GENETIC ENGINEERING and MOLECULAR BIOLOGY RESEARCH CENTER

GE&MBR Center

مركز بحوث الهندسة الوراثية والبيولوجيا الجزيئية

Workshop On

Basic Principles of PCR (Level I)

23 - 24/3/2016

PCR Troubleshooting

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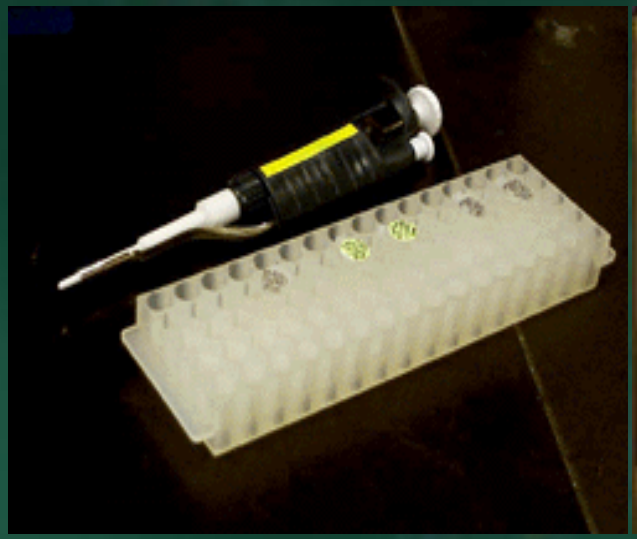
Basic Experimental Design

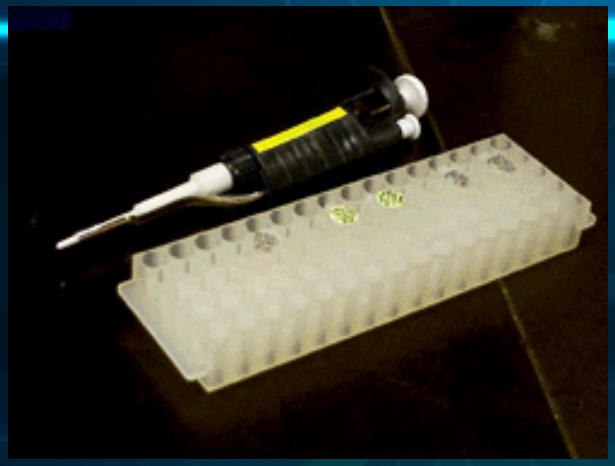
- A well-designed experiment can keep you from getting into trouble!
- A poorly-designed experiment is asking for problems!!!!



The **speed and**
ease of use,
sensitivity,
specificity and
robustness of PCR

has revolutionised molecular biology and made PCR the most widely used and powerful technique with great spectrum of research and diagnostic applications.

PCR Troubleshooting



PCR troubleshooting is a collection of techniques that alter PCR reactions in order to achieve optimum PCR results

