



LIBRARY PREPARATION

NEBNext[®] ChIP-Seq Library Prep Reagent Set for Illumina[®]

Instruction Manual

NEB #E6200S/L
12/60 reactions
Version 6.0 6/16

 NEW ENGLAND
BioLabs[®] Inc.

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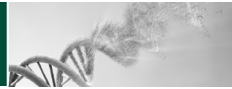







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The Reagent Set Includes:

The volumes provided are sufficient for preparation of up to 12 reactions (NEB #E6200S) and 60 reactions (NEB #E6200L). (All reagents should be stored at -20°C).

- (green) Phosphorylation Reaction Buffer (10X)
- (green) Deoxynucleotide Solution Mix (10 mM each dNTP)
- (green) T4 DNA Polymerase 
- (green) DNA Polymerase I, Large (Klenow) Fragment 
- (green) T4 Polynucleotide Kinase 
- (yellow) Deoxyadenosine 5'-Triphosphate (dATP) (1.0 mM)
- (yellow) Klenow Fragment ($3' \rightarrow 5'$ exo-) 
- (yellow) NEBuffer 2 for Klenow Fragment ($3' \rightarrow 5'$ exo-) (10X)
- (red) Quick T4 DNA Ligase 
- (red) Quick Ligation Reaction Buffer (2X)
- (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix

Required Materials Not Included:

80% Ethanol (freshly prepared)

Nuclease-free Water

0.1X TE, pH 8.0

10 mM Tris-HCl, pH 7.5–8.0

10 mM NaCl (optional)

DNA LoBind Tubes (Eppendorf #022431021)

AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

NEBNext Singleplex or Multiplex Oligos for Illumina (NEB #E7350, #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600)

Magnetic rack/stand

PCR Machine

Applications:

The ChIP-Seq Library Prep Reagent Set for Illumina contains enzymes and buffers that are ideally suited for sample preparation for next-generation sequencing, and for preparation of expression libraries. Each of these components must pass rigorous quality control standards and are Lot Controlled, both individually and as a set of reagents.


Lot Control: The lots provided in the DNA Library Prep Reagent Set for Illumina are managed separately and are qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

Functionally Validated: Each set of reagents is functionally validated together through construction and sequencing of a genomic DNA library on an Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Protocols:

Symbols

 This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

● Colored bullets indicate the cap color of the reagent to be added to a reaction.

Starting Material: 10 ng of chromatin-immunoprecipitated (ChIP) qPCR verified or control DNA, in $\leq 40 \mu\text{l}$ of water or elution buffer.

1.1 End Repair of ChIP DNA

1. Dilute ● (green) DNA Polymerase I, Large (Klenow) Fragment by mixing 1 μl of enzyme with 4 μl of sterile water in a fresh microfuge tube.
2. Mix the following components in a sterile microfuge tube:

ChIP DNA	1–40 μl
● (green) Phosphorylation Reaction Buffer (10X)	5 μl
● (green) T4 DNA Polymerase	1 μl
● (green) T4 Polynucleotide Kinase	1 μl
● (green) dNTPs	2 μl
Diluted DNA Polymerase I, Klenow Fragment	1 μl
Sterile H ₂ O	variable
<hr/>	
Total volume	50 μl

3. Incubate in a thermal cycler for 30 minutes at 20°C.

1.2 Cleanup Using AMPure XP® Beads (Beckman Coulter, Inc.)

1. Vortex AMPure XP Beads to resuspend.
2. Add 90 μl (1.8X) of resuspended AMPure XP Beads to the reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
5. Add 200 μl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.

7. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 40 μ l of 10 mM Tris-HCl or 0.1X TE.
9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 34 μ l of the supernatant to a fresh, sterile microfuge tube.

1.3 dA-Tailing of End Repaired DNA

1. Mix the following components in a sterile microfuge tube:

End Repaired, Blunt DNA	34 μ l
● (yellow) NEBuffer 2 (10X)	5 μ l
● (yellow) Deoxyadenosine 5'-Triphosphate	10 μ l
● (yellow) Klenow Fragment (3'→5' exo-)	1 μ l
Total volume	50 μ l

2. Incubate in a thermal cycler for 30 minutes at 37°C.

1.4 Cleanup Using AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 90 μ l (1.8X) of resuspended AMPure XP Beads to the reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
5. Add 200 μ l of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 15 µl of 10 mM Tris-HCl or 0.1X TE.
9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 10 µl of the supernatant to a fresh, sterile microfuge tube. Sample can be stored at -20°C or proceed directly to Adaptor Ligation.

