User Manual

Advantage® GC 2 PCR User Manual

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I. Introduction & Protocol Overview

Advantage GC 2 PCR Kit (Cat. Nos. 639119 & 639120) and the **Advantage GC 2 Polymerase Mix** (Cat. No. 639114) facilitate PCR amplification of virtually all GC-rich sequences that are difficult or impossible to amplify by conventional methods. Advantage GC 2 combines the ability to amplify GC-rich sequences (up to 90% GC) with the established benefits of Advantage PCR: high efficiency and specificity due to the use of polymerase mixes and "hot-start" antibodies. The enzyme mix contains Titanium® *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and a minor amount of a proofreading polymerase.

The simultaneous use of two different DNA polymerases in a PCR reaction can permit amplification of significantly longer fragments in a process called "long-distance PCR" ([LD PCR]; Barnes, 1994; Cheng *et al.*, 1994). However, the usefulness of two-enzyme systems is not limited to LD PCR. In fact, most PCR reactions can be significantly improved by using a combination of a primary and a proofreading polymerase.

GC-Melt allows amplification of GC-rich sequences

While most DNA fragments can be amplified by standard PCR methods, some genes contain GC-rich regions that prevent their amplification by standard PCR techniques (Chenchik *et al.*, 1996). Because these GC-rich sequences possess strong secondary structure that resists denaturation and prevents primer annealing, PCR often fails to yield any product. Advantage GC 2 PCR Kits successfully amplify these problematic sequences.

Advantage GC 2 includes an optimized buffer containing DMSO, and an additional reagent, GC-Melt, that further weakens base-pairing in GC-rich sequences (Baskaran *et al.*, 1996). Addition of DMSO alone has been shown to improve amplification of some GC-rich sequences by disrupting base-pairing (Pomp *et al.*, 1991). Incorporation of nucleotide analogs may also improve amplification of some sequences (McConlogue *et al.*, 1988; Gelfand *et al.*, 1990), but these analogs interfere with product analysis by ethidium bromide staining. In contrast, the Advantage GC 2 PCR Kit includes a standard nucleotide mix, permitting staining and analysis by standard techniques. GC-Melt destabilizes DNA secondary structure and effectively makes AT and GC base pairs equally stable. This allows efficient amplification of virtually all of the GC-rich sequences that are resistant to PCR by standard techniques.

Uniform amplification of moderately complex DNAs

The efficient amplification of GC-rich templates by the Advantage GC 2 PCR Kit results in more uniform amplification of moderately complex DNA populations. This ability to amplify DNA fragments with equal efficiency regardless of base composition is useful in multiplex PCR, differential display, sequencing, and other applications requiring uniform amplification of moderately complex cDNA.

I. Introduction & Protocol Overview *continued*

Figure 1 illustrates an experiment in which the Advantage GC 2 PCR Kit was used to amplify a fragment of the insulin-like growth factor receptor II (IGFR II) which contains a 90% GC-rich region. The expected 510 bp fragment was amplified with high efficiency in the presence of 0.5 M and 1.0 M GC-Melt and DMSO-containing buffer. This fragment is amplified less efficiently in the absence of GC-Melt and DMSO. The efficiency of amplification varied with the concentration of GC-Melt.

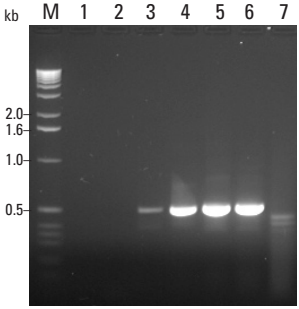

Figure 1. Amplification of a portion of the IGFR II gene with GC-Melt and DMSO. Lane 1: empty. Lane 2: Negative Control (no template). Lane 3: 2 M GC-Melt. Lane 4: 1.5 M GC-Melt. Lane 5: 1 M GC-Melt. Lane 6: 0.5 M GC-Melt. Lane 7: 0 M GC-Melt. Lane M: 1 kb ladder size marker.