- **Main point:**

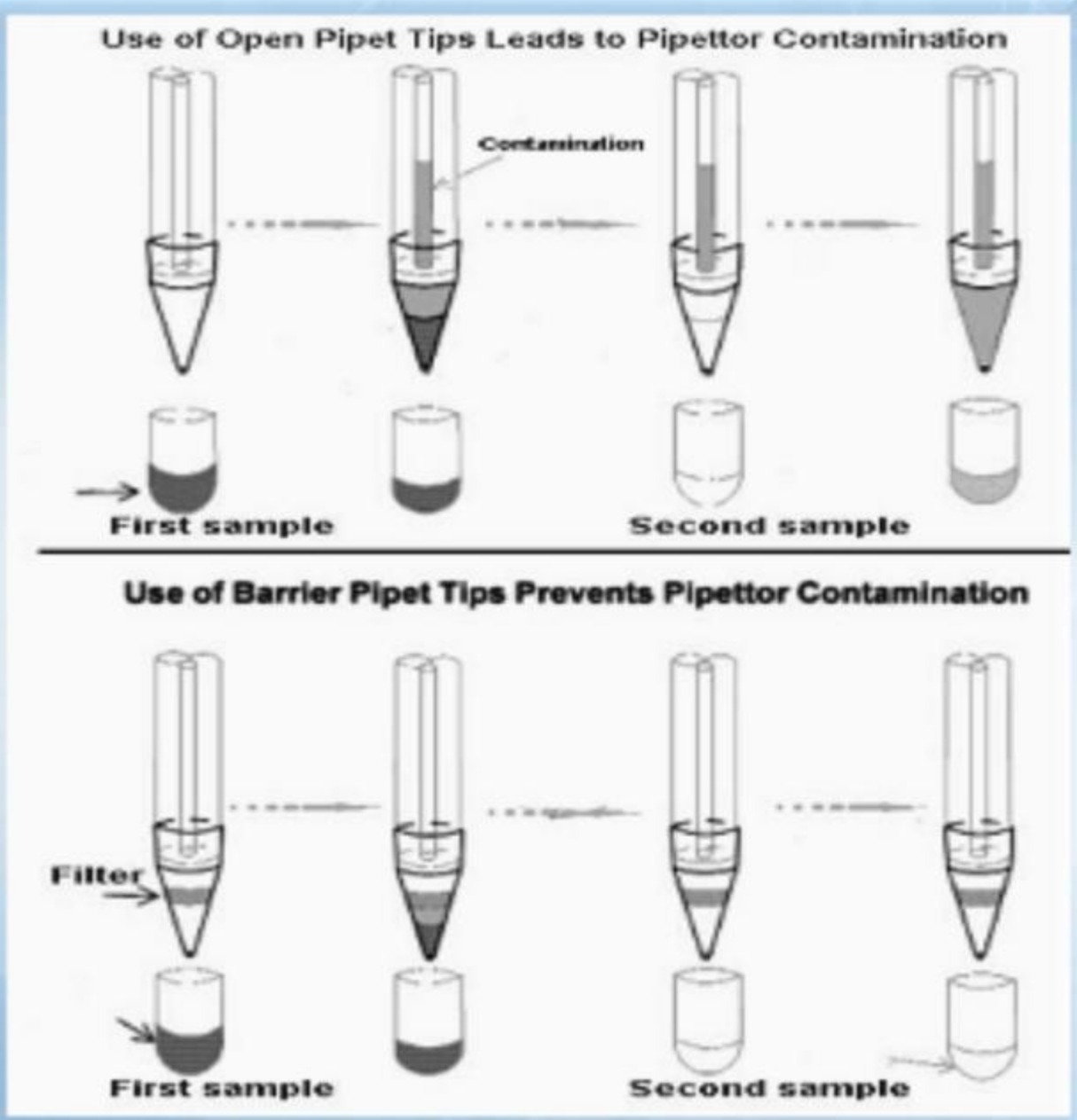
  Always use CONTROLS

- **Positive control**
  - **So you'll know what a successful result looks like.**
- **Negative control**
  - **Lets you know if you have contamination.**

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 and each reaction set-up.

The use of tips with aerosol filters for both DNA sample and reaction mixture preparation, is strongly recommended.



- Autoclaving of all solutions, except dNTPs, primers and *Taq* DNA Polymerase is recommended.

- A control reaction, omitting template DNA, should always be performed, to confirm the absence of contamination.

Factors Influencing PCR Success

- The effect of each component
- PCR Cycling Parameters
- Quantity and quality of DNA
- Primer specificity
- Length of the DNA fragment to be amplified
- Tissue type used for DNA extraction

