

Store at  
-20°C  
**#56795**

# DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN)

1 Kit  
(24 assays)Support: +1-978-867-2388 (U.S.)  
cellsignal.com/supportOrders: 877-616-2355 (U.S.)  
orders@cellsignal.com**For Research Use Only. Not for Use in Diagnostic Procedures.**

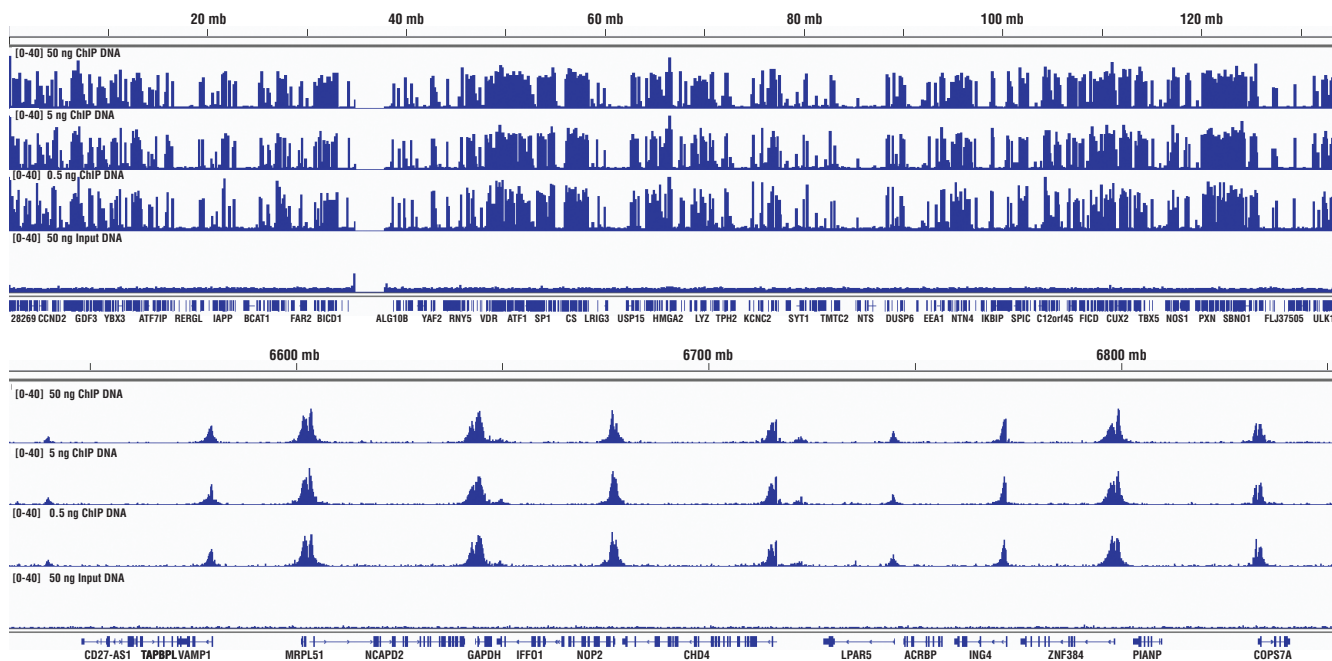
Products Included	Product #	Quantity
End Prep Enzyme Mix	73416	72 µl
End Prep Reaction Buffer	93209	168 µl
Ligation Enhancer	30147	24 µl
Ligation Master Mix	46719	720 µl
Q5 PCR Master Mix	67488	600 µl

**Description:** Next generation sequencing (NG-seq) is a high throughput method that can be used downstream of chromatin immunoprecipitation (ChIP) and Cleavage Under Targets and Release Using Nuclease (CUT&RUN) assays to identify and quantify target DNA enrichment across the entire genome. The DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) contains all of the enzymes and buffers necessary to generate high quality DNA sequencing libraries from ChIP or CUT&RUN DNA for next-generation sequencing on the Illumina Systems platform. The fast, user-friendly workflow minimizes hands-on time needed for generation and purification of DNA libraries. This product must be used in combination with Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538.

This product provides sufficient amounts of reagents for 24 reactions and is compatible with both enzymatic- or sonication-fragmented, ChIP-enriched DNA. Distinct protocols are provided for DNA library preparation from ChIP and CUT&RUN DNA. This product is compatible with SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003, SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005, SimpleChIP® Plus Sonication Chromatin IP Kit #56383, and CUT&RUN Assay Kit #86652. This product is not compatible with SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002 and SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads) #9004 because agarose beads are blocked with sonicated salmon sperm DNA, which will contaminate DNA library preps and NG-seq.

**Storage:** Store all components at -20°C. This product is stable for 12 months if stored properly.

**Specificity/Sensitivity:** This kit has been validated in combination with Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538 to generate qualified DNA libraries using as little as 0.5 ng ChIP DNA or as little as 0.1 ng CUT&RUN DNA as starting materials.



**Figure 1.** ChIP-seq data generated using Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and different starting amounts of ChIP DNA, as described in Table 1. The figure shows binding across chromosome 12 (upper), including GAPDH (lower), a known target gene of H3K4me3. As shown, the peak localizations and heights are similar for all three starting amounts of ChIP DNA.