Automatic hot start with TaqStart® Antibody

Advantage GC 2 contains built-in, hot-start PCR from TaqStart Antibody included in the polymerase mix. Antibody-mediated hot start with TaqStart has been shown to significantly improve the efficiency and specificity of PCR amplifications by reducing background DNA synthesis (Kellogg *et al.*, 1994). Specifically, this antibody reduces or eliminates nonspecific amplification products and primer-dimer artifacts created prior to the onset of thermal cycling.

TaqStart is a neutralizing monoclonal antibody that recognizes both native and N-terminal deletion mutant forms of *Taq* polymerase. The antibody inhibits enzymatic activity during PCR reaction setup at ambient temperatures. Polymerase activity is restored at the onset of thermal cycling because the antibody is denatured at high temperatures. The loss of inhibition is complete and irreversible, so the polymerase regains its full enzymatic activity for PCR.

Besides increased specificity and sensitivity, the built-in hot start in the Advantage GC 2 Polymerase Mix offers convenience. Other methods of hot start require extra steps such as the addition and premelting of wax beads or the addition of a critical component after the initial denaturation. These extra steps are inconvenient and introduce a potential source of cross-contamination. In contrast, TaqStart provides automatic hot-start PCR

I. Introduction & Protocol Overview *continued*

with virtually no risk of cross-contamination. Thus, TaqStart provides all the advantages of hot-start PCR—with none of the disadvantages of other hot-start methods. The antibody comes already included in the Advantage GC 2 Polymerase Mix; there is no need to add it as a separate reagent during PCR setup.

Which Advantage PCR Kit should you use?

The Advantage GC 2 PCR Kit and Polymerase Mix are designed to provide efficient amplification of moderate-complexity sequences with high GC content, such as the 5' ends of many mammalian cDNAs, that cannot be amplified using conventional PCR techniques. Advantage GC 2 is specially formulated for use in various applications including RACE, RT-PCR, and differential display. It is recommended for use with the following Clontech products:

- SMART™ cDNA Library Construction Kit (Cat. No. 634901)
- SMARTer™ PCR cDNA Synthesis Kit (Cat. No. 634926)
- SMARTer RACE cDNA Amplification Kit (Cat. No. 634924)
- Marathon® cDNA Amplification Kit (Cat. No. 634913)
- Marathon-Ready cDNAs (many)
- Clontech PCR-Select™ cDNA Subtraction Kit (Cat. No. 637401)
- Clontech PCR-Select Bacterial Genome Subtraction Kit (Cat. No. 637404)
- Advantage RT-for-PCR Kit (Cat. No. 639505)
- MTC Panels (many)
- QUICK-Clone™ cDNAs (many)

For amplifying **GC-rich genomic** templates, use our Advantage GC 2 PCR Kit (Cat. Nos. 639119 & 6391120) or Polymerase Mix (Cat. No. 639114). These kits are recommended for use with Clontech's GenomeWalker™ Kits (Cat. Nos. 638901 & 638904) and several LD-Insert Screening Amplimer Sets.

For amplifying **non–GC-rich cDNA** and genomic templates, use the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) or Polymerase Mix (Cat. No. 639201).

Clontech also offers the **Advantage-HF 2 PCR Kit** (Cat. Nos. 639123 & 639124), a Titanium *Taq*-based system which delivers very high fidelity in the amplification of cDNA or genomic templates.

II. List of Components

Advantage GC 2 PCR Kit (Cat. Nos. 639119 [100 rxns] & 639120 [10 rxns])

Store all components at –20°C. Enough reagents are supplied for 100 or 10 PCR reactions of 50 µl each.

10 rxns	100 rxns	
• 10 µl	100 µl	**50X Advantage GC 2 Polymerase Mix**

Includes Titanium *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart Antibody (1.1 µg/µl)—and a minor amount of a proofreading polymerase in the following storage buffer.

