PCR Troubleshooting

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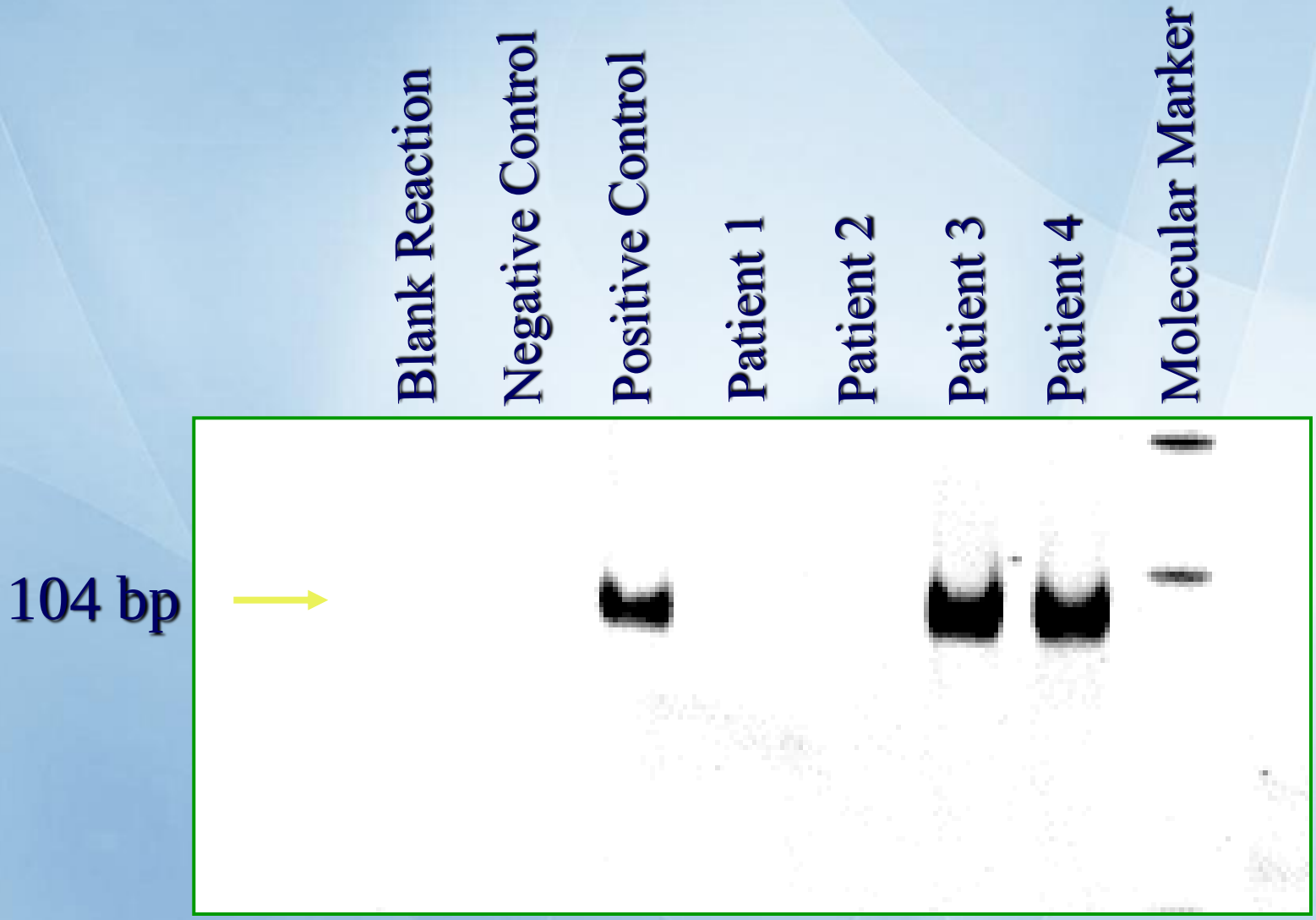
Experiment design