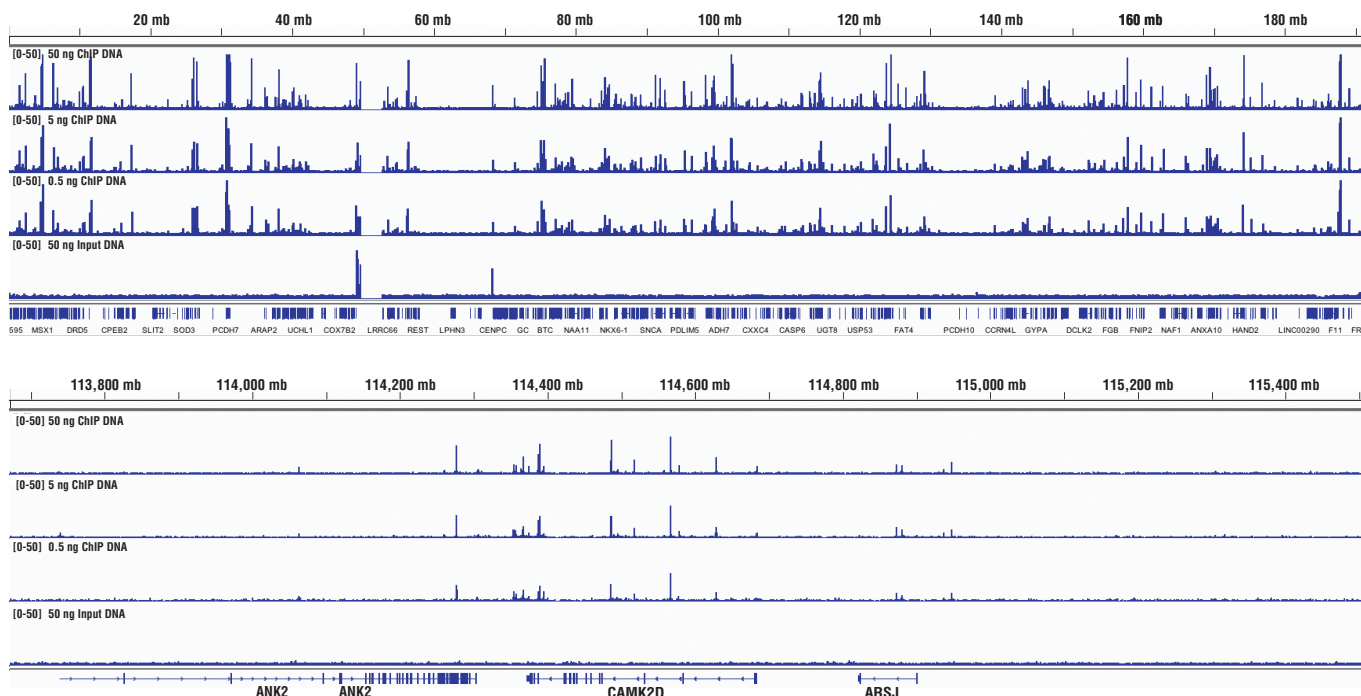
All other trademarks are the property of their respective owners. Visit [cellsignal.com/trademarks](http://cellsignal.com/trademarks) for more information.

**cellsignal.com**

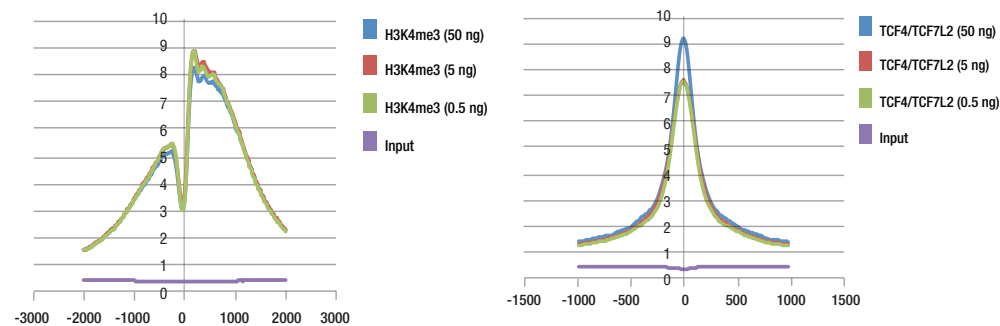
© 2024 Cell Signaling Technology, Inc.

SimpleChIP and Cell Signaling Technology are trademarks of Cell Signaling Technology, Inc.

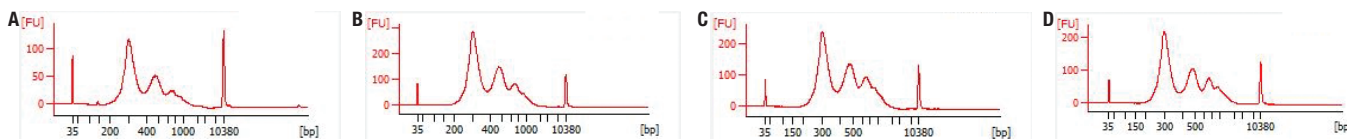
**Applications:** W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry FC-FP—Flow cytometry-Fixed/Permeabilized FC-L—Flow cytometry-Live E-P—ELISA-Peptide  
**Species Cross-Reactivity:** H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse  
**All**—all species expected. Species enclosed in parentheses are predicted to react based on 100% homology.



**Figure 2.** ChIP-seq data generated using TCF4/TCF7L2 (C48H11) Rabbit mAb #2569, as described in Table 1. The figure shows binding across chromosome 4 (upper), including CaMK2D (lower), a known target gene of TCF4/TCF7L2. As shown, the peak localizations and heights are similar for all three starting amounts of ChIP DNA.



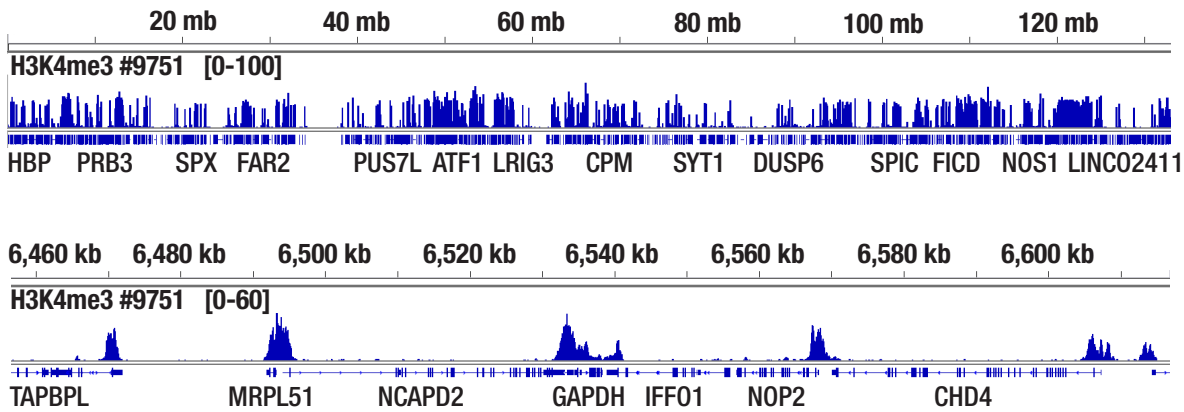
**Figure 3.** Metagene analysis of ChIP-seq data generated using different amounts of starting ChIP DNA. Analyses of both Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 (left) and TCF4/TCF7L2 (C48H11) Rabbit mAb #2569 (right) ChIP-seq data show that the signal-to-noise ratio for peaks identified across entire genome are similar for all three starting amounts of ChIP DNA.