Concentration in 50X mix	Component	Final rxn. Concentration
50 %	Glycerol	1.0 %
15 mM	Tris-HCl (pH 8.0)	0.3 mM
75 mM	KCl	1.5 mM
0.05 mM	EDTA	1.0 µM

• **100 µl** **2 x 600 µl** **5X Advantage GC 2 PCR Buffer**

Concentration in 5X mix	Component	Final rxn. Concentration
200 mM	Tricine-KOH (pH 9.2 at 25°C)	40 mM
75 mM	KOAc1	5 mM
17.5 mM	$Mg(OAc)_2$	3.5 mM
25 %	Dimethyl Sulfoxide (DMSO)	5 %
18.75 µg/ml	Bovine serum albumin	3.75 µg/ml
0.025 %	Nonidet P-40	0.005 %
0.025 %	Tween-20	0.005 %

• **200 µl** **2 x 1 ml** **GC-Melt** (5.0 M)

• **15 µl** **120 µl** **50X dNTP Mix** (10 mM each of dATP, dCTP, dGTP, and dTTP; 1X concentration: 0.2 mM each)

• **10 µl** **30 µl** **Control cDNA Template—IGFR II** (100 attomoles/µl)

• **10 µl** **40 µl** **Control Primer Mix** (10 µM each)
5' primer: 5'–TCCCGCTCCGTCTCCACCTCCGC–3'
3' primer: 5'–ACAGGAAGGCAATGCTGCTCTGGA–3'

• **500 µl** **3 x 1.25 ml** **PCR-Grade H_2O**

II. List of Components *continued*

Advantage GC 2 Polymerase Mix (Cat. No. 639114)

Store all components at –20°C. Enough reagents are supplied for 100 PCR reactions of 50 µl each.

- **100 µl Advantage GC 2 Polymerase Mix (50X)**
  (See previous page for component concentrations.)
- **2 x 600 µl 5X GC 2 PCR Buffer**
  (See previous page for component concentrations.)
- **2 x 1 ml GC-Melt** (5.0 M)

III. Additional Materials Required

The following reagents are not supplied.

- Optional: **Mineral oil** (We recommend Sigma Cat. No. M-3516)
- **0.5-ml PCR reaction tubes** (We recommend Applied Biosystems GeneAmp 0.5-ml reaction tubes [Cat. Nos. N801-0737 or N801-0180].)
- **Thermal cycler** (Applied Biosystems GeneAmp 9700 or equivalent)
- **Pipettors dedicated for PCR**
- **PCR pipette tips** suitable for use with the above pipettors and preferably equipped with hydrophobic filters.
- **DNA size markers** (See Section IV.E)
- **5X Stop/loading buffer** (Sambrook *et al.* [2001] provides several recipes.)

In addition, if you have purchased the Advantage GC 2 Polymerase Mix (Cat. No. 639114), you will need the following:

- **50X dNTP mix** (10 mM each of dATP, dCTP, dGTP, and dTTP)
- **Control DNA template**
- **Control primer mix**
- **PCR-grade deionized H_2O** (filter-sterilized). At Clontech, we use Millipore-filtered H_2O for most PCR applications. **Do not use autoclaved H_2O for PCR.** The recycled steam in some autoclaves can introduce salts and other contaminants that may interfere with PCR reactions.

IV. Advantage®-GC 2 PCR Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

1. Primer design

   Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. *Always check and recheck your primer design before constructing or ordering primers.*

   Length and GC content: The Advantage GC 2 Polymerase Mix can be used in a wide variety of PCR applications, and the constraints on primer design will vary from one application to the next. In general, however, for amplification of GC-rich sequences, primers should have a T_m of at least 70°C to achieve optimal results in a two-step cycling program with a 68°C annealing/extension step. Therefore, whenever possible, primers should be at least 22 nucleotides (nt) long (25–30-mers are preferred) and should have a GC content of 50–60%.

2. Template quality

   Because PCR amplification proceeds exponentially, many conventional PCR applications work well with templates of average or even low quality. In many applications, the Advantage GC 2 Polymerase Mix will also tolerate a wide range of template quality.

   However, the longer or more complex the target, the more important template quality becomes. This is because the number of unnicked, full-length targets decreases as the target length increases, so poor- quality DNA will have very few large, unnicked targets. Furthermore, some depurination occurs when DNA is denatured during thermal cycling, and this can lead to truncated products. Therefore, it is particularly important to prepare high-quality, high molecular-weight DNA when amplifying long targets.

   Template quality is also important when the highest possible sensitivity is needed. In cDNA applications such as RACE and other RT-PCR protocols, incomplete reverse transcription can lead to an absence of product, truncated products, or a mix of truncated and full-length products, resulting in a smeared band on a gel. This problem can be minimized by ensuring that your starting material is of the highest quality.

