Qualitative polymerase chain reactions

Polymerase chain reaction (PCR) can reveal the presence of specific DNA sequences or structural variations such as insertions and deletions when combined with gel-based detection of the resulting amplicon. Even single-nucleotide polymorphisms (SNPs) can be detected in a qualitative manner with allele-specific primer design or restriction fragment analysis. This approach is routinely used to screen bacterial or plasmid clones, or to distinguish known genetic variants in research or diagnostic development contexts.

Two common protocols are described: a Taq polymerase–based method for rapid, low-cost colony screening (*Alternative A*), and a high-fidelity protocol with lower error rates and improved specificity, particularly for GC-rich or long templates. The high-fidelity approach is also compatible with downstream amplicon sequencing (*Alternative B*). Note that relative band intensities cannot be interpreted quantitatively due to variability in template input, enzyme activity, and amplification efficiency.

Buffers and cycling conditions may require optimization depending on the application.

Risk assessment

▷ Wear gloves, safety glasses, lab coat

Reviewed: Jun 8, 2025

Procedures

›› ***Primer design***

(1.) Design primers to flank the region of interest, ideally producing an amplicon between 100–1 500 bp.

- Avoid placing a T at the 3′ end if possible; it has the weakest stability during extension.
- Avoid long stretches of G or C at the 3′ end.

  This is why: G/C runs of 2 bp or longer often result in mispriming and primer dimer formation.
- Aim for a melting temperature of 55–65 °C, with both primers within 2 °C of each other.
- Check for the absence of significant primer-dimer formation or strong hairpin structures.

Hint: For colony screening, aim for an amplicon size between 200–600 bp to aid amplification.

Hint: For large insertions, use primer pairs where one primer binds within the altered region and the other outside. This can result in the presence or absence of a band, or a size shift on the gel.

(2.) *Optional:* For SNP detection, design allele-specific primers with the variable base positioned at the 3′ end of the forward or reverse primer.

Hint: To improve allele discrimination, introduce an additional deliberate mismatch 1–3 bases upstream of the 3′ end to further destabilize off-target amplification.

(3.) *Optional:* For multiplex PCR, design multiple primer pairs with similar annealing temperatures. Avoid cross-reactivity or overlapping product sizes.

Critical: Always validate each primer pair in single-plex reactions before combining them into a multiplex reaction.

[NGH+89]

A › *Colony PCR using Taq DNA polymerase*

- ☐ 10 × Long incubation buffer, 45 mL ⟨*R*⟩

(1.) Pick a small amount of a bacterial (or yeast) colony using a sterile pipette tip or toothpick and resuspend it in 40 µL aqueous buffer. Alternatively, dilute a saturated culture about 5- to 10-fold.
Using the same tip, transfer a small amount of the cell suspension into the PCR reaction mix.

> ***Critical:*** Avoid transferring excess amounts of biomass. Overloading the reaction with DNA may inhibit polymerase activity.

> ***This is why:*** The cell suspension can be stored short-term to recover positive clones after PCR screening.

(2.) Prepare 10 µL PCR master mix for each sample:

Ingredient	Stock	Final	For 1 sample	For 8 samples
Long incubation buffer	10 ×	1 ×	1.0 µL	8.0 µL
Betaine	5 M	500 mM	1.0 µL	8.0 µL
$MgCl_2$	50 mM	2.5 mM	0.5 µL	4.0 µL
Forward primer	10 µM	500 nM	0.5 µL	2.0 µL
Reverse primer	10 µM	500 nM	0.5 µL	2.0 µL
dNTP mix	10 mM	400 nM	0.4 µL	3.2 µL
Taq DNA polymerase	5 U/µL	0.1 U/µL	0.2 µL	1.2 µL
Water, reagent-grade			5.0 µL	40.0 µL
Cell suspension, dilute			1.0 µL	8.0 µL

> ***Critical:*** Taq DNA polymerase lacks hot-start inhibition and may begin extending misprimed or partially annealed products if the reaction is assembled at room temperature. Prepare master mixes on ice and begin thermocycling promptly after setup.

> ***Hint:*** Commercial Taq polymerase master mixes or reaction buffers may be substituted. The long incubation buffer provided here is broadly compatible with both self-made and commercial Taq in most applications.

> ***This is why:*** Betaine reduces secondary structure formation, particularly in GC-rich templates, and can improve amplification robustness.

