Takara Bio USA, Inc.

DNA SMART™ ChIP-Seq Kit User Manual

Cat. Nos. 634865, 634866, 634867
(101617)

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I. Introduction

DNA SMART ChIP-Seq for Illumina® Sequencing Platforms

DNA SMART ChIP-Seq Kit generates Illumina-compatible sequencing libraries from 100 pg–10 ng of single-stranded (ss) or double-stranded (ds) DNA resulting from chromatin immunoprecipitation (ChIP) experiments. ChIP sequencing (ChIP-seq) is a technique for identifying the location of protein–DNA interactions across the entire genome. Preparation of ChIP-seq libraries is challenging due to the small amount of DNA recovered after ChIP, particularly when using a small number of cells or low abundance transcription factors for immunoprecipitation. Additionally, many library preparation methods use ligation to add sequencing adapters. These methods require dsDNA inputs, which limits the use of faster and simpler ChIP methods that generate ssDNA. By modifying template switching technology for use with DNA, the DNA SMART ChIP-Seq Kit provides a ligation-free method for the addition of sequencing adapters, expanding both the amount and type of DNA available for ChIP-seq library preparation.

Takara Bio's template switching technology, known as SMART® (**S**witching **M**echanism **a**t 5' End of **R**NA **T**emplate), has been used as a basis for cDNA synthesis for next-generation sequencing (NGS) applications (Chenchik *et al*., 1998). SMART technology allows for single-step adapter addition, and has the inherent sensitivity to accurately amplify picogram quantities of nucleic acids. Until now, SMART technology has been limited to use with RNA samples; with the DNA SMART ChIP-Seq Kit, this template switching technology is now able to accommodate DNA templates.

To generate ChIP-seq libraries with the DNA SMART ChIP-Seq Kit, a priming site is first added to the 3' end of the DNA template using the Terminal Deoxynucleotidyl Transferase. This is followed by annealing of a proprietary DNA SMART Poly(dA) Primer, which anneals to the T-tail added by the Terminal Deoxynucleotidyl Transferase. This primer is then used by the SMARTScribe™ Reverse Transcriptase (RT) to copy the DNA strand. When the SMARTScribe RT reaches the 5' end of the DNA template, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the newly synthesized DNA. The carefully designed DNA SMART Oligonucleotide base-pairs with these additional non-template nucleotides and creates an extended template, enabling the SMARTScribe RT to continue replicating to the end of the oligonucleotide. Sequencing libraries are then generated by PCR-mediated addition of Illumina adapters using primers compatible with regions on the DNA SMART Poly(dA) Primer and the DNA SMART Oligonucleotide (Figure 1).

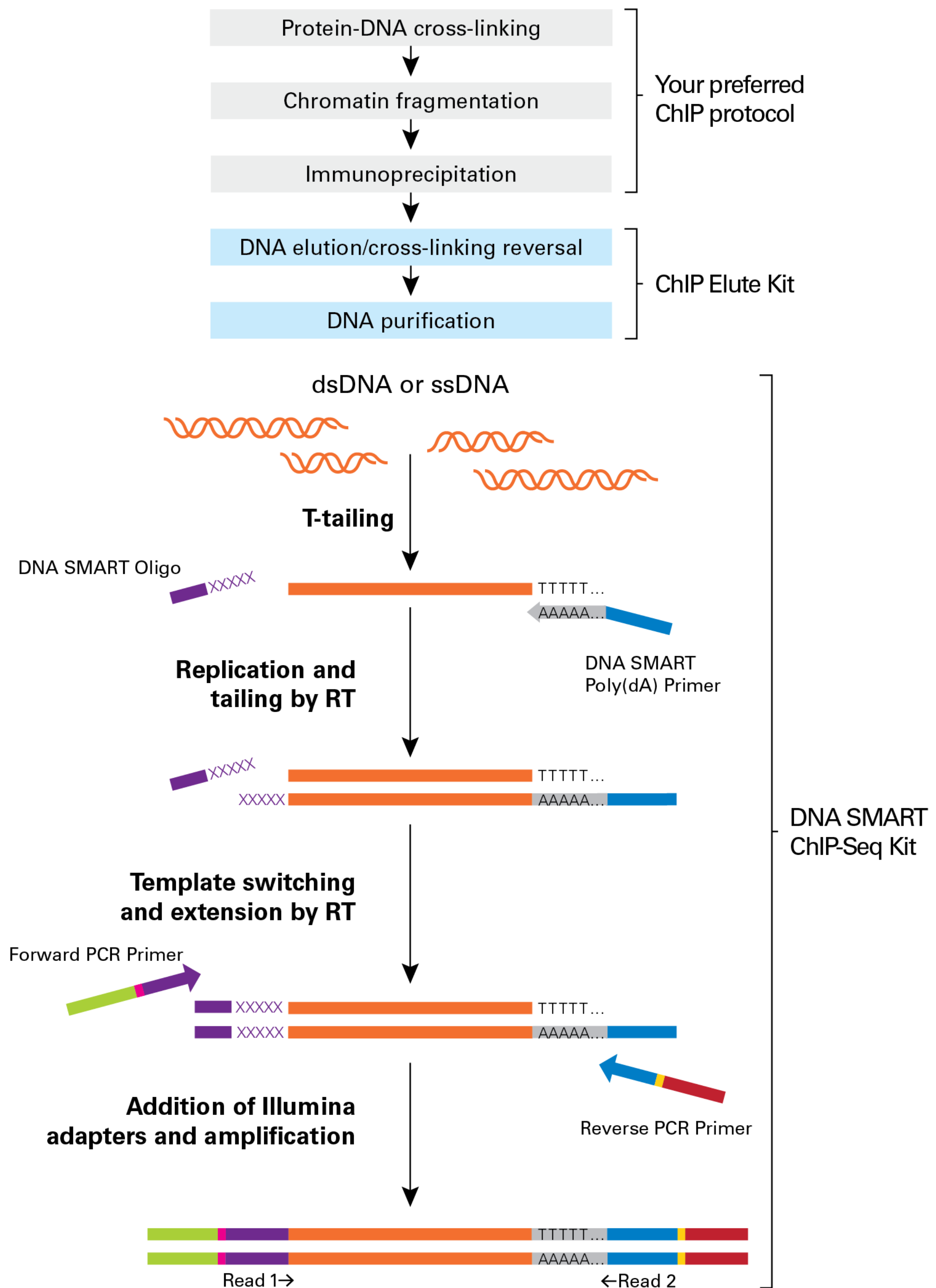

Figure 1. Flowchart of technology in the DNA SMART ChIP-Seq Kit.
This single-tube workflow allows users to generate Illumina-compatible libraries for ChIP-seq experiments. After library size selection and purification, the total time from input DNA to ChIP-seq library is approximately four hours. DNA SMART technology eliminates the need for an adapter ligation step and associated clean-up, reducing the loss of limited input DNA. The Index 1 (i7) sequence is found on the Reverse PCR Primer HT (indicated in yellow) while the Index 2 (i5) sequence is found on the Forward PCR Primer HT (indicated in pink).

