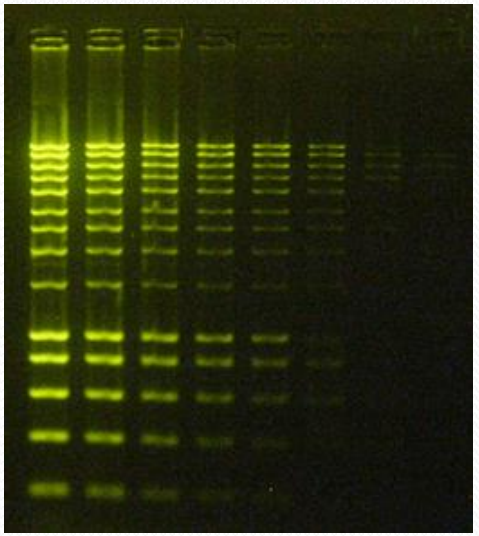
PCR and Gel Electrophoresis



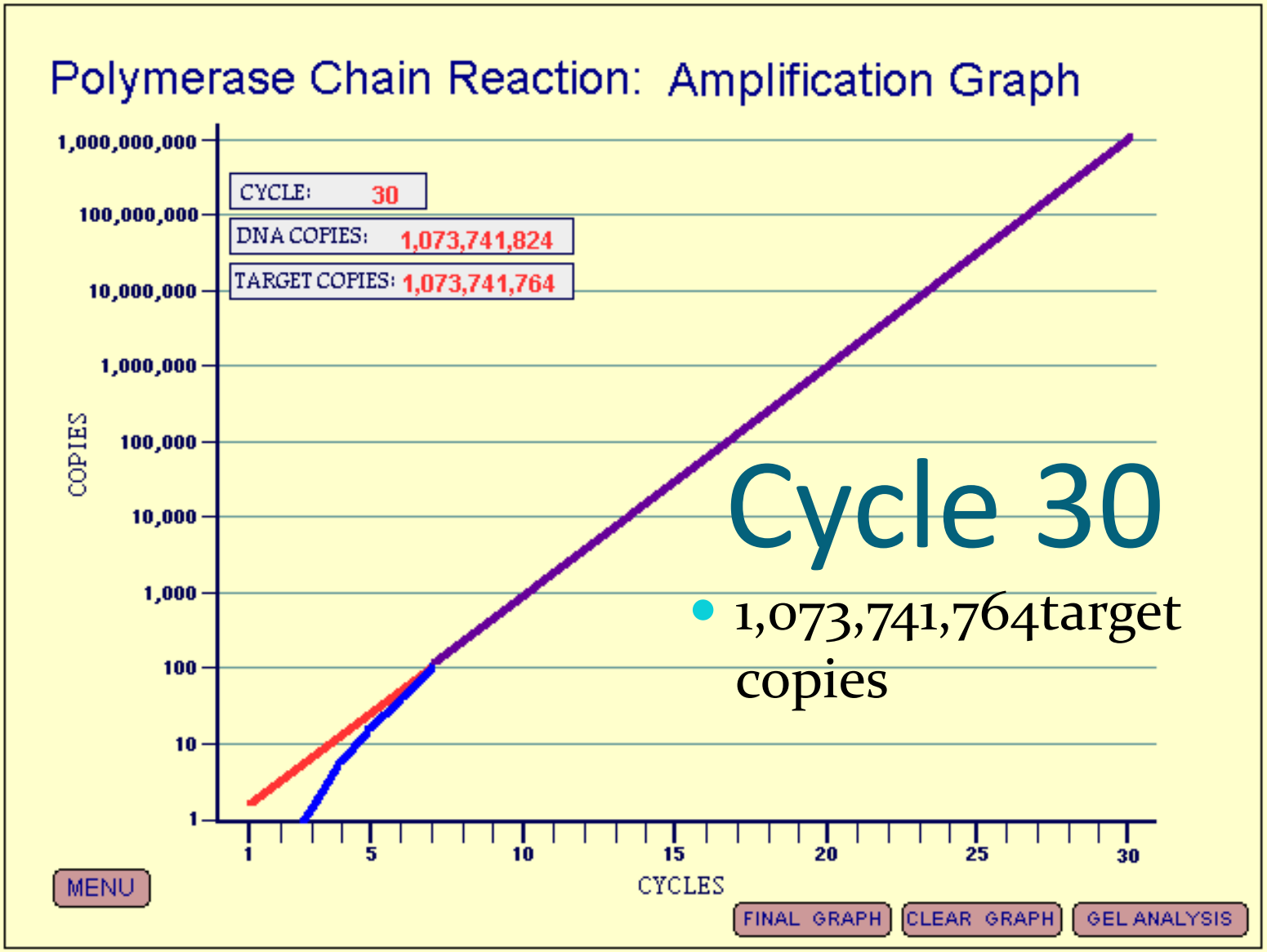
By: Dr Goudarzi
Ph.D in Biotechnology

PCR: Polymerase Chain Reaction

- A method to allow amplification of specific DNA molecules (fragments) in vitro through cycles of enzymatic DNA synthesis
- The most popular and widely used technique in all fields of biological studies probably, Why?

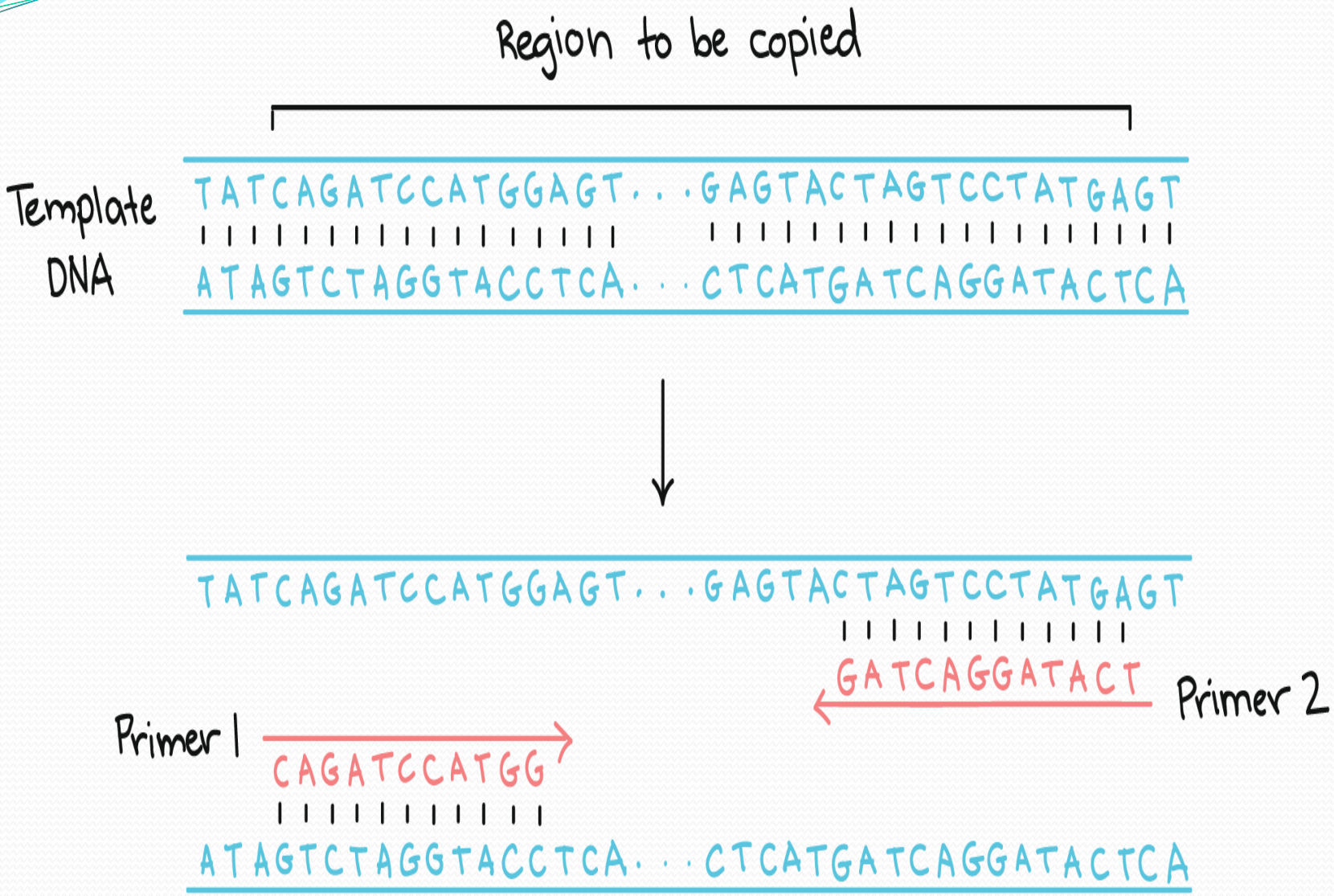
PCR

- 1. simple
- 2. fast
- 3. powerful
  - A. sensitive – sensitivity
  - B. Specific – specificity
  - C. Reliable – fidelity



Cycle 30

- 1,073,741,764target copies


Region to be copied
Template DNA
TATCAGATCCATGGAGT...GAGTACTAGTCCTATGAGT
ATAGTCTAGGTACCTCA...CTCATGATCAGGATACTCA
TATCAGATCCATGGAGT...GAGTACTAGTCCTATGAGT
GATCAGGATACT
Primer 2
Primer 1
CAGATCCATGG
ATAGTCTAGGTACCTCA...CTCATGATCAGGATACTCA

The Nobel Prize in Chemistry 1993

Kary B. Mullis

"for contributions to the developments of methods within DNA-based chemistry"
"for his invention of the polymerase chain reaction (PCR) method"