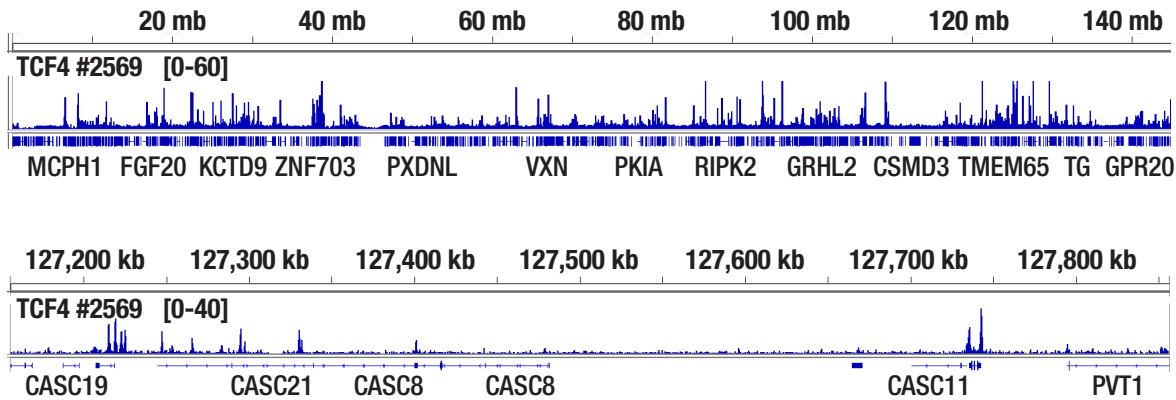
**Figure 4.** Agilent Bioanalyzer System profiles of DNA libraries prepared from different starting amounts of ChIP DNA generated using the SimpleChIP<sup>®</sup> Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared from 50 ng (A), 5 ng (B), and 0.5 ng (C) of enriched ChIP DNA and 50 ng input DNA (D). As shown, each DNA library preparation shows a similar DNA fragment size (expected range 300 bp to 900 bp).

Libraries	Library Yield (ng)	Total Reads (Millions)	% Reads mapped to genome	Identified Peaks
TCF4/TCF7L2 #2569 (50 ng)	140.8	39.8	97.8	9425
TCF4/TCF7L2 #2569 (5 ng)	187.8	34.4	97.8	10506
TCF4/TCF7L2 #2569 (0.5 ng)	67.3	33.7	96.0	9416
H3K4me3 #9751 (50 ng)	110.7	29.4	98.5	27967
H3K4me3 #9751 (5 ng)	120.5	33.7	98.2	28665
H3K4me3 #9751 (0.5 ng)	44.2	29.4	94.1	32221
Input DNA (50 ng)	112.2	26.8	97.9	Not Available

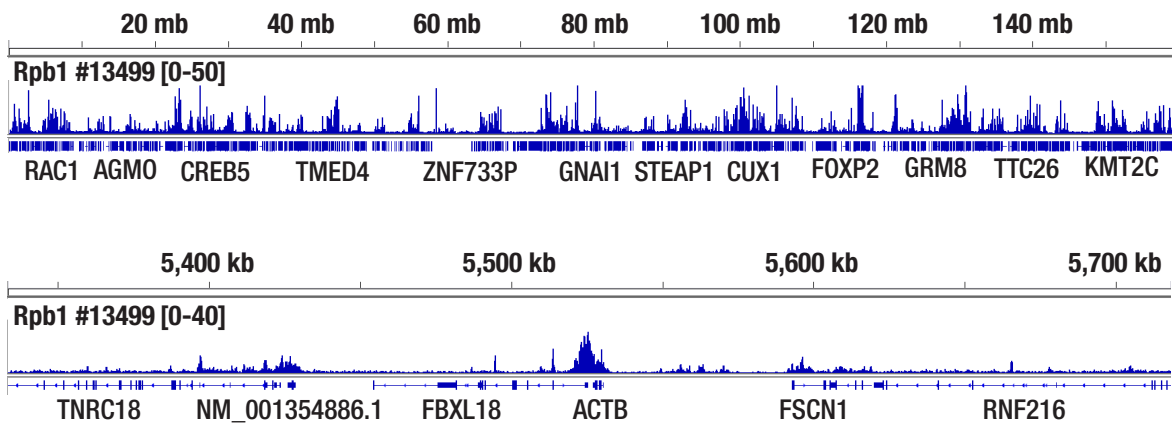
**Table 1.** Comparison of DNA sequencing libraries prepared from different starting amounts of ChIP DNA generated using the SimpleChIP<sup>®</sup> Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. ChIP was performed using 4 x 10<sup>6</sup> HCT 116 cells and either TCF4/TCF7L2 (C48H11) Rabbit mAb #2569 or Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751. Libraries were generated using dual index primers provided in Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538, pooled into one sample, and sequenced on an Illumina Systems Next-Seq platform. As shown, the number of identified peaks is similar for all three starting amounts of ChIP DNA.



**Figure 5.** CUT&RUN NGS data generated using Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and 3.6 ng of CUT&RUN DNA, as described in Table 2. The figure shows binding across chromosome 12 (upper), including GAPDH (lower), a known target gene of H3K4me3.