1.5 Adaptor Ligation of dA-Tailed DNA

Dilute the ● (red) NEBNext Adaptor for Illumina* (15 µM) 10-fold in 10 mM Tris-HCl or 10 mM Tris-HCl with 10 mM NaCl to a final concentration of 1.5 µM.

1. Mix the following components in a sterile microfuge tube:

dA-Tailed DNA	10 µl
● (red) Quick Ligation Reaction Buffer (2X)	15 µl
Diluted NEBNext Adaptor (1.5 µM)	1 µl
● (red) Quick T4 DNA Ligase	4 µl
Total volume	30 µl

*The NEBNext adaptor can be found in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

2. Incubate in a thermal cycler for 15 minutes at 20°C.
3. Add 3 µl of ● (red) USER™ Enzyme Mix by pipetting up and down, and incubate at 37°C for 15 minutes.

Note: This step is only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 and #E7600) Oligos for Illumina.

4. Proceed to Cleanup Using AMPure XP Beads in Section 1.6



A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while performing cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.

1.6 Cleanup of Adaptor Ligated DNA

1. Vortex AMPure XP Beads to resuspend.
2. Add 54 µl of resuspended AMPure XP Beads to the ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute the DNA target by adding 105 µl of 10 mM Tris-HCl or 0.1 X TE to the beads for bead-based size selection.

Note: For size selection using E-Gel size select gels or standard 2% agarose gels, elute the DNA target at desired volume.

9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Transfer 100 µl of supernatant (or desired volume) to a new tube/well, and proceed to bead based size selection.

1.7 Size Select Adaptor Ligated DNA Using AMPure XP Beads

Insert Size	150 bp	200 bp	250 bp	300 bp	400 bp	500 bp	700 bp
Total library size (insert + adaptor)	270 bp	320 bp	370 bp	420 bp	530 bp	660 bp	820 bp
Bead: DNA ratio* 1st bead selection	0.9X	0.8X	0.7X	0.6X	0.55X	0.5X	0.45X
Bead: DNA ratio* 2nd bead selection	0.2X	0.2X	0.2X	0.2X	0.15X	0.15X	0.15X

Table 1.1: Recommended conditions for dual bead-based size selection.



The following size selection protocol is for libraries with 150 bp inserts only. For libraries with different size fragment inserts, please optimize bead: DNA ratio according to Table 1.1 above.

Note: (X) refers to the original sample volume of 100 μ l

1. Add 90 μ l (0.9X) resuspended AMPure XP Beads to 100 μ l DNA solution. Mix well on a vortex mixer or by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Place the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant to a new tube/well (**Caution: do not discard the supernatant**). Discard beads that contain the large fragments.
4. Add 20 μ l (0.2X) resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
5. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard beads**).
6. Add 200 μ l of freshly prepared 80% ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
7. Repeat Step 6 once.
8. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

9. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 22 μ l of 10 mM Tris-HCl or 0.1X TE.
10. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
11. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 20 μ l of the supernatant to a clean PCR tube and proceed to enrichment.

1.8 PCR Enrichment of Adaptor Ligated DNA



Follow Section 1.8A if you are using the following oligos (10 µM primer):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 1.8B if you are using **NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)**.

1.8A PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	20 µl
● (blue) Index Primer/i7 Primer*, **	2.5 µl
● (blue) Universal PCR Primer/i5 Primer*, ***	2.5 µl
● (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
Total volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

*** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	15
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

3. Proceed to Cleanup Using AMPure XP Beads in Section 1.9

1.8B PCR Enrichment of Adaptor Ligated DNA

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	15 μ l
● (blue) Index/ Universal Primer Mix*	10 μ l
● (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 μ l
Total volume	50 μ l

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	15
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