The DNA SMART ChIP-Seq Kit features an easy workflow that can be completed in approximately four hours (Figure 2). DNA template switching technology in the DNA SMART ChIP-Seq Kits provides a robust and reliable tool for ChIP-seq applications, particularly at low input levels.

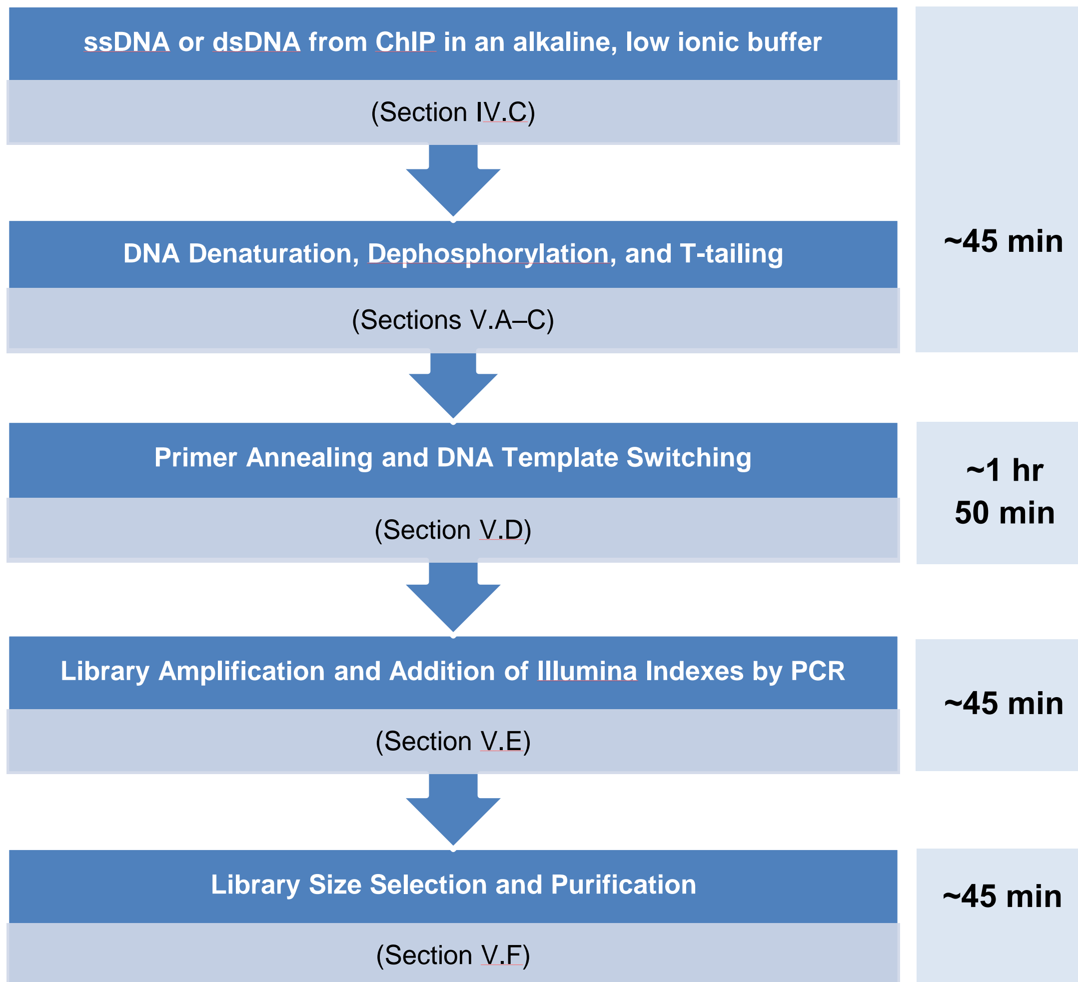

Figure 2. Workflow for the DNA SMART ChIP-Seq Kit.
Illumina-compatible sequencing libraries for ChIP-seq can be generated in around four hours.

II. List of Components

The DNA SMART ChIP-Seq Kit consists of the DNA SMART ChIP-Seq Kit Components (not sold separately), the Indexing Primer Set HT for Illumina - 12, 48 A, or 48 B (not sold separately), and SeqAmp™ DNA Polymerase. These components have been specifically designed to work together and are optimized for this particular protocol. **Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

There are many reagents included with this kit. **Please read the reagent labels carefully to ensure that the correct reagent is used at each step of the protocol**. For convenience, the color of the tube cap for each of the DNA SMART ChIP-Seq Kit Components is indicated in the list of components below.

DNA SMART ChIP-Seq Kits		634865 (12 rxns)	634866 (48 rxns)	634867 (48 rxns)
	SeqAmp DNA Polymerase (Store at –20°C.)			
	SeqAmp DNA Polymerase	50 µl	200 µl	200 µl
	SeqAmp PCR Buffer (2X)	1.25 ml	4 x 1.25 ml	4 x 1.25 ml
	DNA SMART ChIP-Seq Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)			
Cap Color	Package 1 (Store at –70°C.)			
Purple	DNA SMART Oligonucleotide Mix[a]	72 µl	288 µl	288 µl
	Package 2 (Store at –20°C.)			
Blue	DNA Dilution Buffer (5 mM)	1 ml	2 x 1 ml	2 x 1 ml
Red	DNA SMART Buffer	111 µl	444 µl	444 µl
Red	Shrimp Alkaline Phosphatase (1 U/µl)	9 µl	36 µl	36 µl
Green	Terminal Deoxynucleotidyl Transferase	12 µl	48 µl	48 µl
Green	DNA SMART T-Tailing Mix	12 µl	48 µl	48 µl
Yellow	DNA SMART Poly(dA) Primer[b]	24 µl	96 µl	96 µl
Purple	SMARTScribe Reverse Transcriptase (100 U/µl)	48 µl	192 µl	192 µl
Pink	Library Elution Buffer	500 µl	2 x 2 ml	2 x 2 ml
Clear	Control Fragmented Human gDNA (5 ng/µl)	10 µl	10 µl	10 µl
Clear	DNA SMART Custom Read2 Seq Primer (100 µM)	40 µl	120 µl	120 µl

[a] Contains the DNA SMART template switching oligonucleotide with Takara Bio's proprietary sequence

[b] Takara Bio proprietary sequences

The Illumina primers you receive will depend on your particular kit:

DNA SMART ChIP-Seq Kit - 12 (Cat. No. 634865; 12 rxns)

Indexing Primer Set HT for Illumina - 12
(Not sold separately. Store at –20°C.)

