

USER GUIDE

invitrogen[™]
by *life* technologies[™]

MAGnify[™] Chromatin Immunoprecipitation System

For tissue and cells

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For Research Use Only. Not for use in diagnostic procedures.

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Kit Contents and Storage

Kit Components and Storage

Sufficient components are provided for up to 24 reactions (including input control reactions). The kit is shipped in 4 boxes. Boxes 1 and 2 are shipped on wet ice, and Boxes 3 and 4 are shipped on dry ice. See the table below for storage temperatures.

Module 1	Quantity	Storage
Glycine (1.25 M)	2 × 1 mL	4°C
Dynabeads® Protein A/G (do not freeze!)	250 µL	4°C
Reverse Crosslinking Buffer	1.4 mL	4°C
DNA Purification Magnetic Beads (do not freeze!)	500 µL	4°C
DNA Purification Buffer	1.4 mL	4°C
Proteinase K (20 mg/mL)	200 µL	Room temp or 4°C
Module 2	Quantity	Storage
IP Buffer 1	10 mL	4°C
IP Buffer 2	7.5 mL	4°C
DNA Wash Buffer	8.0 mL	4°C
DNA Elution Buffer	7.2 mL	4°C
Module 3	Quantity	Storage
Protease Inhibitors (200X)	100 µL	-20°C
Mouse IgG (1 µg/µL)	15 µL	-20°C
Rabbit IgG (1 µg/µL)	15 µL	-20°C
Module 4	Quantity	Storage
Dilution Buffer	8.0 mL	-20°C
Lysis Buffer	3.6 mL	-20°C



Important

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

Product Qualification

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

Product Use

For research use only. Not for use in diagnostic procedures.

Materials Supplied by the User

Additional Reagents Needed

The following reagents are required for use of the kit. Ordering information for many of these products is provided in **Additional Products** on page 39.

- Cells or fresh or frozen tissue
- For cells: Trypsinizing reagent (e.g., TrypLE™ Express Stable Trypsin Replacement Enzyme)
- For fresh or frozen tissue:
 - Clean razor blades
 - 1.5-inch 18G and 21G needles (a 1.5-inch 16G needle may also be needed for muscular tissues such as heart)
 - 1-mL luer lock syringes
- Antibody of interest (e.g., ChIP-qualified antibodies from Life Technologies, available at www.lifetechnologies.com/chipantibody).
- Nuclease-free water
- Formaldehyde, 37%, Molecular Biology Grade
- PBS or D-PBS (e.g., Phosphate Buffered Saline, 7.4, 1X liquid, or Dulbecco's Phosphate-Buffered Saline, 1X liquid)
- qPCR SuperMix (e.g., EXPRESS qPCR Supermixes and EXPRESS SYBR® GreenER™ qPCR Supermixes)
- qPCR primers for the sequence of interest
- 100-bp DNA ladder or 50-bp ladder (e.g., TrackIt™ 100-bp DNA Ladder, Invitrogen 10488-058; or Invitrogen 50-bp ladder, Invitrogen 10416-014)
- **Optional:** Control primers for qPCR (e.g., MAGnify™ SAT2 Primers, Invitrogen 49-2026; RARβ1 Primers, Invitrogen 49-2027; ERα Primers, Invitrogen 49-2028; and c-Fos Primers, Invitrogen 49-2029)

Continued on the next page

Materials Supplied by the User, continued

Additional Equipment Needed

The following equipment is required for use of the kit. Ordering information for many of these products is provided in **Additional Products** on page 39.

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

Description of the System

MAGnify™ 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, 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, optimized assay for the enrichment of chromatin/protein complexes and DNA recovery using magnetic bead capture technology. The isolated DNA is ready for downstream analysis by methods such as PCR- or qPCR-based assays, or massive parallel DNA sequencing.

This kit enables researchers to start with lower sample amounts than traditional ChIP workflows, thereby preserving precious samples, and the protocol can be completed in a single day, compared with 2–3 days for a traditional ChIP assay.

The kit can be used with a suite of ChIP-validated antibodies from Life Technologies, and is also complementary with MethylCode™ and NCode™ products for downstream epigenetics research.

Applied Biosystems SOLiD™ Workflow

The SOLiD™ ChIP-Seq Kit is available separately from Life Technologies for ChIP analysis using the Applied Biosystems SOLiD™ 4 System. Visit the www.lifetechnologies.com for more information.