3. Proceed to Cleanup Using Ampure XP Beads in Section 1.9

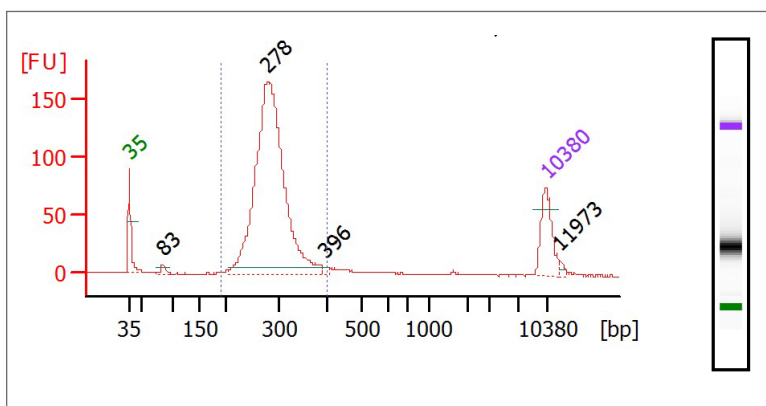
1.9 Cleanup Using AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 45 μ l (0.9X) of resuspended AMPure XP Beads to the PCR reactions (~50 μ l). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/ PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
5. Add 200 μ l of freshly prepared 80% ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 30 μ l of 0.1X TE.
9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
10. Put the tube/PCR plate in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 25 μ l of the supernatant to a clean LoBind® (Eppendorf AG) tube. Libraries can be stored at -20°C .
11. Dilute 2–3 μ l of the library 20 fold with 10 mM Tris-HCl or 0.1X TE and assess the library quality on a Bioanalyzer® (Agilent Technologies, Inc.) high sensitivity chip. Check that the electropherogram shows a narrow distribution with a peak size approximately 275 bp.

Figure 1.1: Bioanalyzer traces of final library.



Phosphorylation Reaction Buffer

#E6201A: 0.06 ml

Concentration: 10X

#E6201AA: 0.3 ml

Store at -20°C

1X Phosphorylation Reaction Buffer:

50 mM Tris-HCl

10 mM MgCl₂

10 mM DTT

1 mM ATP

pH 7.5 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of this reaction buffer at a 1X concentration with 1 µg of φX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this reaction buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

Deoxynucleotide Solution Mix

#E6202A: 0.024 ml 10 mM each dNTP
#E6202AA: 0.120 ml

Store at -20°C

Description: Deoxynucleotide Solution Mix is an equimolar solution of ultrapure dATP, dCTP, dGTP and dTTP.

Supplied in: Milli-Q® (Millipore Corporation) water as a sodium salt at pH 7.5.

Concentration: Each nucleotide is supplied at a concentration of 10 mM. (40 mM total nucleotide concentration).

Quality Assurance: Nucleotide solutions are certified free of nucleases and phosphatases.

Notes: Storing nucleotide triphosphates in solutions containing magnesium promotes triphosphate degradation.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing a minimum of 2 mM dNTPs and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing a minimum of 2 mM dNTPs and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

RNase Activity: Incubation of 1 mM dNTPs with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 5 mM dNTPs in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

HPLC: dNTP purity is determined by HPLC to be > 99%.

Functional Activity (PCR): The dNTPs are tested in 25 cycles of PCR amplification generating 0.5 kb, 2 kb, and 5kb amplicons from lambda DNA.

Lot Controlled

T4 DNA Polymerase

#E6203A: 0.015 ml

#E6203AA: 0.06 ml



Store at -20°C

Description: T4 DNA Polymerase catalyzes the synthesis of DNA in the 5' → 3' direction and requires the presence of template and primer. This enzyme has a 3' → 5' exonuclease activity which is much more active than that found in DNA Polymerase I. Unlike *E. coli* DNA Polymerase I, T4 DNA Polymerase does not have a 5' → 3' exonuclease function.

Source: Purified from a strain of *E. coli* that carries a T4 DNA Polymerase overproducing plasmid.

Supplied in: 100 mM KPO₄ (pH 6.5), 1 mM DTT and 50% glycerol.

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

Endonuclease Activity: Incubation of a minimum of 50 units of this enzyme with 1 µg of φX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 30 units of this enzyme in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Functional Activity (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X T4 DNA Polymerase Reaction Buffer with 33 µM dNTPs including [³H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

Lot Controlled

References:

1. Tabor, S. and Struhl, K. (1989). DNA-Dependent DNA Polymerases. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl (Eds.), *Current Protocols in Molecular Biology* (pp. 3.5.10–3.5.12). New York: John Wiley & Sons Inc.
2. Sambrook, J. et al. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 5.44–5.47). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

DNA Polymerase I, Large (Klenow) Fragment

#E6004A: 0.015 ml

#E6004AA: 0.06 ml



Store at -20°C

Description: DNA Polymerase I, Large (Klenow) Fragment is a proteolytic product of *E. coli* DNA Polymerase I which retains polymerization and 3'→5' exonuclease activity, but has lost 5'→3' exonuclease activity. Klenow retains the polymerization fidelity of the holoenzyme without degrading 5' termini.