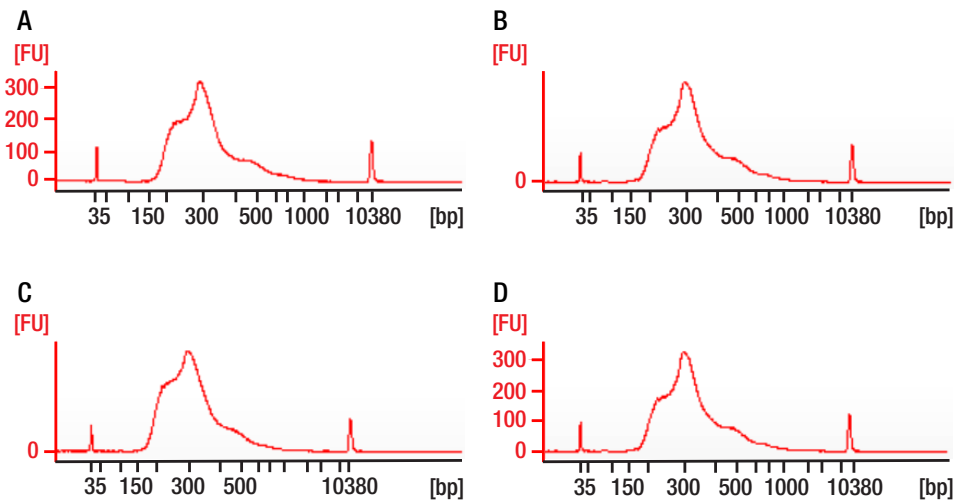
**Figure 6.** CUT&RUN NGS data generated using TCF4/TCF7L2 (C48H11) Rabbit mAb #2569 and 0.7 ng of CUT&RUN DNA, as described in Table 2. The figure shows binding across chromosome 8 (upper), including MYC (lower), a known target gene of TCF4/TCF7L2.



**Figure 7.** CUT&RUN NGS data generated using Phospho-Rpb1 CTD (Ser2) (E1Z3G) Rabbit mAb #13499 and 3.1 ng of CUT&RUN DNA, as described in Table 2. The figure shows binding across chromosome 7 (upper), including ACTB (lower), a known target gene of Phospho-Rpb1.

Libraries	Library Yield (ng)	Total Reads (Millions)	% Reads mapped to genome	Identified Peaks
H3K4me3 #9751 (3.6 ng)	89.2	12.1	96.6	24909
H3K4me3 #9751 (0.5 ng)	44.6	8.2	61.2	13817
TCF4/TCF7L2 #2569 (0.7ng)	32.0	9.0	93.1	936
Phospho-Rpb1 #13499 (3.1 ng)	35.0	9.7	93.8	3893

**Table 2.** DNA sequencing libraries prepared from different starting amount of CUT&RUN DNA generated using the CUT&RUN Assay Kit #86652. CUT&RUN was performed using either Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, TCF4/TCF7L2 (C48H11) Rabbit mAb #2569, or Phospho-Rpb1 CTD (Ser2) (E1Z3G) Rabbit mAb #13499. Libraries were generated using dual index primers provided in Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538, pooled into one sample, and sequenced on an Illumina Systems Next-Seq platform. Peak numbers were identified by MACS2 program.



**Figure 8.** Agilent Bioanalyzer System profiles of DNA libraries prepared from different starting amounts of CUT&RUN DNA generated using the Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751. DNA libraries were prepared from 1 ng (A), 0.5 ng (B), 0.3 ng (C), and 0.1 ng (D) of enriched CUT&RUN DNA. As shown, each DNA library preparation shows a similar DNA fragment size.

## DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN)

Next generation sequencing (NG-seq) is a high throughput method that can be used downstream of chromatin immunoprecipitation (ChIP) and Cleavage Under Targets and Release Using Nuclease (CUT&RUN) assays to identify and quantify target DNA enrichment across the entire genome. The DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) contains all of the enzymes and buffers necessary to generate high quality DNA sequencing libraries from ChIP DNA or CUT&RUN DNA for next-generation sequencing on the Illumina Systems platform. The fast, user-friendly workflow minimizes hands-on time needed for generation and purification of DNA libraries.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of indexed libraries on the Illumina Systems sequencing platform.

This product must be used in combination with Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538. This product provides sufficient amounts of reagents for 24 reactions and is compatible with both enzymatic- or sonication-fragmented, ChIP-enriched DNA, and CUT&RUN DNA (with different protocols for DNA enriched from ChIP or CUT&RUN assays).

### Compatible Assay kits:

SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003

SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005

SimpleChIP® Plus Sonication Chromatin IP Kit #56383

Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580

Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538

CUT&RUN Assay Kit #86652

### Non-Compatible SimpleChIP® kits:

SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002

SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads) #9004

**Note:** Agarose beads are blocked with sonicated salmon sperm DNA, which will contaminate DNA library preps and NG-seq.

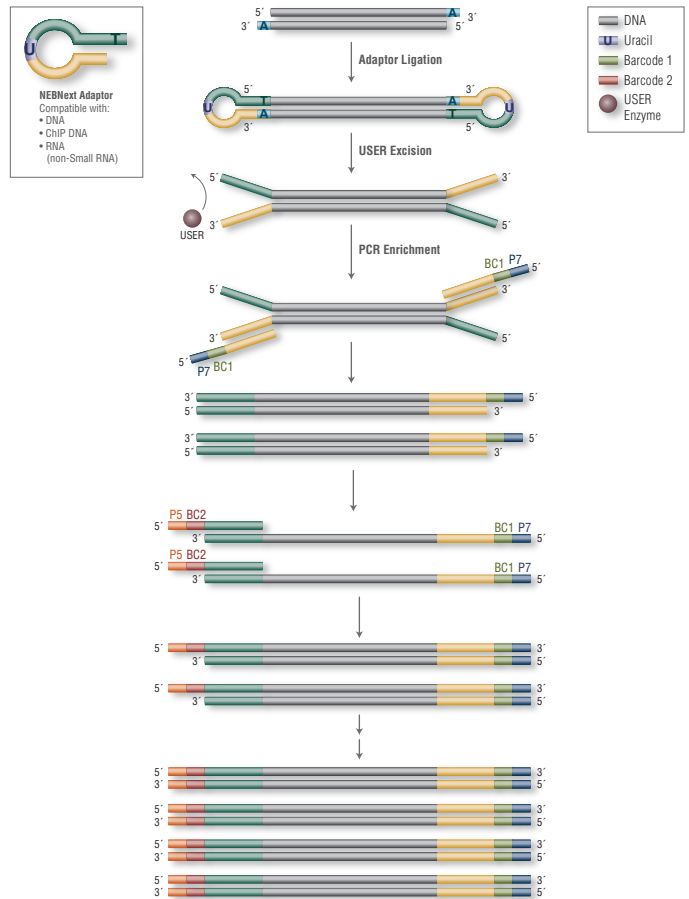
## Required Reagents

Reagents Included:

