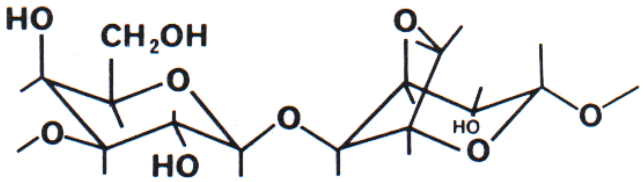
TECHNIQUES IN MOLECULAR BIOLOGY – AGAROSE GELS (HORIZONTAL GEL ELECTROPHORESIS)

DNA gels are used to separate fragments of DNA and RNA. Unlike most protein separations which use acrylamide polymers, use agarose in a submerged horizontal orientation, and at time called horizontal gel electrophoresis. This handout will cover the details of agarose gels, the theory of separation by agarose gel electrophoresis and tips for conducting successful gel electrophoresis.

The basic principle of separation for all electrophoresis is the movement of a charged molecule in a medium subjected to an electric field.

$$v=Eq/f$$

V is the velocity of the molecule subjected to electrophoresis. E is the electrical field in volts/cm, q is the net charge on the molecule and f is the frictional coefficient. The impact of f depends on the mass and shape of the molecule. This equation simply explains that the rate (v) of a particle depends on the electrical field and charge but inversely impacted by the counteracting force generated by the viscous drag. Factors influencing F is of course the size and shape of the molecule. Think of a short linear oligonucleotide vs a large supercoiled plasmid vs long chromosomal DNA. Adding a value to f is the media through which the molecules migrate.



Repeating pattern of agarose

Agarose is a seaweed extract (red algae agar) and is a long polymer of D and L galactose and derivatives in a linear polymer bonded by two different glycosidic bonds. Once hydrated and formed into a gel, the carbohydrate will form helical fibers and aggregates creating channels of 50 to more than 200 nm in diameter. DNA and RNA molecules migrate by 'reptation' (snaking through this matrix).

The mobility (μ) of molecule in gel is related to agarose concentration (ι):

$\log \mu = \log \mu_0 - K_r\iota$

μ_0 – free mobility of DNA in solution

K_r – retardation coefficient (relates to properties of gel and migrating molecules)

ι – agarose concentration

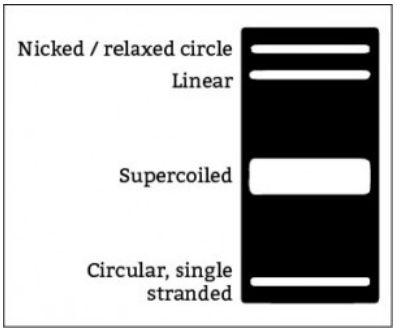
Thus the effect is that the distance migrated in gel decreases as log of molecular weight. What this tells us is the concentration of agarose affects range of effective separation of molecules. The higher the percent of agarose in a gel, the more dense agarose and smaller pores in the solidified gel. Thus

A table describing the separation of DNA is shown below.

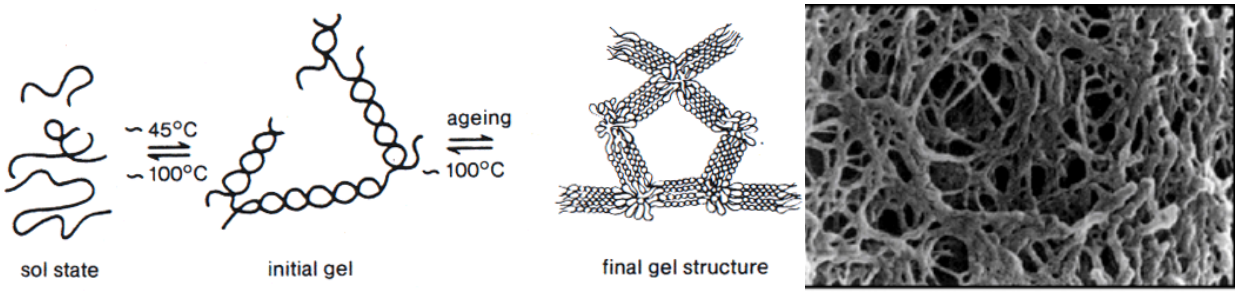
Agarose concentration in gel (%, w/v)	Efficient range of separation of linear DNA molecules (kb)
0.3	5 - 60
0.5	1 - 20
0.7	0.8 - 10
0.9	0.5 - 7
1.2	0.4 - 6
1.5	0.2 - 3
2.0	0.1 - 2

