ion torrent
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Ion ChIP-Seq Library Preparation on the Ion Proton™ System

Publication Number 4473623 **Revision** B

SUBJECT: Life Technologies Demonstrated Protocol: Ion ChIP-Seq Library Preparation

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Description

This user bulletin provides a Life Technologies-demonstrated protocol for the enrichment of chromatin/protein complexes and DNA recovery using magnetic bead capture (MAGnify™) technology, and preparation of libraries from the isolated DNA for sequencing on the Ion Proton™ System. The protocol requires the MAGnify™ Chromatin Immunoprecipitation System (Cat. no. 49-2024) and the DynaMag™-PCR Magnet (Cat. no. 49-2025) for preparation of DNA from chromatin/protein complexes. The Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269) is used for library preparation from the recovered DNA. Barcoded libraries can be prepared using the Ion Xpress™ Barcode Adapters 1-96 (Barcode Cat. no. 4474517) in addition to the Ion Xpress™ Plus Fragment Library Kit.

MAGnify™ ChIP System 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, transcription regulation, 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 MAGnify™ Chromatin Immunoprecipitation System provides a streamlined assay for the enrichment of chromatin/protein complexes and DNA recovery using magnetic bead capture technology. 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 MAGnify™ System can be used with a suite of ChIP-validated antibodies from Life Technologies. Sufficient reagents are provided for 20 ChIP-Seq library preparations.

The isolated DNA is ready for library preparation for next-generation sequencing on the Ion Proton™ System.

ChIP-qualified antibodies

ChIP-qualified antibodies are available separately from Life Technologies. Visit **http://www.lifetechnologies.com/us/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/antibodies-for-chip.html** for more information.

Negative controls

Rabbit IgG and Mouse IgG antibodies are provided in the MAGnify™ ChIP System for use as negative controls, to measure nonspecific binding.

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 Life Technologies for the amplification of common promoter regions analyzed in ChIP experiments.

See page 27 for primer sequences and page 42 for ordering information.

DynaMag™-PCR Magnet

This protocol 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.

ChIP DNA Ion library preparation

Ion Plus Fragment Library Preparation Kit

The Ion Xpress™ Plus Fragment Library Preparation Kit includes reagents for end-repair and library preparation of recovered ChIP DNA. The libraries have insert lengths of approximately 100–250 bp.

Ion Xpress™ Barcode Adapters 1-96 Kit

To prepare barcoded Ion ChIP-Seq libraries, use the Ion Xpress™ Barcode Adapters 1–96 Kit in addition to the Ion Xpress™ Plus Fragment Library Kit. This kit includes P1 adapter and barcoded A adapters that substitute for the non-barcoded adapter mix supplied in the Ion Xpress™ Plus Fragment Library Kit.

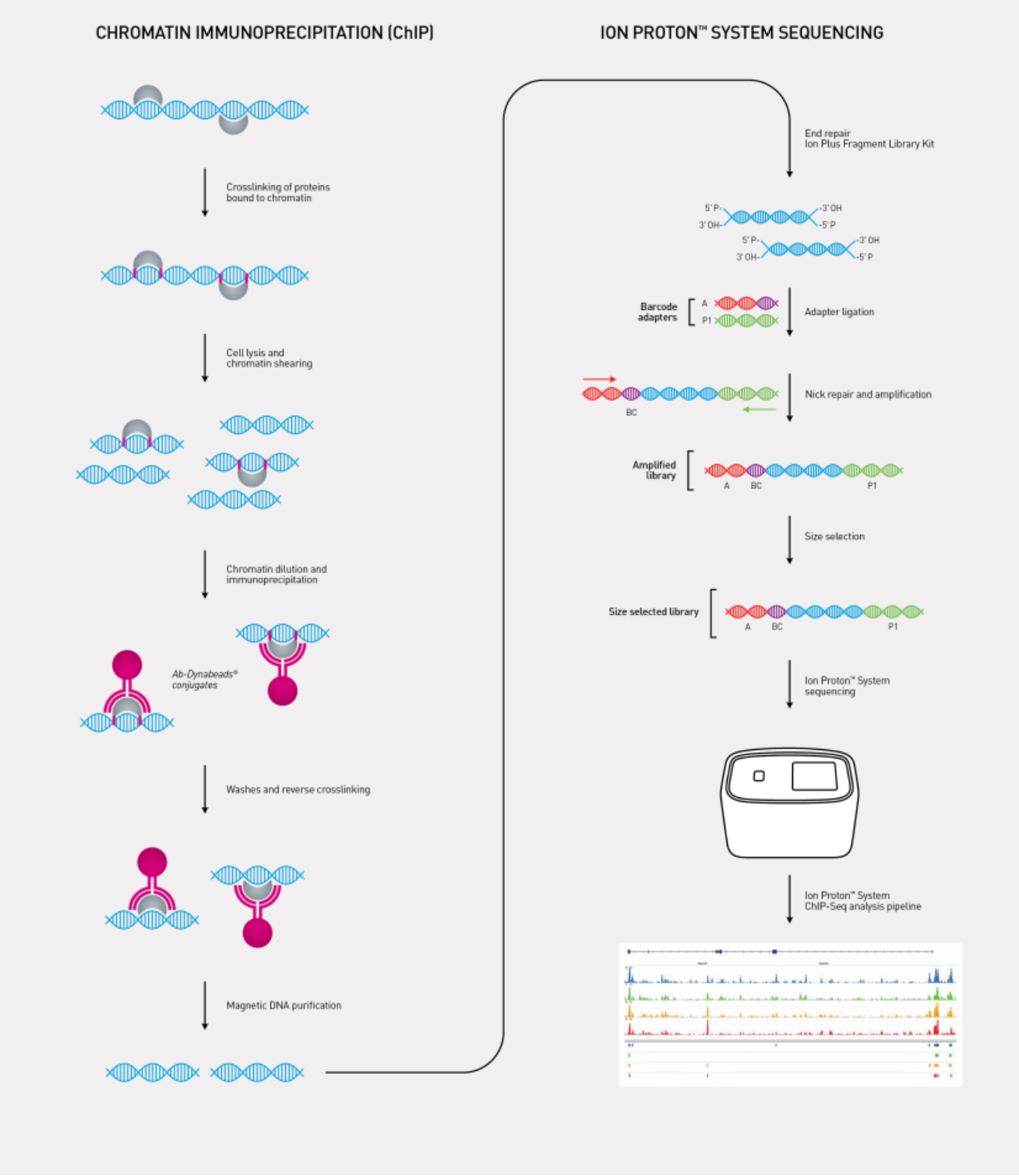
Workflow summary

The workflow is illustrated in Figure 1 on page 4. Using the MAGnify™ Chromatin Immunoprecipitation System, cells or tissue are treated 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.

