Gel Electrophoresis of DNA

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Electrophoresis is used for the separation of DNA, RNA, or other charged molecules like proteins by an electric current applied to a gel matrix. In PCR applications it is usually done for separation and visualization of DNA after PCR or sequencing. The electrophoresis gel is a porous matrix that allows movement of DNA through its pores. DNA fragments with large differences in size are typically separated on agarose gels whereas small differences in DNA fragment sizes are resolved on polyacrylamide gels.

The nucleic acids have a negative charge due to the sugar phosphate backbone of the molecule. This allows movement from the negative to the positive electrode. The double-stranded DNA fragments naturally behave as long rods, so their migration through the gel corresponds to their size. Single-stranded DNA or RNA tends to form secondary structures with complex shapes and migrate through the gel in a complicated manner. This can be overcome by adding denaturing agents like formamide or urea to remove secondary structures and cause them to behave as long rods.

Agarose gel electrophoresis

Agarose is composed of long un-branched chains of carbohydrate. Since there are no cross links between chains these gels have large pores and are suitable for separation of larger molecules of DNA. NuSieve agarose is a special product with smaller pore size and better resolution. The agarose gels are typically run in horizontal tanks after submerging in a buffer solution hence the name submarine gels (Fig. 5.1). The agarose gels are unable to withstand high voltage because the heat generated may cause melting of the gel.

Preparing 2% agarose gel (14 x 10 x 0.5 cm)

1. In a 500 ml beaker take:
    - Agarose 1.6 g
    - 1 x AGB buffer 80 ml
2. Cover with a cling film.
3. Heat in a microwave oven for 2 minutes at high power.
4. Seal the ends of a gel tray with cello-tape.
5. Pour the molten agar on the gel tray.
6. Place the appropriate sample comb in the gel tray.
7. Leave to cool for 30-60 minutes. The gel will solidify and appear opaque grayish in colour.
8. Make sure to remove the scotch tape before running the gel.

To prepare 4% NuSieve agarose gel (14 x 10 x 0.5 cm) take the following in a 500 ml beaker:

a. Agarose: 1.6 g
b. NuSieve agarose: 1.6 g
c. 1 X AGB buffer 80 ml

Running the agarose gel

1. Fill the electrophoresis tank with 1 x TBE buffer.
2. Place the gel in the electrophoresis tank while it is still in the casting tray.
3. Carefully remove the comb(s) from the gel. If the comb is removed hurriedly or the gel has not solidified completely the well floor may be damaged.
2. Prepare the amplified DNA for loading by mixing 20µl amplified product and 5µl loading dye. The amplified DNA must be handled with care as it is a potent source of contamination for other PCR reactions. It is advisable to use a separate pipette marked as "amplified DNA only".
3. Carefully load 20-25µl of the DNA and loading dye mixture in to the wells. The DNA in the sample quickly sinks to the floor of the well because of the glycerol in the loading dye.
4. Start electrophoresis at ~150 volts for 30-60 minutes depending on the size of the DNA fragments to be resolved. Use of higher voltage can cause melting of the gel.
5. Progress of electrophoresis can be monitored by movement of the blue coloured loading dye. Bromophenol blues moves in the agarose gel at approximately the speed of a 200bp DNA fragment.

Staining of agarose gels

The agarose gels are stained with ethidium bromide which is an intercalating dye that binds to double stranded DNA. It fluoresces when exposed to ultraviolet light. The reddish orange fluorescence of ethidium bromide increases 20 fold after binding to double stranded DNA. Ethidium bromide is a carcinogen and mutagen therefore it should be handled after wearing gloves.

1. Add 3-5 drops of 1% ethidium bromide solution to 500 ml of water in a staining tray.
2. Carefully remove the gel from the tray and submerge it in the stain.
3. Keep in the stain for 30 minutes.
4. Transfer the gel to 500 ml clean water and keep for 5-10 minutes. This will wash the excess background satin from the gel.