(3.) *Critical:* Include a background or parental strain as a negative control in each PCR run.

> ***This is why:*** This helps detect non-specific amplification from homologous genomic DNA regions or carryover contamination.

(4.) Spin down the reactions briefly and place directly in the thermocycler. Run the following program:

Cycles	Temperature	Duration	Name	Remarks
1 ×	95 °C	3:00 min	Initial denaturation	Set to 7:00 min to lyse yeast or thick bacterial cell walls
20–25 ×	95 °C	0:30 min	Denaturation	
	50–60 °C	0:30 min	Annealing	Set to 5 °C below the primer melting temperature
	72 °C	1:00 min/kbp	Extension	Adjust for target length
1 ×	72 °C	5:00 min	Final extension	
1 ×	12 °C	∞	Hold	

> ***This is why:*** Taq DNA polymerase has a half-life of 1 h at 95 °C, supporting up to 30 cycles with minimal degradation in most protocols. Avoid long extension times and excess cycling if possible.

(5.) Proceed with analysis of PCR products as described below.

[GS12]

B › *High-fidelity PCR for sequence validation*

- ☐ 5 × High-fidelity PCR buffer, 40 mL ⟨*R*⟩

(1.) Prepare 10 µL PCR master mix for each sample:

Ingredient	Stock	Final	For 1 sample	For 8 samples
High-fidelity PCR buffer	5 ×	1 ×	2.0 µL	16.0 µL
Betaine (*optional*)	5 M	500 mM	1.0 µL	8.0 µL
$MgCl_2$	50 mM	2.5 mM	0.5 µL	4.0 µL
Forward primer	10 µM	500 nM	0.5 µL	2.0 µL
Reverse primer	10 µM	500 nM	0.5 µL	2.0 µL
dNTP mix	10 mM	1.0 mM	1.0 µL	8.0 µL
DNA polymerase, diluted	0.04 U/µL	0.008 U/µL	0.2 µL	1.6 µL
Water, reagent-grade			4.0 µL	32.0 µL
Template	5 ng/µL	1 ng/µL	0.2 µL	1.6 µL

Critical: Avoid exceeding 2 ng of a 5 kbp plasmid per reaction. Excess template can inhibit the polymerase and promote nonspecific amplification. High-fidelity enzymes are effective with as little as 1 pg/µL DNA.

Hint: Titrate polymerase to find the lowest effective concentration. Lower enzyme levels often yield similar results, reduce cost, and often improve specificity. Dilute in 50% glycerol with 1 × reaction buffer.

Hint: Commercial high-fidelity polymerase master mixes or reaction buffers may be substituted. The included high-fidelity buffer recipe performs reliably across a wide range of polymerases and applications. For GC-rich templates, add 1% DMSO or 500 mM betaine. Thermostability and processivity may be altered in modified buffers.

(2.) *Critical:* Include a reaction without the template as negative control in each PCR run.

(3.) Spin down the reactions briefly and place in the thermocycler. Run the following program:

Cycles	Temperature	Duration	Name	Remarks
1 ×	97 °C	1:00 min	Initial denaturation	Set to 2:00 min for GC-rich templates
25–30 ×	97 °C	0:30 min	Denaturation	
	65–68 °C	0:30 min	Annealing	Set to the primer melting temperature
	72 °C	0:15 min/kbp	Extension	Adjust for target length
1 ×	72 °C	5:00 min	Final extension	
1 ×	12 °C	∞	Hold	

Critical: Consult the manufacturer's recommended cycling conditions.

This is why: High-fidelity DNA polymerases such as KOD, VELOCITY, or Phusion remain active for hours at denaturing temperatures, enabling robust amplification of long or GC-rich targets. The error rate is typically $0.8{\times}10^{-6}$–$1.2{\times}10^{-6}$ bp^{-1}.

(4.) Proceed with analysis of PCR products as described below.

(5.) If the amplicon will be used for restriction-ligation cloning SOP 0033, purify over a silica column SOP 0021 or by gel extraction SOP 0022 to remove primers, dNTPs, and polymerase before proceeding with restriction digest.

[RQW+06]

Analyses

- ○ *Critical:* Avoid opening PCR tubes after thermocycling in the same area used for PCR setup.
- ○ *Optional:* Digest PCR product with a restriction enzyme that distinguishes between alleles.

