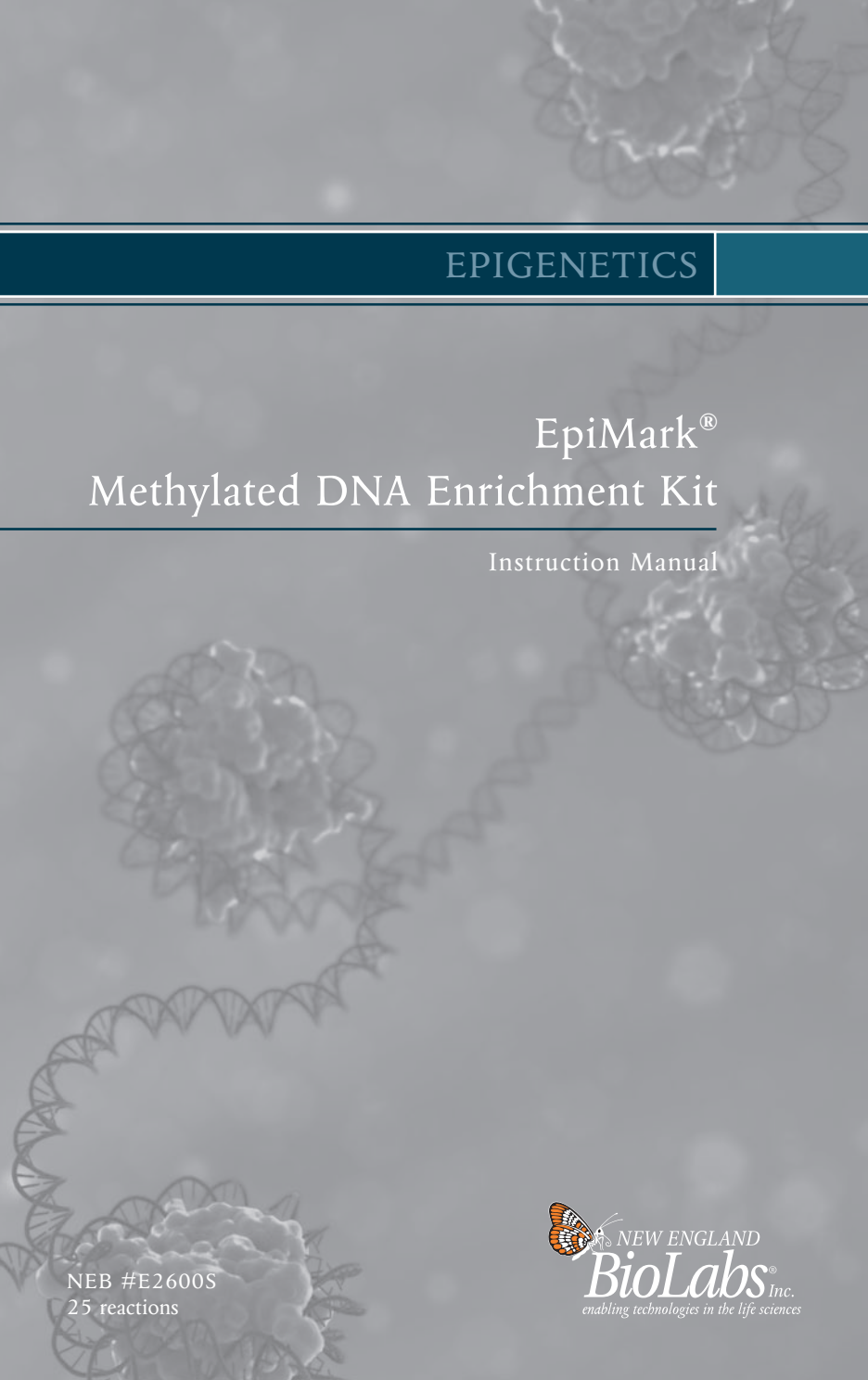




EPIGENETICS

EpiMark® Methylated DNA Enrichment Kit

Instruction Manual

NEB #E2600S
25 reactions

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MBD-Fc and MCIP was developed by Michael Rehli at the University of Regensburg to improve the sensitivity and specificity of conventional CpG binding techniques.