Forward PCR Primer HT Index 2 (F2; 12.5 µM)	2 x 15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

DNA SMART ChIP-Seq Kit - 48 A (Cat. No. 634866; 48 rxns)

Indexing Primer Set HT for Illumina - 48 A
(Not sold separately. Store at –20°C.)

Forward PCR Primer HT Index 1 (F1; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 2 (F2; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 3 (F3; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 4 (F4; 12.5 µM)	2 x 15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

DNA SMART ChIP-Seq Kit - 48 B (Cat. No. 634867; 48 rxns)

Indexing Primer Set HT for Illumina - 48 B
(Not sold separately. Store at –20°C.)

Component	Amount
Forward PCR Primer HT Index 5 (F5; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 6 (F6; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 7 (F7; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 8 (F8; 12.5 µM)	2 x 15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity when sequencing a pool of two or more libraries. Consult the Illumina literature (such as the TruSeq® DNA Sample Preparation Guide) for appropriate pooling guideline information. Compare barcode sequences with Illumina barcodes when in doubt about compatibility.

Indexing Primer Set HT for Illumina sequences:

Index (tube label)	Barcode	Index (tube label)	Barcode
F1	TATAGCCT	**R1**	ATTACTCG
F2	ATAGAGGC	**R2**	TCCGGAGA
F3	CCTATCCT	**R3**	CGCTCATT
F4	GGCTCTGA	**R4**	GAGATTCC
F5	AGGCGAAG	**R5**	ATTCAGAA
F6	TAATCTTA	**R6**	GAATTCGT
F7	CAGGACGT	**R7**	CTGAAGCT
F8	GTACTGAC	**R8**	TAATGCGC
		R9	CGGCTATG
		R10	TCCGCGAA
		R11	TCTCGCGC
		R12	AGCGATAG

III. Additional Materials Required

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Of particular importance are a strong magnetic separation device and nuclease-free thin-wall PCR tubes with strong caps.

For General Use:

- 96-well chiller rack: IsoFreeze (MIDSCI, Cat. No. 5640-T4) or aluminum (Light Labs, Cat. No. A-7079)

For PCR Amplification:

- Thermal cycler
- Nuclease-free thin-wall PCR tubes (0.2 ml; GeneMate; Cat. No. T-3035-1; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (Eppendorf, Cat. No. 022431021; USA Scientific; Cat. No. 1415-2600)

For AMPure XP Bead Purification:

- Agencourt AMPure XP PCR Purification Kit (5 ml Beckman Coulter, Part No. A63880; 60 ml Beckman Coulter, Part No. A63881)
- 80% ethanol—made fresh for each experiment
- Magnetic separation device for 0.2 ml tubes (see Appendix B)

For ChIP-Seq Library Validation:

- Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32866)
- Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854)
- 500 µL thin-walled polypropylene tubes for analysis using the Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32856)
- Agilent High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)

IV. General Considerations

A. General Recommendations

- Please read the entire protocol before starting.
- Extreme care must be taken to avoid contaminating input samples and reagents with foreign DNA. This kit is extremely sensitive; any environmental contaminants (e.g., skin or bacterial DNA) could be incorporated into the libraries. Similarly, contaminating DNases and RNases may compromise the success of the experiment.
- The assay is very sensitive to variations in volume, etc. Ensure all pipettes are calibrated for reliable delivery and that nothing is attached to the outside of the tips.
- Do not increase (or decrease) the amount or concentration of any reagent. The amounts and concentrations have been carefully optimized for this protocol.
- We recommend performing the complete protocol without stopping. However, for convenience, samples can be left overnight at 4°C after the template switching reaction (Step V.D) or after the PCR step (Step V.E).
- When performing this protocol for the first time, it is highly recommended to process a positive control and a negative (no DNA) control along with your samples. Dilute the provided Fragmented Human gDNA control with the DNA Dilution Buffer and use an input concentration similar to that of your estimated ChIP DNA input. For example, if you are starting with 0.5–1 ng of ChIP DNA, use 1 ng of the Fragmented Human gDNA control and process this side-by-side with your samples (including using the same number of PCR cycles). Processing a negative control is particularly important any time you are using 17 or more PCR cycles.

B. Sample Preparation

Input DNA must be free of contamination. We recommend using the ChIP Elute Kit (Cat. No. 634887) for DNA elution and cross-linking reversal at the end of your ChIP protocol. The ChIP Elute Kit significantly decreases the time required for these steps compared to traditional methods. With this kit, ssDNA results from cross-linking reversal. It is then purified with ChIP-grade reagents included in the kit, then eluted in DNA Dilution Buffer (also included), and is directly compatible with the DNA SMART ChIP-Seq Kit.

- ChIP DNA samples not prepared using the ChIP Elute Kit should be cleaned and concentrated with a Macherey-Nagel NucleoSpin Gel and PCR Clean-Up kit (Cat. No. 740609). If you used a standard ChIP elution and reverse cross-linking method involving up to 1% SDS, you will need to use Buffer NTB (available separately; Cat. No. 740595) with the NucleoSpin Gel and PCR Clean-Up kit rather than the standard DNA binding buffer.

  NOTE: For any ChIP DNA isolation method, eluting the DNA in 23 µl of the elution buffer will provide the correct input volume (20 µl) for the DNA SMART ChIP-Seq Kit. If target enrichment verification will be performed prior to library construction (recommended), eluting twice with 15 µl will maximize recovery and provide extra volume for qPCR (see the NucleoSpin Gel and PCR Clean-Up User Manual for more information on how to maximize DNA recovery yield).

Evaluating the amount of DNA obtained from a ChIP experiment can be challenging. We strongly recommend the use of Qubit 2.0 Fluorometer (Life Technologies) along with the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854) or the Qubit ssDNA Assay Kit (Life Technologies, Cat. No. Q10212).

NOTE: Even using these sensitive methods, quantification may still be inaccurate. To determine the number of PCR cycles for library amplification (Step V.E), we recommend performing a pilot library preparation using one or two samples. If the input DNA concentration is too low to be quantified, or quantification is uncertain, use 16 or more PCR cycles. Alternatively, visit our web site at www.takarabio.com and search for "ChIP-Seq Library Preparation" to see examples of experiments conducted with a range of input cells.

