Takara Bio USA

Advantage® 2 PCR Enzyme System User Manual

Cat. Nos. 639207, 639206, 639201, 639202, 639245, 639147, 639148
PT3281-1 (111816)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

I. Introduction

The Advantage 2 Polymerase Mix and Advantage 2 PCR Kit (which includes the Polymerase Mix) produce efficient, accurate, and convenient amplification of DNA from any template.

The Advantage 2 Polymerase Mix is comprised of 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.

Titanium *Taq* provides the most sensitive and robust capabilities of any *Taq*-derived polymerase. Its increased sensitivity and robust nature are especially useful for amplifying a wide size range of DNA fragments, cDNAs of rare transcripts, or products from complex templates.

The higher yields and increased sensitivity that Titanium *Taq* provides translate into two major advantages over conventional polymerases. First, targets can be amplified using fewer PCR cycles, saving time and lowering background in any given experiment. Second, in situations where the amplification target is present at extremely low levels (e.g., amplifying a rare cDNA in an RT-PCR experiment or detecting viral nucleic acid), the high sensitivity obtained with Titanium *Taq* allows successful amplification of your target where other polymerases fail.

Titanium *Taq* allows you to perform PCR without tedious buffer optimization. In any given reaction, Titanium *Taq* tolerates a wide range of Mg^{2+} concentrations resulting in higher yields, especially for longer amplicons. Magnesium is already included at a set concentration in the Advantage 2 PCR Buffer, eliminating the need to add Mg^{2+} as a separate component during reaction setup. In contrast, native *Taq* polymerase only functions well over a narrow [Mg^{2+}] range, and different reactions may require different concentrations of Mg^{2+}. By eliminating the need to perform experiments for determining the optimal Mg^{2+} concentration, Titanium *Taq* saves considerable time and effort. In cases where background amplification is a problem, we recommend the use of 10X Advantage 2 SA PCR Buffer.

The 10X Advantage 2 SA PCR Buffer, available separately (Cat. No. 639147 & 639148), has been optimized for applications using genomic DNA, and in particular with products less than 1–2 kb. We have found yields to be comparable to that obtained with Advantage 2 PCR Buffer for relatively small amplicons; however, for amplicons > 2 kb, we recommend the use of the Advantage 2 PCR Buffer for best results. The 10X Advantage 2 SA PCR Buffer contains Mg^{2+} at a concentration optimized for most applications, particularly those utilizing a genomic DNA template. In addition, the Advantage 2 SA PCR Buffer is recommended when background amplification is present, which may sometimes result as a by-product of the sensitivity and robustness of the Advantage 2 Polymerase Mix.

A. The Advantage Polymerase Systems

The simultaneous use of two different DNA polymerases (primary and proofreading) in a PCR reaction allows amplification of significantly longer fragments in a process known as long and accurate PCR (or 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, the efficiency of most PCR reactions can be significantly improved by using the two-enzyme combination.

The Advantage 2 system offers three primary benefits over conventional, single-polymerase PCR:

- **Increased range.** Whereas the upper limit of conventional PCR using a *Taq* polymerase is ~3 kb (and much lower in many applications), Advantage 2 gives consistent and efficient amplifications of up to 18 kb or more when using two nondegenerate primers of sufficient length to amplify an abundant, noncomplex template. It can also amplify high-complexity (i.e., genomic) DNA templates up to 6 kb. The absolute upper limit in any particular application will depend on the particular primers, the template used, and other experimental variables.

- **Increased fidelity.** The inclusion of a minor amount of a proofreading polymerase results in an error rate that is 3-fold lower than that of conventional PCR using *Taq* alone (Barnes, 1994; Frey et al., 1995; Nelson et al., 1995). In our studies, Advantage 2 exhibits an error rate of 25 errors per 100,000 bp after 25 PCR cycles. Note that the presence of organic solvents or salts in the reaction can decrease fidelity. High fidelity is a particularly important feature when the amplification products will be used in subsequent experiments (e.g., cloning, sequencing, functional assays, expression systems, etc.).

- **Increased efficiency and greater yields.** While range and fidelity are the most commonly noted aspects of long and accurate PCR, the use of a two-polymerase system also increases the efficiency and yield—and therefore the sensitivity—of all PCR assays, even for templates that are well within the range of conventional PCR. While DNA polymerases with proofreading activity offer better accuracy than LD PCR methods when used alone, they lack the increased efficiency and size-range flexibility possible with Advantage 2 system for long and accurate PCR.

