Q5/Phusion PCR

Q5 DNA polymerase PCR setup, thermocycling, optimization, and troubleshooting are described here. The standard reaction protocol and guidelines are largely based on NEB recommendations, and optimization and troubleshooting information are from elsewhere. Q5 is popular because it is characterized to be the highest-fidelity PCR polymerase engineered thus far [(1)] , with a fidelity 280-fold that of *Taq* polymerase. The NEB PCR Fidelity Estimator can be used to estimate the fraction of a PCR product that is mutation-free. The Q5 2× PCR master mix versions combine the enzyme, buffer, and dNTPs, so that only primers, template, and remaining water need be added. The hot-start variants of the polymerase or master mix allow room-temp reaction setup without worry of nonspecific amplification, through an aptamer keeping the polymerase inactivated until the reaction is heated. All information here is also generally applicable to PCR with Phusion polymerase products, and aside from product-specific reaction component and thermocycling details, applicable to PCR in general. Wetlab Calculator can help calculate master mix volumes.

BySHyam Bhakta. Other high-fidelity polymerase manuals: PhantaMax, *Pfu*Ultra.

Reaction Setup

Component	25 µL Rxn	50 µL Rxn	Final Concentration
5× Q5 Reaction Buffer	5 µL	10 µL	1×
10 mM dNTPs	0.5 µL	1 µL	200 µM
10 µM Forward Primer *or 100 µM*	1.25 µL *or 0.15 µL*	2.5 µL *or 0.25 µL*	0.5 µM
10 µM Reverse Primer *or 100 µM*	1.25 µL *or 0.15 µL*	2.5 µL *or 0.25 µL*	0.5 µM
Template DNA	variable	variable	1 ng–1 pg plasmid /viral. 1 ng–1 µg genomic.
Q5 DNA Polymerase	0.25 µL (½ U)	0.5 µL (1 U)	1% with 2 U/µL enzyme. 0.02 U/µL rxn.
(opt) 5× Q5 High GC Enhancer	(5 µL)	(10 µL)	(1×)
Nuclease-Free Water	to 25 µL	to 50 µL	-

1. Assemble all reaction components on ice/cold block in 250 µL "PCR" tubes (unless using a hot-start polymerase which doesn't require keeping cold).
    a. Enzyme must be added after at least buffer and water are mixed.
    b. Make master mixes when possible, as it reduces pipetting steps, reduces errors from pipetting small volumes, and maximizes component precision across reactions. Combine all common components for *n* reac tions together and into reaction tubes aliquot reaction volumes less the volume of the variable component. *e.g.*, primers and templates commonly vary across reactions, so for ten 25 µL reactions containing a combined volume of 0.75 µL (primers + template), combine water, buffer, dNTPs, any enhancers, and polymerase; mix and aliquot 24.25 µL of this master mix across ten tubes, before adding their unique primers and templates. Components may be multichannel-pipetted into reactions for convenience. Wetlab Calculator.
2. After adding last component, mix reaction by pipetting or by flicking and centrifuging tubes to recollect liquid at bottom.
3. Transfer the reactions to a thermocycler optionally preheated to the denaturation temperature (98°C) if not using a hot-start polymerase.
   Bio-Rad thermocyclers: pausing a program right after starting it will pause the protocol after the lid has finished preheating. Pausing the program right after the lid has preheated will hold the block at the step 1 denaturation temp.

Reaction Guidelines

mainly quoting NEB

Thermocycling

Standard Q5/Phusion PCR				
	Step	***Temp***	***Time***	***Notes***
	Initial Denaturation	98°C	30 s–3 min	30 s for most templates (plasmid/linear/*E. coli*). 1–3 min for complex, or to better lyse cells.
25–35 cycles 30–35 genomic	Denaturation	98°C	10 s	5–10 s.
	Annealing	*50–72°C	30 s	10–30 s. *Find T_{anl}
	Extension	72°C	15–40 s /kb	15 s/kb for simple plasmid /bacterial template or <1 kb complex template. 20–40 s/kb for complex genomic/cDNA template. 40–50 s/kb for >6 kb amplicons.
	Final Extension	72°C	2 min	Final holding temp is unnecessary and bad for thermocycler (1) .

Note: If you need to destroy the non-synthetic template (plasmid), you can add 1 µL DpnI per 50 µL completed PCR, and incubate 37C, 15–60 min. DpnI has full activity in Phusion buffer and 50% activity in Q5 buffer NEB .

