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Applied Biosystems SOLiD™ 4 System

SOLiD™ ChIP-Seq Kit Guide

Includes ***NEW*** **reverse crosslinking reaction conditions** and **tissue sample protocol**

ChIP-Seq Library Preparation → Templated Bead Preparation → Instrument Operation

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Kit Contents and Storage

Catalog Numbers

Catalog no. 4449638 — SOLiD™ ChIP-Seq Kit with Magnet
Catalog no. 4449640 — SOLiD™ ChIP-Seq Kit

Kit Components and Storage

Sufficient components are provided for up to 20 reactions (including input control reactions).

Module 1 and 2 are shipped on wet ice; Modules 3, 4, and 6 are shipped on dry ice; and Module 5 is shipped at room temperature. See the table below for storage temperatures.

Module 1	**Quantity**	**Storage**
Glycine (1.25 M)	2 × 1 mL	4°C
Dynabeads® Protein A/G (do not freeze!)	250 µL	4°C
Reverse Crosslinking Buffer	1.4 mL	4°C
DNA Purification Magnetic Beads (do not freeze!)	500 µL	4°C
DNA Purification Buffer	1.4 mL	4°C
Proteinase K (20 mg/mL)	200 µL	Room temp or 4°C
Module 2	**Quantity**	**Storage**
IP Buffer 1	10 mL	4°C
IP Buffer 2	7.5 mL	4°C
DNA Wash Buffer	8.0 mL	4°C
DNA Elution Buffer	7.2 mL	4°C
Module 3	**Quantity**	**Storage**
Protease Inhibitors (200X)	100 µL	–20°C
Mouse IgG (1 µg/µL)	15 µL	–20°C
Rabbit IgG (1 µg/µL)	15 µL	–20°C
Module 4	**Quantity**	**Storage**
Dilution Buffer	8.1 mL	–20°C
Lysis Buffer	3.6 mL	–20°C
Module 5 (included with catalog no. 4449638)	**Quantity**	**Storage**
DynaMag™-PCR Magnet	1	Room temp
Module 6	**Quantity**	**Storage**
5x End Polishing Buffer	2 × 200 µL	–20°C
dNTP (10 mM)	80 µL	–20°C
End Polishing Enzyme 1	2 × 20 µL	–20°C
End Polishing Enzyme 2	160 µL	–20°C
5x Ligase Buffer	1000 µL	–20°C
T4 DNA Ligase	100 µL	–20°C
Platinum® PCR Amplification Mix	4 × 1.125 mL	–20°C

Never freeze the Dynabeads® or the DNA Purification Magnetic Beads, as this will damage the beads.

Product Qualification

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to **www.invitrogen.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Intended Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Materials Supplied by the User

SOLiD™ Fragment Library Barcoding Kits

The SOLiD™ ChIP-Seq Kit may also be used with SOLiD™ Fragment Library Barcoding Kit modules to generate barcoded ChIP-Seq fragment libraries. You can download a protocol at:

http://tools.invitrogen.com/content/sfs/manuals/SOLiD_ChIP-SEQ_Barcode_protocol.pdf

Additional Reagents Needed

The following reagents are required for use of the kit. Ordering information for many of these products is provided in **Additional Products** on page 49.

- Cells or fresh or frozen tissue
- For cells: Trypsinizing reagent (*e.g.*, TrypLE™ Express Stable Trypsin Replacement Enzyme)
- For fresh or frozen tissue:
  - Clean razor blades
  - 1.5-inch 18G and 21G needles (a 1.5-inch 16G needle may also be needed for muscular tissues such as heart)
  - 1-mL luer lock syringes
- Antibody of interest (e.g., ChIP-qualified antibodies from Invitrogen, available at **www.invitrogen.com/chipantibody**.)
- DNase/RNase-free water
- Formaldehyde, 37%, Molecular Biology Grade
- PBS or D-PBS (e.g., Phosphate Buffered Saline, 7.4, 1X liquid, or Dulbecco's Phosphate-Buffered Saline, 1X liquid)
- qPCR SuperMix (e.g., EXPRESS qPCR Supermixes and EXPRESS SYBR® GreenER™ qPCR Supermixes)
- qPCR primers for the sequence of interest
- Trypsinizing reagent (e.g., TrypLE™ Express Stable Trypsin Replacement Enzyme)
- Agencourt® AMPure® or AMPure® XP Magnetic Particles (i.e., Agencourt® AMPure® XP 5-mL Kit, catalog no. A63880; or Agencourt® AMPure® XP 60-mL Kit, catalog no. A63881)
- Ethanol, absolute, 200 proof, Molecular Biology Grade
- ABI SOLiD™ Fragment Library Oligos Kit (Applied Biosystems 4401151)
  - SOLiD™ Library Oligos Kit 1 P1 Adaptor (ds)
  - SOLiD™ Library Oligos Kit 1 P2 Adaptor (ds)
  - SOLiD™ Library Oligos Kit 1 Library PCR Primer 1
  - SOLiD™ Library Oligos Kit 1 Library PCR Primer 2
- Microcentrifuge tubes, RNase/DNase-free, low retention (e.g., Eppendorf 1.5-mL LoBind tubes)
- 100-bp DNA ladder or 50-bp ladder (e.g., TrackIt™ 100-bp DNA Ladder, Invitrogen 10488-058; or Invitrogen 50-bp ladder, Invitrogen 10416-014)
- **Optional:** Control primers for qPCR (e.g., MAGnify™ SAT2 Primers, Invitrogen 49-2026; RARβ1 Primers, Invitrogen 49-2027; ERα Primers, Invitrogen 49-2028; and c-Fos Primers, Invitrogen 49-2029)

Additional Equipment Needed

The following materials are required for use of the kit. Ordering information for many of these products is provided in **Additional Products** on page 49.

- DynaMag™-PCR Magnet (Invitrogen 49-2025), or other magnet capable of holding 0.2-mL PCR tubes or strip wells (included with catalog no. 4449638)
- Sonicator, e.g., Covaris® S2 System (110 V, Applied Biosystems 4387833; 220 V, Applied Biosystems 4392718) or Bioruptor® UCD-200 (Diagenode UCD-200 xx)
  - Ethylene glycol (American Bioanalytical AB00455-01000)
  - Covaris® S2 System Pump Kit, with water fill level label (Covaris 500165)
  - Covaris® MicroTubes with AFA fiber (Covaris 520045)
  - Covaris®-2 series Machine Holder for (one) microTube–6mm (Covaris 500114)
- qPCR instrument (e.g., Applied Biosystems StepOnePlus™, 7500 Fast, 7500, 7900HT, 7500, ViiA™ 7 Instruments)
- qPCR plates
- Microcentrifuge (4°C)
- Microcentrifuge for 0.2-mL PCR tubes or strip wells
- Microcentrifuge tubes, RNase/DNase-free
- 200-µL PCR tubes, RNase/DNase-free, individual or 8-tube strips and caps
- 55°C and 95°C heat sources (e.g., Applied Biosystems GeneAmp® PCR System, or a hybridization oven, water bath, etc.)
- PCR Thermocycler (e.g., Applied Biosystems 96-well GeneAmp® PCR System 9700 N8050200 (Base) and 4314443 (Block))
- Rotating mixer capable of holding 0.2-mL PCR tubes or strip wells
- Cell counter (e.g., Countess® Automated Cell Counter, Invitrogen C10227, or a hemacytometer)
- For confirming DNA fragment sizes:
  - 1.5–2.0% agarose gel (e.g., 2% E-Gel® or 2% E-Gel® EX Gel)
  - Agarose gel apparatus (e.g., E-Gel® iBase™ Power System, Invitrogen G6400; or E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit, Invitrogen G6465)
- Magnetic rack for 1.5-mL tubes
- Nutator
- **Optional:** Qubit® Fluorometer (Invitrogen Q32857).
  - Quant-iT™ DNA Assay Kit, High Sensitivity (0.2–100 ng) (Invitrogen Q33120) or Quant-iT™ dsDNA HS Assay Kits—for use with the Qubit® fluorometer (0.2–100 ng) (Invitrogen Q32851 or Q32854)
- **Optional:** 2100 Bioanalyzer™ (Agilent Technologies G2938C)

Description of the System

SOLiD™ ChIP-Seq Kit Overview

Chromatin immunoprecipitation (ChIP) is a powerful technique for studying the association of certain proteins with specific regions of the genome. These sequence-specific DNA-binding proteins are believed to play a role in such cellular processes as DNA replication, recombination, repair, and segregation; chromosomal stability; cell-cycle progression; and epigenetic silencing. In a standard ChIP assay, a cell is fixed via formaldehyde treatment and the chromatin is sheared and immunoprecipitated via a highly specific antibody. The researcher then analyzes the DNA to identify the genomic regions where the chromatin-associated proteins bind to the chromatin *in vivo*.

The SOLiD™ ChIP-Seq Kit provides a streamlined assay for the enrichment of chromatin/protein complexes and DNA recovery using magnetic bead capture technology, and also includes reagents for preparing the isolated DNA for next-generation sequencing on the Applied Biosystems SOLiD™ System.

This kit uses lower sample amounts than traditional ChIP workflows, thereby preserving precious samples such as primary cells, stem cells, and biopsies. In addition, the ChIP assay portion of this protocol can be completed in a single day, compared with 2–3 days for a traditional ChIP assay. The kit includes reagents for SOLiD™ fragment library preparation (adaptors are sold separately), and can be used with a suite of ChIP-validated antibodies from Invitrogen. Sufficient reagents are provided for 20 ChIP-Seq library preparations.

SOLiD™ Fragment Library Barcoding Kits

The SOLiD™ ChIP-Seq Kit may also be used with SOLiD™ Fragment Library Barcoding Kit modules to generate barcoded ChIP-Seq fragment libraries. You can download a protocol from our website at:

http://tools.invitrogen.com/content/sfs/manuals/SOLiD_ChIP-SEQ_Barcode_protocol.pdf

ChIP-Qualified Antibodies

ChIP-qualified antibodies are available separately from Invitrogen. Visit **www.invitrogen.com/chipantibody** for more information.

Negative Controls

Rabbit IgG and Mouse IgG antibodies are provided in the kit for use as negative controls to measure nonspecific binding. The concentration of the negative control antibodies is 1 µg/µL. Add 1 µL of negative control antibody per ChIP. Preparation of a chromatin input control (non-immunoprecipitated) is also strongly recommended as a reference control for the qPCR checkpoint as well as for ChIP DNA sequencing.

MAGnify™ PCR Primer Pairs

MAGnify™ PCR Primers are available separately from Invitrogen for the amplification of common promoter regions analyzed in ChIP experiments. See page 34 for sequences and page 49 for ordering information.

