INSTRUCTION MANUAL

NEW ENGLAND BioLabs Inc. be INSPIRED drive DISCOVERY stay GENUINE

# Phusion® High Fidelity PCR Kit

NEB #E0553S/L 50/200 reactions

Version 2.0_1/20

## Table of Contents

Introduction ....3
Protocol for a Routine PCR Reaction ....3
Protocol for PCR Optimization ....4
Amplification of Control Template ....5
Troubleshooting Guide ....7
Quality Controls ....7
References ....8
Ordering Information ....8
Revision History ....9

## Kit Components

*This kit contains a sufficient supply of polymerase, buffers, deoxynucleotides, magnesium chloride, DMSO, and DNA size standard to perform 50 PCR reactions (NEB #E0553) or 200 PCR reactions (NEB #E0553L). Control template and primers are provided for 20 control reactions.*

**Phusion DNA Polymerase (NEB #M0530)** 50/200 units (2 units/µl)
Supplied in: 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 µg/ml BSA and 50% glycerol.

Unit definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes at 74°C.

Unit assay conditions: 25 mM TAPS-HCl (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 µM dNTPs including [$^3$H]-dTTP and 15 nM primed M13 DNA.

**Deoxynucleotide Solution Mix (NEB #N0447)** 100/400 µl (10 mM of each dATP, dCTP, dGTP and dTTP)
Deoxynucleotide Solution Mix is an equimolar solution of ultrapure dATP, dCTP, dGTP and dTTP. These deoxynucleotides are supplied at a 10 mM concentration in Milli-Q® water as a sodium salt at pH 7.5.

**Phusion HF Buffer (NEB #B0518)** 1.5/4.5 ml (5X)
5X Phusion HF Buffer contains 7.5 mM $MgCl_2$ (1.5 mM at 1X dilution). HF buffer is recommended as the default buffer for high-fidelity amplification.

**Phusion GC Buffer (NEB #B0519)** 1.5/1.5 ml (5X)
5X Phusion GC Buffer contains 7.5 mM $MgCl_2$ (1.5 mM at 1X dilution). For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve enzyme performance. GC buffer should be used in experiments where HF buffer does not work.

**$MgCl_2$ Solution (NEB #B0510)** 1.5/1.5 ml (50 mM)
When used at the recommended concentration, both Phusion buffers contain 1.5 mM $MgCl_2$. Additional $MgCl_2$ is provided to optimize polymerase activity if necessary. As Phusion DNA Polymerase is quite sensitive to changes in $MgCl_2$, the solution should be diluted with water to 10 mM to avoid pipetting errors.

**Control Lambda Template (NEB #N3010)** 40/40 µl (0.5 ng/µl)
Bacteriophage lambda DNA (GenBank #NC_001416, 48,502 bp) is provided as a control template. It is supplied in TE buffer at 0.5 ng/µl.

**1.3 and 10 kb Primers (NEB #S0535/S0536)** 50/50 µl (4 µM)
The 1.3 kb Control Primer Mix is a mix of primers in water for amplification of a 1.3 kb fragment using the Control Lambda Template. Primers are provided at a concentration of
4 µM each.

Sequences are as follows:
Primer 1 (27-mer): 5´ GTC ACC AGT GCA GTG CTT GAT AAC AGG 3´ $T_m$ 71.0°C, coordinates in lambda: 30,006–30,032

Primer 2 (28-mer): 5´ GAT GAC GCA TCC TCA CGA TAA TAT CCG G 3´ $T_m$ 73.2°C, coordinates in lambda: 31,325–31,352