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Kit Components:

Each kit contains sufficient reagents for the enrichment of methylated DNA from up to 100 µg of fragmented input DNA. If starting with 5 ng to 10 µg of input DNA per experiment, the kit provides sufficient reagents for 25 reactions. Store at 4°C. For long-term storage > 6 months, MBD2-Fc protein should be stored at -20°C.

MBD2-Fc Protein (2 mg/ml)	25 µl
Protein A Magnetic Beads	250 µl
Bind/Wash Buffer (5X)	2 x 25 ml
High-Salt Elution Buffer (5 M)	10 ml
Fragmented HeLa DNA (100 µg/ml)	20 µl
Line Element Primers for Methylated-locus Controls (100 µM each, supplied as a mix)	20 µl
RPL30 Primers for Unmethylated Locus Controls (100 µM ea., supplied as a mix)	20 µl
MirA Locus Control Primers (100 µM each, supplied as a mix)	20 µl

Required Materials Not Included

50 bp DNA Ladder (NEB #N3236)

6-tube Magnetic Separation Rack (NEB #S1506)

Taq DNA Polymerase with Standard *Taq* Buffer (NEB #M0273)

Deoxynucleotide Solution Mix (NEB #N0447)

Nuclease-free Water

Introduction:

The EpiMark Methylated DNA Enrichment Kit will selectively bind and enrich double-stranded methyl-CpG DNA from fragmented genomic DNA with as little as 5 ng of input DNA.

Methylated DNA is isolated from fragmented genomic DNA (5 ng–25 µg) by binding to the methyl-CpG binding domain of human MBD2 protein fused to the Fc tail of human IgG1 (MBD2-Fc), which is coupled to paramagnetic hydrophilic protein A beads (MBC2-Fc/Protein A Magnetic Bead). Two Fc domains can be bound to one site on protein A with high affinity ($K_d=10^{-7}$). As the Fc fragment is a dimer, four MBD2 domains are exposed to the solvent per molecule of protein A, increasing the relative equilibrium constant 100-fold. This stable complex will selectively bind double-stranded methylated CpG containing DNA. After simple wash steps followed by magnetic capture, the enriched DNA sample is easily eluted in a small volume of nuclease-free water by incubation at 65°C. The sample is immediately ready for downstream analysis by a variety of methods including:

- Endpoint and real-time PCR assays
- Bisulfite conversion followed by DNA amplification
- Cloning and sequencing
- Direct sequencing
- Library preparation for high-throughput sequencing
- Labeling for DNA microarray analysis
- Methylation-sensitive restriction enzyme-based assays

MBD-Fc and MCIP were originally developed by Michael Rehli at the University of Regensburg to improve the sensitivity and specificity of conventional CpG binding techniques.

Advantages:

- High-affinity binding provides greater sensitivity
- Elution in a small volume simplifies downstream applications.
- Easy-to-use protocol yields enriched fractions in less than 2 hours.
- Enriched methylated DNA fractions can be easily ligated to double-stranded adaptors for Next Generation Sequencing.
- Highly pure product from a wide range of input DNA concentrations.

Method Overview:

Step I—Fragment Genomic DNA

DNA must be fragmented by sonication, nebulization or enzymatic treatment to an average size of less than 1,000 bp.

Step II—Combine MBD2-Fc and Protein A Magnetic Beads in 1X Bind/Wash Buffer.

Incubate the reaction for 15 minutes at room temperature. Wash beads two times in Bind/Wash Reaction Buffer.