The crosslinked protein of interest is immunoprecipitated and isolated 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 Ion Proton™ System library preparation. The DNA is end-repaired and purified. The end-repaired DNA is ligated to Ion-compatible adapters, followed by nick repair to complete the linkage between barcode adapters and DNA inserts. The library is amplified by PCR and purified with two rounds of AMPure® XP bead capture to size-select fragments for downstream clonal amplification on Ion Sphere™ Particles (template preparation) and sequencing on the Ion Proton™ sequencer.

Figure 1 Ion ChIP-Seq overview



Kit contents and storage conditions

MAGnify™ Chromatin Immuno-precipitation System

The MAGnify™ Chromatin Immunoprecipitation System (Cat. no. 49-2024) includes sufficient components for up to 24 reactions (including input control reactions).

Modules 1 and 2 are shipped on wet ice; Modules 3 and 4 are shipped on dry ice. See the table below for storage temperatures.

IMPORTANT! Never freeze the Dynabeads® Protein A/G or the DNA Purification Magnetic Beads, as this will damage the beads.

Module 1	Quantity	Storage
Glycine (1.25 M)	2 × 1 mL	2°C to 8°C
Dynabeads® Protein A/G (do not freeze!)	250 µL	2°C to 8°C
Reverse Crosslinking Buffer	1.4 mL	2°C to 8°C
DNA Purification Magnetic Beads (do not freeze!)	500 µL	2°C to 8°C
DNA Purification Buffer	1.4 mL	2°C to 8°C
Proteinase K (20 mg/mL)	200 µL	Room temp or 2°C to 8°C

Module 2	Quantity	Storage
IP Buffer 1	10 mL	2°C to 8°C
IP Buffer 2	7.5 mL	2°C to 8°C
DNA Wash Buffer	8.0 mL	2°C to 8°C
DNA Elution Buffer	7.2 mL	2°C to 8°C

Module 3	Quantity	Storage
Protease Inhibitors (200X)	100 µL	–30°C to –10°C
Mouse IgG (1 µg/µL)	15 µL	–30°C to –10°C
Rabbit IgG (1 µg/µL)	15 µL	–30°C to –10°C

Module 4	Quantity	Storage
Dilution Buffer	8.1 mL	–30°C to –10°C
Lysis Buffer	3.6 mL	–30°C to –10°C

Ion Xpress™ Plus Fragment Library Kit

The Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269) includes sufficient reagents for preparation of up to 20 libraries.

Component	Cap color	Quantity	Volume	Storage
5X End Repair Buffer	Red	1 tube	400 µL	–30°C to –10°C
End Repair Enzyme	Orange	1 tube	20 µL	
10X Ligase Buffer	Yellow	1 tube	200 µL	
DNA Ligase	Blue	1 tube	40 µL	
Nick Repair Polymerase	Clear	1 tube	160 µL	
dNTP Mix	Violet	1 tube	40 µL	
Adapters	Green	1 tube	100 µL	
Platinum® PCR SuperMix High Fidelity	Black	2 tubes	1 mL each	
Library Amplification Primer Mix	White	1 tube	100 µL	
Low TE	Clear	2 tubes	1.25 mL each	Room temperature (15°C to 25°C) or –30°C to –10°C

Ion Xpress™ Barcode Adapters 1–96 Kit

One or more Ion Xpress™ Barcode Adapters Kits are required for preparing barcoded libraries. Each kit includes reagents sufficient for preparing up to 20 Ion ChIP-Seq libraries per barcode (20 × 16 libraries). Substitute Ion Xpress™ Barcode Adapters for standard Ion AmpliSeq™ Adapters as described in this user guide.

- Ion Xpress™ Barcode Adapters 1–16 Kit (Cat. no. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 Kit (Cat. no. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 Kit (Cat. no. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 Kit (Cat. no. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 Kit (Cat. no. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 Kit (Cat. no. 4474521)

And the complete set of adapters:

- Ion Xpress™ Barcode Adapters 1-96 (Cat. no. 4474517)

Ion Xpress™ Barcode Adapters Kits (each kit includes 16 individually numbered barcodes)				
Component	**Cap color**	**Quantity**	**Volume**	**Storage**
Ion Xpress™ P1 Adapter	Violet	1 tube	320 µL	–30°C to –10°C
Ion Xpress™ Barcode X	White	16 tubes (1 per barcode)	20 µL each	

*Shipped at ambient temperature. Store as indicated.

Product qualification

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

Required materials and equipment (not provided)

Ordering information for many of these products is provided in Appendix D on page 42.