   For 5' and 3' RACE and general PCR from cDNA, you can ensure the quality of your cDNA by using Marathon-Ready cDNA from Clontech.

IV. Advantage®-GC 2 PCR Protocol *continued*

3. Good PCR practices
   a. Prepare reactions with dedicated pipettors in a dedicated workspace

      Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can produce nonspecific amplification; in some instances, contaminants can cause DNA bands even in the absence of added template DNA. We recommend that you use small aliquots of starting material to avoid contaminating your stocks. You should set up your PCR reactions in a dedicated lab area or noncirculating containment hood and use dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. Finally, perform post-PCR analysis in a separate area using a separate set of pipettors.

   b. Pipetting

      Because of the small volumes used in PCR experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture, deliver the contents from the pipette tip into the mixture, and pipet up and down several times.

   c. Use a Master Mix

      Assembling a Master Mix, which contains the appropriate volumes of all reagents required for multiple PCR reactions, saves time and greatly reduces tube-to-tube variation. If multiple templates are being tested with the same primers, include the primers in the Master Mix. If one template is being tested with multiple primer sets, include the template in the Master Mix. If you are setting up several sets of parallel samples, assemble multiple Master Mixes (e.g., each with a different set of primers). *The Master Mix should be thoroughly mixed before use (i.e., vortexed without bubbling).*

   d. Always include positive and negative controls (i.e., H_2O instead of DNA template).

4. Touchdown PCR

   "Touchdown" PCR can significantly improve the specificity of many PCR reactions in a wide variety of applications (Don *et al.*, 1991; Roux, 1995). Briefly, touchdown PCR involves using an annealing/extension temperature that is several degrees (typically 3–10°C) higher than the T_m of the primers during the initial PCR cycles (typically 5–10 cycles). The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles.

IV. Advantage®-GC 2 PCR Protocol *continued*

5. TaqStart Antibody provides automatic hot-start PCR

   The use of a manual hot start or wax bead-based hot start is not required when using Advantage GC 2. As discussed in the Introduction, hot start is automatic because the enzyme mix already contains TaqStart Antibody.

6. Use of additives

   TaqStart Antibody binds N-terminal deletion mutant *Taq* polymerase with high affinity under the conditions described in this protocol. The DMSO in the reaction buffer will not interfere with TaqStart function. However, the addition of formamide or other cosolvents may disrupt TaqStart function. Furthermore, excessive glycerol, solutes (e.g., salts), pH extremes, or other deviations from the recommended reaction conditions may reduce the effectiveness of the antibody and/or DNA polymerases.

7. Advantage GC 2 is not intended for certain applications

   Because of the improved fidelity from long and accurate PCR, Advantage GC 2 is not recommended for mutagenesis protocols involving so-called "sloppy" PCR.

IV. Advantage®-GC 2 PCR Protocol *continued*

B. Determining the Optimal Concentration of GC-Melt

The preparation of PCR reactions with Advantage GC 2 Polymerase Mix will vary depending on your specific application. The following example, using a 50-µl reaction volume, is intended as a guide for determining the optimal concentration of GC-Melt for amplification of your specific sequence. The PCR parameters given here (i.e., amount of template and primer, cycling recommendations) are intended as guidelines only. Refer to Section V (Troubleshooting) for suggestions on improving results.

This optimization routine should be performed initially with any new GC-rich sample that you intend to amplify using the Advantage GC 2 Polymerase Mix. The concentration of GC-Melt that yields maximal amplification of the desired product may then be used in all subsequent reactions involving that sample.

On many templates, we have found a GC-Melt concentration of **0.5 M** to be optimal and, with experience, you may find this to be a satisfactory starting point for most applications.

1. Place all components at room temperature and allow to thaw completely. Mix each component thoroughly before use.
2. Combine the following reagents in a 0.5-ml PCR tube (all component amounts are in µl; 50 µl final reaction volume). See Section II for final concentrations of enzyme and buffer components.

