

# PCR amplification assays:RAPD

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## Introduction

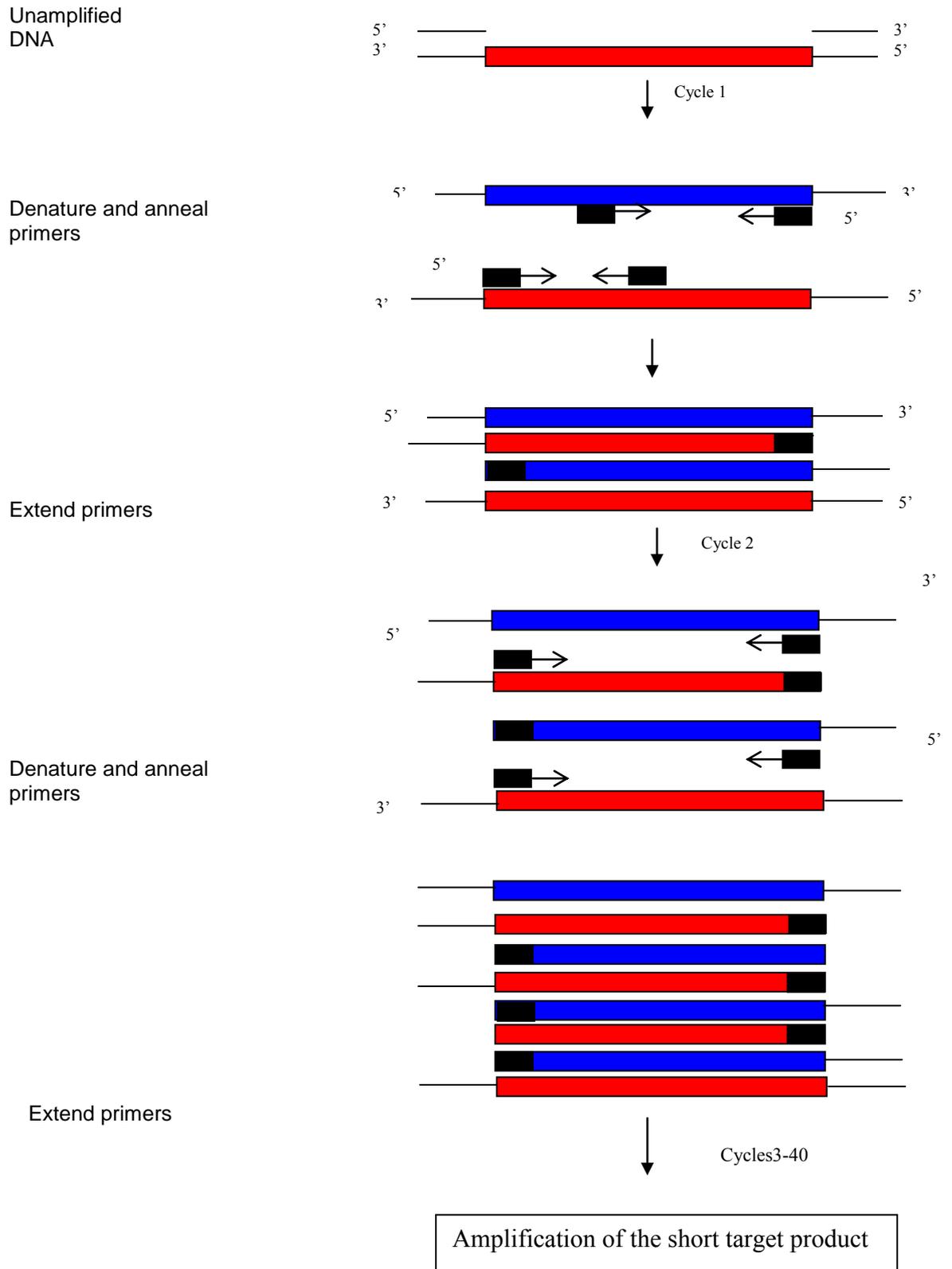
The quality of DNA, obtained by various protocols and subjected to various storage conditions can be judged by comparing the PCR amplification profiles. The most commonly used technique that can be accessed by almost any laboratory is RAPD .

Random amplified polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation. It involves the use of a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. The technique was developed independently by two different laboratories (Williams et. al., 1990; Welsh and McClelland, 1990) and called as RAPD and AP-PCR (Arbitrary primed PCR) respectively. This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular RAPD band. Such polymorphisms thus behave as dominant genetic markers.

## Principle

The RAPD technique is based on the polymerase chain reaction (PCR). A target DNA sequence is exponentially amplified with the help of arbitrary primers, a thermostable DNA polymerase, dideoxy nucleotide tri - phosphates, magnesium and reaction buffer. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step (fig 1). In the first step the DNA is made single stranded by raising the temperature to 94°C (denaturation). In the second step, lowering of the temperature to about 40 to 65°C results in annealing of the primer to their target sequences on the template DNA (annealing step). In the third cycle, temperature is chosen where the activity of the thermostable Taq DNA polymerase is optimal, i.e., usually 72<sup>0</sup> C.





**Figure 1. Schematic diagram of the PCR process**

The polymerase now extends the 3' ends of the DNA-primer hybrids towards the other primer binding site. Since this happens at both primer-annealing sites on both the DNA strands, the target fragment is completely replicated. Repeating these three step cycles 40 to 50 times results in the exponential amplification of the target between the 5' ends of the two primer binding sites. Amplification products are separated by gel electrophoresis and visualized by ethidium bromide staining.