(from p. 6.5, Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

It simple to consider the two equations above of rate and mobility that between two strands of DNA, one much smaller than the other, that there will be a greater charge in the larger DNA. However, the charge to mass ratio for both DNA strands will be the same. While the negative charge of the phosphate backbone will attract the nucleotide to the positive electrode, the size and shape of the DNA will impact its mobility through the gel pores. Lets consider a 2.5 kB plasmid. A gel electrophoresis of a purified plasmid will revel three or four bands (see figure to right). As created by *E. coli,* plasmid DNA is double stranded and tightly wound. Think of an overwound rubber band; this is called supercoiled. Supercoiled DNA, while having the same mass will take a much smaller volume of space than relaxed DNA and thus run as if it were a smaller size. The two larger, slower migrating bands, are the result if one of the strands of a supercoiled plasmid were cut and the plasmid unwound and relaxed. It is still a circular plasmid but as a fully relaxed nucleotide will have a retarded run through the agarose gel pores and migrate at a higher more expected molecular weight. If both strands are cut by mechanical or enzymatic means, the DNA will no longer be circular and will run at its "true" molecular mass as a linear piece of DNA. The smallest band is a single strand that is remains circular. During alkaline lysis, the low pH of the solution can defeat the hydrogen bonds maintaining the complimentary strands together IF the DNA is exposed to the low pH for too long. This results in a permanently denatured single stranded closed circles of DNA that migrates much faster than the supercoiled DNA.



Additionally, if there is RNA contamination, it will run as a wide band at a much smaller molecular mass than plasmid or genomic DNA. This is because there is no one specific size for the many types of RNA and mRNA has a range of sizes (depending on the ORF!) and thus does not run as a discrete tight band... It pays to know what your looking at and why!



Types of Agarose. Agarose in not just agarose. There are many types of agarose. Standard agarose has a high strength and high melting temperature. Melting temp: 85-95°C; gelling tem: 34-42°C. While low melting temperature agaroses melt at 63-65°C and gel at 25-35°C. A quality agarose will have a low electroendo-osmosis (EEO). That is the tendency of negatively charged sulfates in agarose to induce positive ions in the buffer causing the DNA to migrate in the opposite direction.

Some of the differences are the preparation and purity (sulfate and other salts), while other agarose preparations have been modified to have different physical characteristics. One example is low melting agarose, often used for retrieving DNA from agarose after electrophoresis, is modified by hydroxyethlation to reduce the number of hydrogen bonds needed to melt the solidified agarose. BioRad has a nice explanation of the different types of agarose available to purchase. An abbreviated table is shown below:

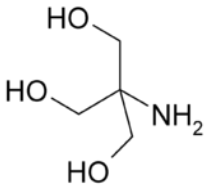
Molecular biology agarose**:** This is a general-purpose agarose that has a high exclusion limit. This type of agarose has high gel strength and is easy to handle at low percentages. Molecular biology agarose is GQT (genetic quality tested) grade, making it ideal for preparative gels and recovery of DNA. Analytical separation is >1,000 base pairs (bp).

Low-melt agarose**:** The main use of low-melt agarose is for preparative electrophoresis. It is ideal for in-gel applications such as ligation, PCR, restriction enzyme digestion, transformation, and sequencing. Other applications include pulsed field electrophoresis of megabase DNA and embedding chromosomes. This agarose has a gelation temperature of 26°C and high resolving capacity, >1,000 kb.