DynaMag™-PCR Magnet

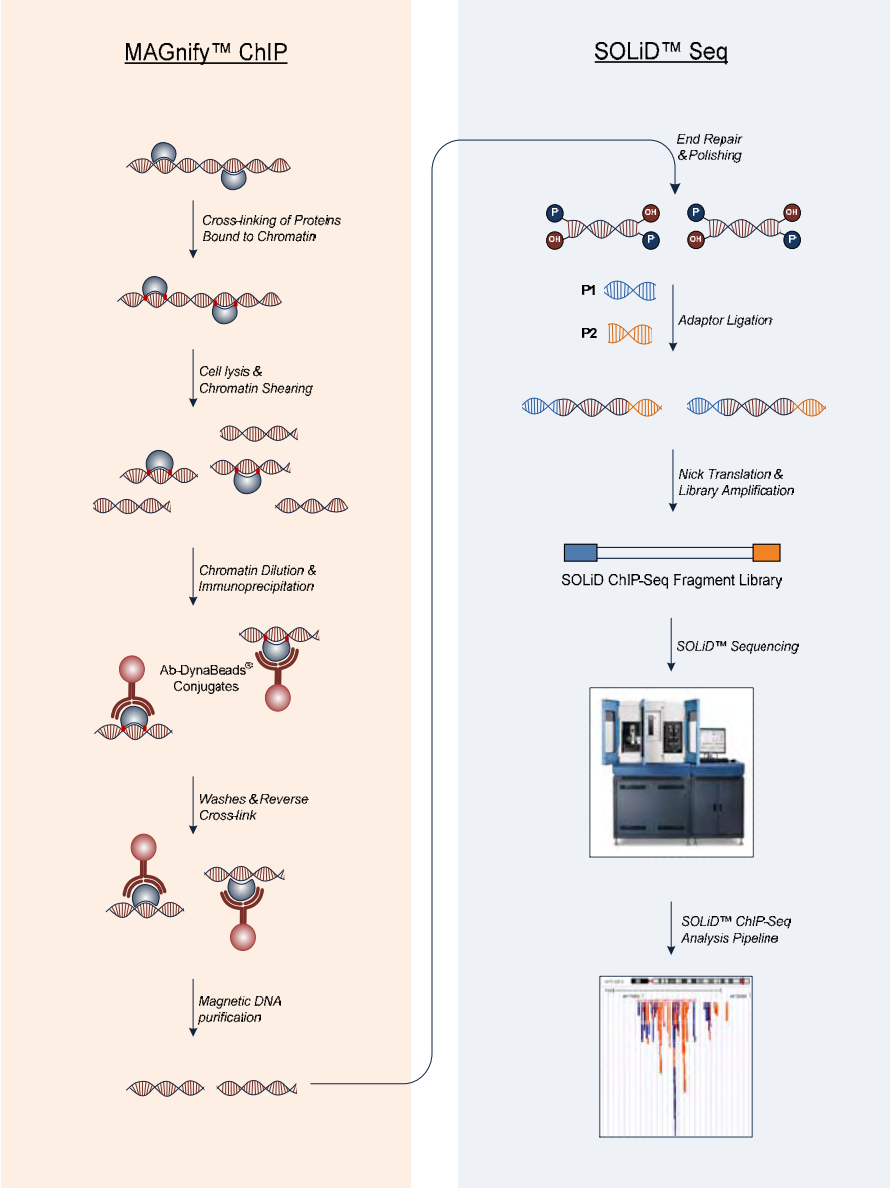
The SOLiD™ ChIP-Seq Kit requires the use of a magnetic tube holder that can be used with 0.2-mL PCR tubes. The DynaMag™-PCR Magnet holds up to 16 0.2-mL tubes, in individual or strip-well format. The magnet is included with catalog no. 4449638 and sold separately for catalog no. 4449640. See page 49 for ordering information.

Workflow Summary

Using the SOLiD™ ChIP-Seq Kit, you treat cells or tissue with formaldehyde to generate protein-protein and protein-DNA crosslinks between molecules in close proximity within the chromatin complex. The cells are then lysed, and the chromatin is released from the nuclei and sheared by sonication to reduce average DNA fragment size to ~100–300 bp.

You then immunoprecipitate and isolate the crosslinked protein of interest using a specific ChIP-qualified antibody conjugated to Dynabeads® Protein A/G. The formaldehyde crosslinking is reversed by heat treatment, and the DNA associated with that protein is purified.

The ChIP DNA is then ready for SOLiD™ System library preparation. The DNA is end-repaired and polished, P1 and P2 Adaptors are ligated to the sheared DNA and the fragment library is nick translated and amplified using primers specific to the P1 and P2 Adaptors. Multiple purification steps are performed to isolate the adaptor-ligated and amplified fragments prior to SOLiD™ sequencing.



Methods

Before Starting

Amount of Starting Material

For each ChIP reaction, we recommend using 10,000–300,000 cells or 0.167–5 mg of tissue. To ensure consistency and decrease experimental variability, we recommend preparing a common chromatin batch suitable for multiple ChIP experiments.

Note that following lysis, samples are at a concentration of 1 million cells/50 µL.

Number of ChIP Reactions Per Sample

The number of ChIP reactions per sample depends on (1) the total starting amount of cells or tissue and (2) the amount used per ChIP reaction. The table below shows some typical experimental scenarios:

Total Starting Amount of Cells/Tissue	Amount of Lysis Buffer	Amount Per ChIP Reaction	Total Number of ChIP Reactions
1 million cells	50 µL	100,000	10
1 million cells	50 µL	10,000	100
3 million cells	150 µL	100,000	30
3 million cells	150 µL	10,000	300
50 mg tissue (= 3 million cells)	150 µL	100,000	30
50 mg tissue (= 3 million cells)	150 µL	10,000	300

ChIP Antibody Selection

Selecting the right ChIP antibody is critical for successful ChIP-Seq experiments. Whenever possible, use an antibody that is qualified for ChIP. A selection of ChIP-qualified antibodies is provided on our website at **www.invitrogen.com/chipantibody**.

If a ChIP-qualified antibody is unavailable, there are some factors that **may** indicate that an antibody will be acceptable for ChIP. Antibodies should be specific and well-characterized. Characteristics such as purity, titer (determined by ELISA), and cross-reactivity (determined by dot blot) are good indicators of specificity. Western blot analysis, immunohistochemistry (IHC), and immunoprecipitation (IP) can also help determine an antibody's suitability for ChIP.

An antibody **may** have greater success in ChIP if it is affinity-purified, polyclonal (i.e., containing a population of antibodies that recognize different epitopes), and recognizes native protein conformations (qualified by immunoprecipitation).

When testing an antibody in ChIP, you should always include positive control antibodies for repressive and active genomic regions, such as H3-K9Me3 and H3-K9Ac respectively, and negative control antibodies such as Rabbit IgG and Mouse IgG (see the next page for details).

ChIP Controls

Negative controls: Rabbit IgG and Mouse IgG antibodies are provided in the kit for use as negative controls to measure non-specific binding. The concentration of the negative control antibodies is 1 µg/µL. Add 1 µL of negative control antibody per ChIP.

Positive control (not included in the kit): For a positive control, select an antibody that consistently binds chromatin-associated proteins under a wide variety of cellular conditions. For example, we observe consistent enrichment of heterochromatin markers such as H3-K9Me3 at the satellite repeat locus (SAT-2) and H3-K9Ac, which is often associated with actively transcribed genes such as the c-Fos gene. The amount of positive control to use varies depending on the antibody. Visit **www.invitrogen.com/chipantibody** for more information.

Negative control PCR primers (not included in the kit): Primers designed for a sequence that is not enriched by your ChIP procedure. They can detect contamination of your ChIP preparation by non-immunoprecipitated sample. These primers must be designed and ordered separately.

Input Control: This is DNA obtained from chromatin that has been reverse-crosslinked but has not been immunoprecipitated. It is reserved in **Step 4. Diluting the Chromatin**, on page 27, and then analyzed by qPCR.

Handling Dynabeads®

DynaMag™-PCR Magnet

The SOLiD™ ChIP-Seq Kit utilizes a novel magnet for processing Dynabeads® that is compatible with 0.2-mL PCR strip tubes. The DynaMag™-PCR Magnet (included with catalog no. 4449638; also available separately) is optimized for efficient magnetic separation of the small sample volumes used in ChIP experiments, and allows for the processing of multiple ChIP assays using a multi-channel pipettor. The magnet holds up to 16 0.2-mL PCR tubes, in individual or strip-tube format.

Always keep the following in mind when working with Dynabeads®:

- Never mix Dynabeads® by vortexing, as this will damage the beads.
- Never freeze Dynabeads®, as this will damage the beads.
- When removing liquid from Dynabeads®, avoid touching the beads with the pipette tip. This will disturb the bead pellet.
- Do not allow the beads to dry out. Resuspend the beads within 1 minute of removing any liquid from them.

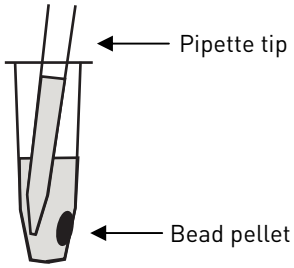
Resuspending Dynabeads®

To resuspend Dynabeads®, use gentle up-and-down pipetting while taking care to avoid creating air bubbles. ***Never mix the beads by vortexing.***

After resuspension, mix the beads by gently inverting the tube using continuous slow rotation.

Removing Liquid from Dynabeads®

To remove liquid from Dynabeads®:



1. Place the PCR tube or strip tubes containing the beads in the DynaMag™-PCR Magnet and allow to stand for at least 1 minute. During this time, the beads will concentrate as a pellet along the inner surface of the tube wall.
2. Open the tube without displacing it from the rack or disturbing the bead pellet and carefully extract the liquid volume with a pipette tip *without touching the bead pellet*. Angle the pipette tip away from the bead pellet to avoid contact.
3. After the liquid has been removed, remove the tube from the rack and quickly and gently resuspend the beads with the volume of appropriate solution. *Do not allow the beads dry out.* Add the next solution within 1 minute.

Step 1. Coupling the Antibody to Dynabeads®

Introduction

In this step, you wash the Dynabeads® and then couple them to your antibody of interest and control antibodies

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Antibody of interest
- Positive control antibody
- DynaMag™-PCR Magnet
- Rotating mixer, refrigerated
- 0.2-mL PCR tubes or strip tubes
- Ice
- Pipettor and pipette tips

ChIP Antibody Controls

Negative controls: Rabbit IgG and Mouse IgG antibodies are provided in the kit at a concentration of 1 µg/µL. Add 1 µL (1 µg) of negative control antibody per ChIP.

Positive control: For a positive control, select an antibody that consistently binds chromatin-associated proteins under a wide variety of cellular conditions. For example, we observe consistent enrichment of heterochromatin markers such as H3-K9Me3 at the satellite repeat locus (SAT-2) and H3-K9Ac, which is often associated with actively transcribed genes such as the c-Fos gene. The amount of positive control to use varies depending on the antibody (see below).

Amount of Antibody to Use

The amount of antibody required for a ChIP assay must be determined empirically and can vary considerably depending on the antibody. In general, 1–10 µg of antibody is a typical starting range.

Place the magnet, tubes, and buffers on ice before use, to cool them down before use. Rotate the tubes at 4°C during the coupling procedure.

Coupling the Antibodies to the Dynabeads®

Place the magnet, tubes, and buffers on ice before performing the following steps, to cool them down.