Development/Invention of PCR Technique

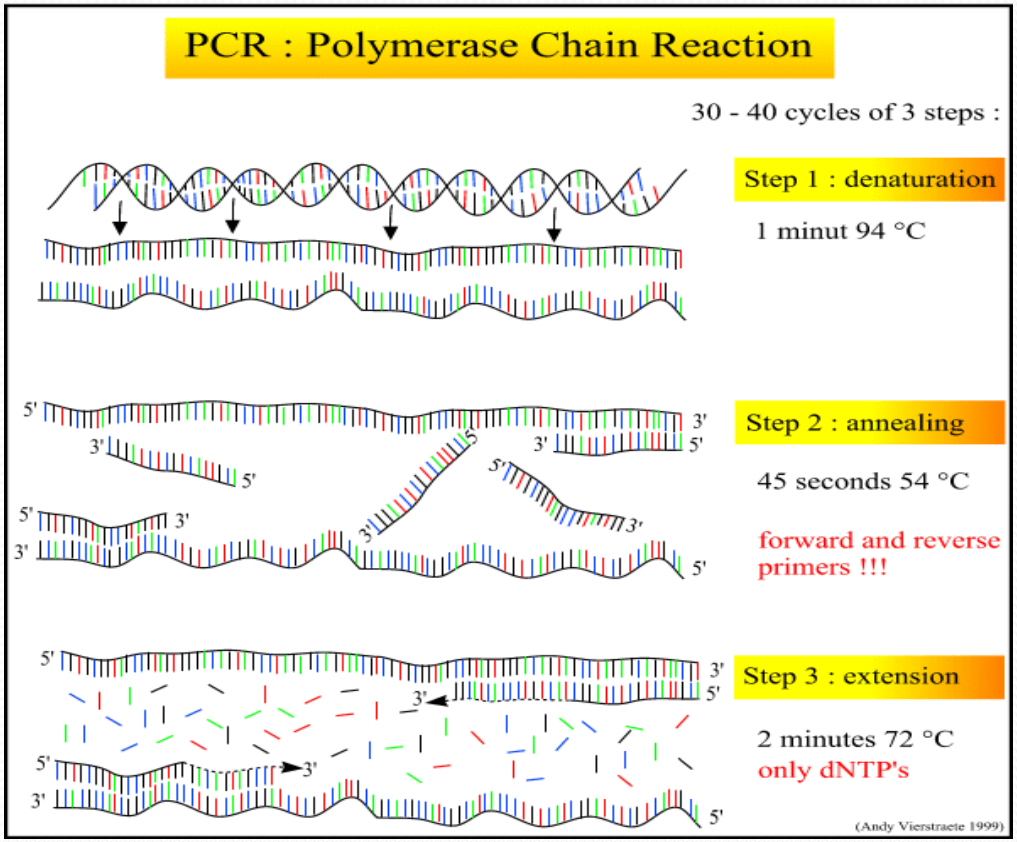
1993 Nobel Prize in Chemistry

Overview of PCR

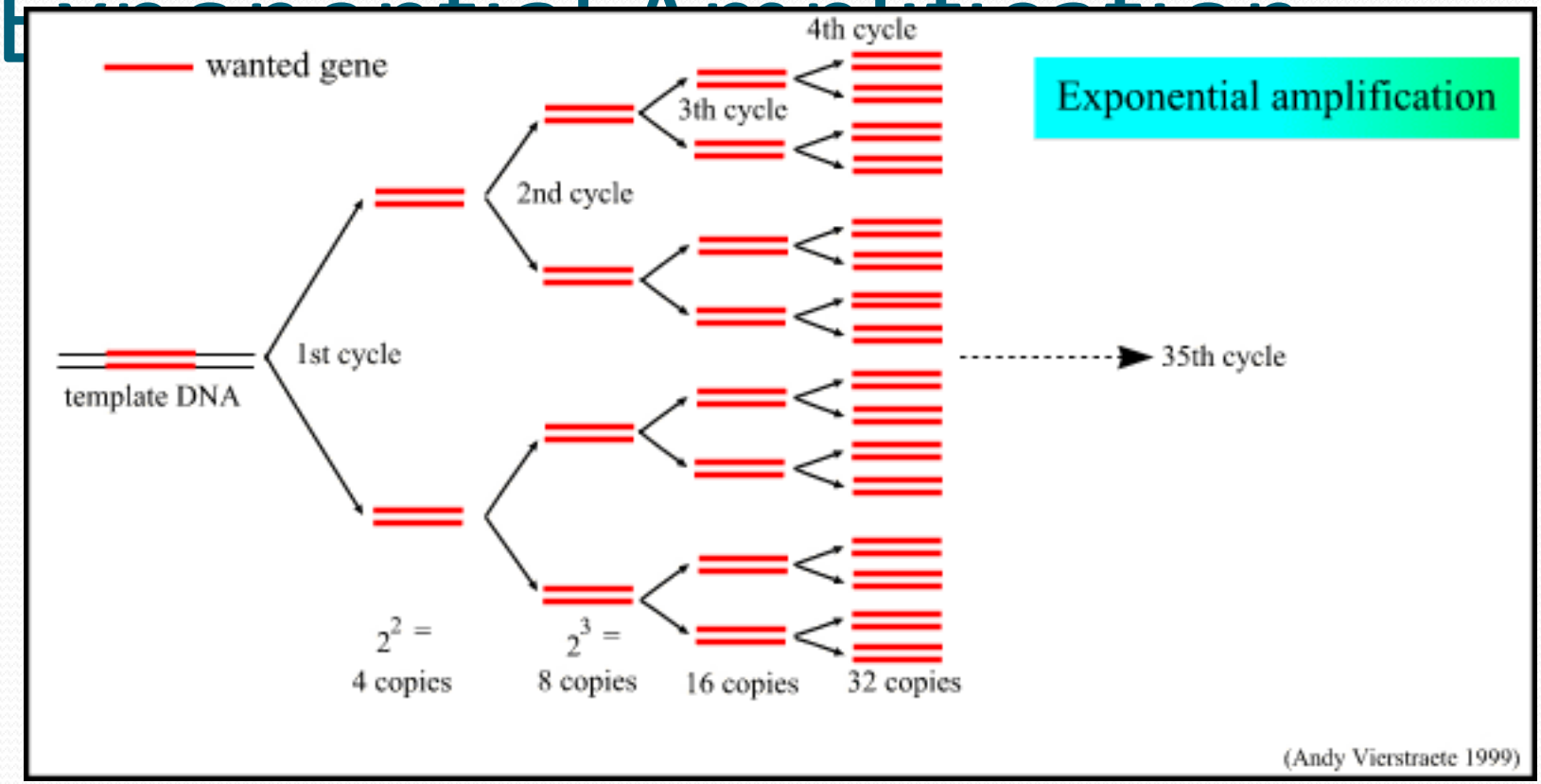
1. Temperature Cycling

Denaturation	94°
Annealing	55°
Extension	72°

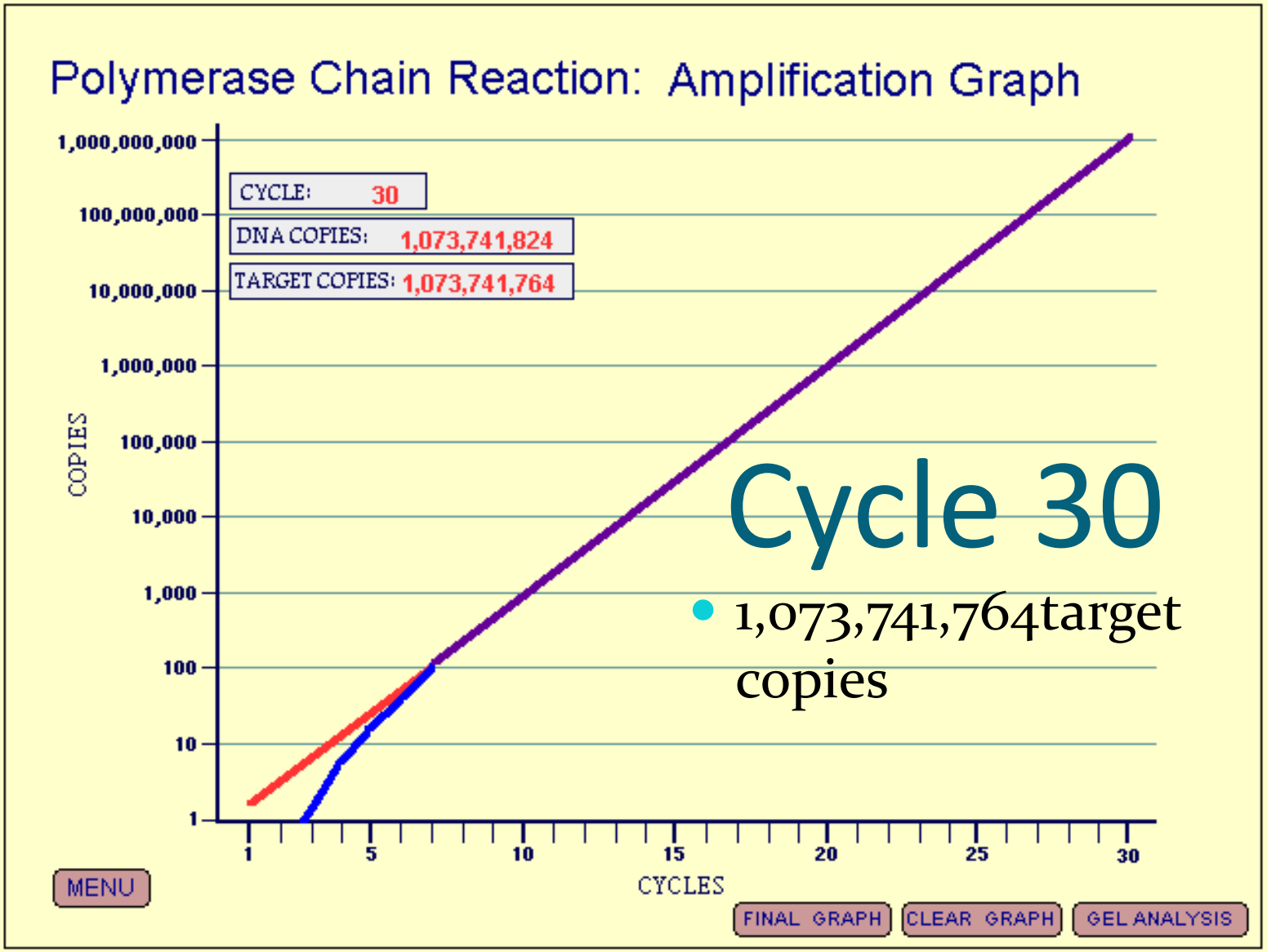
2. Every cycle DNA between primers is duplicated



Exponential Amplification

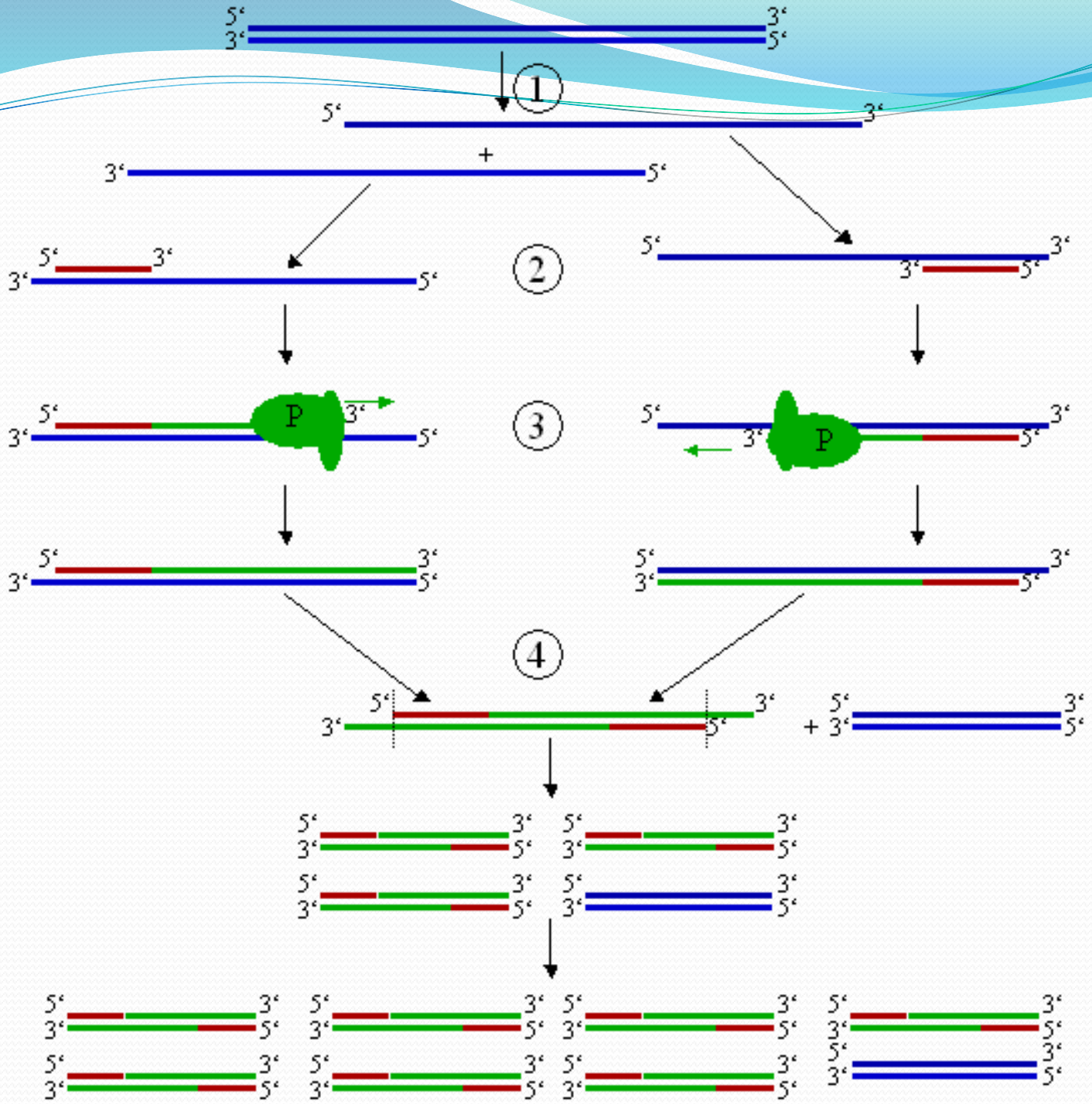


30 cycles --- 1 billion copies in theory



Cycle 30

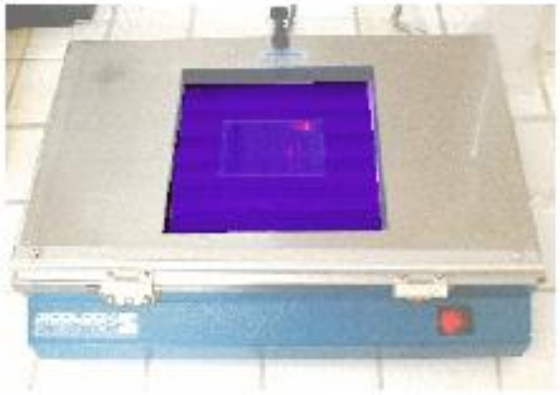
- 1,073,741,764target copies


5'
3'
1
+
2
P
3
4

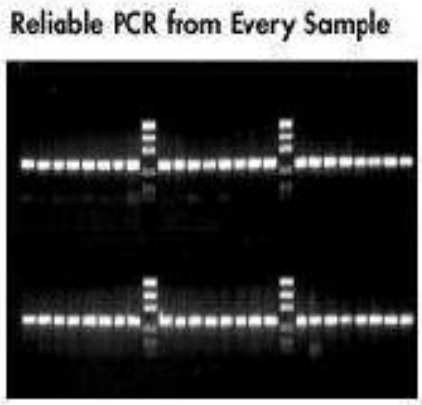
PCR

Agarose gel electrophoresis

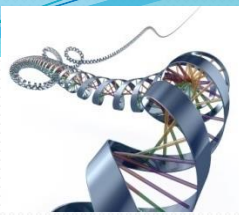
3-4 hours



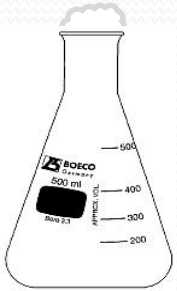
UV visualisation



The final product



DNA EXTRACTION: *E. COLI*



200 µl

5000 rpm
2 min

Discard liquid phase

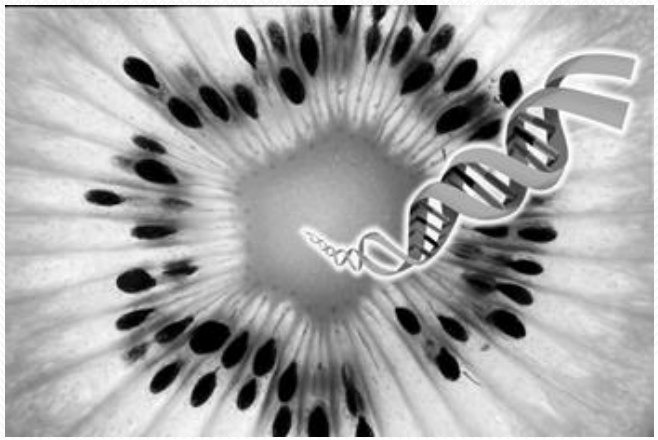
Step 1

Add 100µl dH2O

resuspend

Step 2

Add 30 µl dH2O



95 ºC
10 min

10000 rpm
2 min

DNA is in liquid phase

NanoDrop-2000c-in-the-lab_jpg-650_wdp.jxr

Components of PCR Reaction

- Template DNA
- Flanking Primers
- Thermo-stable polymerase
  - Taq Polymerase
- dNTP
  - (dATP, dTTP, dCTP, dGTP)
- PCR Buffer (mg^{++})
- Thermocyler

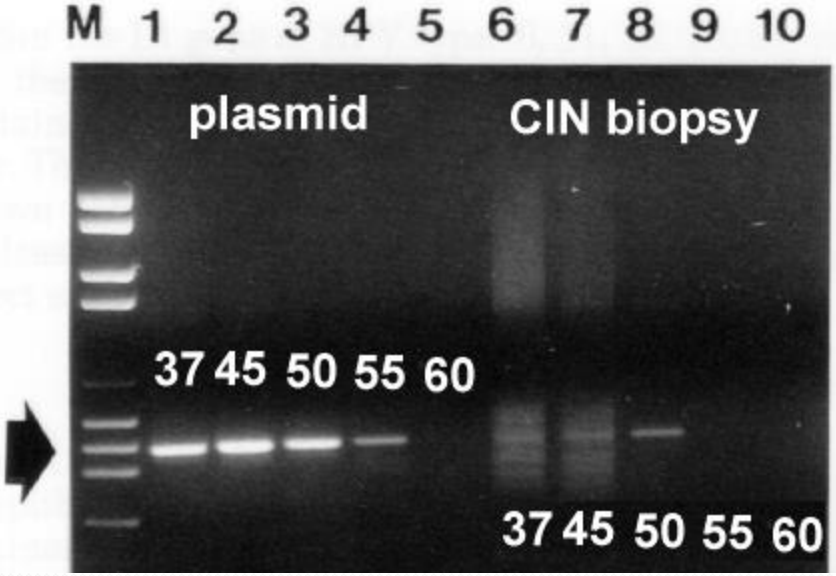
Thermus aquaticus

PCR Variables

1. Temperature
2. Cycle Times and Temps
3. Primer
4. Buffer
5. Polymerase

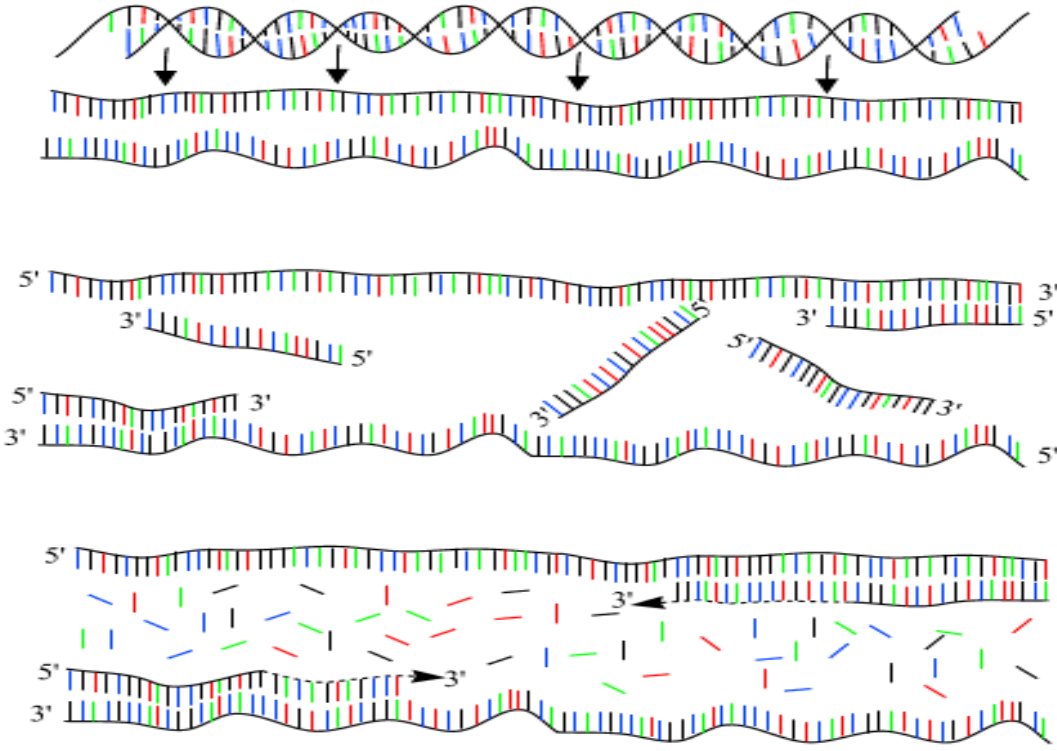
Temperature

- Denaturation
  - Trade off between denaturing DNA and not denaturing Taq Polymerase
    - Taq half-life 40min at 95 °, 10min at 97.5°
  - 95°
- Annealing
  - Trade off between efficient annealling and specificity
  - 2-5 ° below Tm
- Extension
  - Temperature optimum for Taq Polymerase
  - 72 °



PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

1 minut 94 °C

Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

Step 3 : extension

2 minutes 72 °C

only dNTP's

Cycle Times and Temps

Typical PCR Run

Step	Time/Temp
1	3 min at 95°
2	30 sec at 95°
3	1 min at 55°
4	2 min at 72°
5	Go to step 2 - 29 times
6	10 min 72°
7	5 min 4°
8	End

Primers

- Paired flanking primers
- Length (17-28bp)
- GC content 50-60%
- Tm's between 55-80
- Avoid simple sequences – e.g. strings of G's
- Avoid primer self complementary
  - e.g. hairpins, homodimers, heterodimers

PCR Buffer

- **Basic Components**
  - 20mM Tris-HCL pH 8.4
  - 50mM KCl
  - 1.5 mM $MgCl_2$
- **Magnesium** – Since Mg ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of MgCl2 has to be selected for each experiment. Too few Mg2+ ions result in a low yield of PCR product, and too many increase the yield of non-specific products and promote misincorporation.
- **Potential Additives**
  - **Helix Destabilisers** - useful when target DNA is high G/CWith NAs of high (G+C) content.
    - dimethyl sulphoxide (DMSO),
    - dimethyl formamide (DMF),
    - urea
    - formamide
  - **Long Targets** >1kb. Formamide and glycerol
  - **Low concentration of template**: Polyethylene glycol (PEG)

PCR Polymerases

- Taq, Vent, Pfu, others
- Native or Cloned
- Half-life
  - e.g. Taq 40 min half-life, Vent 7 hour half-life at 95 c
- 3'-5' Exo nuclease – proofreading
- Fidelity (Error Rate)
  - Taq 1/10,000nt, Pfu 1/1,000,000
- Processivity
- Extra bases at end

TABLE 8-1 Properties and Applications of Thermostable DNA Polymerases

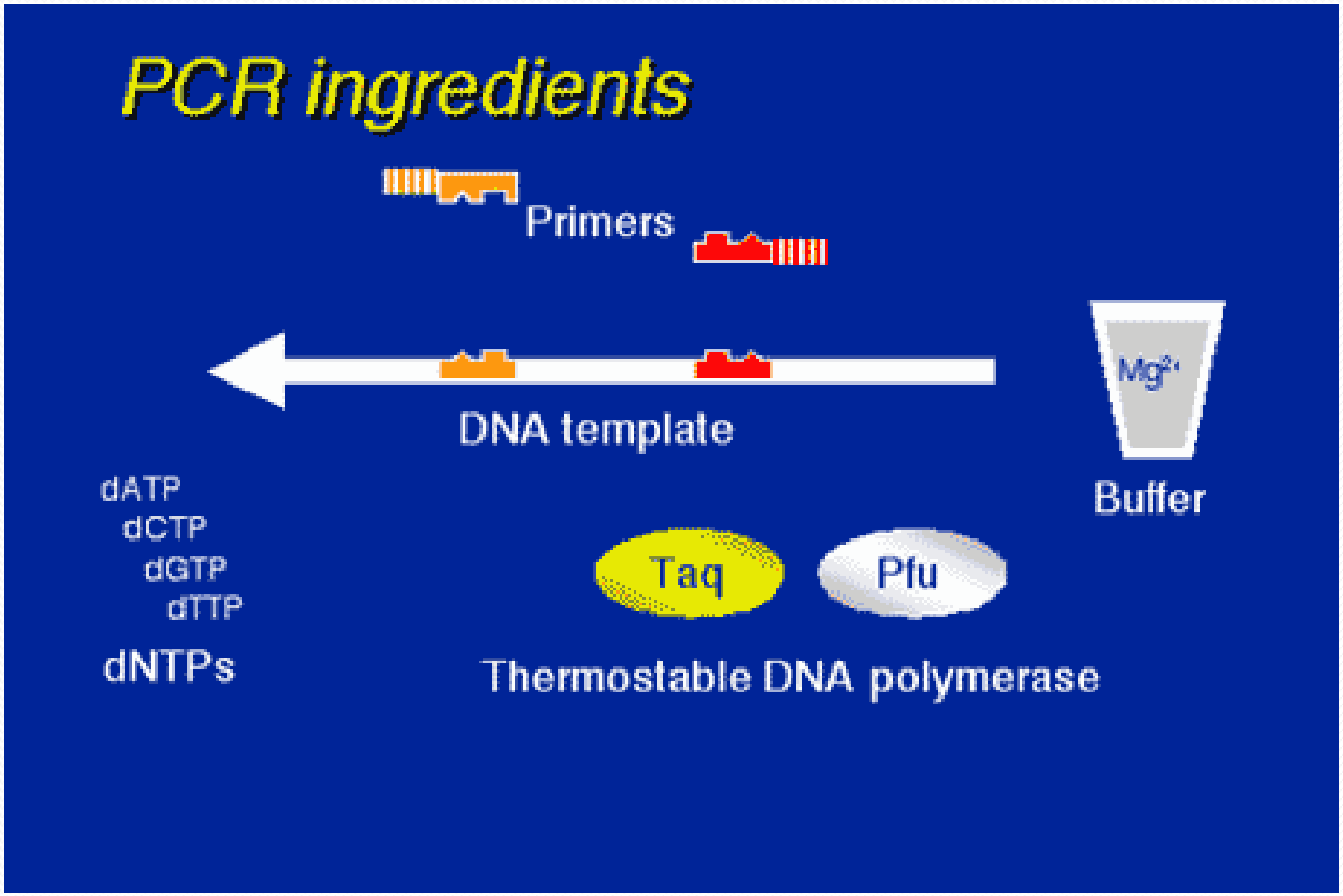
Enzyme	Manufacturer[a]	Organism	Optimum Temp. (°C)	Exonuclease Activity	Fidelity	Stability (Minutes at Specified Temperature)	K_M dNTP (μM)	K_{cat}	Comments
Taq	BM, LT, Pro, Strat, P-E, T	*T. aquaticus*	75–80	5′–3′	low	9 min at 97.5°C	10–16	60–150	°C
Taq Stoffel fragment	P-E	*T. aquaticus*	75–80	none	low	21 min at 97.5°C	–	130	The Stoffel fragment consists of the 544 carboxy-terminal amino acid residues of *Taq* polymerase. The processivity of the Stoffel fragment is ~10-fold lower than the processivity of full-length *Taq* polymerase.
rTth	BM, ET, P-E	*T. thermophilus*	75–80	5′–3′	low	20 min at 95°C	115	25	r*Tth*, the recombinant form of *Tth* DNA polymerase, can use Mg^{2+} or Mn^{2+} as a cofactor. In the presence of Mn^{2+}, the reverse transcriptase activity of r*Tth* is enhanced.
Tfl	Pro	*T. flavus*	70	none	low	120 min at 70°C	63	–	Useful for amplification of large segments of DNA.
Hot Tub	Amr	*T. ubiquitus*	–	none	low	–	–	–	–
Tbr	Amr, Finnz	*T. brockianus*	75–80	5′–3′	low	150 min at 96°C	–	–	–
UlTma	P-E, Roche	*Thermotoga maritima*	75–80	3′–5′	high	50 min at 95°C	–	–	–
rBst	ET	*Bacillus sterothermophilus*	60–65	5′–3′ (3′–5′)[b]	–	–	–	–	Conflicting data exist in the literature about exonuclease activities associated with *Bst* DNA polymerase. Two widely differing estimates have been published for the K_m of the enzyme for dNTPs. *Bst* DNA polymerase is used mainly for DNA sequencing. The enzyme, which will readily accept nucleotide analogs (e.g., dITP) as substrates, is used to sequence regions of DNA rich in GC and/or containing a high degree of secondary structure.
Isotherm *Bst* large fragment	ET, Bio-Rad	*Bacillus sterothermophilus*	60–65	–	high	–	7–85	–	The large fragment, which consists only of the polymerase domain, is obtained from the *Bst* holoenzyme

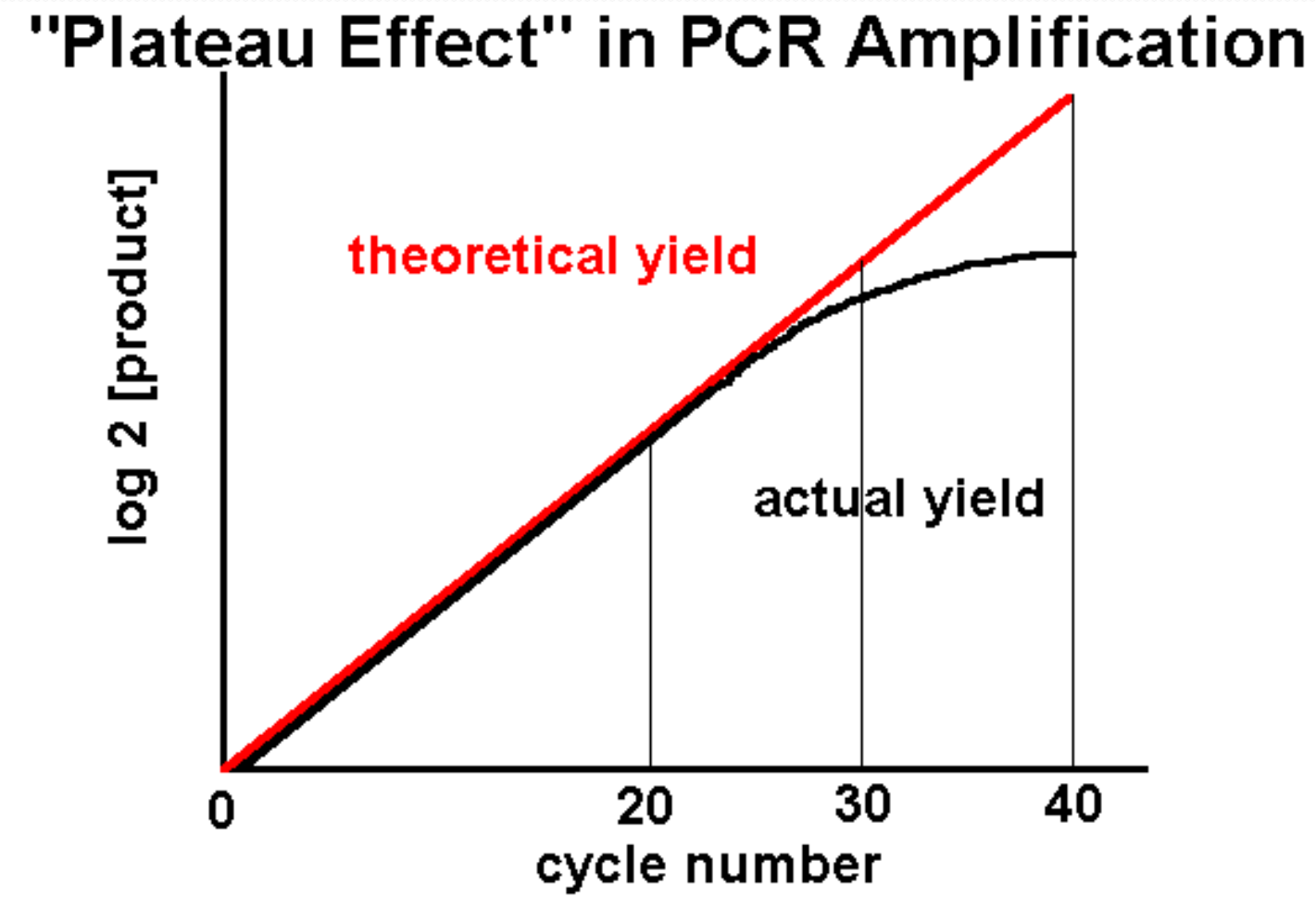
									by proteolytic digestion, and is used for sequencing difficult tracts of DNA.
Pwo	BM	*Pyrococcus woesei*	60–65	3′–5′	high	>2 hr at 100°C	–	–	*Pwo* generates blunt-ended DNA fragments and is recommended for cloning of PCR products.
Tli	Pro	*Thermococcus litoralis*	70–80	3′–5′	low	100 min at 100°C	66	60	*Vent* and *Tli* are suitable for primer-extension and PCRs requiring a high-fidelity highly stable DNA polymerase. Most of the products generated in PCRs are blunt-ended, which simplifies cloning. *Vent* is useful for long PCR. Neither *Vent* nor *Tli* is recommended for DNA sequencing. However, derivatives of *Vent* with reduced exonuclease activity have been generated and are sold by NEB. These enzymes are used as catalysts in cycle-sequencing reactions.
DeepVent	NEB	*Pyrococcus* strain GB-D	70–80	3′–5′	high	480 min at 100°C	50	–	*DeepVent* is suitable for primer-extension and PCRs requiring a high-fidelity highly stable DNA polymerase. Enzymes with reduced exonuclease activity have been generated and are sold by NEB.
Pfu	Strat	*Pyrococcus furiosus*	72–78	3′–5′	high	240 min at 95°C	–	–	*Pfu* is suitable for primer-extension reactions and PCRs requiring a high-fidelity highly stable DNA polymerase. The published error rate of *Pfu* per nucleotide is the lowest of any DNA polymerase (Cline et al. 1996). A variant of *Pfu* lacking exonuclease activity has been generated and is sold by Stratagene. This enzyme is well-suited as a catalyst in cycle-sequencing reactions. Stratagene also markets *PfuTurbo*, which is a mixture of *Pfu* and a newly discovered factor that enhances both yield and size of the amplified product.