- (green) End Prep Enzyme Mix #73416
- (green) End Prep Reaction Buffer #93209
- (red) Ligation Master Mix #46719
- (red) Ligation Enhancer #30147
- (blue) Q5 PCR Master Mix (2X) #67488

Reagents Not Included:

- Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538
- Nuclease-free Water #12931
- AMPure XP Beads (Beckman Coulter, Inc. #A63881) or SPRIselect Reagent Kit (Beckman Coulter, Inc. #B23317)
- 80% Ethanol (freshly prepared)
- 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 10 mM Tris-HCl (pH 8.0-8.5)
- Magnetic rack/stand
- Agilent Bioanalyzer Systems and Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.)
- PCR tubes and PCR Machine



**Figure 9.** Workflow demonstrating the use of DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) in combination with the Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538.



**Safe Stop** This is a safe stopping point in the protocol, if stopping is necessary.

## I. End Preparation:

When performing ChIP-seq, it is necessary to generate a control DNA sequencing library that can be used to determine any experimental bias in DNA enrichment that is introduced during the ChIP assay and DNA library preparation. DNA purified from input chromatin (i.e. the chromatin that was used for the IP) is typically used to generate the control DNA library.

Starting Material: 500 pg –1 µg of ChIP DNA. To ensure optimal diversity of the DNA sequencing libraries, we recommend using 5 ng of ChIP-enriched DNA for transcription factor or co-factor ChIP-seq, 50 ng of ChIP-enriched DNA for total histone or histone modification ChIP-seq, and 50 ng of input DNA for the control DNA sequencing library. If necessary, less than 5 ng of ChIP-enriched DNA can be used for library generation; however, this may result in a lower diversity of library due to PCR bias during amplification.

### Before starting:

- Thaw End Prep Reaction Buffer (●) at room temperature.
  - Prepare 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
1. Prepare 0.5-50 ng of ChIP DNA and relative input DNA (control DNA) in a sterile nuclease-free tube. Add 1X TE to generate a final volume of 50 µl for each DNA sample.
  2. Add 3 µl of End Prep Enzyme Mix (●) and 7 µl of End Prep Reaction Buffer (●) to each DNA sample to make a total reaction volume of 60 µl.
  3. Thoroughly mix the reactions by pipetting up and down at least 10 times and perform a quick spin to collect all liquid from the sides of the tube. The presence of a small amount of bubbles will not interfere with performance.
  4. Place in a thermocycler and run the following program:
    - 30 minutes at 20°C
    - 30 minutes at 65°C
    - Hold at 4°C
  5. Proceed to Adaptor Ligation (Section II). If necessary, samples can be stored at -20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

## II. Adaptor Ligation:

### Before starting:

- Both Adaptor for Illumina Systems (●) and USER enzyme (●) can be found in either Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538.
- Thaw Adaptor for Illumina Systems (●) at room temperature.
- Mix the Ligation Master Mix by pipetting up and down several times.
- Prepare approximately 2.5 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.

1. Dilute the Adaptor for Illumina Systems (●) in 10 mM Tris-HCl (pH 8.0-8.5). If starting DNA is 5 ng to 100 ng, dilute the adaptor 1:10 to generate a working concentration of 1.5 µM. If starting DNA is less than 5 ng, dilute the adaptor 1:25 to generate a working concentration of 0.6 µM.
2. Add 30 µl Ligation Master Mix (●), 1 µl Ligation Enhancer (●), and 2.5 µl diluted Adaptor for Illumina Systems directly to the 60 µl End Prep Reaction Mixture from step 5 in Section I.
 

**Note:** Preparing a reaction premix containing the diluted adaptor ahead of time is NOT recommended.
3. Thoroughly mix the ligation reaction by pipetting up and down at least 10 times and perform a quick spin to collect all liquid from the sides of the tube. The Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.
4. Incubate at room temperature for 15 minutes.
5. Add 3 µl of USER Enzyme (●) to the ligation mixture. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.
6. Proceed to Cleanup of Adaptor-ligated ChIP DNA (Section III). **(Safe Stop)** Alternatively, samples can be stored overnight at -20°C.

## III. Cleanup of Adaptor-ligated ChIP DNA without Size Selection

Size selection is NOT recommended during the Cleanup Adaptor-ligated ChIP DNA phase, because it results in a dramatic decrease in both yield and diversity of ChIP-seq DNA libraries.

### Before starting:

- If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.
- Resuspend AMPure XP Beads or SPRIselect beads by tube inversion or pipetting up and down.
- Prepare 400 µl of 80% ethanol for each sample.
- Prepare 17 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.

1. Add 87 µl (0.9X) resuspended AMPure XP beads or SPRIselect beads to each adaptor ligation reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
2. Incubate samples on bench top for at least 5 minutes at room temperature.
3. Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
4. Once the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
6. Repeat step 5 once for a total of two washes. Be sure to remove all visible liquid after the second wash.
7. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
 

**Note:** Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still glossy looking, but when all visible liquid has evaporated. When the beads start to crack, they are too dry.
8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl (pH 8.0-8.5) per sample. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.
9. Place the tube/plate on the magnetic stand and wait for 5 minutes. Once the solution is clear, transfer 15 µl of supernatant containing the DNA targets to a new PCR tube. Proceed to PCR Enrichment of Adaptor-ligated ChIP-DNA (Section IV). **(Safe Stop)** Alternatively, samples can be stored at -20°C.