Low-melt agarose is also used to secure immobilized pH gradient (IPG) strips for isoelectric focusing, the second dimension of 2-D SDS-PAGE. This agarose contains bromophenol blue for monitoring electrophoresis.

PCR agarose**:** This high-strength agarose forms gels that are easy to handle and remain flexible even at high gel percentages, reducing the risk of cracking or breaking. Gelation temperature is 40°C. This agarose has excellent sieving properties and the highest gel strength of all the agaroses.

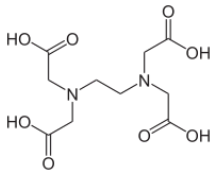
PCR low-melt agarose has a high sieving capacity. It is ideal for preparative electrophoresis and in-gel applications such as restriction enzyme digests, ligation, and transformation. PCR agaroses are recommended for DNA fragments <1,000 bp.



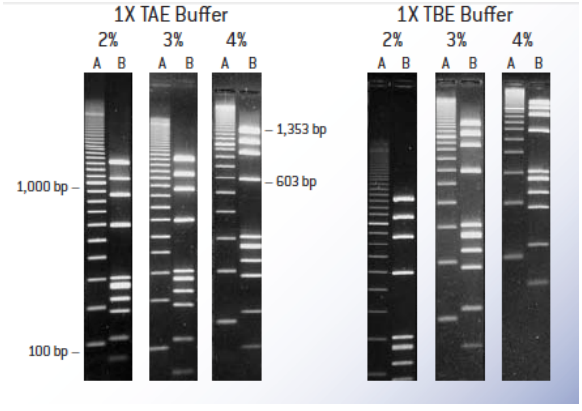
Tris pKa 8.07 at 25oC. Note the amino group responsible for proton donation or accepting as a buffer.

Buffers - There are a few basic buffers for running DNA gels. ***TAE, TBE, TPE and Borate buffers*** (not the same as a TBE buffer). Each has a different use.

T= Tris or Trizma; a buffer to maintain pH of the solution. Other buffering compounds used in DNA gel buffers are A - acetate or B - borate. During electrophoresis, protons are generated at the anode and hydroxyl ions at the cathode. Thus a pH buffered system is critical to maintain the pH of the system. At a neutral pH range the buffers ensure the phosphate groups of DNA and RNA are charged and will migrate towards the anode.



E = EDTA; ethylenediaminetetraacetic acid. This is a chelator of divalent cations. Specifically Fe^{2+}, Ca^{2+} and Mg^{2+}. It is used to remove such metals from solution which are important for the activity of most DNA involved enzyme reactions and limit metal-induced oxidation during electrophoresis. *PS- EDTA is not soluble until titrated to pH~8.0. Depending on the concentration you are preparing, it may take quite a bit of NaOH to titrate EDTA into solubility. Ensure your additions don't overshoot the required final volume!*

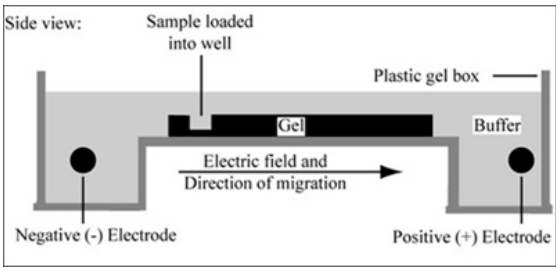


Impact of % gel and buffer selection on resolution (separation) of DNA. Lane A 100 bp ENA ladder (4-7.5 ng/band) Lane B. *Hae III* DNA digest (0.25 ng/band) From Lonza Inc.