Typical PCR mix

In a thin wall Eppendorf tube assemble the following

PCR components	Amount
Template DNA (50-1000 ng)	1 ul
10 X PCR buffer(1X final)	2 uL
10 mM dNTPs (200 uM final)	0.4 uL
50 mM MgCl2 (1.5 mM final)	0.6 uL
10 uM forward primer (0.5 uM final)	1 uL
10 uM reverse primer (0.5 uM final)	1 uL
5 units/uL Taq DNA polymerase (1 units)	0.2 uL
dH20	13.8 ul
Final Volume	20 uL


PCR ingredients
Primers
DNA template
Mg2+
Buffer
dATP
dCTP
dGTP
dTTP
dNTPs
Taq
Pfu
Thermostable DNA polymerase


"Plateau Effect" in PCR Amplification
log 2 [product]
theoretical yield
actual yield
0
20
30
40
cycle number

Master Mix

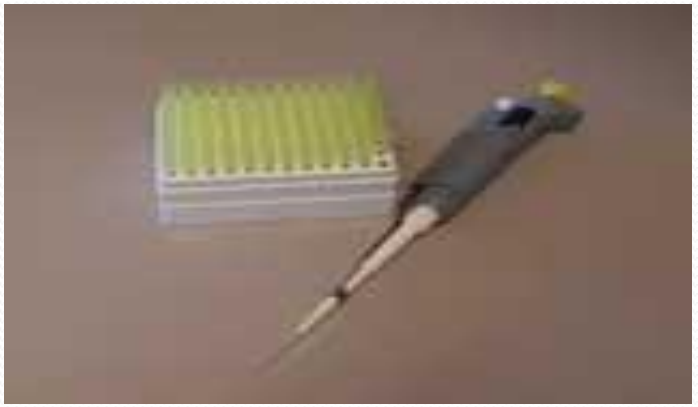
- **1 volume master mix**
  - 31.5 µl dH2O
  - 5 µl 10 X PCR buffer
  - 1 µl 200 µM dNTP
  - 1 µl 1.5mM MgCl2
  - 0.5 µl Taq Pol (5 Units/ µl)

  39 µl Total Volume

- **To set up 4 reactions prepare 4.4 volumes of reaction master mix**
  - 143 µl dH2O
  - 22 µl 10 X PCR buffer
  - 4.4 µl 200 µM dNTP
  - 1 µl 1.5mM MgCl2
  - 2.2 µl Taq Pol (5 Units/ µl)

- **Individual reactions**
  - 39 µl master mix
  - 0.5 µl Left primer
  - 0.5 µl Right primer
  - 10 µl worm lysate

Consumables



PCR Inhibitors

Almost anything will inhibit PCRs if present in excess.

The common inhibitors include:

- Proteinase K
- phenol
- EDTA.
- ionic detergents
- polyanions such as spermidine
- hemoglobin,
- gel-loading dyes such as bromophenol blue and xylene cyanol

- In many cases, the chief cause of low yields are contaminants in the template DNA, which is often the only component of the reaction supplied by the investigator.

- Many problems with PCR can be cured simply by cleaning up the template by dialysis, ethanol precipitation, extraction with chloroform, and/or chromatography through a suitable resin.

PCR optimization – empirical technic

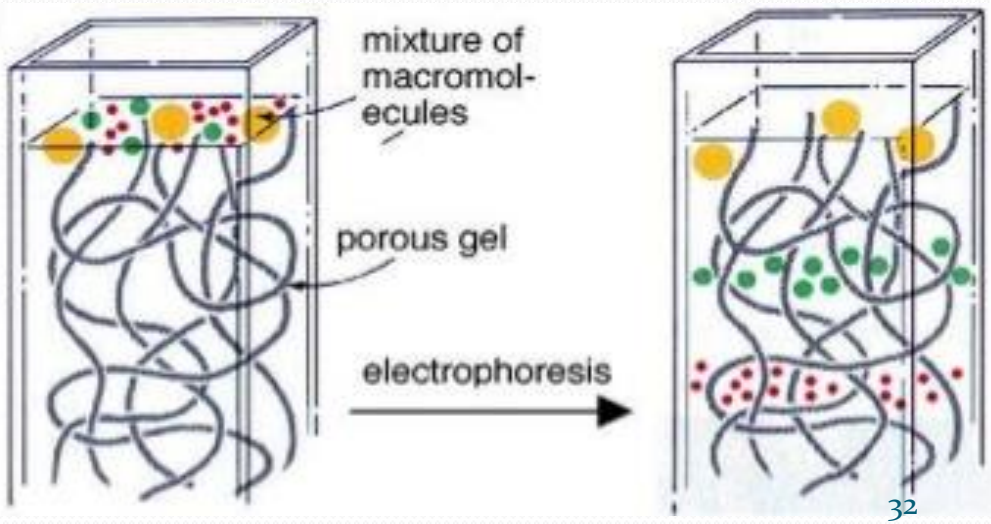
1.)Generalities
Components, PCR cycle, vials
2.)Choosing PCR primers
How to design the primers
3.)Reaction volumes
Do they influence the results?
4.)Number of PCR products Multiplexing. How ?
5.)Primer amount
How much primer?
6.)PCR buffers
Comparisons and concentration
7.)Salt (KCl) concentration
Essential optimization factor
8.)Designing PCR programs
Customize them for your purpose
9.)Annealing time and temperature
10.)Extension time and temperature
11.)DNA template concentration
12.)Taq polymerase(s)
Comparisons and concentration
13.)dNTP concentration
Interaction with magnesium
14.)MgCl2 concentration
Interaction with dNTP and salt (PCR buffer)
15.)Gel electrophoresis
Agarose and polyacrylamide (PAA)
16.)Adjuvants in PCR
DMSO, glycerol and BSA

Gel Electrophoresis

How does electrophoresis work?

- Linear double stranded DNA fragments basically have the same rod shape, so shape is not a factor in the separation.
- All DNA has identical charge to mass ratio, one negatively charged phosphate per nucleotide.
- As DNA fragments increase in length, charge increases preserving the charge to mass ratio.

- Forcing DNA to travel through a porous matrix will allow the smaller fragments to run faster while slowing the rate of larger molecules.

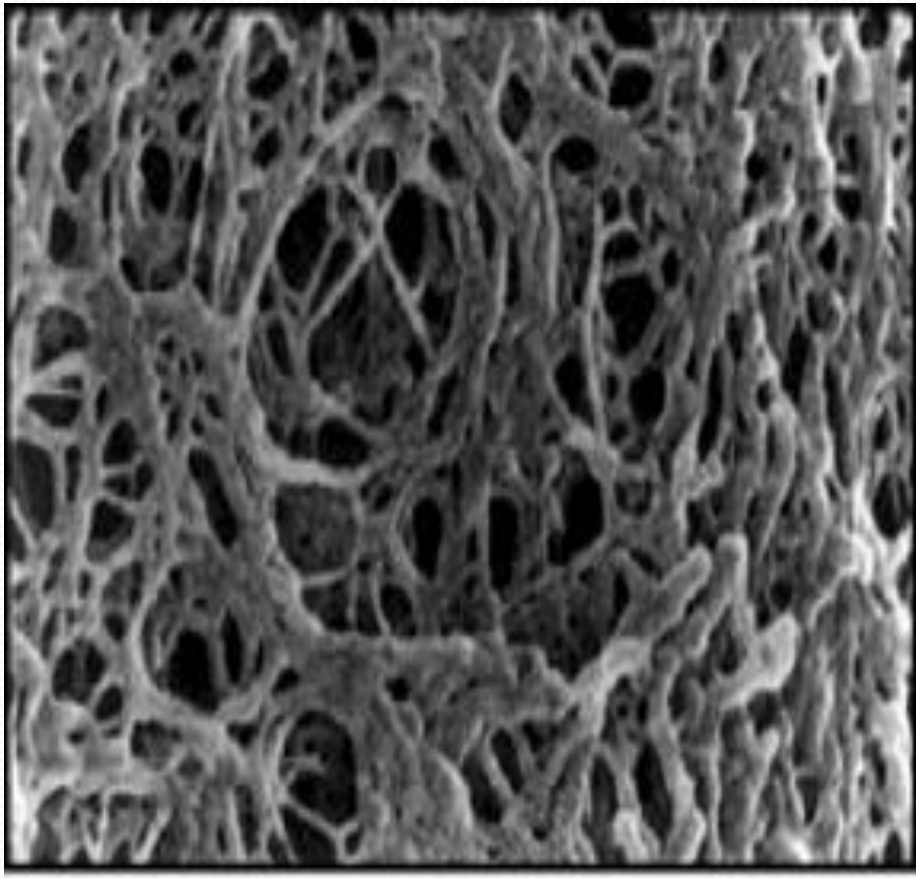


Scanning Electron Micrograph of Agarose

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

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

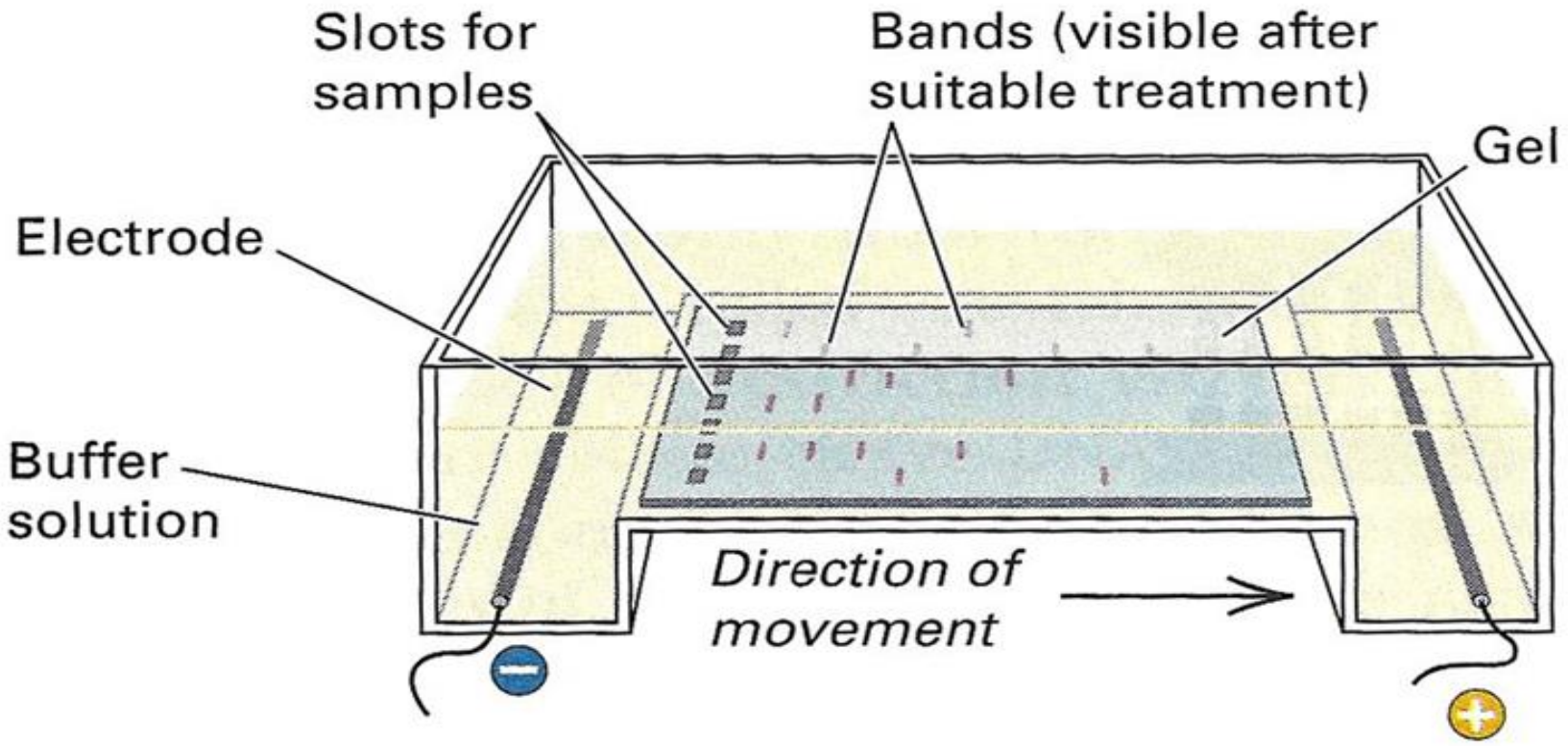
→



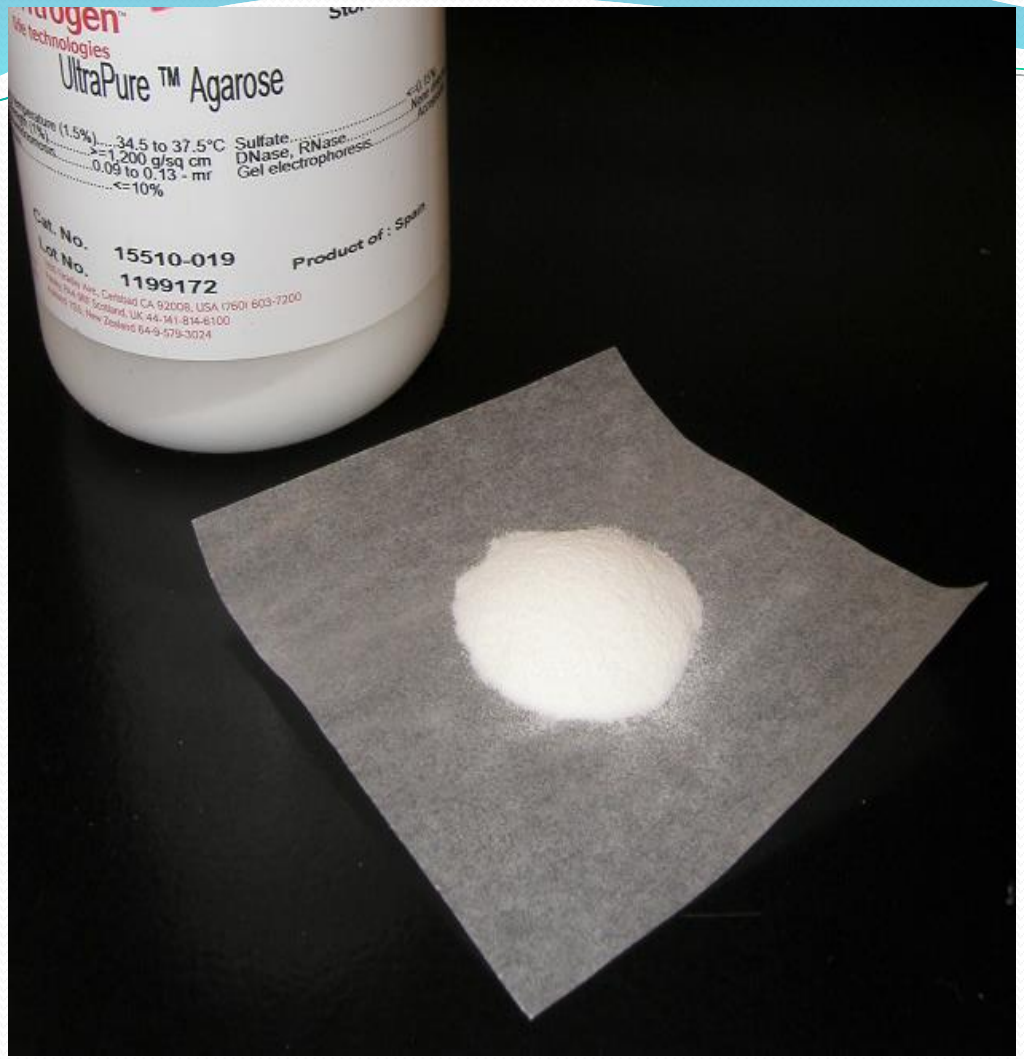
Applications of Agarose Gel Electrophoresis

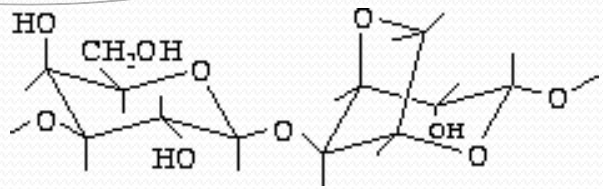
- Separation of restriction enzyme digested DNA including genomic DNA, prior to Southern Blot transfer. It is also often used for separating RNA prior to Northern transfer.
- Analysis of PCR products after polymerase chain reaction to assess for target DNA amplification.
- Allows for the estimation of the size of DNA molecules using a DNA marker or ladder which contains DNA fragments of various known sizes.
- Allows the rough estimation of DNA quantity and quality.