PCR Reaction Components

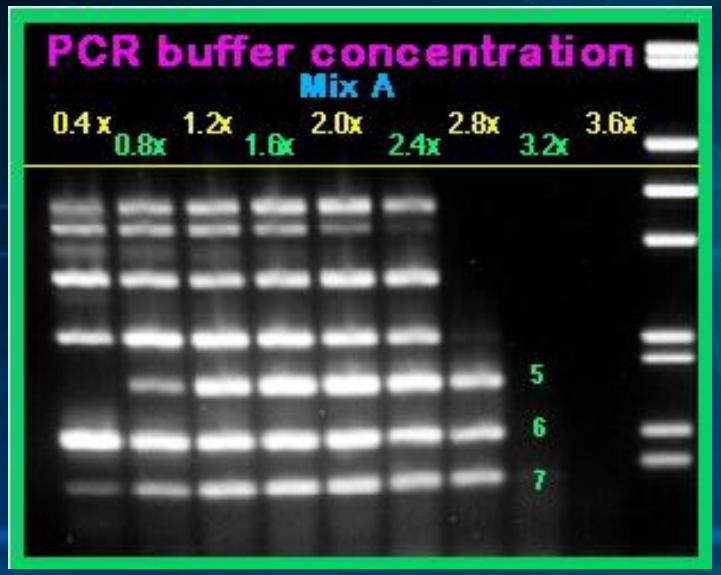
- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg++ ions
- DNA Polymerase

Water

- Purity
- Contamination
  - Amplification Products

Buffer

- Must match polymerase
- Typically contain KCl and Tris
- Can vary over a slight range:
  - Not much difference in range from 0.8 X to 2.0 X
  - Primer efficiency reduced outside this range



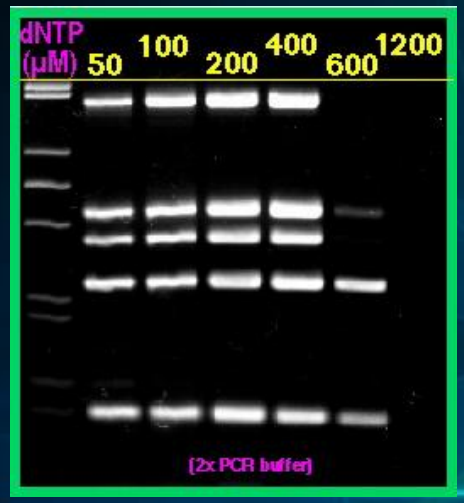
DNA template

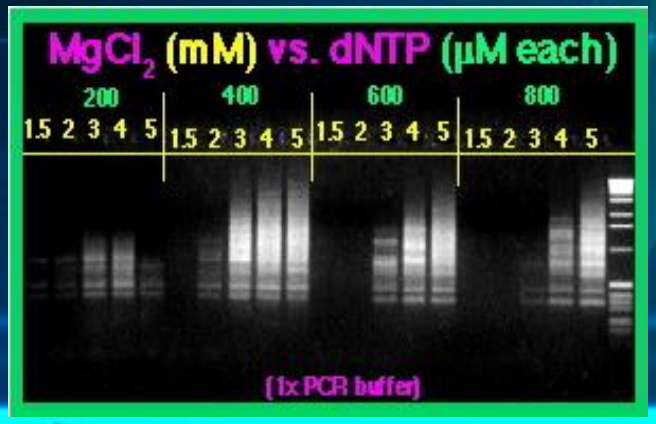
- **Amount of DNA present**
  - Less DNA means more cycles
- **Complexity of DNA**
  - Plasmid vs. whole genome
- **Purity**
  - Interfering factors, enzymes, salts
- **Degradation**
  - PCR more forgiving of degraded DNA
- **Contamination**
  - Amplification products

Primers

- Age
- Number of freeze-thaws
- Contamination
- Amount
  - Can vary over a wide range (50X)
  - 100-500 nM typical
  - Too low: low amplification
  - Too high: low amplification

