Universal Annealing Temperature in PCR and its Impact on Amplification Results

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INTRODUCTION

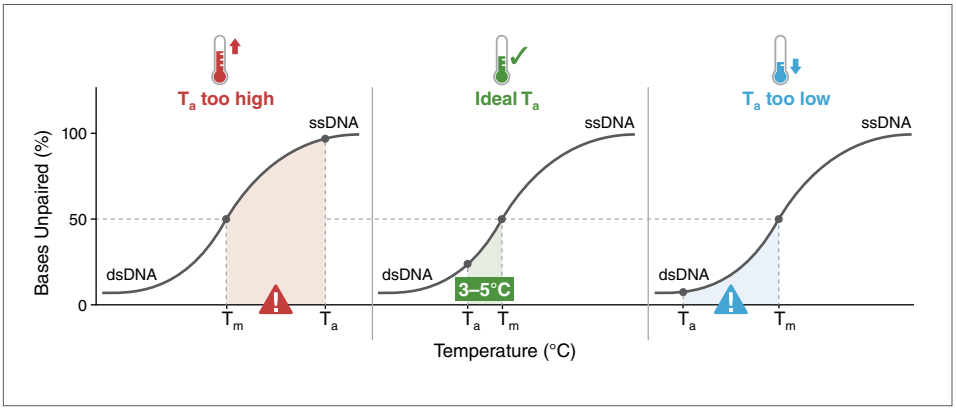
The polymerase chain reaction (PCR) is a powerful molecular biology technique used to amplify DNA using target-specific primers, a thermostable polymerase, and a series of temperature cycles. Thermocycling conditions for routine PCR often use a three-step protocol to enable denaturation, annealing and extension. The optimal annealing temperature (Ta) is one of the key thermocycling parameters that must be determined to achieve best assay performance for a particular primer pair[1]. The ideal Ta for an assay is driven by the melting temperature (Tm) of the PCR primers, which is a measure of duplex stability and indicates the temperature at which half of the primer-template duplex dissociates to become single-stranded (Figure 1). Proper annealing of the primer to the template DNA is critical for the polymerase to initiate synthesis. Factors that influence the Tm of the primers and thus impact the optimal Ta of the PCR assay are the length, sequence, and concentration of the primers. The reaction buffer composition also impacts primer Tm and therefore, the optimal Ta is affected. The optimal Ta for a primer pair can be determined empirically by running a gradient PCR or theoretically by using an online calculator tool. General PCR guidelines recommend setting the Ta to be 3–5°C lower than the Tm of the less stable primer-template pair. If the Ta is too high, the efficiency of primer binding is reduced, leading to poor amplification because the target amplicon cannot be duplicated during each PCR cycle. If the Ta is too low, primers may bind DNA nonspecifically, resulting in off-target amplification (Figure 2, page 2).

Although guidelines for establishing the best Ta are helpful, using a Ta that is 3–5°C lower than the Tm is not optimal for all workflows. For example, multiplex assays or high-throughput processes involve multiple PCRs that must use a single thermocycling protocol. Using one annealing temperature and extension time for all assays minimizes the number of sequential thermocycling runs and can result in significant time savings. Fortunately, the thermodynamics of primer binding and best practices for PCR primer design often make it possible to use a single (i.e., "universal") annealing temperature, as there is a wide temperature range surrounding the primer Tm that will result in stable primer-template duplex formation. This means a range of annealing temperatures can successfully support primer binding and enable DNA synthesis. For example, a study optimizing the annealing temperature of PCR showed that successful amplification was achieved using primers with calculated Tm values of 56.8°C and 46.9°C across an annealing temperature range of 44°C to 64°C[2]. Additionally, guidelines for PCR primer design typically specify that the optimal primer length is 18 to 24 nucleotides with an average GC content between 40 to 60%. Following these best practices means that a 20-nucleotide primer with 50% GC content will frequently have a Tm between 56°C to 62°C[3]. PCR primer design guidelines also suggest minimizing the difference in Tm values between the forward and reverse primer, with a maximum difference of 5°C. Given that a wide range of annealing temperatures can support DNA duplex formation, and that best-practice primer design results in similar primer Tm values, using a single annealing temperature to amplify multiple different targets simultaneously is often possible.