Thermocycling Guidelines

mainly quoting NEB

Denaturation

An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 5 min) for templates that require it.

During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

Annealing

Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The NEB Tm Calculator should be used to determine the annealing temperature when using this enzyme. Typically, use a 10–30 second annealing step at 3°C above the T_m of the lower T_m primer. A temperature gradient across a strip of aliquotted reactions can also be used to optimize the annealing temperature for each primer pair.

2-step PCR

Template

Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template are:

DNA	Concentration
Genomic or cDNA	1 ng–1 µg per 50 µL PCR
Plasmid or Viral	1 pg–1 ng per 50 µL PCR

Low quality genomic DNA preparation

Low quality genomic DNA is present in cell lysate and is often sufficient for cloning PCR or strain genotyping. Simply "boil" a cell solution in a thermocycler tube for 10 min, centrifuge the cell debris, and use the supernatant as genomic DNA. The cell solution can be a saturated liquid culture or a mass of cells resuspended in TE (elution buffer). This lysate can be frozen and reused many times as a PCR template.

Primers

Oligonucleotide primers are generally 17–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 (in Benchling) can be used to design or analyze primers. The best results typically come from reactions with 0.5 µM each primer.

For small reactions, the 100 µM primer volume can be <0.2 µL and thus not accurately pipettable. Using a minimum of 0.2 µL, though excess, is typically still successful and convenient as it avoids making primer dilutions. Primer dilutions often waste time and freezer space, given that they aren't typically needed once the immediate PCR is complete.

Buffers

The 5× Q5 Reaction Buffer provided with the enzyme is recommended as the first-choice buffer for robust, high-fidelity amplification.

The 5× Q5 Reaction Buffer is detergent-free and contains the optimal 2.0 mM $MgCl_2$ at the final (1×) concentration. Shyam deduced based on safety data sheets that Q5 Reaction Buffer, but not Phusion buffer, contains glycerol, which reduces DNA secondary structure, and tetramethylammonium, which increases primer stringency.

Store buffers at 20°C for long-term. Once you claim an aliquot, you may store it at 0–4°C until it is used up, preventing the need for thawing.

Deoxynucleotides

The final concentration of dNTPs is typically 200 M of each deoxyribonucleotide, typically mixed and stored as a 10 mM solution at -20°C. Freeze-thaw cycles of dNTPs must be limited to preserve the triphosphate moiety, just as with ATP-containing solutions. dNTP stocks are thus aliquoted in 0.25 or 0.6 mL tubes in 20 µL volumes. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.

Q5 DNA Polymerase concentration

Q5 High-Fidelity DNA Polymerase is recommended to be used at a final concentration of 20 U/mL (1.0 U/50 l reaction). However, the optimal concentration of Q5 High-Fidelity DNA Polymerase may vary from 10–40 U/mL (0.5–2 U/50 l reaction) depending on amplicon length and difficulty. Do not exceed 2 U/50 l reaction, especially for amplicons longer than 5 kb.

The hot-start formulation, Q5 Hot-Start DNA Polymerase, inhibits the robust exonuclease (and polymerase?) activity of the enzyme, allowing for convenient room temperature reaction setup. The aptamer/inhibitor is released from the enzyme during normal cycling conditions, so no separate activation step is required.

When primers with an annealing temperature 70°C are used, a 2-step thermocycling protocol (combining annealing and extension into one 72° step) is possible.

Touchdown/Touch-up PCR

See *Troubleshooting*

Extension

The recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb for complex, genomic samples, but can be reduced to 10 seconds per kb for simple templates (plasmid, *E. coli*, etc.) or complex templates < 1 kb. Extension time can be increased to 40 seconds per kb for cDNA or long, complex templates, if necessary. When amplifying products > 6 kb, it is often helpful to increase the extension time to 40–50 seconds/kb.

A final extension of 2 minutes at 72°C is recommended.

Cycle number

Generally, 25–35 cycles yield sufficient product. For genomic amplicons, 30-35 cycles are recommended.

PCR product

The PCR products generated using Q5 High-Fidelity DNA Polymerase have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.

PCR Additives

Difficult PCRs with GC-rich sequences or secondary structure in the amplified DNA or primers may be improved by the addition of Q5 High GC Enhancer at a final 10–20% (from the 5–10× stock). It is not a reaction buffer itself and cannot be used alone, only to be added to reactions along with reaction buffer when other conditions have failed. NEB says use of the Q5 High GC Enhancer often lowers the range of temperatures at which specific amplification can be observed, but that the normal, unmodified T_{anl} generally still supports specific amplification.
It is unclear how most PCR additives below influence polymerase fidelity.