## IV. PCR Enrichment of Adaptor-ligated ChIP DNA

### Before starting:

- Universal PCR Primer for Illumina Systems (●) and twelve Index Primers for Illumina Systems (●) are provided in Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580. For use with Single Index Primers (#29580), use only one Index Primer per PCR reaction. Refer to #29580 data sheet for valid barcode combinations and tips for setting up PCR reactions.
- Eight Index 5 Primers for Illumina Systems (white caps) and twelve Index 7 Primers for Illumina Systems (orange caps) are provided in Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538. For use with Dual Index Primers (#47538), use only one Index 5 primer and one Index 7 primer per reaction. Refer to #47538 data sheet for valid barcode combinations and tips for setting up PCR reactions.
- Thaw primers and the purified adaptor-ligated ChIP DNA fragments (from step 9, Section III) at room temperature.

1. Add the following components to a sterile PCR tube:

Reagents	Volume for 1 PCR Reaction (50 µl)
Purified adaptor ligated ChIP-DNA fragments (from step 9, Section III)	15 µl
Q5 PCR Master Mix (●)	25 µl
Single Index Primer for Illumina Systems (●) (or Dual Index 7 Primer for Illumina Systems [orange cap] for dual indexing)	5 µl
Universal PCR Primer for Illumina Systems (●) (or Dual Index 5 Primer for Illumina Systems [white cap] for dual indexing)	5 µl

2. Thoroughly mix the reaction by pipetting up and down 10 times and perform a quick spin to collect all liquid from the sides of the tube.
3. Place the tube on a thermo cycler and perform PCR amplification using the following PCR cycling conditions:

a.	Initial Denaturation	98°C for 30 sec
b.	Denaturation	98°C for 10 sec
c.	Anneal and Extension	65°C for 15 sec
d.	For starting material of 50 ng ChIP DNA, repeat steps b and c for a total of 6 cycles. For starting material of 5 ng ChIP DNA, repeat steps b and c for a total of 10 cycles. For starting material of 0.5 ng ChIP DNA, repeat steps b and c for a total of 13 cycles.	
e.	Final Extension	65°C for 3 min
f.	Hold	4°C

4. Proceed to Cleanup of PCR Amplification (Section V). **(Safe Stop)** Alternatively, samples can be stored at -20°C.

### V. Cleanup of PCR Amplification

#### Before starting:

- If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.
  - Resuspend AMPure XP Beads or SPRIselect beads by tube inversion or pipetting up and down.
  - Prepare 400 µl of 80% ethanol for each sample.
  - Prepare approximately 40 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.
1. Add 45 µl (0.9X) resuspended AMPure XP beads or SPRIselect beads to 50 µl PCR reaction from step 4 in Section IV. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
  2. Incubate samples on bench top for at least 5 minutes at room temperature.
  3. Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
  4. Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
  5. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
  6. Repeat Step 5 once for a total of two washes. Be sure to remove all visible liquid after the second wash.
  7. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
 

**Note:** Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still glossy looking, but when all visible liquid has evaporated. When the beads start to crack, they are too dry.
  8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 10 mM Tris-HCl (pH 8.0-8.5) per sample. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.
  9. Place the tube/plate on the magnetic stand and wait for 5 minutes. Carefully transfer 30 µl of supernatant containing the DNA targets to a new tube. **(Safe Stop)** Libraries can be stored at -20°C.
  10. Measure the concentration of library DNA by Nanodrop or Picogreen assay. The concentration of the DNA library should be in the range of 10-40 ng/µl.
  11. Dilute 1 µl of the library DNA with 10mM Tris-HCl to a final concentration of 5-10 ng/µl. Use the diluted library DNA to determine size distribution using an Agilent Bioanalyzer High Sensitivity DNA chip, according to the manufacturer's instructions.
  12. If adaptor dimer (~128 bp for Single Index Primers or ~146 bp for Dual Index Primers) contamination is observed, repeat cleanup steps 1-10. Residual adaptors and/or adaptor dimers will strongly contaminate the sequencing reaction.
  13. Dilute final purified library samples with 10mM Tris-HCl for high throughput sequencing. Refer to Illumina Systems sequencing manual for optimal concentration and volume of library DNA required for NG-seq.

**SAFE STOP** This is a safe stopping point in the protocol, if stopping is necessary.

## I. End Preparation

When performing CUT&RUN-seq, it is necessary to generate a control DNA sequencing library that can be used to determine any experimental bias in DNA enrichment that is introduced during the CUT&RUN assay and DNA library preparation. DNA purified from input DNA (refer to Section V of CUT&RUN Assay Kit #86652 Protocol) is typically used to generate the control DNA library.

**Starting Material:** 0.5 ng–1 µg of CUT&RUN DNA. The typical yield for CUT&RUN DNA is 0.6 to 6 ng per reaction with 100,000 starting cells. To ensure optimal diversity of the DNA sequencing libraries, we recommend using as much CUT&RUN DNA as you can obtain from a CUT&RUN reaction. If necessary, as little as 0.1 ng of CUT&RUN DNA can be used for library generation; however, this may result in a lower diversity of library due to PCR bias during amplification. For the control DNA sequencing library using input DNA, 5 - 10 ng is a good starting point. Please note that a Picogreen assay is needed to determine the concentration of CUT&RUN DNA because Nanodrop alone is not sensitive enough.

