Touchdown PCR for increased specificity and sensitivity in PCR amplification

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Published online 21 August 2008; doi:10.1038/nprot.2008.133

Touchdown (TD) PCR offers a simple and rapid means to optimize PCRs, increasing specificity, sensitivity and yield, without the need for lengthy optimizations and/or the redesigning of primers. TD-PCR employs an initial annealing temperature above the projected melting temperature (T_m) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles. Any difference in T_m between correct and incorrect annealing will produce an exponential advantage of twofold per cycle. TD-PCR has found wide applicability in standard PCR protocols, including reverse transcriptase-dependent PCR, as well as in the generation of cDNA libraries and single nucleotide polymorphism screening. TD-PCR is particularly useful for templates that are difficult to amplify but can also be standardly used to enhance specificity and product formation. The procedure takes between 90 and 120 min, depending on the template length.

INTRODUCTION

Since the first papers on PCR were published[1–3], it has become one of the most standard and basic tools in the biological and medical sciences, and advances in engineering and science have increased its scope and usability. However, the widespread use of PCR as a diagnostic and preparative technique belies the frustrations that many users have experienced, particularly with regard to the consumption of time and resources in refining and troubleshooting the procedure when spurious products are generated. The generation of multiple products is often caused by poor melting temperature (T_m) estimations and improper annealing conditions, in response to which many different methods have been suggested to increase the specificity of the reaction, ranging from the addition of additives to the reaction mix (e.g., DMSO, formamide, betaine and others), variations in salt concentration and altering the annealing temperature, to name a few[4,5]. However, although these elements can be successful to greater or lesser degrees, their efficacy is largely dependent on trail and error, as what proves effective for one primer set may have little, or even a detrimental, outcome on another primer pair reaction. An empirical solution to this problem and a far simpler method to employ is TD-PCR[6], which was developed to abrogate mispriming and the production of nonspecific products. TD-PCR deals specifically with the limitations inherent in T_m calculations and largely alleviates the need for optimization of reactions and redesigning of primers[6,7].

TD-PCR (and its sister technique, stepdown PCR (SD-PCR); see below) has no inherent limitations that are not encountered with most PCR methods, as it simply represents an empirical approach to favoring the most specific primer–template interactions. TD-PCR can be incorporated as a standard part of any PCR to enhance specificity and product formation; however, it also has the potential to largely overcome problems associated with high annealing temperatures required for some primer–template combinations and is particularly useful for difficult-to-amplify templates such as those with extensive secondary structures, high % G + C islands in genomes and targets from organisms with >60% G + C content. In these circumstances, it is especially recommended that TD-PCR be supplemented with a 'hot-start' method[5], outlined in more detail in TROUBLESHOOTING, wherein an essential reaction component is omitted before the first denaturing step or a reversible inhibitor of the polymerase is added, as this makes the amplification of difficult templates more feasible.

The only inherent limitation of the technique is that it is not suitable for quantitative assessment of target concentration, which is ideally performed by real-time PCR approaches under optimized conditions[8].

T_m and primer annealing

The performance of any experiment predicated on hybridization between double-stranded nucleic acids is dependent on the experimental value of the melting temperature (T_m) (ref. 9). This is the temperature at which half the molecules are single stranded and half are double stranded, and the overall fidelity of any successful PCR usually relies on the accurate calculation of this variable, as an incorrect estimation can lead to inappropriate hybridization performance and the amplification of nonspecific products due to spurious priming on related sequences.

The most widely used method of calculating the T_m of oligonucleotide primers is the 4(G + C) + 2(A + T) formula, but although this basic formula has become the most common way of estimating T_m due to its simplicity, it is also considered the least preferred method due to its limitations[10]. This is because the kinetics of DNA duplex formation—particularly with regard to primer–template hybridization—are determined by additional factors beyond nucleotide content, such as the sequence arrangement of nucleotides, the concentrations of each ssDNA, solvent conditions (i.e., salt concentrations and ionic strength) and the concentration of denaturants in the buffer[4,9,11,12]. Attempts at addressing these factors to more accurately estimate the 'true' T_m under particular conditions have yielded more complicated algorithms incorporating additional parameters[11,13–15], but despite either the simplicity or sophistication of these different methods, the experience of many laboratories is that theoretically calculated