MATERIALS

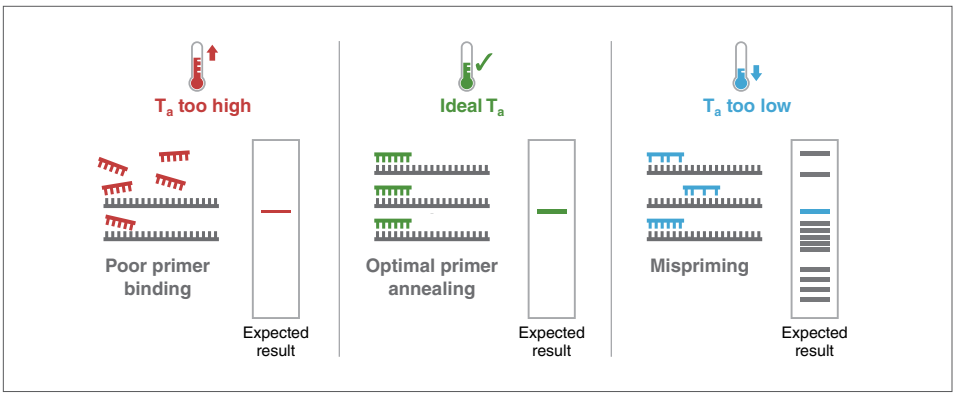
- Q5® High-Fidelity DNA Polymerase (NEB #M0491)
- Q5 High-Fidelity 2X Master Mix (NEB #M0492)
- Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493)
- Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)
- Q5 Quick-Load™ High-Fidelity 2X Master Mix (NEB #M0578)
- Q5 Quick-Load Hot Start High-Fidelity 2X Master Mix (NEB #M0580)

FIGURE 1: **Annealing temperature relative to the melting temperature of a PCR primer**



Typical PCR guidelines suggest setting the annealing temperature (Ta) to be 3–5°C lower than the lowest melting temperature (Tm) primer of the assay. This ensures stable primer-template duplex (dsDNA) formation while minimizing non-specific primer binding.

FIGURE 2: **The impact of annealing temperature on primer binding during PCR.**



Using an ideal annealing temperature (Ta) ensures optimal primer binding to support amplification of the intended target. Poor primer binding occurs when the Ta is too high relative to the ideal Ta and can result in low to no product yields. Mispriming can occur if the Ta is too low relative to the ideal Ta and can result in non-specific amplification.

Here we provide guidelines for using a single annealing temperature in PCR with Q5 High Fidelity DNA Polymerase. Q5 is an ultra-low error rate, thermostable family B DNA polymerase with enhanced processivity conferred by the Sso7d DNA binding domain. One of the standout features of Q5 is its fidelity, which is approximately 280 times higher than that of *Taq* DNA Polymerase[4]. This high fidelity makes it ideal for PCR applications where accuracy is critical, such as site-directed mutagenesis, cloning, and next-generation sequencing. We also evaluate two other commercially available high-fidelity DNA polymerases: Phusion™ Plus (PN #F630S) and Platinum™ SuperFi™ II (PN #12361010). Both polymerases use a universal annealing temperature of 60°C in their default three-step thermocycling protocol, and the product literature claims that most PCR assays can be performed at this temperature, eliminating the need to optimize the annealing temperature for each primer pair of interest[5,6]. However, the data presented herein for all three PCR polymerases show that while a protocol using a single annealing temperature supports successful amplification for many assays, the best PCR results are frequently achieved using the optimal Ta value for a given primer pair, and that no annealing temperature is truly universal.

RESULTS

Guidelines for using a single annealing temperature with Q5 Hot Start High-Fidelity DNA Polymerase

To establish general recommendations for using a single annealing temperature in a three-step thermocycling protocol for Q5, we first determined the optimal annealing temperature for a number of different PCR primer pairs using the New England Biolabs online calculator (NEB Tm Calculator). Unlike other calculators, the NEB Tm Calculator takes into consideration NEB buffer components that influence primer melting temperatures, as well as empirical observations, when calculating the optimal annealing temperature. Using the NEB Tm Calculator is critical for establishing accurate Ta recommendations when using Q5, which typically requires higher annealing temperatures than other PCR-based DNA polymerases. We selected eight amplicons (Table 1) with Ta values for Q5 ranging from 56°C to 72°C (Calculator setting: Q5 Hot Start High-Fidelity DNA Polymerase, 500 nM primer).

Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493) was tested on these eight amplicons using 25µl reactions with 0.4ng/µl human genomic DNA (Jurkat; BioChain #D1255815) in 1X Q5 Reaction Buffer (NEB #B9027). Q5 High GC Enhancer (NEB #B9028) was added for targets with GC content above 65%. Standard extension times were chosen based on the amplicon length (30s/kb). We determined that an annealing temperature of 62°C provides robust amplification across a broad range of targets with varying optimal Ta values using Q5 Hot Start High-Fidelity DNA Polymerase (Figure 3, page 3). In several cases, the best performance (i.e., yield and specificity) was achieved using the optimal annealing temperature for each primer pair. This was also true for Phusion Plus DNA Polymerase and Platinum SuperFi II DNA Polymerase, two commercially available hot-start polymerases that recommend a universal annealing temperature of 60°C regardless of primer Tm values (Figure 3, page 3).

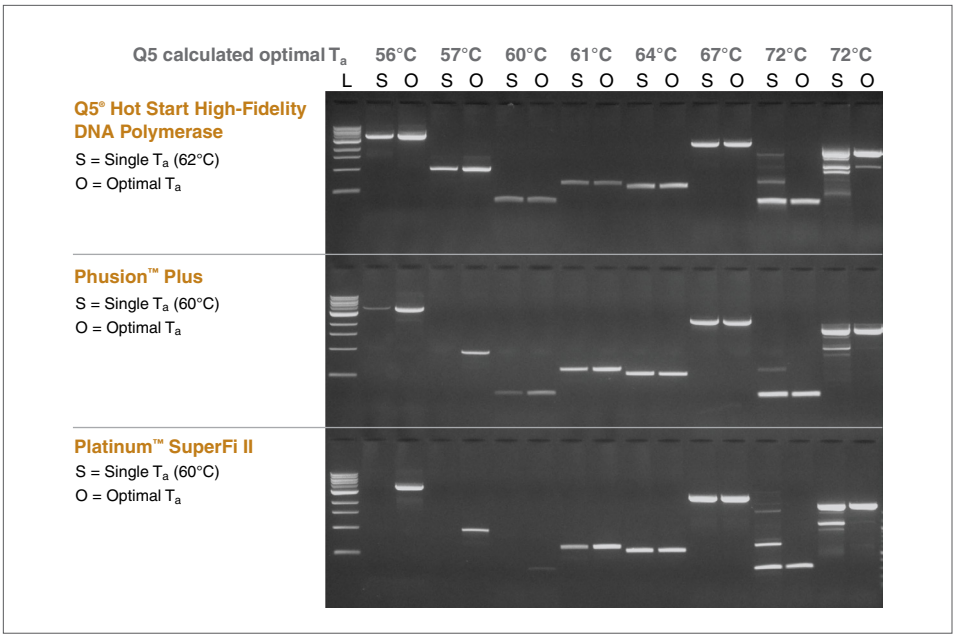
Enabling multiplex PCR using Q5 and a single annealing temperature

While using the optimal Ta often gave better results than using a single pre-defined Ta for all three DNA polymerases, using the optimal annealing temperature for each primer pair is not feasible in all workflows. As discussed previously, this is particularly true for multiplex PCR where multiple primer pairs are used in a single reaction simultaneously. In addition to a shared annealing temperature, multiplex requires a single extension time, regardless of amplicon length. To evaluate whether the previously determined annealing temperature of 62°C is suitable for multiplexing with Q5, we performed a multiplex PCR experiment. Four primer pairs corresponding to amplicons 2, 3, 5, and 6 from Table 1 were selected, representing a range of Ta values for Q5 (between 57°C and 67°C), and a multiplex PCR was run using a 62°C annealing step.

TABLE 1: **Amplicon details**

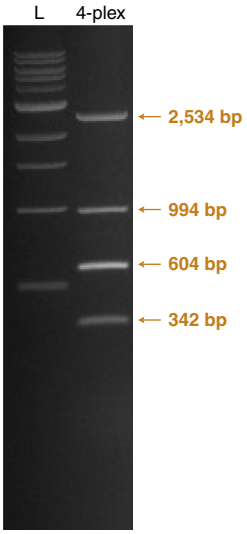
AMPLICON	OPTIMAL TA FOR Q5 HS (°C)	F, R PRIMER TMS (°C)	GC CONTENT (%)	SIZE (BP)
1	56	55, 59	29	3864
2	57	56, 61	27	994
3	60	62, 59	24	342
4	61	61, 60	47	665
5	64	63, 65	41	604
6	67	66, 67	55	2534
7	72	77, 77	75	322
8	72	75, 77	70	1839

FIGURE 3: **Comparison of using a single annealing temperature (S) versus optimal annealing temperature (O) during thermocycling in PCR**



Resulting PCR assays were conducted using a single annealing temperature for all eight amplicons (S) or the optimal annealing temperature (O) for each amplicon. All PCR products were visualized by ethidium bromide staining on 1.2% TAE agarose gel.
Best product yield and specificity was typically observed using the optimal annealing temperature for each specific polymerase.