C. Sample Requirements

- This kit can generate sequencing libraries from 100 pg–10 ng of ssDNA or dsDNA up to 2 kb in length, but it has been optimized specifically for ChIP DNA with an average size of 200–400 bp. If the size of your starting material is significantly longer, you may need to increase the number of PCR cycles to obtain sufficient yield after size selection.
- Starting material should be in 1–20 µl of an alkaline, low ionic buffer such as the DNA Dilution Buffer (5 mM Tris-HCl pH 8.5) provided in both the ChIP Elute Kit and the DNA SMART ChIP-Seq Kit, or water. The optimal pH range of samples is 7.5–8.5. Avoid EDTA-containing buffers.

D. Recommendations for a Smooth Workflow

- **Master mixes:** Always prepare enough master mix for the number of samples, plus an extra 10% to allow for pipetting errors. Prepare and keep all master mixes on ice. While adding the master mix to the samples, keep samples on ice, or place them back on ice immediately after adding the master mix. We strongly recommend the use of a 96-well chiller rack for best results. If using an aluminum chiller rack, simply keeping it on top of ice will keep it cold.
- **Mixing:** Adequate mixing is required after the addition of components at each step. We recommend a quick, **gentle** vortexing (avoiding the creation of bubbles as much as possible) or flicking the tubes with your fingers for mixing. Although gentle pipetting up and down can be used for mixing, we found that it can lead to a lower yield. However, **we do not recommend vortexing the samples when adding the AMPure XP beads**.
- **Thermal cycling:** Pre-program the thermal cycler with all five programs used in this protocol before starting:
    - ChIP-94 (page 12; to use in Section V.A and Section V.D)
    - ChIP-B (page 12; to use in Section V.B)
    - ChIP-C (page 13; to use in Section V.C)
    - ChIP-D (page 14; to use in Section V.D)
    - ChIP-PCR (page 15; to use in Section V.E)
- **AMPure XP Beads:** Aliquot beads into 1–2 ml aliquots. Before use, beads should be brought to room temperature for 30 min and mixed well to disperse.

V. Protocols

A. PROTOCOL: Denaturation of DNA

During this step, dsDNA is converted to ssDNA. **Do not skip the denaturation step even if your starting material is already single stranded.** We recommend performing the positive control reaction using the provided Fragmented Human gDNA the first time you use this kit.

For this step you will need the following components: DNA Dilution Buffer (blue cap)

1. Aliquot appropriate amounts of DNA (100 pg to 10 ng) in a maximum volume of 20 µl in PCR tubes. If necessary, add DNA Dilution Buffer (5 mM Tris-HCl pH 8.5) to bring the total volume to 20 µl.

1–20 µl	dsDNA or ssDNA (0.1–10 ng)
0–19 µl	DNA Dilution Buffer (blue cap)
20 µl	Total volume

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler (ChIP-94) for exactly 2 min.

ChIP-94	94°C	hold

3. Immediately remove the samples and place on ice for at least 2 minutes.
4. Spin the tubes briefly to bring down any condensation.
   NOTE: Proceed immediately to the next step.

B. PROTOCOL: Dephosphorylation of 3’ Ends

The 3’ end of ssDNA is dephosphorylated by Shrimp Alkaline Phosphatase in preparation for T-tailing.

For this step you will need the following components: DNA SMART Buffer (red cap), Shrimp Alkaline Phosphatase (red cap)

1. Prepare enough Dephosphorylation Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

3.25 µl	DNA SMART Buffer (red cap)
0.75 µl	Shrimp Alkaline Phosphatase (red cap)
4 µl	Total volume per reaction

NOTE: Add the Shrimp Alkaline Phosphatase to the buffer immediately prior to use. Mix well by gently vortexing then spin the tubes briefly in a microcentrifuge. **Keep the DNA SMART Buffer on ice** until its next use in Step V.D.

2. Add 4 µl of the Dephosphorylation Master Mix to each reaction tube from Step V.A. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
3. Place the tubes in a preheated thermal cycler and run program ChIP-B. Leave the tubes at 4°C until the next step.

ChIP-B	37°C	10 min
	65°C	5 min
	4°C	hold

NOTE: During the 65°C incubation step, prepare the T-Tailing Master Mix in Step V.C.1 below.

C. PROTOCOL: T-Tailing

A poly(T) tail is added to the ssDNA, providing a priming site for the DNA SMART Poly(dA) Primer.

For this step you will need the following components: DNA SMART T-Tailing Mix (green cap) Terminal Deoxynucleotidyl Transferase (green cap)

1. Prepare enough T-Tailing Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

1 µl	DNA SMART T-Tailing Mix (green cap)
1 µl	Terminal Deoxynucleotidyl Transferase (green cap)
2 µl	Total volume per reaction

2. Add 2 µl of the T-Tailing Master Mix to each reaction tube. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
3. Place the tubes in a preheated thermal cycler and run program ChIP-C. Leave the tubes at 4°C until the next step.

ChIP-C	37°C	20 min
	70°C	10 min
	4°C	hold

D. PROTOCOL: Primer Annealing and Template Switching Reaction

The DNA SMART Poly(dA) Primer anneals to the ssDNA template followed by DNA replication and template switching. At the end of this step, the DNA is double stranded with partial adapters on both ends.

For this step you will need the following components: DNA SMART Poly(dA) Primer (yellow cap), DNA SMART Buffer (red cap), DNA SMART Oligonucleotide Mix (purple cap), SMARTScribe Reverse Transcriptase (100 U/µl) (purple cap)

NOTE: Prepare the Template Switching Master Mix (Step V.D.5) prior to starting the primer annealing step below.

1. **Primer Annealing:** Add 2 µl of the DNA SMART Poly(dA) Primer (yellow cap) to each reaction tube. Mix the contents of the tubes by gently vortexing then spin the tubes briefly.
2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler (ChIP-94) for exactly 1 min.

   ChIP-94 94°C hold

3. Immediately remove the samples and place on ice for at least 2 minutes.
4. Spin the tubes briefly to bring down any condensation.

   NOTE: Proceed immediately to the next step.

5. Prepare enough Template Switching Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

6 µl	DNA SMART Buffer (red cap)
6 µl	DNA SMART Oligonucleotide Mix (purple cap)
4 µl	SMARTScribe Reverse Transcriptase (100 U/µl)* (purple cap)
16 µl	Total volume per reaction

* DO NOT add the SMARTScribe Reverse Transcriptase to the buffer until immediately prior to use (Step V.D.6). Mix well by gently vortexing then spin the tubes briefly in a microcentrifuge.