Final GC-Melt conc. (M)				
0	**0.5**	**1.0**	**1.5**	
35	30	25	20	PCR-grade H_2O
10	10	10	10	5X GC 2 PCR Buffer
0	**5**	**10**	**15**	**GC Melt (5 M)**
1	1	1	1	DNA template (~100 ng/µl)
2	2	2	2	Primer mix (10 µM each)
1	1	1	1	50X dNTP mix (10 mM each)
1	1	1	1	Advantage GC 2 Pol. Mix (50X)
50	**50**	**50**	**50**	**Total volume**

3. Mix well and spin the tube briefly to collect all the liquid in the bottom of the tube.
4. If your thermal cycler does not have a "hot lid", add 1–2 drops of mineral oil to prevent evaporation during cycling. A good "seal" of mineral oil should have a well-defined meniscus between the two phases. Cap the PCR tubes firmly.
5. Commence thermal cycling. For a guide to cycling parameters, refer to Section IV.C.

IV. Advantage®-GC 2 PCR Protocol *continued*

C. Control PCR Reactions

The following PCR reactions can be performed in parallel with your experiments as controls to ensure that the Advantage GC 2 Polymerase Mix is working properly.

1. Place all components at room temperature and allow to thaw completely. Mix each component thoroughly before use.
2. Combine the following reagents in a 0.5-ml PCR tube (50-µl final reaction volume). See Section II for final concentrations of enzyme and buffer components.

Positive Control	Negative Control	[Optional] Negative Control*	
30 µl	31 µl	35 µl	PCR-grade H_2O
10 µl	10 µl	10 µl	5X GC 2 PCR Buffer
5 µl	5 µl	–	GC-Melt (5 M)
1 µl	–	1 µl	Control DNA template
2 µl	2 µl	2 µl	Control primer mix
1 µl	1 µl	1 µl	50X dNTP mix
1 µl	1 µl	1 µl	50X Advantage GC 2 Pol. Mix
50 µl	50 µl	50 µl	Total volume

*To observe the benefit conferred by the use of GC-Melt (as shown in Figure 1), this negative control reaction, *without* GC-Melt, may be prepared.

Note: Since this kit is intended for the amplification of GC-rich sequences that fail to amplify with other PCR kits or enzyme mixes/PCR reaction buffers, another negative control reaction may be prepared with your DNA template and a conventional enzyme mix and PCR reaction buffer.

3. Mix well and spin the tube briefly to collect all the liquid in the bottom of the tube.
4. If your thermal cycler does not have a "hot lid", add 1–2 drops of mineral oil to prevent evaporation during cycling. A good "seal" of mineral oil should have a well-defined meniscus between the two phases. Cap the PCR tubes firmly.
5. Commence thermal cycling using the following parameters:
   - 94°C for 3 min
   - 25-30 cycles*
     94°C for 30 sec
     68°C for 1.5 min
   - 68°C for 3 min

*25–30 cycles with a 1.5-min annealing/extension time is sufficient for amplification of the positive control template provided in the kit. Other templates may require more cycles and different annealing/extension times. (See Section IV.D.)

IV. Advantage®-GC 2 PCR Protocol *continued*

6. Transfer a 5-µl sample of your PCR reaction to a fresh tube and add 1 µl of 5X stop/loading buffer. Analyze your sample(s), along with suitable DNA size markers, by electrophoresis on a 1.5% agarose/EtBr gel.

Expected results: If you are using the positive control reagents provided in the kit, the reaction should produce a major band of 510 bp. No bands should be generated in the negative (i.e., no DNA template) control.

D. Recommended Cycling Parameters

Use the following guidelines when setting up your initial experiments with the Advantage GC 2 Polymerase Mix. These are general guidelines—the optimal parameters may vary with different thermal cyclers and will depend on your particular primers and templates, and on other experimental variables.