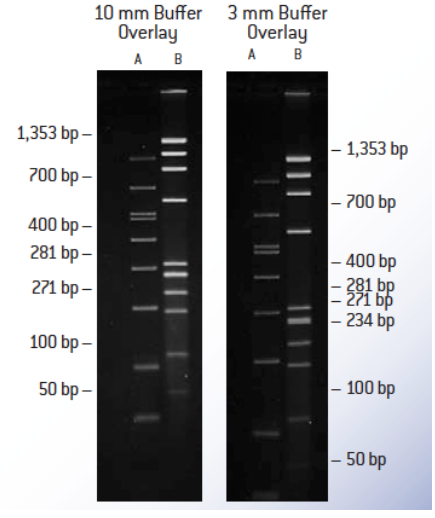
Each gel and buffer should match the correct use. TAE is best used if recovering DNA from gel slice, while TBE is better for smaller (<1kB) DNA strands. See figure and table for details.

TAE Buffer	TBE Buffer	TPE Buffer	Na Borate Buffer
• Use when DNA is to be recovered • Use for large > 12kB DNA • Low ionic strength • Low buffering capacity - may need to recirculate for extended runs	• Used for <1kB DNA - provides tighter bands with higher % gels • Decreased DNA mobility • High ionic strength • High buffering capacity • Not best buffer if recovering DNA after run	• High buffering capacity • Will work for recovering DNA after run • Good for long runs • Used for analysis of single-stranded DNA • Will interfere with phosphate-sensitive reactions of recovered DNA	• Used for high voltages providing faster runs • Limited resolution • Best for quick analytical gels of purified DNA or restriction digests
50X TAE Stock • 242.0 g Tris Base • 57.1 ml Glacial Acetic Acid • 18.61g $Na_2EDTA{\cdot}2H_2O$ • QS to 1.0 liter with water - do not adjust pH, but check... *note: can use stock EDTA to make buffer, will need to check and adjust pH depending on stock (100 ml of 0.5 M EDTA at ph 8.0)* 1X=40 mM Tris pH 7.6-8.0, 20 mM acetic acid, 1 mM EDTA	**10X TBE Stock** • 108.0 g Tris Base • 55.0g boric acid • 40 ml 0.5M EDTA (pH 8.0) • QS to 1.0 liter with water 1X=89 mM Tris pH 8.3, 89 mM boric acid, 2 mM EDTA	**10X TPE Stock** • 108.0 g Tris Base • 15.5 ml 85% Phosphoric acid • 7.44 g $Na_2EDTA{\cdot}2H_2O$ • QS to 1.0 liter with water 1X=89 mM Tris pH 8.3, 89 mM boric acid, 2 mM EDTA	**1X Na Borate (SB)** • Prepare 1M boric acid (6.1 g/100 ml water). • Carefully add 1.0 ml of 10 M NaOH to 500 ml water with stirring • Adjust pH of NaOH solution to pH 8.5 using a 1M Boric Acid slurry in a dropwise fashion. *note: Boric acid may not go into solution easily, slightly warm and/or use as a well mixed slurry. Biotechniques 36:214-216 2004*

OTHER IMPORTANT FACTORS:



Buffer Depth and Depletion: For any buffer the depth of buffer over the gel should range from 3 to 5 mm. Too much buffer will distort bands and cause heating and partial melting of the gel. Too little buffer and the gel is likely to partially dry out. Gel melting and band smearing is a tell-tale sign that the pH capacity of the buffer/gel has been depleted. This is mostly observed in longer runs in larger gels. Most mini-gels will not have this problem.



Effect of buffer depth on DNA gel. Notice loss of loss of intensity and bands with a deep (10 mm) buffer overlay.

Edge Effects / Smiling Gels: An uneven gel will cause issues with the electrical current subjected to the gel. Increased thickness of gel at the edge decreases resistance. Higher current causes more rapid migration of DNA at edges. Both of these will cause a smiling or sad (frowning) shape to the DNA gel. Often the outer gel lanes are avoided, as this effect hard to prevent.