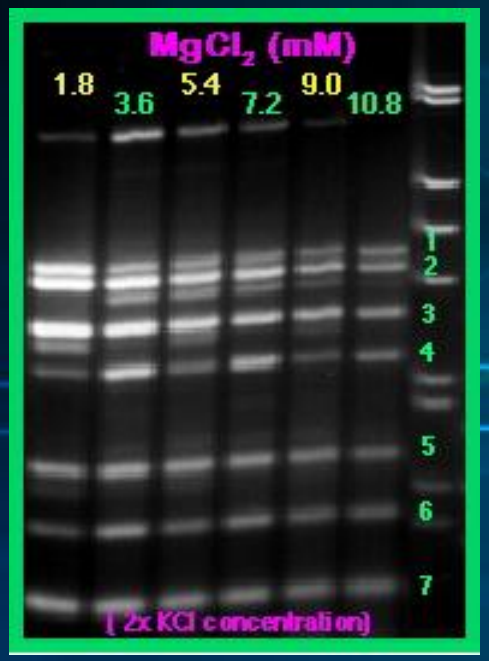
Nucleotides





- 20-400 uM works well
  - Too much: can lead to mispriming and errors
  - Too much: can scavenge Mg++
  - Too low: faint products
- Age
- Number of freeze-thaws
  - Just 3-5 cycles is enough to make PCRs not work well
- Dilute in buffer (eg. 10mM Tris pH 8.0 to prevent acid hydrolysis)
- Contamination

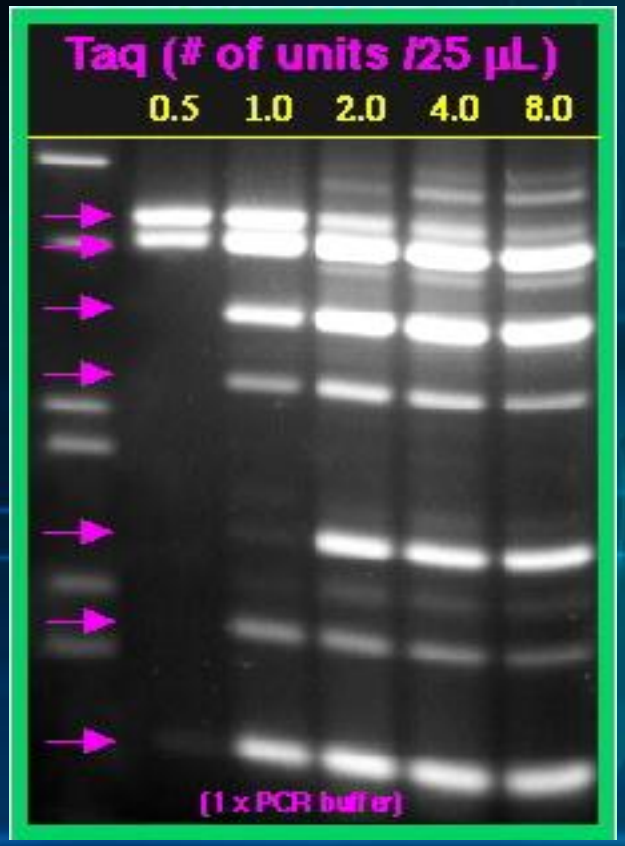
Mg++ ions



- Mg is an essential cofactor of DNA polymerase
- Amount can vary
  - 0.5 to 3.5 uM suggested
  - Too low: Taq won't work
  - Too high: mispriming

- **Bottom Line:**
  - **All components work over a wide range.**
  - **Need to avoid contamination.**
  - **Optimization by trial-and-error.**

DNA Polymerase



- Thermostable?
  - Activity declines with time at 95C
- Matches buffer?
- Age
- Contamination
- Concentration: Typically 0.5 to 1.0 U/rxn

PCR Cycling Parameters

- Denaturation Temp
- Annealing Temp
- Extension Temp
- Time
- Number of Cycles
- Reaction Volume
- “Odd” Protocols

Denaturation Step

Must balance DNA denaturation with Taq damage

95C for 30 - 60s typically is enough to denature DNA

Even 92C for 1s can be enough

Taq loses activity at high temps:

- Half-life at 95C: 40 min
- Half-life at 97.5C: 5 min

Annealing Step

- Most critical step
- Calculate based on Tm
  - Often does not give expected results
- Trial-and-Error
  - Almost always must be done any way
  - Too hot: no products
  - Too cool: non-specific products
- Gradient thermo cyclers very useful
- Typically only 20s needed for primers to anneal

Extension Step

- Temperature typically 72C
  - Reaction will also work well at 65C or other temps
- Time (in minutes) roughly equal to size of the largest product in kb
  - Polymerase runs at 60bp/s under optimum conditions
- Final “long” extension step mostly unnecessary