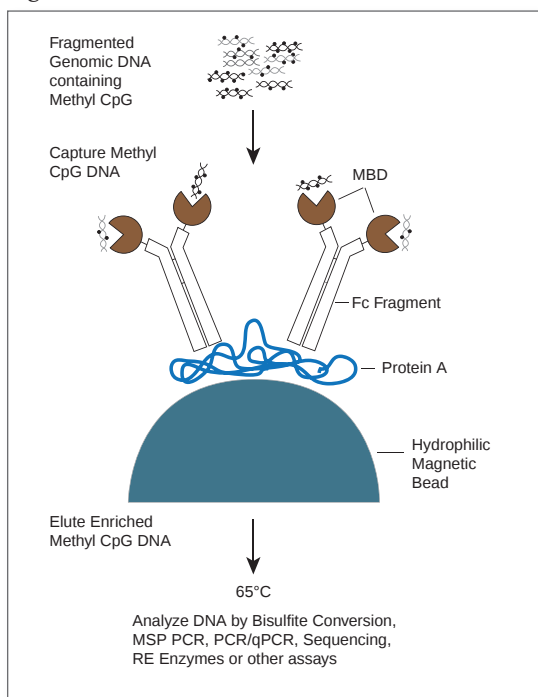
Step III—Add fragmented DNA to MBD2-Fc / Protein A Magnetic Beads.

Incubate the reaction for 20 minutes at room temperature. Wash beads three times at room temperature for five minutes each to remove unbound DNA.

Step IV—Elute enriched methylated CpG DNA from beads

Incubate the sample at 65°C for 15 minutes in DNase-free water.

Figure 1. Enrichment workflow



Protocols:

DNA Fragmentation

DNA may be fragmented using sonication, nebulization, enzymatic treatment or by other methods. Fragments must average less than 1,000 bp and should be in DNase-free TE buffer (pH 7.5). Determine the approximate size distribution of the DNA by agarose gel electrophoresis of the sample alongside a DNA marker (e.g., 50 bp DNA Ladder, NEB #N3236). A sample of fragmented HeLa genomic DNA of the size range of 100–1000 bp is included as a control. 4 μ l of this DNA standard (~400 ng) is sufficient to visualize on an agarose gel alongside the experimental sample. The desired fragment size should be appropriate for the desired downstream analysis. For example, DNA fragmented to an average length of ~250 bp is suitable for assay by real-time quantitative PCR (qPCR). Smaller fragments of 150 bp or less are suitable for linker addition in Next Generation Sequencing platforms. It is also important to quantitate the amount of DNA in the experimental sample by A_{260} measurement using a spectrophotometer, Nanodrop™ instrument or Bioanalyzer®.

Prebind MBD2-Fc to Protein A Magnetic Beads

Before proceeding, determine the number of samples to be analyzed. As a general rule of thumb, use 1 μ l of MBD2-Fc protein and 10 μ l Protein A Magnetic Beads for up to 10 μ g of input DNA. For larger amounts of input DNA, scale the reaction accordingly (e.g., for an experiment using 100 μ g of input DNA, add 10 μ l MBD2-Fc protein and 100 μ l Protein A Magnetic Beads).

Resuspend Protein A Magnetic Beads by gently pipetting the slurry up and down until suspension is homogeneous. Alternatively, rotate the tube gently for 30 minutes.

Prepare 1X Bind/Wash Buffer by diluting 1 part of 5X Bind/Wash Buffer with 4 parts of DNase-free water. One individual reaction from start to finish will require ~5 mls of 1X Bind/Wash Buffer.

1. In one tube, add 10 μ l of beads to 1 μ l of MBD2-Fc. The MBD2-Fc/Protein A Magnetic Bead mixture is stable for up to 1 week at 4°C.
2. Add 11 μ l of 1X Bind/Wash Buffer.
3. Mix the bead-protein mixture by placing the tube in a rotating mixer for 15 minutes at room temperature.
4. Add 1000 μ l of 1X Bind/Wash Buffer to the tube to wash the beads.
5. Mix the beads on a rotating mixer for 3 minutes at room temperature.
6. Place the tube on the magnetic rack for 2–5 minutes to concentrate all of the beads on the inner wall of the tube.

7. Carefully remove the supernatant with a pipette without disturbing the beads.
8. Repeat steps 4–7.
9. Remove the tube from the rack and add 11 μ l of Bind/Wash Reaction Buffer.

Capture Methylated CpG DNA

1. Add 20 μ l of 5X Bind/Wash Buffer to a clean 1.7 ml DNase-free microcentrifuge tube.
2. Add 5 ng-10 μ g of fragmented sample DNA, up to a total volume of 70 μ l.
3. Add 10 μ l of well-mixed MBD2-Fc / Protein A Magnetic Beads.
4. Bring the total volume of the reaction to 100 μ l with DNase-free water.
5. Incubate the reaction for 20 minutes at room temperature with rotation.