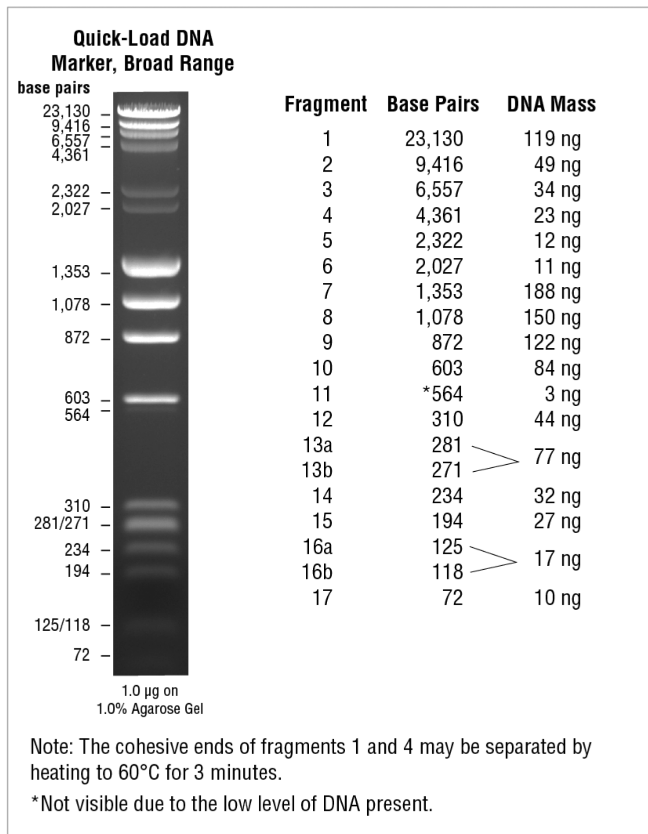
The 10 kb Control Primer Mix is a mix of primers in water for amplification of a 10 kb fragment using the Control Lambda Template. Primers are provided at a concentration of 4 µM each.

Sequences are as follows:
Primer 1 (22-mer): 5´ CAG TGC AGT GCT TGA TAA CAG G 3´ $T_m$ 63.0°C, coordinates in lambda: 30,011–30,032

Primer 2 (20-mer): 5´ GTA GTG CGC GTT TGA TTT CC 3´ $T_m$ 62.7°C, coordinates in lambda: 40,024–40,043

**DMSO (NEB #B0515)** 0.5/0.5 ml (100%)
DMSO can be used as an additive in the reaction when amplifying difficult targets. Recommended concentration is generally 3%, but can range from 1%–10%.

**Quick-Load® DNA Marker, Broad Range (NEB #N0303)** 200/400 µl (100 µg/ml)
The Quick-Load DNA Marker, Broad Range is a mix of HindIII digest of Lambda DNA and HaeIII digest of ϕX174 DNA. It is supplied in 3.3 mM Tris-HCl (pH 8.0), 11 mM EDTA, 2.5% Ficoll 400, 0.017% SDS and 0.015% bromophenol blue. Recommended usage is 10 µl (1 µg) per gel lane.




| Fragment | Base Pairs | DNA Mass |
|---|---|---|
| 1 | 23,130 | 119 ng |
| 2 | 9,416 | 49 ng |
| 3 | 6,557 | 34 ng |
| 4 | 4,361 | 23 ng |
| 5 | 2,322 | 12 ng |
| 6 | 2,027 | 11 ng |
| 7 | 1,353 | 188 ng |
| 8 | 1,078 | 150 ng |
| 9 | 872 | 122 ng |
| 10 | 603 | 84 ng |
| 11 | *564 | 3 ng |
| 12 | 310 | 44 ng |
| 13a | 281 | 77 ng |
| 13b | 271 | |
| 14 | 234 | 32 ng |
| 15 | 194 | 27 ng |
| 16a | 125 | 17 ng |
| 16b | 118 | |
| 17 | 72 | 10 ng |

Note: The cohesive ends of fragments 1 and 4 may be separated by heating to 60°C for 3 minutes.

*Not visible due to the low level of DNA present.

## Introduction

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (1). PCR amplifies specific DNA sequences exponentially by using multiple cycles of a three-step process. First, the double-stranded DNA template is denatured at a high temperature. Sequence-specific primers are then annealed to sites flanking the target sequence. A thermostable DNA polymerase (2–6) extends the annealed primers, thereby doubling the amount of the original DNA sequence. This newly synthesized product then becomes an additional template for subsequent cycles of amplification. These three steps are repeated for 20 to 30 cycles, resulting in a $10^5$–$10^9$ fold increase in target DNA concentration.

High Fidelity DNA Polymerases are important for applications in which the DNA sequence needs to be correct after amplification. Manufactured and quality controlled at New England Biolabs, Thermo Scientific® Phusion High-Fidelity DNA Polymerase offers both high fidelity and robust performance, and thus can be used for all PCR applications. Its unique structure, a novel *Pyrococcus*-like enzyme fused with a processivity-enhancing domain, increases fidelity and speed. Phusion DNA Polymerase is an ideal choice for cloning and can be used for long or difficult amplicons. With an error rate 50-fold lower than that of *Taq* DNA Polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA Polymerase (7), Phusion is one of the most accurate thermostable polymerases available. Phusion DNA Polymerase possesses 5´→ 3´ polymerase activity, 3´→ 5´ exonuclease activity and will generate blunt-ended products.