Agarose gel electrophoresis of DNA



Agarose





D-galactose

3,6-anhydro L-galactose

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

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

Agarose is a linear polymer extracted from seaweed.

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

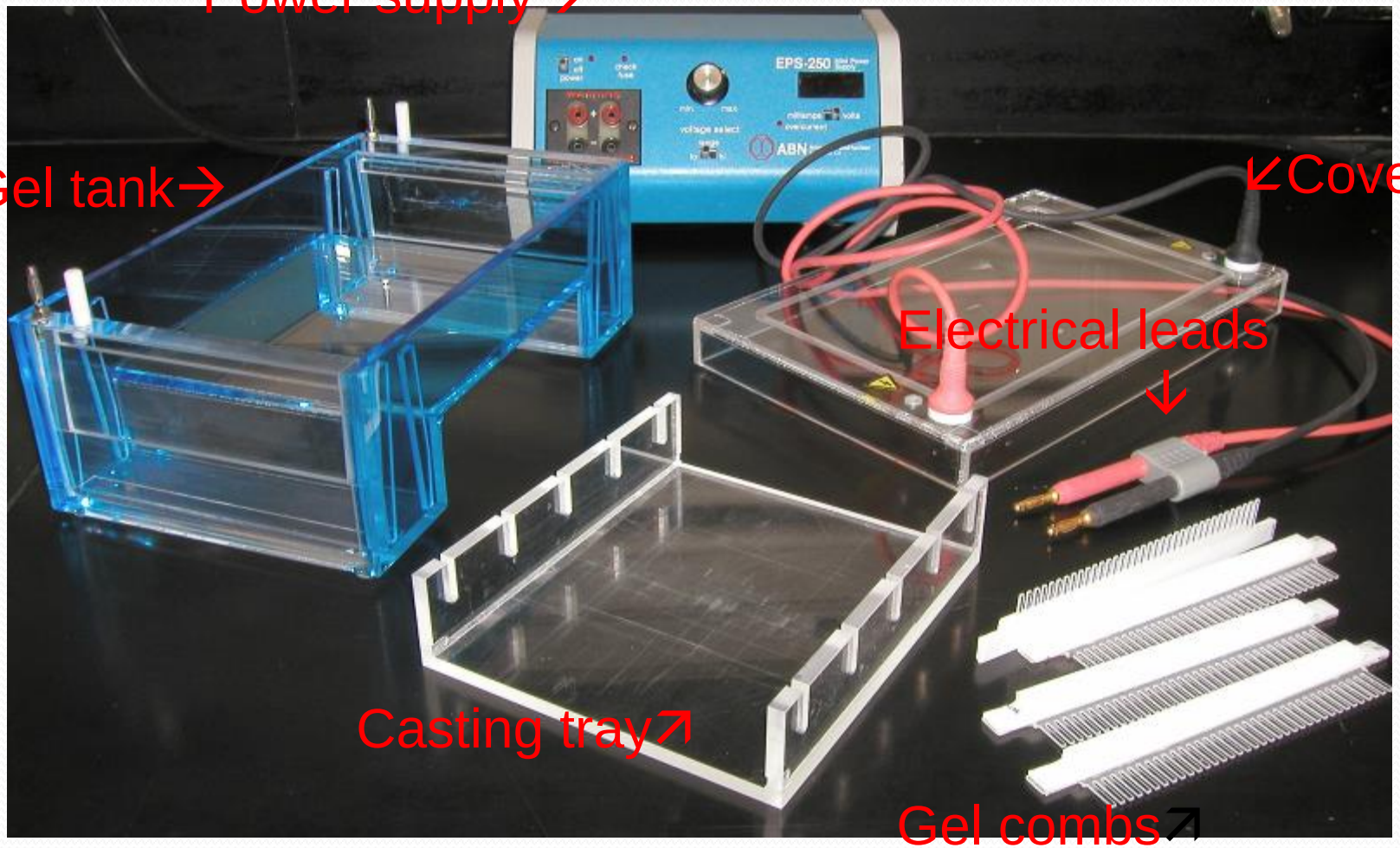
TBE Buffer(10x)
Tris base(121.14 g/mol) 890mM
Boric acid (61.83 g/mol) 890mM
EDTA (292.24 g/mol) 25mM
Add H2O to 1000ml adjust pH to 8.

Resolving DNA Fragments

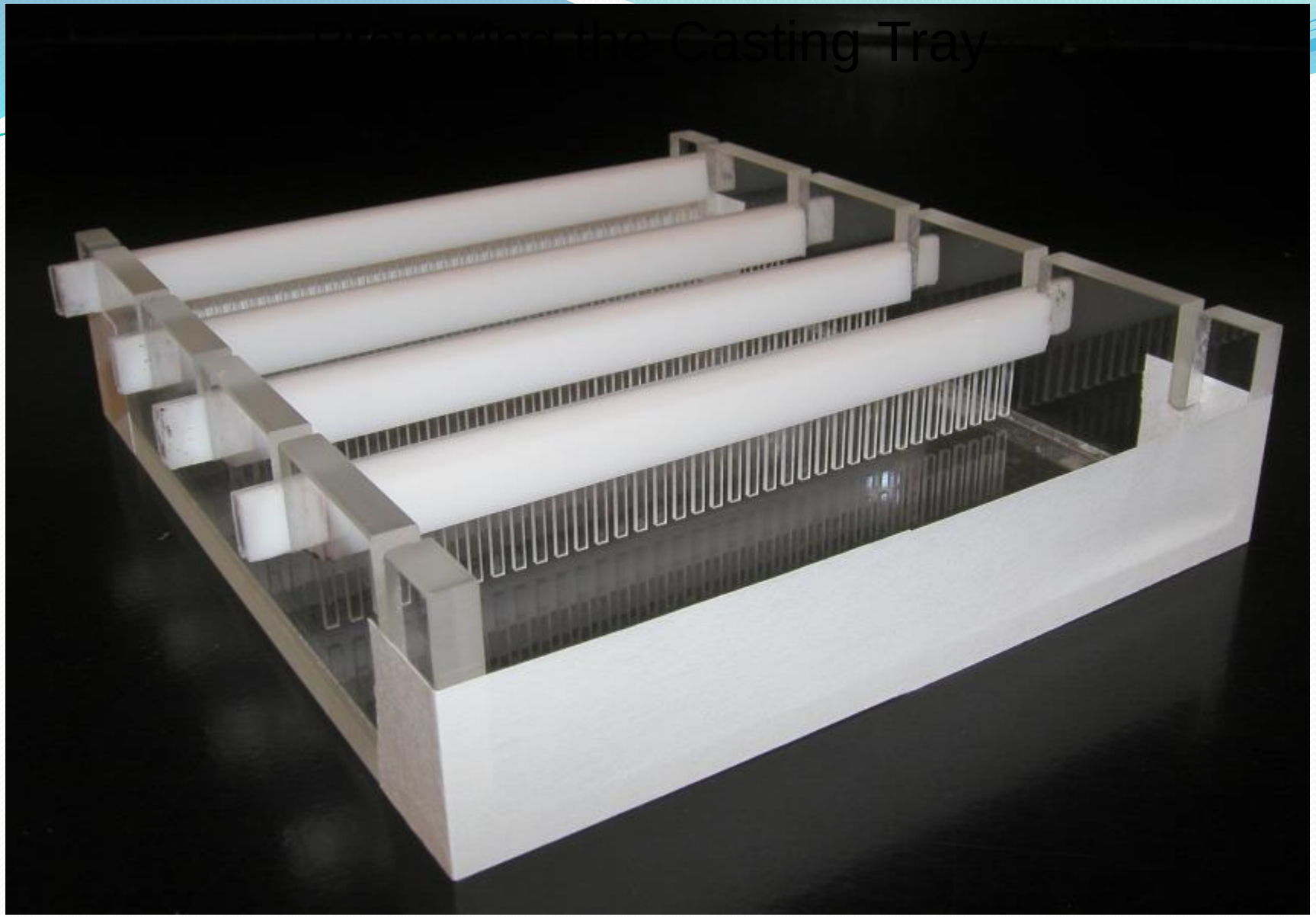
Percent Agarose Gel (w/v)	DNA Size Resolution(kb = 1000)
0.5%	1 kb to 30 kb
0.7%	800 bp to 12 kb
1.0%	500 bp to 10 kb
1.2%	400 bp to 7 kb
1.5%	200 bp to 3 kb
2.0%	50 bp to 2 kb

Table 1: Correct Agarose Gel Concentration for Resolving DNA Fragments

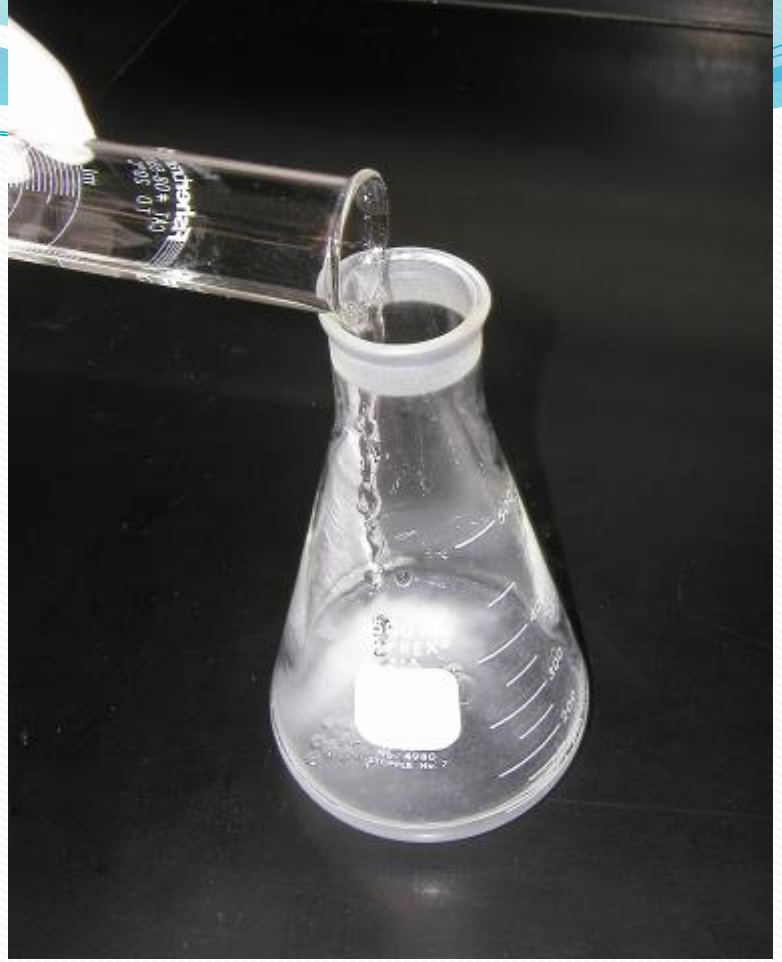
Electrophoresis Equipment



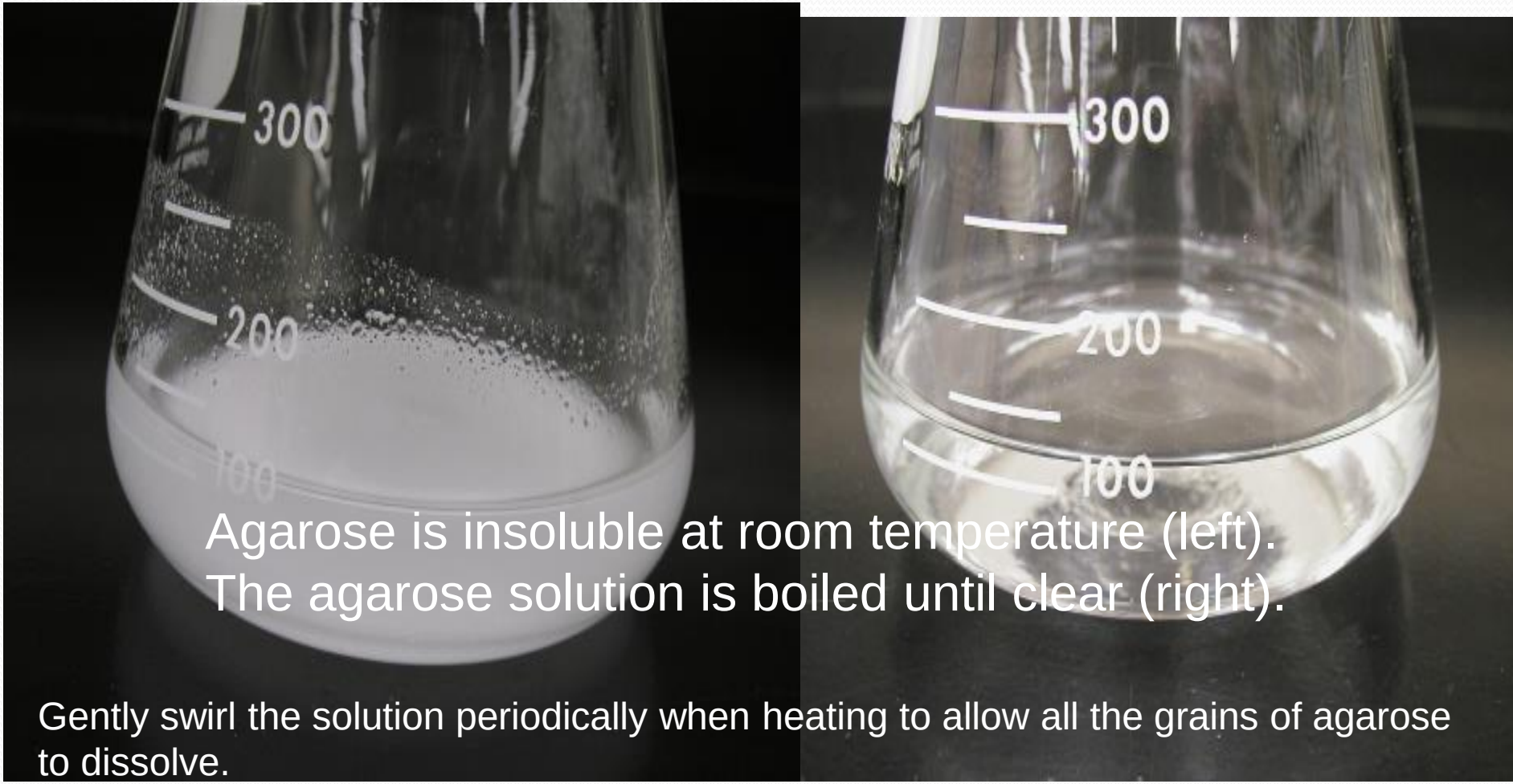
Preparing the Casting Tray



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.