Q5 reaction buffer appears to contains glycerol, which reduces DNA secondary structure, and tetramethylammonium, which increases primer stringency; and Q5 High-GC Enhancer contains DMSO and glycerol to do more of the same (Shyam).

Of these PCR Additives, 5% DMSO (with a 3° lower T_{anl}) is Shyam's most common first-choice when a PCR fails without high GC-content, followed by 1 M betaine. For high GC, you might try 1 M betaine or 0.5 M trehalose with 3° lower T_{anl}, or the Horakova or preCES-II mixes.

- **DMSO:** reduces DNA secondary structure. (1,2,4,5,6,13) Use at 3–10% final, adjusting in 2% increments. 10% DMSO lowers T_m 5.5–6°C. DMSO reduces the activity of *Taq* polymerase, which may need to be increased with high DMSO. Does not seem to be a mutagen. (9) In combination with BSA for GC-rich templates.(14)
- **Glycerol:** reduces DNA secondary structure. Found in Q5 buffer. Use at 5–10% final. (2,5)
- **Betaine / Betaine·HO:** greatly reduces the high T_m bias of G:C over A:T pairs, reversing the bias slightly at lower T_ms. Especially useful for GC-rich and structured templates. Use at 1–3 M final. (1,2,4,7,8,9,13) Or use 1 M final. (8) Can inhibit amplification of some templates. Don't use betaine·HCl. Does not seem to be a mutagen.(9)
- **Formamide:** increases the stringency of primer annealing, resulting in less non-specific priming and increased amplification efficiency. Use at 1-10%. (1,2,4,11) In combination with BSA for GC-rich templates. (14)
- **Tetramethylammonium** chloride: increases the stringency of primer annealing, resulting in less non-specific priming and increased amplification efficiency. Use at 10–100 mM final. (1,2) Found in Q5 buffer.
- **Triton X-100, Tween-20/-40 or NP-40:** reduce DNA secondary structure, but can increase non-specific amplification. Use at 0.1–1% final. (1)(2) TWEEN can neutralize SDS left over from template DNA preparation that would inhibit the reaction. Use at 0.25–1% final. (1,2)
- **BSA:** prevents reaction components adhering to the tube and reduces the effect of PCR inhibitors. Use at 0.8 mg/mL final. (2,5) It might already be in manufacturers' PCR buffers, as it is found in restriction enzyme reaction buffers.

  The following can improve success of GC-rich template amplification:
- **1,2-propanediol:** Use at 0.8 M final. (3, 9) Or combine with trehalose. (7) Does not seem to be a mutagen. (9)
- **1,2-ethanediol / ethylene glycol:** Use at 1 M final. (3,9) Does not seem to be a mutagen. (9)
- **Sulfolane (tetramethylene sulfoxide/sulfone):** Use at 0.4 M final.(6,10)
- **7-deaza-2-deoxyguanosine:** a dGTP analogue. Found to work up to 83% GC. Use a 1:3 ratio of dGTP:7-deaza-2-deoxyguanosine. (2,12)
- **Trehalose:** Use 0.4 M final or 0.2–1 M. (8) Reduces Tm by a few °C (8). Or combine with 1,2-propanediol. (7)
- **Dithiothreitol (DTT):** (4,5)
- **DMSO+BSA** or **Formamide+BSA**: 6–10 µg/µL final BSA + 5–10% DMSO or formamide. (14) More BSA can be

supplemented every 10th cycle.

Or you can try a published GC-enhancer mix:

- **preCES-II, 5× :** 4 M betaine, 10 mM DTT, 10% DMSO. [4]
- **"Horakova universal mix, 5×"** : 5 M 1,2-propanediol, 1 M trehalose. [7]
- **"Nagai universal mix, 5×"**: 5 mg/mL BSA, 50 mM DTT, 25% glycerol.[5]

Sources: (1) Bitesize Bio, (2) Bitesize Bio, (3) Bitesize Bio, (4) Ralser, (5) Nagai, (6) CSH, (7) Horakova, (8) Spiess, (9) Zhang, (10) Chakrabarti, (11) Sarkar, (12) Shore, (13) Jensen, (14) Farell