1. Resuspend the Dynabeads® using gentle up-and-down pipetting while taking care to avoid creating air bubbles.
2. Add 100 µL of cold Dilution Buffer to each tube (individual 0.2-mL PCR tubes or 8-tube strip wells may be used).
3. Add 10 µL of fully resuspended Dynabeads® Protein A/G to each tube, and pipet up and down gently 5 times to mix.
4. Place the tubes in the DynaMag™-PCR Magnet and wait at least 30 seconds, or until the beads form a tight pellet.
5. With the tubes on the magnet, remove and discard the liquid, being careful not to disturb the bead pellet (see figure on previous page).
6. Remove the tube containing the pelleted magnetic beads from the magnet and add 100 µL of cold Dilution Buffer to each tube.
7. Add the antibody of interest to the appropriate experimental tubes. (The amount of antibody must be determined empirically.)
8. Add any positive control antibodies to the appropriate control tubes.
9. Add 1 µL of negative control antibody (provided in the kit) to the appropriate control tubes. The concentration of the negative control antibodies is 1 µg/µL.
10. Cap the tubes and flick gently to resuspend the beads.
11. Rotate the tubes end-over-end at 4°C for 1 hour.

While the Antibody/Dynabeads® mixture is incubating, proceed to either **Step 2A. Preparing Cells** (next page) or **Step 2B. Preparing Tissue** (page 20). When the Antibody-Dynabeads® have finished mixing, hold the tube at 4°C until use.

Step 2A. Preparing Cells

Introduction

This section provides instructions for preparing cells for ChIP analysis. First, you cross-link the cells with formaldehyde to preserve the chromatin structure, then you lyse the cells.

For instructions on preparing tissue samples for analysis, see Step 2B.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Cells, unstimulated or treated as desired
- Trypsinizing reagent, such as TrypLE™ Express Stable Trypsin Replacement Enzyme
- PBS or D-PBS (1X), liquid
- 37% formaldehyde
- Cell counter, either automated (*e.g.*, the Countess® Automated Cell Counter) or manual
- Microcentrifuge at 4°C
- Vortex mixer
- Ice
- Pipettor and pipette tips

Crosslinking the cells with formaldehyde ensures that the chromatin structure is preserved during the isolation and ChIP procedure. Separate protocols are provided for:

- Collecting and crosslinking adherent cells
- Collecting and crosslinking cells in suspension
- Crosslinking adherent cells directly in a dish.

- The 1.25 M glycine must be at room temperature before use.
- Follow the instructions for formaldehyde treatment carefully, since too little crosslinking will not sufficiently preserve the chromatin structure and too much crosslinking will hamper the ChIP procedure.
- Keep the formaldehyde incubation time and method consistent between samples that you want to compare, to maintain consistency and reproducibility of results.

Determining the Optimal Amount of Crosslinking

In the following protocol, we recommend a 10-minute crosslinking step using formaldehyde at a 1% final concentration. However, you may choose to perform a time course experiment to optimize crosslinking conditions.

Too much crosslinking can lead to less protein bound to the DNA and fewer epitopes or changes in epitopes available for antibody binding.

Collecting and Crosslinking Adherent Cells

Use the following method to collect adherent cells prior to crosslinking. Alternatively, you can fix the cells directly, as described on the following page.

1. For adherent cells, aspirate the media and wash cells with 10 mL of room-temperature 1X PBS (or D-PBS).
2. Aspirate the PBS and add enough trypsinizing reagent to cover the cells. *Example for a T-175 Flask:* Add 4 mL of TrypLE™ Express Stable Trypsin Replacement Enzyme.
3. Incubate at 37°C for ~3 minutes or until cells dislodge from the plate surface.
4. When all the cells have detached, add 10 mL of room-temperature PBS and pipet the cells gently up and down to mix.
5. Transfer the cell suspension to a centrifuge tube and spin at 200 × *g* for 5 minutes to pellet.
6. Discard the supernatant and resuspend the pellet in room-temperature PBS. (Estimate the resuspension volume so the cell density is more concentrated than your planned dilution.) Mix the cell solution gently.
7. Collect a small aliquot to verify that the cells are at the desired concentration. Determine cell density electronically using an automated cell counter or manually using a hemacytometer chamber.
8. Determine the volume of cell suspension required for the total number of immunoprecipitations (IPs) planned (number of cells per IP times the total number of IPs). Transfer this volume to a new tube.
9. *If the volume is ≤500 µL,* bring the final volume to 500 µL with room-temperature PBS.

   If the volume is >500 µL, spin the cell suspension at 200 × *g* for 5 minutes, aspirate the supernatant, and resuspend the pellet in 500 µL of PBS.
10. Add 13.5 µL of 37% formaldehyde to the 500 µL of sample, for a final concentration of 1%. Invert the tube to mix, and incubate for 10 minutes at room temperature (or perform a time course to determine optimal time).
11. To stop the reaction, add 57 µL of room-temperature 1.25 M glycine to the sample. Invert the tube to mix, and incubate for 5 minutes at room temperature.
12. In a cold centrifuge at 4°C, spin the crosslinked cells at ~200 × *g* for 10 minutes. From this point, keep all tubes on ice.
13. Remove and discard the supernatant, leaving ~30 µL behind so as to not disturb the pellet.
14. Resuspend the cells in 500 µL of cold PBS, and spin at 200 × *g* for 10 minutes at 4°C to pellet.
15. Aspirate the PBS and resuspend once more in 500 µL of cold PBS. Spin cells at 200 × *g* for 10 minutes at 4°C to pellet.
16. Aspirate the PBS, leaving 10–20 µL behind. Make sure not to disturb the cell pellet.

Proceed to **Preparing the Lysis Buffer with Protease Inhibitors**, page 19.

Collecting and Crosslinking Cells in Suspension

1. For cells in suspension, transfer the cell suspension to a centrifuge tube and spin at 200 × *g* for 5 minutes at room temperature to pellet.
2. Aspirate the media and add 10 mL of room-temperature PBS. Spin at 200 × *g* for 5 minutes to pellet.
3. Aspirate the supernatant and resuspend the pellet in room-temperature PBS. (Estimate the resuspension volume so the cell density is more concentrated than your planned dilution.) Mix the cell solution gently.
4. Collect a small aliquot to verify that the cells are at the desired concentration. Count the cells electronically using an automated cell counter or manually using a hemacytometer chamber.
5. Determine the volume of cell suspension required for the total number of immunoprecipitations (IPs) planned (number of cells per IP times the total number of IPs). Transfer this volume to a new tube.
6. *If the volume is ≤500 µL,* bring the final volume to 500 µL with room-temperature PBS.

   If the volume is >500 µL, spin the cell suspension at 200 × *g* for 5 minutes, aspirate the supernatant, and resuspend the pellet in 500 µL of PBS.
7. Add 13.5 µL of 37% formaldehyde to the 500 µL of sample, for a final concentration of 1%. Invert the tube to mix, and incubate for 10 minutes at room temperature.
8. To stop the reaction, add 57 µL of room-temperature 1.25 M glycine to the sample. Invert the tube to mix, and incubate for 5 minutes at room temperature.
9. In a cold centrifuge at 4°C, spin the crosslinked cells at ~200 × *g* for 10 minutes. From this point, keep all tubes on ice.
10. Aspirate the supernatant, leaving ~30 µL behind so as to not disturb the pellet.
11. Gently resuspend the cells in 500 µL of cold PBS, and spin at ~200 × *g* for 10 minutes at 4°C to pellet.
12. Aspirate the PBS and wash once more in 500 µL cold PBS. Spin the cells at 200 × *g* for 10 minutes at 4°C to pellet.
13. Aspirate the PBS, leaving 10–20 µL behind. Make sure not to disturb the cell pellet.

Proceed to **Preparing the Lysis Buffer with Protease Inhibitors**, next page.

Crosslinking Cells Directly in a Dish

Use the following method to crosslink cells directly in a 10-mm dish. You can scale the reaction accordingly, depending on the dish size.

Note: Sufficient reagents are provided for 4 crosslinking reactions in 10-mm dishes, as described below.

1. When cells reach ~80% confluency, remove media and replace with 5 mL of complete media (for a 10-mm dish).
2. Add 135 µL of 37% formaldehyde (final concentration = 1%) and incubate at room temperature for 10 minutes.
3. To stop the reaction, add 0.5 mL of room-temperature 1.25 M glycine to the sample and incubate for 5–10 minutes at room temperature.
4. Aspirate the media and carefully wash the cells two times with 5 mL cold PBS at 4°C. (Cells will still be attached, so simply overlay with PBS and aspirate without disturbing the cell layer.) For this point on, keep all tubes on ice.
5. Use a cell scraper to scrape the cells into a 1.5-mL tube on ice.
6. Count the cells electronically using an automated cell counter or manually using a hemacytometer chamber.
7. Centrifuge the cells ~200 × *g* for 10 minutes at 4°C to pellet.
8. Aspirate the PBS, leaving 10–20 µL behind. **Do not disturb the pellet.**

Proceed to preparing the lysis buffer for cell lysis below.

Lysis Buffer Guidelines

- The Lysis Buffer must be at room temperature and fully resuspended before use. Vortex briefly to resuspend.
- Prepare only enough Lysis Buffer with Protease inhibitors for the amount of cells you will need on that day.
- The final concentration of cells in Lysis Buffer will be 1 million cells/ 50 µL.

Preparing the Lysis Buffer with Protease Inhibitors

Prepare 50 µL of Lysis Buffer containing Protease Inhibitors for every 1 million cells to be lysed (prepare fresh each day). The Protease Inhibitors are necessary to prevent protein degradation.

Vortex the Lysis Buffer briefly to resuspend, and then add Protease Inhibitors (200X) to achieve a final concentration of 1X. For example, to prepare 200 µL of Lysis Buffer with Protease Inhibitors, add 1 µL of 200X Protease Inhibitors to 199 µL of stock Lysis Buffer.

Lysing the Cells

1. Add Lysis Buffer with Protease Inhibitors (prepared as above) to the cell pellet from the previous pages. Use 50 µL of prepared Lysis Buffer per 1 million cells (e.g., add 100 µL for 2 million cells or 150 µL for 3 million cells).
2. Resuspend by mild pulses on the vortex mixer.
3. Incubate the tube on ice for at least 5 minutes.

Proceed to **Chromatin Shearing**, next page, or snap-freeze cells in liquid nitrogen or on dry ice and store at –80°C until use.

Step 2B. Preparing Tissue

Introduction

This section provides instructions for preparing fresh and frozen tissue for ChIP analysis.

For instructions on preparing cells for analysis, see Step 2A.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Tissue samples, fresh or frozen, 50–1000 mg
- Clean razor blades
- 1.5-inch 18G and 21G needles (a 1.5-inch 16G needle may also be needed for muscular tissues such as heart)
- 1-mL luer lock syringes
- 50-mL sterile conical tube
- D-PBS (1X), liquid— ice-cold and room temperature
- 37% formaldehyde
- Microcentrifuge at 4°C
- Vortex mixer
- Ice
- Pipettor and pipette tips

- The 1.25 M glycine must be at room temperature before use.
- The Lysis Buffer must be at room temperature and fully resuspended before use. Vortex briefly to resuspend.
- Prepare only enough Lysis Buffer with Protease inhibitors for the amount of tissue you will need on that day.