MAGnify™ Workflow Summary

Using the MAGnify™ system, you treat cells or tissue with formaldehyde to generate protein-protein and protein-DNA crosslinks between molecules in close proximity within the chromatin complex. The cells are then lysed, and the chromatin is released from the nuclei and sheared by sonication to reduce the average DNA fragment size to 200–500 bp for analysis by quantitative real-time PCR (qPCR) or 100–300 bp for analysis by massive parallel DNA sequencing.

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

The DNA is now ready for downstream analyses such as end-point PCR or quantitative PCR (qPCR), genome-wide analyses using promoter-tiling arrays, or next-generation sequencing. In PCR/qPCR analysis, primers are designed to span the desired DNA sequence of interest, and the data demonstrates whether the specific protein of interest is associated *in vivo* with that DNA region.

Negative Controls

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

Preparation of a chromatin input control (non-immunoprecipitated) is also strongly recommended as a reference control for ChIP-qPCR checkpoint as well as for ChIP DNA sequencing.

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Description of the System, continued

ChIP-Qualified Antibodies

ChIP-qualified antibodies are available separately from Life Technologies. Visit www.lifetechnologies.com/chipantibody for more information.

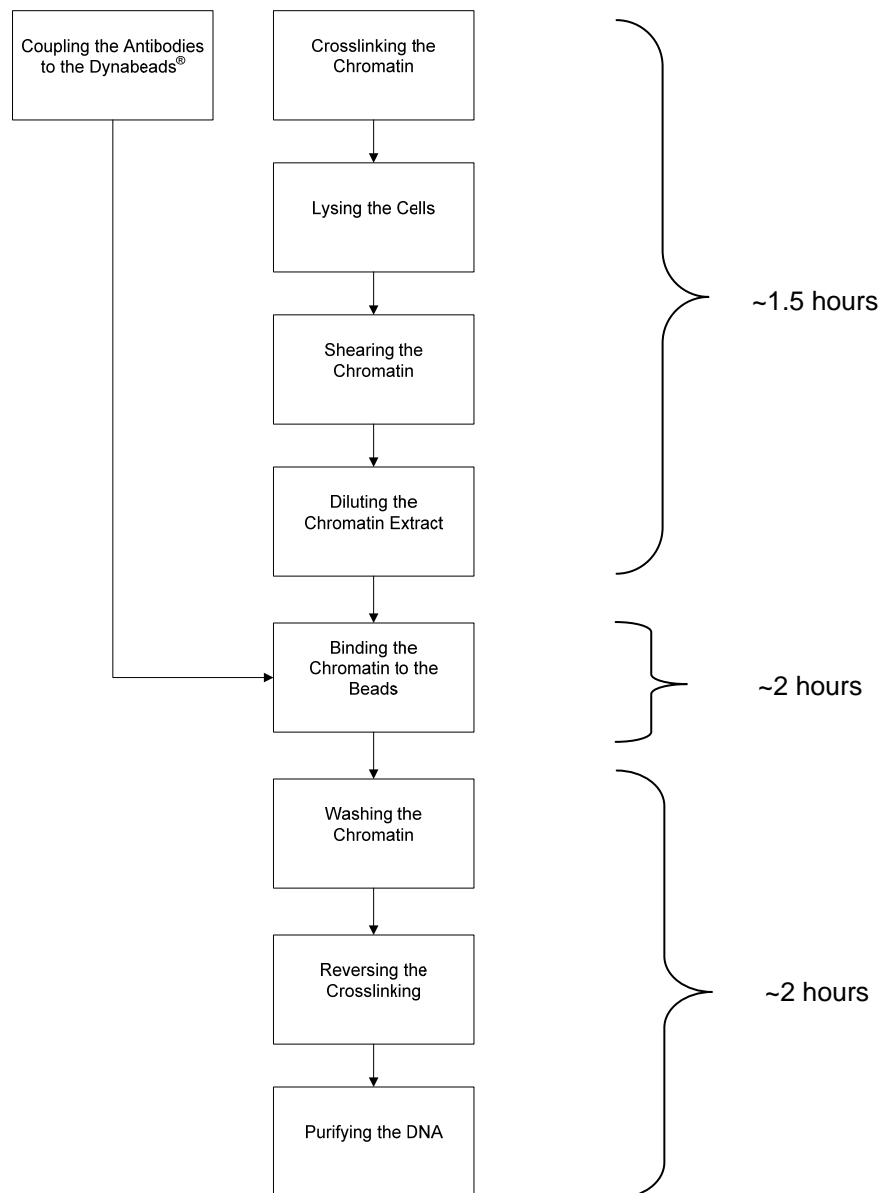
MAGnify™ PCR Primer Pairs

MAGnify™ PCR Primers are available separately from Life Technologies for the amplification of common promoter regions analyzed in ChIP experiments. See page 33 for sequences and page 35 for ordering information.