Troubleshooting/optimizing a PCR

- Recheck primer annealing temperatures and GC-content.
- Add 5% DMSO or high GC-enhancer or other additives. See Additives section. A lower, 3% DMSO can be added as a preventative measure, or if significant homodimerization or heterodimerization of primers is expected, a few DMSO concentrations can be tested (0/5% or 3/6% or 3/6/9%) with a 2–5° lower T_{anl}.
- To increase specificity (remove spurious products), use the touchdown thermocycling protocol. See below.
- To increase specificity (remove spurious products or obtain a missing product), use the touch-up thermocycling protocol. See below.
- To increase specificity (remove spurious products or obtain a missing product), try a range of annealing temperatures. See *Annealing* section.
- Remake your primer stocks.
- If strong spurious template, ensure reagents are not contaminated. *E. coli* genomic contamination is common in cheaply purified polymerase.
- If no products at all, ensure polymerase, buffer, and dNTPs are functional in a positive control reaction with known-to-work primers on a trusted template.

Touchdown PCR

To enhance amplification specificity, a touchdown thermocycling protocol can be used, which starts at a higher, stringent T_{anl} and ramps it down across successive cycles to a steady, permissive T_{anl}, ensuring high specificity of primer binding in initial products at the elevated T_m, which have a head start in amplification. If the desired band is not visible at all under standard protocol, touch-up PCR must be used. (Don 1991, Hecker 1996, Korbie 2008)

A typical protocol consists of (after initial denaturation) 10 PCR cycles with the expected T_{anl} **+5°C** decrementing 0.5°C per cycle, followed by 25 PCR cycles with T_{anl}. The $T_{anl,i}$ must be at most the extension temperature. The constant, $T_{anl,f}$ ought to be at least the expected T_{anl} and can be set as high as the T_{anl} of the entire primer oligo pair for ensured sustained high stringency. The number of ramping cycles must equal the T_{anl} elevation divided by the T_{anl} decrement, and the number of constant T_{anl} cycles must be 25 cycles to ensure sufficient amplification after ramping.

$n_{cycles,ramp}$ = (10–15 cycles) = ($T_{anl,i} - T_{anl}$) / T
T = ($T_{anl,i} - T_{anl}$) / (10–15 cycles)

Q5 Touchdown PCR Thermocycling				
	Step	***Temp***	***Time***	***Notes***
	Initial Denaturation	98°C	30 s–3 min	30 s for most templates 1–3 min for complex, or to better lyse *E. coli*.
10–15	Denaturation	98°C	10 s	

Primer Design

Primers are the template-binding portion of oligonucleotides that are extended by a DNA (or RNA) polymerase. The entire oligo is also often called a primer. For any PCR primer design, the priming region is ideally 16–30 nt long, 55° annealing temperature with 5° Tm difference between primers, 40–60% GC content with ~uniform distribution, ending in 2–3 G/C bases among the final (3) five bases, a "GC clamp", though not more than this to reduce nonspecific annealing.

Primers ought to be absent of complimentary regions within a primer or between the two primers that could otherwise create primer structure that inhibits template binding or allows primers to prime themselves and form primer monomer or dimer products that take over a reaction. Primers ought not bind the template in unintended locations that would allow unwanted products to be amplified.

All these factors contribute to a PCR's sensitivity (yield of a particular product) and specificity (fraction of products correct). The simpler and smaller the PCR template (*e.g.* monoclonal purified plasmid or a linear DNA), the shorter and less restricted the sequence content of the primer can be (aside from strong 2° structure and template repetitiveness) while still producing a sizeable and specific PCR product, due to the reaction containing less non-target template DNA to throw off the PCR. This is of particular importance because oftentimes PCR products are needed that have fixed, nonnegotiable 5 ends to capture the exact boundary of a sequence, and the only degree of freedom in primer design is how long the 3' end of the primer is designed starting from these fixed positions on the template (*e.g.* amplifying a CDS from precisely the start to stop codons). Not much can be done if such primers have problematic characteristics: if they have substantial predicted structure, a PCR additive such as DMSO may be used to inhibit the structure and raise PCR yield; or if unintended binding sites exist in the template, a touch-down thermocycling protocol can help increase specificity, or the repetitive sequence can omitted in the PCR and be included in the assembly with the PCR product as a separate non-PCRed fragment, often made by oligo assembly or (*e.g.* when amplifying a promoter with multiple copies of an operator).