Preparing the Lysis Buffer with Protease Inhibitors

Prepare 150 µL of Lysis Buffer containing Protease Inhibitors for every 50 mg of tissue (prepare fresh each day). The Protease Inhibitors are necessary to prevent protein degradation.

Vortex the Lysis Buffer briefly to resuspend, and then add Protease Inhibitors (200X) to achieve a final concentration of 1X. For example, to prepare 400 µL of Lysis Buffer with Protease Inhibitors, add 2 µL of 200X Protease Inhibitors to 398 µL of stock Lysis Buffer.

Weighing and Mincing Fresh Tissue

The following protocol is designed for 50–1000 mg of fresh tissue.

1. Add ~10 mL of ice-cold 1X D-PBS to a 50-mL sterile conical tube and weigh the tube.
2. Place the tube on ice. Remove the fresh tissue to be analyzed and immediately place 50–1000 mg of tissue in the tube.
3. Weigh the tube containing the fresh tissue in D-PBS and subtract the original tube weight from Step 1.

Important: Keep the tubes and dishes on ice for the following steps.

4. To a sterile 10-cm culture dish on ice, add 250 µL of ice-cold 1X D-PBS per 50 mg of tissue, up to a maximum of 2 mL (e.g., add 750 µL of D-PBS for 150 mg of tissue, add 2 mL of D-PBS for 1000 mg of tissue, etc.).
5. Transfer the tissue to the 10-cm dish containing D-PBS (tilt the dish slightly if necessary to immerse the tissue in D-PBS).
6. Remove unwanted tissue such as necrotic material and fat from the sample. Remove any connective tissue that could clog the needle. Then use two clean razor blades to quickly mince the tissue into the smallest pieces possible (less than 1 mm cubed).
7. With a 2-mL pipette, transfer all minced tissue and D-PBS into a 50-mL conical tube on ice. Mash the tissue as much as possible with the pipette.
8. Proceed immediately to **Homogenizing the Tissue**.

Weighing and Mincing Frozen Tissue

The following protocol is designed for 50–1000 mg of frozen tissue.

1. Weigh an empty 50-mL sterile conical tube.
2. Place the tube on ice. Remove the fresh tissue to be analyzed and immediately place 50–1000 mg of tissue in the tube.
3. Immediately freeze the tube containing the tissue in liquid nitrogen and store at –80ºC.
4. When you are ready to prepare the frozen tissue for ChIP analysis, remove the tube from –80ºC and weigh the tube and sample while still frozen. Subtract the original tube weight from Step 1.

Important: Keep the tubes and dishes on ice for the following steps.

5. To a sterile 10-cm culture dish on ice, add 250 µL of ice-cold 1X D-PBS for every 50 mg of tissue, up to a maximum of 2 mL (e.g., add 750 µL of D-PBS for 150 mg of tissue, add 2 mL of D-PBS for 1000 mg of tissue, etc.).
6. Transfer the still-frozen tissue to the 10-cm dish containing D-PBS (tilt the dish slightly if necessary to immerse the tissue in D-PBS).
7. Remove unwanted tissue such as necrotic material and fat from the sample. Remove any connective tissue that could clog the needle. Then use two clean razor blades to quickly mince the tissue into the smallest pieces possible (less than 1 mm cubed).
8. With a 2-mL pipette, transfer all minced tissue and D-PBS into a 50-mL conical tube on ice. Mash the tissue as much as possible with the pipette.
9. Proceed immediately to **Homogenizing the Tissue**.

Homogenizing the Tissue

Note: The following steps use 18G and 21G needles; however, for muscular tissue such as heart, you may need to start with a 16G needle and then proceed to an 18G needle followed by a 21G needle.

1. Attach a 1.5-inch 18G needle in its plastic sheath to a sterile 1-mL syringe. With the needle still in the plastic sheath, carefully mash the tissue with the tip of the sheath.
2. Remove the plastic sheath and pipette the tissue up and down 10 times using the needle. If the syringe becomes clogged, pull out the stopper, re-insert it, and then push to expel the clog.
3. Attach a 1.5-inch 21G needle to a new syringe, and pass the tissue up and down 20 times to homogenize. If the syringe becomes clogged, pull out the stopper, re-insert it, and then push to expel the clog.
4. Immediately proceed to **Crosslinking the Chromatin**.

Crosslinking the Chromatin

1. Take the tube with the homogenized tissue in D-PBS off the ice. Add additional **room-temperature** 1X D-PBS to the tube up to a total D-PBS volume of 450 µL per 50 mg of tissue.

Initial Tissue in D-PBS	+ Additional D-PBS	Final D-PBS
50 mg in 250 µL D-PBS	+ 200 µL	= 450 µL
150 mg in 750 µL D-PBS	+ 600 µL	= 1350 µL
1000 mg in 2000 µL D-PBS	+ 7000 µL	= 9000 µL

2. Add 37% formaldehyde to a final concentration of 1% (e.g., add 13.5 µL of 37% formaldehyde for every 50 mg of tissue).
3. Swirl the tube gently to mix and incubate for exactly 10 minutes at room temperature, swirling the tube gently every 2 minutes during incubation.
4. Add 1.25 M glycine to a final concentration of 0.125 M (e.g., add 57 µL of 1.25 M glycine for every 50 mg of tissue).
5. Swirl the tube gently to mix evenly and incubate for 5 minutes at room temperature, swirling gently every ~2 minutes during incubation.
6. Aliquot the mixture into 1.5-mL LoBind tubes, adding 570 µL per tube (the equivalent of 50 mg of tissue per tube).
7. In a cold centrifuge at 4°C, spin the LoBind tubes at ~200 × g for 10 minutes. Transfer the tubes to ice and keep them on ice for all subsequent steps.
8. Remove and discard the supernatant from each tube, leaving ~30 µL so as to not disturb the pellet.
9. Add 500 µl of cold PBS to each tube and resuspend the sample by flicking it with your finger.
10. Spin at 200 × g for 10 minutes at 4°C to pellet.
11. Aspirate the PBS and resuspend the sample once more in 500 µL of cold PBS. Spin at 200 × g for 10 minutes at 4°C to pellet.
12. Aspirate the PBS, leaving 10–20 µL behind. **Do not disturb the cell pellet.**

Proceed to **Lysing the Cells**, next page.

For all subsequent steps, 50 mg of starting tissue is the equivalent of 3 million cells. When each pellet from the previous procedure is resuspended in 150 µL of Lysis Buffer as below, the resulting is concentration is 1 million cells per 50 µL.

Lysing the Cells

1. Add 150 µL of Lysis Buffer prepared with Protease Inhibitors (see page 20) to each pellet from the previous page. This is the equivalent of 50 µL of Lysis Buffer per 1 million cells.
2. Resuspend by mild pulses on the vortex mixer.
3. Incubate the tube on ice for at least 5 minutes.

Proceed to **Step 3. Shearing the Chromatin** or snap-freeze the sample in liquid nitrogen or on dry ice and store at –80°C until use.

Step 3. Shearing the Chromatin

Introduction

In this step, you shear the chromatin from the previous page into fragments of 100–300 bp.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Sonicator, e.g., the Covaris® S2 System or Bioruptor® UCD-200
- For confirming DNA fragment sizes:
  - 1.5–2.0% agarose gel (e.g., 2% E-Gel® or 2% E-Gel® EX Gel)
  - 100-bp DNA ladder (e.g., TrackIt™ 100-bp DNA Ladder)
- Ice
- Liquid nitrogen or dry ice
- Microcentrifuge
- Microcentrifuge tubes, RNase/DNase-free, low retention (e.g., Eppendorf 1.5-mL LoBind tubes)
- Pipettor and pipette tips

Fragment Size

Shearing the chromatin into 100–300 bp fragments is required for downstream sequencing on the SOLiD™ system.

Sonication is the preferred method for shearing chromatin when using formaldehyde crosslinking, as crosslinking restricts the access of MNase to chromatin.

Keep the cell lysate cooled on ice during sonication, as heat released by the sonication probe can reverse the crosslinks. If using probe sonication, place the sample on ice between cycles.

Sonication Conditions

ChIP-Seq sonication conditions have been optimized for the Covaris® S2 or the Bioruptor® UCD-200. Sonication conditions should be determined independently for each cell type. The sonication efficiency varies to some extent with changes in sample volume and tube size.

For probe sonicators, the tip of the sonicator probe should be kept as deep as possible in the tube while not touching the tube wall, and no more than a few millimeters above the bottom of the tube. This is important for two reasons:

- Continuous contact between the probe and tube wall will lead to reduced efficiency of chromatin shearing.
- Positioning the tip of the probe too close to the sample surface will lead to foaming and inefficient sonication.

If foaming is not eliminated by taking these precautions, try reducing the sonicator output energy.

Sonication Optimization

Sonication is a critical step in the ChIP-Seq procedure. We recommend testing various sonication conditions on your cells of interest, and running treated chromatin lysates on a 1.5–2.0% agarose gel (e.g., a 2% E-Gel® or 2% E-Gel® EX Gel) with a 100-bp ladder to determine fragment length.

In general, when starting with 1 million cells per 50 µL, add 1 µL of Proteinase K to 10 µL of chromatin input and incubate at 55°C for 20 minutes prior to pelleting the cell debris by spinning at 20,000 × *g* at 4°C for 5 minutes. Transfer the chromatin to a new tube and run ~5 µL of chromatin input per well on a 2% E-Gel®.

Alternatively, add 0.25 µL of Proteinase K to 2.5 µL of chromatin input and incubate at 55°C for 20 minutes prior to pelleting the cell debris by spinning at 20,000 × *g* at 4°C for 5 minutes. Transfer the chromatin to a new tube and run ~0.5–1 µL of treated chromatin input on a 2% E-Gel® EX Gel.

Storing Sheared Chromatin

The sheared chromatin may be used directly in ChIP or snap-frozen in liquid nitrogen or on dry ice. Store frozen aliquots of sheared chromatin at –80°C.

Sonication Using the Covaris® S2

Below is an example sonication procedure using the Covaris® S2 to shear chromatin into 100–300 bp fragments. Refer to the instrument manual for setup and maintenance.

1. Program the instrument as follows:

Duty Cycle: 5%	**Cycles:** 10
Intensity: 2	**Temperature (bath):** 4°C
Cycles per Burst: 200	**Power mode:** Frequency Sweeping
Cycle Time: 60 seconds	**Degassing mode:** Continuous

2. Fill the water level to 15 and degass the instrument for 30 minutes.
3. Load up to 100 µL of cell lysate (from **Lysing the Cells**, page 19 or 23) into a Covaris® MicroTube with AFA fiber. Insert the tube into a Covaris®-2 series Machine Holder for one 6-mm MicroTube.
4. Sonicate the sample using the program specified above.
5. Transfer the freshly sonicated chromatin into a new, sterile microcentrifuge tube.
6. Pellet the cell debris by spinning at 20,000 × g at 4°C for 5 minutes.
7. **The supernatant contains the chromatin.** Aliquot the freshly sonicated chromatin into new, sterile tubes.