Source: A genetic fusion of the *E. coli* *polA* gene, that has its 5'→3' exonuclease domain genetically replaced by maltose binding protein (MBP). Klenow Fragment is cleaved from the fusion and purified away from MBP. The resulting Klenow fragment has the identical amino and carboxy termini as the conventionally prepared Klenow fragment.

Supplied in: 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol and 50% glycerol.

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

16-Hour Incubation: 50 µl reactions containing a minimum of 5 units of this enzyme and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing a minimum of 5 units of this enzyme and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a minimum of 50 units of this enzyme with 1 µg of φX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 50 units of this enzyme in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

RNase Activity: Incubation of a minimum of 5 units of this enzyme with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Functional Activity (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X NEBuffer 2 with 33 µM dNTPs including [³H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

Lot Controlled

References:

1. Sambrook, J. et al. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 5.40–5.43). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

T4 Polynucleotide Kinase

#E6205A: 0.015 ml

#E6205AA: 0.06 ml



Store at -20°C

Description: Catalyzes the transfer and exchange of P_i from the γ position of ATP to the 5'-hydroxyl terminus of polynucleotides (double- and single-stranded DNA and RNA) and nucleoside 3'-monophosphates. Polynucleotide Kinase also catalyzes the removal of 3'-phosphoryl groups from 3'-phosphoryl polynucleotides, deoxynucleoside 3'-monophosphates and deoxynucleoside 3'-diphosphates (1).

Source: An *E. coli* strain that carries the cloned T4 Polynucleotide Kinase gene. T4 Polynucleotide Kinase is purified by a modification of the method of Richardson (1).

Supplied in: 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 μ M ATP and 50% glycerol.

Quality Assurance: Free of exonuclease, phosphatase, endonuclease and RNase activities. Each lot is tested under 5'-end-labeling conditions to assure maximal transfer of [32 P].

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

16-Hour Incubation: 50 μ l reactions containing a minimum of 10 units of this enzyme and 1 μ g of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 μ l reactions containing a minimum of 10 units of this enzyme and 1 μ g T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a minimum of 200 units of this enzyme with 1 μ g of ϕ X174 RF I DNA in assay buffer for 4 hours at 37°C in 50 μ l reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 100 units of this enzyme in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

RNase Activity: Incubation of a minimum of 100 units of this enzyme with 2 μ g MS2 phage RNA for 1 hour at 37°C in 50 μ l 1X T4 Polynucleotide Kinase Reaction Buffer followed by agarose gel electrophoresis shows no degradation. Incubation of 10 units of this enzyme with 40 ng of a FAM- labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Exonuclease Activity: Incubation of 300 units of enzyme with 1 μ g sonicated [3 H]DNA (10^5 cpm/ μ g) for 4 hours at 37°C in 50 μ l reaction buffer released < 0.1% radioactivity.

Functional Activity (Labeling): 32 P end labeling of 5'-hydroxyl terminated d(T) $_8$ with a minimum of 50 units of this enzyme for 30 minutes at 37°C in 50 μ l 1X T4 Polynucleotide Kinase Buffer followed by 20% acrylamide gel electrophoresis reveals that less than 1% of the product has been degraded by exonuclease or phosphatase activities.

Lot Controlled

References:

1. Richardson, C.C. (1981). In P.D. Boyer (Ed.), *The Enzymes* Vol. 14, (pp. 299-314). San Diego: Academic Press.
2. Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 10.59-10.67, 11.31-11.33). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Deoxyadenosine 5'- Triphosphate (dATP)

#E6006A: 0.12 ml

Concentration: 1.0 mM

#E6006AA: 0.6 ml

Store at -20°C

Supplied in: Milli-Q water as a sodium salt at pH 7.5.

Concentration: dATP is supplied at a concentration of 1mM.

Quality Assurance: Nucleotide solutions are certified free of nucleases and phosphatases.

Notes: Storing nucleotide triphosphates in solutions containing magnesium promotes triphosphate degradation. Nucleotide concentrations are determined by measurements of absorbance.