  Note: Primers must span a polymorphic restriction site.
- ○ Analyze 5–10 µL of the PCR mix on an agarose gel SOP 0032.

  Note: If the reaction contains betaine, it can be directly loaded into the wells.
- ○ When amplified with a high-fidelity DNA polymerase, purify the reaction for DNA sequencing.

Reporting standards

Please document all essential (E) and critical desirable (D) experimental parameters to ensure reproducibility and compliance with reporting standards expected by journals and repositories. Provide the minimal (5) or extended (3) level of information.

Parameter	E/D	Description	Standard
Primer sequences	E	◦ Nucleotide sequences	MIQE
		⋆ Database reference or reference to synthesis batch documentation	
Polymerase and buffer	E	◦ Enzyme name and vendor	MIQE
		⋆ Lot number, buffer formulation, and enzyme units used	
Thermal cycling program	E	◦ Cycle number, temperature, and duration	MIQE
		⋆ Cycler model and heated lid settings	
Template DNA	E	◦ Source (colony, plasmid)	
		⋆ DNA concentration, estimated size, and preparation method	
Thermal cycler	D	◦ Brand and model	
		⋆ Calibration date or error margin	
Gel electrophoresis	E	◦ Agarose percentage, ladder, staining method	
		⋆ Voltage, run time, and gel image reference	

Troubleshooting

Colony PCR using Taq DNA polymerase

In Step 1:

– Smearing or low-intensity band at correct size
  - ◦ Use a smaller colony. Excess cell material in colony PCR inhibits polymerase. Prepare a dilute lysate or use heat-treated extract as template to reduce inhibitory substances.

In Step 2:

– Non-specific bands
  - ◦ Decrease the magnesium chloride content to improve specificity. Typical final concentrations range from 1.5–3.0 mM.
  - ◦ Optimize primer design, target length, and template quality.
– Faint bands, weak amplification
  - ◦ Increase the magnesium chloride content to increase yield.

High-fidelity PCR for sequence validation

In Step 4:

– Amplification product in water-only control
  - ◦ Check primer sequences for dimer formation.
  - ◦ Prepare new primers or aliquot stocks into small volumes.
  - ◦ Clean pipettes and use filtered tips.
  - ◦ Set up reactions in a clean area physically separate from post-PCR analysis to avoid aerosol contamination of primers or template.

Recipes

Long incubation buffer, pH 8.8, 10 ×

Amount	Ingredient	Stock	Final
20 mL	Tris-Cl, pH 8.0 R0055	1 M	200 mM
1.5 mL	Potassium chloride (KCl) R0046	3 M	100 mM
595 mg	Ammonium sulfate ($(NH_4)_2SO_4$) [7783-20-2]	132.14 g/mol	100 mM
To 45 mL	Water, reagent-grade		

Adjust pH with potassium hydroxide. Filter sterilize. Dispense into 1 mL aliquots. Store at −20 °C.

10 × Long incubation buffer

20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, pH 8.8 *[At 1 × dilution]*

Date: Sign: R0193

High-fidelity PCR buffer, pH 8.8, 5 ×

Amount	Ingredient	Stock	Final
20 mL	Long incubation buffer, pH 8.8 R0193	10 ×	5 ×
2 mL	Triton™ X-100 R0057	10%	0.5%
0.2 mL	Bovine serum albumin (BSA) R0005	10%	0.05%
To 40 mL	Water, reagent-grade		

Filter sterilize and aliquot. Store at −20 °C.

5 × High-fidelity PCR buffer

1 × Long incubation buffer, 0.1% Triton™ X-100, 0.01% BSA, pH 8.8 *[At 1 × dilution]*

Date: Sign: R0194

List of references

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M. Ralser, R. Querfurth, H.-J. Warnatz, H. Lehrach, M.-L. Yaspo, and S. Krobitsch, *Biochem. Biophys. Res. Commun.* **347**(3), 747—751 (2006).

Change log

2011-11-08	Matthias Meurer; Michael Knop lab	Colony PCR from yeast cells with self-made Taq DNA polymerase.
2014-08-08	Matthias Meurer; Michael Knop lab	Generic buffers for high-fidelity DNA polymerases (VELOCITY).
2025-06-08	Benjamin C. Buchmuller	Adaptation as SOP.