer has greater buffering capacity than a 1 X TAE buffer

Agarose Gel Electrophoresis

- The best separation will apply voltage at no more than 5V/cm of gel length.

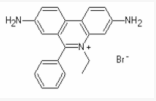
Agarose Gel Electrophoresis

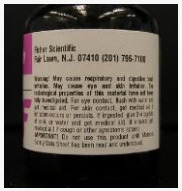
- To make DNA fragments visible after electrophoresis, the DNA must be stained
- The favorite—ethidium bromide
  - When bound to DNA it fluoresces under ultraviolet light
  - Quite sensitive
- Two big problems
  - UV light can damage your eyes----and many students don't wear safety gogles!!!!!
  - Ethidium bromide is a mutagen!!!!!

Staining the Gel

• Ethidium bromide binds to DNA and fluoresces under UV light, allowing the visualization of DNA on a Gel.

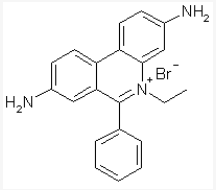
• Ethidium bromide can be added to the **gel** and/or **running buffer** before the gel is run or the gel can be stained after it has run.





***CAUTION! Ethidium bromide is a powerful mutagen and is moderately toxic. **Gloves should be worn at all times**.

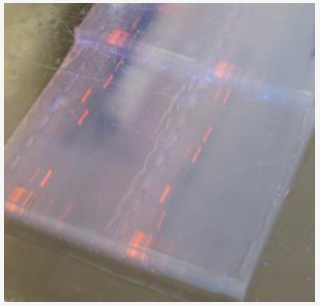
Agarose Gel Electrophoresis



- In the Ames test, 90ug of EtBr is as mutagenic as the smoke from one cigarette.
- The standard concentration used in staining DNA in gels is 0.5-1ug/mL

Agarose Gel Electrophoresis

- Convenient because it can be added directly to the gel
- Sensitive—detects 0.1ug of DNA
- Inexpensive--$0.02 per gel
- Stains in 10 minutes/or immediate if in gel
- Re-useable



Agarose Gel Electrophoresis

- Wear gloves when preparing solutions, handling gels.
- Powder is especially dangerous due to possible inhalation and so premade solutions are always purchased

Safer alternatives to Ethidium Bromide

- Methylene Blue
- BioRAD - Bio-Safe DNA Stain
- Ward's - QUIKView DNA Stain
- Carolina BLU Stain

…others

Agarose Gel Electrophoresis

Alternatives to EtBr

Methylene Blue

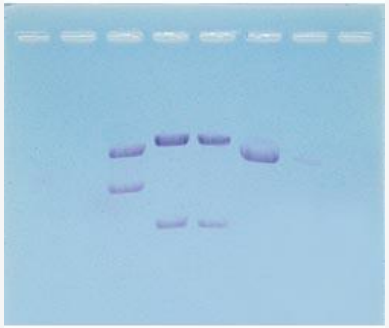
- Sensitivity –Better than 0.5ug DNA
- Stain time 30 minute
- Can't be added to gel
- Stain solution can be reused
- Cost $0.20/gel
- Used white light

SYBER Safe

- Sensitivity –Better than 0.1ug DNA
- Stain time 30 minute
- Can't be added to gel
- Stain solution can't be reused
- Cost $0.50/gel
- Used UV light

Agarose Gel Electrophoresis

A gel stained with Methylene blue



De-staining is accomplished by soaking the gel in an excess of water for about an hour.