- **Blank reaction**
  - Controls for contamination
  - Contains all reagents except DNA template
- **Negative control reaction**
  - Controls for specificity of the amplification reaction
  - Contains all reagents and a DNA template lacking the target sequence
- **Positive control reaction**
  - Controls for sensitivity
  - Contains all reagents and a known target-containing DNA template

Experimental design

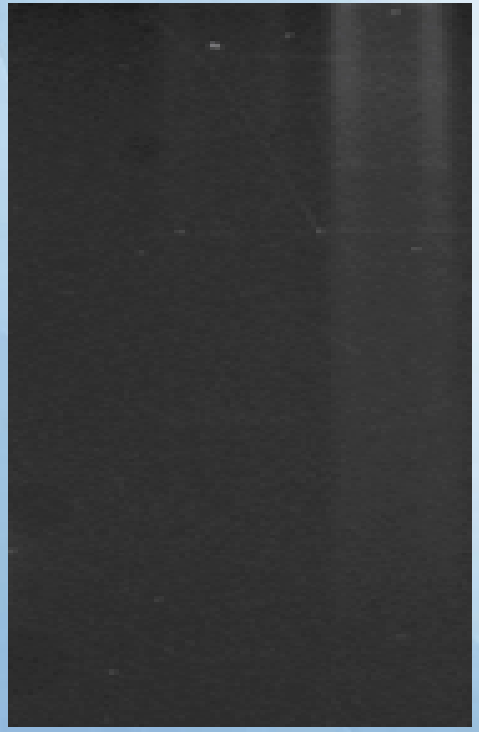


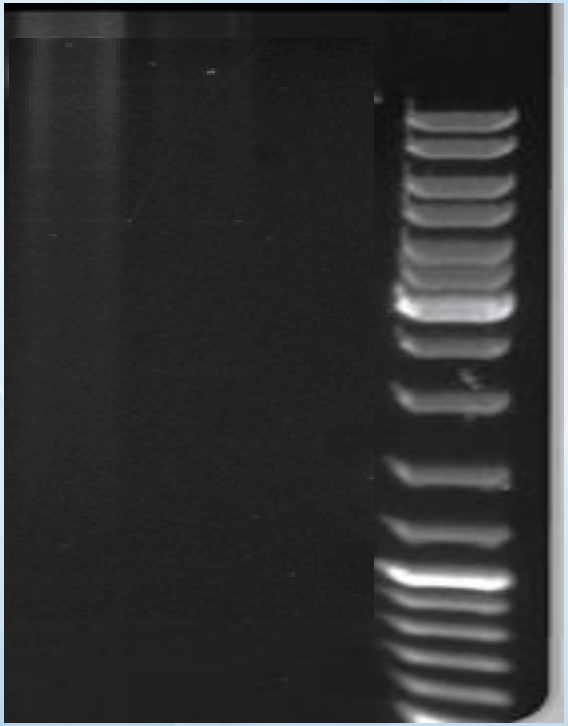
Avoiding Contamination

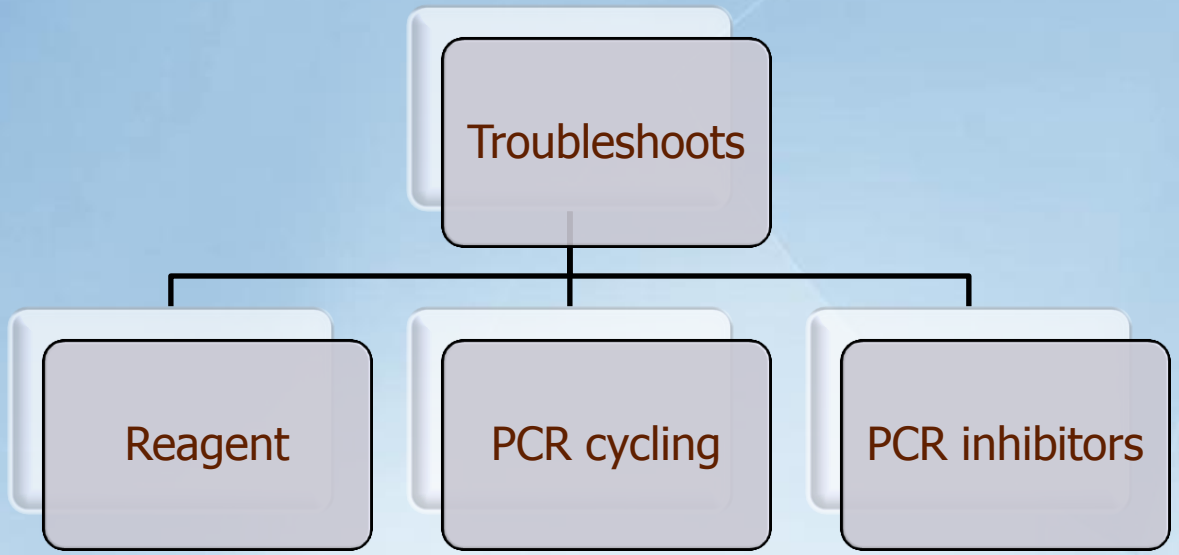
- DNA sample preparation, reaction mixture assemblage should be performed in separate areas.
- A Laminar Flow Cabinet with a UV lamp is recommended for preparing the reaction mixture.
- New gloves should be used for DNA purification.
- The use of tips with filters for both DNA sample and reaction mixture preparation
- Autoclaving of all buffers is recommended.

Troubleshooting PCR