melting temperatures remain rough approximations. Indeed, a comparison of the different methods used to estimate T_m seems to reinforce this conclusion, demonstrating large and significant differences between each method[9]. This uncertainty, combined with experimental observations that successful PCRs often occur at annealing temperatures divergent from the theoretical limit, has led some to advocate annealing steps at invariant temperatures (usually 55 °C) in spite of T_m calculations, whereas others advise that the annealing step should be individually optimized for each reaction and primer pair, making it unclear which methodology one should use[4,5].

TD-PCR

Touchdown PCR eliminates many of these uncertainties by empirically addressing the limited approximations made when calculating the melting temperature of PCR primers, regardless of the method used[6]. The key principle in TD-PCR is to employ successively lower annealing temperatures, beginning first with an annealing temperature above the projected T_m, then transitioning to a lower, more permissive temperature over the course of 10–15 cycles. This approach exploits the exponential nature of PCR, where the first stages of annealing and amplification are the most critical in producing the desired product. By decreasing the annealing temperature of the reaction 0.5 °C per cycle, for example, any difference in T_m between correct and incorrect annealing will produce a twofold advantage per cycle, or fourfold per °C; for example, a 5 °C difference would produce a 4^5 (i.e., 10^3) change[6], with the appearance of additional misprimed products reduced or effectively abrogated. This method compensates for the limitations inherent in T_m calculations as well as stochastic variations in PCR that can affect yield and specificity[16], providing a simple and robust method to increase the stringency of the reaction without the need to screen different enhancers, salt concentrations or annealing temperatures. Even for those reactions that are already optimized, TD-PCR continues to yield specific amplicons despite using T_ms below the optimized annealing temperature, suggesting that TD-PCR has the potential to greatly benefit reactions that require an increase in stringency, while having little-to-no deleterious effect on reactions that are already ideal. TD-PCR also has applicability when the degree of complementarity between primers and template is uncertain[17–20], especially when degenerate primers are used[21–24], in reverse transcriptase-dependent PCR[21,25,26], and appears particularly efficient and robust when used in single-nucleotide polymorphism typing[7,27]. This protocol also has the potential to largely overcome the problems associated with high annealing temperatures required for some primer–template combinations and is particularly useful for difficult-to-amplify templates, such as those with extensive secondary structures, high % G + C islands in genomes and targets from organisms with >60% G + C content.

Stepdown PCR

The sister technique of TD-PCR is SD-PCR[16,28]. The only difference between these two techniques is that SD-PCR employs cruder (i.e., larger) blocks of annealing-temperature change, as opposed to the smaller graduations of temperature decrease used in TD-PCR. SD-PCR has been used as an alternative to TD-PCR to eliminate the long and protracted manual programming of each temperature change in early thermocyclers, which lacked an option to automatically adjust temperatures after each annealing step. As most modern thermocyclers now have an option to automatically increase/decrease the temperature after each cycle, SD-PCR is largely unnecessary.

Experimental design

PCR set-up and optimization. A comprehensive review of PCR setup and methodology is beyond the scope of this article, and a basic familiarity with PCR is assumed. However, for a more detailed breakdown of PCR mechanics and parameters, the reader is referred to *Current Protocols in Molecular Biology*[5] or *Methods in Molecular Biology*[4]. Additives or enhancers can also be employed with TD-PCR if higher specificity is required; *Current Protocols in Molecular Biology*[5] and *Methods in Molecular Biology*[4] have compiled a detailed list of these, which can be referred to if necessary. Higher specificity in TD-PCR may also by achieved by using lower concentrations of primers, dNTPs, hot-start PCR or some combination of these. However, care should be taken when altering the concentration of dNTPs and/or primers, as this can affect the amount of free magnesium.

Template preparation. Genomic DNA or cDNA can be used as a template in TD-PCR protocols. To prepare genomic DNA, readers are referred to Strauss in *Current Protocols in Molecular Biology*[29] for a standard method. cDNA sample preparation requires RNA isolation followed by reverse transcription; the acid guanidinium method[30] or commercially available reagents such as Trizol (Invitrogen) are both adequate for RNA isolation, and reverse transcriptase kits such as Superscript III (Invitrogen) are sufficient for cDNA generation, with the methodology performed as directed by the manufacturers' protocols.