Reagents

- Cells or fresh or frozen tissue
- Antibody of interest
- For cells: Trypsinizing reagent (for example, TrypLE™ Express, Cat. no. 12604-013 or 12605-010)
- 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
  - 50-mL sterile conical tubes
- Formaldehyde, 37%, Molecular Biology Grade
- PBS or D-PBS (for example, Phosphate Buffered Saline, pH 7.4, 1X liquid, or Dulbecco's Phosphate-Buffered Saline, 1X liquid)
- qPCR SuperMix (for example, EXPRESS qPCR Supermixes and EXPRESS SYBR® GreenER™ qPCR Supermixes)
- qPCR primers for the sequence of interest
- 100-bp DNA ladder or 50-bp ladder (for example, TrackIt™ 100-bp DNA Ladder, Part no. 10488-058; or 50-bp ladder, Cat. no. 10416-014)
- Agencourt® AMPure® XP Kit (Beckman Coulter A63880 or A63881)
- Ethanol, absolute, 200 proof, Molecular Biology Grade
- Nuclease-free Water (Cat. no. AM9932)
- (*Optional*) Positive control antibody (see "ChIP controls" on page 9)
- (*Optional*) Control primers for qPCR (for example, MAGnify™ SAT2 Primers, Cat. no. 49-2026; RARβ1 Primers, Cat. no. 49-2027; ERα Primers, Cat. no. 49-2028; and c-Fos Primers, Cat. no. 49-2029)

Materials and equipment

- DynaMag™-PCR Magnet (Cat. no. 49-2025), or other magnet capable of holding 0.2-mL PCR tubes or strip wells
- Magnetic rack for 1.5 mL tubes, such as DynaMag™-2 magnet (Cat. no. 123-21D)
- Cell counter (for example, Countess® Automated Cell Counter, Cat. no. C10227, or a hemacytometer)
- Sonicator, for example, Bioruptor® UCD-200 (Diagenode UCD-200 xx) or Bioruptor® Pico (Diagenode B01060002)
- qPCR instrument (for example, Applied Biosystems® StepOnePlus™, 7500 Fast, 7500, 7900HT, 7500, ViiA™ 7 Instruments)

- Thermal cycler (for example, Applied Biosystems® 96-well GeneAmp® PCR System 9700 [Cat. nos. N8050200 (Base) and 4314443 (Block)] or the Veriti® 96-Well Thermal Cycler (Cat. no. 4375786)
- Microcentrifuge (4°C)
- Microcentrifuge for 0.2-mL PCR tubes or strip wells
- 55°C and 65°C heat sources (for example, Applied Biosystems® GeneAmp® PCR System, or a hybridization oven, water bath, etc.)
- Rotating mixer capable of holding 0.2-mL PCR tubes or strip wells
- Vortex mixer
- qPCR plates
- Microcentrifuge tubes, RNase/DNase-free, low retention (for example, Eppendorf 1.5-mL LoBind® tubes)
- 0.2-mL PCR tubes, RNase/DNase-free, individual or 8-tube strips and caps
- For confirming DNA fragment sizes after sonication:
  - 1.5–2.0% agarose gel (for example, 2% E-Gel® agarose gel or 2% E-Gel® EX Gel)
  - Agarose gel apparatus (for example, E-Gel® iBase™ Power System, Cat. no. G6400; or E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit, Cat. no. G6465)
- For assessing final library size:
  - Agilent 2100 Bioanalyzer™ instrument
  - Agilent High Sensitivity DNA Kit (Agilent 5067-4626)
- Wet ice
- Liquid nitrogen or dry ice
- Pipettors and nuclease-free pipette tips
- For quantitation of recovered ChIP DNA, Qubit® Fluorometer (Cat. no. Q32857) and one of the following:
  - Quant-iT™ DNA Assay Kit, High Sensitivity (0.2–100 ng) (Cat. no. Q33120)
  - Quant-iT™ dsDNA HS Assay Kits (0.2–100 ng) (Cat. no. Q32851 or Q32854)

Important procedural guidelines

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

Total starting amount of cells/ tissue	Amount of Lysis Buffer	Amount per ChIP reaction	Total number of ChIP reactions
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. Visit **http://www.lifetechnologies.com/us/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/antibodies-for-chip.html** for a selection of ChIP-qualified antibodies.

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 (that is, 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 section 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.

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

Input control DNA is DNA obtained from chromatin that has been reverse-crosslinked but has not been immunoprecipitated. It is reserved in "Step 4. Dilute the chromatin" starting on page 21 and then analyzed in "Step 9. Analysis of the ChIP DNA using qPCR" or "Step 10. Ion ChIP-Seq library preparation".

Handling Dynabeads® Protein A/G

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

- Do not mix Dynabeads® by vortexing, as this will damage the beads.
- Do not freeze Dynabeads®, as this will damage the beads.
- When removing liquid from Dynabeads® after magnetic capture, 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.

DynaMag™-PCR Magnet

This protocol utilizes a novel magnet for processing Dynabeads® that is compatible with 0.2 mL PCR strip tubes. The DynaMag™-PCR Magnet (Cat. no. 49-2025) 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.

Resuspending Dynabeads® Protein A/G

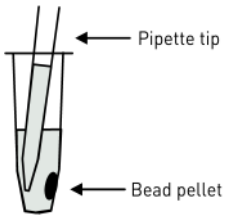
To resuspend Dynabeads®, use gentle up-and-down pipetting while taking care to avoid creating air bubbles. Do not mix the beads by vortexing.

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

Removing liquid from Dynabeads® Protein A/G

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. Couple the Antibody to Dynabeads® Protein A/G

Introduction

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

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

- Antibody of interest
- Positive control antibody
- DynaMag™-PCR Magnet
- Rotating mixer, refrigerated (for example: a standard end-over-end rotating mixer can be placed in a refrigerated deli case or cold room)
- 0.2-mL PCR tubes or strip tubes
- Ice
- Pipettor and pipette tips

Important procedural guidelines

ChIP antibody controls

Use appropriate negative and positive control antibodies. See "ChIP controls" on page 9.

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.

Couple the antibodies to the Dynabeads® Protein A/G

IMPORTANT! 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.

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 the illustration on page 10).

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.
12. When the Antibody-Dynabeads® have finished mixing, hold the tube at 4°C until use.

While the Antibody-Dynabeads® mixture is incubating, proceed either to"Step 2A. Prepare cultured cells: crosslink and lyse cells" in the following section or to "Step 2B. Prepare tissue: homogenize, crosslink, and lyse tissue cells" on page 16.

Step 2A. Prepare cultured cells: crosslink and lyse 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. Crosslinking the cells with formaldehyde ensures that the chromatin structure is preserved during the isolation and ChIP procedure.