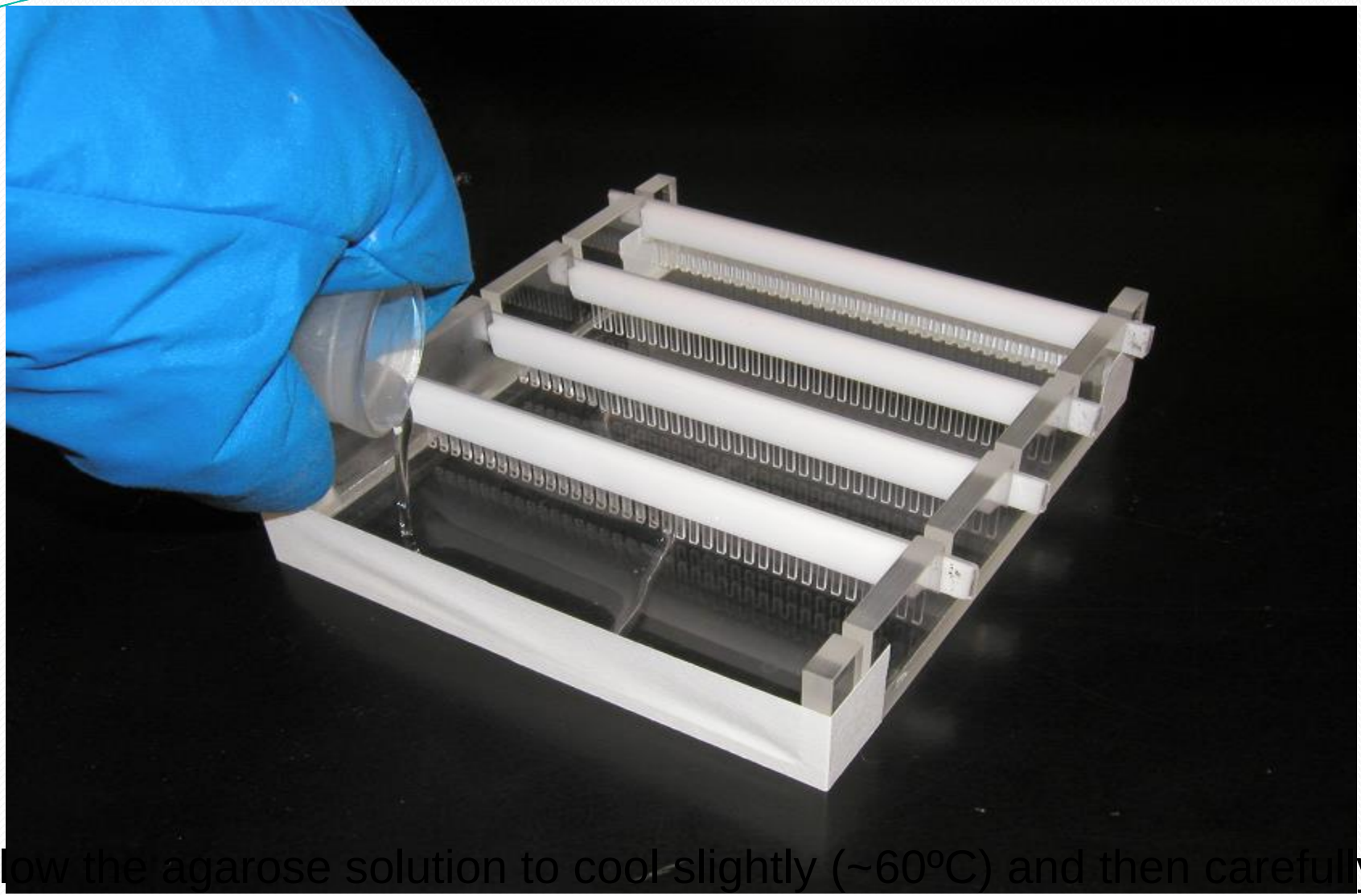
Melting the Agarose



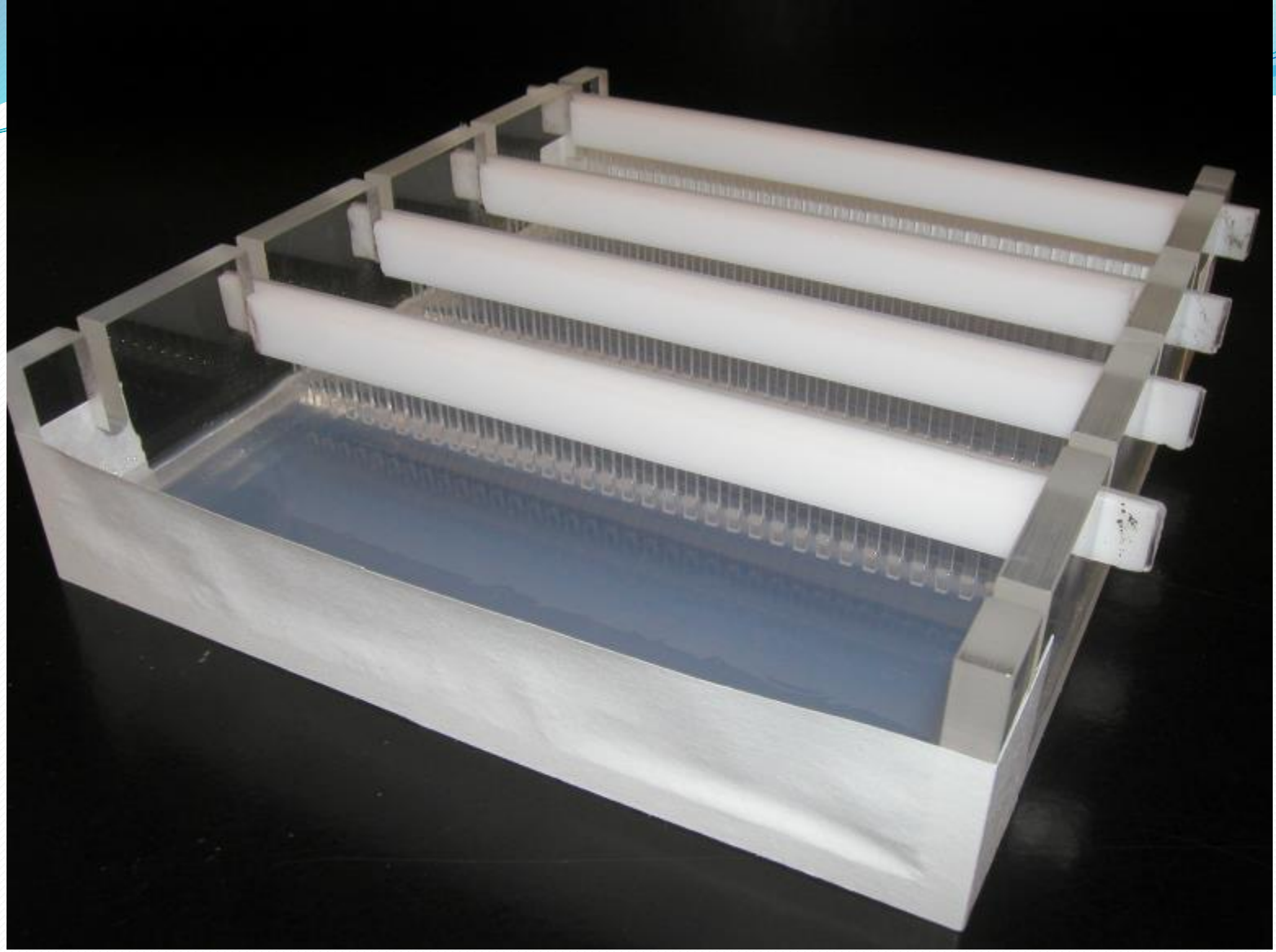
Agarose is insoluble at room temperature (left).
The agarose solution is boiled until clear (right).

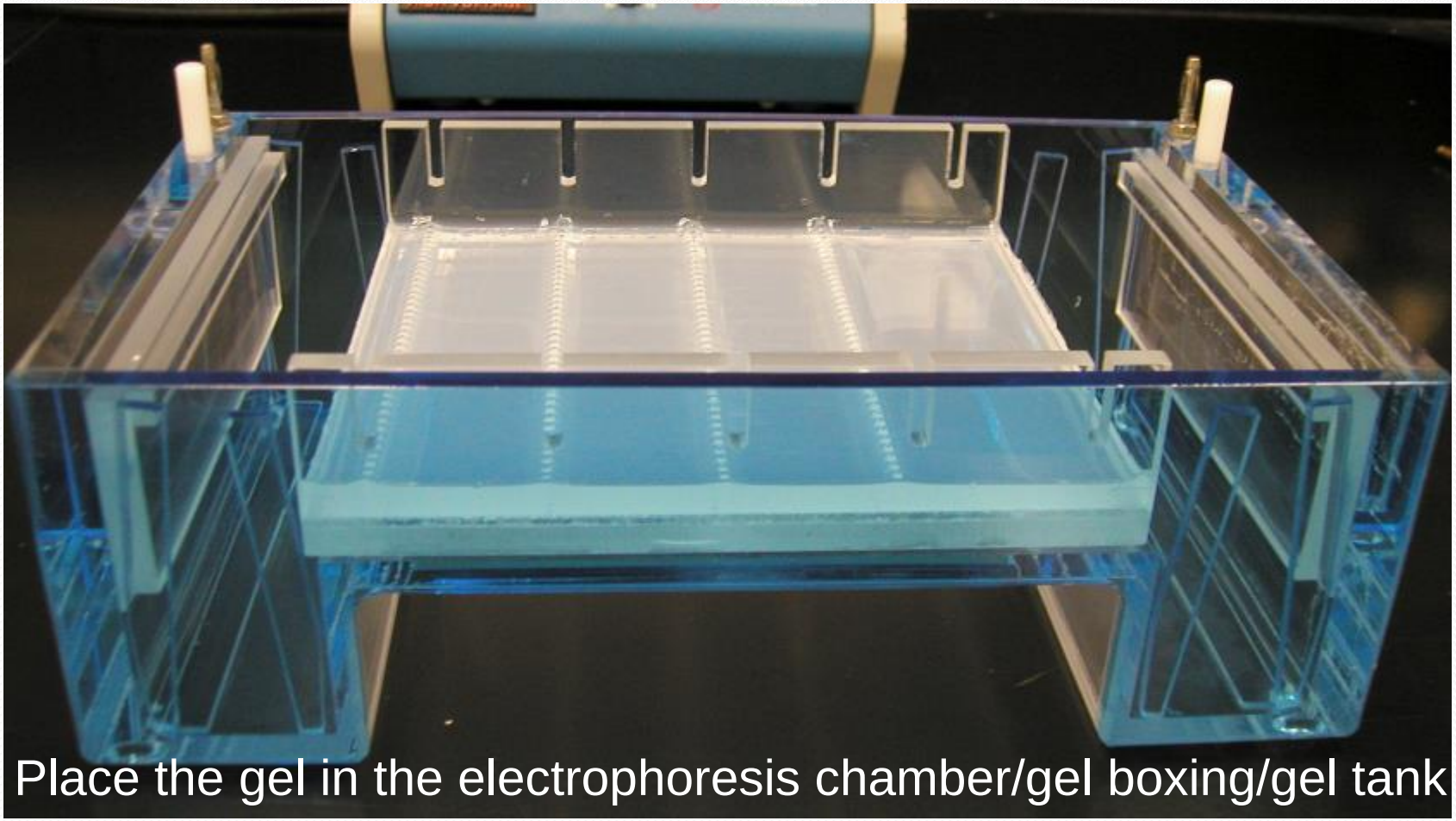
Gently swirl the solution periodically when heating to allow all the grains of agarose to dissolve.

Pouring the gel

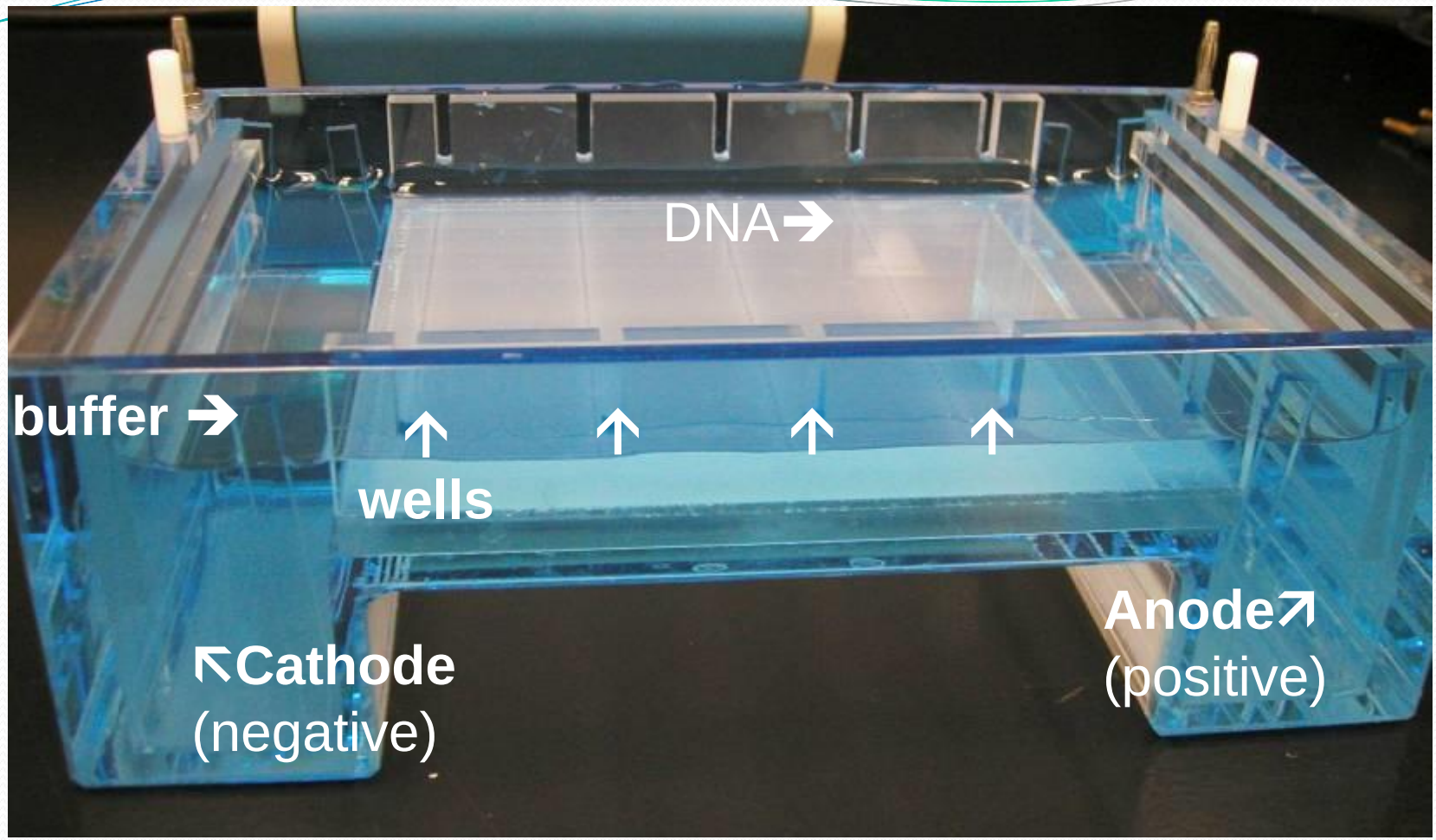


Allow the agarose solution to cool slightly (~60ºC) and then carefully pour the melted agarose solution into the casting tray. Avoid air bubbles.