DynaMag™-PCR Magnet

The MAGnify™ system 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. See page 35 for ordering information.

System Workflow Diagram



Methods

Before Starting

Amount of Starting Material

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

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

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 μ L	100,000	10
1 million cells	50 μ L	10,000	100
3 million cells	150 μ L	100,000	30
3 million cells	150 μ L	10,000	300
50 mg tissue (= 3 million cells)	150 μ L	100,000	30
50 mg tissue (= 3 million cells)	150 μ L	10,000	300

ChIP Antibody Selection

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

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

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

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

Continued on the next page

Before Starting, continued

ChIP Controls

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

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

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

Input Control: This is DNA obtained from chromatin that has been reverse-crosslinked but has not been immunoprecipitated. It is reserved in **Step 4. Diluting the Chromatin**, on page 26, and then analyzed by qPCR.

Handling Dynabeads®

DynaMag™ -PCR Magnet

The MAGnify™ ChIP system utilizes a novel magnet for processing Dynabeads® that is compatible with 0.2-mL PCR strip tubes. The new DynaMag™ -PCR Magnet (sold separately) is optimized for efficient magnetic separation of the small sample volumes used in ChIP experiments, and allows for the processing of multiple ChIP assays using a multi-channel pipettor. The magnet holds up to 16 0.2-mL PCR tubes, in individual or strip-tube format.



Important

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

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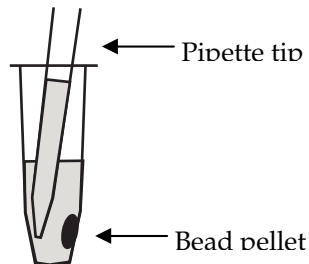
Resuspending Dynabeads®

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

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

Removing Liquid from Dynabeads®

To remove liquid from Dynabeads®:



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

Step 1. Coupling the Antibody to Dynabeads®

Introduction

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

Materials Needed

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

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

ChIP Antibody Controls

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

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

Amount of Antibody to Use

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



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.

Continued on next page

Step 1. Coupling the Antibody to Dynabeads[®], continued

Coupling the Antibodies to the Dynabeads[®]

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

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

While the Antibody/Dynabeads[®] mixture is incubating, proceed to either **Step 2A. Preparing Cells** (next page) or **Step 2B. Preparing Tissue** (page 19). When the Antibody-Dynabeads[®] have finished mixing, hold the tube at 4°C until use.

Step 2A. Preparing Cells

Introduction

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

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

Materials Needed

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

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



Note

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

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



Important

- 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.

Continued on next page

Step 2A. Preparing Cells, continued

Collecting and Crosslinking Adherent Cells

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

1. For adherent cells, aspirate the media and wash cells with 10 mL of room-temperature 1X PBS (or D-PBS).
2. Aspirate the PBS and add enough trypsinizing reagent to cover the cells. *Example for a T-175 Flask:* Add 4 mL of TrypLE™ Express Stable Trypsin Replacement Enzyme.
3. Incubate at 37°C for ~3 minutes or until cells dislodge from the plate surface.
4. When all the cells have detached, add 10 mL of room-temperature PBS and pipet the cells gently up and down to mix.
5. Transfer the cell suspension to a centrifuge tube and spin at 200 × g for 5 minutes to pellet.
6. Discard the supernatant and resuspend the pellet in room-temperature PBS. (Estimate the resuspension volume so the cell density is more concentrated than your planned dilution.) Mix the cell solution gently.
7. Collect a small aliquot to verify that the cells are at the desired concentration. Determine cell density electronically using an automated cell counter or manually using a hemacytometer chamber.
8. Determine the volume of cell suspension required for the total number of immunoprecipitations (IPs) planned (number of cells per IP times the total number of IPs). Transfer this volume to a new tube.
9. *If the volume is ≤500 μL, bring the final volume to 500 μL with room-temperature PBS.*
If the volume is >500 μL, spin the cell suspension at 200 × g for 5 minutes, aspirate the supernatant, and resuspend the pellet in 500 μL of PBS.
10. Add 13.5 μL of 37% formaldehyde to the 500 μL of sample, for a final concentration of 1%. Invert the tube to mix, and incubate for 10 minutes at room temperature (or perform a time course to determine optimal time).
11. To stop the reaction, add 57 μL of room-temperature 1.25 M glycine to the sample. Invert the tube to mix, and incubate for 5 minutes at room temperature.
12. In a cold centrifuge at 4°C, spin the crosslinked cells at ~200 × g for 10 minutes. From this point, keep all tubes on ice.
13. Remove and discard the supernatant, leaving ~30 μL behind so as to not disturb the pellet.
14. Resuspend the cells in 500 μL of cold PBS, and spin at 200 × g for 10 minutes at 4°C to pellet.
15. Aspirate the PBS and resuspend once more in 500 μL of cold PBS. Spin cells at 200 × g for 10 minutes at 4°C to pellet.
16. Aspirate the PBS, leaving 10–20 μL behind. Make sure not to disturb the cell pellet.