NOTE: The DNA SMART Buffer is also used in Step V.B.1.

6. **DNA Replication and Template Switching:** Add the SMARTScribe Reverse Transcriptase (purple cap) to the Template Switching Master Mix prepared in Step V.D.5, then add 16 µl of the Template Switching Master Mix to each reaction tube. Mix the contents of the tubes by gently vortexing then spin the tubes briefly.
7. Place the tubes in a preheated thermal cycler and run program ChIP-D. Leave the tubes at 4°C until the next step.

ChIP-D	42°C	90 min
	70°C	15 min
	4°C	hold

NOTE: If desired, the reactions can be left overnight at 4°C.

E. PROTOCOL: ChIP-Seq Library Amplification by PCR

The dsDNA produced in the Primer Annealing and Template Switching Reaction (Step V.D) is directly amplified into ChIP-seq libraries using SeqAmp DNA Polymerase and the Forward and Reverse Primers from the Indexing Primer Set HT for Illumina. No pre-PCR purification or size selection is necessary.

For this step you will need the following components: SeqAmp DNA Polymerase, SeqAmp PCR Buffer (2X), Indexing Primer Set HT for Illumina - 12, 48 A, or 48 B (depending on your particular kit)

IMPORTANT:

- **Optimal cycling parameters may vary with different templates and thermal cyclers.** The guidelines in Table 1 (below) are optimized for DNA fragments with an average size between 200–400 bp. To determine the optimal number of cycles for your samples and conditions, we strongly recommend that you perform a range of cycles.
- **The final yield will vary depending on the stringency of the size selection.** If you are planning to perform a stringent size selection (Step V.F Option 3), you will need to use more PCR cycles than if you choose other size selection options (Table 2).

1. Prepare enough PCR Master Mix for all reactions, plus 10%. Combine the following reagents *in the order shown*, then mix well and spin the tube briefly in a microcentrifuge.

50 µl	SeqAmp PCR Buffer (2X)
2 µl	Forward PCR Primer (12.5 µM)
2 µl	Reverse PCR Primer (12.5 µM)
2 µl	SeqAmp DNA Polymerase
56 µl	Total volume per reaction

NOTE: Separate PCR Master Mixes should be prepared for each combination of Forward and Reverse Primers. If each sample will receive a different index/set of primers, prepare a master mix excluding the forward and reverse primers (total volume per reaction will be 52 µl), and add 2 µl each of forward and reverse primers directly to each reaction tube. **Not all indexes can be pooled for multiplexing! Consult Section II and Illumina literature for more information.**

2. Add 56 µl (52 µl if forward and reverse primers are excluded) of PCR Master Mix to each reaction tube.
3. OPTIONAL: If the forward and reverse PCR primers were not added to the PCR Master Mix, add 2 µl of the primers to each reaction tube.
4. Mix the contents of the tubes by gently vortexing then spin the tubes briefly.
5. Place the tubes in a preheated thermal cycler and run program ChIP-PCR. Leave the tubes at 4°C until the next step.

ChIP-PCR	94°C		1 min
	X[a] cycles:		
		98°C	15 sec
		55°C	15 sec
		68°C	30 sec
	4°C		hold

[a] The number of cycles depends on the amount of input DNA. See Table 1 (below) for guidelines.

Table I. Cycling Guidelines Based on Amount of Starting Material.

Amount of Input DNA (ng)*	Typical Number of PCR Cycles
10	12
1	15
0.1	18

* Quantification of input DNA amount may be inaccurate; when in doubt, add additional PCR cycles, up to a maximum of 18 cycles.

NOTE: If desired, the reactions can be left overnight at 4°C or frozen at –20°C for one week.

F. PROTOCOL: Library Size Selection and Purification

The ChIP-seq library is size selected and purified using AMPure XP beads. The protocol involves a double size selection that removes primers, primer dimers, and PCR products containing large inserts.

In the first step of the size selection, fragments larger than 500–600 bp are removed from the supernatant onto the beads. In the second step of the size selection, the supernatant is added to fresh beads so that fragments of the desired size are then immobilized. The beads are then washed with 80% ethanol and eluted in Library Elution Buffer.

There are four options for size selection and purification:

- Option 1 selects for PCR products ranging between approximately 250–600 bp (depending on the size of the starting material). This option generates consistent sequencing while preserving excellent yield and library complexity and should give good results for most applications.
- If a more stringent selection is desired, you may use Option 2 or Option 3. However, more stringent selection will lead to a significant reduction in total yield and may also reduce library complexity.
- If no upper size selection is desired, or if the input amount is very low, you may use the alternative single size-selection protocol: Option 4. This option will only remove primers, primer dimers, and PCR products with very small inserts. This option should be used if low yield is anticipated.

Table II. Alternative Volumes of AMPure XP Beads to Use for Library Size Selection and Purification.

	Option	First bead volume (µl)	Second bead volume (µl)	Expected approximate size range (bp)
Double size selection	1	65	30	250–600
	2	75	25	250–500
	3	90	20	200–400
Single size selection	4	–	90	>250 (no upper size selection)

NOTE: The exact size range will be influenced by the size of the starting material (e.g., if the original input DNA was between 100–300 bp, Options 1 and 2 will generate libraries with fragments no larger than ~450 bp as library construction adds 153 bp).

For this step you will need the following components: Agencourt AMPure XP PCR Purification Kit, 80% ethanol (made fresh), a magnetic separation device, Library Elution Buffer (pink cap)

NOTE: Before use, bring AMPure XP bead aliquots to room temperature for at least 30 minutes and mix well by vortexing to disperse the beads.

Double Size Selection Protocol (Options 1–3)

1. Add 65 µl of AMPure XP beads to each reaction tube. If performing Option 2 or 3, refer to Table 2 (above) for the volume of AMPure XP beads. Mix by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly. DO NOT VORTEX, this will generate bubbles making subsequent handling of the beads difficult.

2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Place the reaction tubes on the Magnetic Separation Device for 10–20 min or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear as any carryover of beads into the next step will decrease the efficiency of size selection. There is no disadvantage in separating the samples for more than 20 min.