5. Place the gel on UV transilluminator. The amplified DNA fragments are seen as reddish orange bands (Fig. 5.2).
6. A permanent record of the gel is kept by taking a picture with a camera or gel documentation system.

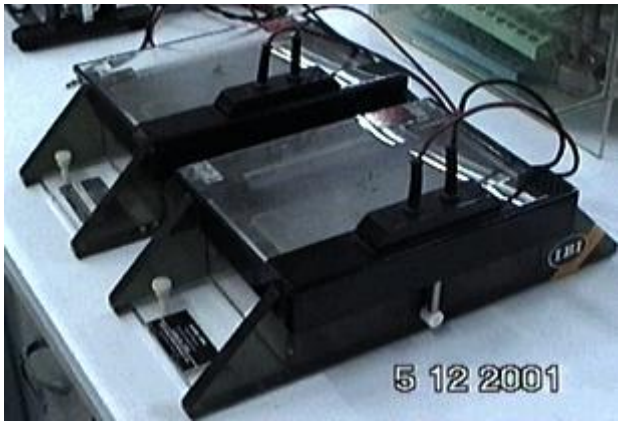

Fig. 5.1. Submarine gel electrophoresis tank.

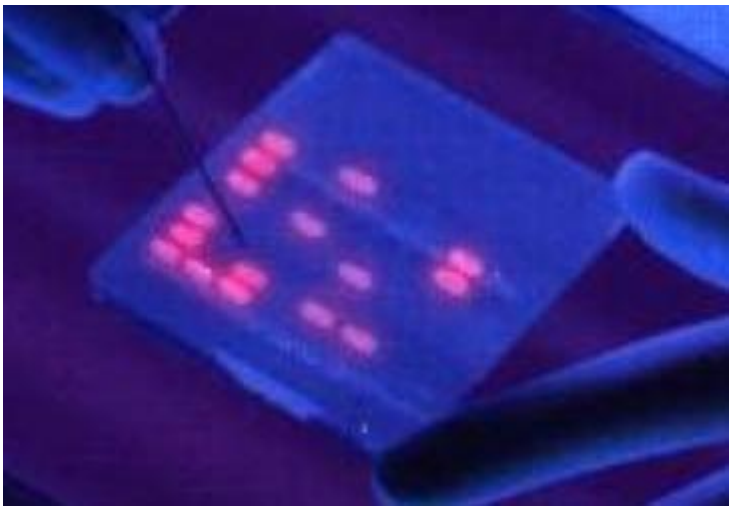

Fig. 5.2 Ethidium bromide stained agarose gel. The amplified DNA appears as reddish orange bands.

AGB Buffer (10 X)

Tris: 48.4 g

$NaAC.3H_2O$:	27.2 g
Disodium EDTA:	0.744 g
Glacial acetic acid:	12 ml
Deionized water:	up to 1L

TBE Buffer (10 X)

Tris:	108 g
Boric acid:	55 g
Na_2EDTA:	9.3 g
Deionized water:	up to 1L

Loading dye for agarose gels

• Bromophenol blue	5 mg
• Glycerol	10 ml
• Deionized water	up to 100 ml

Ethidium bromide (1% stock solution)

• Ethidium bromide	1 g
• Distilled water	100 ml

Acrylamide gel electrophoresis

Acrylamide is a white crystalline powder that forms polymers after dissolving in water. The acrylamide polymers can be cross-linked by addition of bisacrylamide. As a result of cross-linking the pore size of the gels also decreases. This makes acrylamide gels most suitable for separation and resolution of DNA molecules that may differ in size by only one base pair. The acrylamide gels can withstand high temperatures and hence are suitable for applications requiring high voltage. Acrylamide gels are typically run in a vertical position using discontinuous buffer compartments (Fig. 5.3).

