

Frequently asked questions

USB® HotStart-IT® Fidelity™ DNA Polymerase and Master Mix [PN 71155/56]

Why should HotStart-IT Fidelity DNA Polymerase be used?

HotStart-IT Fidelity DNA Polymerase should be used whenever it is necessary to generate products that are longer than 5 kb. HotStart-IT Fidelity enzyme is a mixture of Taq DNA Polymerase and a proofreading polymerase. Taq and proofreading polymerases by themselves have difficulty generating longer products. HotStart-IT Fidelity DNA Polymerase should also be used when higher fidelity than Taq alone is necessary. In addition, the built-in hot start feature enhances PCR reactions by providing maximum sensitivity and specificity.

When should the stand-alone polymerase or the master mix be used?

Use the stand-alone polymerase for maximum control over PCR reactions, for example, if one wishes to alter the buffer and nucleotide concentrations. Use the master mix for maximum convenience, as the mix has all the necessary components for PCR except template, primers, and water.

What are the maximum product sizes that have been amplified?

Affymetrix scientists have generated a 35 kb product from lambda DNA and a 23 kb product from human genomic DNA. Some optimization is generally required for targets longer than 10 kb. The 35 kb product from lambda DNA was generated using 10 ng of template and 0.2 μM primers with the following PCR profile:

95°C	20 seconds
25 cycles of:	
95°C	5 seconds
68°C	20 minutes plus extra 3 seconds per cycle

The 23 kb product from human genomic DNA was generated using 100 ng of template and 0.2 μM primers with the following PCR profile:

92°C	2 minutes
30 cycles of:	
92°C	10 seconds
60°C	30 seconds
68°C	20 minutes plus extra 3 seconds per cycle

What is the error rate of HotStart-IT Fidelity DNA Polymerase?

Up to a 6-fold increase in accuracy for mixtures of Taq DNA Polymerase and a proofreading polymerase over Taq DNA Polymerase alone has been previously reported⁽¹⁻⁴⁾. Using a *lacI* complementation assay⁽⁵⁾ Affymetrix scientists determined the error rate of Fidelity DNA Polymerase was 1.33×10^{-5} mutations per bp per duplication or 1 mutated base per 75,000 bases synthesized in a single round of amplification. This assay yielded an error rate for Taq DNA Polymerase alone of 4.37×10^{-5} mutations per bp per duplication or 1 mutated base per 23,000 bases synthesized in a single round of amplification. Thus, in this particular assay, Fidelity enzyme had over 3-fold greater fidelity than Taq DNA Polymerase alone. It is noted that fidelity measurements are not entirely absolute, as they are assay dependent.

How many clones should I sequence when using HotStart-IT Fidelity DNA Polymerase?

Usually, 2-3 clones are enough to ensure that one is entirely error-free.

Should HotStart-IT Fidelity PCR products be cloned into TA or blunt-end vectors?

PCR products may be cloned into either TA or blunt-end cloning vectors. This is because the enzyme blend generates PCR products whose 3'-ends are a mixture of blunt-ends and A-tailed ends. However, Affymetrix scientists have found that A-tailed ends are favored over blunt ends in an approximately 3 to 1 ratio. Thus, higher cloning efficiencies generally occur using TA-cloning vectors rather than blunt-end vectors.

Is it necessary to purify the PCR products before the cloning reactions?

No. Minor amounts of unincorporated nucleotides and primers do not inhibit cloning reactions, whether they are ligation-based or TOPO®-based. Keep the volume of PCR product in the cloning reaction to less than 25% of the total volume. Gel purification is recommended only in those situations where non-specific products appear following gel electrophoresis.

Can dUTP be used with HotStart-IT Fidelity DNA Polymerase?

No. There is a minor amount of a proofreading polymerase from an archaebacterium in the Fidelity mixture. These types of polymerases are strongly inhibited by dUTP, as they do not efficiently replicate DNA that has uracil incorporated into it^(6, 7).

Is an extension temperature of 68°C essential to generate products longer than 5 kilobases in length?

Yes. Temperatures higher than 68°C will cause a marked reduction in product yield.

How should I design my primers?

Longer oligonucleotides which are greater than 25 bases in length and have melting temperatures above 60°C are recommended to achieve more specific and robust amplification. We also strongly recommend using a computer program such as Primer3 from the Whitehead Institute (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>).

What is an easy estimate of a primer's melting temperature (T_m)?

The formula: $T_m (^{\circ}\text{C}) = 2*(\text{A}+\text{T}) + 4*(\text{G}+\text{C})$, known as the "Wallace Rule", may be used⁽⁸⁾.

Are there any additives that can be used to help amplify difficult targets?

Yes. If one encounters difficulties with longer targets (> 10 kb), those with a high G+C content (> 60%), or those from complex templates (genomic DNA), adding certain supplements such as DMSO, glycerol, trehalose, and/or betaine to the PCR reaction may improve the results. DMSO and glycerol may be added at final concentrations ranging from 1% to 10% (v/v). Trehalose may be added to 0.6 M final concentration. Betaine (PN 77507, 5 M Stock Solution) may be added at 0.5 M to 2.0 M final concentration. Note that all of these solvents tend to decrease the T_m values for double-stranded DNA, thus their presence in reactions may result in a decrease in the optimum annealing temperature by several degrees⁽⁹⁻¹²⁾.

What are some remedies for reactions that generate faint product or no product at all?

