

PCR

The Polymerase Chain Reaction

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Polymerase Chain Reaction

PCR – first described in mid 1980's, Mullis Nobel prize in 1993

An *in vitro* method for the enzymatic synthesis of specific DNA sequences

Selective amplification of target DNA from a heterogeneous, complex DNC/cDNA population

Requires

Two specific oligonucleotide primers

Thermostable DNA polymerase

dNTP's

Template DNA

Sequential cycles of (generally) three steps (temperatures)

Initially PCR used the Klenow fragment of *E. coli* DNA polymerase - inactivated by high temperatures
Kleppe, Ohtsuka, Kleppe, Molineux, Khorana. 1971. *J. Mol. Biol.* 56:341.

Required a thermostable DNA polymerase - *Taq*
DNA polymerase from *Thermus aquaticus*
a thermophilic eubacterial microorganism
isolated from a hot spring in Yellowstone
National Park

$K_{cat} = 150$ nucleotides/sec/enzyme (at T_{opt})

$Taq_{1/2} =$	92.5 °C	➡	130 min
	95.0 °C	➡	40 min
	97.5 °C	➡	5 min

PCR - before the thermocycler



95° C
5 min



55° C
3 min



72° C
5 min



8 **BORING** hours per PCR!



Thermocyclers



heated lids
adjustable ramping times
single/multiple blocks
gradient thermocycler blocks



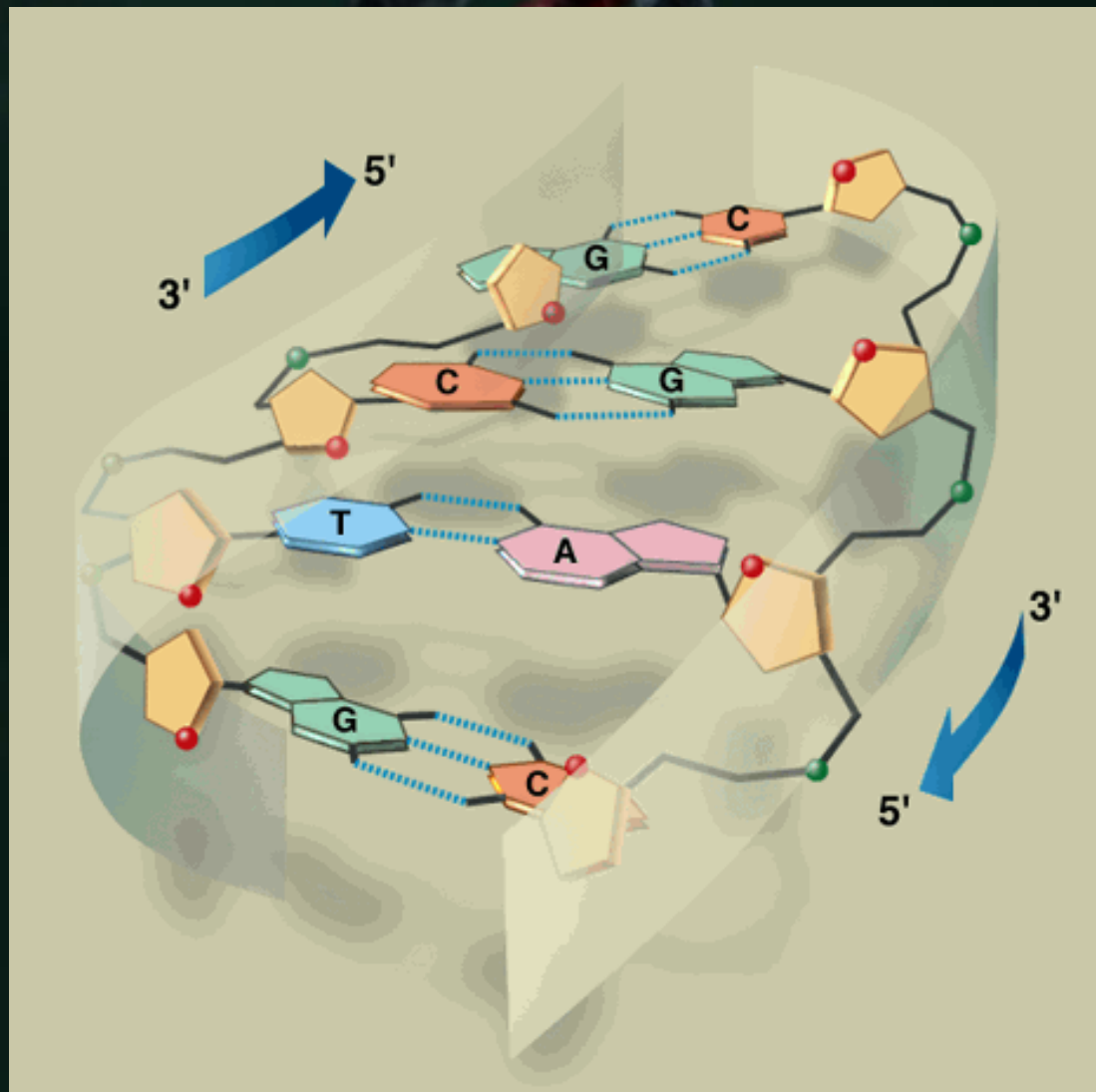


**standard tube, ↑ volume, ↑ cost
evaporation & heat transfer concerns**



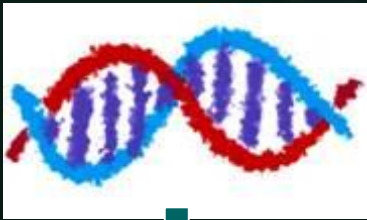
**thin walled tube, ↓ volume, ↓ cost
↓ evaporation & heat transfer concerns**