Target Size	Cycle Parameters
< 5 kb:	• 94°C for 3 min • 25–35 cycles[A] 94°C 30 sec [B] 68°C 3 min [C] • 68°C for 3 min [D]
5–9 kb:	• 94°C for 3 min • 25–35 cycles[A] 94°C 30 sec [B] 68°C 6 min [C] • 68°C for 6 min [D]
10–20 kb:	• 94°C for 3 min • 25–35 cycles[A] 94°C 30 sec [B] 68°C 12 min [C] • 68°C for 12 min [D] • Soak at 15°C

[A] Initially, run samples for 25 cycles. If the expected product is not observed upon analysis, run an additional 5–10 cycles. For most applications, we prefer two-step cycles (denaturation at T_1 followed by annealing and extension at T_2) instead of three-step cycles (denaturation at T_1 followed by annealing at T_2 followed by extension at T_3). Three-step cycles will be necessary when the T_m of the primers is less than 60–65°C and in certain special protocols.

[B] Use the minimal possible denaturation time. In some cases, better results may be obtained by using a 15-sec denaturation at 94°C. With fast cyclers (e.g., the PE 9600), denaturation times as short as 10 sec may be used. Exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to truncation. High temperature also leads

IV. Advantage®-GC 2 PCR Protocol *continued*

to gradual loss of enzyme activity. Minimizing denaturation time is particularly important in experiments with very large templates where total cycling time can exceed 12 hrs.

[C] Use the maximum possible annealing/extension temperature. See Note A.

We recommend using 1 min per kb of expected target.

[D] Optional: This final extension may reduce background in some cases.

E. Recommendations for Electrophoresis

We recommend that you transfer a 5-µl sample of your PCR reaction to a fresh tube and add 1 µl of 5X stop/loading buffer. (The remaining 45 µl of the reaction mixture can be subjected to further cycling if you do not see a product.) Analyze your sample(s), along with suitable DNA size markers, by electrophoresis on a suitable agarose gel containing 0.1 µg/ml EtBr. The percentage agarose and the DNA size markers you choose will depend on the expected range of insert sizes. You may wish to refer to the following general guidelines before assembling your gel.

Recommendations for agarose gels:

Expected insert size range	Recommended % agarose	Recommended DNA size markers
0.3 – 1.5 kb	1.5	ϕX174/*Hae* III
0.5 – 10 kb	1.2	1-kb DNA ladder
> 5 kb	0.8	λ/*Hin*d III

V. Troubleshooting Guide

The following **general** guidelines apply to most PCR reactions. However, no attempt has been made to address troubleshooting for all of the many applications for which Advantage GC 2 Polymerase Mix can be used. When using the mix with a Clontech companion product, additional, application-specific troubleshooting information can be found in the relevant *Protocol*.

A. No product observed

Concentration of GC-Melt is too high or too low	The range of GC-Melt recommended in this protocol is suitable for most sequences. The optimal concentration of GC-Melt will differ for every individual sequence. Initially, it is crucial to perform the optimization reactions outlined in Section IV.B.
PCR component missing or degraded	Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent.
Too few cycles	Increase cycle number (3–5 additional cycles at a time).
Annealing temp. too high	Decrease the annealing temperature in increments of 2–4°C. **Note:** GC-Melt can reduce the T_m of PCR primers by several degrees.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt, try using a longer primer. The optimal primer length for use with the Advantage GC 2 Kits is 25–30 nt. If the original primer(s) had a GC content of less than 50%, try to design a primer with a GC content of 50–60%.
Not enough template	Repeat PCR using a higher concentration of DNA (after trying more cycles).
Poor template quality	Check template integrity by electrophoresis on a standard TBE-agarose gel. If necessary, repurify your template using methods that minimize shearing and nicking.
Denaturation temp. too high or low	Optimize denaturation temperature by decreasing or increasing it in 1°C increments. (A denaturation temperature that is too high can lead to degradation of the template, especially for long target sequences.)