Place the gel in the electrophoresis chamber/gel boxing/gel tank


DNA➔
buffer ➔
wells
↖Cathode
(negative)
Anode↗
(positive)

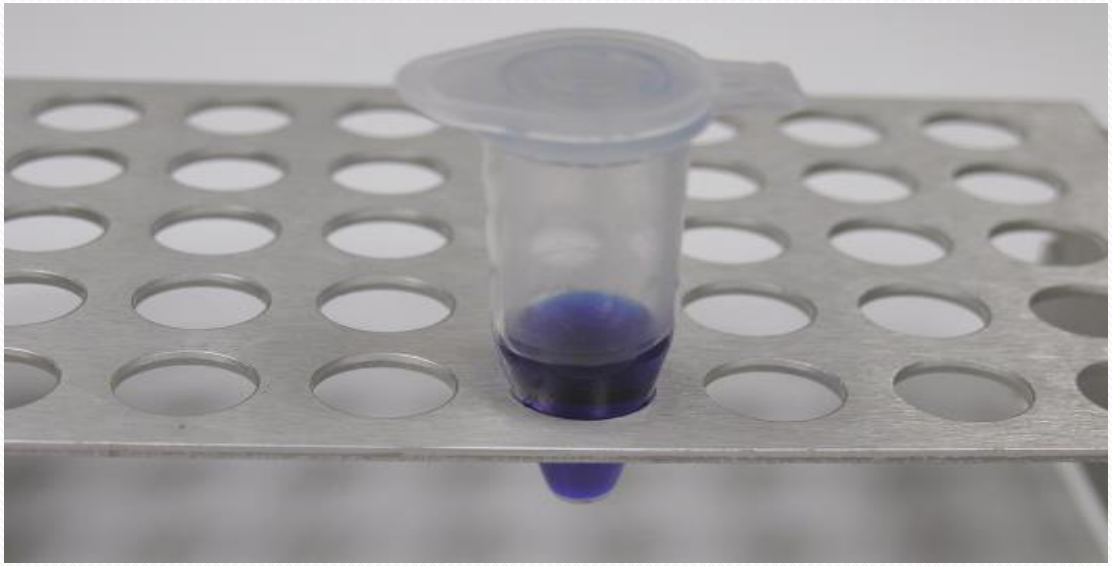
Sample Preparation

❑Mix the samples of DNA with the 6X sample loading buffer . 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. give it color and density

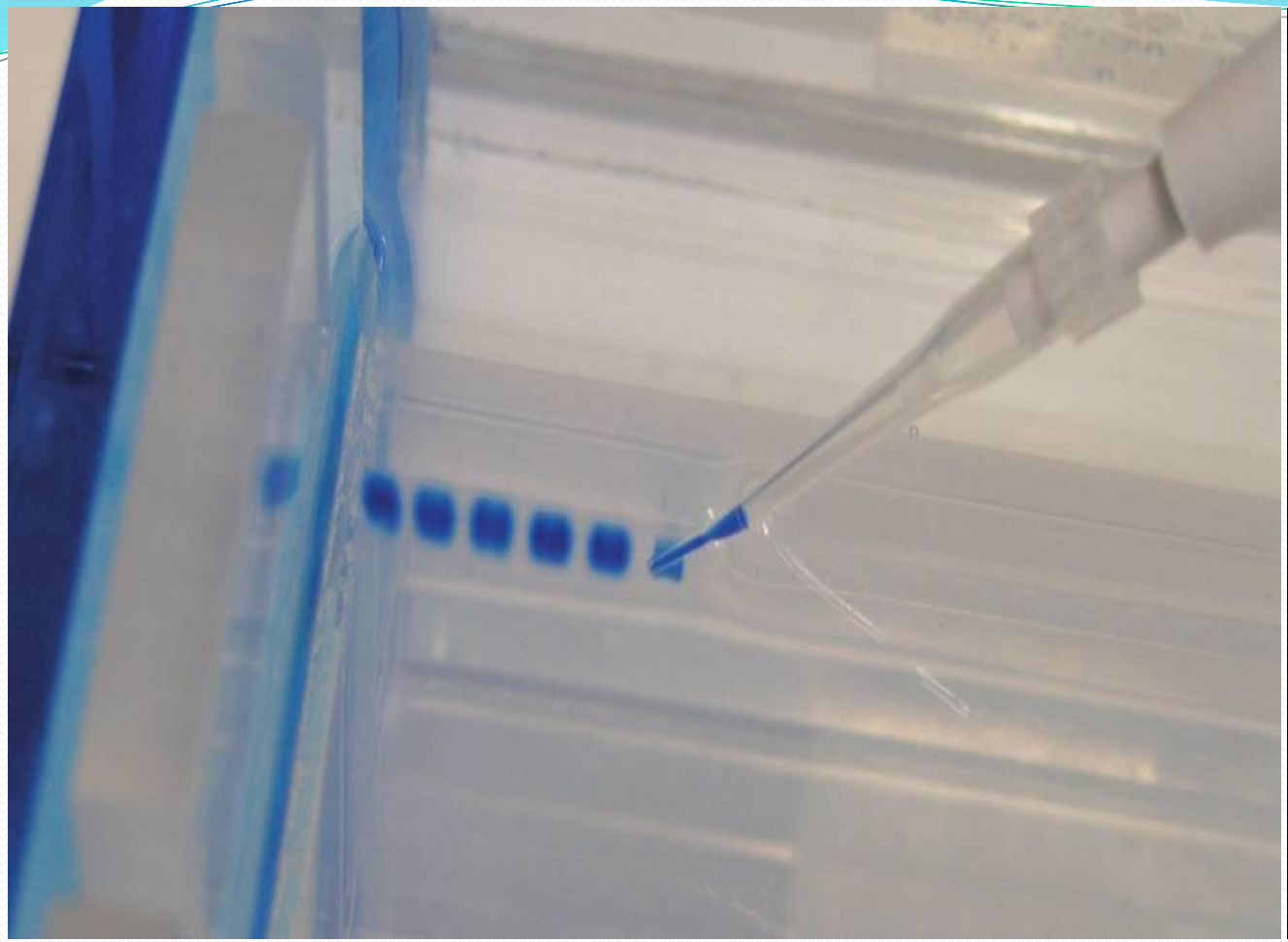
6X Loading Buffer: →

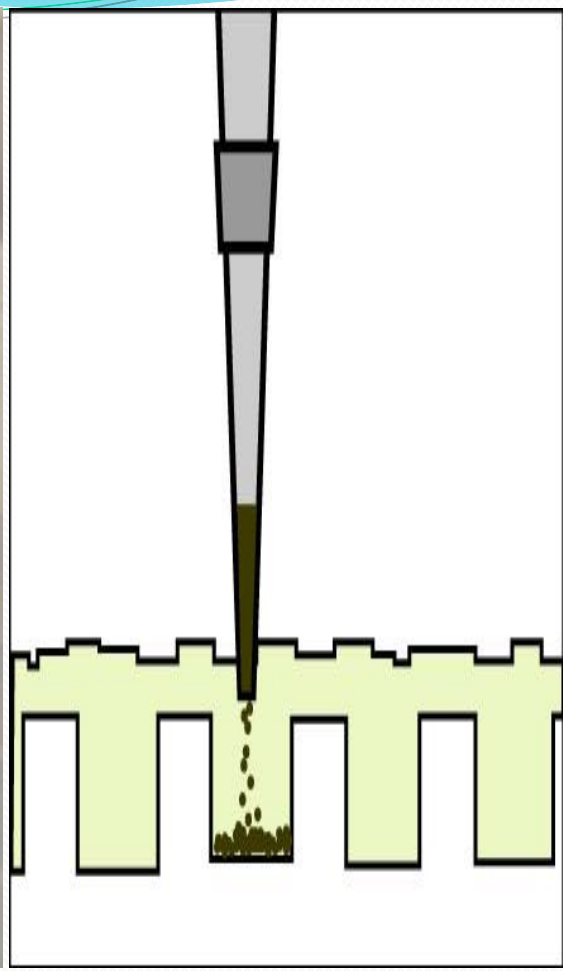
- Bromophenol Blue: 0.25gr
- Glycerol,sucrose: 40gr

  ddw: up to 100ml



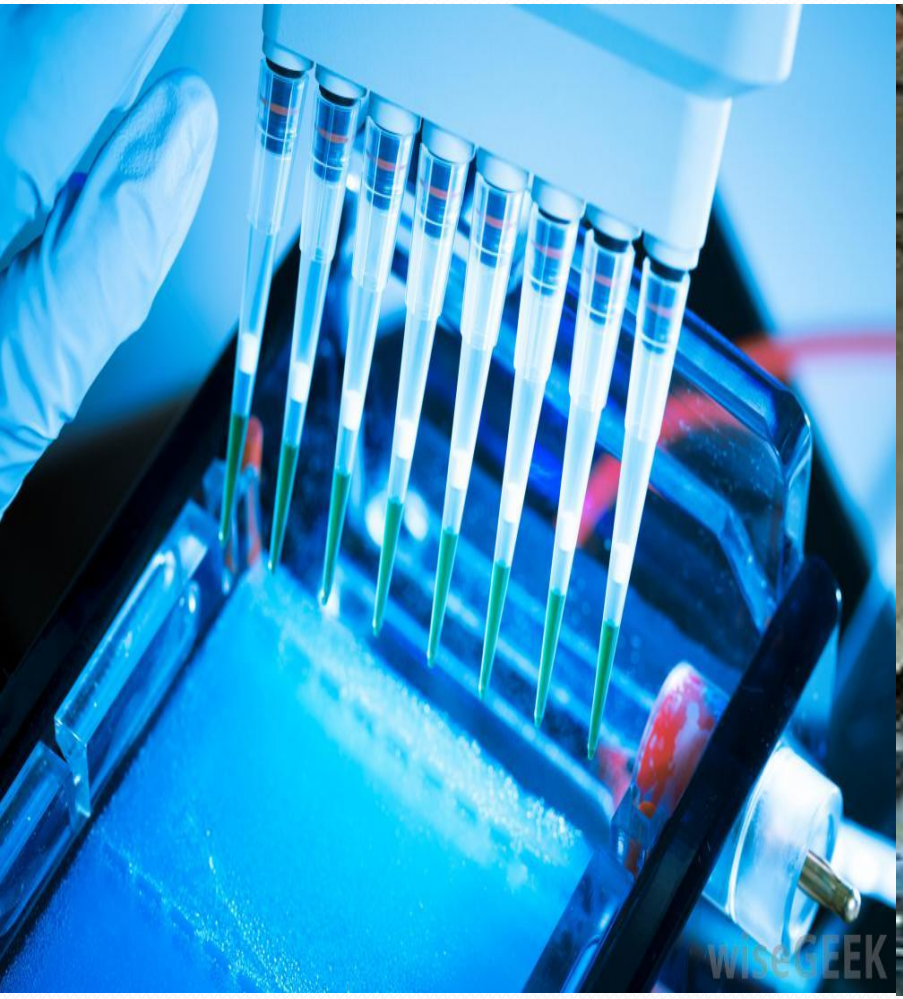
Sample Loading

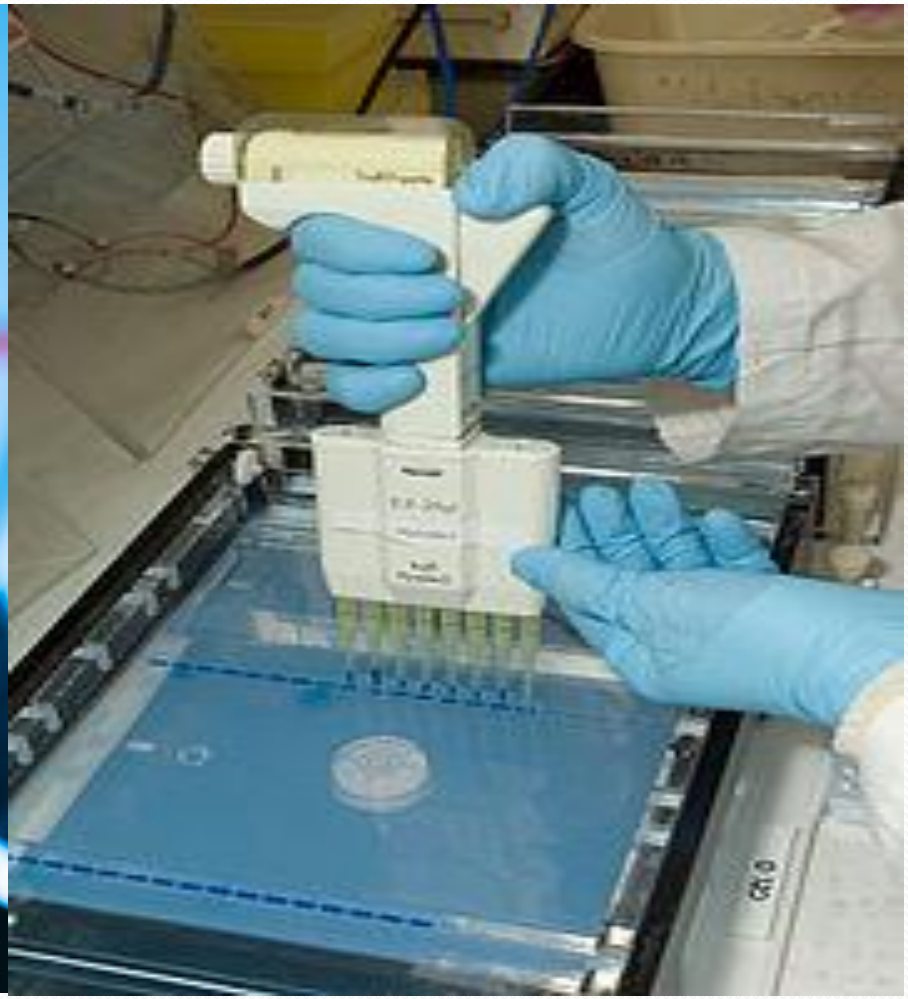




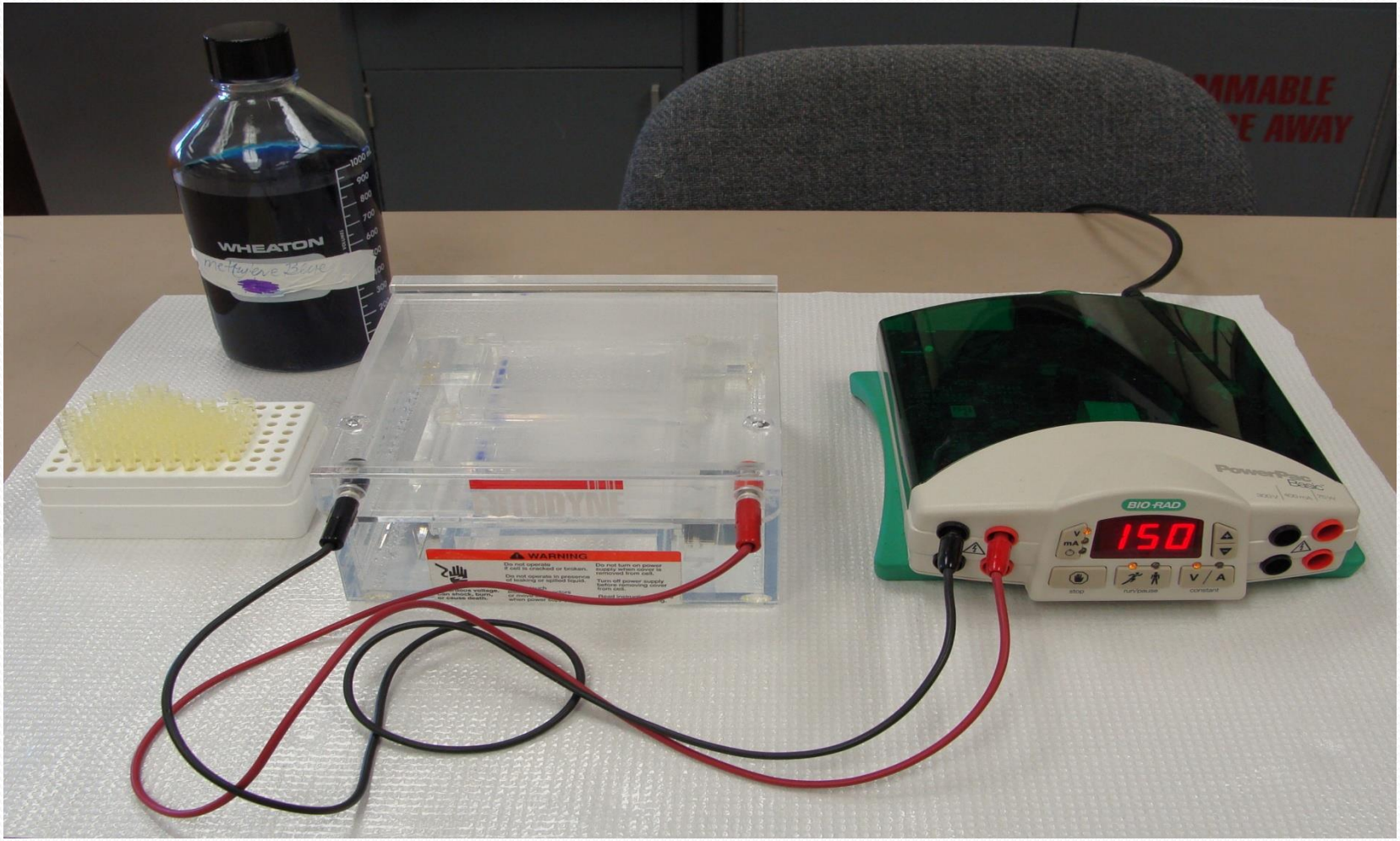
Carefully place the pipette tip over a well and gently expel the sample. The sample should sink into the well. Be careful not to puncture the gel with the pipette tip.