Quality Control Assays

Phosphatase Activity: Incubation of a minimum of 1 mM dATP in protein phosphatase assay buffer (1M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

16-Hour Incubation: 50 µl reactions containing a minimum of 0.2 mM dATP and 1 µg of HindIII digested Lambda DNA incubated for 16 hours results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 0.2 mM dATP and 1 µg T3 DNA incubated for 16 hours also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a minimum of 0.1 mM dATP with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

HPLC: dATP purity is determined by HPLC to be > 99%.

Functional Activity (PCR): This dATP in a pool of dNTPs is tested in 25 cycles of PCR amplification generating 0.5 kb, 2 kb, and 5kb amplicons from lambda DNA.

Lot Controlled

Klenow Fragment (3'→5' exo⁻)

#E6207A: 0.015 ml

#E6207AA: 0.06 ml



Store at -20°C

Description: Klenow Fragment (3'→5' exo⁻) is an N-terminal truncation of DNA Polymerase I which retains polymerase activity, but lacks the 5'→3' exonuclease activity and contains mutations (D355A, E357A), which abolish the 3'→5' exonuclease activity (1).

Source: An *E. coli* strain containing a plasmid with a fragment of the *E. coli* polA (D355A, E357A) gene starting at codon 324.

Supplied in: 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT and 50% glycerol.

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

16-Hour Incubation: 50 µl reactions containing a minimum of 5 units of this enzyme and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing a minimum of 5 units of this enzyme and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a minimum of 50 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 50 units of this enzyme in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

RNase Activity: Incubation of a minimum of 5 units of this enzyme with 40 ng of a FAM- labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Exonuclease Activity: Incubation of a minimum of 200 units of this enzyme with 1 µg sonicated [³H]DNA (10⁵ cpm/µg) for 4 hours at 37°C in 50 µl reaction buffer releases < 0.1% radioactivity.

3'→5' Exonuclease Activity: Incubation of a minimum of 50 units of enzyme in 20 µl of a 10 nM solution of a fluorescent 5'-FAM labeled oligonucleotide for 30 minutes at 37°C yields no detectable 3'→5' degradation as determined by capillary electrophoresis.

Functional Activity (Nucleotide Incorporation): One unit of this enzyme incorporates 10 nmol of dNTP into acid-precipitable material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X NEBuffer 2 with 33 µM dNTPs including [³H]-dTTP, 70 µg/ml denatured herring sperm DNA and 50 µg/ml BSA.

References:

1. Derbyshire, V. et al. (1988) *Science* 240, 199-201.

NEBuffer 2 for Klenow Fragment (3' → 5' exo⁻)

#E6008A: 0.06 ml

Concentration: 10X

#E6008AA: 0.3 ml

Store at -20°C

1X NEBuffer 2:

50 mM NaCl

10 mM Tris-HCl

10 mM MgCl₂

1 mM DTT

pH 7.9 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of this reaction buffer at a 1X concentration with 1 µg of φX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this reaction buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

Quick T4 DNA Ligase

#E6209A: 0.048 ml

#E6209AA: 0.24 ml



Store at -20°C

Source: Purified from *E. coli* C600 pC1857 pPLc28 lig8 (2).

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

16-Hour Incubation: 50 µl reactions containing a minimum of 2,000 units of this enzyme and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing a minimum of 2,000 units of this enzyme and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a minimum of 3,200 units of this enzyme with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 20,000 units of this enzyme in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

RNase Activity: Incubation of a minimum of 2,000 units of this enzyme with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Exonuclease Activity: Incubation of a minimum of 3,200 units of this enzyme with 1 µg sonicated [³H] DNA (10⁵ cpm/µg) for 4 hours at 37°C in 50 µl reaction buffer releases < 0.1% radioactivity.

Functional Activity (Blunt End Ligation): 50 µl reactions containing a 0.5 µl Quick T4 DNA Ligase, 18 µg HaeIII digested ϕX174 and 1X T4 DNA Ligase Buffer incubated at 16°C for 7.5 min results in > 95% of fragments ligated as determined by agarose gel electrophoresis.

Functional Activity (Cohesive End Ligation): 20 µl reactions containing 0.5 µl Quick T4 DNA Ligase, 12 µg HindIII digested lambda DNA and 1X T4 DNA Ligase Buffer incubated at 37°C overnight results in > 95% of fragments ligated as determined by agarose gel electrophoresis. Redigestion of the ligated products, 50 µl reactions containing 6 µg of the ligated fragments, 40 units HindIII, and 1X NEBuffer 2 incubated at 37°C for 2 hours, results in no detectable undigested fragments as determined by agarose gel electrophoresis.