Directional Synthesis



“Xeroxing” DNA

1 copy



PLUS dNTPs, buffer,
salts, Taq pol,
primers



Cycle 1



2 copies

Cycle 2



4 copies

Cycle 3



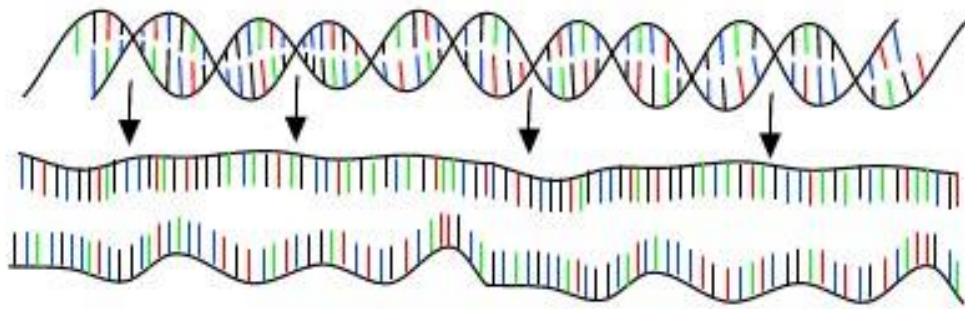
8 copies

Cycle 35

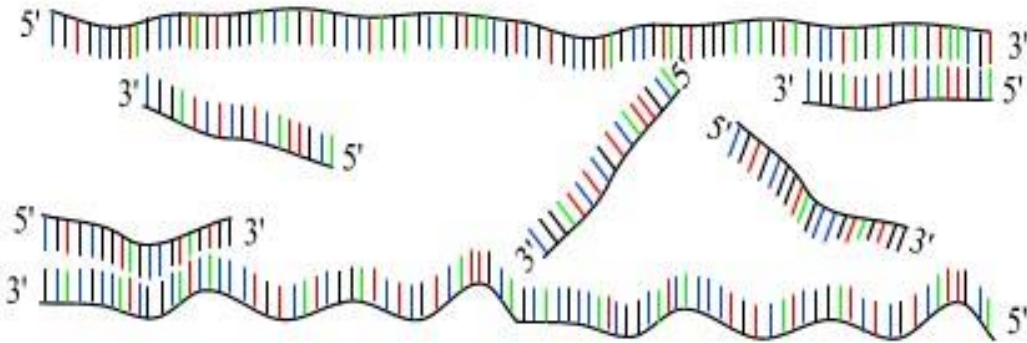
$n^{36} = 68,719,476,736$ copies in ~ 2 hrs

A simple thermocycling protocol

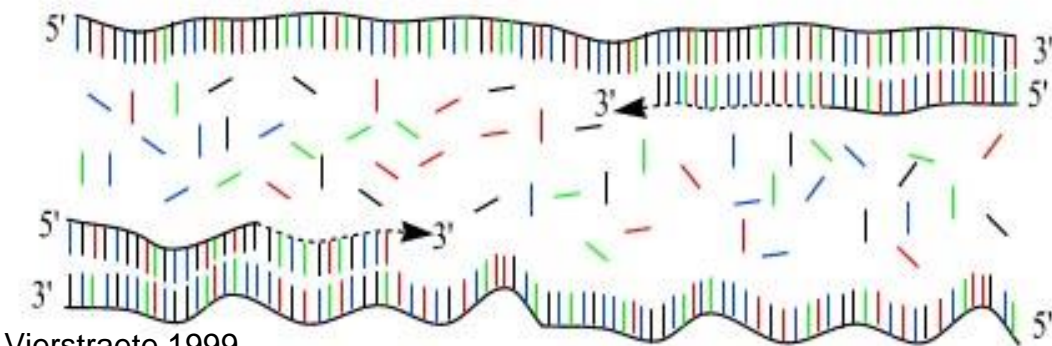




Step 1:
Denaturation
dsDNA to ssDNA



Step 2:
Annealing
Primers onto template



Step 3:
Extension
dNTPs extend 2nd strand

extension products in one cycle serve as template in the next

Basic Components of PCR

- **Template DNA (0.5 - 50 ng)**
< 0.1 ng plasmid DNA, 50 ng to 1 µg gDNA for single copy genes
- **Oligonucleotide primers (0.1 – 2.0 µM)**
- **dNTP's (20 –250 µM)**
- **Thermostable DNA pol (0.5 – 2.5 U/rxn)**
- **MgCl₂ (1 – 5 mM)** *affects primer annealing and Taq activity*
- **Buffer (usually supplied as 10X)**