B. Automatic hot start with TaqStart Antibody

The Advantage 2 system contains built-in, hot-start PCR from TaqStart Antibody included in the polymerase mix. Antibody-mediated hot start using TaqStart Antibody 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 *Taq* and N-terminal deletions such as Titanium *Taq*. 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 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 with virtually no risk of cross-contamination. Thus, TaqStart Antibody 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 2 Polymerase Mix; there is no need to add it as a separate reagent during PCR setup.

C. Recommended uses for Advantage 2 Products

The Advantage 2 Mix and Kit are the recommended polymerase systems for use in applications involving RACE, RT-PCR, cDNA synthesis and library construction, cDNA subtraction and differential display, high-performance cloning, and RNA fingerprinting. The Advantage 2 System has been optimized for use with all Clontech PCR-based application kits, including SMART® cDNA Library Construction, SMARTer® PCR cDNA Synthesis, SMARTer RACE cDNA Amplification, Clontech PCR-Select™ Subtraction, Marathon® cDNA Amplification, and GenomeWalker™ Kits.

II. List of Components

Advantage 2 PCR Kit (Cat. Nos. 639207 & 639206)

Store all components at –20°C. Enough reagents are supplied for 30 or 100 PCR reactions of 50 µl each.

639207	639206	
30 rxns	**100 rxns**	
• **30 µl**	**100 µl**	**50X Advantage 2 Polymerase Mix** Includes Titanium *Taq* DNA Polymerase, a small amount of proofreading polymerase, and TaqStart Antibody (1.1 µg/µl) in the following storage buffer:

Concentration in 50X mix	Component	Final rxn concentration
50%	Glycerol	1%
15 mM	Tris-HCl (pH 8.0)	0.3 mM
75 mM	KCl	1.5 mM
0.05 mM	EDTA	1.0 µM

• **200 µl** **600 µl** **10X Advantage 2 PCR Buffer**

Concentration in 10X mix	Component	Final rxn concentration
400 mM	Tricine-KOH (pH 8.7 at 25°C)	40 mM
150 mM	KOAc	15 mM
35 mM	$Mg(OAc)_2$	3.5 mM
37.5 µg/ml	BSA	3.75 µg/ml
0.05%	Tween 20	0.0005%
0.05%	Nonidet-P40	0.0005%

- **200 µl** **600 µl** **10X Advantage 2 SA PCR Buffer**
- **50 µl** **120 µl** **50X dNTP Mix** (10 mM each of dATP, dCTP, dGTP, and dTTP; final rxn concentration: 0.2 mM each)
- **30 µl** **100 µl** **Control DNA Template** (100 ng/µl)
  Calf Thymus DNA
- **30 µl** **100 µl** **Control Primer Mix (10 µM each)**
  5' primer: 5'–GCAACTGCAGGAAGAGCAAGAAATGCA–3'
  3' primer: 5'–TGGCACGGCCATAAGAGGTAGATGTCA–3'
- **2.5 ml** **5.0 ml** **PCR-Grade Water**

Advantage 2 Polymerase Mix (Cat. Nos. 639201, 639202 & 639245)

Store all components at –20°C. Enough reagents are supplied for 100, 500 or 2,000 PCR reactions of 50 µl each.

639201 100 rxns	639202 500 rxns	639245 2,000 rxns	
100 µl	**5 x 100 µl**	**2 x 1 ml**	**50X Advantage 2 Polymerase Mix** See previous page for component concentrations
600 µl	**5 x 600 µl**	**1 x 12 ml**	**50X Advantage 2 PCR Buffer** See previous page for component concentrations
600 µl	**5 x 600 µl**	**1 x 12 ml**	**10X Advantage 2 SA PCR Buffer**

10X Advantage 2 SA PCR Buffer (Cat. Nos. 639147 & 639148)

Store at –20°C. Enough buffer is supplied for 240 or 2,000 PCR reactions of 50 µl each.

639147 240 rxns	639148 2,000 rxns	
• **2 x 600 µl**	**10 ml**	**10X Advantage 2 SA PCR Buffer** Buffer alone.

Concentration in 10X mix	Component	Final rxn concentration
100 mM	Tris-HCl (pH 8.5)	10 mM
500 mM	KCl	50 mM
20 mM	$MgCl_2$	2 mM

III. Additional Materials Required

The following reagents are not supplied.