Proceed to **Step 4. Diluting the Chromatin**, page 27, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

Sonication Using the Bioruptor® UCD-200

Below is an example sonication procedure using the Bioruptor® UCD-200 from Diagenode for shearing chromatin into fragments ranging from 100 to 300 bp. Refer to the instrument manual for setup and maintenance.

1. Pre-cool the water reservoir with ice.
2. Remove most of the ice and add ice-cold water to the water level mark.
3. Transfer the cell lysate (from **Lysing the Cells**, page 19 or 23) into 1.5-mL Eppendorf LoBind tubes. Use a final volume of 50–150 µL per tube.
4. Place one of the tubes in the Bioruptor® UCD-200 and set it to High Power.

   Note: Sonicating one tube at a time will ensure consistent results and proper size distribution.
5. Sonicate the tube for 8 cycles of 30 seconds ON, 30 seconds OFF. After 8 cycles, cool the water in the reservoir with ice, then remove and add ice-cold water up to the water mark.
6. Sonicate the tube for another 8 cycles.
7. Repeat Steps 5–6 for each of the remaining tubes.
8. Pellet the cell debris by spinning at 20,000 × *g* at 4°C for 5 minutes.
9. **The supernatant contains the chromatin.** Aliquot the freshly sonicated chromatin into new, sterile tubes.

Proceed to **Step 4. Diluting the Chromatin**, next page, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

Step 4. Diluting the Chromatin

Introduction

In this step, you dilute the sheared chromatin based on the number of cells you want to assay in each immunoprecipitation (IP) reaction.

This kit contains sufficient reagents for 20 ChIP-Seq library preparations.

Preparing Dilution Buffer with Protease Inhibitors

To prepare the Dilution Buffer, add the Protease Inhibitors (200X) provided in the kit to a final concentration of 1X. For example, to prepare 1,000 µL of Dilution Buffer with Protease Inhibitors, add 5 µL of 200X Protease Inhibitors to 995 µL of stock Dilution Buffer.

The prepared Dilution Buffer should be cold before use.

Diluting the Chromatin

Dilute the sheared chromatin from the previous section in cold Dilution Buffer prepared with Protease Inhibitors. The starting concentration of the chromatin is 1 million cells/50 µL. The ratio of chromatin to Dilution Buffer is based on the number of cells you want to use in each IP reaction (see table below).

The final dilution volume is 100 µL per IP reaction. Prepare an extra dilution for each sample for use as an Input Control, as described below.

Example Volumes of Chromatin and Dilution Buffer

Cells per IP	# of IPs	Amount of Chromatin	Amount of Dilution Buffer with Protease Inhibitors	Total Volume
50,000	1	2.5 µL	97.5 µL	100 µL
100,000	1	5 µL	95 µL	100 µL
200,000	1	10 µL	90 µL	100 µL

Reserve the Input Control

For each sample you are analyzing, prepare an extra 100-µL dilution. Pipet up and down gently to fully mix, and save 10 µL of this dilution in a separate 0.2-µL PCR tube. This is your Input Control.

You will reverse-crosslink this control sample (**Step 7. Reversing the Crosslinking,** page 30) and isolate the DNA without performing immunoprecipitation. This isolated DNA will be used as a positive control and can also be used for data normalization using qPCR, as described on page 34.

Step 5. Binding Chromatin to the Beads

Introduction

In this step, you bind the sheared chromatin to the Antibody-Dynabeads® complexes.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Microcentrifuge
- DynaMag™-PCR Magnet
- Rotating mixer, refrigerated
- Ice
- Pipettor and pipette tips

- The binding time in the following protocol is 2 hours, which is sufficient for most applications. However, you may optimize this time based on the characteristics of your particular antibodies and samples.
- Remember to reserve at least one 10-µL aliquot of diluted chromatin for each sample as an Input Control. This Input Control will not be bound to the beads, and will be used directly in the **Reverse Crosslinking** procedure on page 30.

Binding the Chromatin

When the Antibody-Dynabeads® from page 15 have finished mixing, proceed with the following. Keep the magnet, tubes, and buffers cold during these steps.

1. Spin the tubes briefly to remove any liquid trapped in the caps, then place in the DynaMag™-PCR Magnet.
2. Let stand for at least 30 seconds, or until the beads form a tight pellet.
3. With the tubes on the magnet, remove and discard the liquid from each tube, being careful not to disturb the bead pellet.
4. Remove the tubes from the magnet and immediately add 100 µL of diluted chromatin extract (from **Diluting the Chromatin**, previous page) to each tube containing the appropriate Antibody-Dynabeads® complex.
5. Cap the tubes and flick gently to resuspend the beads.
6. Rotate the tubes end-over-end at 4°C for 2 hours.

After rotation, proceed to **Step 6. Washing the Bound Chromatin,** next page.

Step 6. Washing the Bound Chromatin

Introduction

In this procedure, you wash the Chromatin-Antibody-Dynabeads® complexes to remove any unbound product.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- DynaMag™-PCR Magnet
- Rotating mixer, refrigerated
- Ice
- Pipettor and pipette tips

Begin warming the Reverse Crosslinking Buffer and DNA purification beads and buffers to room temperature before beginning the wash steps. These components must be at room temperature before use.

Washing with IP Buffer 1

After the tubes from **Binding the Chromatin**, previous page, have finished mixing, proceed with the following.

Keep the magnets, tubes, and IP Buffer 1 cold during the following procedure.

1. Spin the tubes briefly to remove any liquid trapped in the caps, and then place the tubes in the DynaMag™-PCR Magnet.
2. Let stand for at least 30 seconds, or until the beads form a tight pellet.
3. With the tubes in the magnet, remove and discard the liquid from each tube, being careful not to disturb the bead pellet.
4. Remove the tubes from the magnet and add 100 µL of IP Buffer 1 to each tube. Cap the tubes and flick gently to resuspend the beads.
5. Rotate the tubes end-over-end at 4°C for 5 minutes.
6. Repeat Steps 1–5 two more times.

After rotation, proceed immediately to **Washing with IP Buffer 2** below.

Washing with IP Buffer 2

Keep magnet and tubes cold during the following steps. IP Buffer 2 can remain at room temperature.

1. Spin the tubes briefly to remove any liquid trapped in the caps, and then place the tubes in the DynaMag™-PCR Magnet.
2. Let stand for at least 30 seconds, or until the beads form a tight pellet.
3. With the tubes in the magnet, remove and discard the liquid from each tube, being careful not to disturb the bead pellet.
4. Remove the tubes from the magnet and add 100 µL of IP Buffer 2 to each tube. Cap the tubes and flick gently to resuspend the beads.
5. Rotate the tubes end-over-end at 4°C for 5 minutes. (During rotation, prepare the Reverse Crosslinking Buffer with Proteinase K as described on the following page.)
6. Repeat Steps 1–5 one more time.

Proceed immediately to **Step 7. Reversing the Crosslinking**, next page.

Step 7. Reversing the Crosslinking

Introduction

In this procedure, you reverse the formaldehyde crosslinking of the chromatin.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- DynaMag™-PCR Magnet
- Thermal cycler, hybridization oven, water bath, or other heat source
- Sterile 0.2-mL PCR tubes or strip tubes
- Pipettor and pipette tips

Preparing Reverse Crosslinking Buffer with Proteinase K

Note: The Reverse Crosslinking Buffer must be at room temperature before use.

To prepare the final Reverse Crosslinking Buffer for your IP samples, you must add Proteinase K to the stock buffer provided in the kit. Prepare 54 µL of buffer per IP reaction as follows:

Component	1 reaction	12 reactions
Stock Reverse Crosslinking Buffer	53 µL	636 µL
Proteinase K	1 µL	12 µL
Final Volume	54 µL	648 µL

Preparing the Input Controls

Because the Input Controls are not bound to Dynabeads®, they are prepared separately from the IP samples as follows. However, the reverse-crosslinking incubation steps are the same as the IP samples and should be performed at the same time (see Steps 4 and 7 on the next page).

1. To each tube containing 10 µL of Input Control from **Reserve the Input Control**, page 27, add 43 µL of Reverse Crosslinking Buffer, for a total volume of 53 µL.
2. Add 1 µL of Proteinase K to the tube.
3. Vortex briefly to mix, and immediately proceed to **Reverse Crosslinking**, next page.

Reverse Crosslinking

All tubes and buffers should be at room temperature, unless otherwise indicated.

1. Place the tubes from **Washing with IP Buffer 2**, page 29, in the DynaMag™-PCR Magnet and wait at least 30 seconds for a pellet to form.
2. With the tubes in the magnet, remove and discard the liquid from each tube, being careful not to disturb the bead pellet.
3. Remove the tubes from the magnet and add 54 µL of Reverse Crosslinking Buffer prepared with Proteinase K to each tube. Vortex lightly to fully resuspend the beads.
4. Incubate the IP sample tubes and Input Control tubes (from previous page) at 55°C for 15 minutes in a thermal cycler, hybridization oven, water bath, or other heat source of choice. Spin tubes briefly, and then proceed directly through Steps 5–7.
5. Place the IP sample tubes in the DynaMag™-PCR Magnet and wait at least 30 seconds for a pellet to form.
6. ***Do not discard the liquid—the liquid contains your sample.*** With the tubes in the magnet, carefully transfer the liquid (~50 µL) to new, sterile 0.2-mL PCR tubes or strip tubes. Be careful not to disturb the bead pellet. Proceed immediately to Step 7.
7. Spin the IP sample tubes and Input Control tubes briefly, and then incubate at 65°C for 15 minutes.
8. Cool the tubes on ice for ~5 minutes.
9. Discard the used magnetic beads. Do not reuse.

Proceed to **Step 8. Purifying the DNA**, next page.

Step 8. Purifying the DNA

Introduction

In this procedure, you purify the un-crosslinked DNA using the DNA Purification Magnetic Beads and buffers in the kit.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- DynaMag™-PCR Magnet (provided with catalog no. 4449638; to order separately see page 49)
- Pipettor and pipette tips

- The DNA Purification Magnetic Beads pellet may appear more spread out on the magnet than the previous Dynabeads® pellet. Remove liquid slowly when the beads are on the magnet to minimize bead loss.
- Leave ~5 µL in the well after each wash and DNA elution to avoid disturbing the pellet.
- A precipitant may form with the Input Controls. This will not affect purification.

Preparing the DNA Purification Magnetic Beads with Dilution Buffer

All beads and buffers should be at room temperature before use.

1. Briefly vortex the DNA Purification Magnetic Beads to resuspend.
2. Prepare 70 µL of beads per sample by adding 50 µL of DNA Purification Buffer to 20 µL of resuspended DNA Purification Magnetic Beads. Scale accordingly based on your number of samples (including Input Controls).
3. Pipet up and down gently 5 times to mix.

Binding and Washing the DNA

All beads and buffers should be at room temperature before use.