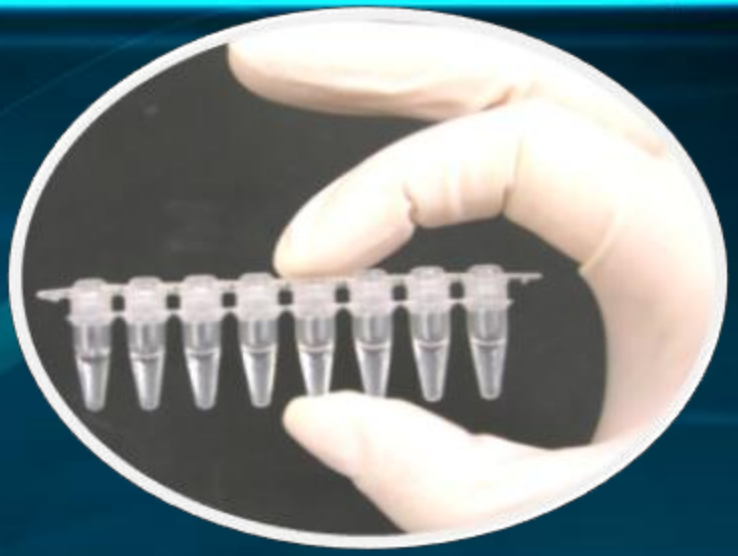
Number of Cycles

- Number of source molecules:
  - >100,000: 25-30
  - >10,000: 30-35
  - >1,000: 35-40
  - <50: 20-30 fb. nested PCR
- Do not run more than 40
  - Virtually no gain
  - Extremely high chance of non-specific products
- Best optimized by trial-and-error