- **Thermal cycler**
- **Dedicated pipettors** (1–2 µl, 1–10 µl, 1–20 µl, 20–200 µl, 200–1000 µl)
- **PCR pipette tips** suitable for use with the above pipettors and preferably equipped with hydrophobic filters.
- **DNA size markers** (See Section V.D.)
- **5X Stop/loading buffer** (Sambrook & Russell [2001] provides several recipes)
- **PCR-grade water**
- Avoid using autoclaved H_2O because recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- **PCR reaction tubes**
- [Optional]: **Mineral oil** (We recommend Sigma Cat. No. M3516.)

If you have purchased the **Advantage 2 Polymerase Mix** alone (Cat. Nos. 639201 & 639202), you will need the following:

- dNTP mix
- appropriate control template and primers

If you have purchased the **Advantage 2 SA PCR Buffer** alone (Cat. Nos. 639147 & 639148), you will need the following:

- heat-stable DNA Polymerase
- dNTP mix
- appropriate control template and primers

IV. General Considerations

A. 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 G-C content: The Advantage 2 system 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, primers should have a Tm of approximately 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 G-C content of 45–60%. Furthermore, the 3'-terminal ends of each primer should not be complementary to each other and should contain a low G-C content.

B. Template Quality

Because PCR amplification proceeds exponentially, many conventional PCR applications work well with templates of average or even low quality. In many applications (such as screening cDNA inserts with LD Insert Screening Amplimers), long and accurate PCR with Advantage 2 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 large targets.

Template quality is also important when the highest possible sensitivity is needed. In cDNA applications such as RACE and RT-PCR protocols, incomplete reverse transcription can lead to an absence of product, truncated products, or a mix of truncated and full-length product, 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 Takara Bio.

C. Amplicon Size

Yields are unaffected when amplifying products less than 2 kb, whether the 10X Advantage 2 SA PCR Buffer or the Advantage 2 PCR Buffer is used. We recommend initial use of the 10X Advantage 2 PCR Buffer; however, if a problem arises, in particular with amplicons less than 2 kb in length, then we recommend switching to the 10X Advantage 2 SA PCR Buffer. For products greater than 2 kb, we recommend the original Advantage 2 PCR Buffer be used (rather than the SA PCR Buffer).

D. Background Amplification

Due to the sensitivity and robustness of the Advantage 2 Polymerase Mix, non-specific background amplification may sometimes result. The 10X Advantage 2 Short Amplicon (SA) PCR Buffer has been specifically engineered to address this issue. We recommend the use of this buffer in those applications where some background amplification may result.

E. Good PCR Practices

1. Prepare reactions with dedicated pipettors in a dedicated work space

   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. When performing PCR, you should wear gloves and set up your reactions in a dedicated lab area or noncirculating containment hood using dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend setting up a negative control reaction that does not contain any template. Finally, perform post-PCR analysis in a separate area using a separate set of pipettors.

2. 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.

3. 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).

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

F. Additional Considerations

1. 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 Tm of the primers during the initial PCR cycles (typically 5–10 cycles). The annealing/extension temperature is then reduced to the primer Tm for the remaining PCR cycles.

2. 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 the Advantage 2 system. As discussed in the Introduction, hot start is automatic because the enzyme mix already contains TaqStart Antibody.

3. Half-life

   The half-life of Titanium *Taq* depends on the specific reaction conditions used, but generally ranges from 20–40 min at 95°C.

4. Use of additives

   TaqStart Antibody binds Titanium *Taq* DNA Polymerase with high affinity under the conditions described in this protocol. The addition of 2–5% DMSO will not interfere with TaqStart antibody function and may improve results in some instances. However, the addition of formamide or other cosolvents may disrupt TaqStart antibody 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.

5. Advantage 2 Polymerase Mix is not intended for certain applications

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

6. T/A Cloning

   Titanium *Taq* PCR products are compatible with T/A cloning methods. However, for optimal ligation efficiencies, we recommend that you use PCR products immediately (<1 day) after amplification. The single 3' A-overhangs on the PCR products will degrade over time, reducing the efficiency. For best results, please observe the T/A cloning tips in Section V.C.

V. Advantage 2 PCR Procedure

Please read the entire protocol before starting.

A. Control PCR Reactions

The following control PCR reactions should be performed in parallel with your experiments to ensure that the Advantage 2 Polymerase Mix is working properly. A positive control template and primers are provided in the Advantage 2 PCR Kit. When using the Advantage 2 Polymerase Mix with our other PCR-based applications kits, use the positive controls provided with those kits.