The following solutions are provided:

- Use more DNA template; verify template concentration.
- Increase the concentration of primers, for example from 0.2 μM to 0.5 μM or higher.
- Increase the number of cycles, up to 45 cycles for low-copy targets.
- Increase the MgCl₂ final concentration in 0.25 mM increments.
- Test a range of annealing temperatures. Start with an annealing temperature 5-10°C below the lowest calculated primer T_m and increase in 1-2°C increments. Annealing temperatures that are either too high or too low can result in absence of product.
- For targets longer than 10 kb, decrease the initial and subsequent denaturation time to 10 seconds or less and/or decrease the denaturation temperature to 92°C. This will limit the amount of temperature-induced depurination of template DNA during the reaction.
- For targets longer than 5 kb, use an extension time of at least one minute per kb. If this fails, increase the extension time to two minutes per kb and also use the auto-extend feature on the thermal cycler. In addition, always use 68°C as the extension temperature.
- If all else fails, design new primers. Use a primer design computer program.

What are some remedies for reactions that generate background or nonspecific products?

The following solutions are provided:

- Use less DNA template; verify template concentration.
- Decrease the concentration of primers, for example to 0.2 μM or less.
- Reduce the number of cycles, in 5 cycle increments.
- Increase the annealing temperature in 1-2°C increments.
- For targets longer than 10 kb, decrease the initial and subsequent denaturation time to 10 seconds or less and/or decrease the denaturation temperature to 92°C. This will limit the amount of temperature-induced depurination of template DNA during the reaction.
- Use the supplements as suggested for difficult targets in a previous answer.
- If all else fails, design new primers. Use a primer design computer program.

What is the composition of the 10X PCR Buffer for the stand-alone polymerase?

100 mM Tris-HCl, pH 8.6, 500 mM KCl, and 15 mM MgCl₂.

What is the Affymetrix HotStart method and how does it work?

The HotStart-IT Binding Protein is the active component in the novel hot start technology designed and developed at Affymetrix. In general, hot start PCR methods reduce or eliminate non-specific primer-extension products formed at lower temperatures. During assembly of PCR reactions, the binding protein sequesters primers making them unavailable for use by the polymerase. This primer-sequestration technique effectively blocks DNA synthesis from mis-priming events at lower temperatures. Following the initial denaturation step during PCR, the protein is inactivated and the primers are free to participate in the amplification reaction. This technique has the advantages of a short initial denaturation step (unlike chemical methods) and a low risk of introducing contaminating mammalian genomic DNA into the reactions (unlike antibody methods).

References:

1. Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2216-2220.
2. Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5695-5699.
3. Barnes, W. M. (1992) *Gene* **112**, 29-35.
4. Cline, J., Braman, J. C., and Hogrefe, H. H. (1996) *Nucl. Acids Res.* **24**, 3546-3551.
5. Lasken, R. S., Schuster, D. M., and Rashtchian, A. (1996) *Journal Biol. Chem.* **271**, 17692-17696.
6. Frey, B., and Suppmann, B. (1995) *Biochemica* **2**, 8-9.
7. Hogrefe, H. H., Hansen, C. J., Scott, B. R., and Nielson, K. B. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 596-601.
8. Suggs, S. V., Hirose, T., Miyake, T., Kawashima, E. H., Johnson, M. J., Itakura, K., Wallace, R. B. (1981) "Developmental Biology Using Purified Genes", Edited by: Brown D and Fox, C. F. New York: Academic Press Inc., 683-693.
9. Carninci, P., Nishiyama, Y., Westover, A., Itoh, M., Nagaoka, S., Sasaki, N., Okazaki, Y., Muramatsu, M., and Hayashizaki, Y. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 520-524.
10. Sambrook, J. and Russell, D. W. (2001) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, 8.9.
11. Santoro, M. M., Liu, Y., Khan, S. M., Hou, L. X., and Bolen, D. W. (1992) *Biochemistry* **31**, 5278-5283.
12. Spiess, A. N. and Ivell, R. (2002) *Anal. Biochem.* **301**, 168-174.

For research use only. Not for use in diagnostic procedures.

USB and HotStart-IT are registered trademarks of Affymetrix, Inc. FidelityTaq is a registered trademark of Affymetrix, Inc. HotStart-IT Taq DNA Polymerase—Methods for using this product may be covered by US Patent No. 7,700,281. Taq DNA Polymerase—sold under licensing arrangements with Applied Biosystems. Purchase is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, *i.e.*, an authorized thermal cycler. TOPO is a registered trademark of Life Technologies.

Affymetrix, Inc.

USB® Products

26111 Miles Road
Cleveland Ohio 44128
Tel: 800-321-9322 | 216-765-5000
Fax: 800-535-0898 | 216-464-5075
USBcustomerserv@affymetrix.com
usb.affymetrix.com

Affymetrix UK Ltd.

USB® Products

Voyager, Mercury Park,
Wycombe Lane, Wooburn Green,
High Wycombe HP10 0HH, United Kingdom
Tel: +44 (0)1628 55 2600
Fax: +44 (0)1628 55 2675
USBcustomerserveurope@affymetrix.com

Affymetrix Pte Ltd

USB® Products

7 Gul Circle, #2M-01
Keppel Logistics Building
Singapore 629563
Tel: +65 63957301
Fax: +65 63957300
USBcustomerserv@affymetrix.com