- **No PCR product**
  - **Marker + no product**







Template DNA

Primers

dNTP's

Taq DNA Polymerase

Buffer

MgCl2

Thermal cycler.

Detergent

Phenol

Heparin

Heme

Dyes (bromphenol blue)

Urine

➢DNA Influencing PCR Success

- Tissue type used for DNA extraction
- Quantity and quality of DNA
- Length of the DNA fragment to be amplified

Template Considerations

- **Amount:**

  1µg of 1kb dsDNA = 9.12×10^{11} molecules
  1µg of pGEM® Vector DNA = 2.85×10^{11} molecules
  1µg of lambda DNA = 1.9×10^{10} molecules
  1µg of *E. coli* genomic DNA = 2×10^{8} molecules
  1µg of human genomic DNA = 3.04×10^{5} molecules

- **Quality:**

  Inhibitors of DNA polymerases: salts, guanidine, proteases, organic solvents and SDS, etc.

  "Spiking" Experiment

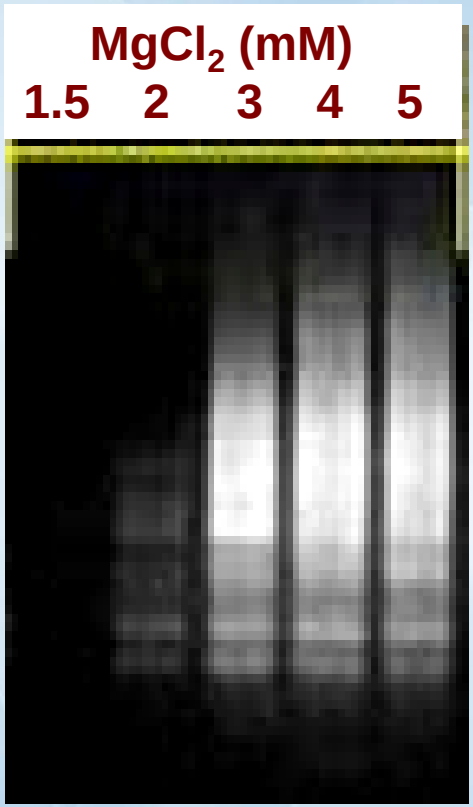
  Ethanol precipitation of the nucleic acid sample

➢ **Template DNA:**

Larger template DNA amounts usually increase the yield of non-specific PCR products.