1. Place all components on ice and allow to thaw completely. Mix each component thoroughly before use.
2. Combine the following reagents in a PCR tube.

Positive Control	Negative Control	
40 µl	41 µl	PCR-Grade Water
5 µl	5 µl	10X Advantage 2 PCR Buffer
1 µl	—	Control DNA Template (100 ng/µl)
2 µl	2 µl	Control Primer Mix (10 µl ea.)
1 µl	1 µl	50X dNTP Mix (10 mM ea.)
1 µl	1 µl	50X Advantage 2 Polymerase Mix
50 µl	**50 µl**	**Total volume**

3. Mix well and spin tube briefly to collect all the liquid in bottom of tube.
4. [Optional]: 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:

   - 95°C for 1 min
   - 30 cycles*
     95°C for 15 sec
     68°C for 3 min

   * 30 cycles with a 3-min annealing/extension time is sufficient for amplification of the positive control template provided in the kit. Other templates may require more or less cycles and different annealing/extension times (See Section V.B.).

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 1.2% agarose/EtBr gel.

   Expected results: If you are using the positive control reagents provided in the kit, the reaction should produce a single major fragment of 3.5 kb, derived from the gene for the bovine pancreatic trypsin inhibitor. No bands should be generated in the negative (i.e., no DNA template) control.

B. Recommended Cycling Parameters for all Advantage 2 PCR Products

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

NOTES:

- When using the Advantage 2 Polymerase Mix with our other PCR-based application kits (such as SMART cDNA Library Construction, SMARTer PCR cDNA Synthesis, Clontech PCR-Select Subtraction Kits, Marathon cDNA Amplification, or Marathon-Ready cDNAs, use the parameters recommended in the protocol for that kit.
- If you intend to capture your PCR product by T/A cloning, we recommend that you add an additional 10-min extension at 70°C, and then immediately clone or freeze the PCR product. Do not store the reaction at 4°C. These steps will help ensure the incorporation and preservation of 3' A-overhangs.

Target Size	Cycle Parameters
<1 kb:	• 95°C for 1 min • 25–35 cycles[a] 95°C for 30 sec[b] 68°C for 1 min[c] • 68°C for 1 min[d]
1–5 kb:	• 95°C for 1 min • 25–35 cycles[a] 95°C for 30 sec[b] 68°C for 1 min[c] • 68°C for 1 min[d]
5–9 kb:	• 95°C for 1 min • 25–35 cycles[a] 95°C for 30 sec[b] 68°C for 6 min[c] • 68°C for 6 min[d]
10–20 kb:	• 95°C for 1 min • 25–35 cycles[a] 95°C for 30 sec[b] 68°C for 12 min[c] • 68°C for 12 min[d]

a 25 cycles for multiple-copy genes or medium-to-high abundance cDNAs; 30–35 cycles for single- or low-copy-number genes or rare cDNAs. For most applications, we prefer two-step cycles (denaturation at T1 followed by annealing and extension at T2) instead of three-step cycles (denaturation at T1 followed by annealing at T2 followed by extension at T3). Three-step cycles will be necessary when the Tm 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 modifying the denaturation step (94°C for 15 sec). Exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to strand scission. High temperature also leads 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 hr.

c Use the maximum possible annealing/extension temperature. See "Note a". Some researchers prefer to use an annealing/extension time equal to the expected target size (in kb) plus two minutes. We recommend using 1 min per kb of expected target.

d Optional: This final extension may reduce background in some cases.

C. 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. Place the remaining 45 µl of the reaction mixture on ice; it 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/HaeIII
0.5–10 kb	1.2	1 kb DNA ladder
>5 kb	0.8	λ/Hind III

D. Using 10X Advantage 2 SA PCR Buffer

In cases where non-specific background amplification is a problem, we strongly recommend the 10X Advantage 2 SA PCR Buffer be used as the buffer of choice.

Yields are unaffected when amplifying products less than 2 kb, whether the 10X Advantage 2 SA PCR Buffer or the Advantage 2 PCR Buffer is used. We recommend initial use of the 10X Advantage 2 PCR Buffer; and if background amplification arises, in particular with amplicons less than 2 kb in length, then we recommend switching to the 10X Advantage 2 SA PCR Buffer.

VI. 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 the Advantage 2 Polymerase Mix can be used. When using the enzyme with one of Clontech companion products, additional application-specific troubleshooting information can be found in the relevant User Manual.