Proceed to preparing the lysis buffer for cell lysis, page 17.

Continued on next page

Step 2A. Preparing Cells, continued

Collecting and Crosslinking Cells in Suspension

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

Proceed to preparing the lysis buffer for cell lysis on the next page.

Continued on next page

Step 2A. Preparing Cells, continued

Crosslinking Cells Directly in a Dish

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

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

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

Proceed to preparing the lysis buffer for cell lysis below.

Lysis Buffer Guidelines

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

Preparing the Lysis Buffer with Protease Inhibitors

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

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

Continued on next page

Step 2A. Preparing Cells, continued

Lysing the Cells

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

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

Step 2B. Preparing Tissue

Introduction

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

For instructions on preparing cells, see Step 2A.

Materials Needed

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

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



Important

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

Preparing the Lysis Buffer with Protease Inhibitors

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

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

Continued on next page

Step 2B. Preparing Tissue, continued

Weighing and Mincing Fresh Tissue

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

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

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

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

Weighing and Mincing Frozen Tissue

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

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

Continued on next page

Step 2B. Preparing Tissue, continued

Homogenizing the Tissue

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

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

Crosslinking the Chromatin

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

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

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

Continued on next page

Step 2B. Preparing Tissue, continued



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 .

Lysing the Cells

1. Add 150 μL of Lysis Buffer prepared with Protease Inhibitors (see page 19) to each pellet from the previous page. This is the equivalent of 50 μL of Lysis Buffer per 1 million cells.
2. Resuspend by mild pulses on the vortex mixer.
3. Incubate the tube on ice for at least 5 minutes.

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

Step 3. Shearing the Chromatin

Introduction

In this step, you shear the chromatin into 200–500-bp fragments for analysis by quantitative real-time PCR (qPCR) or 100–300-bp fragments for analysis by massive parallel DNA sequencing.

Materials Needed

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

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



Note

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

Fragment Size

Chromatin should be sheared into ~200–500 bp fragments for use in qPCR analysis and ~100–300 bp fragments for use in massive parallel DNA sequencing analysis. We have used the Bioruptor® UCD-200 to generate ~200–500 bp fragments and the Bioruptor® UCD-200 and the Covaris® S2 to generate ~100–300 bp fragments.

In general, sonication conditions should be optimized for each sample type and instrument. We recommend performing pilot experiments using different settings and times and, after decrosslinking, evaluating the size of the DNA by agarose gel electrophoresis or Bioanalyzer analysis.



Important

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

Continued on next page

Step 3. Shearing the Chromatin, continued

Using Probe Sonicators

As noted previously, keep the sample cooled on ice during sonication to avoid reversing the crosslinks. In addition, 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 MAGnify™ procedure. We recommend testing various sonication conditions on your samples of interest and running treated chromatin lysates on a 1.5–2.0% agarose gel (e.g., a 2% E-Gel® or 2% E-Gel® EX Gel) with a 100-bp ladder to determine fragment length.

For example, 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 $\times g$ at 4°C for 5 minutes. Transfer the chromatin to a new tube and run ~5 μL of chromatin input per well on a 2% E-Gel®.

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

Storing Sheared Chromatin

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

200–500 bp Fragments Using the Bioruptor® UCD-200

Below is an example sonication procedure using the Bioruptor® UCD-200 to shear chromatin into 200–500 bp fragments for analysis by qPCR. Refer to the instrument manual for setup and maintenance.