Primer design. There are many computer programs available that can be used to design PCR primers, and their use is strongly recommended, as these programs tend to incorporate several different parameters that the manual design of primers may fail to consider (i.e., T_m, intra- and/or intermolecular complementarity, mispriming libraries, etc.). We have had success using Primer3 (ref. 31), which is available for use as an online tool (see http://frodo.wi.mit.edu/primer3/input.htm) as well as being freely available for download. For a more comprehensive review of the parameters of primer design (of which there are many), readers may find it useful to refer to *PCR Primer Design* from Humana Press[32].

T_m calculation. Despite the inherent limitations in T_m calculations previously outlined, it is still necessary to estimate T_m to design an effective thermal cycling program. A useful online tool for T_m calculation and oligonucleotide properties is OligoCalc[10] (http://www.basic.northwestern.edu/biotools/oligocalc.html), which offers basic, salt-adjusted and nearest-neighbor T_m estimations. However, despite the utility of OligoCalc, users should be aware that it still suffers from the limitations inherent in T_m estimations mentioned previously. Regardless of the method used, it is important to calculate T_m in the same manner every time to achieve consistent results.

Cycling program. The cycling program involves two separate phases. Phase 1 is the touchdown phase, which starts with an annealing temperature above the T_m of the primers being used and transitions to a lower annealing temperature over the course of successive cycles. For general applicability, it is recommended that

the first cycling phase begin with an annealing temperature of T_m + 10 °C (where the T_m is calculated according to the user's preferred method; see above), and then decrease the annealing temperature by 1 °C per cycle until the T_m of the primers is reached or slightly bypassed (i.e., T_m 5 °C), for a total of 10–15 cycles.

Phase 2 of the TD program is a generic amplification stage of 20 or 25 cycles using the final annealing temperature reached in Phase 1. Ideally, the total number of cycles of Phases 1 and 2 should not exceed 30 or 35 cycles, as additional cycles beyond this risks the creation of nonspecific products and/or primer dimers.

Controls. Positive and negative control reactions should also be set up using, for example, a plasmid template containing the sequence of interest as a positive control and a reaction lacking template as the negative control.

MATERIALS

REAGENTS

- Template DNA (see Experimental design)
- dNTP mix (dATP, dCTp, dGTP and dTTP) (Promega, cat. no. PROM1145; however, various companies such as Bio-Rad, Invitrogen, Sigma, etc. supply suitable dNTP sets) (see REAGENT SETUP)
- 25 mM $MgCl_2$ solution (Sigma-Aldrich, cat. no. M8266)
- Any commercially available thermostable DNA polymerase, for example, *Taq* DNA Polymerase, native (Invitrogen, cat. no. 18038-042)
- 10× PCR buffer ▲ CRITICAL Most commercially available thermostable DNA polymerases include a 10× PCR buffer, usually containing minor variations of: 100 mM Tris-HCl, 500 mM KCl, pH 8.4–9.0. However, users should be aware that different companies may employ different solvent formulations, which can in turn affect stringency and specificity.
- Oligonucleotide primers. Custom DNA primer synthesis is available from many different suppliers
- Sodium borate (SB) (Sigma-Aldrich, cat. no. 71996)
- Sodium hydroxide (Sigma-Aldrich, cat. no. S5881)
- Trizol (Invitrogen, cat. no. 15596-018)
- Superscript III (Invitrogen, cat. no. 18080-51)
- Xylene cyanol (Sigma-Aldrich, cat. no. X4126)
- Bromophenol blue (Sigma-Aldrich, cat. no. B8062-5G)
- Sodium dodecyl sulfate (Sigma-Aldrich, cat. no. L4390)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- Ficoll 400 (Sigma-Aldrich, cat. no. F4375)
- Orange G (Sigma-Aldrich, cat. no. O3756)
- Agarose (Invitrogen, cat. no. 15510-027)
- Ethidium bromide (Sigma-Aldrich, cat. no. E7637) ! CAUTION Ethidium bromide is a known mutagen and suspected carcinogen. When handling, gloves should be worn at all times, and appropriate care should be taken to avoid skin contact.