Follow the appropriate protocol for your starting cell material:

- Collect and crosslink adherent cells (page 13)
- Collect and crosslink cells in suspension (page 14)
- Crosslink adherent cells directly in a dish (page 15)

For instructions on preparing tissue samples for analysis, see "Step 2B. Prepare tissue: homogenize, crosslink, and lyse tissue cells" on page 16.

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

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

Important procedural guidelines

- 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.

Collect and crosslink adherent cells

Use the following method to collect adherent cells prior to crosslinking. Alternatively, you can fix the cells directly, as described on page 15.

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.
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 that 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.

    Note: 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 "Prepare the Lysis Buffer with Protease Inhibitors" on page 15.

Collect and crosslink 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 ≤500µL*, 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. Add 57 µL of room-temperature 1.25 M glycine to the sample to stop the reaction. 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.

   Note: 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 "Prepare the Lysis Buffer with Protease Inhibitors" on page 15.

Crosslink 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. Add 0.5 mL of room-temperature 1.25 M glycine to the sample to stop the reaction. 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.)

   Note: 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 "Prepare the Lysis Buffer with Protease Inhibitors" in the following section.

Prepare 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.

- 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.

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.

Lyse the cells

1. Add Lysis Buffer with Protease Inhibitors (prepared as above) to the cell pellet collected as described in the previous sections. Use 50 µL of prepared Lysis Buffer per 1 million cells (for example, 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 "Step 3. Shear the chromatin" on page 19, or snap-freeze the lysed cells in liquid nitrogen or on dry ice and store at –80°C until use.

Step 2B. Prepare tissue: homogenize, crosslink, and lyse tissue cells

Introduction

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

For instructions on preparing cells for analysis, see "Step 2A. Prepare cultured cells: crosslink and lyse cells" on page 12.

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

- Tissue samples, fresh or frozen, 50–1000 mg
- Clean unused 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
- 1.5-mL LoBind tubes
- D-PBS (1X), liquid- ice-cold and room temperature
- 37% formaldehyde
- Microcentrifuge at 4°C
- Vortex mixer
- Ice
- Pipettor and pipette tips

Important procedural guidelines

- 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.

Prepare 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.

Weigh and mince 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 (for example, 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. Mince the tissue:
   a. Remove unwanted tissue such as necrotic material and fat from the sample.
   b. Remove any connective tissue that could clog the needle.
   c. 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 "Homogenize the tissue" on page 18.

Weigh and mince 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 –80°C.
4. When you are ready to prepare the frozen tissue for ChIP analysis, remove the tube from –80°C 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 (for example, 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. Mince the tissue:

a. Remove unwanted tissue such as necrotic material and fat from the sample.

b. Remove any connective tissue that could clog the needle.

c. 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 "Homogenize the tissue" in the following section.

Homogenize 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. Proceed immediately to "Crosslink the chromatin".

Crosslink 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% (for example, 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 (for example, 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 Eppendorf LoBind® tubes, adding 570 µL per tube (the equivalent of 50 mg of tissue per tube).

7. Spin the Eppendorf LoBind® tubes at ~200 × *g* for 10 minutes at 4°C. 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 "Lyse the cells" in the following section.

Lyse the cells

Note: 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 concentration is 1 million cells per 50 µL.

1. Add 150 µL of Lysis Buffer prepared with Protease Inhibitors (see page 15) 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. Shear the chromatin" or snap-freeze the sample in liquid nitrogen or on dry ice and store at -80°C until use.

Step 3. Shear the chromatin

Introduction

In this step, you shear the chromatin from Step 2 into fragments 100–300 bp in length.

Shearing the chromatin into 100–300 bp fragments is required for downstream sequencing on the Ion Proton™ System.

Note: Sonication is the preferred method for shearing chromatin when using formaldehyde crosslinking, as crosslinking restricts the access of Micrococcal nuclease to chromatin.

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

- Sonicator, for example, the Covaris® S2 System, Bioruptor® UCD-200, or Bioruptor® Pico
- For confirming DNA fragment sizes:
  - 1.5–2.0% agarose gel (for example, 2% E-Gel® agarose gel or 2% E-Gel® EX Gel)
  - 100-bp DNA ladder (for example, TrackIt™ 100-bp DNA Ladder)
- Ice
- Liquid nitrogen or dry ice
- Microcentrifuge

- Microcentrifuge tubes, RNase/DNase-free, low retention (for example, 1.5-mL Eppendorf LoBind® tubes)
- Pipettor and pipette tips

Important procedural guidelines

IMPORTANT! Keep the cell lysate cooled on ice during sonication, because heat released by the sonication 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 Bioruptor® Pico, described in this section, and for the Covaris® S2 sonicator, described in Appendix C. You can use other fragmentation methods, but be sure to optimize sonication conditions for your experimental samples.

We recommend optimizing sonication conditions for each cell type (see the following section). 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 (for example, a 2% E-Gel® agarose gel or 2% E-Gel® EX Gel) with a 100-bp ladder to determine fragment length.

Assessing sheared chromatin length during optimization

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® agarose 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.

Alternatively, use 1 µL for Bioanalyzer.

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.

Sonicate using the Bioruptor® Pico sonicator

Below is an example sonication procedure using the Bioruptor® Pico sonicator from Diagenode for shearing chromatin into fragments 100–300 bp in length. Refer to the instrument manual for setup and maintenance.

1. Fill water reservoir to fill mark.
2. Pre-cool the water reservoir to 4°C.
3. Transfer the cell lysate (from page 15 or page 19) into manufacturer supplied tubes. Use a final volume appropriate to the sonication device used as described by the manufacturer.
4. Place tubes in the Bioruptor® Pico sonicator filling any unused portions with empty tubes.

   Note: Filling all spaces in the sonication device ensures consistent results and proper size distribution.
5. Sonicate the tube for 9 cycles of 30 seconds ON, 30 seconds OFF. After 9 cycles, remove samples and pulse spin to pool solution to the bottom of the tube and place back in the sonication adapter.
6. Sonicate the tube for another 9 cycles of 30 seconds ON, 30 seconds OFF.
7. The supernatant contains the chromatin. Aliquot the freshly sonicated chromatin into new, sterile tubes.