1. Pre-cool the water reservoir with ice.
2. Remove the ice and add ice-cold water to the water level mark.
3. Set the Bioruptor® UCD-200 to High Power, and sonicate the cell lysate (from page 18 or 22) for 16 cycles of 30 seconds ON, 30 seconds OFF. Make sure the water in the reservoir does not overheat.
4. Pellet the debris by spinning at 20,000 $\times g$ at 4°C for 5 minutes.
5. **The supernatant contains the chromatin.** Aliquot the freshly sonicated chromatin into new, sterile tubes.

Proceed with **Step 4. Diluting the Chromatin**, page 26, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

Continued on next page

Step 3. Shearing the Chromatin, continued

100–300 bp Fragments Using the Covaris® S2

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

1. Program the instrument as follows:

Duty Cycle: 5%	Cycles: 10
Intensity: 2	Temperature (bath): 4°C
Cycles per Burst: 200	Power mode: Frequency Sweeping
Cycle Time: 60 seconds	Degassing mode: Continuous
2. Fill the water level to 15 and degass the instrument for 30 minutes.
3. Load up to 100 µL of cell lysate (from **Lysing the Cells**, page 18 or 22) 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 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 with **Step 4. Diluting the Chromatin**, next page, or snap-freeze the chromatin in liquid nitrogen or on dry ice. Store frozen aliquots at –80°C.

100–300 bp Fragments Using the Bioruptor® UCD-200

Below is an example sonication procedure using the Bioruptor® UCD-200 to shear chromatin into 100–300 bp fragments for analysis by massive parallel DNA sequencing. Refer to the instrument manual for setup and maintenance.

1. Pre-cool the water reservoir with ice.
2. Remove most of the ice and add ice-cold water to the water level mark.
3. Transfer the cell lysate (from **Lysing the Cells**, page 18 or 22) into 1.5-mL Eppendorf LoBind tubes. Use a final volume of 50–150 µL per tube.
4. Place one of the tubes in the Bioruptor® UCD-200 and set it to High Power.
Note: Sonicating one tube at a time will ensure consistent results and proper size distribution.
5. Sonicate the tube for 8 cycles of 30 seconds ON, 30 seconds OFF. After 8 cycles, cool the water in the reservoir with ice, then remove and add ice-cold water up to the water mark.
6. Sonicate the tube for another 8 cycles.
7. Repeat Steps 5–6 for each of the remaining tubes.
8. Pellet the debris by spinning at 20,000 × g at 4°C for 5 minutes.
9. **The supernatant contains the chromatin.** Aliquot the freshly sonicated chromatin into new, sterile tubes.

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

Step 4. Diluting the Chromatin

Introduction

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

Preparing Dilution Buffer with Protease Inhibitors

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

The prepared Dilution Buffer should be cold before use.

Diluting the Chromatin

Dilute the sheared chromatin from page 24 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. Prepared an extra dilution for each sample for use as an Input Control, as described below.

Example Volumes of Chromatin and Dilution Buffer: Starting Concentration = 1 million cells per 50 μL Lysis 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
	12	30 μL	1170 μL	1200 μL
100,000	1	5 μL	95 μL	100 μL
	12	60 μL	1140 μL	1200 μL
200,000	1	10 μL	90 μL	100 μL
	12	120 μL	1080 μL	1200 μL

Reserve the Input Control

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

You will reverse-crosslink this control sample (**Step 7. Reversing the Crosslinking**, page 29) 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 33.

Step 5. Binding Chromatin to the Beads

Introduction

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

Materials Needed

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

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



Note

- 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- μ L 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 29.
-

Binding the Chromatin

When the Antibody-Dynabeads® from page 13 have finished mixing, proceed with the following. Keep the magnet, tubes, and buffers cold during these steps.