Sample Loading

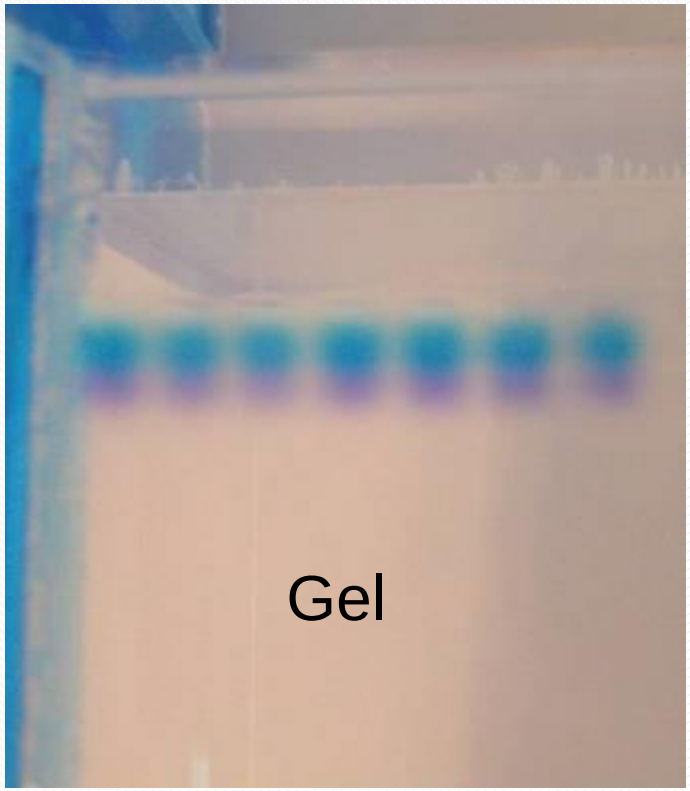




Running the Gel



Cathode
(-)



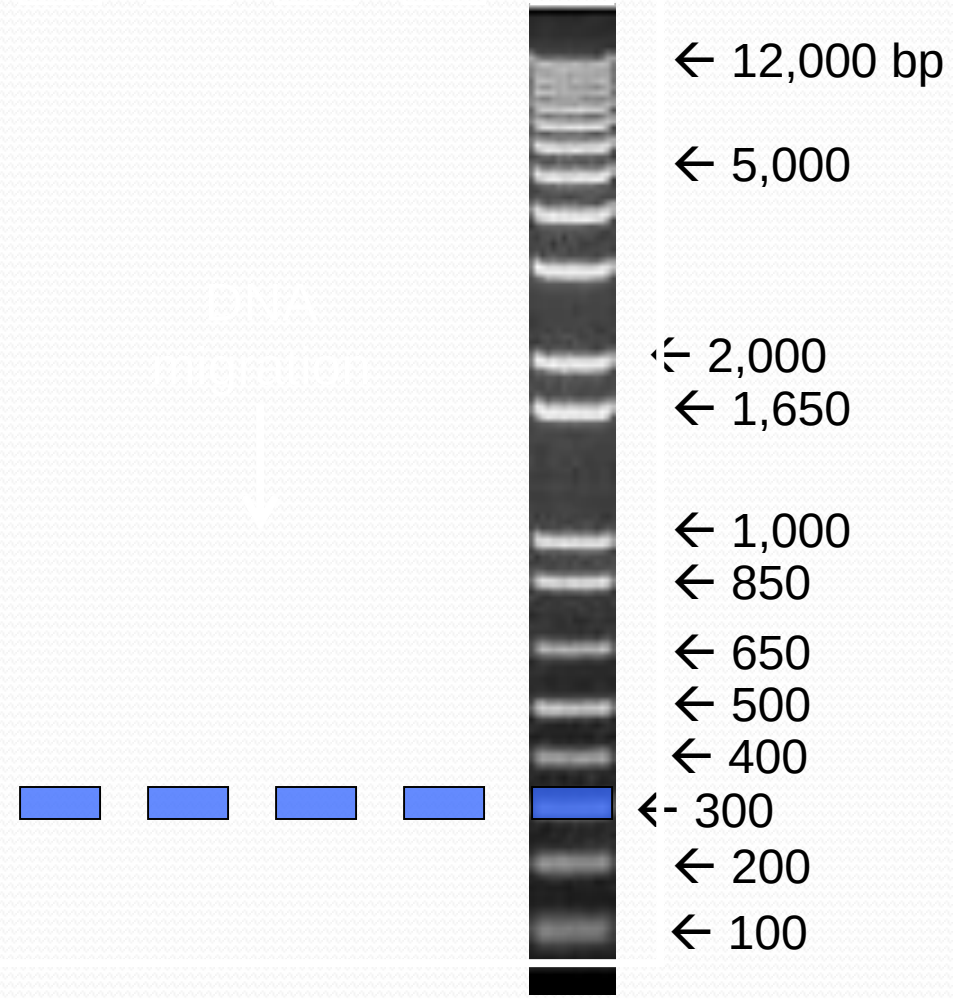
← wells
← Bromophenol Blue

DNA
(-)
↓

Anode
(+)

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 Ladder Standard



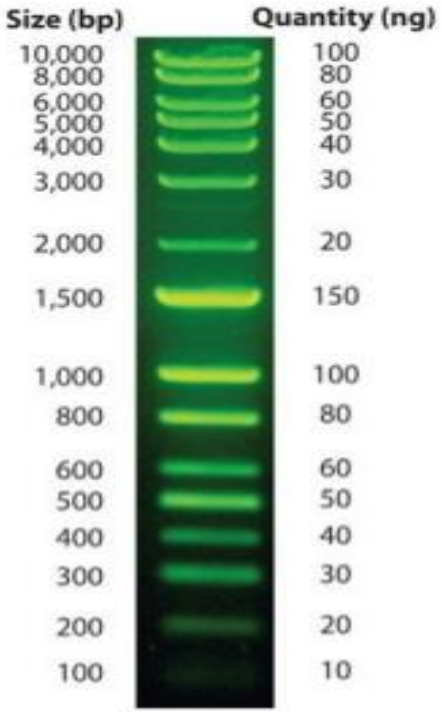
Note: bromophenol blue migrates at approximately the same rate as a 300 bp DNA molecule

bromophenol blue

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

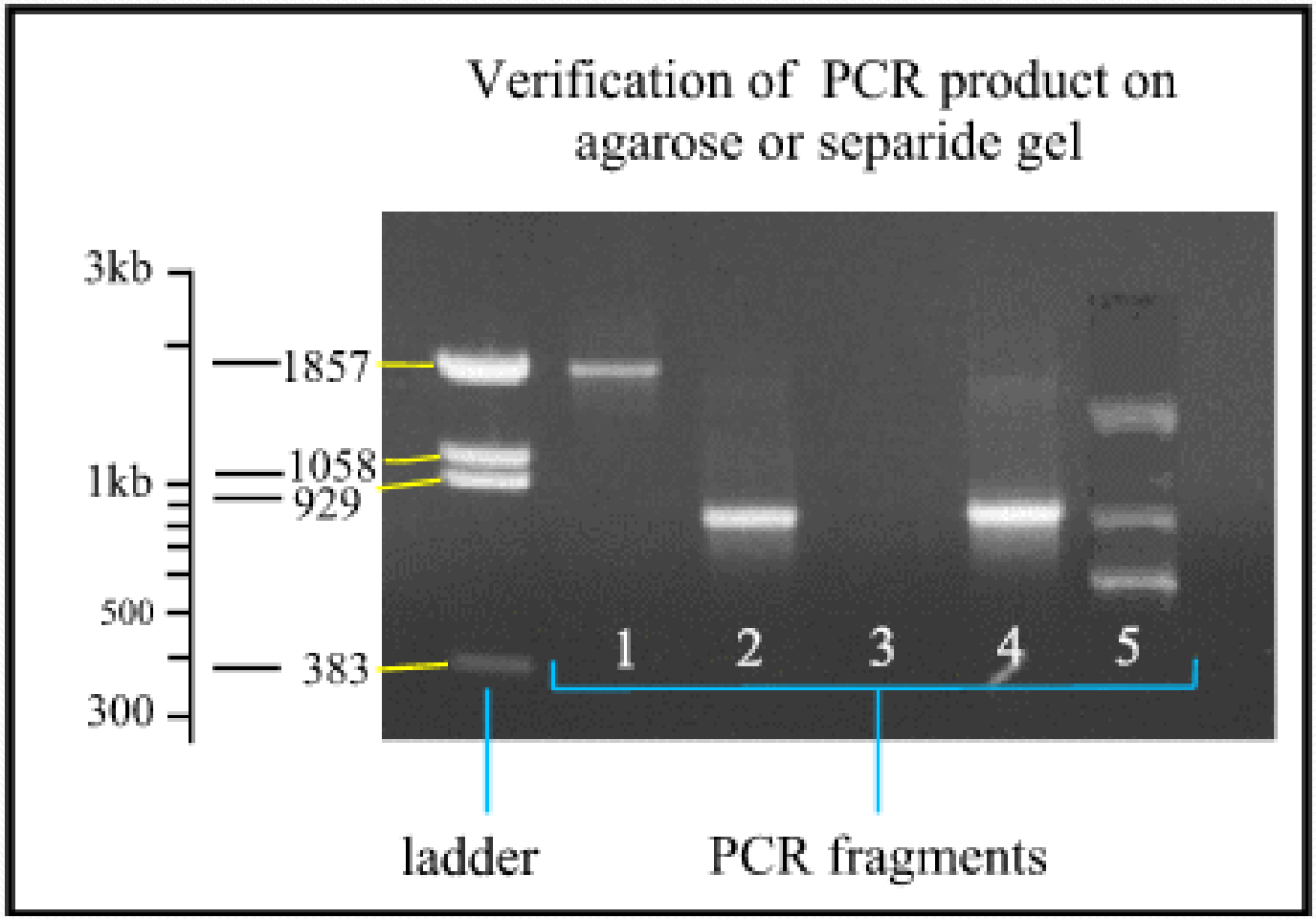
DNA ladder

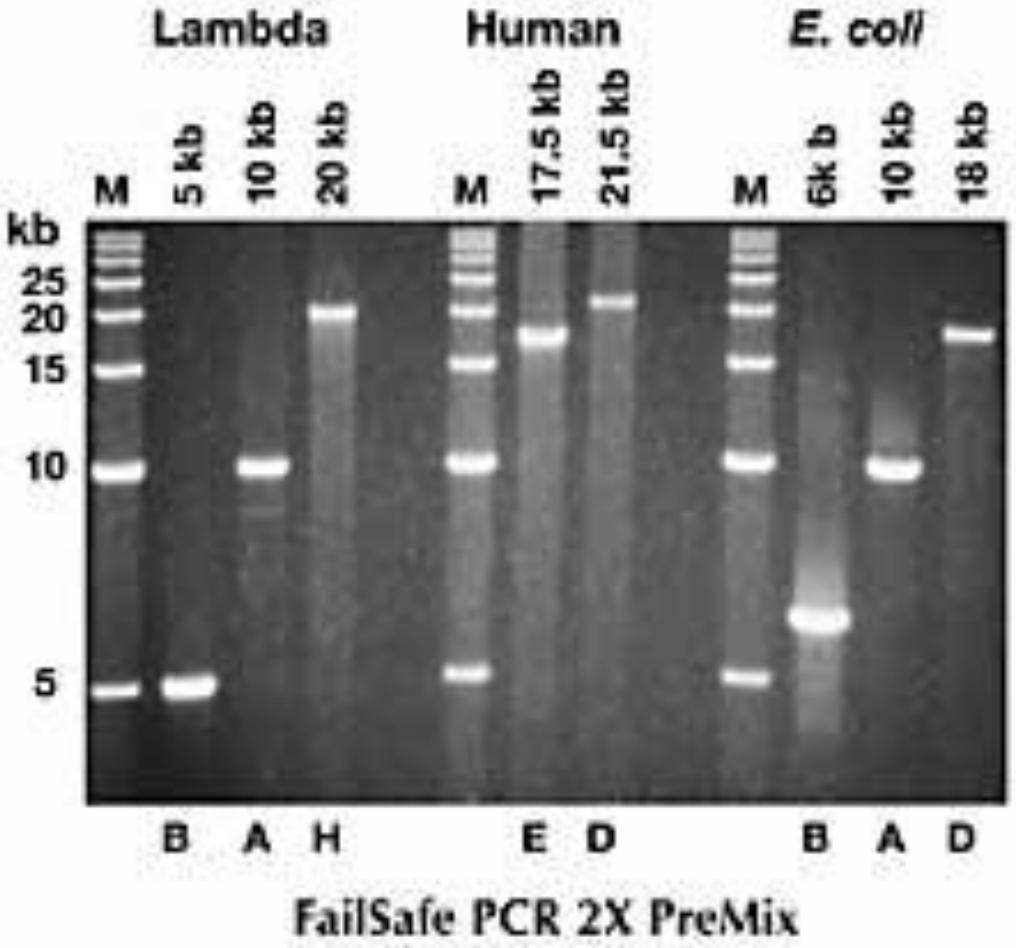
DNA ladder



- It is a solution of DNA molecules of different length
- DNA Ladder consists of known DNA sizes used to determine the size of an unknown DNA sample.
- The DNA ladder usually contains regularly spaced sized samples which when run on an agarose gel looks like a "ladder".

Visualization of the DNA fragments


Verification of PCR product on agarose or separide gel
3kb
1kb
500
300
1857
1058
929
383
1
2
3
4
5
ladder
PCR fragments


Lambda
Human
E. coli
M
5 kb
10 kb
20 kb
M
17.5 kb
21.5 kb
M
6k b
10 kb
18 kb
kb
25
20
15
10
5
B
A
H
E
D
B
A
D
FailSafe PCR 2X PreMix

The END