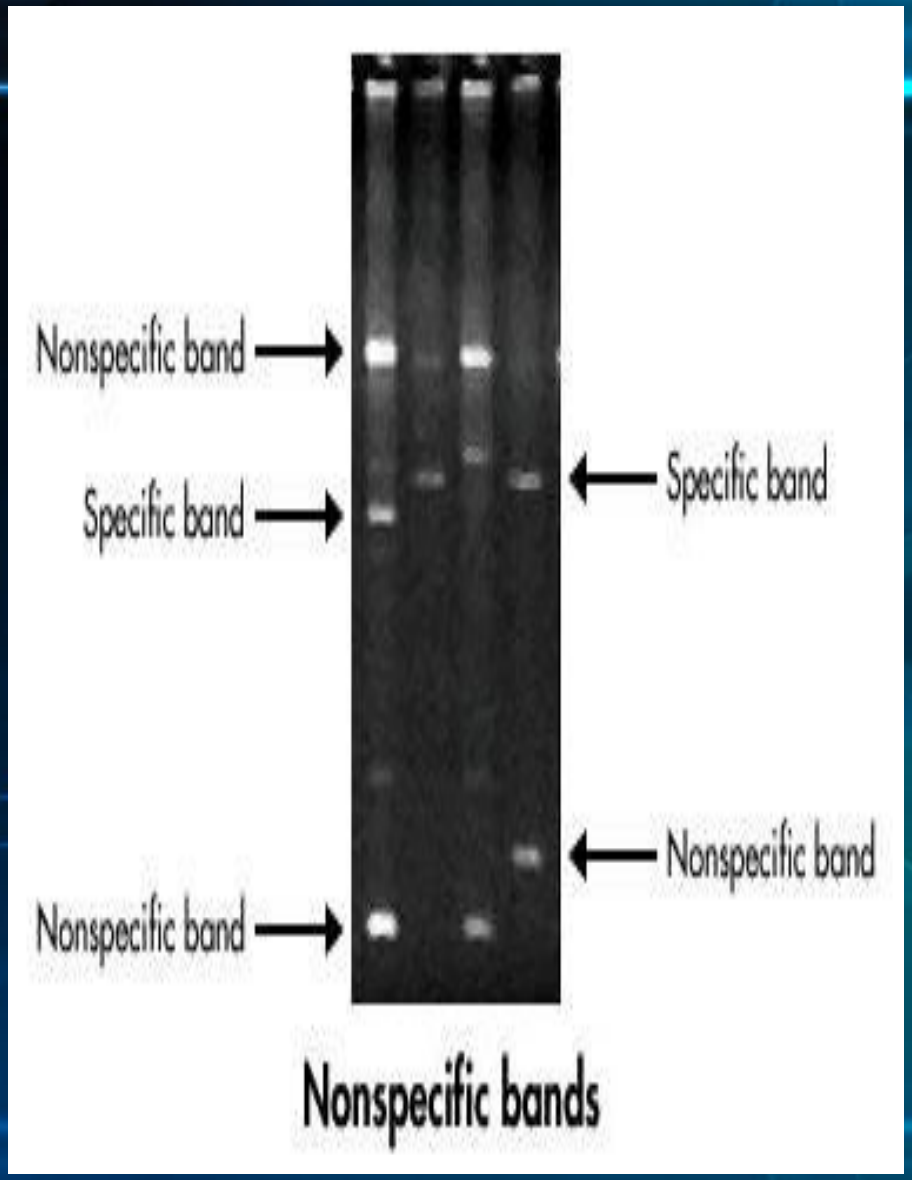
Reaction Volume

- Doesn’t affect PCR results as long as volume is within limits.
- Heated lid important.
- 5ul, 20ul, 100ul all work.
- Slightly higher yield with lower volumes.

- Using right plastic?
- Using right seals?
- Heated lid enabled?

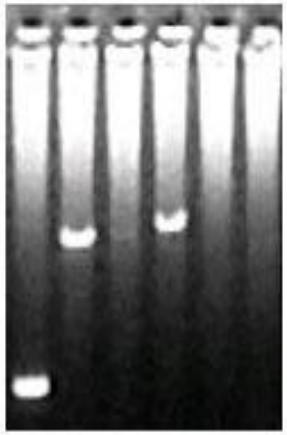


- Right protocol entered?
- Is power reliable?
- Proper reaction volume?



Smeared Bands

Low molecular weight smears



High molecular weight smears

Problem	Possible Causes	Actions
No Amplicon	Error in set up	Repeat the experiment, checking all reagents are added in correct volumes. Use master mix to ensure all components added correctly.
	Error in cycling	Check program is correct on thermal cycler and that cycling starts and finishes correctly
	Error in gel analysis	Check wells on gel loaded correctly, correct loading buffer was added to samples, EtBr is added to gel and UV settings are correct
	Incorrect annealing temperature	Run a temperature gradient in 2°C increments
	Incorrect $MgCl_2$ concentration	Run a $MgCl_2$ gradient of 0.5mM increments between 1.5 and 4.0mM
	Insufficient template	Increase template concentration
	Primer dimers	Increase temperature and/or decrease $MgCl_2$. Check self complementarity of primers on primer design software. Redesign primers.
	Primer design error	Blast primers. Check primer parameters on primer design software. Redesign primers
	DNA not clean or contains inhibitors	Check template is clean. Check all ethanol was evaporated from DNA extractions. If inhibitors are present diluting DNA can improve the reaction.
	Secondary structure in template	Use touchdown PCR, add adjuvant such as DMSO, BSA or Betaine or use a hot-start *Taq* DNA polymerase

Low Yield	Annealing temperature not optimal	Run a temperature gradient in 2°C increments
	$MgCl_2$ concentration not optimal	Run a $MgCl_2$ gradient of 0.5mM increments between 1.5 and 4.0mM
	Buffer not optimal	Use a NH_4 based buffer instead of KCl based buffer for greater yield
	Insufficient template	Increase template concentration
	Insufficient primers	Increase primer concentration
	Insufficient cycles	Increase amount of cycles
	Secondary structure in template	Use touchdown PCR, add adjuvant such as DMSO, BSA or Betaine, or use a hot-start *Taq* DNA polymerase
	GC-rich template	Add adjuvant such as DMSO, BSA or Betaine, or use Thermo-Start DNA Polymerase with High Performance Buffer.
	Extension time too short	For long products (>2kb), extension time (in mins) should be approximately equal to the number of kb in the amplicon.
	Long denaturation inactivating enzyme	Only use a 2 minute denaturation time for polymerases which do not require a hot-start.
	DNA not clean or contains inhibitors	Check template is clean. Check all ethanol was evaporated from DNA extractions. If inhibitors are present diluting DNA can improve the reaction.
	Sample evaporating during thermal cycling	Check levels in wells after cycling. Ensure screw-down lid is pressing firmly on plate. Use high quality adhesive seals and rigid PCR plates.