Description of Problem	Possible Explanation	Solution
A. No product observed	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 the number of cycles (3–5 additional cycles at a time).
	Annealing temp. too high	Decrease the annealing temperature in increments of 2–4°C.
	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 long, try using a longer primer. If the original primer(s) had a G-C content of less than 45%, try to design a primer with a G-C content of 45–60%.
	Not enough template	Repeat PCR using a higher concentration of DNA (after trying more cycles).
	Poor quality template	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.)
	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.

Description of Problem	Possible Explanation	Solution
A. No product observed (continued)	Too little enzyme	The Advantage 2 Polymerase Mix is 50X for most applications; however, a 1X final reaction concentration of the enzyme mix may be too low for some 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 final concentration to >2X in the reaction mix is likely to lead to higher background levels.
	[Mg^{2+}] is too low	The Advantage 2 Polymerase Mix performs well at a broad range of Mg2+ 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 Mg2+ concentration. However, high concentrations of EDTA or other metal chelators in the template stock solution can reduce the effective concentration of Mg2+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. Since we have gone up to 35 kb with the Advantage Genomic PCR Kit, it is unlikely that dNTPs are limiting. Note that if you increase the concentration of dNTPs, you will also need to increase the [Mg2+] proportionately.
	Difficult target	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt long, try using a longer primer. If the original primer(s) had a G-C content of less than 45%, try to design a primer with a G-C content of 45–60%.

Description of Problem	Possible Explanation	Solution
B. Multiple products	Too many cycles	Reducing the cycle number may eliminate nonspecific bands.
	Annealing temp. too low	Increase the annealing/extension temperature in increments of 2–3°C.
	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 long, try using a longer primer. If the original primer(s) had a G-C content of less than 45%, try to design a primer with a G-C content of 45–60%.
	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 Tm of the primers during the initial PCR cycles. The annealing/extension temperature is then reduced to the primer Tm for the remaining PCR cycles. The change can be performed either in a single step or in increments over several cycles.
	Contamination	See Section E below.
C. Low yield	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.
D. Products are smeared on gel	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.
	Non-specific priming	Use the 10X Advantage SA PCR Buffer which has been specifically engineered to address this (rather than the 10X Advantage 2 PCR Buffer).

D. Products are smeared on gel (continued)	Too much enzyme	The Advantage 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.
	Too much template	Try a lower concentration of DNA template in the PCR reaction.
	Contamination	See Section E below.
E. Dealing with contamination	Contamination most often results in extra bands or smearing. It is important to include a negative control (a control that replaces the DNA template with PCR-grade H_2O but 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 1N HCl followed by 1N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) and rinse with ddH_2O. We advise using commercially available aerosol-free pipette tips. An enzymatic method has been published 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). When performing PCR directly on phage plaques or bacterial colonies, failure to isolate single plaques or colonies will also produce multiple bands.	

VII. References

Barnes, W. M. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91**:2216–2220.

Cheng, S., Fockler, C., Barnes, W. M. & Higuchi, R. (1994) Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA* **91**:5695–5699.

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. & Mattick, J. S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**:4008.

Frey, B. & Suppmann, B. (1995) Demonstration of the Expand™ PCR system's greater fidelity and higher yields with a *lacI*-based PCR fidelity assay. *Biochemica* **2**:8–9.

Kellogg, D. E., Rybalkin, I., Chen, S., Mukhamedova, N., Vlasik, T., Siebert, P. & Chenchik, A. (1994) TaqStart Antibody: Hotstart PCR facilitated by a neutralizing monoclonal antibody directed against *Taq* DNA polymerase. *BioTechniques* **16**:1134–1137.

Longo, M. C., Berninger, M. S. & Hartley, J. L. (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93**:3749.

Nelson, K., Brannan, J. & Kretz, K., (1995) The fidelity of TaqPlus™ DNA Polymerase in PCR. *Strategies Mol. Biol.* **8**:24–25.

Roux, K. H. (1995) Optimization and troubleshooting in PCR. *PCR Methods Appl.* **4:**5185–5194.

Sambrook, J. & D. W. (2001) *Molecular Cloning: A Laboratory Manual, Third Edition* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com	web: takarabio.com
e-mail: ordersUS@takarabio.com	e-mail: techUS@takarabio.com

Notice to Purchaser

Our products are to be used for research purposes only. They may not be used for any other purpose, including, but not limited to, use in drugs, *in vitro* diagnostic purposes, therapeutics, or in humans. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without prior written approval of Takara Bio USA, Inc.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

This document has been reviewed and approved by the Quality Department.