### Before starting:

- Thaw the End Prep Reaction Buffer #93209 (●) at room temperature. Vortex if DTT is precipitated.
  - Prepare 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
1. Prepare 0.5-10 ng of CUT&RUN DNA and relative input DNA (control DNA) in separate sterile nuclease-free tubes. Add 1X TE to generate a final volume of 50 µl for each DNA sample.
  2. Add 3 µl of End Prep Enzyme Mix (●) and 7 µl of End Prep Reaction Buffer (●) to each DNA sample to make a total reaction volume of 60 µl.
  3. Thoroughly mix the reactions by pipetting up and down at least 10 times and perform a quick spin to collect all liquid from the sides of the tube. The presence of a small amount of bubbles will not interfere with performance.
  4. Place in a thermocycler and run the following program:
    - ◇ 30 minutes at 20°C
    - ◇ 30 minutes at 50°C
    - ◇ Hold at 4°C
  5. Proceed to Adaptor Ligation (Section II). If necessary, samples can be stored at -20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

## II. Adaptor Ligation

### Before starting:

- Both Adaptor for Illumina Systems (●) and USER enzyme (●) can be found in either Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538.
  - Thaw Adaptor for Illumina Systems (●) at room temperature.
  - Mix the Ligation Master Mix by pipetting up and down several times.
  - Prepare approximately 2.5 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.
1. Dilute the Adaptor for Illumina Systems (●) in 10 mM Tris-HCl (pH 8.0-8.5) as indicated in the table to avoid the contamination of library DNA by excess adaptors.

Starting DNA	Adaptor Dilution	Working Adaptor Concentration
100 ng – 1 µg	No Dilution	15 µM
5 ng – 100 ng	1:10	1.5 µM
2.5 ng – 5 ng	1:25	0.6 µM
1.25 ng – 2.5 ng	1:50	0.3 µM
Less than 1.25 ng	1:125	0.12 µM

2. Add 30 µl Ligation Master Mix (●), 1 µl Ligation Enhancer (●), and 2.5 µl diluted Adaptor for Illumina Systems directly to the 60 µl End Prep Reaction Mixture from step 5 in Section I.
 

**Note:** Preparing a reaction premix containing the diluted adaptor ahead of time is NOT recommended. However, a premix of Ligation Master Mix and Ligation Enhancer is allowed if placed at 4°C for < 8 hours.
3. Thoroughly mix the ligation reaction by pipetting up and down at least 10 times and perform a quick spin to collect all liquid from the sides of the tube. The Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.
4. Incubate at room temperature for at least 15 minutes.
5. Add 3 µl of USER Enzyme (●) to the ligation mixture. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.
6. Proceed to Cleanup of Adaptor-ligated CUT&RUN DNA (Section III). **(Safe Stop)** Alternatively, samples can be stored overnight at -20°C.

## III. Cleanup of Adaptor-ligated CUT&RUN DNA without Size Selection

Size selection is NOT recommended during the Cleanup Adaptor-ligated CUT&RUN DNA phase, because it results in a dramatic decrease in both yield and diversity of DNA libraries.

### Before starting:

- If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.
  - Resuspend AMPure XP Beads or SPRIselect beads by tube inversion or pipetting up and down.
  - Prepare 400 µl of 80% ethanol for each sample.
  - Prepare 17 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.
1. Add 106 µl (1.1X) resuspended AMPure XP beads or SPRIselect beads to each adaptor ligation reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
  2. Incubate samples on bench top for at least 5 minutes at room temperature.
  3. Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
  4. Once the solution is clear, carefully remove and discard the supernatant. Be careful to remove all liquid residues but not to disturb the beads that contain DNA targets.
  5. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
  6. Repeat step 5 once for a total of two washes. Be sure to remove all visible liquid after the second wash.
  7. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
 

**Note:** Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still glossy looking, but when all visible liquid has evaporated. If the beads start to crack, they are too dry.
  8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl (pH 8.0-8.5) per sample. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.
  9. Place the tube/plate on the magnetic stand and wait for 5 minutes. Once the solution is clear, transfer 15 µl of supernatant containing the DNA targets to a new PCR tube. Proceed to PCR Enrichment of Adaptor-ligated ChIP-DNA (Section IV). **(Safe Stop)** Alternatively, samples can be stored at -20°C.



## IV. PCR Enrichment of Adaptor-ligated CUT&RUN DNA

### Before starting:

- Universal PCR Primer for Illumina Systems (●) and twelve Index Primers for Illumina Systems (●) are provided in Multiplex Oligos for Illumina Systems (Single Index Primers) (ChIP-seq, CUT&RUN) #29580. For use with Single Index Primers (#29580), use only one Index Primer per PCR reaction. Refer to #29580 data sheet for valid barcode combinations and tips for setting up PCR reactions.
- Eight Index 5 Primers for Illumina Systems (white caps) and twelve Index 7 Primers for Illumina Systems (orange caps) are provided in Multiplex Oligos for Illumina Systems (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538. For use with Dual Index Primers (#47538), use only one Index 5 primer and one Index 7 primer per reaction. Refer to #47538 data sheet for valid barcode combinations and tips for setting up PCR reactions.
- Thaw primers and the purified adaptor-ligated CUT&RUN DNA fragments (from step 9, Section III) at room temperature.

### 1. Add the following components to a sterile PCR tube:

Reagents	Volume for 1 PCR Reaction (50 µl)
Purified adaptor ligated CUT&RUN-DNA fragments (from step 9, Section III)	15 µl
Q5 PCR Master Mix (●)	25 µl
Single Index Primer for Illumina Systems (●) (or Dual Index 7 Primer for Illumina Systems [orange cap] for dual indexing)	5 µl
Universal PCR Primer for Illumina Systems (●) (or Dual Index 5 Primer for Illumina Systems [white cap] for dual indexing)	5 µl

2. Thoroughly mix the reaction by pipetting up and down 10 times and perform a quick spin to collect all liquid from the sides of the tube.
3. Place the tube on a thermo cycler and perform PCR amplification using the following PCR cycling conditions:

<b>a.</b>	Initial Denaturation	98°C for 30 sec
<b>b.</b>	Denaturation	98°C for 10 sec
<b>c.</b>	Anneal and Extension	65°C for 13 sec
<b>d.</b>	For 50 - 100 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 6 - 7 cycles
	For 5 - 50 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 8-12 cycles
	For 1 - 5 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 13 - 15 cycles
	For 0.5 - 1 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 16 - 17 cycles
	For 0.2 - 0.5 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 18 - 19 cycles
	For 0.1 - 0.2 ng starting CUT&RUN DNA	Repeat steps b and c for a total of 20 cycles
<b>e.</b>	Final Extension	65°C for 3 min
<b>f.</b>	Hold	4°C

4. Proceed to Cleanup of PCR Amplification (Section V). **(Safe Stop)** Alternatively, samples can be stored at -20°C.

## V. Cleanup of PCR Amplification

### Before starting:

- If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.
- Resuspend AMPure XP Beads or SPRIselect beads by tube inversion or pipetting up and down.
- Prepare 400 µl of 80% ethanol for each sample.
- Prepare approximately 40 µl of 10 mM Tris-HCl (pH 8.0-8.5) for each sample.