Acrylamide in solution becomes viscous but does not form a gel. The cross-linked acrylamide polymers can be solidified to form a gel by adding ammonium persulphate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) in equimolar ratio. The concentration of APS and TMED determine the rate of gel formation and its turbidity and elasticity.

Acrylamide should be stored in a cool dark and dry place to reduce autopolymerisation and hydrolysis. Acrylamide is neurotoxic that is absorbed through skin. Its effect is cumulative and toxicity may develop over prolonged exposure. Gloves must be worn when using acrylamide.

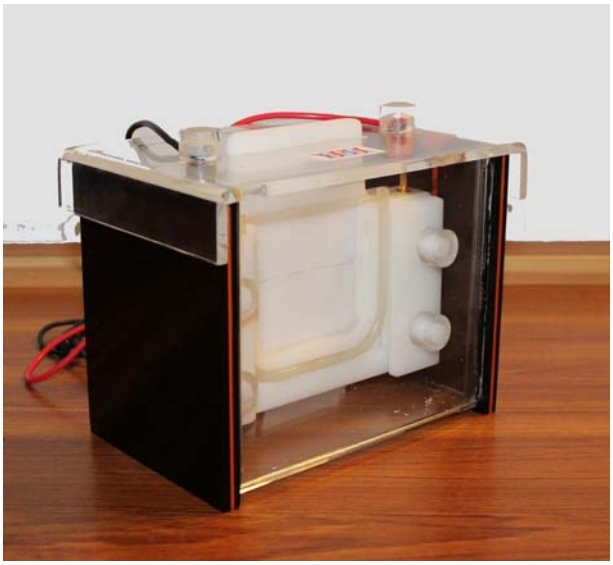

Fig. 5.3. Vertical polyacrylamide gel electrophoresis tank.

Preparation of 6% polyacrylamide mini gel (10 x 10 x 0.1 cm)

1. Make 6% acrylamide solution by mixing the following:
   - Acrylamide 28.5 g
   - N, N' methyl bis-acrylamide 1.5 g
   - TBE buffer (10 X) 50 ml
   - Distilled water up to 500 ml
2. Wash 10 x 10 cm glass plates with soap and water.
3. Dry the plates completely with cotton gauze.
4. Put 1-2 ml ethanol on the surface of the plates facing the gel and wipe the surfaces thoroughly with dry piece of gauze to remove any grease etc.
5. Assemble the glass plates as per instructions of the manufacturer.
6. Place the required comb between the plates.
7. Take 10 ml 6% acrylamide in a small beaker.
8. Add 100µl 10% ammonium persulphate (APS) not older than one week.
9. Add 20µl TEMED.
10. Gently mix and fill the polymer in a 10 ml syringe.
11. Attach a 21 gauge butterfly needle set and gently push the plunger to fill the polymer in the tubing. Remove bubbles in the tube if necessary.

12. Gently pour the acrylamide between the plates before the gel polymerization starts (within 2-3 minutes).
13. Remove bubbles if any by gently tapping the plates.
14. Allow up to 30 minutes for complete polymerization to occur.
15. For larger polyacrylamide gels increase the polymer and the catalysts accordingly. For example 16 x 20 x 0.1 cm gel would take 32 ml polymer. Make about 35 ml polymer in a beaker and add 350µl APS and 70µl TMED.
16. The larger gel plates must be cleaned thoroughly to leave no trace of grease or dirt on the plates. When preparing very large gels one of the plates (not both) may be coated with silicon to allow easier separation at later stage.

Running acrylamide gel

1. Remove the gel plates from the casting assembly and fix it on the gel tank as recommended by the manufacturer.
2. Fill the upper and the lower buffer compartments with 1x AGB buffer.
3. Gently pull the comb out and wash the wells with buffer to remove any residual un-polymerized acrylamide. A syringe with 21 gauge butterfly needle may be used for washing wells.
4. Prepare the samples for loading by mixing 2-4µl amplified product (depending on the quality of amplification) and 2-3µl of loading dye (0.05% xylene cyanol and bromophenol blue in formamide). The samples may be prepared in 96 well ELISA plates or 0.2ml plastic tubes.
5. Load 3-4µl of the prepared sample with a long nose plastic tip. The amplified DNA must be handled with care as it is a potent source of contamination for other PCR reactions. Use separate pipette marked as "amplified DNA only".
6. Run the gel at 150-200 volts for 15-45 minutes depending on the fragment size of the amplified products.

