



Primer design for PCR and Sanger sequencing

How to design primers that give a single sharp band and clean Sanger reads: target choice, design tools, key parameters, dimer checks and troubleshooting.

A well-designed primer gives one sharp band on the gel and a clean Sanger read. A poor primer gives extra bands, no product or mixed peaks.

1. Choose the target region

- Start from genomic DNA, a plasmid or the gene of interest, and know the exact region to amplify (for example a gene, 16S rRNA or a plasmid insert).
- Use the sequence in FASTA format in the design tools.

2. Use a design tool

- **NCBI Primer-BLAST** (design plus specificity check): ncbi.nlm.nih.gov/tools/primer-blast
- **Primer3** (flexible, many options): primer3.ut.ee

3. Recommended parameters

Parameter	Recommended	Why
Primer length	18–24 nt	Specific, yet binds efficiently
Melting temperature (T _m)	55–65 °C, ideally 58–60 °C; pair within 2 °C	Stable annealing
GC content	40–60%	Too high binds too strongly; too low too weakly
GC clamp	1–2 G or C at the 3' end	Firm start for the polymerase
Amplicon size	100–1,000 bp; best 300–800 bp for sequencing	Clear on the gel, fully readable by Sanger
3' complementarity	None between or within primers	Prevents primer-dimers
Repeats	No runs of more than 4 identical bases	Prevents slipping and hairpins

4. Check dimers and hairpins

Paste both primers into a tool such as IDT OligoAnalyzer. Aim for hairpin ΔG above -2 kcal/mol and self- or cross-dimer ΔG above -5 kcal/mol.

5. Check specificity

BLAST the primer sequences to make sure they do not bind elsewhere, especially in complex genomes or mixed samples.

6. Add tags only if needed

For cloning, add restriction sites or adaptors; for sequencing, universal sites such as M13 or T7. Make sure overhangs do not bind inside the amplicon.

PCR and gel tips

Tip	Effect
Use good-quality template DNA	Degraded DNA gives weak or no bands
Start annealing at $T_m - 3\text{ }^{\circ}\text{C}$, then optimise by gradient PCR	Specific, efficient amplification
Use a hot-start polymerase	Fewer non-specific products
Adjust Mg^{2+}	Too much: extra bands; too little: no band
Use 2% agarose for short amplicons	Sharper separation

Troubleshooting

Problem	Likely cause	Solution
Several bands	Non-specific binding, low annealing temperature	Raise annealing temperature or redesign primers
No band	Poor design or degraded DNA	Check T_m and sequence; use fresh DNA
Smear	Too much template or degraded product	Reduce template; optimise extension time
Mixed sequencing peaks	Mixed product or primer-dimers	Gel-purify the band; clean up the PCR

Checklist

- ✓ Length 18–24 nt; T_m 58–60 $^{\circ}\text{C}$; GC 40–60%; GC clamp at the 3' end
- ✓ No strong dimers or hairpins
- ✓ Product 300–800 bp for sequencing
- ✓ Specificity checked by BLAST
- ✓ Sequencing primer = your forward or reverse PCR primer (or an internal primer)

Questions before sending samples? WhatsApp / phone +880 1308-222824 · bdgenome@yahoo.com ·
bdgenomes.com/guides/primer-design/