EQUIPMENT

- PCR tubes
- Thermal cycler
- Electrophoresis apparatus
- Power supply
- UV transilluminator

REAGENT SETUP

Oligonucleotide primers Prepare stock solutions of primers (e.g., 100 µM) using sterile DNase/RNase-free water. Prepare aliquots of 10× working solution (e.g., 10 µM) and store at −20 °C to prevent contamination of stock and repeat freeze–thaw cycles. Under proper storage conditions, active working solutions of primers that are subject to freeze–thawing should be stable for several weeks, and reserve solutions of primers should be stable for at least a year.

dNTP stock solutions Our lab prefers a 2 mM stock of dNTPs (dATP, dCTp, dGTP and dTTP) in ddH_2O, as opposed to higher concentrations (e.g., 10 mM). We find that this more dilute stock offers greater control, as minor variations in pipetting 10 mM dNTP stock can affect final dNTP concentration, which in turn can influence the free Mg^{2+} concentration and the fidelity of the reaction.

SB electrophoresis buffer 20× SB buffer is prepared with 45 g of SB and 8 g of NaOH in 1 liter of dH_2O, pH 8.5. ▲ CRITICAL We recommend SB buffer as it offers increased conductivity, speed and resolution with decreased heat generation[33,34]. Standard TBE/TAE buffers also perform well.

1–3% (wt/vol) agarose gel Depending on the size of PCR product to be analyzed, it is prepared with the electrophoresis buffer of choice.

DNA gel loading dye A basic 10× recipe is as follows: 6.25 ml of dH_2O, 0.025 g of xylene cyanol, 0.025 g of bromophenol blue, 1.25 ml of 10% (wt/vol) SDS and 12.5 ml of glycerol. For most PCR products, a standard 10× bromophenol blue/xylene cyanol dye is sufficient. Alternately, a 6× dye of 15% (wt/vol) Ficoll 400 and 0.9% (wt/vol) Orange G can be used for those situations where the bromophenol blue or xylene cyanol dye obscures the product of interest.

PROCEDURE

PCR setup ● TIMING 30 min

1| For each primer pair, set up a reaction as follows on ice:

	Initial concentration	Final concentration	Final volume per 50 µl reaction
PCR buffer	10×	1×	5 µl
$MgCl_2$	25 mM	1.5 mM	3 µl
Template DNA[a]	—	—	X µl
dNTP mix	2 mM	200 µM	5 µl
Forward primer	10 µM	200 nM	1 µl
Reverse primer	10 µM	200 nM	1 µl
DNA polymerase	5 U $µl^{-1}$	1.25 U per 50 µl	0.25 µl
dH_2O			Up to 50 µl

[a]The total amount of template added to the reaction is dependent on the starting material being used and will need to be determined empirically by the user. Broadly speaking, the total amount of template added depends on the sequence complexity of the DNA, and can range from picogram, nanogram to microgram amounts when using plasmid, bacterial or mammalian DNA, respectively.

Thermal cycling ● TIMING 90–120 min

2| Program a standard thermocycler to run the reaction using the following parameters:

Phase 1	Step	Temperature	Time
1	Denature	95 °C	3 min
2	Denature	95 °C	30 s
3	Anneal	T_m + 10 °C[a]	45 s
4	Elongate	72 °C	60 s or more[b]
Repeat steps 2–4 (10–15 times)			
Phase 2	**Step**	**Temperature**	**Time**
5	Denature	95 °C	30 s
6	Anneal	T_m or (T_m−5 °C)	45 s
7	Elongate	72 °C	60 s or more[b]
Repeat steps 5–7 (20–25 times)			
Termination	**Step**	**Temperature**	**Time**
8	Elongate	72 °C	5 min
9	Halt reaction	4 °C	15 min
10	Hold	23 °C (room temperature)	Until removed from machine

[a]Every time steps 2–4 are repeated, the annealing temperature should be decreased by 1 °C/cycle, until the estimated T_m of the primers being used is reached or slightly bypassed (approximately 10–15 cycles). [b]An elongation (synthesis) step of 60 s is usually sufficient for target amplicons up to 1 kb in size; for amplicons larger than this, the general rule is 60 s of extension time per kb of product (i.e., a 2.5-kb amplicon would require an extension time of 150 s).