Working concentrations

KCL (10 – 50 mM)

Tris-HCl (10 mM, pH 8.3)

NaCl₂ (sometimes)

Taq polymerase

DNA template

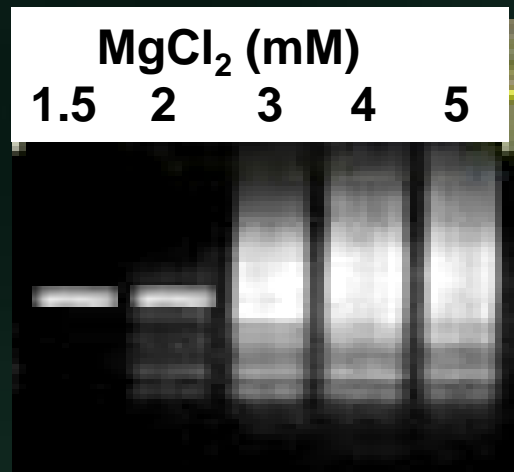
Buffer

Primers



dNTPs

MgCl₂



Magnesium Chloride

(MgCl₂ - usually 0.5-5.0mM)

Magnesium ions have a variety of effects

Mg²⁺ acts as cofactor for *Taq* polymerase

Required for *Taq* to function

Mg²⁺ binds DNA - affects primer/template interactions

Mg²⁺ influences the ability of *Taq* pol to interact with primer/template sequences

More magnesium leads to less stringency in binding

PCR Problems

Taq is active at low temperatures

At low temperatures mis-priming is likely

Temp	Extension Rate	
55° C	24 nt/sec	
37° C	1.5 nt/sec	
22° C	0.25 nt/sec	➔ 150 nucleotides in 10 min

“Cures” for mis-priming

- **“Cheap” fixes**
 - Physical separation – “DNA-in-the-cap”
 - Set up reactions on ice
- **Hot-start PCR –holding one or more of the PCR components until the first heat denaturation**
 - Manually - delay adding polymerase
 - Wax beads
 - Polymerase antibodies
- **Touch-down PCR – set stringency of initial annealing temperature high, incrementally lower with continued cycling**
- **PCR additives**
 - 0.5% Tween 20
 - 5% polyethylene glycol 400
 - betaine
 - DMSO

Primer Design



1. Typically 20 to 30 bases in length
2. Annealing temperature dependent upon primer sequence (~ 50% GC content)
3. Avoid secondary structure, particularly 3'
4. Avoid primer complementarity (primer dimer)
5. The last 3 nucleotides at the 3` end is the substrate for DNA polymerase - G or C
6. Many good freeware programs available

Primer Design Software



Many free programs available online

OLIGO

PRIMER

PrimerQuest

Primer Dimers

- Pair of Primers

5'-ACGGATACGTTACGCTGAT-3'

5'-TCCAGATGTACCTTATCAG-3'

- Complementarity of primer 3' ends

5'-ACGGATACGTTACGCTGAT-3'

3'-GACTATTCCATGTAGACCT-5'

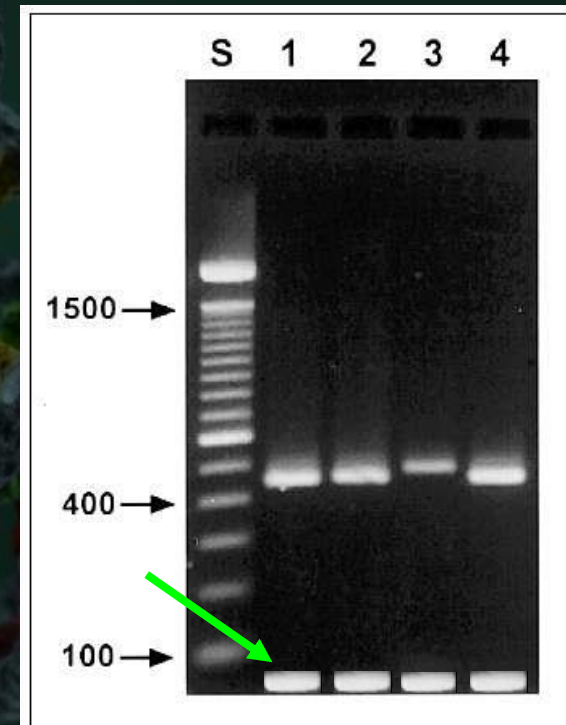
- Results in PCR product

Primer 1

5'-ACGGATACGTTACGCTGATAAGGTACATCTGGA-3'

3'-TGCCTATGCAATGCGACTATTCCATGTAGACCT-5'

Primer 2



Rules of thumb for PCR conditions

- Add an extra 3-5 minute (longer for Hot-start *Taq*) to your cycle profile to ensure everything is denatured prior to starting the PCR reaction
- Approximate melting temperature (T_m) = $[(2 \times (A+T)) + (4 \times (G+C))]$ °C
 - If GC content is $< 50\%$ start 5°C beneath T_m for annealing temperature
 - If GC content $\geq 50\%$ start at T_m for annealing temperature
- Extension @ 72°C: rule of thumb is ~500 nucleotide per minute. Use 3 minutes as an upper limit without special enzymes
- “Special” PCR cycling protocols
 - Touchdown PCR
 - Step-up PCR
 - Gradient cycling

Common PCR additives

BSA (usually at 0.1 to 0.8 $\mu\text{g}/\mu\text{L}$ final concentration)

Stabilize *Taq* polymerase & overcome PCR inhibitors

DMSO (usually at 2-5% v/v, inhibitory at $\leq 10\%$ v/v)

Denaturant - good at keeping GC rich template/primer strands from forming secondary structures.