➢MgCl2 concentration.

- Too few Mg2+ ions result in a low yield of PCR product
- Too many will increase the yield of non-specific products.



- **Taq DNA polymerase.**
  - Higher Taq polymerase concentrations than needed may cause synthesis of non-specific products.

No PCR product---Taq Expiered

dNTPs.

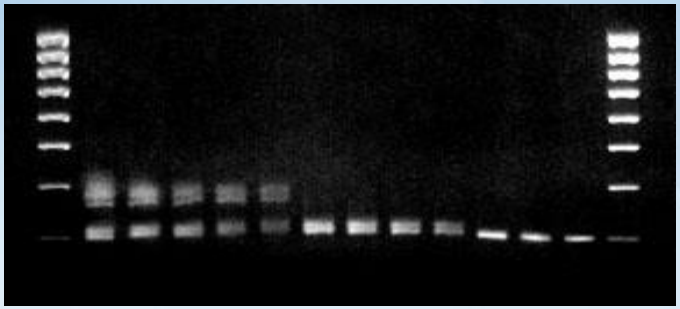
The concentration of 4 dNTPs (dATP, dCTP, dGTP, dTTP) should be equal in the reaction mixture.

Common problem during PCR

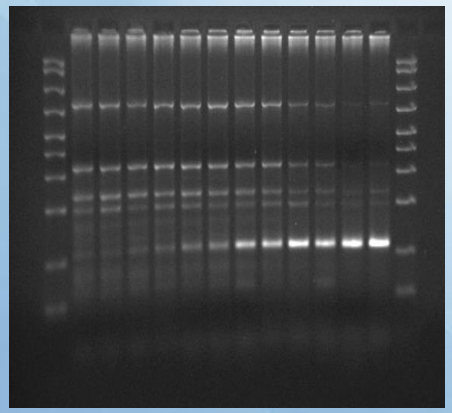
Primers.

- The primer should not be self-complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimers and hairpin formation.

Tm



- **Too many bands** – Low Tm



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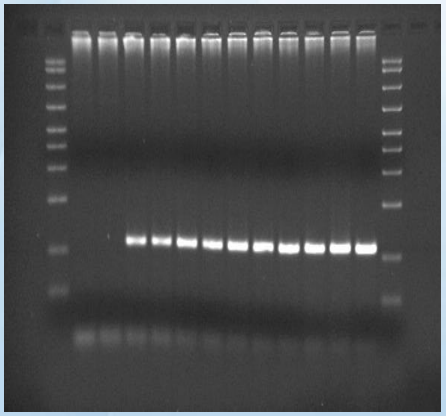
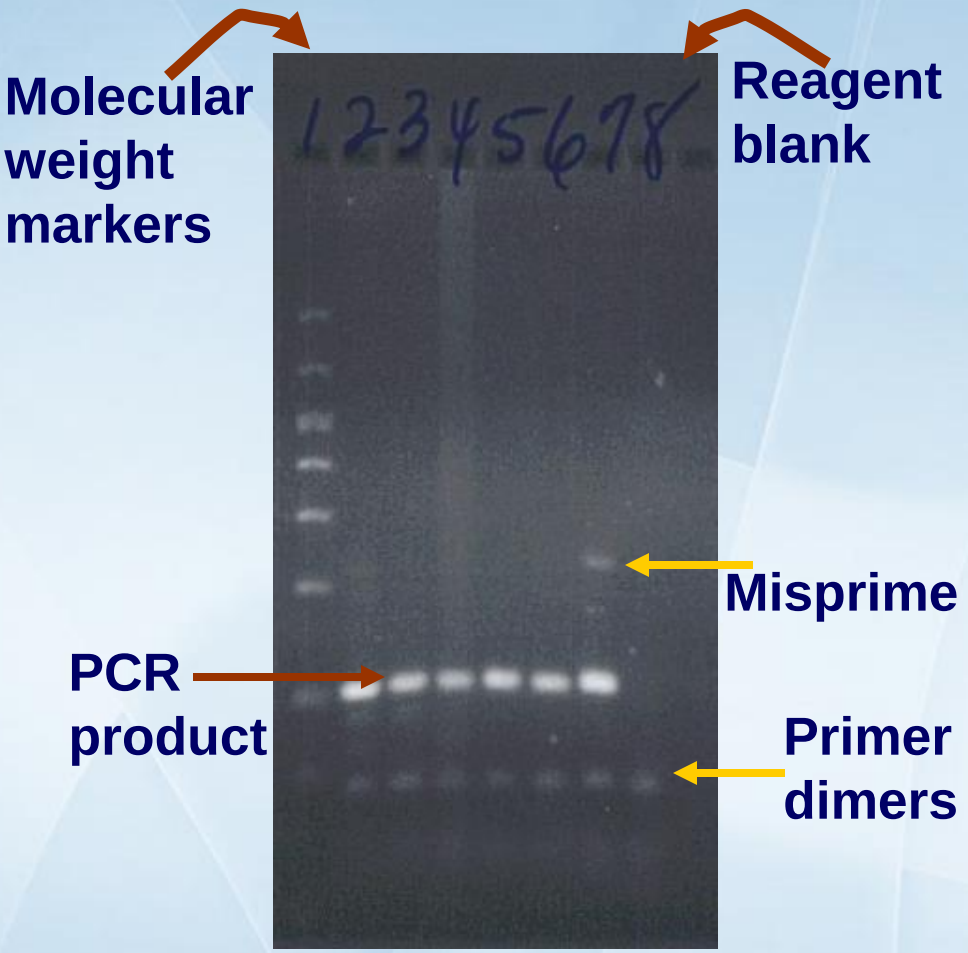