Gel Loading Dyes: The dyes **xylene cyanol FF (XC)** and **bromophenol blue (BB)** plus **30% glycerol** in water are often used to help visualize where your samples are during loading the gel and to find the "leading edge" and middle sized samples of your samples during electrophoresis. The glycerol makes the final solutions dense so they sink to the bottom of the wells. Using both dyes is helpful to see separation, BB is purpled and migrates at about the same rate as a linear double-stranded **400 bp** DNA fragment whereas XC is blue-

green and migrates at about the same rate as a **8000 bp** DNA fragment. When BB reaches the end of the gel (about 2/3 of the way down the gel) the electrophoresis run is finished. See table below for the approximate migration of these dyes in other concentrations of agarose gels (using 1X TBE running buffer). Some buffers include both dyes. [Source: New England Biolabs (http://circuit.neb.com/neb/products/nucleic/N3272.html)]

10X DNA loading dye

- 0.025g Xylene cyanol FF (0.25% w/v)
- 0.025 g Bromophenol Blue (0.25% w/v)
- 5.0 ml glycerol * (5%)
- 2 ml 500 mM EDTA ** optional (10 mM)
- QS to 10 ml with TAE buffer.

* Sucrose (40%), Ficol (25-30%) or glycerol (30-50%) can all substitute for each other.

**Optional: 1 ml of 10% SDS added before QS with TAE can be included to eliminate protein-DNA interactions, preventing appearance of additional bands (protein-DNA complexes).
100 mM (in 10X stock) EDTA can be added to avoid enzymatic degradation of DNA/RNA

% agarose	xylene cyanol FF	bromophenol blue
0.5	20-40 kb	4,000 bp
0.8	8,000 bp	400 bp
1.0	4,000 bp	300 bp
1.3	1,800 bp	150 bp
1.5	1,200 bp	100 bp
2.0	700 bp	65 bp

Preparing Gels: Agarose undergoes a series of steps when it is dissolved; dispersion, hydration and melting/dissolution. Dispersion simply refers to the separation of particles by the buffer without clumping. Clumping occurs when the agarose starts to dissolve before it is completely dispersed, coating itself with a gelatinous layer which inhibits the penetration of water and keeps the powder from dispersing. Hydration is the surrounding of agarose by an aqueous (water/running buffer) solution. In part, this occurs because hydration is time dependent and microwave ovens bring up the temperature rapidly. The problem is exacerbated by the fact that the agarose is not being agitated to help dilute the highly concentrated solution around each particle and dissolution is slowed. Melting and dissolution: Melting can be done in a microwave or a hot plate. As the particles hydrate they become small, highly concentrated gels. The melting temperature of a standard agarose gel is 93oC. All must be boiled to fully hydrate and melt the gels. (From Lonza Bench Guides).

Microwave instructions:

- Choose a flask that is 2-4 tiimes to volume of the solution
- Add room temperature buffer and stir bar to the flask
- Sprinkle in the agarose powder while solution is rapidly stirred
- REMOVE stir bar!!!!
- Cover with plastic wrap and puncture hole for ventilation
- Weigh the mass of flask (without the stir bar).
- Heat flask on high (did you remove the stir bar?) until bubbles appear.
- Remove beaker and GENTLY swirl the beaker to resuspend any settled bowder and gel pieces. BE CAREFUL!!! A microwaved solution can become overheated and when agitated can bump molten hot agar onto your hand. Wear SAFETY GLOVES AND EYE PROTECTION.
- Reheat the flask until the solution comes to a boil. It may be safe to use short time periods depending on the microwave. Hold the boil for about one minute.
- Gently swirl the flask (USING SAFTEY OVEN MITS/GLOVES) to mix the agarose solution

- Add hot distilled water to obtain the initial weight and mix thoroughly.
- Cool solution to 60oC before adding stain and casting.

NOTE: *Unused and unstained agarose can be stored and re-melted (without stain) but the percent will be slightly higher each time unless you measure the mass before and after heating while replacing with hot water as above.*

Stain:

Ethidium Bromide [CAUTION]

Staining with ethidium bromide (EtBr) is a rapid, sensitive, and highly reliable method for visualizing DNA in gels. The stained gel is illuminated from below ('transillumination') with short- or medium-wavelength of UV light causing the EtBr, bound to DNA, to fluoresce brightly. As a molecule that binds DNA, however, EtBr is a mutagen and likely carcinogen. EtBr should be handled with appropriate caution, and its use restricted to limited areas. Gloves and a lab coat should always be worn when using EtBr or handling items that might be contaminated with EtBr (such as gel staining containers).