Glycerol (usually at 5-10% v/v)

Increases apparent concentration of primer/template mix, and often increases PCR efficiency at high temperatures.

Stringency enhancers (Formamide, Betaine, TMAC)

Concentrations used vary by type

Enhances yield and reduces non-specific priming

Non-ionic detergents (Triton X, Tween 20 or Nonidet P-40) (0.1–1%)


NOT SDS (0.01% SDS cuts *Taq* activity to $\sim 10\%$ of normal)

Stabilize *Taq* polymerase & suppress formation of 2° structure

PCR additives - Literature

Additive	References
<p>DMSO (dimethyl sulfoxide)</p>	<p><i>Amplifications</i> 5: 16 <i>Gene</i> 140: 1 <i>Nucleic Acids Research</i> 18: 1666</p>
<p>Betaine (N,N,N-trimethylglycine = [carboxymethyl] trimethylammonium)</p>	<p><i>Biochemistry</i> 32: 137 <i>BioTechniques</i> 21: 1102 <i>Genome Research</i> 6: 633 <i>Nucleic Acids Research</i> 25: 3957 <i>Proceedings of the National Academy of Sciences of the United States of America</i> 70: 298 <i>Trends in Biochemical Science</i> 22: 225</p>
<p>Formamide</p>	<p><i>Nucleic Acids Research</i> 18: 7465</p>
<p>Non-ionic detergents e.g. Triton X-100, Tween 20 or Nonidet P-40 (NP-40)</p>	<p><i>Biotechniques</i> 12: 332 <i>Nucleic Acids Research</i> 18: 1309</p>
<p>TMAC (tetramethylammonium chloride)</p>	<p><i>Nucleic Acids Research</i> 18: 4953 <i>Nucleic Acids Research</i> 23: 3343</p>
<p>dC⁷GTP (7-deaza-2'-deoxyguanosine)</p>	<p><i>Nucleic Acids Research</i> 16: 3360</p>
<p>BSA (bovine serum albumin)</p>	<p><i>Applied and environmental microbiology</i> 62:1102-1106 <i>BioTechniques</i> 23:504 <i>BioTechniques</i> 25:564 <i>Nucleic Acids Research</i> 16: 9775</p>

Typical PCR Temps/Times

Initial denaturation	90° – 95° C	1 – 3 min	
Denature	90° – 95° C	0.5 – 1 min	
Primer annealing	45° – 65° C	0.5 – 1 min	
Primer extension	70° – 75° C	0.5 – 2 min	
Final extension	70° – 75° C	5 – 10 min	
Stop reaction	4° C or 10 mM EDTA	hold	

Gel electrophoresis

Workhorse method of the biotech laboratory

Separation through a matrix (agarose or acrylamide)

Analytical or preparative method

Separates fragments with large range of molecular weights

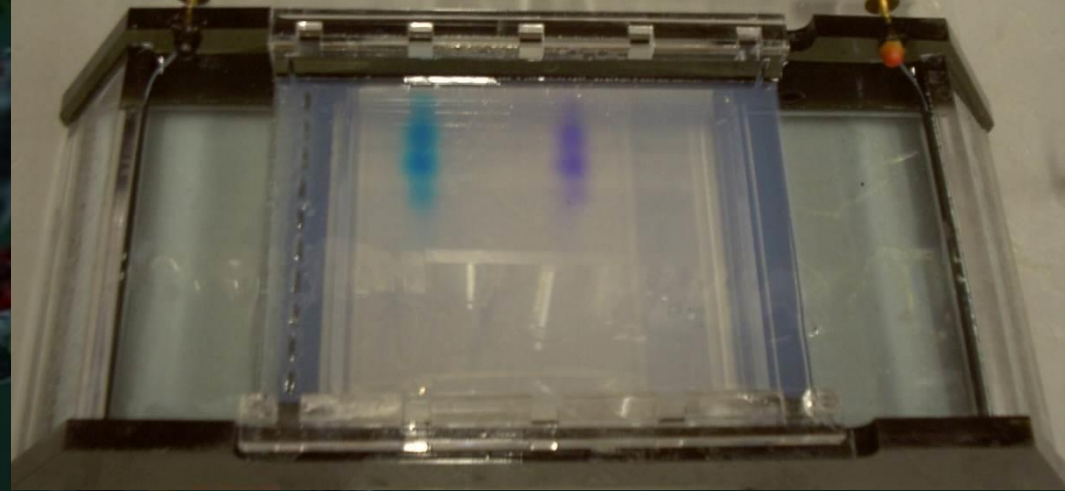
Driven by simple current and the fact nucleic acids are uniformly negatively charged

Sample buffer (SB) or tracking dye (TD) or loading buffer - used to keep sample in the well and visualize run



An aside: **Ohm's law**

$$V = IR$$



voltage = current x resistance
(electric field) (milliamps) (ohms)

What does this REALLY mean to you?