The most crucial factors that need to be optimized in a RAPD reaction are the magnesium concentration, enzyme concentration, DNA concentration and annealing temperature of the primer. The G+C content of the primers should be 40-60% and care should be taken to avoid sequences that produce internal secondary structures. The annealing temperature for a PCR cycle is generally 3-5° below the melting temperature (T<sub>m</sub>) of the primer. The T<sub>m</sub> is calculated as

$$T_m = 81.5 + 16.6 \cdot (\log_{10}[\text{Na}^+]) + 0.41 \cdot (\%G+C) - 675/n$$

Where [Na<sup>+</sup>] is the molar salt concentration ; [K<sup>+</sup>] = [Na<sup>+</sup>]

and n = number of base in the oligonucleotide

As a thumb rule the following formula can be used

$$T_m = 2(A+T) + 4(G+C)$$

Other useful formulae are

Nanogram of primer = picomole of primer x 0.325 x # bases

MicroMolar concentration of primer = picomoles of primer/ volume (μL) in which the primer is dissolved.

## Material and reagents

Instruments:

PCR machine (Perkin Elmer 9600), microcentrifuge, 100V power supply, Gel electrophoresis tank, gel mould and slot former, UV transilluminator, Camera, autopipettes, vortex.

Reagents:

1. *Taq* DNA polymerase
2. Genomic DNA (5ng/μl)
3. dNTP mix (2mM each of dATP, dCTP, dGTP and dTTP)
4. MgCl<sub>2</sub> (25mM)
5. Buffer for DNA polymerase
6. 10-mer oligonucleotide primers (5μM)
7. Sterile distilled water
8. Electrophoresis grade agarose
9. 0.5X TBE buffer
10. Ethidium bromide solution (10mg/ml)

11. DNA length marker
12. Loading buffer

Miscellaneous: Thin walled PCR tubes, tips, tissue paper.

### Protocol

1. Each 10 $\mu$ l of reaction mix contains

Component	Volume	Final Concentration
Genomic DNA	3.0 $\mu$ l	15ng
Buffer	1.0 $\mu$ l	1X
dNTPs	1.0 $\mu$ l	0.2mM
Primer	0.6 $\mu$ l	0.6 $\mu$ M
Taq DNA pol	0.2 $\mu$ l	1 unit
MgCl <sub>2</sub>	1.0 $\mu$ l	2.5mM
Water		to 10 $\mu$ l

Prepare a mastermix (for all samples + control) that contains all the above components except the DNA.

- Thaw all components completely
- Vortex the MgCl<sub>2</sub> vigorously
- Vortex the mastermix to mix all components before aliquoting

2. Aliquot into PCR tubes and add the template DNA. Mix well.
3. Place the PCR plate carrying the reaction tubes in the sample block of the thermocycler.
4. Carry out an initial denaturation step at 94°C for 4min followed by 40 cycles with the following cycle parameters:
  - Step 1        94°C for 1min
  - Step 2        35°C for 1min
  - Step 3    72°C for 2min
 Extend the 72°C step of the final cycle by 5min
5. When the amplification has finished, add 3 $\mu$ l of the loading dye to each sample.
6. Prepare a 1.2% agarose gel in 0.5X TBE buffer containing ethidium bromide (5 $\mu$ g/ml of gel). Load the DNA length marker and the samples. Run the gel in 0.5 x TBE buffer at 55V for 4 h.
7. Visualize the gel on a UV transilluminator. If required the gel can be photographed using Polaroid 665 or 667 film and analysed further.

### Trouble shooting

Problems	Suggested solutions
Erratic or no amplification	<ul style="list-style-type: none"> <li>-A component is missing from the reaction; repeat the reaction</li> <li>-Inhibitors of PCR may have co-purified with the DNA; Vary DNA concentration, include a clean up step during DNA isolation</li> <li>-Make fresh DNA dilutions</li> <li>-Make new primer stock</li> <li>-Use a different primer</li> <li>-Increase concentration of <i>Taq</i> polymerase per reaction</li> </ul>
Poor amplification resulting in bands being fuzzy/ indistinct	<ul style="list-style-type: none"> <li>-Replace <i>Taq</i> polymerase buffer</li> <li>-Check the primer; ensure primer is properly stored</li> <li>-Use another primer; all primers may not give amplification products</li> </ul>

	<ul style="list-style-type: none"> <li>-Check activity of <i>Taq</i> polymerase by comparing between different batches</li> <li>-Vary DNA concentration</li> </ul>
Single, monomorphic intense band in all samples and in the control	Product may be a primer artifact. Decrease primer concentration
High molecular weight smears > 4 kb	<ul style="list-style-type: none"> <li>-Decrease DNA concentration</li> <li>-Decrease <i>Taq</i> polymerase concentration</li> <li>-Ensure gels are made with the correct buffer</li> <li>-Conduct electrophoresis at lower voltage</li> </ul>
Gel has strong background after staining	<ul style="list-style-type: none"> <li>-Decrease staining time</li> <li>-Destain gel for longer</li> <li>-Too much <i>Taq</i> or DNA in the PCR reactions</li> </ul>
Inadequate separation of low molecular weight products	-Separate products on higher concentrations of agarose gels, specialist agaroses or polyacrylamide gels

## References

1. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* **18**: 6531-6535.
2. Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**: 7213-7218.
3. Cateno-Anolles G, Bassam BJ, Gresshoff PM (1991) High resolution DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology* **9**: 553-557