The 5 ends of primer oligos can augment a PCR product with new, non-complimentary sequence that allow the further manipulation of the PCR product. Restriction endonuclease sites can be added to the ends, for downstream use of the PCR product in restriction-ligation or Golden Gate assemblies. Homology arms or site-specific recombinase sites can be added for homologous recombination (*e.g.* genomic integration or yeast assembly), *in vitro* homology-based assembly (*e.g.* isothermal /Gibson, CPEC, SLiC/SLiCE), or recombinase-based assembly (*e.g.* Gateway). Mutations can likewise also be added, by primers having bases non-complimentary to the template, generally no more than a few, and not within 3 nt from the primer's 3 end. When estimating the T_{anl} for such primers, the non-complimentary bases may simply be omitted*, just as non-complimentary 5 ends must be omitted as these are not designed to bind the template. *Current Tm algorithms consider enthalpy /entropy parameters of dinucleotides, so instead of only deleting internal non-complimentary bases from the calculation, try also replacing it with A, T, AT, and TA, and use the minimum Tm amongst the five cases.

Benchling T_m Prediction

cycles	**Ramping Annealing**	**T_{anl} + *x***	30 s	**Ramp by –*T* every cycle, such that *T* = *x*/(10–15 cycles). *x* = 5–10°C. For *x* = 7.5°C elevation, *T* = –0.5°C over 15 cycles.**
	Extension	72°C	10–40 s /kb	10–15 s/kb for simple plasmid/*E. coli* template. 20–40 s/kb for complex genomic /cDNA template. 30–40 s/kb for >6kb amplicons
25 cycles	Denaturation	98°C	10 s	
	Annealing	*50–72° C = ***T_{anl}**	30 s	Find T_{anl} , 3° above T_m of lower T_m primer
	Extension	72°C	10–40 s /kb	10–15 s/kb for simple plasmid/*E. coli* template. 20–40 s/kb for complex genomic /cDNA template. 30–40 s/kb for >6kb amplicons
	Final Extension	72°C	2 min	Holding temp is unnecessary and bad for thermocycler (1) .

Touch-up PCR

While the touchdown thermocycling enhances amplification specificity by imposing a more stringent initial annealing temperature and ramping to a more permissive T_{anl} across cycles, touch-up thermocycling does the opposite, by starting at the permissive (expected) T_{anl} and ramping up across successive cycles to a steady, higher, stringent T_{anl}, ensuring high specificity of primer binding in later products which are selectively amplified for over successive cycles from non-specific amplicons at the initial permissive T_{anl}. If the desired band does not appear at the expected T_{anl}, the initial T_{anl} can be lower so as to better ensure the correct amplicon is part of the initial selection pool. (Rowther 2012)

A typical protocol can consist of (after initial denaturation) 10–15 PCR cycles with a lower-than-expected $T_{anl,i}$, incrementing 0.5–1°C to a final $T_{anl,f}$ at least 5–10°C higher and as high as that of the T_{anl} of the entire primer oligos. This is followed by a second, regular cycling PCR phase using a $T_{anl,const}$ equal to $T_{anl,f}$. $T_{anl,f}$ can (and perhaps should) be increased 2–5°C above $T_{anl,con}$ for higher stringency, followed by the constant $T_{anl,con}$ cycles not having that additional increase for better product priming. If the desired $T_{anl,const}$ is 70–72°C, the constant phase can eliminate the annealing step (2-step PCR). If the primers do not have a non-annealing 5 end or if the entire oligo pair T_{anl} is not substantially higher than that of just the annealing regions, then the $T_{anl,i}$ cannot be elevated much without the artificial higher threshold $T_{anl,f}$ being used for sufficient selectivity.

The 10–15 incrementing T_{anl} cycles must equal the T_{anl} elevation divided by the T_{anl} increment, and the number of constant T_{anl} cycles should be 20–25 cycles.

$n_{cycles,\ ramping}$ = (10 to 15 cycles) = $(T_{anl,f} - T_{anl,i})/T$
$T = (T_{anl,f} - T_{anl,i})/$(10 to 15 cycles)

Q5 Touch-Up PCR Thermocycling				
	Step	***Temp***	***Time***	***Notes***
	Initial Denaturation	98°C	30 s–3 min	30 s for most templates, 1–3 min for complex or to better lyse *E. coli*.
10–15 cycles	Denaturation	98°C	10 s	

Shyam found these Benchling T_m thermodynamic parameters to give rather good estimates (within rounding) for Q5 T_ms, compared to the NEB Tm Calculator, especially with moderate GC-content primers. For the PCR T_{anl}, simply use the *lower* of the two primers' T_ms.
Compared to NEB, these parameters seem to need slight adjustment according to GC content: **subtract 1–2° T_m for 30% GC** sequence and **add 1–2° T_m for 70% GC** sequence. This correction can generally be ignored, as it is within the range for a cloning PCR to work well with simple, non-repetitive templates.