FIGURE 4: **Multiplex PCR using an annealing temperature of 62°C with Q5 Hot Start High-Fidelity DNA Polymerase**



A four-plex reaction, with amplicons ranging in length from 342 bp to 2.5 kb, was successfully conducted using an annealing temperature of 62°C and an extension time of 60 seconds.

The extension time was set based on the largest amplicon in the multiplex PCR assay, and the thermocycling protocol used was as follows: 1 cycle of 98°C/30s, 30 cycles of (98°C/10s, 62°C/20s, 72°C/60s), 1 cycle of 72°C/5 min. Analysis by gel electrophoresis highlighted that all four targets, ranging from 342 bp to 2.5 kb were successfully amplified using a single protocol that employed the 62°C annealing temperature (Figure 4).

DISCUSSION

Amplifying multiple DNA targets simultaneously using a single annealing temperature is regularly used in many PCR-based workflows. Multiplex PCR assays must employ a single thermocycling protocol, regardless of the optimal Ta value for each primer pair or the varying amplicon lengths. The annealing temperature is often set based on the lowest Ta primer pair in the multiplex while the extension time supports the longest amplicon in the assay. Previous work on multiplexing with Q5 showed successful amplification in a four-plex assay using an annealing temperature between 60 and 67°C, despite the primer pairs having optimal calculated Ta values ranging from 66°C to 72°C[7]. More recent work using Q5 for ARTIC SARS-CoV-2 sequencing relied on two multiplex primer pools to amply the entire 29 kb genome of SARS-CoV-2 using a single thermocycling protocol[8,9].

While multiplex PCR must always rely on one annealing temperature, there are many other common PCR workflows that use a single annealing temperature for convenience. For instance, real-time PCR (qPCR) assays using *Taq* DNA polymerase routinely use one annealing temperature in a two-step thermocycling protocol that cycles between 95°C and 60°C, thus combining annealing and extension into a single step[10]. For these workflows, users must ensure that all primers and probes are compatible with this temperature or alter the thermocycling parameters accordingly. Additionally, two-step thermocycling protocols are commonly recommended when PCR primers have high Tm values (≥ 68°C). This can occur with primers that are GC-rich or are longer in length, assuming full complementarity with the target sequence. In the case of Q5, a combined annealing and extension step at 72°C is recommended when primers with annealing temperatures ≥ 72°C are used (e.g., amplicons 7 & 8 in Table 1, page 2). Similar recommendations apply to Phusion Plus and Platinum SuperFi II when the primers are greater than 30 nucleotides in length[5,6].

In our evaluation, we found that an annealing temperature of 62°C worked best with Q5 Hot Start High Fidelity DNA Polymerase in a three-step cycling protocol on amplicons with optimal Q5 Ta values ranging from 56°C to 72°C. Phusion Plus and Platinum SuperFi II, both of which are marketed as compatible with universal annealing at 60°C in three-step protocols, were also tested. Both polymerases supported amplification for many targets using a Ta of 60°C. However, we typically observed improved reaction efficiency (i.e., higher yield) and specificity when using the optimal annealing temperature for all three polymerases tested (Figure 3). Primer pairs with optimal Ta values lower than the experimental single annealing temperature (optimal Ta < experimental Ta) frequently showed reduced or no product yield, particularly for Phusion Plus and Platinum SuperFi II. Conversely, when the optimal Ta value was higher than the experimental single annealing temperature used (optimal Ta > experimental Ta), non-specific amplification occurred. These results are in line with theoretical PCR expectations and highlight that no single annealing temperature is truly universal.

The product literature for Phusion Plus and Platinum SuperFi II highlights the inclusion of isostabilizing molecules that help promote duplex DNA formation and enable universal annealing[5,6]. The use of specific PCR buffer components, such as monovalent salts (e.g., K^+), have long been known to stabilize the DNA phosphodiester backbone and promote duplex formation[11]. Similarly, additives like DMSO, which help destabilize secondary structures in GC-rich regions, reduce the primer Tm and thus lower the optimal Ta[12]. As a result, buffer components that affect melting temperature are taken into account when Ta value recommendations are generated by the NEB Tm Calculator.

Relying on a single annealing temperature can simplify PCR assay design and streamline workflows involving multiple targets. Using a shared thermocycling protocol – one annealing temperature and one extension time for the longest amplicon – saves time and resources. The data presented herein show there is occasionally a tradeoff for this convenience, and it can sometimes result in complete PCR failure.