Non-Specific Amplification – Multiple Products	Priming starting during set up	Set up reaction on ice or use a hot-start *Taq* DNA polymerase
	Annealing temperature not optimal	Run a temperature gradient in 2°C increments
	$MgCl_2$ concentration not optimal	Run a $MgCl_2$ gradient of 0.5mM increments between 1.5 and 4.0mM
	Buffer not optimal	Use a KCl based buffer instead of a NH_4 based buffer for greater specificity
	Primers not specific	Blast primers to check specificity. Redesign primers.
	Overabundance of primer	Decrease primer concentration
	Overabundance of template	Decrease template concentration
	Annealing time too long	Decrease time of annealing step
	Contamination	Check no template control (NTC) for bands

Non-Specific Amplification – Smeared Product	Priming starting during set up	Set up reaction on ice or use a hot-start *Taq* DNA polymerase
	Annealing temperature not optimal	Run a temperature gradient in 2°C increments
	$MgCl_2$ concentration not optimal	Run a $MgCl_2$ gradient of 0.5mM increments between 1.5 and 4.0mM
	Buffer not optimal	Use a KCl based buffer instead of a NH_4 based buffer for greater specificity
	Primers not specific	Blast primers to check specificity. Redesign primers.
	Overabundance of primer	Decrease primer concentration
	Overabundance of template	Decrease template concentration
	Annealing time too long	Decrease time of annealing step
	Template degraded	Minimize freeze thawing of DNA. Run template on agarose gel to check integrity.
	Extension time too short	For long products (>2kb), extension time (in mins) should be approximately equal to the number of kb in the amplicon.
Band in No Template Control (NTC) - Contamination	Contaminated reagents	Use a fresh aliquot of reagents
	Pipettes contaminated	Clean and sterilize pipettes. Use filter tips. Use different pipettes for pre- and post-PCR.
	Work area contaminated	Clean work bench or move areas. Use a different area for pre- and post-PCR.
	Aerosol contamination	Use a master mix to minimize pipetting steps, use filter tips, close lids on all tubes and expel reagents carefully. Change gloves regularly.

Wrong Size Band Amplified	Contamination	Check no template control for bands
	Wrong primers or template added	Check primers and template vials have been labeled correctly and selected correctly during set up.
	Different gene form	Check gene for isoforms or splice variants.
Reaction Not Reproducible or Reaction Stopped Working	Different cycling conditions	Use the same thermal cycler for optimization and all future experiments. Different cyclers can vary in ramping speeds and temperature.
	dNTPs degraded	dNTPs are very susceptible to freeze thawing. Replace with a fresh aliquot.
	Error in set up	Repeat - checking correct reagents added and correct thermal cycler program used.
	Change in component	Check any new components that have been added (eg. new batch of primers)
	Inhibitors in template	Decrease template concentration, dilute template or clean template.

Common PCR inhibitors

SDS (denature polymerase. 0.01% SDS cuts *Taq* activity to ~10% of normal)

Ca (calcium ions inhibit PCR by competing with the magnesium ions as a cofactor for the DNA polymerase).