Well optimized

Troubleshooting PCR

- **Primer dimers and misprime:**
  - Annealing temp. too low (dimers) or too high (misprime).
  - excess primers
  - Design primers carefully
  - Size is the sum of two primer lengths.



Cycle parameters

- **Annealing temperature**
  - **starting approximately 5°C below calculated T_m**
- **Extension time**
  - **every 1kb of amplicon: 1 minute (Is it always true?)**
- **Number of cycles**
  - **25-40 cycles**

Yet More Troubleshooting

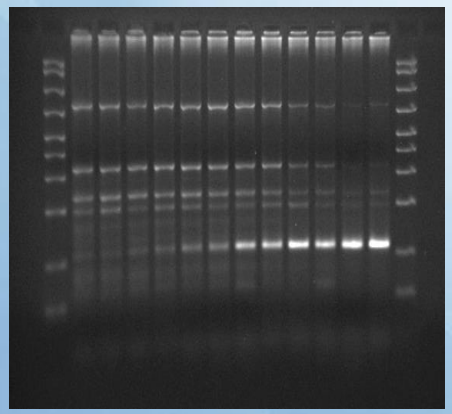
- Thermocyclers can loose calibration over time,
- Some blocks on thermocyclers do not support “fast cycling” protocols
- Older thermocyclers need mineral oil layer over reactions

PCR Inhibitors

- Detergent
- Phenol
- Heparin
- Heme
- Dyes (bromphenol blue)
- Urine
- High concentration of DNA

Too many bands

- Specificity of primers.
- Annealing temp too low.
- Contamination
- Primer Conc.
- Decrease cycles
- Decrease MgCl2 concentration



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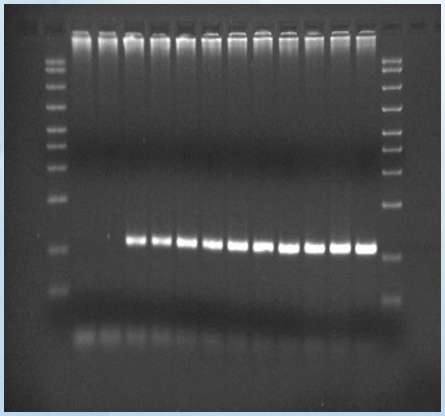