1. Add 50 µl (1.0X) resuspended AMPure XP beads or SPRIselect beads to 50 µl PCR reaction from step 4 in Section IV. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
2. Incubate samples on bench top for at least 5 minutes at room temperature.
3. Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
4. Carefully remove and discard the supernatant. Be careful to remove all liquid residues but not to disturb the beads that contain DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
6. Repeat Step 5 once for a total of two washes. Be sure to remove all visible liquid after the second wash.
7. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
 

**Note:** Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still glossy looking, but when all visible liquid has evaporated. If the beads start to crack, they are too dry.
8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 10 mM Tris-HCl (pH 8.0-8.5) per sample. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.
 

**Note:** A smaller volume of Tris-HCl (pH 8.0-8.5) (eg. 17 µl) can be used, if a higher concentration of library DNA is desired.
9. Place the tube/plate on the magnetic stand and wait for 5 minutes. Carefully transfer 30 µl of supernatant containing the DNA targets to a new tube. **(Safe Stop)** Libraries can be stored at -20°C.
 

**Note:** Transfer only 15 µl supernatant containing the DNA targets to a new tube if 17 µl of Tris-HCl (pH 8.0-8.5) is used in Step 8.
10. Measure the concentration of library DNA by Nanodrop or Picogreen assay. The concentration of the DNA library should be in the range of 10-40 ng/µl.
11. Dilute 1 µl of the library DNA with 10mM Tris-HCl to a final concentration of 5-10 ng/µl. Use the diluted library DNA to determine size distribution using an Agilent Bioanalyzer High Sensitivity DNA chip, according to the manufacturer's instructions.
12. If adaptor dimer (~128 bp for Single Index Primers or ~146 bp for Dual Index Primers) contamination is observed, repeat cleanup steps 1-10. Residual adaptors and/or adaptor dimers will strongly contaminate the sequencing reaction.
13. Dilute final purified library samples with 10mM Tris-HCl for high throughput sequencing. Refer to Illumina Systems sequencing manual for optimal concentration and volume of library DNA required for NG-seq.

### I. End Prep Enzyme Mix (•)

#### Description

End Prep Enzyme Mix is optimized to convert 500 pg-1 µg of fragmented DNA to repaired DNA having 5'-phosphorylated, 3'-dA-tailed ends.

#### Quality Control Assays

1. SDS-PAGE Purity: SDS-PAGE analysis of each individual enzyme indicates > 95% enzyme purity.
2. Endonuclease Activity: Incubation of a minimum of 10 µl of this enzyme mix 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.
3. Phosphatase Activity: Incubation of a minimum of 10 µl of this enzyme mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) 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.
4. Functional Activity (Nucleotide Incorporation, Phosphorylation and dA-Tailing): 1 µl of this enzyme mix repairs and phosphorylates the ends of > 95% of 0.5 µg of DNA fragments containing both 3' and 5' overhangs with 20 minutes at 25°C, in 1X End Repair Reaction buffer, as determined by capillary electrophoresis.

### II. End Prep Reaction Buffer (•)

#### Quality Control Assays

1. 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.
2. 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.
3. 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.
4. 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 MgCl<sub>2</sub>) 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.

### III. Ligation Master Mix (•)

#### Description

Ligation Master Mix is a ready-to-use solution of T4 DNA Ligase, proprietary ligation enhancer, and optimized reaction buffer.

#### Quality Control Assays

1. 16-Hour Incubation: 50 µl reactions containing Ligation Master Mix 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 Ligation Master Mix 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.
2. Endonuclease Activity: Incubation of Ligation Master Mix 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.
3. RNase Activity: Incubation of Ligation Master Mix 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.

4. Transformation Assay: LITMUSTM 28 vector is cut with EcoRV (blunt), treated with calf intestinal phosphatase and gel purified. Blunt inserts from a HaeIII digest of φX174 DNA are ligated into the vector at a 3:1 insert:vector ratio using the Ligation Master Mix Protocol. Ligation products are transformed as described. Each lot exceeds the following standards:

#### Efficiency (transformants/µg)

	Recircularization	Insertion
Blunt ends	> 1 x 10 <sup>7</sup>	> 2.5 x 10 <sup>6</sup>
Uncut vector	> 1 x 10 <sup>8</sup>	N/A

### IV. Ligation Enhancer (•)

#### Quality Control Assays

1. 16-Hour Incubation: 50 µl reactions containing 1 µl Ligation Enhancer 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 1 µl NEBNext 5' SR Adaptor 3 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.
2. Endonuclease Activity: Incubation of a 50 µl reaction containing 1 µl Ligation Enhancer with 1 µg of φX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.
3. RNase Activity: Incubation of a 10 µl reaction containing 1 µl Ligation Enhancer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.
4. Phosphatase Activity: Incubation of 1 µl Ligation Enhancer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) 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.

### V. Q5 PCR Master Mix (2X) (•)

#### Description

The Q5 PCR Master Mix (2X) 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 Q5 PCR Master Mix ideal for NGS library construction. This convenient 2X master mix contains dNTPs, Mg<sup>++</sup> 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

1. 16-Hour Incubation: A 50 µl reaction containing Q5 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 Q5 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.
2. Phosphatase Activity: Incubation of Q5 PCR Master Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM MgCl<sub>2</sub>) 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.
3. 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 Q5 PCR Master Mix result in the four expected amplicons and no inhibition of amplification in the presence of the beads.