1. After the tubes from **Reverse Crosslinking**, previous page, have cooled, spin them briefly to collect the contents.
2. Add 70 µL of DNA Purification Magnetic Beads prepared with DNA Purification Buffer to each tube (including Input Controls).
3. Pipet up and down gently 5 times to mix. Incubate at room temperature for 5 minutes.
4. Place the tubes in the DynaMag™-PCR Magnet and wait at least 1 minute for a pellet to form.
5. With the tubes in the magnet, remove and discard the liquid from each tube, leaving ~5 µL at the bottom to avoid disturbing the beads.
6. Remove the tubes from the magnet and add 150 µL of DNA Wash Buffer to each tube. Pipet up and down gently 5 times to mix.
7. Repeat Steps 4–6 one time.

Proceed to **Eluting the DNA**, next page.

Eluting the DNA

1. Place the tubes from **Binding and Washing the DNA**, previous page, in the DynaMag™-PCR Magnet and wait at least 1 minute for a pellet to form.
2. With the tubes in the magnet, remove and discard the liquid from each tube, leaving ~5 µL at the bottom to avoid disturbing the beads.
3. Remove the tubes from the magnet and add 60 µL of DNA Elution Buffer to each tube. Pipet up and down gently 5 times to mix.
4. Incubate at 55°C for 20 minutes in a thermal cycler, hybridization oven, water bath, or other heat source of choice.
5. Spin the tubes briefly to collect the contents. Place the tubes in the DynaMag™-PCR Magnet and wait at least 1 minute for a tight pellet to form.
6. ***Do not discard the liquid—the liquid contains your purified sample.*** With the tubes in the magnet, carefully transfer the liquid to new, sterile tubes. Leave ~5 µL at the bottom to avoid disturbing the beads.

   Note: If you have accidentally aspirated beads during the transfer step, the eluate may appear discolored. In this case, return the liquid to the tube containing the beads and repeat Steps 5–6.
7. Discard the used magnetic beads. Do not reuse.

Store the purified DNA at –20°C or proceed immediately to **Step 9. Analysis of the ChIP DNA Using qPCR,** next page. Avoid repeatedly freezing and thawing the DNA.

Quantification of ChIP DNA

The amount of ChIP DNA you will recover is dependent on many factors, including antibody epitope accessibility and protein binding-site accessibility. The Quant-iT™ DNA Assay Kit, High Sensitivity, and Quant-iT™ dsDNA HS Assay Kits for use with the Qubit® fluorometer provide accurate quantitation of most chromatin input samples (see ordering information on page 49). However, some targets may still be too dilute to be accurately quantified.

Step 9. Analysis of the ChIP DNA Using qPCR

Introduction

This section provides guidelines for analyzing the purified ChIP DNA using real-time quantitative PCR (qPCR).

qPCR Materials and Guidelines

Applied Biosystems and Invitrogen have a wide range of reagents, instruments, and other products for qPCR, including TaqMan® probes, SYBR® GreenER™, and EXPRESS qPCR SuperMixes. Visit **www.appliedbiosystems.com** and **www.invitrogen.com** for details.

The instructions provided with the reagent kits provide specific qPCR guidelines and parameters for the enzymes and dyes provided in those kits.

MAGnify™ PCR Primer Pairs

The following primer pairs are available separately from Invitrogen for the amplification of common promoter regions analyzed in ChIP experiments. See page 49 for ordering information.

Primer Pair	Sequences	Catalog no.
MAGnify™ SAT2 Primers	CTGCAATCATCCAATGGTCG GATTCCATTCGGGTCCATTC	49-2026
MAGnify™ RARβ1 Primers	GGCATTTGCATGGCATCCA CCGCGGTACACGCAAAA	49-2027
MAGnify™ ERα Primers	TGAACCGTCCGCAGCTCAAGATC GTCTGACCGTAGACCTGCGCGTTG	49-2028
MAGnify™ c-Fos Primers	TTAGGACATCTGCGTCAGCAGGTT TCTCGTGAGCATTTCGCAGTTCCT	49-2029

Replicates

In general, individual samples should be run in triplicate. Obvious outliers occur with some frequency, generally at <5%. Triplicate analysis of samples permits removal of those outliers while still allowing for inclusion of two accurate measurements for each sample. While this reduces the number of different samples that can be run at any given time, the resulting data is much more reliable and accurate.

qPCR of the Input Control DNA

For each primer pair, run the Input Control DNA alongside the immunoprecipitated samples. Amplification efficiencies among different primer pairs vary slightly on a per-cycle basis, but these slight variations in efficiency can translate into substantially different amounts of amplified material. Precise quantitation of relative binding cannot be accurately performed without data from the Input Controls for each primer pair.

Determining Amplification Efficiency using the Input Control

We recommend determining the amplification efficiency of your qPCR reaction using 10-fold serial dilutions of the Input Control DNA in the DNA Elution Buffer provided in the kit (e.g., 1:1 to 1:100). An acceptable efficiency range is ~1.9–2.1. This efficiency range corresponds to qPCR standard curve slopes of –3.1 to –3.6. Amplification efficiency (AE) is calculated by the formula AE = 10^(-1/slope).

Data Normalization Using the Input Control

1. Export the qPCR data to a spreadsheet program such as Microsoft Excel by using built in filters. The file should not contain omitted wells and should be in a column format containing well positions, descriptors, and CT values for each selected well.
2. Open the exported file. Average the replicate measurements for each IP reaction in a new column (AVERAGECT IP).
3. For each primer pair, calculate the adjusted CT for the Input Controls. For an Input Control that was 10% of the IP reaction, then the dilution factor (DF) is 10 and you should subtract 3.32 cycles (i.e., log2 of 10) from the Ct value of the Input Control. Then average the Input Control replicates (AVERAGE CT INPUT).
4. Subtract the AVERAGECT INPUT from AVERAGECT IP in a new column. This number is the dCT. This value represents the difference in cycles between the immunoprecipitated sample and the input DNA.
5. Normalized input is calculated by:

   100 × AE^(AVERAGECT INPUT – AVERAGECT IP)

   Where AE is the amplification efficiency as calculated above.

Fold Enrichment as Calculated by Signal Over Background

With this method, signals from the IP reactions are divided by the signals from the negative antibody control reaction (i.e., the Rabbit IgG or Mouse IgG antibody provided in the kit). This represents the IP signal as the fold increase in signal relative to the background signal.

The assumption of this method is that the level of background signal is reproducible between different primer sets, samples and replicate experiments, even though background signal levels can vary due to these factors.

Step 10. SOLiD™ ChIP-Seq Library Preparation

Introduction

Following purification, you are ready to prepare SOLiD™ ChIP-Seq libraries from the ChIP DNA, as described in this section.

Materials Needed

In addition to materials provided in the kit, you will need the following:

- Agencourt® AMPure® or AMPure® XP Beads (e.g., Agencourt® AMPure® XP 5-mL Kit, A63880; or Agencourt® AMPure® XP 60-mL Kit, A63881)
- Magnetic rack for 1.5-mL tubes
- Ethanol, absolute, 200 proof, Molecular Biology Grade
- ABI SOLiD™ Fragment Library Oligos Kit (Applied Biosystems 4401151)
  - SOLiD™ Library Oligos Kit 1 P1 Adaptor (ds)
  - SOLiD™ Library Oligos Kit 1 P2 Adaptor (ds)
  - SOLiD™ Library Oligos Kit 1 Library PCR Primer 1
  - SOLiD™ Library Oligos Kit 1 Library PCR Primer 2
- Microcentrifuge tubes, RNase/DNase-free, low retention (e.g., Eppendorf 1.5-mL LoBind tubes)
- PCR tubes
- Microcentrifuge
- Nutator mixer
- qPCR instrument
- For confirming DNA fragment sizes:
  - 1.5–2.0% agarose gel (e.g., 2% E-Gel® or 2% E-Gel® EX Gel)
  - 100-bp DNA ladder (e.g., TrackIt™ 100-bp DNA Ladder)
- Pipettor and pipette tips

The following procedure can use **either** AMPure® or AMPure® XP beads. The text refers to AMPure® XP beads because the original AMPure® beads will be discontinued in 2010.

- Do not freeze Agencourt® AMPure® beads. Store at 4°C.
- Allow Agencourt® AMPure® beads to warm to room temperature before use. To mix, invert by hand several times until the beads appear homogenous and consistent in color.

Volume of Beads Needed

For each ChIP sample that you convert into a SOLiD™ library, you will perform four separate purification procedures. A total of approximately 864 µL of AMPure® XP beads per library prep will be needed.

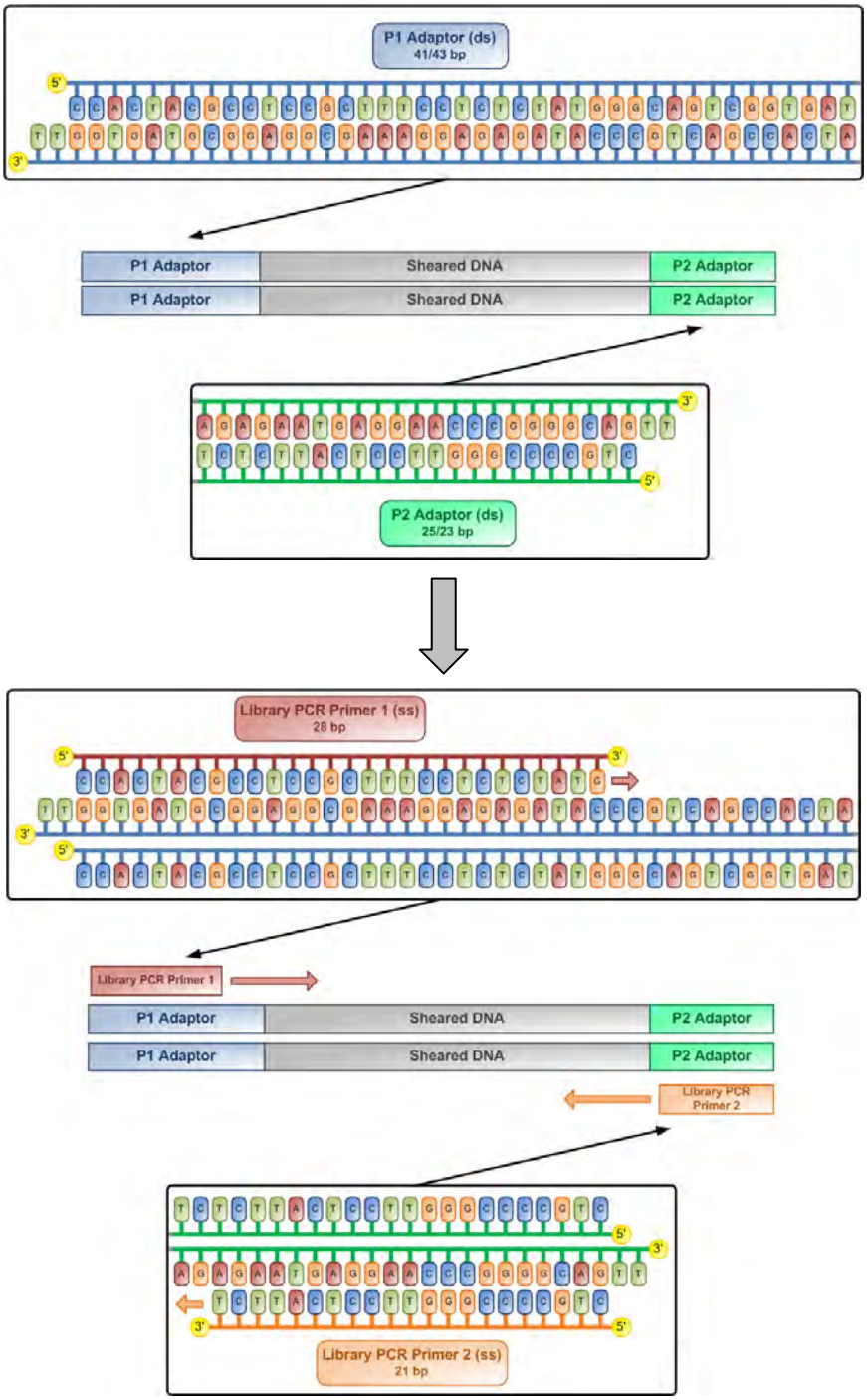
Preparing a Reference Control Library

To detect differential enrichment, we suggest preparing a reference control library using the Input Control (the non-immunoprecipitated control sample).