Best results will most often be achieved using the optimal annealing temperature for a given primer pair. The extent to which a single cycling protocol will work across multiple different PCR assays will be amplicon and workflow dependent. In some cases, reduced reaction efficiency may not be evident, depending on the template input amount and number of PCR cycles used relative to when the reaction reaches plateau[2]. For Q5 High-Fidelity DNA Polymerase, if a single annealing temperature is desired to simplify an assay or workflow, we recommend starting with an annealing temperature of 62°C. This annealing temperature can then be paired with a single extension time that supports the longest product such that a single thermocycling protocol can be used. However, if a primer pair fails to give acceptable results using an annealing temperature of 62°C, an alternative temperature can possibly be identified to support the workflow. In this case, a temperature gradient may be useful to empirically determine the optimal annealing temperature.

While the results here highlight the use of the stand-alone Q5 enzyme, a single annealing temperature can also be used successfully with Q5 Master Mixes (NEB #M0492/M0494/M0578/M0580). In this case, we recommend starting with a Ta of 60°C. This slightly lower temperature is due to the master mix formulation, which is optimized for robust amplification of GC-rich templates (≥60% GC) and better disrupts secondary structure compared to Q5 Reaction Buffer alone (Figure 5). This enhanced disruption can lead to slightly lower primer Tm values, regardless of whether the amplicon is AT- or GC-rich. The NEB Tm Calculator doesn't account for this because, as mentioned previously, multiple temperatures can support primer binding and the NEB Tm Calculator allows users to switch between Q5 products without requiring a change to thermocycling protocols. Using a 60°C annealing temperature and an extension time that accommodates the longest amplicon may allow a single thermocycling protocol to be used for multiple assays in a single PCR workflow.

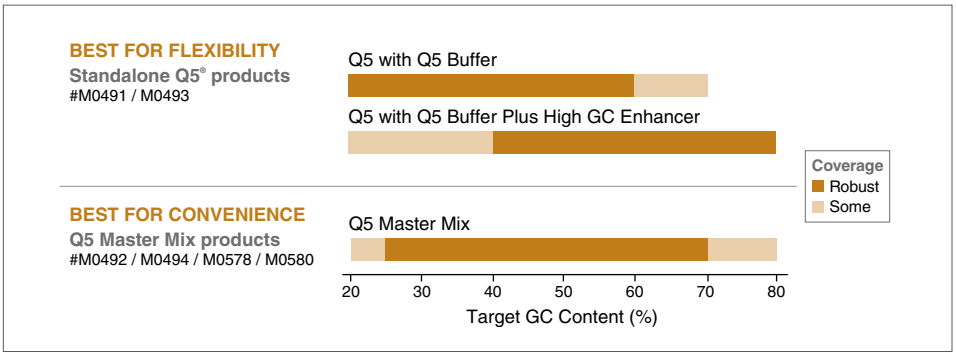
Recommended single annealing temperature protocol for Q5

RECOMMENDED 3-STEP CYCLING PROTOCOL		
STEP	**TEMPERATURE**	**TIME**
Initial Denaturation	98°C	30 seconds
25–35 cycles	98°C	5–10 seconds
	62°C (NEB #M0491/M0493) or 60°C (NEB #M0492/M0494/M0578/M0580)	20 seconds
	72°C	20–30 seconds/kb*
Final Extension	72°C	2 minutes
Hold	4–10°C	

RECOMMENDED 2-STEP CYCLING PROTOCOL (WHEN ALL PRIMER Ta ≥ 72°C)		
STEP	**TEMPERATURE**	**TIME**
Initial Denaturation	98°C	30 seconds
25–35 cycles	98°C 72°C	5–10 seconds 20–30 seconds/kb*
Final Extension	72°C	2 minutes
Hold	4–10°C	

**The extension time can be set to accommodate the longest amplicon in the workflow.*

FIGURE 5: **Q5-High Fidelity 2X Master Mix formats allow robust amplification of a broad range of targets with a single formulation**



CONCLUSION

A single annealing temperature and extension time can be used successfully with Q5 to support multiple different PCR assays simultaneously, offering significant assay flexibility and convenience. However, as with other commercially available polymerases, when the experimental annealing temperature deviates from the optimal primer annealing temperature, reduced product yield (i.e., reaction efficiency) or poor specificity may occur. As a result, when trying to achieve the *best performance* in PCR, we recommend using the optimal annealing temperature as determined by a temperature gradient or the NEB Tm Calculator. However, when *convenience* is desired or necessary, a single annealing temperature for multiple different primer sets can be used.

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