Proceed to "Step 4. Dilute the chromatin" in the following section, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

Step 4. Dilute 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.

Prepare 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.

Dilute 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-mL PCR tube. This is your Input Control.

You will reverse-crosslink this control sample in "Step 7. Reverse the crosslinking" 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 28.

Step 5. Bind 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 MAGnify™ System, you will need the following:

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

Important procedural guidelines

- 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 24.

Bind the chromatin

When the Antibody-Dynabeads® complexes from page 11 have finished the coupling reaction, proceed with the following.

Note: Keep the magnet, tubes, and buffers cold during these steps.

1. Briefly spin the tubes with the coupled Antibody-Dynabeads® complexes 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 "Step 4. Dilute the chromatin" in the previous section) 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 or up to O/N.

Proceed to "Step 6. Wash the bound chromatin" in the following section.

Step 6. Wash 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 MAGnify™ System, you will need the following:

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

Important procedural guideline

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.

Wash with IP Buffer 1

Note: 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.

Proceed immediately to "Wash with IP Buffer 2" in the following section.

Wash 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 this step, prepare the Reverse Crosslinking Buffer with Proteinase K as described in the following section.
6. Repeat steps 1–5 one more time.

Proceed immediately to "Step 7. Reverse the crosslinking" in the following section.

Step 7. Reverse the crosslinking

Introduction

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

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

- DynaMag™-PCR Magnet
- Thermal cycler, hybridization oven, water bath, or other heat sources at 55°C and 65°C
- Sterile 0.2-mL PCR tubes or strip tubes
- Pipettor and pipette tips

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

Prepare the Input Controls

Because the Input Controls are not bound to Dynabeads® beads, 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 in the next section "Reverse the crosslinking of the IP reactions").

1. To each tube containing 10 µL of reserved Input Control from page 20, 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 in the following section.

Prepare Reverse Crosslinking Buffer with Proteinase K

To prepare the final Reverse Crosslinking Buffer for your IP samples, add Proteinase K to the stock buffer provided in the kit. Scale accordingly based on the number of samples (excluding Input Controls which will be processed differently). Prepare 54 µL of buffer per IP reaction as follows:

Component	1 reaction
Stock Reverse Crosslinking Buffer	53 µL
Proteinase K	1 µL

Component	1 reaction
Final Volume	**54 µL**

Reverse the crosslinking of the IP reactions

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

1. Place the tubes from "Wash with IP Buffer 2" on page 23 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 the previous section) at 55°C for 15 minutes. Spin tubes briefly, and 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 supernatant—the supernatant contains your sample.** With the tubes in the magnet, carefully transfer 50 µL of the liquid to a 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 to inactivate Proteinase K.
8. Cool the tubes on ice for ~5 minutes.
9. Discard the used Dynabeads®. Do not reuse.

Proceed to "Step 8. Purify the DNA" in the following section.

Step 8. Purify the DNA

Introduction

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

Materials needed

In addition to materials provided in the MAGnify™ System, you will need the following:

- DynaMag™-PCR Magnet
- Pipettor and pipette tips
- Qubit® 2.0 Fluorometer and the Quant-iT™ DNA Assay Kit, High Sensitivity or the Quant-iT™ dsDNA HS Assay Kit

Important procedural guidelines

- 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 precipitate may form with the Input Controls. This will not affect purification.

Prepare 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 using 10% excess (including Input Controls).
3. Pipet up and down gently 5 times to mix. Bind and wash the DNA

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

1. After the tubes from "Reverse the crosslinking of the IP reactions" in the previous section 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 supernatant 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 more time.

Proceed to "Elute the DNA" in the following section.

Elute the DNA

1. Place the tubes 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 75 µL of DNA Elution Buffer to each tube. Pipet up and down gently 5 times to mix.
4. Pulse-spin the tubes. 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 supernatant—the supernatant 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.

STOPPING POINT Store the purified DNA at –15°C to –20°C or proceed immediately to the next section, "Quantify the recovered ChIP DNA". Avoid repeatedly freezing and thawing the DNA.

Quantify the recovered ChIP DNA

We recommend using the Qubit® 2.0 Fluorometer and the Quant-iT™ DNA Assay Kit, High Sensitivity or the Quant-iT™ dsDNA HS Assay Kit to quantify the recovered ChIP DNA (see ordering information on page 42).

The amount of ChIP DNA that is recovered 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® 2.0 Fluorometer provide accurate quantitation of most chromatin input samples. 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).

Materials needed

qPCR materials and guidelines

Life Technologies has a wide range of reagents, instruments, and other products for SYBR® Green-based or TaqMan®-based qPCR.

For SYBR® Green-based qPCR with the MAGnify™ PCR Primer Pairs described below, we suggest SYBR® GreenER™ or EXPRESS qPCR SuperMixes. Visit **www.lifetechnologies.com** for details.

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

MAGnify™ PCR Primer Pairs

The following primer pairs for SYBR® Green-based qPCR targeting common promoter regions analyzed in ChIP experiments are available separately from Life Technologies.

See page 42 for ordering information.

Primer Pair	Sequences	Cat. no.
MAGnify™ SAT2 Primers	CTGCAATCATCCAATGGTCG GATTCCATTCGGGTCCATTC	49-2026

Primer Pair	Sequences	Cat. no.
MAGnify™ RARβ1 Primers	GGCATTTGCATGGCATCCA CCGCGGTACACGCAAAA	49-2027
MAGnify™ ERα Primers	TGAACCGTCCGCAGCTCAAGATC GTCTGACCGTAGACCTGCGCGTTG	49-2028
MAGnify™ c-Fos Primers	TTAGGACATCTGCGTCAGCAGGTT TCTCGTGAGCATTTCGCAGTTCCT	49-2029

Procedural guidelines

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 are 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.