For a given current, decreasing the thickness of the gel or ionic strength of the running buffer increases the mobility of the nucleic acid fragments

**Manipulate this when possible to speed up the “pay-off” ...
Did your PCR work?!!**

Electrophoresis

Variations on a Theme

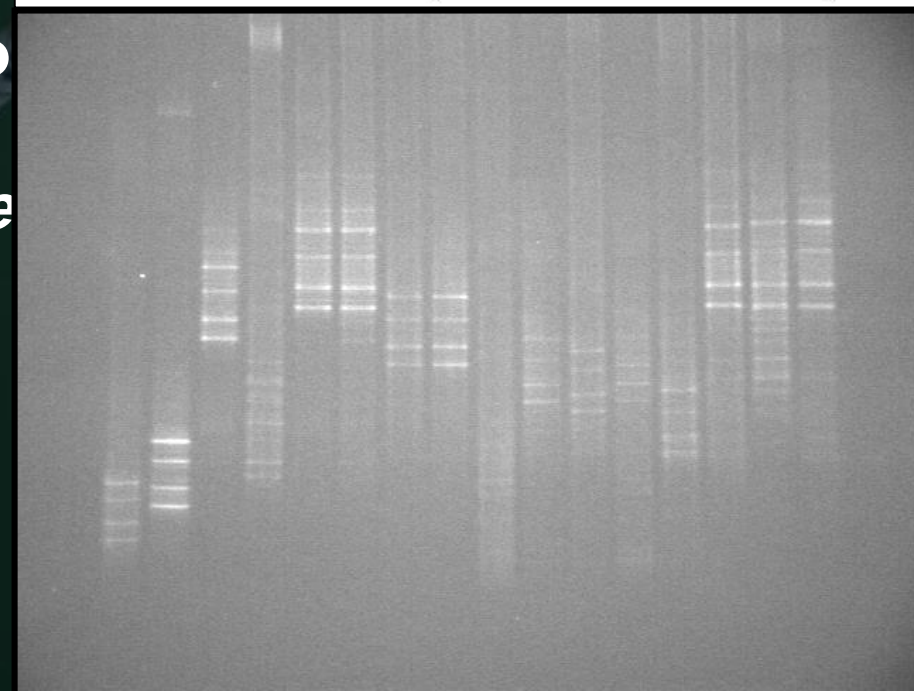
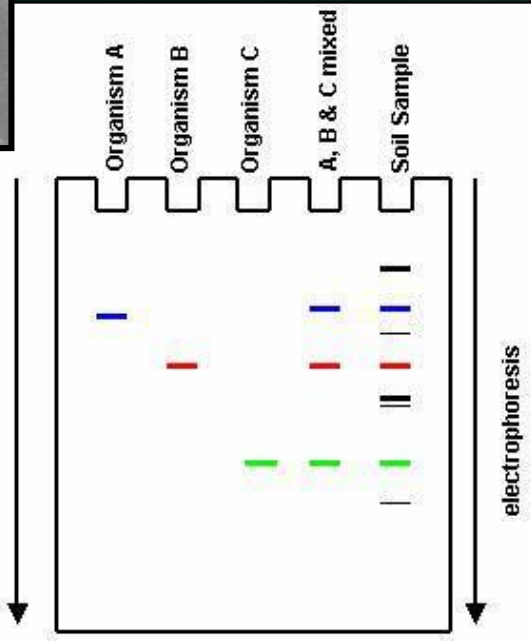
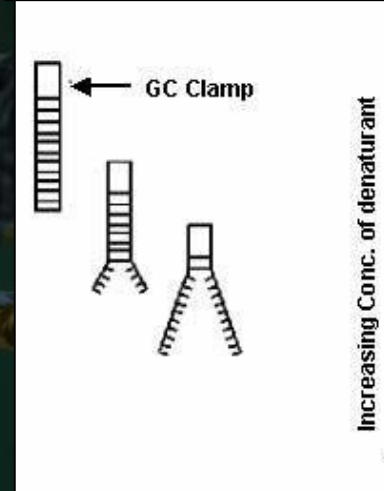
DGGE

Denaturing gradient gel electro

TGGE

Temperature gradient gel electro

Allows separation of single base polymorphisms



Pulsed field gel electrophoresis (PFGE)

Schwartz and Cantor (1984) Cell 37:67-75

DNA (from cells or DNA, undigested or digested)