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

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

Step 6. Washing the Bound Chromatin

Introduction

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

Materials Needed

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

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



Important

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

Washing with IP Buffer 1

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

Keep the magnets, tubes, and buffers 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, then proceed immediately to **Washing with IP Buffer 2** below.
-

Washing with IP Buffer 2

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

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

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

Step 7. Reversing the Crosslinking

Introduction

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

Materials Needed

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

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

Preparing Reverse Crosslinking Buffer with Proteinase K

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

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

<u>Component</u>	<u>1 reaction</u>	<u>12 reactions</u>
Stock Reverse Crosslinking Buffer	53 μ L	636 μ L
Proteinase K	<u>1 μL</u>	<u>12 μL</u>
Final Volume	54 μ L	648 μ L

Preparing the Input Controls

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

1. To each tube containing 10 μ L of Input Control from **Reserve the Input Control**, page 26, add 43 μ L of Reverse Crosslinking Buffer, for a total volume of 53 μ L.
 2. Add 1 μ L of Proteinase K to the tube.
 3. Vortex briefly to mix, and immediately proceed to **Reverse Crosslinking**, next page.
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Continued on next page

Step 7. Reversing the Crosslinking, continued

Reverse Crosslinking

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

1. Place the tubes from **Washing with IP Buffer 2**, page 28, 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 μ L of Reverse Crosslinking Buffer prepared with Proteinase K to each tube. Vortex lightly to fully resuspend the beads.
4. Incubate the IP sample tubes and Input Control tubes (from previous page) at 55°C for 15 minutes in a thermal cycler, hybridization oven, water bath, or other heat source of choice.
5. Spin the tubes briefly, and then proceed uninterrupted through the following steps.
6. Place the IP sample tubes in the DynaMag™-PCR Magnet and wait at least 30 seconds for a pellet to form.
7. ***Do not discard the liquid—the liquid contains your sample.*** With the tubes in the magnet, carefully transfer the liquid (~50 μ L) to new, sterile 0.2-mL PCR tubes or strip tubes. Be careful not to disturb the bead pellet, and proceed immediately to the next step.
8. Spin the IP sample tubes and Input Control tubes briefly, and then incubate at 65°C for 15 minutes.
9. Cool the tubes on ice for ~5 minutes.
10. Discard the used magnetic beads. Do not reuse.

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

Step 8. Purifying the DNA

Introduction

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

Materials Needed

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

- DynaMag™-PCR Magnet
 - Pipettor and pipette tips
-



Note

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

Preparing the DNA Purification Magnetic Beads with Dilution Buffer

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

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

Binding and Washing the DNA

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

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

Proceed to **Eluting the DNA**.

Continued on next page

Step 8. Purifying the DNA, continued

Eluting the DNA

1. Place the tubes from **Binding and Washing the DNA**, previous page, in the DynaMag™-PCR Magnet and wait at least 1 minute for a pellet to form.
2. With the tubes in the magnet, remove and discard the liquid from each tube, leaving ~5 µL at the bottom to avoid disturbing the beads.
3. Remove the tubes from the magnet and add 150 µL of DNA Elution Buffer to each tube. Pipet up and down gently 5 times to mix.
4. Incubate at 55°C for 20 minutes in a thermal cycler, hybridization oven, water bath, or other heat source of choice.
5. Spin the tubes briefly to collect the contents. Place the tubes in the DynaMag™-PCR Magnet and wait at least 1 minute for a tight pellet to form.
6. ***Do not discard the liquid—the liquid contains your purified sample.*** With the tubes in the magnet, carefully transfer the liquid to new, sterile tubes. Leave ~5 µL at the bottom to avoid disturbing the beads.

Note: If you have accidentally aspirated beads during the transfer step, the eluate may appear discolored. In this case, return the liquid to the tube containing the beads and repeat Steps 5–6.

7. Discard the used magnetic beads. Do not reuse.

Store the purified DNA at –20°C or use immediately for PCR, qPCR, or other downstream applications. Avoid repeatedly freezing and thawing the DNA.

Quantification of ChIP DNA

The Quant-iT™ DNA Assay Kit, High Sensitivity, and Quant-iT™ dsDNA HS Assay Kits for use with the Qubit® fluorometer provide accurate quantitation of most chromatin input samples (see page 35). However, some targets may still be too dilute to be accurately quantified.

Data Analysis

Introduction

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

qPCR Materials and Guidelines

Life Technologies has a wide range of reagents, instruments, and other products for qPCR, including TaqMan[®] probes, SYBR[®] GreenER[™], and EXPRESS qPCR SuperMixes. Visit www.lifetechnologies.com for details.

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

Primer Specifications

Primer design is one of the most important parameters in qPCR. In general, primers should be 20–30 bases long with a T_m of 55°C to 60°C, and should be designed for an amplicon length of approximately 80–250 bp. A final concentration of 200 nM per primer is effective for most reactions. Optimal results may require a titration of primer concentrations between 100 and 500 nM.