Determine 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 (for example, 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)

Normalize the data using the Input Control

1. Export the qPCR data to a spreadsheet program such as Microsoft® Excel software by using built in filters. The file should not contain omitted wells and should be in a column format containing well positions, descriptors, and C_T 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 C_T 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 (that is, $\log_2$ of 10) from the C_T 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 dC_T. 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.

Calculate fold enrichment as signal over background

With this method, signals from the IP reactions are divided by the signals from the negative antibody control reaction (that is, the Rabbit IgG or Mouse IgG antibody provided in the MAGnify™ System). 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. Ion ChIP-Seq library preparation

Introduction

This section provides instructions for preparation of Ion ChIP-Seq libraries from the ChIP DNA.

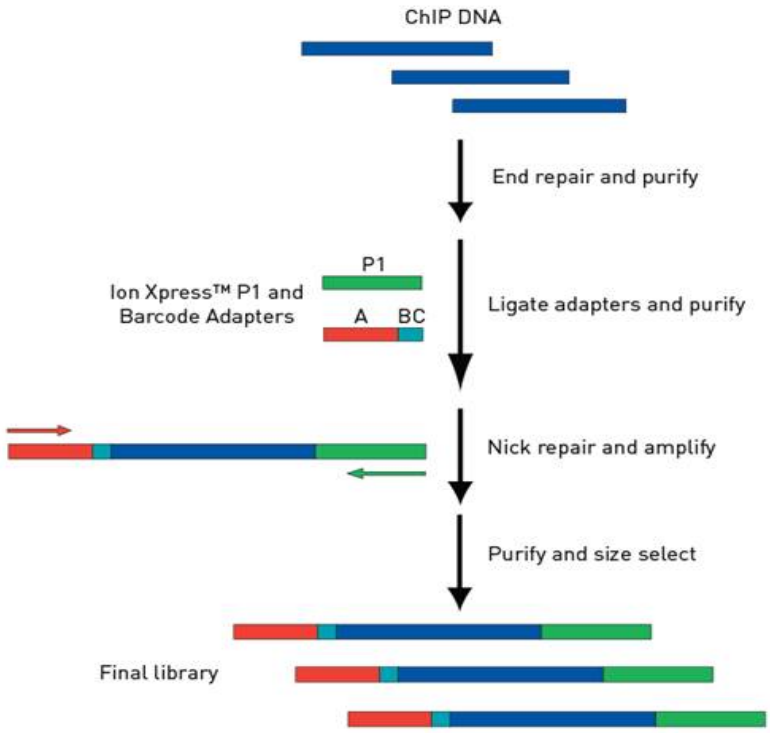
Materials needed

Use the Ion Xpress™ Plus Fragment Kit and the following materials:

- Agencourt® AMPure® XP Kit
- DynaMag™-2 magnet
- Ethanol, absolute, 200 proof, Molecular Biology Grade
- Microcentrifuge tubes, RNase/DNase-free, low retention (for example, 1.5-mL Eppendorf LoBind® tubes)
- 0.2-mL PCR tubes
- Microcentrifuge
- Vortex mixer
- Thermal cycler
- Rotator
- Pipettor and pipette tips
- Nuclease-free Water (Cat. no. AM9932)
- Ion Xpress™ Barcode Adapters 1–96

Overview of library preparation

As illustrated in the following figure, purified ChIP DNA is end-repaired and ligated to Ion-compatible barcode adapters. Barcoded libraries are required in this step and adapters from the Ion Xpress™ Barcode Adapters 1-96 Kit are used. Following ligation the samples are nick repaired to complete the linkage between the adapters and the DNA inserts are are then PCR amplified. The final libraries are purified with two rounds of AMPure® XP bead capture to size select fragments approximately 160—340 bp in length.



Important procedural guidelines

- 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.
- To detect differential enrichment, we strongly recommend preparing a reference control library using the Input Control (the non-immunoprecipitated control sample).
- When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.

End-repair the ChIP DNA

Use ≤10 ng of recovered ChIP DNA, as quantitated by the Qubit® 2.0 Fluorometer in "Step 8. Purify the DNA", per end-repair reaction. When samples are below the limit of detection by Qubit® 2.0 Fluorometer, use all of the ChIP DNA for the end repair reaction.

IMPORTANT! Using more than 10 ng of DNA introduces significant bias into final libraries and will negatively impact analysis.

1. In a 1.5-mL Eppendorf LoBind® Tube, add the following reagents in the indicated order, and mix by pipetting up and down:

Component	Reaction volume
ChIP DNA, ⩽10 ng	variable
Nuclease-free Water	variable
5X End Repair Buffer	20 µL

Component	Reaction volume
End Repair Enzyme	2 µL
Total	**100 µL**

2. Incubate the end-repair reaction for 30 minutes at room temperature.

Purify the end-repaired ChIP DNA

Use the Agencourt® AMPure® XP Kit to purify the end-repaired ChIP DNA.

IMPORTANT! Use **freshly prepared 70% ethanol** (1 mL plus overage per sample) for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

1. Add 180 µL (1.8 × sample volume) of Agencourt® AMPure® XP Reagent beads to the end repair reactions, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.
2. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
6. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
7. Remove the tube from the magnet, and elute in 40 µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new 1.5-ml Eppendorf LoBind® tube without disturbing the pellet.

IMPORTANT! The **supernatant** contains the eluted DNA. **Do not discard!**

STOPPING POINT (*Optional*) Store the DNA at -20°C.

Ligate adapters and purify the ligated ChIP DNA

1. In a 1.5-ml Eppendorf LoBind® tube, combine the reagents as indicated in the table below, and mix well by pipetting up and down.

   Note: Add both Ion P1 Adapter and the desired Ion Xpress™ Barcode X adapter to the ligation reaction.

IMPORTANT! When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.

Component	Volume
End-repaired ChIP DNA	40 µL
10X Ligase Buffer	10 µL
Ion P1 Adapter	1 µL
Ion Xpress™ Barcode X†	1 µL
Nuclease-free Water	46 µL
DNA Ligase	2 µL
Total	**100 µL**

† X = Barcode chosen

2. Incubate at room temperature for 30 minutes.

Purify the adapter-ligated DNA.

IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) for the next steps.

1. Add 150 µL (1.5 × sample volume) of Agencourt® AMPure® XP Reagent to the ligation reactions, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
6. Keeping the tube on the magnetic rack, air-dry the beads at room temperature for ≤5 minutes.
7. Remove the tube from the magnetic rack and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® tube without disturbing the pellet.

IMPORTANT! The supernatant contains the eluted DNA. Do not discard!

STOPPING POINT (Optional) Store the DNA at -20°C.

Nick repair and amplify the ChIP library

1. Combine the following reagents in an appropriately sized tube and mix by pipetting up and down.

Component	Volume
Platinum® PCR SuperMix High Fidelity	225 µL
Library Amplification Primer Mix	5 µL
Ligated ChIP DNA (unamplified library)	20 µL
Total	250 µL

2. Split the 250-µL reaction into three 0.2-mL PCR tubes, each containing about 85 µL.
3. Place the tubes into a thermal cycler and run the following PCR cycling program.

   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, 18 cycles yield sufficient quantity of library material for downstream Template Preparation.

Stage	Step	Temperature	Time
Holding	Nick repair	72°C	20 min
Holding	Denature	95°C	5 min
Cycling (18 cycles; see note above)	Denature	97°C	15 sec
	Anneal	60°C	15 sec
	Extend	70°C	1 min
Holding		70°C	5 min
Holding	—	4°C	∞

4. Combine the previously split PCRs in a new 1.5-mL Eppendorf LoBind® tube.

Purify the amplified library

IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) for the next steps.

1. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5 × sample volume) to each sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.

5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
6. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
7. Remove the tube from the magnetic rack, and add 25 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® tube without disturbing the pellet.

IMPORTANT! The supernatant contains the library. Do not discard!

Size-select the library

Optional: Retain 1 µL of amplified library before size-selection for Bioanalyzer analysis. Refer to "Step 11. Qualify and quantify the library" on page 35.

Use the Agencourt® AMPure® XP Kit to size-select the library with two rounds of binding to AMPure® XP beads followed by wash and elution.

- The **first round** selectively captures DNA >350 BP on the AMPure® XP beads, and DNA <350 bp is retained in the **supernatant.**
- The **second round** uses the first round supernatant and new beads to selectively capture DNA >160 bp. In this round, the **beads** are retained; the captured DNA is 160–350 bp in length. The size-selected DNA is then eluted from the beads.

IMPORTANT! **Use freshly prepared 70% ethanol** (1 mL plus overage per sample) for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

First round

1. Add 75 µL Nuclease-free water to the amplified ChIP DNA to bring the total volume to 100 µL.
2. Add 70 µL of Agencourt® AMPure® XP Reagent to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature for 5 minutes.
3. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears.
4. **Do not discard the supernatant—it contains the desired DNA.** Carefully transfer the supernatant to a new 1.5-mL Eppendorf LoBind® tube. Leave ~5 µL at the bottom to avoid disturbing the bead pellet.
5. Remove the tube with the beads from the magnet and pulse spin. Place the tube back on the magnet and wait for the solution to clear.
6. Remove the remaining liquid, being careful not to distub the pellet, and add this to the saved liquid from step 4. Discard the beads.

Second round

1. Add 80 µL of Agencourt® AMPure® XP Reagent beads to the amplified ChIP DNA, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.
2. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
6. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
7. Remove the tube from the magnet, and elute in 25 µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new 1.5-ml Eppendorf LoBind® tube without disturbing the pellet.

IMPORTANT! The **supernatant** contains the eluted DNA. **Do not discard!**

STOPPING POINT Store the library at –20°C. Before use, thaw on ice. To reduce the number of freeze-thaw cycles, store the library in several aliquots.

Step 11. Qualify and quantify the library

Materials needed

- Agilent 2100 Bioanalyzer™ instrument
- Agilent High Sensitivity DNA Kit
- Qubit® 2.0 Fluorometer
- dsDNA HS Assay Kit

Optional

Analyze amplified libraries before size selection

Analyze 1 µL of amplified library on the Agilent® Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit (Cat. no. 5067-4626).

Analyze size-selected libraries

1. Measure the concentration of the purified DNA with the dsDNA HS Assay Kit and the Qubit® 2.0 Fluorometer.
2. If the library concentration exceeds 1000 pg/µL, dilute the library to 1000 pg/µL and go to the next step. Otherwise, proceed to the next step without dilution.
3. Determine the molar concentration of the amplified library using the Agilent® 2100 Bioanalyzer® software (see Figure 2 on page 37). Ensure that the upper and lower marker peaks are identified and assigned correctly. Briefly:
   a. Select the **Data** icon in the Contexts panel, and view the electropherogram of the sample to be quantified.
   b. Select the **Region Table** tab below, and create a region spanning 160–340 bp. Correct the baseline if needed.
   c. The molarity is automatically calculated and displayed in the table in pmol/L (pM).
4. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.

   For example:

   - The library concentration is 3000 pM.
   - The dilution factor is 3000 pM/100 pM = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 100 pM.
5. Dilute library to ~100 pM as described and proceed to combining libraries or template preparation.