▲ **CRITICAL STEP** Many PCR machines also employ a temperature gradient function, and combining TD-PCR over a gradient offers an additional way to optimize product formation and specificity.

Product electrophoresis ● TIMING 30 min

3| Combine 10–15 µl of the finished reaction with DNA loading dye, resolve the product on an ethidium-bromide-stained agarose gel of the appropriate percentage and visualize with a UV-transilluminator.

? TROUBLESHOOTING

● TIMING

Step 1, PCR setup: 30 min

Step 2, thermal cycling: 60–90 min

Step 3, product electrophoresis and analysis: 30 min

? TROUBLESHOOTING

Lack of expected product, G + C-rich templates and templates with unusual secondary structure

In the case where no product is visible, it is possible that one or more of the reagents used have been omitted and/or are not performing, in which case the performance of the system, and if necessary each individual component, should be checked using a different set of primers and target that are known to amplify reliably.

If the PCR is performing properly, lack of product may be due to poorly designed primers, in which case new primers may be tried. Lack of product may also be due to the presence of unusual secondary structure and/or high G + C content in the template being amplified. One way of dealing with this is to include an additional step in the first cycle where the temperature is spiked to 96 °C or 97 °C for 1 min. Inclusion of this step has been reported to be extremely useful in the amplification of G + C-rich templates, and in many instances, no products were obtained if this spike was omitted[35–38]. The addition of denaturants such as DMSO or formamide to the reaction mix may also assist in amplifying these types of sequences[5,39,40].

In the case of G + C-rich templates or templates with unusual secondary structure, it is especially recommended that TD-PCR be supplemented with a 'hot-start' method, which is outlined in more detail below.

Multiple products, products of incorrect sizes or primer dimers

When spurious products are present, it may be useful to include an additive or enhancer in the reaction mix in an attempt to further increase specificity; *Current Protocols in Molecular Biology*[5] and *Methods in Molecular Biology*[4] have compiled lists of these compounds and their effects. Alternatively, specificity may also be increased by using lower concentrations of primers, dNTPs, using hot-start PCR (discussed in more detail in the following section), reducing the number of cycles or a combination of these. However, for those situations where none of the above are successful, it may be necessary to redesign the primers being used to achieve the required specificity.

Hot-start PCR

Although TD-PCR offers a simple way to enhance any PCR, researchers may find that TD-PCR generates no product, multiple products or products of the incorrect size. In these situations, it is especially recommended that researchers consider using a

'hot-start' method in conjunction with TD-PCR cycling. Hot-start reactions avoid spurious priming by either omitting an essential reaction component before the first denaturing step or by adding a reversible inhibitor of the polymerase. Hot start also increases specificity by avoiding primer-dimer formation in early cycles, making the amplification of difficult templates more feasible. As such, hot-start methods are suitable for situations where no product is visible as well as when spurious products are present. For a more detailed description of the various hot-start methods, of which there are several, readers are referred to *Current Protocols in Molecular Biology*[5] or *Methods in Molecular Biology*[4].

ANTICIPATED RESULTS

Optimally, visualization of a TD-PCR on an ethidium-bromide-stained agarose gel should yield PCR products of the expected size, with reduced or absent nonspecific products as compared with standard PCR cycling. TD-PCR can be used as a standard part of any PCR or reverse transcriptase-dependent PCR and should result in increased specificity and sensitivity, and often yield as well[16,17,19,21]. Even for those reactions that are largely optimized, TD-PCR continues to yield specific amplicons despite using T_ms below the optimized annealing temperature, suggesting that TD-PCR has the potential to greatly benefit reactions that require an increase in stringency, while having little-to-no deleterious effect on reactions that are already optimized. Indeed, the robustness of TD-PCR and the successful experience of our lab with it has led us to incorporate it as a standard part of virtually all of our PCRs.

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