We strongly recommend using a primer design software program such as OligoPerfect[™] at www.lifetechnologies.com/oligos or Primer Express 3.0. In addition to designing primers for optimal efficiency, programs such as these will automatically perform a BLAST search of NCBI databases to ensure that primers are target-specific.

MAGnify[™] PCR Primer Pairs

The following primer pairs are available separately from Life Technologies for the amplification of common promoter regions analyzed in ChIP experiments. See page 35 for ordering information.

Primer Pair	Sequences	Catalog no.
MAGnify [™] SAT2 Primers	CTGCAATCATCCAATGGTTCG GATTCCATTCGGGTCCATTC	49-2026
MAGnify [™] RARβ1 Primers	GGCATTTCATGGCATCCA CCGCGGTACACGCAAAA	49-2027
MAGnify [™] ERα Primers	TGAACCGTCCGCAGCTCAAGATC GTCTGACCGTAGACCTGCGCGTTG	49-2028
MAGnify [™] c-Fos Primers	TTAGGACATCTGCGTCAGCAGGTT TCTCGTGAGCATTTTCGCAGTTCCT	49-2029

EXPRESS qPCR SuperMixes

EXPRESS qPCR SuperMixes are highly robust master mixes for real-time qPCR that can accommodate a wide range of cycling conditions and reaction volumes. They include Platinum[®] Taq DNA polymerase, MgCl₂, heat-labile uracil DNA glycosylase (UDG) and dUTP for cross-contamination prevention, dNTPs, and stabilizers.

EXPRESS SYBR[®] GreenER[™] qPCR SuperMixes include SYBR[®] GreenER[™] in the reaction mix for higher sensitivity and lower PCR inhibition than other fluorescent double-stranded DNA binding dyes.

See page 35 for ordering information.

Continued on next page

Data Analysis, continued

Replicates

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

qPCR of the Input Control DNA

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

Determining Amplification Efficiency using the Input Control

We recommend determining the amplification efficiency of your qPCR reaction using 10-fold serial dilutions of the Input Control DNA in MAGnify™ DNA Elution Buffer (*e.g.*, 1:1 to 1:1000). 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 $AE = 10^{(-1/\text{slope})}$.

Data Normalization Using the Input Control

1. Export the qPCR data to a spreadsheet program such as Microsoft Excel by using built in filters. The file should not contain omitted wells and should be in a column format containing well positions, descriptors, and CT values for each selected well.
 2. Open the exported file. Average the replicate measurements for each IP reaction in a new column (AVERAGECT IP).
 3. For each primer pair, calculate the adjusted CT for the Input Controls. For an Input Control that was 10% of the IP reaction, then the dilution factor (DF) is 10 and you should subtract 3.32 cycles (*i.e.*, \log_2 of 10) from the Ct value of the Input Control. Then average the Input Control replicates (AVERAGE CT INPUT).
 4. Subtract the AVERAGECT INPUT from AVERAGECT IP in a new column. This number is the dCT. This value represents the difference in cycles between the immunoprecipitated sample and the input DNA.
 5. Normalized input is calculated by:
$$100 \times AE^{(\text{AVERAGECT INPUT} - \text{AVERAGECT IP})}$$
Where AE is the amplification efficiency as calculated above.
-

Fold Enrichment as Calculated by Signal Over Background

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

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

Appendix

Troubleshooting

Problem	Possible Cause	Solution
Low level of amplification as detected by PCR/qPCR	Heat released by sonicator reversed crosslinks	Keep samples cool during sonication; place samples on ice between cycles.
	Excessive or inefficient crosslinking	Keep the crosslinking time and temperature consistent across samples. Optimize the length of time for crosslinking by performing a time-course experiment.
	Not enough antibody	Increase the titration of antibody per cell range to determine the window for best enrichment.
	Protein is degraded	Keep samples on ice during lysis. Also, place the dilution buffer, lysis buffer, and IP wash buffer 1 on ice before use, and be sure they are cold before use. Be sure to add the Protease Inhibitors provided in the kit to lysis buffer prior to use as specified in the protocol.
	Chromatin binding incubation time is too short	The kinetics of reaching equilibrium of epitope-antibody binding may be antibody or target dependent. Increasing the incubation time may improve results.
No amplification	qPCR/PCR failure	See the troubleshooting provided with your PCR/qPCR kit. Isolate the problem using Input Control DNA and a control primer such as SAT2. Try increasing the amount of DNA template per reaction.