Algorithm: SantaLucia 1998	
DNA: 1500 nM	**Na^+/K^+:** 75 mM
Mg^{2+}: 2 mM	**dNTP:** 0.2 mM

	Ramping Annealing	**$T_{anl,i} = T_{anl} (-x)$** = 45–65° C	30 s	**Ramp by +*T* each cycle, such that $T_{anl,f}$ is reached in 10–15 cycles** ***i.e.* $T = (T_{anl,f} - T_{anl,i})/(10–15$ cycles)** $T_{anl,f} = T_{anl,con} + y°$; ***y* = 0–2–5°C to raise threshold.** ***x* 5° if band is absent in standard PCR** Find $T_{anl,i}$, 3° above T_m of lower T_m primer
	Extension	72°C	10–40 s /kb	10–15 s/kb for simple plasmid/*E. coli* template. 20–40 s/kb for complex genomic /cDNA template. 30–40 s/kb for >6kb amplicons
20–25 cycles	Denaturation	98°C	10 s	
	Annealing	**$T_{anl,con}$** = 50–72°C	30 s	**$T_{anl,i}$+5–10°C $T_{anl,con}$ $T_{anl,full}$ oligos**
	Extension	72°C	10–40 s /kb	10–15 s/kb for simple plasmid/*E. coli* template. 20–40 s/kb for complex genomic /cDNA template. 30–40 s/kb for >6kb amplicons
	Final Extension	72°C	2 min	Holding temp is unnecessary and bad for thermocycler (1) .

Another kind of touch-up protocol cycles the set of T_{anl} ramping cycles 4–5 times; it has no constant T_{anl} phase.

Cyclic Touch-Up PCR Thermocycling					
		Step	Temperature	Time	Notes
		Initial Denaturation	98°C	30 s–3 min	30 s for most templates, 1–3 min for complex or to better lyse *E. coli*.
4–5 cycles	**10–15 cycles**	Denaturation	98°C	10 s	
		Ramping Annealing	**$T_{anl,i} = T_{anl}$ (−*x*)** = 45–65°C	30 s	**Ramp +T°C each cycle, such that $T_{anl,f}$ is reached in 10–15 cycles** **i.e. $T = (T_{anl,f} - T_{anl,i})/(10–15$ cycles)** **$T_{anl,f} = T_{anl,con} + y°$; *y* = 0–2–5°C to raise threshold.** ***x* 5° if band is absent in standard PCR** Find $T_{anl,i}$, 3° above T_m of lower T_m primer
		Extension	72°C	10–40 s /kb	10–15 s/kb for simple plasmid/*E. coli* template. 20–40 s/kb for complex genomic /cDNA template. 30–40 s/kb for >6kb amplicons
		Final Extension	72°C	2 min	Holding temp is unnecessary and bad for thermocycler (1) .

Band-Stab / Purification PCR

Touch-down and touch-up PCRs can help bias PCR product formation in favor of a desired product amid a mixture of other products, but if a single product is desired from a PCR to improve purification yield and purity, the band-stab method[6] can provide a specific gel band's DNA as template for a second round of 'purification PCR', avoiding gel-purification of bands before secondary PCRs.

An initial PCR is resolved on a gel to obtain at least a faint correct-sized band. While visualizing, the band is stabbed with a pipette tip and ‘eluted’ into a second PCR mix, or into a small volume of eluent which is used as a template in a second PCR. The eluent (water or Tris elution buffer) can be heated to perhaps better release the DNA from the tip and agarose.

The second ‘band-stab PCR’[6] uses the same primers as the first. In cases where the primers add new 5 sequence ‘overhangs’ to the product, *e.g.*, restriction sites for later assembly, the T_{anl} can be recalculated to include the entire oligo sequences, which raises the temp and adds specificity, perhaps even permitting a 2-step PCR.

Though not necessary, a crude purification can also be performed, wherein a select gel band is excised, mashed in a tube, combined with eluent, and heated to extract the DNA out of the agarose. The eluent can be used in secondary purification PCRs.

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