Staining of acrylamide gels

Silver staining

1. Remove the gel from casting assembly.
2. Carefully separate the plates taking care to avoid tearing of the gel.
3. Mark the first well or the side of the gel by cutting a small piece from the left lower corner of the gel.
4. Put the gel in 0.1% silver nitrate solution for 15-20 minutes. The gel may be kept in stain while it is still on the glass plate.
5. Discard the stain and wash the gel in plenty of tap water.

6. The stain may be reused if kept in dark brown bottles. However it looses potency after exposure to bright light.
7. Prepare fresh developing solution by adding 75µl formaldehyde to approximately 100 ml 1.5% NaOH. Stock NaOH is stable at room temperature but once formaldehyde is added it must be used within one hour.
8. Submerge the gel completely in developing solution.
9. In approximately 5-10 minutes the bands of amplified DNA can be seen on the gel. The background of the gel also becomes light yellowish brown. The background colour could become very dark if the gel is kept for too long in the developer solution. The exact developing time can be learnt by trial and error.
10. Discard the developing solution and wash the gel in plenty of water when the DNA bands are clearly seen.
11. Cut a piece of filter paper slightly larger than the gel itself and lay it flat on the gel surface. Gently pick the filter paper along with the gel that sticks to its surface.
12. Place the gel and the filter paper on a gel dryer making sure that the gel faces towards the front.
13. Dry the gel under vacuum for 20-30 minutes at 80^{o}C.
14. The dried gel can be pasted in a record book for long term storage after trimming its margins.
15. The gel may be photographed if a gel dryer is not available.

Ethidium bromide staining

The acrylamide gels can also be stained in ethidium bromide as described in section of staining agarose gels.

Denaturing Gradient Gel Electrophoresis (DGGE)

The single stranded DNA tends to form secondary structures (Chapter 1). The movement of single stranded DNA in acrylamide gels, in addition to the size of the fragments, is also dependent on its base composition. The base composition determines the amount and the shape of the secondary structures.

When a DNA fragment 200-700bp in length is run on acrylamide gel with increasing concentration (gradient) of a denaturant like urea it would initially move according to the molecular weight. As it moves into the higher concentration of the denaturant it reaches a point where the DNA starts to melt (the two strands begin to separate). The resulting single strands of DNA also start to develop secondary structures. The partial melting and formation of secondary structures severely retard the progress of the molecule in the gel. A single base pair change in the DNA can cause significant mobility shift. In this way different alleles can be identified by the differences in mobility on a gel with a gradually increasing denaturant concentration.

Constant temperature of 60^{o}C, formamide (0-40%) and gradually increasing concentration of urea from 0-7 M are usually used as denaturants in DGGE.

Preparation of denaturing gradient gel

Prepare the following stock solutions:

1. Acrylamide (40%) and bis-acrylamide (1.07%)
2. 20 x TAE gel running buffer (pH 7.4) containing 800 mM Tris base, 400 mM sodium acetate, and 20 mM EDTA.
3. 80% denaturant stock solution containing 7% acrylamide, 32% formamide, 5.6 M Urea and 1 x TAE buffer.
4. 0% denaturant stock solution containing 7% acrylamide, and 1 x TAE buffer.

Formamide is deionized before use by gently stirring with Dowex AG50W (20-40) mesh mixed bed resin. The stock solutions of 0% and 80% denaturent are used to prepare the gradients of varying strengths. Gels measuring 20 x 16 cm and 1.5 mm thick are poured by a gradient mixer. Ammonium persulphate (100µl of 10% stock solution) and 10µl of TEMED are used as gel polymerization catalysts.