Wash Off Unbound DNA

1. After incubating the DNA and MBD2-Fc/ Protein A Magnetic Beads, place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the inner wall of the tube.
2. Carefully remove the supernatant with a pipette without disturbing the beads, and save it in a clean microcentrifuge tube. This saved supernatant is the non-captured DNA fraction. Store this sample on ice or at -20°C.
3. Add 1000 μ l of 1X Bind/Wash Buffer to the tube to remove of residual non-captured DNA.
4. Mix the beads on a rotating mixer for 3 minutes at room temperature.
5. Place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the inner wall of the tube. Remove the supernatant.
6. Repeat steps 3–5 two more times.

Elute Captured Methylated CpG DNA

For all downstream techniques, including endpoint and real-time PCR assays, bisulfite conversion, cloning and sequencing, direct sequencing, library preparation for high-throughput sequencing, labeling for DNA microarray analysis or methylation-sensitive restriction enzyme-based assays, heating the sample at 65°C for 15 minutes is the recommended method of choice for elution of DNA. The appendix section also includes a protocol for NaCl multi-fraction elution series, followed by Qiagen spin column cleanup if necessary.

Heat Method

1. Add 50–100 µl of DNase-free water to the sample pellet. Mix beads by gentle vortexing (1200 rpm) or by flicking the tube. Incubate the slurry in a heat block or thermomixer set at 65°C for 15 minutes, with frequent mixing.
2. Briefly centrifuge the sample at 14,000 rpm in a microcentrifuge.
3. Place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the inner wall of the tube.
4. Carefully remove the supernatant to a fresh microcentrifuge tube. Eluent contains enriched methyl CpG containing DNA. Proceed directly to downstream analysis or store the eluted DNA sample at –20°C.

Capture and Elute Step for Control DNA

Included in the kit are control reagents and primers to detect a methylated region, LINE element, an active gene exon, RPL30, and a micro RNA control region, MirA from a sample of fractionated HeLa DNA. (Sequences of the primers and target DNA are listed in the appendix section). A typical control reaction, that will yield a sufficient quantity of eluted material to perform qPCR reactions in triplicate, is outlined below.

1. Perform capture experiment using 5 µl (0.5 µg) of fractionated HeLa Control DNA and 10 µl MBD2-Fc beads as described above.
2. Elute the beads by incubation at 65°C in 100 µl of DNase-free water.

Downstream Analysis

Quantitation of DNA by qPCR (for control fragmented Hela DNA)

1. After magnetic capture, aliquot 10 µl each of the supernatant in triplicate for each methylated, unmethylated and control sample.
2. For input control and saved supernatant of the non-captured DNA fraction, dilute 1 µl of these samples in 1 ml water, aliquot 10 µl each of the sample in triplicate for each methylated, unmethylated and control sample.
3. Add 1 µl of the primer mix specific to each control locus to each tube.
4. Add 10 µl of the desired qPCR Master Mix according to manufacturer's recommendations.
5. Perform qPCR standard reaction program using an annealing temperature of 60°C.
6. Analyze quantitative PCR results using software provided with the real-time PCR machine.

At least a 500–1000 fold enrichment of the methylated locus (LINE) versus the active gene (RPL30) within the methylated eluant sample as compared to the input DNA should be observed. Conversely, no enrichment of the methylated locus should be observed in the saved supernatant of the non-captured DNA fraction.