To limit the generation of waste, the EtBr staining solution can be reused repeatedly until staining in gels begins to fade. Stained gels and the first rinse from destaining will be collected as hazardous waste.

SYBR Safe DNA Stain [Safe Alternative]

SYBR Safe is a cyan based, non-mutagenic dye (no observed in an acute oral toxicity study in rats) form of SYBR Green dye. The dye absorbs in the blue range, fluoresces only when complexes with DNA and then emits in the green (lambda max 520 nm). The dye is purchased as a highly concentrated stock (often 10,000X). Add to cooling agarose gel before pouring into a casting stand.

- SYBR Safe stain can use blue light for visualization avoiding UV damage to DNA
- For 50 ml of gel, 5 ul of 10,000X will be enough for most minigels
- SYBR Safe is slightly less sensitive than ethidium bromide. SYBER Green I is much more sensitive (>60 pg per band) than either stain.
- Will not alter downstream applications and will not ppt in ethanol DNA precipitations.

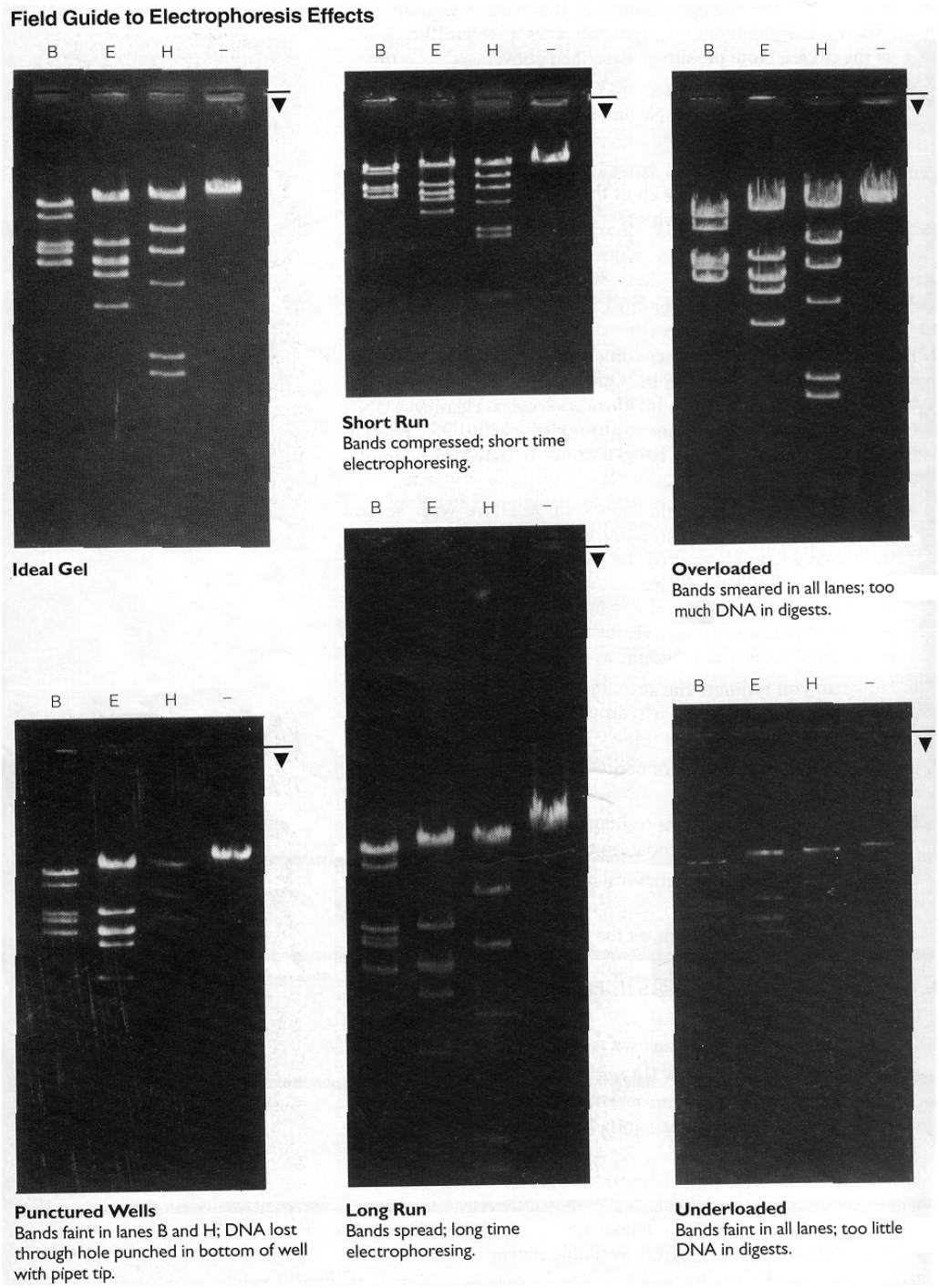
Casting the Gel:

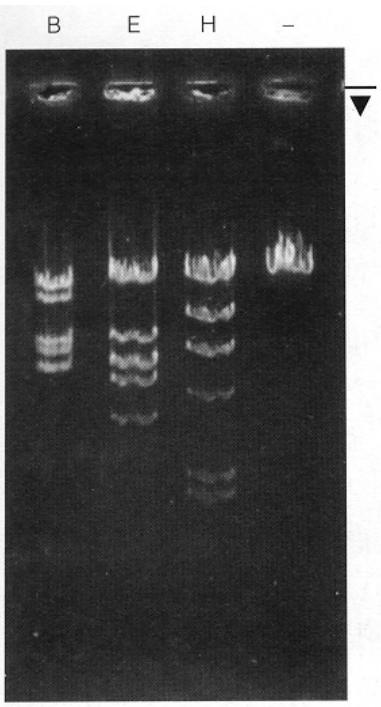
- Measure the required volume for a gel (should be 3-4 mM thick). Thick gels will cause problems during electrophoresis. Smaller DNA fragments will be lost or fuzzy. Thick gels also show much higher background staining. See figure.
- Level and assemble the tray (tape or damns at the end of the tray as appropriate).
- Allow a small 0.5-1.0 mm space between bottom of comb and tray.
- Pour gel and allow to cool at room temperature for 30 min.
- Slowly wiggle and remove the comb
- Place the tray into the gel unit and cover with 3-5 mm buffer. Ensure loose fragments are flushed out of loading wells
- Slowly, keeping positive pressure on the pipetter, dispense DNA into the gel.

Visualization: Depending on stain, follow manufacturer's instructions. If visualizing directly on UV tansluminator, wear protective eyewear or best, face shield to protect from UV damage to eyes and sunburn.

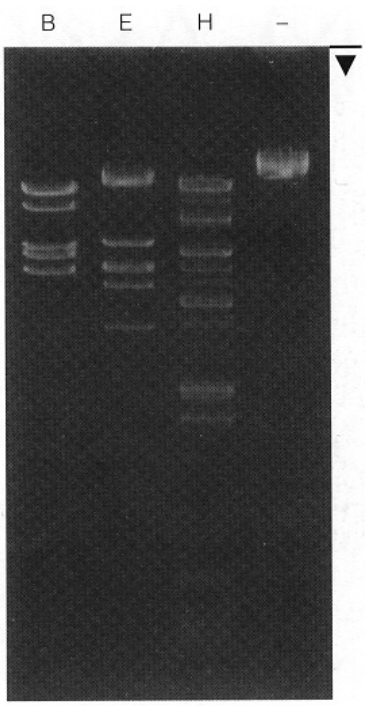
- 1-10 ng of a single, double stranded DNA band should be appropriate. Less is possible with some stains. Do not exceed 100 ng of DNA per band. 1-5 ng per band for ethidium bromide and 3-10 ng per band will give reasonable results.