Overview of Fragment Library Preparation

After the purified ChIP DNA is end-repaired and polished, P1 and P2 Adaptors are ligated to the sheared DNA and the library is nick translated and amplified using primers specific to the P1 and P2 Adaptors.

Multiple purification steps are performed to isolate the adaptor-ligated and amplified fragments prior to SOLiD™ sequencing.



End Repairing the DNA

1. For each purified ChIP DNA sample from **Eluting the DNA**, page 33, combine and mix the following components in an Eppendorf LoBind tube:

Component	Volume
ChIP DNA	50 µL
5X End-Polishing Buffer	20 µL
dNTP Mix (10 mM)	4 µL
End Polishing Enzyme 1	1 µL
End Polishing Enzyme 2	8 µL
Nuclease-free water	17 µL
Total	100 µL

2. Vortex briefly, then incubate at room temperature for 30 minutes.

Proceed to **Binding and Washing the End-Repaired DNA** below.

Use Only Freshly Prepared 70% Ethanol

Prepare 70% ethanol fresh each day for library preparation. Using freshly prepared 70% ethanol is **critical** for the wash procedures, because 70% ethanol is hygroscopic.

Prepare 3.6 mL of 70% ethanol for each library [4 × (3 × 300 µL per purification)].

Note that using a higher percentage of ethanol will result in inefficient washing of smaller-sized molecules and a lower percentage could cause loss of sample.

Binding and Washing the End-Repaired DNA

Use the Agencourt® AMPure® XP Kit to selectively bind the end-repaired DNA. Make sure the beads are at room temperature and have been thoroughly mixed by hand before use (see **Important** note, previous page).

1. Add 70 µL of beads to each 100-µL sample (from **End Repairing the DNA**) and pipet up and down 10 times to mix.
2. Incubate for 10 minutes at room temperature on a nutator.
3. Place the sample tube in a magnetic rack and wait for the solution to clear (~1–3 minutes).
4. ***Do not discard the liquid—the liquid contains your purified sample.*** With the tube in the magnet, carefully transfer the liquid to a new, sterile 1.5-mL LoBind tube. Leave ~5 µL at the bottom to avoid disturbing the beads
5. Remove the tube with the beads from the magnet and briefly pulse spin. Place the tube back on the magnet and wait for solution to clear.
6. Remove the remaining liquid, being careful not to disturb the pellet, and add this to the saved liquid from Step 4. Discard the beads.
7. Place the tube containing the purified sample in the magnetic rack, wait for the solution to clear, and transfer the liquid to a new 1.5-mL LoBind tube. If the solution is still not clear, repeat this step one more time.

Procedure continued on the next page

Continued from the previous page

8. Add 110 µL of Agencourt® AMPure® XP beads to the sample and pipet up and down 10 times to mix.
9. Incubate for 10 minutes at room temperature on a nutator.
10. Place the sample tube in the magnetic rack, and wait for the solution to clear.
11. Remove the supernatant and discard. The sample is bound to the beads.
12. Add 300 µL of **freshly prepared** 70% ethanol. Wash the bead pellet by lifting the tube, turning it 180° and then placing it back in the rack. Turn a total of 6 times to wash thoroughly. Turning the tube allows the magnet to declump the beads and prevents the need for vigorous vortexing.
13. Remove and discard the ethanol without disturbing the bead pellet.
14. Repeat Steps 12 and 13 two more times.
15. After the final ethanol wash, pulse-spin the beads, place them back on the rack, and remove any residual ethanol. Repeat this step 2–3 more times.

Proceed to **Eluting the End-Repaired DNA** below.

Eluting the End-Repaired DNA

1. With the tube cap open and the tube in the magnetic rack, air dry the beads **completely** at room temperature on the bench or in a fume hood (~20–30 minutes on the bench and 10–15 minutes in a hood). **The pellet will form visual cracks on its surface when dry.**
2. Add 30 µL of the DNA Elution Buffer included in the SOLiD™ ChIP-Sep Kit to the sample and vortex for 10 seconds. Pipet the solution up and down several times to ensure homogeneity.
3. Place the tube of sample in the magnetic rack. After the solution clears, transfer the liquid into a new 1.5-mL LoBind tube. Repeat this step two more times, transferring the liquid into a new tube each time.

Proceed to **Ligating P1 and P2 Adaptors** below.

Ligating the P1 and P2 Adaptors

1. Dilute the P1 and P2 adaptors 1:20 in nuclease-free water (2.5 pmol/µL)
2. Mix the components below, then incubate at room temperature for 10 minutes.

Component	Volume
P1 Adaptor (2.5 pmol/µL)	1 µL
P2 Adaptor (2.5 pmol/µL)	1 µL
5X T4 Ligase Buffer	20 µL
DNA in Elution Buffer (from previous page)	30 µL
T4 Ligase (5 U/µL)	5 µL
Nuclease-free water	43 µL
Total	100 µL

Proceed to **Purifying the Adaptor-Ligated DNA**, next page.

Purifying the Adaptor-Ligated DNA

1. Mix Agencourt® AMPure® XP beads thoroughly by hand, and then add 180 µL of beads to each 100-µL sample (from **Ligating the P1 and P2 Adaptors**, previous page) and pipet up and down 10 times to mix.
2. Incubate for 10 minutes at room temperature on a nutator.
3. Place the sample tube in the magnetic rack and wait for the solution to clear.
4. Remove the supernatant and discard.
5. Add 300 µL of **freshly prepared** 70% ethanol. Wash the bead pellet by lifting the tube, turning it 180° and then placing it back in the rack. Turn a total of 6 times to wash thoroughly. Turning the tube allows the magnet to declump the beads and prevents the need for vigorous vortexing.
6. Remove and discard the ethanol without disturbing the bead pellet.
7. Repeat Steps 5 and 6 two more times.
8. After the final ethanol wash, pulse-spin the beads, place them back on the rack, and remove any residual ethanol. Repeat this step 2–3 more times.
9. With the tube cap open and the tube in the magnetic rack, air dry the beads **completely** at room temperature on the bench or in a fume hood (~20–30 minutes on the bench and 10–15 minutes in a hood). **The pellet will form visual cracks on its surface when dry.**
10. Add 30 µL of DNA Elution Buffer to the sample and vortex for 10 seconds. Pipet the solution up and down several times to ensure homogeneity.
11. Place the tube in the magnetic rack. After the solution clears, transfer the liquid into a new 1.5-mL LoBind tube. Repeat this step two more times, transferring the liquid into a new tube each time.

Store the purified DNA at 4°C or proceed immediately to **Nick Translate and Amplify**, next page.

Program the Thermal Cycler

Program the thermal cycler for nick translation and amplification as follows.

Note: Depending on the initial amount of DNA input, you may need to optimize the number of PCR cycles. In general, for 1–10 ng of DNA, 15 cycles yield sufficient quantity of library material visible when analyzed on a 2% E-Gel® EX Gel.

Temperature (°C)	Time	Condition/Note
72	20 min	Nick Translation
95	5 min	Denaturation
95	15 sec	10–15 cycles *(See Note Above)*
62	15 sec	
70	1 min	
70°	5 min	Extension
4	Hold	

Nick Translate and Amplify

1. For each sample, prepare the following PCR reaction mix in a separate LoBind tube on ice:

Component	Volume
Platinum® PCR Amplification Mix	200 µL
Library PCR Primer 1 (50 µM)	5 µL
Library PCR Primer 2 (50 µM)	5 µL
Adaptor-Ligated DNA	30 µL
Nuclease-free water	10 µL
Total	250 µL

2. Divide the reaction mix into two separate sterile PCR tubes (125 µL each) and place them in the thermal cycler.
3. Run the thermal cycler program described previously.
4. After amplification, combine the samples into a new 1.5-mL LoBind tube.
5. **Optional:** Remove 5 µL of the 250-µL amplified sample and run it on a 2% E-Gel® EX Gel to determine the minimum number of cycles needed for library amplification.

Proceed to **Purifying the Amplified DNA** below.

Purifying the Amplified DNA

1. Mix Agencourt® AMPure® XP beads by hand, and add 450 µL of beads to each 250-µL sample from above. Pipet up and down 10 times to mix.
2. Incubate for 10 minutes at room temperature on a nutator.
3. Place the sample tube in the magnetic rack until the solution is clear. Remove the supernatant and discard.
4. Add 300 µL of **freshly prepared** 70% ethanol. Wash the bead pellet by lifting the tube, turning it 180° and then placing it back in the rack. Turn a total of 6 times to wash thoroughly.

   Note: Because of the large volume of beads, check to make sure the pellet is completely in solution during Step 4. You can spin the tube briefly if necessary to fully submerge the beads.
5. Remove and discard the ethanol without disturbing the bead pellet.
6. Repeat Steps 4 and 5 two more times.
7. After the final ethanol wash, pulse-spin the beads, place them back on the rack, and remove any residual ethanol. Repeat this step 2–3 more times.
8. With the tube cap open and the tube in the magnetic rack, air dry the beads **completely** at room temperature on the bench or in a fume hood, until visible cracks form in the pellet surface (~30–40 minutes on the bench and 20–25 minutes in a hood).
9. Add 30 µL of DNA Elution Buffer to the sample and vortex for 10 seconds. Pipet the solution up and down several times to ensure homogeneity.
10. Place the tube in the magnetic rack. After the solution clears, transfer the liquid into a new 1.5-mL LoBind tube. Repeat this step two more times, transferring the liquid into a new tube each time.

Proceed to **Purifying the Amplified DNA a Second Time**, next page.