V. Troubleshooting Guide *continued*

Denaturation time too long or too short	Optimize denaturation time by decreasing or increasing it in 10-sec increments. (A denaturation time that is too long can lead to degradation of the template, especially for long target sequences.)
Extension time too short	Especially for longer templates, increase the extension time in 1-min increments.
Too little enzyme	The Advantage GC 2 Polymerase Mix is 50X for most applications. Therefore, try to optimize the cycle parameters as described above before increasing the enzyme concentration. In rare cases, the yields can be improved by increasing the concentration of the enzyme mix. However, increasing the concentration >2X is likely to lead to higher background levels.
[Mg^{2+}] is too low	The Advantage GC 2 Polymerase Mix performs well over a broad range of Mg^{2+} concentration. Therefore, as long as you use the buffer included with the mix and a final concentration of 0.2 mM of each dNTP, it is unlikely that a lack of product is due to problems with the Mg^{2+} concentration. However, high concentrations of EDTA or other metal chelators in the template stock solution can reduce the effective concentration of Mg^{2+} to below a minimum level.
[dNTPs] is too low	When used as recommended, the 50X dNTP mix provided with the kit gives a final concentration of 0.2 mM of each dNTP. In our experience, this concentration of dNTPs is suitable for a wide range of applications. If you are preparing your own dNTPs, be sure that your final concentration of each dNTP in the reaction is 0.2 mM. Some manufacturers recommend using concentrations higher than 0.2 mM of each dNTP when amplifying large templates. However, we have had no trouble amplifying large templates using 0.2 mM for each dNTP. We have gone up to 35 kb with the Advantage Genomic PCR Kit, so it is unlikely that dNTPs are limiting. Note that if you increase the concentration of dNTPs, you will also need to increase the [Mg^{2+}] proportionately.

V. Troubleshooting Guide *continued*

B. Multiple products

Too many cycles	Reducing the cycle number may eliminate non-specific bands.
Annealing temp. too low	Increase the annealing/extension temperature in increments of 2–3°C.
Touchdown PCR needed	"Touchdown" PCR significantly improves the specificity of many PCR reactions in various applications (Don *et al.*, 1991; Roux, 1995). Touchdown PCR involves using an annealing/extension temperature that is several degrees *higher* than the T_m of the primers during the initial PCR cycles. The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles. The change can be performed either in a single step or in increments over several cycles.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt, try using a longer primer. If the original primer(s) had a GC content of less than 50%, try to design a primer with a GC content of 50–60%.
Contamination	See Section V.D.

C. Products are smeared

Too many cycles	Reduce the cycle number by 3–5 to see if non-specific bands go away.
Denaturation temp. too low	Try increasing the denaturation temperature in increments of 1°C.
Extension time too long	Decrease the extension time in 1–2-min increments.
Poor template quality	Check template integrity by electrophoresis on a denaturing agarose gel. Repurify your template if necessary.
Touchdown PCR needed	See "Touchdown PCR needed" under previous section.
Too much enzyme	The Advantage GC 2 Polymerase Mix is 50X for most applications; however, a 1X final concentration of the enzyme mix may be too high for some applications. If smearing is observed, first try optimizing the cycle parameters as described above, then try reducing the enzyme concentration to 0.5–0.2X.

V. Troubleshooting Guide *continued*

$[Mg^{2+}]$ is too high	Titanium *Taq* DNA Polymerase performs well over a broad range of $[Mg^{2+}]$ with no loss of efficiency. Therefore, as long as you have used the buffer supplied in the kit and a final concentration of 0.2 mM of each dNTP, it is unlikely that smearing is due to problems with the Mg^{2+} concentration.
Too much template	Try a lower concentration of DNA template in the PCR reaction.
Contamination	See Section D below.

D. Dealing with contamination

Contamination most often results in extra bands and/or smearing. It is important to include a negative control (i.e., a control using deionized H_2O instead of the DNA template but that still includes the primers) in every PCR experiment to determine if the PCR reagents, pipettors or PCR reaction tubes are contaminated with previously amplified targets.

If possible, set up the PCR reaction and perform the post-PCR analysis in separate laboratory areas with separate sets of pipettors.

Laboratory benches and pipettor shafts can be decontaminated by depurination. Wipe surfaces with 1 N HCl followed by 1 N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) and rinse with distilled H_2O.

We advise using commercially available aerosol-free pipette tips.

There is an enzymatic method for destroying PCR product carryover (Longo *et al.*, 1990). It involves incorporation of dUTP into the PCR products and subsequent hydrolysis with uracil-N-glycosylase (UNG).

If contamination problems persist, it may be necessary to design new PCR primers and repeat your experiments.

When performing PCR directly on phage plaques or bacterial colonies, failure to isolate single plaques or colonies will also produce multiple bands.

VI. References

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