To make a 25-50% gradient of the denaturant the required volumes of 0% and 80% stock solutions are shown in Table 5.1.

Denaturant	25% (lower limit)	50% (upper limit)
0% denaturant	13.8 ml	7.5 ml
80% denaturant	6.2 ml	12.5 ml
Total volume	20 ml	20 ml

Table. 5.1. The volumes of 0% and 80% denaturant solutions to be used for preparation of denaturing gradient gels.

Making gradient gel

The gradient mixer is made of two chambers connected through a tube close to the bottom. The connection is normally closed. One of the chambers (downstream) has an outlet where tubing for the out flowing polymer is attached. This outlet is also kept closed. The required amount of high denaturant mix (e.g. 50%) is added to the out flowing chamber and the low denaturant mix (e.g. 25%) is added to the other chamber. It is important that the volumes of the two denaturant mixtures should be equal. A small magnetic stirrer is placed in the out flowing chamber. The gradient mixer is placed at a height of about 12 inches above the gel casting assembly. A 23 gauge butterfly needle is attached at the outlet.

Before pouring the gel the tap between the two chambers is opened and the magnetic stirrer is started Ammonium persulphate (100µl of 10% stock solution) and 10µl of TEMED are added to the polymer in each of the chambers. The tap at the outlet orifice is opened and

bubbles in the needle are removed. The butterfly needle is placed between the two glass plates and the polymer is allowed to fill the space between the plates. As the polymer flows from the upstream chamber to the out flowing chamber the polymer in the two chambers is gradually mixed and this forms a gradual gradient with highest concentration at the bottom of the gel and the lowest concentration at the top of the gel.

Running conditions for DGGE

Electrophoresis is carried out on a vertical polyacrylamide gel running system. A specially designed acrylic chamber to hold the gel assembly, heater and buffer circulation device is used (Fig 5.4). The samples are loaded on the gel in the usual way. In each well up to 12µl of the sample and loading dye (0.05% xylene cyanol in formamide) can be used. Electrophoresis is carried out at 50V for 16 hours. Throughout the procedure buffer temperature is maintained at 60^{o}C. At the end of the run the gel is removed and it is stained with silver nitrate or ethidium bromide (Fig 5.5).

Fig. 5.4. Apparatus for denaturing gradient gel electrophoresis. The main buffer compartment contains an immersion heater and a buffer circulation pump that maintains a constant temperature of 60^{o}C throughout the electrophoresis run.

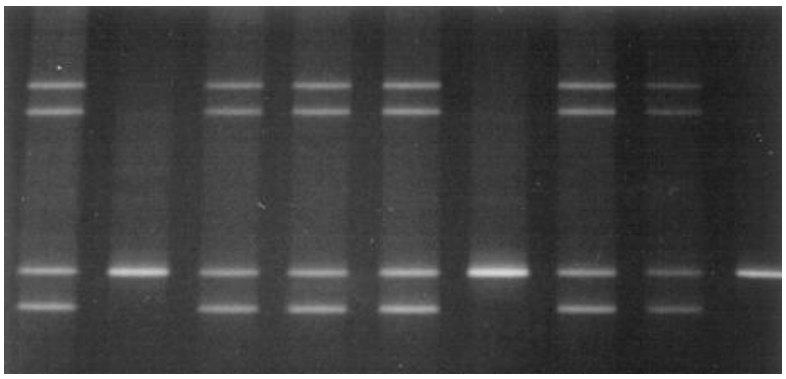

Fig. 5.5. Ethidium bromide stained DGGE.

Single Strand Conformation Polymorphism (SSCP)

Single stranded DNA tends to form secondary structures which are dependent on its nucleotide composition. The secondary structures interfere with the mobility of the strand in a polyacrylamide gel. Two single-stranded DNA fragments with only a single nucleotide difference would migrate at different rates. This forms the basis of SSCP.