Purifying the Amplified DNA a Second Time

1. Mix Agencourt® AMPure® XP beads by hand, then add 54 µL of beads to each 30-µL sample (from **Purifying the Amplified DNA,** previous page). Pipet up and down 10 times to mix.
2. Incubate for 10 minutes at room temperature on a nutator.
3. Place the sample tube in the magnetic rack until the solution is clear. Remove the supernatant and discard.
4. Add 300 µL of **freshly prepared** 70% ethanol. Wash the bead pellet by lifting the tube, turning it 180° and then placing it back in the rack. Turn a total of 6 times to wash thoroughly.
5. Remove and discard the ethanol without disturbing the bead pellet.
6. Repeat Steps 4 and 5 two more times.
7. After the final ethanol wash, pulse-spin the beads, place them back on the rack, and remove any residual ethanol. Repeat this step 2–3 more times.
8. With the tube cap open and the tube in the magnetic rack, air dry the beads **completely** at room temperature on the bench or in a fume hood, until visible cracks form in the pellet surface (~10 minutes on the bench and ~5 minutes in a hood)
9. Add 30 µL of DNA Elution Buffer to the sample and vortex for 10 seconds. Pipet the solution up and down several times to ensure homogeneity.
10. Place the tube in the magnetic rack. After the solution clears, transfer the liquid into a new 1.5-mL LoBind tube. Repeat this step two more times, transferring the liquid into a new tube each time.

Proceed to **Optional Gel Analysis**, next page.

Optional Gel Analysis

Remove ~1 µL of purified amplified product and run it on a 2% E-Gel® EX Gel to confirm the removal of any primer dimers, or analyze the sample using the Agilent High Sensitivity DNA Kit.

Note: If you are using the Agilent kit, keep samples on the magnet while loading the High Sensitivity DNA chip to prevent the potential loading of any residual beads.

Quantitation by qPCR

Quantitative PCR (qPCR) is the preferred method for determining the amount of amplifiable template in a SOLiD™ library. Quantitative PCR provides the high level of specificity required by emulsion PCR (ePCR), and can accurately measure extremely low quantities of DNA, allowing the user to dilute SOLiD™ libraries to very low concentrations for quantitation.

The SOLiD™ Library TaqMan® Quantitation Kit is recommended in the *Applied Biosystems SOLiD™ System Library Preparation Guide*, *Appendix B*, and contains the following validated reagents for qPCR:

- SOLiD™ Library qPCR Mix, an optimized master mix of qPCR reagents including DNA polymerase and dNTPs
- TaqMan® Assay for SOLiD™ Library Quantification, a fluorogenic probe-based qPCR detection assay
- SOLiD™ Library qPCR Standard, a validated, pre-quantified, ready-to-use standard specifically designed for quantifying SOLiD™ libraries in qPCR

The kit is designed to quantify libraries accurately regardless of size, can be used on any real-time instrument, and is compatible with both fast and standard cycling programs.

Templated Bead Preparation

Following qPCR quantitation, the SOLiD™ ChIP-Seq fragment library is now ready for templated beads preparation. Either store the DNA in Elution Buffer at 4ºC, or proceed directly to emulsion PCR as described in the *Applied Biosystems SOLiD™ System Templated Bead Preparation Guide.*

Data Analysis

Software Tools

After the SOLiD™ ChIP-Seq and reference input control libraries are created, the samples are sequenced on the SOLiD™ System. Then the sequence reads are mapped against known genomic sequences using BioScope™. The aligned reads can then be analyzed using a variety of publicly available tools.

Details on how to use these tools with SOLiD™ data are provided on the SOLiD™ Software Community website at: **http://info.appliedbiosystems.com/solidsoftwarecommunity**.

Data can then be visualized with a tool such as the University of California, Santa Cruz Genome Browser (**http://genome.ucsc.edu/cgi-bin/hgGateway**) to identify and quantify the sequence regions that bind to the protein of interest.

Appendix

Troubleshooting

Problem	Possible Cause	Solution
Low level of amplification as detected by PCR/qPCR	Heat released by sonicator reversed crosslinks	Keep samples cool during sonication; place samples on ice between cycles.
	Excessive or inefficient crosslinking	Keep the crosslinking time and temperature consistent across samples. Optimize the length of time for crosslinking by performing a time-course experiment.
	Not enough antibody	Increase the titration of antibody per cell range to determine the window for best enrichment.
	Protein is degraded	Keep samples on ice during lysis. Also, place the dilution buffer, lysis buffer, and IP wash buffer 1 on ice before use, and be sure they are cold before use. Be sure to add the Protease Inhibitors provided in the kit to lysis buffer prior to use as specified in the protocol.
	Chromatin binding incubation time is too short	The kinetics of reaching equilibrium of epitope-antibody binding may be antibody or target dependent. Increasing the incubation time may improve results.
No amplification	qPCR/PCR failure	See the troubleshooting provided with your PCR/qPCR kit. Isolate the problem using Input Control DNA and a control primer such as SAT2. Try increasing the amount of DNA template per reaction.
Low yield of DNA following library preparation steps	Residual ethanol from insufficient drying of AMPure® XP beads	Make sure the bead pellet dries completely, with visible cracks in its surface, for full DNA recovery
	AMPure® XP beads were not at room temperature prior to use	Allow AMPure® beads to come to room temperature prior to use.
	AMPure® XP beads not mixed properly prior to use.	Mix the beads well by inverting multiple times before use. The bead solution should appear homogenous and consistent in color.
	Improper storage of AMPure® XP beads	**Do not freeze the beads.** Store at 4°C.
	Freshly prepared 70% ethanol was not used	Use freshly prepared 70% ethanol (Note: 70% ethanol is hygroscopic. Fresh 70% ethanol should be prepared daily for optimal results).

Problem	Possible Cause	Solution
Antibody not working in ChIP	Antibody not ChIP-qualified	Whenever possible, use an antibody that is qualified for ChIP. A selection of ChIP-qualified antibodies is provided on our website at: **www.invitrogen.com/chipantibody**.
	Antibody not acceptable for use in ChIP even when it functions in other applications	See the guidelines for selecting an antibody on page 11.

Frequently Asked Questions

How do I know if cross-linking is necessary for my particular DNA binding protein of interest?

As a general guideline, crosslinking is recommended for all non-histone DNA-binding proteins. Histones generally do not require crosslinking because they are already tightly associated with DNA. However, you may need to empirically determine whether you need to crosslink.

For example, some histone proteins may be less tightly associated with DNA and require crosslinking in order to maintain their association with DNA. Likewise, some non-histone proteins may be tightly associated with the DNA and not require crosslinking.

How do I cross-link?

We use 1% formaldehyde, as the links it forms are reversible. UV crosslinking is irreversible.

How long should I cross-link?

Crosslinking is a time-critical procedure and optimization may be required. Typically, we crosslink for 10 minutes minimum. If you are uncertain, perform a time-course experiment to optimize conditions. Excessive cross-linking can lead to a decrease in the amount of protein bound to the DNA and reduction in the availability of epitopes/changes in epitopes for antibody binding. In turn, this leads to reductions in the material bound/antigen available in your sample.

What is the optimal fragment size?

Shearing of the chromatin into 100–300 bp fragments is required to ensure good resolution for SOLiD™ ChIP-Seq reactions. If your average fragment size is greater than 300 bp, further optimization may be required. The sonication conditions need to be assessed for each cell type examined.

Sonication efficiency varies to some extent with each type of sonicator. We recommend testing your sonicator using different settings and times, and then checking the size of the DNA by agarose gel electrophoresis or on an Agilent Bioanalyzer™.

How do I know if my antibody is compatible with ChIP?

Antibodies are used in ChIP to capture the DNA/protein complex. Performing a successful ChIP assay requires that the antibody recognizes fixed protein that is bound to the chromatin complex. Antibodies used for ChIP should be fully characterized. However, even fully characterized antibodies may not function, as the effects of cross-linking can dramatically alter protein epitopes. In general, a polyclonal antibody population may recognize a number of different epitopes, rather than a monoclonal antibody that only recognizes a single epitope.

What concentration of antibody should I use in my ChIP experiment?

The amount of each antibody will need to be empirically determined. However, if you are uncertain, 1–10 µg of antibody per ChIP assay is a general recommended starting point.

How do I QC my ChIP DNA prior to Library Construction?

- Optimize sonication conditions prior to ChIP (see page 24)
- Use proper negative and positive control antibodies to assess specific enrichment (see page 12)
- Use an Input Control (see page 12)
- Test enrichment of the ChIP sample relative to the Input Control by qPCR following the guidelines in this manual (see page 34)

What controls should I use?

We recommend the following controls:

Negative Control Antibody: Either do not use a primary antibody, or use the normal rabbit IgG or mouse IgG that is provided in the kit.

Positive Control Antibody: This control ensures that each step of the procedure is working. For example, we observe consistent enrichment of heterochromatin markers such as H3-K9Me3 at the satellite repeat locus (SAT-2).

Negative Control PCR Primer: This control is designed against a sequence that would not be enriched by your chromatin IP procedure.

Input DNA Control: Input DNA is DNA obtained from chromatin that has not been immunoprecipitated and has been reversed crosslinked similar to your samples. It is a control for PCR effectiveness and utilized in ChIP-sequencing data analysis.

Additional Products

ChIP-Qualified Antibodies

Invitrogen has a wide range of ChIP-qualified antibodies for use with the SOLiD™ ChIP-Seq Kit. Visit our website at **www.invitrogen.com/chipantibody** for a complete list and details.

Real-Time qPCR Instruments and Reagents

Applied Biosystems and Invitrogen have a wide range of industry-standard instruments, plates, reagents, and other products for real-time qPCR. Visit **www.appliedbiosystems.com** and **www.invitrogen.com** for more information.

Additional Products

The following additional products are available separately from Invitrogen. For more information or to place an order, visit our website at **www.invitrogen.com** or contact Technical Support (page 51).

Product	Quantity	Catalog no.
MAGnify™ SAT2 Primers	100 µL	49-2026
MAGnify™ RARβ1 Primers	100 µL	49-2027
MAGnify™ ERα Primers	100 µL	49-2028
MAGnify™ c-Fos Primers	100 µL	49-2029
DynaMag™-PCR Magnet (holds up to 16 0.2-mL PCR tubes)	1 magnet	49-2025
Phosphate Buffered Saline (PBS) 7.4 (1X), liquid	500 mL 10 × 500 mL	70011-044 70011-069
Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	500 mL 10 × 500 mL	14190-144 14190-250
TrypLE™ Express Stable Trypsin Replacement Enzyme without Phenol Red	100 mL 500 mL	12604-013 12604-021
Countess® Automated Cell Counter	1 unit	C10227
E-Gel® EX Gel, 2%	Starter Kit 10 pak 20 pak	G6512ST G4010-02 G4020-02
E-Gel® 2% with SYBR® Safe™	Starter Kit 18 pak	G6206-02 G5218-02
E-Gel® 2% with Ethidium Bromide	Starter Kit 18 pak	G6000-02 G5018-02
E-Gel® iBase™ Power System	1 unit	G6400
100-bp DNA Ladder	50 µg	15628-019
TrackIt™ 100 bp DNA Ladder	100 applications	10488-058
UltraPure™ DEPC-treated Water	1 liter	750023
Quant-iT™ DNA Assay Kit, High Sensitivity (0.2–100 ng)	1000 assays	Q33120
Quant-iT™ dsDNA HS Assay Kits—for use with the Qubit® fluorometer (0.2–100 ng)	100 assays 500 assays	Q32851 Q32854
Qubit® Fluorometer	1 unit	Q32857

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