4. While the solution clears, add 30 µl AMPure XP beads to new PCR strip tubes. If performing Option 2 or 3, refer to Table 2 (above) for the volume of AMPure XP beads.
5. While the reaction tubes are sitting on the magnetic stand, transfer the supernatant (which contains your library) to the clean PCR strip tubes pre-filled with beads. Mix by pipetting the entire volume up and down at least 10 times.
6. Incubate at room temperature for 8 min to let the DNA bind to the beads.
7. Place the PCR strip tubes on the Magnetic Separation Device for 10 min or until the solution is completely clear.
8. While the PCR strip tubes are sitting on the magnetic stand, remove the supernatant. (The library is now bound to the beads.)
9. Keep the tubes on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully remove the supernatant. DNA will remain bound to the beads during the washing process.
10. Repeat step 9 one more time.
11. OPTIONAL: Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
12. Let the sample tubes rest open at room temperature for ~3–5 min until the pellet appears dry, without any shine. You may see a tiny crack in the pellet.

    NOTE: Be sure to dry the pellet enough.

    - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet it may take longer to rehydrate.

13. Once the pellet has dried, remove the tubes from the magnetic stand and add 20 µl of Library Elution Buffer (pink cap) to cover the beads. Mix thoroughly by pipetting the beads up and down to ensure complete dispersion.

    NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube.

14. Incubate at room temperature for at least 5 min to rehydrate.

15. Place the sample tubes on the Magnetic Separation Device for 2 min or longer, until the solutions are completely clear.
16. Transfer the clear supernatant, containing the purified ChIP-seq library, from each tube to a labeled nuclease-free nonsticky tube.

Single Size Selection Protocol (Option 4)

This option will only remove primers, primer dimers, and PCR products with very small inserts.

1. Add 90 µl of AMPure XP beads to each reaction tube. Mix by pipetting the entire volume up and down at least 10 times.
   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly. DO NOT VORTEX, this will generate bubbles making subsequent handling of the beads difficult.
2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Place the reaction tubes on the Magnetic Separation Device for 20 min or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.
   NOTE: Ensure that the solution is completely clear as any carryover in beads into the next step will decrease the efficiency of size selection. There is no disadvantage in separating the samples for more than 20 min. The pellet will not be as tight as with Options 1–3.
4. While the tubes are sitting on the magnetic stand, remove the supernatant. (The library is bound to the beads.)
5. Keep the tubes on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully remove the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat step 5 one more time.
7. OPTIONAL: Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for 12 min or until the pellet appears dry. You may see a tiny crack in the pellet.
   NOTE: Be sure to dry the pellet enough.
   - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry. It will take longer to dry the pellet using Option 4 than with Options 1–3.
   - If you over-dry the pellet it may take longer to rehydrate
9. Once the pellet has dried, remove the tubes from the magnetic stand and add 20 µl of Library Elution Buffer (pink cap) to cover the beads. Mix thoroughly by pipetting the beads up and down to ensure complete dispersion.
   NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube.
10. Incubate at room temperature for at least 7 min to rehydrate.
11. Place the tubes on the Magnetic Separation Device for 5 min or longer, until the solution is completely clear.

12. OPTIONAL: Transfer the clear supernatant from each tube to a new PCR strip tube. Place the PCR strip tubes on the Magnetic Separation Device for 2-3 min, until the solutions are completely clear.
    NOTE: The second removal of magnetic beads ensures that no beads are left in the sample, which may happen due to the large amount of beads used for purification in Option 4. Beads carried over in your sample may affect the profile obtained with the Agilent 2100 Bioanalyzer.
13. Transfer the clear supernatant containing the purified ChIP-seq library from each tube to a labeled nuclease-free nonsticky tube.

G. PROTOCOL: Library Validation

The ChIP-seq library is quantified using a Qubit 2.0 Fluorometer, and the quality of the library is determined using an Agilent 2100 Bioanalyzer.

1. Use 1–2 µl of the amplified ChIP-seq libraries for quantification using the Qubit 2.0 Fluorometer (Life Technologies) with the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854). See the Qubit dsDNA HS Assay Kits User Manual for instructions.
2. Evaluate the library concentration in ng/µl. If the library is more than 2 ng/µl, dilute in water or Library Elution Buffer so that the concentration is between 1–2 ng/µl.
3. Use 1 µl of the diluted sample for validation with the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit Guide for instructions.
4. Compare the results for your sample and controls to determine whether the sample is suitable for sequencing.

VI. References

1. Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis.* Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319.

VII. Example Results

A successful DNA SMART assay and amplification should yield no product in the negative control (Figure 3, Panel A), and a distinct Bioanalyzer electropherogram spanning 175–900 bp, with a peak around 275-375 bp (Figure 2, Panel B). A small amount of PCR product may be visible between 125–250 bp in the no DNA control if using 18 cycles of PCR. This background will increase in this control if more PCR cycles are performed. Occasionally, products <100 bp, corresponding to primers or primer dimers, may also be visible, but these small products do not interfere with sequencing. The yield of a ChIP-seq library generated with this kit should be >10 nM. Both the yield and size distribution will depend on the input DNA (amount and size), number of PCR cycles, and size selection.