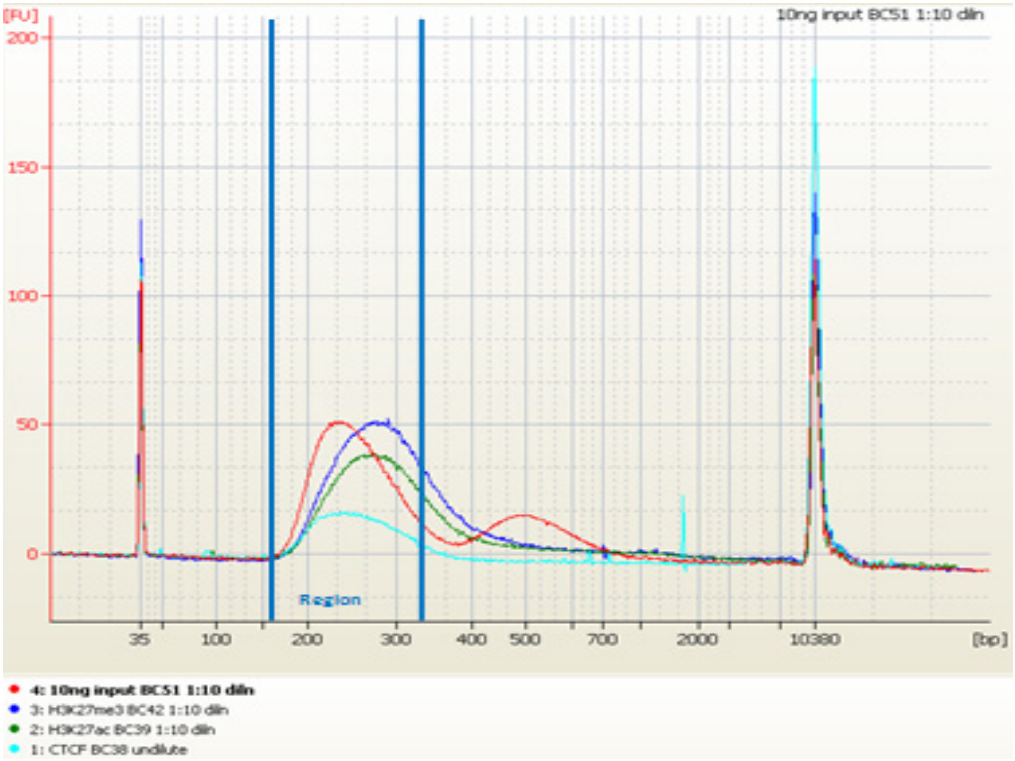


Figure 2 Molar concentration and size distribution of amplified libraries prepared from immunoprecipitated samples (CTCF, H3K27ac, H3K27me3) or an input control. The molarity of each sample between the region 160–340 bp is used to calculate the template dilution for Proton sequencing.

Proceed to Template Preparation

The ChIP-Seq libraries are ready for the downstream Template Preparation procedure for clonal amplification on Ion Sphere™ using the Ion PI™ Template OT2 200 Kit v3 (Cat. no. 4488318). Refer to the *Ion PI™ Template OT2 200 Kit User Guide* (Pub. no. MAN0007624), or *Ion PI™ Template OT2 200 KIt v3 User Guide* (Pub. no. MAN0009133) available at the Ion Torrent users community **http://ioncommunity.iontorrent.com/** and **www.appliedbiosystems.com/iontorrent**.

Data analysis

After the ChIP-Seq and reference input control libraries are created, the libraries are used for template preparation and sequencing on the Ion Proton™ System.

A Sanger FASTQ sequencing and quality file is generated automatically after the Ion Proton™ sequencing run by Torrent Suite™ Software. This file can be used in a variety of publicly available tools for mapping. If the reference genome is identified at the time of the Ion Proton™ sequencing run setup, the sequencing reads are mapped against known genomic sequences automatically after the Ion Proton™ sequencing run by the Torrent Suite™ Software, producing BAM files. The aligned reads can then be analyzed using a variety of publicly available tools.

Visit the Ion Community website at **http://ioncommunity.iontorrent.com/** for further information on available analysis tools and details on using them with Ion Proton™ data.

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 A Troubleshooting

Observation	Possible cause	Recommended action
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 SAT-2. Try increasing the amount of DNA template per reaction.
Low yield of DNA following library preparation steps	AMPure® XP beads were not at room temperature prior to use	Allow AMPure® XP 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).
Antibody not working in ChIP	Antibody not ChIP-qualified	Whenever possible, use an antibody that is qualified for ChIP. See "ChIP antibody selection" on page 9.
	Antibody not acceptable for use in ChIP even when it functions in other applications	See the guidelines for selecting an antibody on page 9.

Appendix B 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 the chromatin into 100–300 bp fragments is required to ensure good resolution for Ion 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.

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 20)
- Use proper negative and positive control antibodies to assess specific enrichment (see page 9)
- Use an Input Control (see page 22)
- Test enrichment of the ChIP sample relative to the Input Control by qPCR following the guidelines "Calculate fold enrichment as signal over background" on page 29.

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 MAGnify™ System.
- **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.

Appendix C Sonicate the chromatin with the Covaris® S2 System

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

Note: The Covaris® S220 instrument manual gives guidelines for converting Covaris® S2 conditions to the Covaris® S220 conditions; these conditions have not been validated by Life Technologies.

Materials needed

- Covaris® S2 System (110 V, Cat. no. 4387833; 220 V, Cat. no. 4392718))
- 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)

Procedure

1. Program the Covaris® S2 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 "Lyse the cells" on page 15 or on page 19) 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. Dilute the chromatin" on page 21, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

Appendix D Additional products

ChIP-qualified antibodies

Life Technologies has a wide range of ChIP-qualified antibodies for use in ChIP DNA preparation. Visit **http://www.lifetechnologies.com/us/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/antibodies-for-chip.html** for a complete list and details.

Real-time qPCR instruments and reagents

Life Technologies has a wide range of industry-standard instruments, plates, reagents, and other products for real-time qPCR. Visit **www.lifetechnologies.com** for more information.

Additional products

The following additional products are available separately from Life Technologies. For more information or to place an order, visit **www.lifetechnologies.com**.

Product	Quantity	Cat. 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 X 500 mL	70011-044 70011-069
Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	500 mL 10 X 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% Gels with SYBR® Safe™	Starter Kit 18 pak	G6206-02 G5218-02
E-Gel® 2% Gels 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

Product	Quantity	Cat. no.
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® 2.0 Fluorometer	1 unit	Q32857

Appendix E Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and support

Customer and technical support

Visit **www.lifetechnologies.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support
- Order and web support
- Product documentation, including:
  - User guides and manuals
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from third-party manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA
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19 August 2014