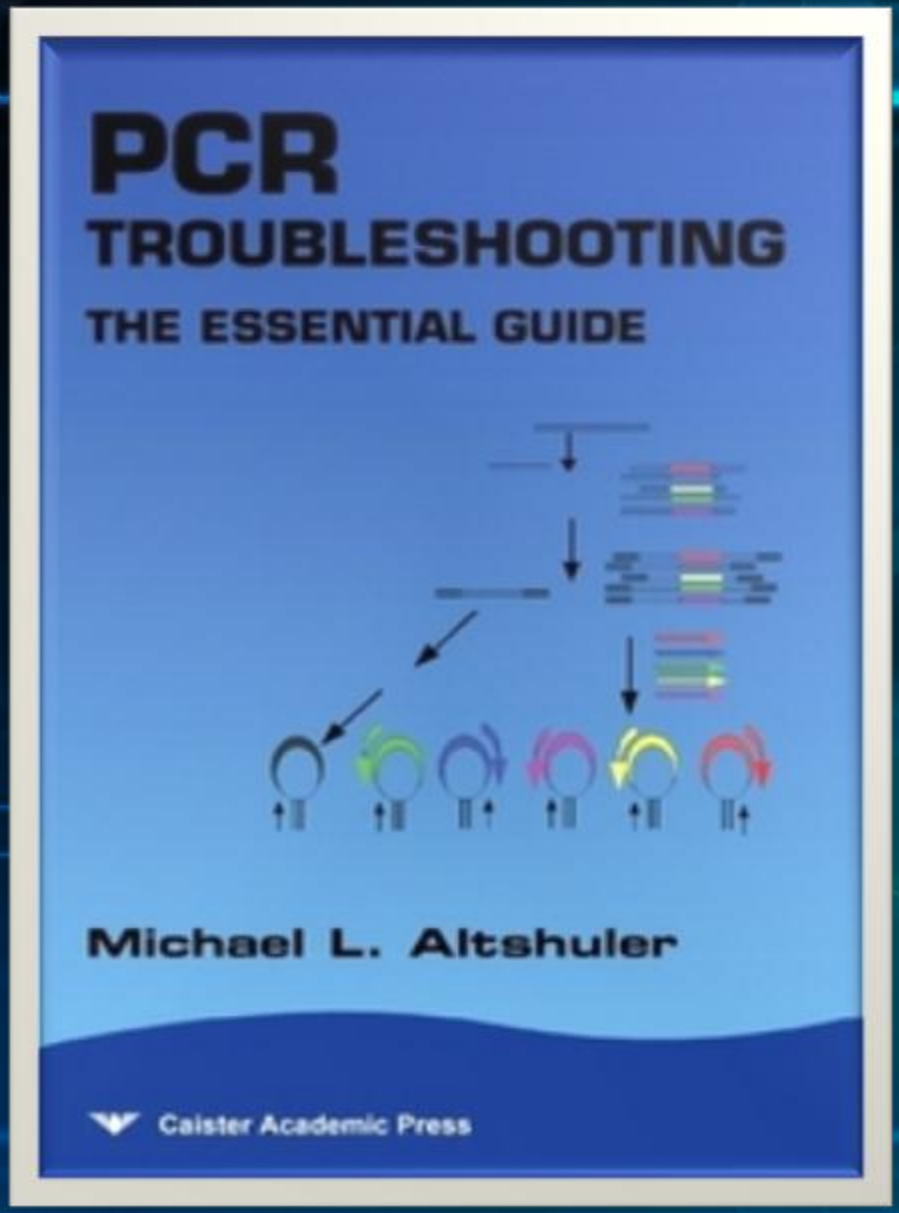
EDTA (chelating the Mg2+ necessary for the activity of DNA polymerase. Therefore, use of an increased magnesium ion concentration has been employed to maintain PCR activity in the presence of chelating agents.)

Blood (PCR inhibitors originating from the starting material include heparin (>0.15mg/mL), proteins such as hemoglobin and lactoferin (>1mg/mL), immunoglobulin

polysaccharides, chlorophylls, melanin, humic acids, etc. Contaminants from the nucleic acid extraction phase include SDS (>0.01% w/v), phenol (>0.2% w/v), ethanol (>1%), proteinase K, guanidinium, and sodium acetate (>5mM).

BSA, provides some resistance to inhibitors

Diluting your sample can solve the problem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