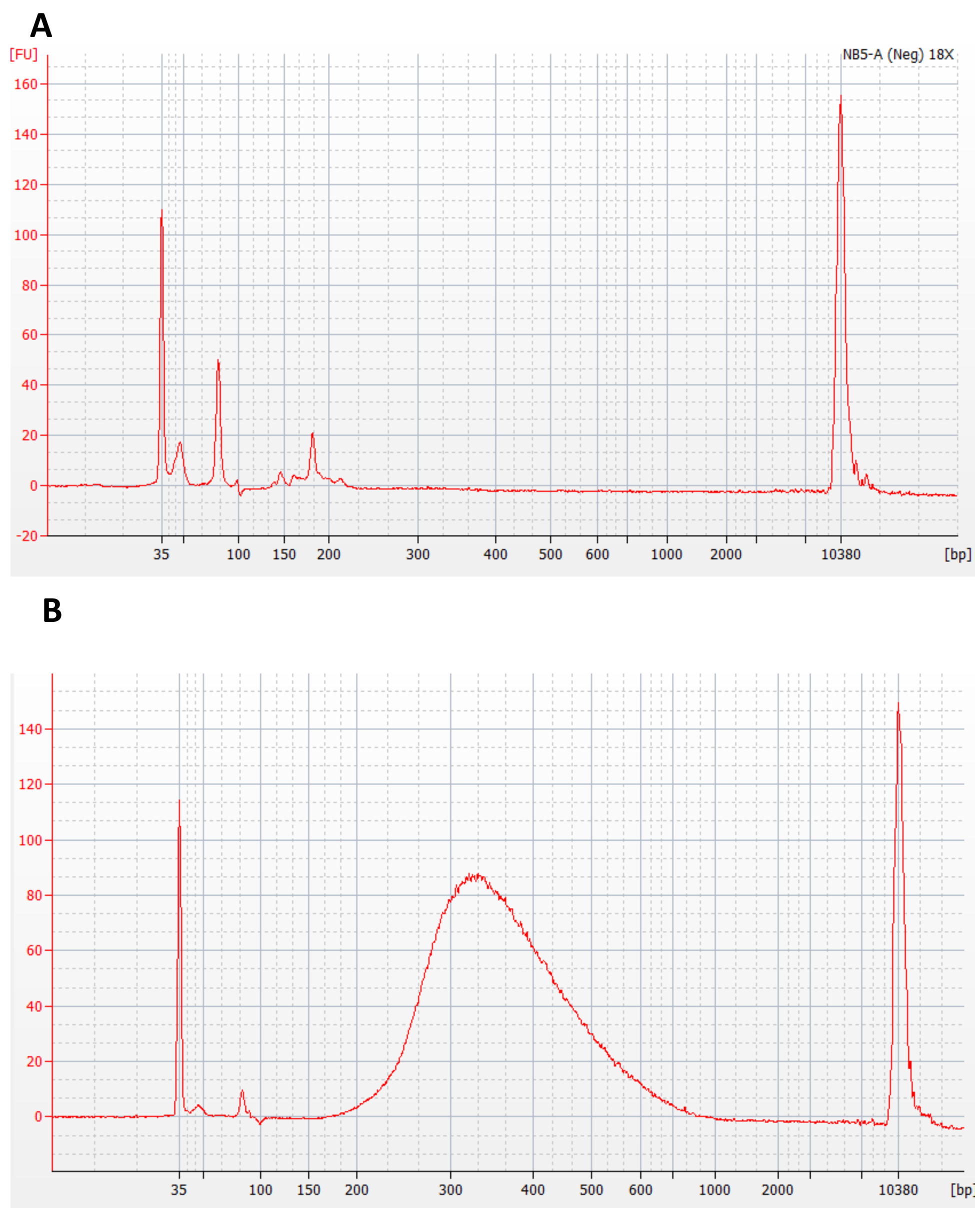

Figure 3. Example electropherograms of control reactions.
Panel A, shows an example of a no input DNA control reaction generated with the DNA SMART ChIP-Seq Kit using 18 cycles of PCR. **Panel B**, shows an example of a positive control reaction generated with the DNA SMART ChIP-Seq Kit using 1 ng of Fragmented Human gDNA Control as input with 14 cycles of PCR. Library size selection and purification Option 4 was used for both reactions.

Figure 4 gives examples of ChIP-seq library profiles generated from ChIP experiments using anti-CTCF (Panel A) or anti-H3K4me3 (Panel B) antibodies. ChIP-seq libraries are shown for different size selection protocols.

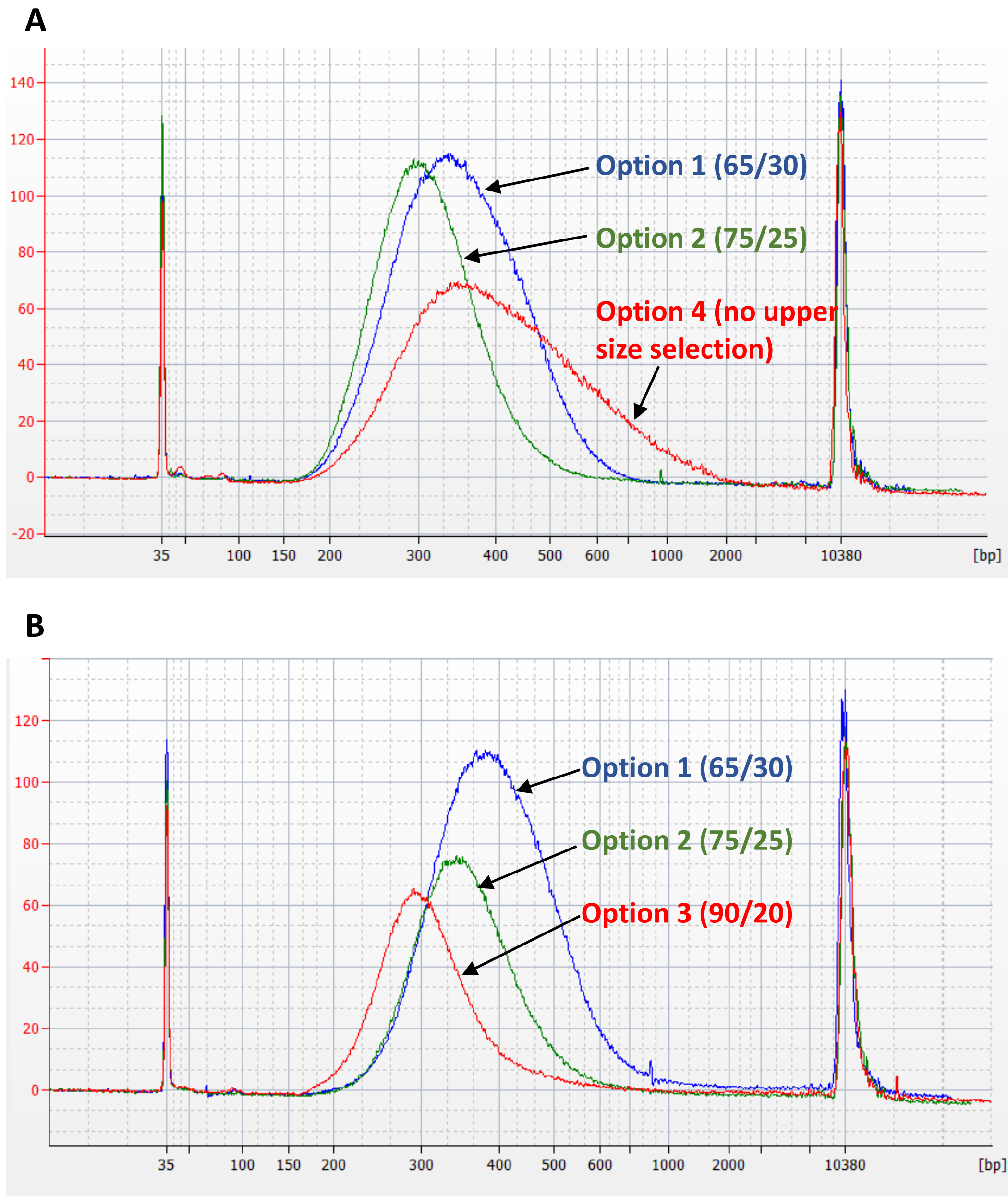

Figure 4. Example electropherograms of ChIP-seq libraries generated with DNA SMART ChIP-Seq Kits. Electropherograms showing libraries made with the DNA SMART ChIP-Seq Kit from input ChIP DNA generated from HEK293T cells using antibodies against CTCF (**Panel A**) or H3K4me3 (**Panel B**). The numbers in parentheses refer to the amount of AMPure XP beads (µl) used in the first and second size selection step for each option.

