PCR Optimisation

IMBB 2014
PCR

• PCR has become a widespread research technique.
• Popularity of PCR is primarily due to its apparent simplicity and high probability of success.
• However, PCR is a relatively complicated and, as yet, incompletely understood biochemical brew
  – constantly changing kinetic interactions among the several components determine the quality of the products obtained.
### Basic conditions: ideal starting conditions when trying a new PCR

Set up PCR in a thin-walled 0.2 ml PCR tube. Prepare a Master Mix to avoid pipetting errors.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity, for 25 µl PCR</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>To final volume of 25 µl</td>
<td>10X</td>
</tr>
<tr>
<td>10X Taq buffer</td>
<td>2.5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.5</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>20 mM dNTP mix</td>
<td>0.25 µl</td>
<td>200 µM of each</td>
</tr>
<tr>
<td>Primer F (2 µM)</td>
<td>3.125 µl</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>Primer R (2 µM)</td>
<td>3.125 µl</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5 u/µl)</td>
<td>0.125 µl</td>
<td>0.625 units/25 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>variable</td>
<td>10 pg-0.5 µg/25 µl</td>
</tr>
</tbody>
</table>
Basic conditions: ideal starting cycling parameters when trying a new PCR

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial</strong></td>
<td><strong>Denaturation</strong></td>
<td><strong>95°C</strong></td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td><strong>95°C</strong></td>
<td><strong>30 sec</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
<td><strong>Tm -5°C</strong></td>
<td><strong>30 sec</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Extension</strong></td>
<td><strong>72°C</strong></td>
<td><strong>30 sec</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Final Extension</strong></td>
<td><strong>72°C</strong></td>
<td><strong>7 min</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Can also try 94°C

1 Normally between 1 and 5 min, but 2 min is a good starting point.
2 Optimal Ta should be determined empirically by testing Tm+/- 5°C in ~1-2°C steps, but Tm minus 5°C is a good starting Ta. If your PCR primers have different Tm, then first try the lowest Tm minus 5°C.
3 Number of cycles is determined empirically, but is normally between 30-40 cycles. Initially try 30 or 35 cycles.
4 30 sec is suitable for a product up to 500 bp. From 500-1000 bp product use 1 min. For each additional 500 bp add 30 sec.
Cycling Parameters

• Primer sequence is a major factor that determines the optimal Ta (annealing temperature), which is often within 5°C of the melting temperature of the primers

• Most commonly altered cycling parameters
  – annealing temperature
  – number of cycles
  – extension time
Annealing temperature (Ta)

- In many cases, non-specific amplification and primer-dimer formation can be reduced through optimization of Ta.
- Using a Ta slightly higher than the primer $T_m$ can minimize non-specific primer annealing and decrease the amount of non-specific products synthesized.
- Using a Ta lower than the primer $T_m$ can result in higher yields, as the primers anneal more efficiently at the lower temperature. However, non-specific products can become a problem.
- Recommend testing several Ta in 1°C to 3°C steps, starting 5°C below the lowest $T_m$ of the primer pair, to determine the best annealing conditions.
Extension time

• Standard Taq pol extends at ~1 kb/minute
• Decreasing extension time can help to reduce high molecular weight non-specific products.
  – Example: reducing extension time of a 400 bp reaction from 2 minutes to 30 seconds could help to remove a smear or high molecular weight products.
Other cycling parameters

• Cycle number
  • Increasing the cycle number can increase yield for low efficiency assays or low copy targets.
  • Optimizing the PCR conditions by changing other parameters is preferable i.e. annealing temperatures, Mg, redesigning primers, changing DNA extraction protocol etc
Magnesium concentration

• Magnesium is a required cofactor for thermostable DNA polymerases

• Magnesium concentration is a crucial factor that can affect amplification success

• Free Mg\(^{2+}\) is affected by
  – template DNA concentration
  – chelating agents present in the sample (e.g., EDTA)
  – dNTP concentration
  – presence of proteins
Magnesium concentration

• In the absence of adequate free magnesium, *Taq* DNA polymerase is inactive

• Excess free magnesium
  – reduces enzyme fidelity (i.e. it may incorporate wrong bases)
  – may increase the level of non-specific amplification
  – may inhibit PCR amplification

• For these reasons, researchers should empirically determine the optimal magnesium concentration for each PCR.
Magnesium concentration

• Many thermostable DNA polymerases are supplied with a magnesium-free reaction buffer and a tube of 25mM MgCl$_2$ so that you can adjust the Mg$^{2+}$ concentration to the optimal level for each reaction.

• Set up a series of PCRs containing 0.5–5.0mM Mg$^{2+}$ in 0.5-1mM increments

• Determine which magnesium concentration gives
  – highest yield of product
  – minimal amount of nonspecific product
MgCl$_2$ titration (mM)

Meisa1 PCR with cassava gDNA
MgCl$_2$ titration (mM)

Group 2: Twiga

Ethanol fixed animal muscle samples $\rightarrow$ PureLink extraction of gDNA $\rightarrow$ CO1 PCR with individual components, DreamTaq Pol and Mg titration $\rightarrow$ 1.8% agarose gel