Well optimized

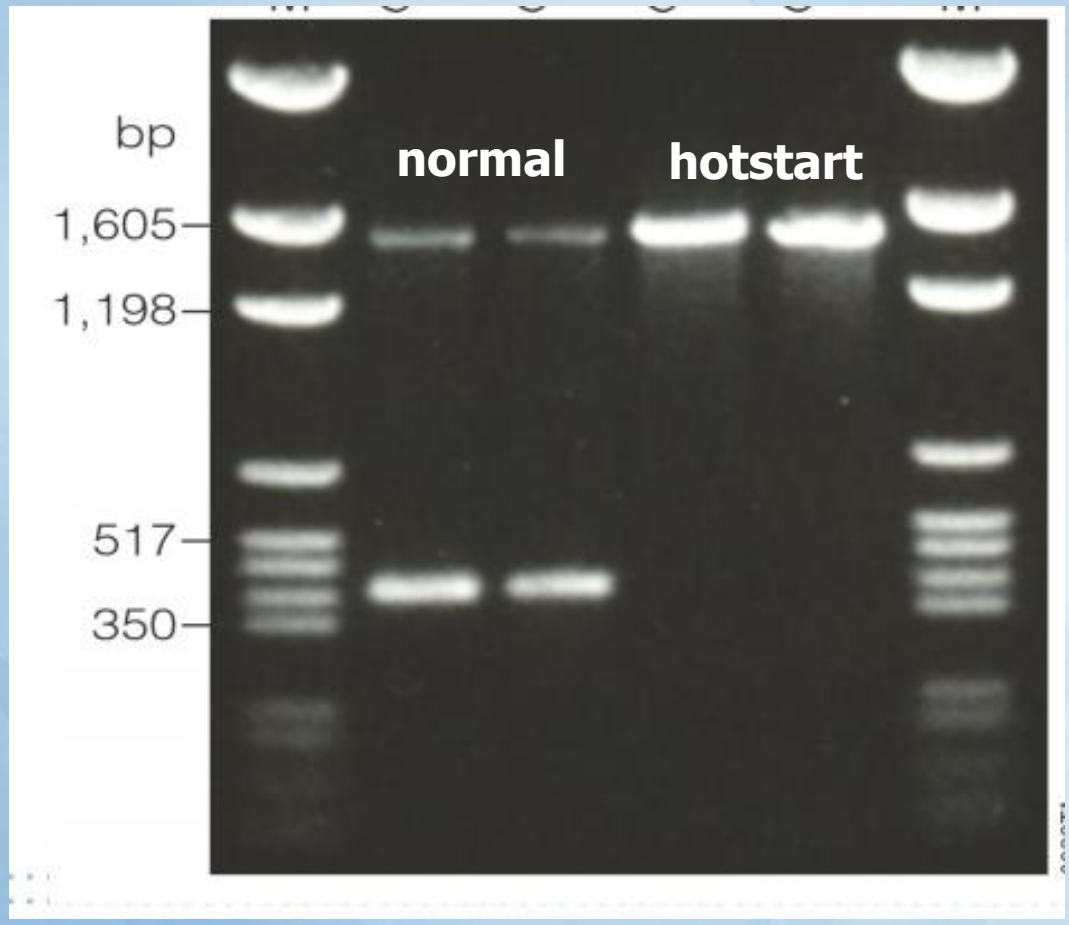
Hot-Start PCR

What is hot-start PCR?

- In hot-start PCR the amplification reaction is rendered inactive until the temperature reaches 60-95°C eliminating or minimizing the binding of the primers to each other or at non-homologous locations in the target DNA.

How?

- Separate reaction components until reactions reach high temperature
- Inactivate components until reactions reach high temperature


bp
1,605
1,198
517
350
normal
hotstart