Embedded into agarose plugs

Electrical field constantly changes direction (non-continuous)
allows for increased resolution of very h.m.w. gDNA
(Kb to Mb ranges)

chiller, pump, flatbed electro4 box
can use digested or undigested

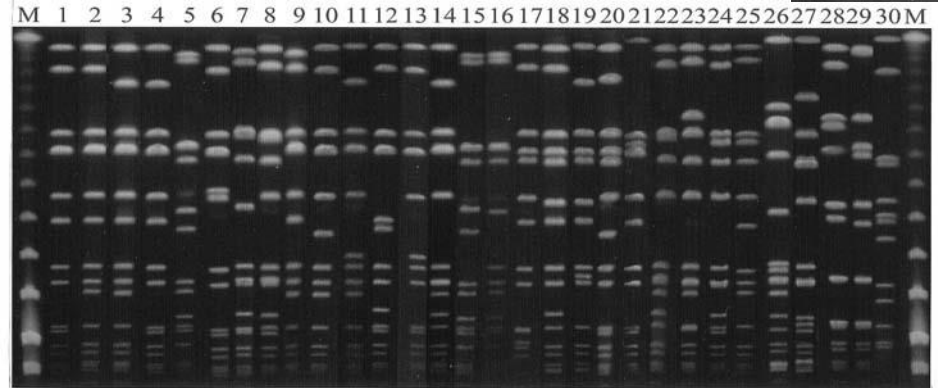
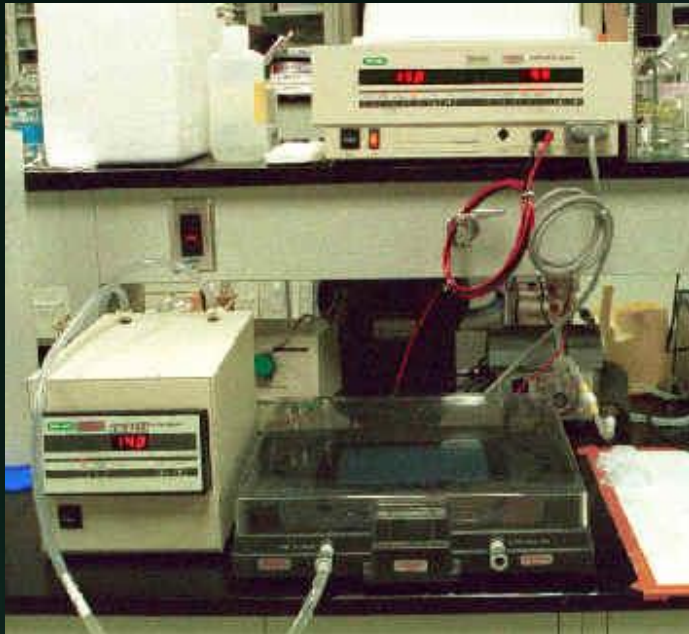
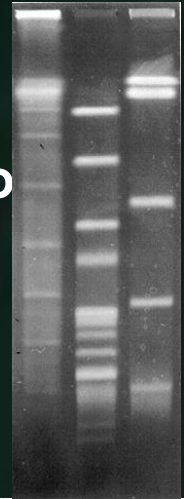
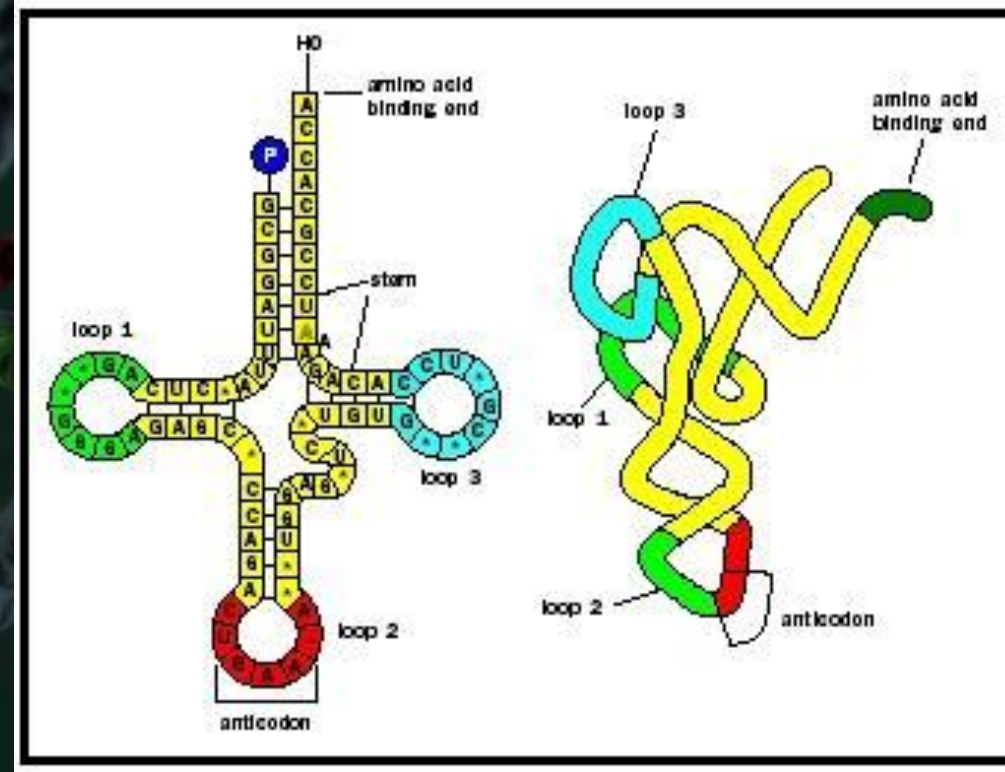


Fig. 1. Pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA from MRSA isolates. M: low range PFG Marker. Lanes 1 to 30: MRSA isolates with different PFGE patterns A1 to T shown in Fig. 2B.