Quantitation of DNA by Standard PCR Reaction (for control fragmented DNA)

Perform capture experiment as described previously. Elute sample by incubation at 65°C in 50 µl of DNase-free water. Prepare a master reaction mix as described below:

REAGENT	VOLUME FOR 1 PCR REACTION (20 µl)
DNA Samples (or 1:1000 diluted input DNA)	13.5 µl
Standard <i>Taq</i> Buffer (10X)	2.0 µl
dNTP Mix (4 mM)	1.0 µl
Primers (MyoD, RPL30, MirA, 10 µM)	1.0 µl
<i>Taq</i> DNA Polymerase	0.5 µl

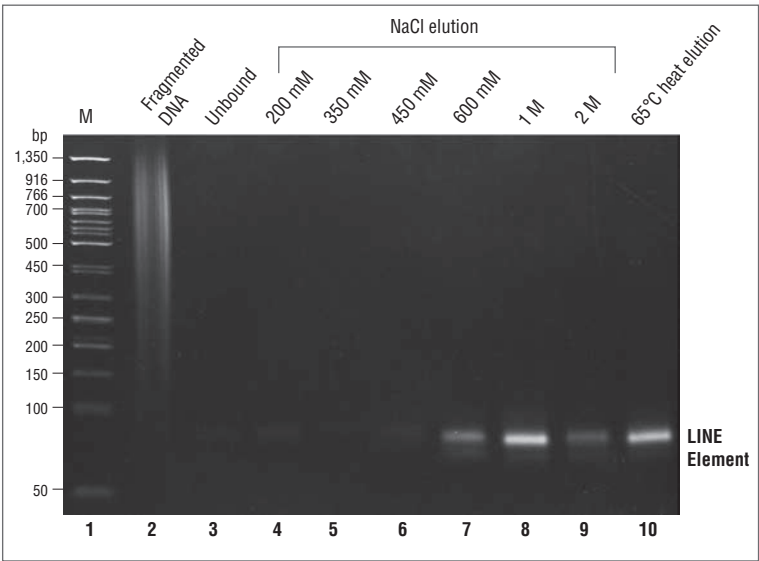
Transfer PCR tubes to a thermocycler:

Thermocycling conditions for a routine PCR:

CYCLE STEP	TEMP	TIME
Initial Denaturation	95°C	5 Seconds
25 cycles	95°C	15 Seconds
	60°C	15 Seconds
	72°C	30 Seconds
Final Extension	72°C	5 Minutes
Hold	4°C	

Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% polyacrylamide gel electrophoresis using the 50 bp DNA Ladder (NEB #N3236) as a standard. The expected size of all the amplicons is ~75 bp. Alternatively, the eluted sample DNA from the qPCR reaction tube may be used.

Figure 2: Endpoint PCR of a methylated locus (LINE) that has been purified using the EpiMark methylated DNA Enrichment Kit



Analyzed fractions are described above gel.

Troubleshooting Guide:

Observation	Possible Cause(s)	Solution(s)
No or poor DNA target detection by PCR-based method in unbound and/or elution fraction(s)	DNA is degraded	Take precautions to prevent DNA degradation by maintaining a nuclease-free environment. Increase EDTA concentration of sample to 10 mM.
	Not enough target DNA	Verify target DNA concentration by nanodrop instrument, or other sensitive DNA detection system. Run target DNA on agarose gel to determine quantity, quality and size.
	DNA did not elute from the MBD2-Fc beads.	Raise the temperature of the elution to 98°C, mindful that this will render the sample single-stranded.
Unable to clone eluted DNA fragments.	DNA ends are frayed due to sonication or nebulization, or DNA has been rendered single-stranded.	Repair DNA ends using a blunt-end repair kit (e.g., Quick Blunting Kit, NEB #E1201).
Controls did not work, did not see bands as expected on gel.	DNA is degraded.	See above for DNA precautions.
	DNA did not elute from the MBD2-Fc beads.	See above for elution at 98°C.
	PCR did not work.	Verify that all of the components have been added to the PCR reaction mix. Lower the annealing temperature of the reaction to 55°C.
Controls did work, but did not see my gene of interest	DNA target does not contain sufficient amounts of CpG methylation.	Raise input DNA concentration to at least 1 µg.
	PCR did not work.	Optimize PCR conditions for your target sequence.

Appendix:

NaCl Elution Protocol

In this step, the captured methylated CpG containing DNA will be eluted with NaCl, either as a single fraction using 2 M NaCl, or multiple fractions. Before proceeding, determine the step-wise elution series to be used to elute the DNA based on the number of methylation sites per molecule. The Bind/Wash Buffer contains 150 mM NaCl. To elute captured CpG-methylated DNA into distinct fractions, start with the lowest NaCl concentration (e.g., 200 mM NaCl). Follow this with the next higher NaCl concentration (e.g., 350 mM NaCl). Continue this process until all the desired fractions have been collected.