## Protocol for Routine PCR Reaction:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last, in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity. Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. **Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.**

1. Set up the appropriate reactions on ice:

| | 20 µl REACTION | 50 µl REACTION | FINAL CONCENTRATION |
|---|---|---|---|
| Nuclease-Free Water | to 25 µl | to 50 µl | |
| 5X Phusion HF or GC Buffer | 4 µl | 10 µl | 1X |
| 10 mM dNTPs | 0.4 µl | 1 µl | 200 µM |
| 10 µM Forward Primer | 1 µl | 2.5 µl | 0.5 µM |
| 10 µM Reverse Primer | 1 µl | 2.5 µl | 0.5 µM |
| Template DNA | variable | variable | < 250 ng |
| DMSO (optional) | (0.6 µl) | (1.5 µl) | 3% |
| Phusion DNA Polymerase | 0.2 µl | 0.5 µl | 1.0 units/50 µl PCR |

2. Gently mix the reaction and spin down in microcentrifuge.

*If the thermocycler does not have a heated lid, overlay the sample with mineral oil.*

3. Cycling Conditions for a Routine PCR Reaction:

| CYCLE STEP | CYCLES | TEMP | TIME |
|---|---|---|---|
| Initial Denaturation | 1 | 98°C | 30 seconds |
| Denaturation | 30 | 98°C | 5-10 seconds |
| Annealing | | 45-72°C* | 10-30 seconds |
| Extension | | 72°C | 15-30 seconds/kb |
| Final Extension | 1 | 72°C | 5-10 minutes |
| Hold | 1 | 4°C | ∞ |

## Protocol for PCR Optimization:

The following guidelines are provided to ensure successful PCR using Phusion DNA Polymerase. These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations or longer amplicons may require further optimization.

1. Template:
   Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

   | DNA | AMOUNT |
   |---|---|
   | Genomic | 50 ng–250 ng |
   | Plasmid or Viral | 1 pg–10 ng |

   If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

2. Primers:
   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 (http://frodo.wi.mit.edu/primer3) can be used to design or analyze primers. The final concentration of each primer in a PCR reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

3. $Mg^{++}$ and additives:
   $Mg^{++}$ is critical to achieve optimal activity with Phusion DNA Polymerase. The final $Mg^{++}$ concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive $Mg^{++}$ can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal $Mg^{++}$ concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. If the chelators (e.g., EDTA) are present, it may be necessary to increase the $Mg^{++}$ concentration. $Mg^{++}$ can be optimized in 0.5 mM increments using the $MgCl_2$ provided.

   Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer $T_m$ (8). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides:
   The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template.

5. Phusion DNA Polymerase Concentration:
   We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 µl reaction). However, the optimal concentration of Phusion DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

6. Buffers
   5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. HF buffer is recommended as the default buffer for high-fidelity amplification. For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve reaction performance. GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g., microarray, DHPLC).

7. Denaturation:
   An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

   During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

8. Annealing:
   Annealing temperatures required for use with Phusion tend to be higher than with other PCR polymerases. **The NEB $T_m$ Calculator (www.neb.com/TmCalculator) should be used to determine the annealing temperature when using Phusion**. Typically, primers greater than 20 nucleotides in length anneal for 10–30 seconds at 3°C above the $T_m$ of the lower $T_m$ primer. If the primer length is less than 20 nucleotides, an annealing temperature equivalent to the $T_m$ of the lower primer should be used. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For two-step cycling, the gradient can be set as high as the extension temperature.

   For high $T_m$ primer pairs, two-step cycling without a separate annealing step can be used.

9. Extension:
   The recommended extension temperature is 72°C. Extension times are dependent on amplicon length and complexity. Generally, an extension time of 15 seconds per kb can be used. For complex amplicons, such as genomic DNA, an extension time of 30 seconds per kb is recommended. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary.

10. Cycle number:
    Generally, 25–35 cycles yields sufficient product.

11. 2-step PCR:
    When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

    Thermocycling conditions for a routine 2-step PCR:

| STEP | TEMP | TIME |
|---|---|---|
| Initial Denaturation | 98°C | 30 seconds |
| 25-35 Cycles | 98°C | 5-10 seconds |
| | 72°C | 15-30 seconds/kb |
| Final Extension | 72°C | 5-10 minutes |
| Hold | 4°C | ∞ |

12. PCR product:
    The PCR products generated using Phusion DNA Polymerase have blunt ends; if cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, then DNA should be purified prior to A-addition, as Phusion DNA Polymerase will degrade any overhangs generated.

    Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo$^{-}$ (NEB #M0212).

## Amplification of the Control Template:

1. Set up the appropriate reactions on ice:

| | 20 µl REACTION | 50 µl REACTION | FINAL CONCENTRATION |
|---|---|---|---|
| Nuclease-Free Water | 13.6 µl | 34 µl | |
| 5X Phusion HF Buffer | 4 µl | 10 µl | 1X |
| 10 mM dNTPs | 0.4 µl | 1 µl | 200 µM |
| Primers* | 1 µl | 2.5 µl | 0.2 µM |
| Control Template DNA | 0.8 µl | 2 µl | 0.02 ng/µl |
| Phusion DNA Polymerase | 0.2 µl** | 0.5 µl | 0.02 U/µl |

* Reaction can be set up using either the 1.3 or 10 kb primer set
** Dilute polymerase with 1X reaction buffer to avoid pipetting errors

2. Recommended cycling conditions for the 1.3 kb fragment using a 2-step protocol:

| CYCLE STEP | TEMP | TIME |
|---|---|---|
| Initial Denaturation | 98°C | 1 Minute |
| 25 Cycles | 98°C | 5 seconds |
| | 72°C | 20 seconds |
| Final Extension | 72°C | 10 minutes |
| Hold | 4°C | ∞ |

3. Recommended cycling conditions for the 10 kb fragment using a 3-step protocol. Alternatively, this program can be used to amplify both fragments simultaneously.

| CYCLE STEP | TEMP | TIME |
| --- | --- | --- |
| Initial Denaturation | 98°C | 1 Minute |
| 25 Cycles | 98°C | 5 seconds |
| | 60°C* | 15 seconds |
| | 72°C | 2 Minutes 30 Seconds |
| Final Extension | 72°C | 10 minutes |
| Hold | 4°C | ∞ |

Note: Controls have been shown to work in a variety of conditions.

## Troubleshooting Guide:

| OBSERVATION | PROBABLE CAUSE(S) | SOLUTION(S) |
|---|---|---|
| No Amplification Product | Incorrect annealing temperature | • Verify annealing temperature using NEB $T_m$ Calculator<br>• Test temperature gradient |
| | Poor primer design | • Verify that primers are non-complementary, both internally and to each other.<br>• Increase length of primer |
| | Poor primer specificity | • Verify that oligos are complementary to proper target sequence. |
| | Insufficient primer concentration | • Increase primer concentration to 0.5 µM. |
| | Missing reaction component | • Repeat reaction setup |
| | Target sequence not present in template DNA | • Try other sources of template DNA |
| | Poor reaction conditions | • Optimize [$Mg^{++}$], annealing temperature and extension time<br>• Check primer concentrations<br>• Titrate enzyme concentration |
| | Questionable template quality | • Analyze DNA via gel electrophoresis after incubation with $Mg^{++}$ |
| | Inhibitory substance in reaction | • Decrease sample volume<br>• Try alcohol precipitation or drop dialysis to further purify DNA |
| | Insufficient number of cycles | • Rerun the reaction with more cycles |
| | Incorrect thermocycler programming | • Check program, verify times and temperatures |
| | Inconsistent block temperature | • Test calibration of heating block |
| | Reaction tubes or solutions contaminated | • Autoclave tubes prior to use to eliminate biological inhibitors |
| | Complex template | • Try Phusion GC buffer. Add DMSO to the reaction |
| Multiple or non-specific products | Premature replication | • Set up reactions on ice with chilled components. Add samples to pre-heated (98°C) thermocycler. |
| | Primer annealing temperature too low | • Test temperature gradient<br>• Verify annealing temperature using NEB $T_m$ calculator |
| | Insufficient mixing of reaction buffer | • Reaction buffer must be thoroughly mixed |
| | Improper $Mg^{++}$ concentration | • Adjust $Mg^{++}$ concentration in 0.5 mM increments |
| | Poor primer design | • Verify that primers have no complementary regions – either internally or to each other<br>• Try longer primers<br>• Avoid GC-rich 3′ ends |
| | Excess primer | • Reduce primer concentration to 0.1–0.5 µM |
| | Contamination with exogenous DNA | • Use positive displacement pipettes or non-aerosol tips<br>• Set-up dedicated work area and pipettor for reaction setup<br>• Wear gloves during reaction setup |
| | Multiple target sequences in template DNA | • Redesign primers with higher specificity to target sequence |
| | Poor reaction conditions | • Reduce enzyme concentration<br>• Shorten extension time<br>• Titrate template<br>• Reduce number of cycles |

## Quality Controls

**7.5 kb Genomic DNA PCR:**

30 cycles of PCR amplification in a 50 µl reaction containing 50 ng genomic DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 7.5 kb product.