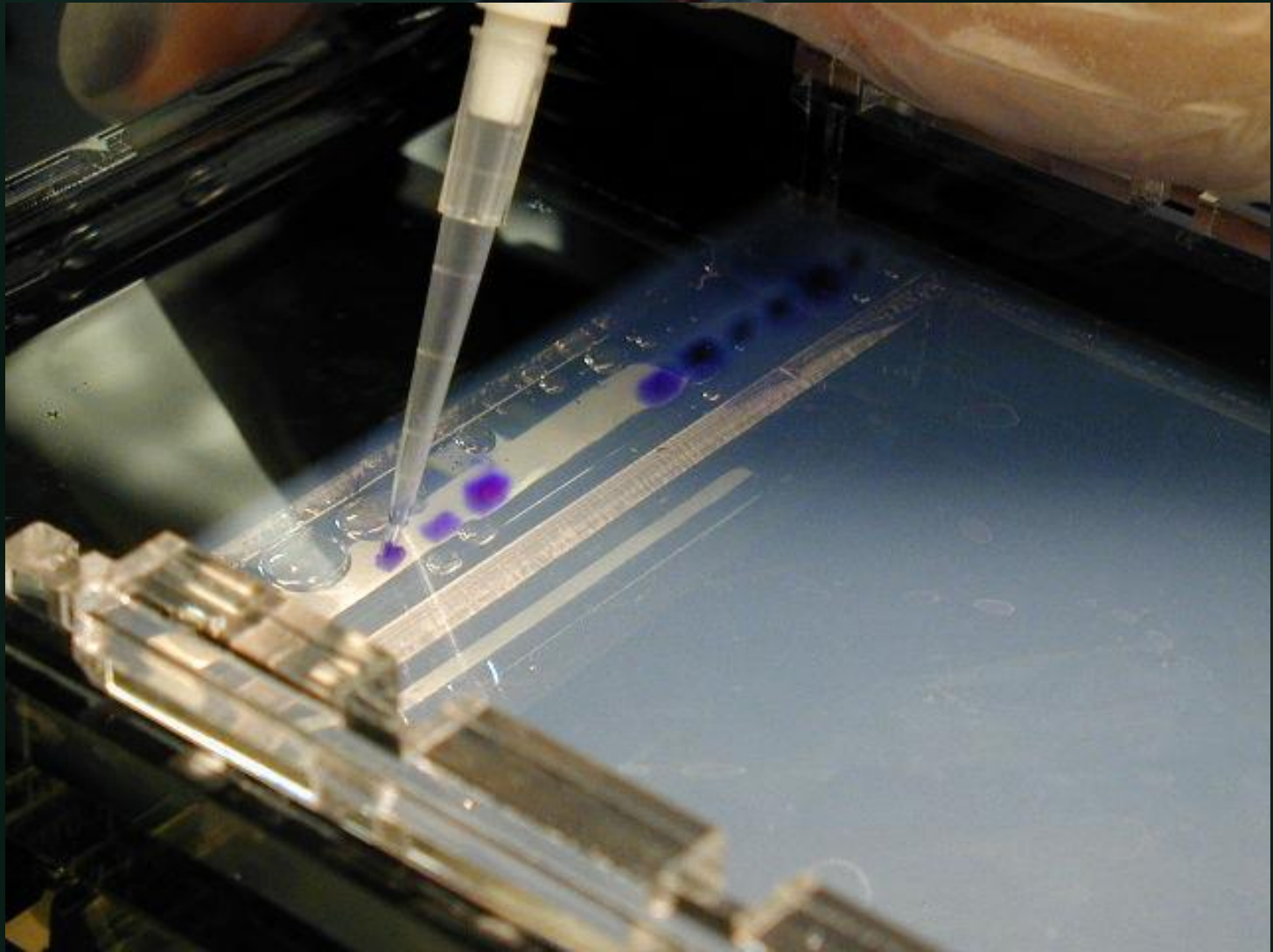
RNA electrophoresis

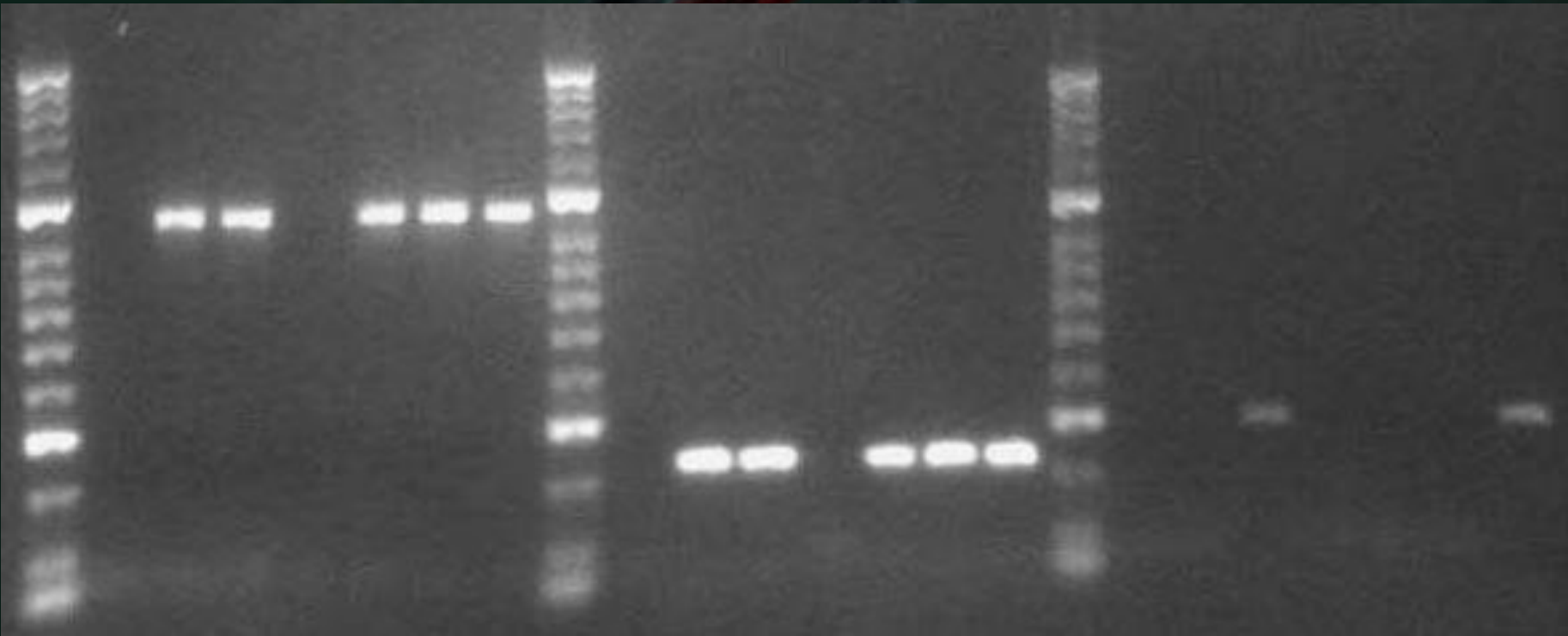
Requires special solution treatment to protect RNA from degradation or from folding in on itself



RNA is denatured and run on agarose gels containing formaldehyde. Formaldehyde forms unstable Schiff bases with the single imino group of guanine bases. This maintains RNA in a denatured state so it electrophoreses properly according to it's molecular weight.

Uses same gel box and power supply as traditional DNA electrophoresis





Houston, we have a PCR product...



BUT what if you don't...



Troubleshooting PCR

Non-specific bands on your gel

Reagents, set-up

Template concentration inappropriate

Annealing temp too low

Extension time too short

Cycle number too high

Primer design not appropriate

Primer concentration too high

Non-specific priming

MgCl₂ concentration too high

GC-rich template, ↑ 2° structure

Contaminating DNA

Run negative control

Review guidelines

Optimize by gradient PCR

↑↑ time for longer products

Review guidelines

↑ specificity

Optimize by titration

↑↑ specificity, Hot Start

Optimize by titration

PCR additives

Decontaminate work area:

use ARTs, wear gloves,

pipettor, reagents,

UV treat plastics

Troubleshooting PCR

Diffuse smearing on your gel

Template concentration inappropriate

Taq concentration too high

Extension time inappropriate

Cycle number too high

Primer design not appropriate