Prepare Elution Buffers:

1. For each capture reaction, prepare 1 ml elution buffers using the ratios below:

ELUTION BUFFER 1 ml	NaCl CONCENTRATION	AMOUNT BIND/WASH	AMOUNT 5 M NaCl
Wash	150 mM	1 ml	0 ml
Elution 1	200 mM	990 μ l	10 μ l
Elution 2	350 mM	960 μ l	40 μ l
Elution 3	450 mM	940 μ l	60 μ l
Elution 4	600 mM	910 μ l	90 μ l
Elution 5	1000 mM	830 μ l	170 μ l
Elution 6	2000 mM	630 μ l	370 μ l

2. Immediately following the Wash Off Unbound DNA Protocol, elute the captured DNA by resuspending the beads in 200 μ l of Elution 1 Buffer.
3. Incubate the beads on a rotating mixer for 3 minutes. Place the tube on the magnetic rack for 2–5 minutes to allow the solution to clear.
4. Carefully remove the supernatant to a fresh microcentrifuge tube labeled Elution 1 and store on ice.
5. Resuspend the beads in 200 μ l of the next Elution buffer and repeat the above process until all of the elution steps are completed.
6. Desalt and purify samples using Qiagen or other DNA purification kit following manufacturer's recommendations.

qPCR Primer Sequences and Amplicons

Methylated Sequence: LINE Element

GAAATGCAGAAATCA CCCGTCTT forward
CGGAGGCCGAATAGG AACAGCTCCG reverse

Amplicon-

GAAATGCAGAAATCACCC**CG**TCTTCTGCAT**CG**CTCACACTGGGAGCTGTAGAC
CGGAGCTGTTCTATT**CG**GCCT**CG** 5 CpG

Unmethylated sequence: RPL30

CAAGGCAAAGCGAAATTGGT forward
GCCCCTTCAGTCTCTTCGATT reverse

Amplicon-

GCC**CG**TTTCAGTCTCTT**CG**ATTACCTCAAAGCTGGGCAGTTGTTAG**CG**AGAAT
GACCAATTT**CG**CTTTGCCCTG 4 CpG

Control Sequence: MirA

TTTAAACAGGATATTTACGTTCTGC forward
GAGGAAATCTTCACATCCACG reverse

Amplicon-

TTTAAACAGGATATTTA**CG**TTCTGCTACAATTGACTGATAACACTTG
AAGTGTAGTCTGAACAGTAATTTTGTTAATCATTTCAACAAGTATTT
GCTAAGTGAAGCCAGAAGAGGAGGAAAATGTTTTGCCA**CG**TGGATG
TGAAGATTTCTC 2 CpG

References:

1. Cross, S.H., Charlton, J.A., Nan, X., and Bird, A.P. (1994) *Nat. Genet.* 6, 236–244.
2. Fraga, M.F. et al. (2003) *Nucleic Acids Res.* 31, 1765–1774.
3. Gebhard, C. et al. (2006) *Nucleic Acids Res.* 34,e82.
4. Gebhard, C. et al. (2006) *Cancer Research*, 66, 6118–6128
5. Jacinto, F.V., Ballestar, E. and Esteller, M. (2008) *Biotechniques*, 44, 35, 37, 39 passim.
6. Lister, R. et al. (2008) *Cell*, 133, 523–536.
7. Rauch, T. and Pfeifer, G.P. (2005) *Lab Invest.*, 85, 1172–1180.
8. Schilling, E. and Rehli, M. (2007) *Genomics*, 90, 314–323.
9. Weber, M. et al. (2005) *Nat. Genet.*, 37, 853–862.

Ordering Information

PRODUCT	NEB #	SIZE
EpiMark Methylated DNA Enrichment Kit	E2600S	25 reactions
COMPANION PRODUCTS		
50 bp DNA Ladder	N3236S/L	100-200/500-1,000 gel lanes



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