Functional Activity (Adapter Ligation): 50 µl reactions containing 0.125 µl Quick T4 DNA Ligase, 8 nmol 12 bp adapter, and 1X T4 DNA Ligase Buffer incubated at 16°C overnight results in no detectable unligated adapter as determined by agarose gel electrophoresis.

Functional Activity (Transformation): After a five-minute ligation of linearized, dephosphorylated LITMUS™ 28 (containing either blunt [EcoRV] or cohesive [HindIII] ends) and a mixture of compatible insert fragments, transformation into chemically competent *E. coli* DH-5 alpha cells yields a minimum of 1 x 10⁶ recombinant transformants per µg plasmid DNA.

Lot Controlled

References:

1. Engler, M. J. and Richardson, C. C. (1982). In P. D. Boyer (Ed.), *The Enzymes* Vol. 5, (p. 3). San Diego: Academic Press.
2. Remaut, E., Tsao, H. and Fiers, W. (1983) *Gene* 22, 103-113.

Quick Ligation Reaction Buffer

#E6210A: 0.18 ml

Concentration: 2X

#E6210AA: 0.9 ml

Store at -20°C

1X Quick Ligation Reaction Buffer:

66 mM Tris-HCl

10 mM MgCl₂

1 mM dithiothreitol

1 mM ATP

7.5% Polyethylene glycol (PEG 6000)

pH 7.6 @ 25°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of this reaction buffer at a 1X concentration with 1 µg of φX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this reaction buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration in protein phosphatase assay buffer (1M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

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NEBNext Q5 Hot Start HiFi PCR Master Mix

E6630A: 0.3 ml

Concentration: 2X

E6630AA: 0.75 ml (2 vials provided)

Store at -20°C

Description: The NEBNext Q5 Hot Start HiFi PCR Master Mix is specifically optimized for robust, high-fidelity amplification of next-generation sequencing (NGS) libraries, regardless of GC content. The polymerase component of the master mix, Q5 High-Fidelity DNA Polymerase, is a novel thermostable DNA polymerase that possesses 3'→5' exonuclease activity, and is fused to a processivity-enhancing Sso7d domain. Q5 also has an ultra-low error rate (> 100-fold lower than that of *Taq* DNA Polymerase and ~12-fold lower than that of *Pyrococcus furiosus* (Pfu) DNA Polymerase). The buffer component of the master mix has been optimized for robust amplification, even with GC-rich amplicons and offers enhanced compatibility with a variety of beads used in typical NGS workflows. These features make the NEBNext Q5 Hot Start HiFi PCR Master Mix ideal for NGS library construction. This convenient 2X master mix contains dNTPs, Mg⁺⁺ and a proprietary buffer, and requires only the addition of primers and DNA template for robust amplification. The inclusion of the hot start aptamer allows convenient room temperature reaction set up.

Quality Control Assays

16-Hour Incubation: A 50 µl reaction containing NEBNext Q5 Hot Start HiFi PCR Master Mix and 1 µg of HindIII digested λ DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing NEBNext Q5 Hot Start HiFi PCR Master Mix and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of NEBNext Q5 Hot Start HiFi PCR Master Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl₂) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Functional Activity (Multiplex PCR, Bead Inhibition): 30 cycles of PCR amplification of 20 ng genomic DNA with and without carboxylated magnetic beads in a 50 µl reaction containing 0.5 µM 4-plex primer mix and 1X NEBNext Q5 Hot Start HiFi PCR Master Mix result in the four expected amplicons and no inhibition of amplification in the presence of the beads.

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Revision History:

Revision #	Description
4.0	<p>Include protocol for use with NEBNext Q5 Hot Start HiFi PCR Master Mix. Include protocol for changes in concentration of NEBNext Singleplex and Multiplex Oligos for Illumina.</p> <p>Changed all AMPure Bead drying times after ethanol washes to 5 minutes.</p> <p>Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Changed ratio of AMPure Beads to 0.9X in final cleanup after PCR reaction. Added 2 minute incubation after eluting DNA from AMPure Beads.</p>
5.0	<p>Remove protocol for use with NEBNext High-Fidelity 2X PCR Master Mix. Include protocol for use with NEBNext Multiplex Oligos (96 Index Primers, NEB #E6609).</p>
6.0	<p>Protocol updated to include NEB #E7710 and NEB #E7730. Section C in the PCR setup step was removed because all of the 25 μM primers are now expired.</p>

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