  - This is not total DNA - account for all potential bands!
- Loading the smallest volume will give the best results
- The recommended voltage [actually *electrical field strength*] is 4–10 V/cm ([Total voltage divided by the] distance between anode and cathode, not the length of the gel) in the gel electrophoresis unit. If the voltage is too low, then the mobility is reduced and band broadening will occur due to diffusion. If the voltage is too high, the band resolution is reduced, mainly because of gel overheating.
- Typical runs in mini-gel format are 50-100 volts.
- Run until bromophenol blue dye is 2/3 of the way through the gel.

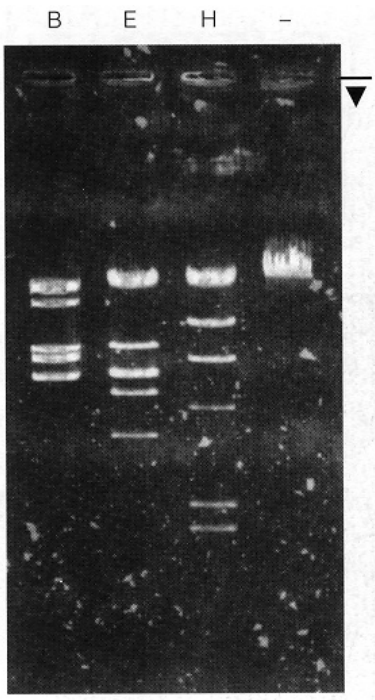




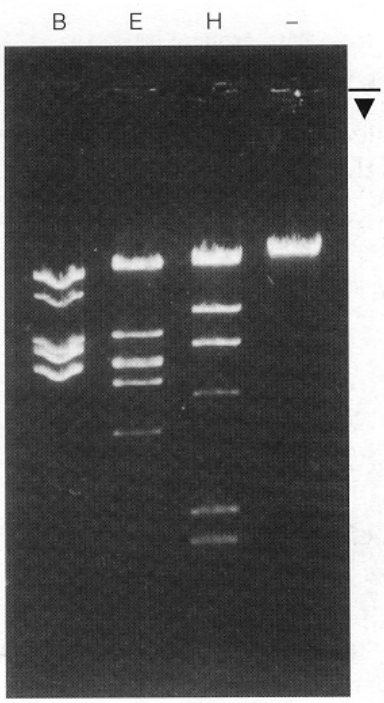
Poorly Formed Wells
Wavy bands in all lanes; comb removed before gel was completely set.



Enzymes Mixed
Extra bands in Lane H; *Bam*HI and *Hin*dIII mixed in digest.

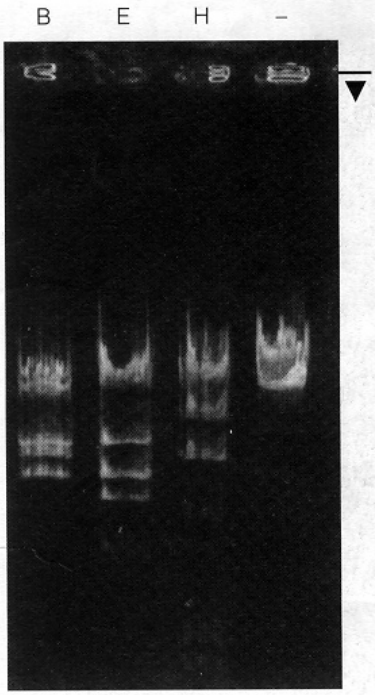


Precipitate
Precipitate in TBE buffer used to make gel.



Bubble in Lane
Bump in band in lane B; bubble in lane.

Incomplete Digest
Bands faint in lane H; very little *Hin*dIII in digest. Also, extra bands are present in lanes B and E.



Gel Made with Water
Bands smeared in all lanes; gel made with water or wrong concentration of TBE buffer.