**20 kb Lambda DNA PCR:**

22 cycles of PCR amplification in a 50 µl reaction containing 10 ng Lambda DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 20 kb product.

### Endonuclease Activity

Incubation of a 50 µl reaction in NEBuffer 2 containing a minimum of 10 units of Phusion DNA Polymerase with 200 µM dNTPs and 1 µg of supercoiled ϕX174 DNA for 4 hours at either 37°C or 72°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

### Reaction Buffers

The supplied Phusion reaction buffers and supplements are free of detectable nucleases.

### Deoxynucleotide Solution

Deoxynucleotide solutions are certified free of detectable nucleases and phosphatases.

## References


1. Saiki, R.K. et al. (1985) *Science,* 230, 1350–1354.
2. Chien, A., Edgar, D.B. and Trela, J.M. (1976) *J. Bact.,* 127, 1550-1557.
3. Kaledin, A.S., Sliusarenko, A.G. and Gorodetskii, S.I. (1980) *Biokhimiya*, 45, 644-651.
4. Lawyer, F.C. et al. (1993) *PCR Method and App*l., 2, 275-287.
5. Longley, M.J., Bennett, S.E. and Mosbaugh D.W. (1990) *Nucleic Acids Res*., 18, 7317-7322.
6. Lyamichev, V., Brow, M.A. and Dahlberg, J.E. (1993) *Science*, 260, 778-783.
7. Frey, B. and Suppman, B. (1995) *Biochimica*, 2, 34–35.
8. Chester, N. and Marshak, D.R. (1993) *Analytical Biochemistry*, 209, 284–290.


## Ordering Information

| NEB # | PRODUCT | SIZE |
|---|---|---|
| E0553S/L | Phusion High-Fidelity PCR Kit | 50/200 reactions |
| KIT COMPONENTS SOLD SEPARATELY | | |
| M0530S/L | Phusion DNA Polymerase | 100/500 reactions |
| N0447S/L | Deoxynucleotide Solution Mix | 8/40 µmol of each |
| B0518S | Phusion HF Buffer Pack | 6.0 ml |
| B0519S | Phusion GC Buffer Pack | 6.0 ml |

COMPANION PRODUCTS

| NEB # | PRODUCT | SIZE |
|---|---|---|
| N0446S | Deoxynucleotide Solution Set | 25 µmol of each |
| B9021S | Magnesium Chloride ($MgCl_2$) Solution | 6.0 ml |
| M0531S/L | Phusion High-Fidelity PCR Master Mix with HF Buffer | 100/500 reactions |
| M0532S/L | Phusion High-Fidelity PCR Master Mix with GC Buffer | 100/500 reactions |
| N3011S/L | Lambda DNA | 250/1,250 µg |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.1 | | 6/11 |
| 1.2 | | 5/12 |
| 1.3 | | 7/12 |
| 1.4 | N/A | 2/15 |
| 2.0 | Updated to new manual format | 1/20 |

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

This product is covered by one or more patents, trademarks and/or copyrights owned or controlled by New England Biolabs, Inc. For more information about commercial rights, please email us at busdev@neb.com While NEB develops and validates its products for various applications, the use of this product may require the buyer to obtain additional third party intellectual property rights for certain applications.

Phusion® DNA Polymerase was developed by Finnzymes Oy, now a part of Thermo Fisher Scientific.

Licenses/Patents/Disclaimers:
Notice to purchaser: Limited license.
The purchase price of this product includes a limited, non-transferable license under U.S. and foreign patents owned by Bio-Rad Laboratories, Inc., to use this product. No other license under these patents is conveyed expressly or by implication by purchase of this product.

B CORPORATION™ is a trademark of B Lab, Inc.
PHUSION® is a registered trademark and property of Thermo Fisher Scientific.
THERMO SCIENTIFIC® is a registered trademark of Thermo Fisher Scientific.
MILLI-Q® is a registered trademark of Millipore Corporation.

© Copyright 2020, New England Biolabs, Inc.; all rights reserved

be INSPIRED
drive DISCOVERY
stay GENUINE

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com