Primer concentration too high

Non-specific priming

MgCl₂ concentration too high

GC-rich template, ↑ 2° structure

Contaminating DNA

Review guidelines

Optimize by titration

Review guidelines

Reduce, review guidelines

↑ specificity

Optimize by titration

use Hot Start

Optimize by titration

PCR additives

Decontaminate work area:

use ARTs, wear gloves,
pipettor, reagents,
UV treat plastics

Troubleshooting PCR

Poor or no amplification of bands

Problem with thermocycler, set-up, reagents

Enzyme concentration low

Annealing temp too low

Extension time too short

Cycle number too low

Primer design not appropriate

Primer concentration too high

Non-specific priming

MgCl₂ concentration too low

GC-rich template, ↑ 2° structure

Run positive control

↑ Concentration

Optimize by gradient PCR

↑ Time for longer products

Review guidelines

↑ Specificity

Optimize by titration

↑ Specificity, Hot Start

Optimize by titration

PCR additives



Troubleshooting PCR Prioritizing Approaches

“Pilot” error (set-up errors common in the interim between training with someone and working independently)

Template dilution error (concentration matters!)

Thermocycling parameter errors (temps/times)

Bad reagents (1. dNTPs, 2. primers, 3. *Taq*)

Unique template or template structure issues

BAD KARMA (don't believe it!)

Don't get discouraged...validating PCRs can be tricky



Questions?

شکرا لِحضورکم و اہتمامکم