Continued on the next page

Troubleshooting, continued

Antibody not working in ChIP	Antibody not ChIP-qualified	Whenever possible, use an antibody that is qualified for ChIP. A selection of ChIP-qualified antibodies is provided on our website at: www.lifetechnologies.com/chipantibody
	Antibody not acceptable for use in ChIP even when it functions in other applications	Antibodies should be specific and well-characterized. It is ideal to determine purity, titer (via ELISA), and cross reactivity (via dot blot), and perform western blot analysis and immunohistochemistry (IHC) or immunoprecipitation (IP). An antibody MAY have greater success in ChIP if it is affinity-purified, polyclonal, and qualified by immunoprecipitation. 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 c-Fos.

Frequently Asked Questions

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

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

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

How do I cross-link?

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

How long should I cross-link?

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

What is the optimal fragment size?

Shearing of the chromatin into appropriate fragments is required to ensure good resolution for MAGnify™ ChIP-Seq reactions.

Target ChIP sonication conditions yielding ~200–500 bp fragments for use in downstream qPCR analysis and ChIP sonication conditions yielding ~100–300 bp fragments for use in massive parallel DNA sequencing downstream analysis.

- Conditions for sonication need to be assessed for each cell and tissue type.
 - Efficiency of sonication varies to some extent with type of sonicator.
 - We recommend testing different sonication settings and times on your type of samples, then checking the size of the DNA by agarose gel electrophoresis or on a Bioanalyzer.
-

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

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

Continued on the next page

Frequently Asked Questions, continued

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

The amount of each antibody must be empirically determined. A general recommended starting point is 1–10 μg of antibody per ChIP assay.

What controls should I use?

We recommend the following controls:

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

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

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

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

Additional Products

Ordering from Invitrogen by Life Technologies

The following additional products are available separately from Life Technologies. For more information or to place an order, visit our website at www.lifetechnologies.com or contact Technical Support (page 40).

Product	Quantity	Catalog no.
MAGnify™ SAT2 Primers	100 µL	49-2026
MAGnify™ RARβ1 Primers	100 µL	49-2027
MAGnify™ ERα Primers	100 µL	49-2028
MAGnify™ c-Fos Primers	100 µL	49-2029
EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	200 rxns 1,000 rxns	11784-200 11784-01K
EXPRESS SYBR® GreenER™ qPCR SuperMix with Premixed ROX™	200 rxns 1,000 rxns	11794-200 11794-01K
EXPRESS qPCR SuperMix Universal	200 rxns 1,000 rxns	11785-200 11785-01K
EXPRESS qPCR SuperMix with Premixed ROX™	200 rxns 1,000 rxns	11795-200 11795-01K
DynaMag™-PCR Magnet (holds up to 16 0.2-mL PCR tubes)	1 magnet	49-2025
Phosphate Buffered Saline (PBS) 7.4 (1X), liquid	500 mL 10 × 500 mL	70011-044 70011-069
Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	500 mL 10 × 500 mL	14190-144 14190-250
TrypLE™ Express Stable Trypsin Replacement Enzyme without Phenol Red	100 mL 500 mL	12604-013 12604-021
Countess® Automated Cell Counter	1 unit	C10227
E-Gel® EX Gel, 2%	10 pak 20 pak	G4010-02 G4020-02
E-Gel® iBase™ Power System	1 unit	G6400
100-bp DNA Ladder	50 µg	15628-019
TrackIt™ 100 bp DNA Ladder	100 applications	10488-058
UltraPure™ DEPC-treated Water	1 liter	750023
Quant-iT™ DNA Assay Kit, High Sensitivity (0.2–100 ng)	1000 assays	Q33120
Quant-iT™ dsDNA HS Assay Kits—for use with the Qubit® fluorometer (0.2–100 ng)	100 assays 500 assays	Q32851 Q32854
Qubit® Fluorometer	1 unit	Q32857

ChIP-Qualified Antibodies

Life Technologies has a wide range of ChIP-qualified antibodies for use with the MAGnify™ system. Visit our website at www.lifetechnologies.com/antibody for a complete list and details.

Applied Biosystems Real- Time PCR

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.

Technical Support

Web Resources



Visit the Life Technologies website at www.lifetechnologies.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
 - Complete technical support contact information
 - Access to the Life Technologies Online Catalog
 - Additional product information and special offers
-

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.lifetechnologies.com).

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.lifetechnologies.com/msds.

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