Appendix A: Constructing a Magnetic Separation Device for 0.2 ml PCR Tubes

It can be difficult to find magnetic separation devices designed specifically to handle 0.2 ml PCR strip tubes. Often, one can place strip tubes in a column/row of a magnetic separation device designed for use with 96-well plates. However, with the larger volumes used in this kit, a 96-well separation device may not adequately separate your samples. Alternatively, one can construct a suitable low-cost separation device from common laboratory materials.

Building a 0.2 ml tube magnetic separation device from rare earth bar magnets and a tip rack

As seen in Figure 5, neodymium bar magnets are taped together on the underside of the top section of a 20 µl tip rack (Panel A), and the rack is inverted so the tubes can be inserted (Panel B).

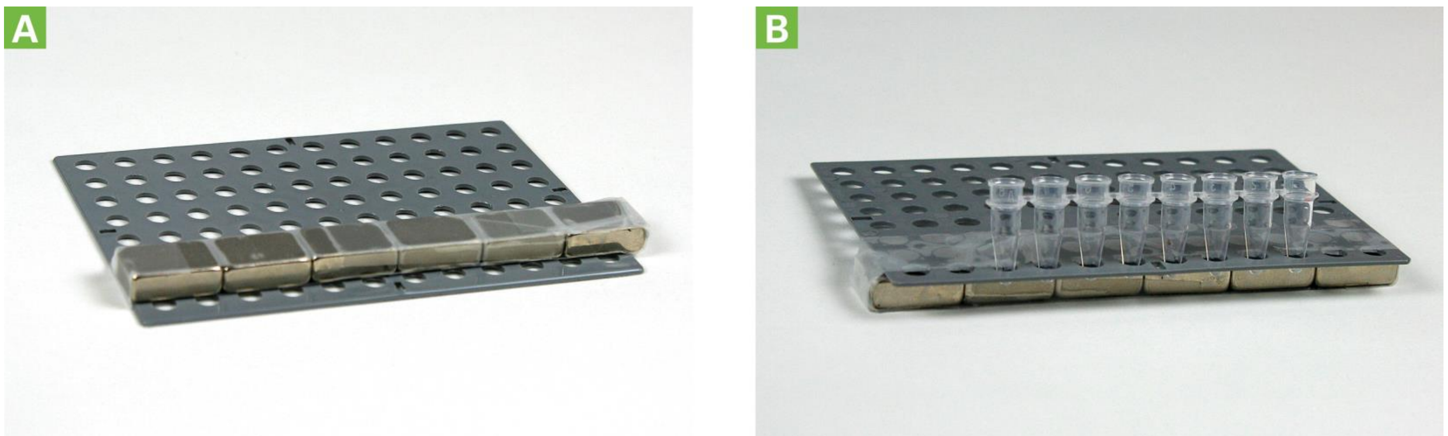

Figure 5. Constructing a magnetic separation device for 0.2 ml tubes from rare earth magnets.
Panel A shows six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets Model # NB026) taped together on the underside of the top section of a 20 µl tip rack. **Panel B** shows the upright rack, into which an 8-tube strip of 0.2 ml tubes has been inserted.

Appendix B: Sequencing Guidelines

Pooling Recommendations

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained with the Qubit with the average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion: http://www.molbiol.edu.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using our Library Quantification Kit (Cat. No. 638324).

Most Illumina sequencing library preparation protocols require libraries with a final concentration of 2 nM or 4 nM, depending on the sequencing platform. Lower concentrations can also be accommodated, depending on the instrument.

Prepare a pool of 2 nM (or 4 nM) as follows:

1. Dilute each library to 2 nM (or 4 nM) in nuclease-free water. To avoid pipetting error, use at least 2 µl of each original library for dilution.
2. Pool the diluted libraries by combining an equal amount of each library in a low-bind 1.5 ml-tube. Mix by vortexing at low speed or by pipetting up and down. Use at least 2 µl of each diluted library to avoid pipetting error.
3. Depending on the Illumina sequencing library preparation protocol, use a 5 µl aliquot (for the 4-nM concentration) or a 10 µl aliquot (for the 2-nM concentration) of the pooled libraries. Follow the library denaturation protocol according to the latest edition of your Illumina sequencing instrument's User Guide.

If you are planning to include a PhiX control spike-in, make sure to combine the aliquot with an appropriate amount of the PhiX control.

- See our recommendations below (Table 3) regarding the amount of PhiX control to include with DNA SMART ChIP libraries.
- Follow Illumina guidelines on how to denature, dilute and combine a PhiX control library with your own pool of libraries.

PhiX Control Spike-In Recommendations

Illumina cluster detection algorithms are optimized around a balanced representation of A, T, G, and C nt. DNA SMART ChIP libraries can have a lower than average pass filter rate due to the low complexity observed in the first three cycles. To alleviate this issue, libraries should be combined with a PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in. Make sure to use a fresh and reliable stock of the PhiX control library (see Table 3).

Table III. PhiX Control Spike-in Guidelines for Various Illumina Sequencing Instruments.

Sequencing instrument	PhiX (%)
MiSeq	5–10 (optional[1])
HiSeq 1500/2000/2500	10
HiSeq 3000/4000	20
NextSeq/MiniSeq	20

[1] A typical MiSeq run generates a high passing filter rate. While the inclusion of a PhiX spike-in can be beneficial, it does not significantly improve overall performance.

Sequencing Analysis Guidelines

- The first three nucleotides of the first sequencing read (Read 1) are derived from the template-switching oligo. Consequently, these three bases need to be trimmed prior to mapping.
- If you are performing paired-end sequencing, we highly recommend using the DNA SMART Custom Read2 sequencing primer for the second sequencing read (Read 2). This custom primer will allow sequencing to start right after the priming site of the DNA SMART Poly(dA) Primer, thus avoiding reading through the A/T tail created on the 3' end of ssDNA molecules during Step V.C Sequencing through the stretch of adenine bases may result in low sequencing performance due to low complexity.

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