Single stranded DNA can be produced by first amplifying DNA and then denaturing it to form single strands. Alternatively single stranded DNA can be produced by asymmetric PCR in which one primers of a pair is used in excess. In the initial cycles of PCR double stranded DNA is produced but as the primer with lower concentration is consumed the primer in excess causes extension of only a single strand. The usual fragment size for SSCP is between 150-300 bases. SSCP is done on non-denaturing polyacrylamide gels that are stained in silver nitrate.

SSCP is a useful technique for screening of SNPs and unknown mutations. SSCP is unable to tell the base composition of DNA unless control DNA fragments of known composition are also run parallel to the unknown samples.

Capillary electrophoresis

Electrophoresis of DNA can be done in very narrow bore long and flexible capillaries. The capillaries are filled with an electrolytic solution and its ends are dipped in buffer chambers. The sample injection and running is done by electric current. The electrophoresis is usually done for short periods at 5000 to 30,000 volts (Fig. 5.6).

Capillary electrophoresis is especially useful for automated fragment analysis and genomic sequencing of fluorescent labeled amplified products. Many automated genetic analyzers are commercially available that allow simultaneous running of several samples. These analyzers are extremely efficient in fragment analysis and genomic sequencing applications.

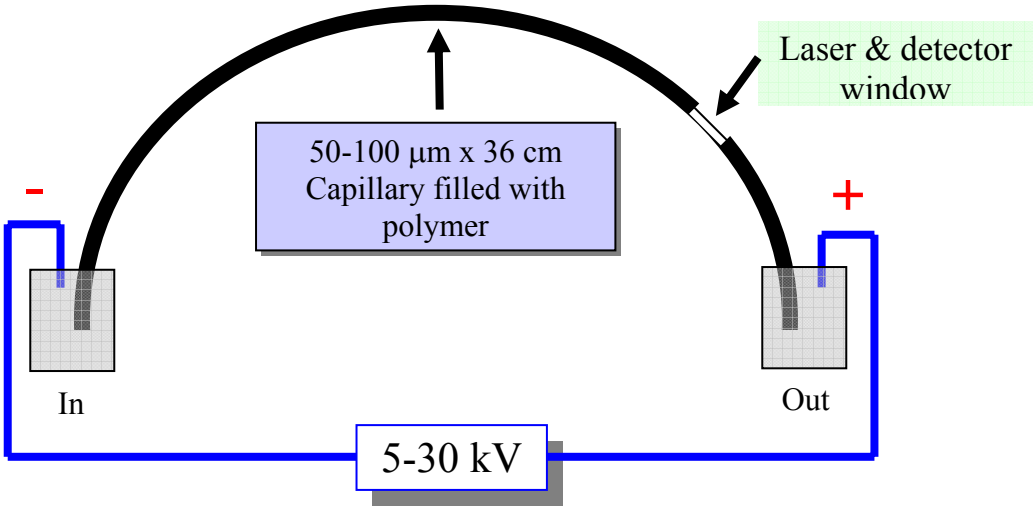

Fig. 5.6. Diagrammatic representation of capillary electrophoresis.

Bibliography

1. Fodde R and Losekoot M (1994) Mutation detection by denaturing gradient gel electrophoresis (DGGE). *Hum Mutation* 3: 83-94.
2. Grompe M (1993) The rapid detection of unknown mutations in nucleic acids. *Nature Genetics* 5: 111-117.
3. Cai SP, Kan YW (1990) Identification of the multiple β-thalassaemia mutations by Denaturing Gradient Gel Electrophoresis. *J Clin Invest* 85: 550-553.
4. Constantinos Z (2009, January 18) Capillary Electrophoresis. *SciTopics*. Retrieved July 2, 2012, from http://www.scitopics.com/Capillary_Electrophoresis.html