Instruction Manual
for
EZ-Zyme™ Chromatin Prep Kit

Catalog # 17-375

Sufficient reagents for 22 enzymatic chromatin preparations per kit.

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I. INTRODUCTION

Chromatin Immunoprecipitation (ChIP) is a widely utilized experimental technique to monitor the association of proteins with specific DNA sequences. This technique has been used to study patterns of histone modifications and to map binding sites of various proteins on a genome-wide scale with the aid of microarray technology. ChIP has also been used to determine the temporal regulation underlying the occupation of the particular chromatin locus by multiple proteins. In addition, because formaldehyde efficiently crosslinks protein to DNA as well as protein to protein, this method can, in principle, detect proteins that bind DNA directly as well as those that bind DNA indirectly through other factors.

When performing ChIP, chromatin from cells and tissues needs to be fragmented so that it becomes soluble and resolution can be achieved in detecting protein-DNA interaction at specific loci. Sonication is a common method for producing sheared chromatin. Although this method is very efficient in releasing chromatin into the solution, it has a number of limitations. Sonication conditions can be physically harsh and can negatively impact immunoprecipitation efficiency. For example, foaming and overheating associated with sonication can result in protein denaturation or incomplete chromatin fragmentation. These and other limitations in the procedure can make sample sonication difficult to optimize and perform reproducibly.

The EZ-Zyme™ Chromatin Prep kit allows ChIP analysis at nucleosome resolution by performing complete or partial digestions with a proprietary enzymatic cocktail to obtain chromatin fragments of on average one to a few nucleosomes in length. Compared to sonication, this procedure not only allows mapping the protein-DNA association at a higher resolution, but also renders the subsequent immunoprecipitation more efficient due to its mild digestion condition. In addition, enzymatic shearing is compatible with non-crosslinked ChIP (native ChIP), which allows analysis of histone modifications not only on cultured cells, but also on freshly dissected or frozen tissues.

Kit Description

Quantity: One box containing the necessary reagents to perform 22 enzymatic chromatin preparations.

Storage and Stability: Upon receipt, store components at the temperatures indicated on the labels. Storage temperatures are also indicated on page 3 of this manual.

Use: The EZ-Zyme™ kit contains reagents optimized for generating enzymatically processed chromatin from mammalian cells for use in chromatin immunoprecipitation. While the kit contains a full complement of buffers required for chromatin shearing, careful attention must be paid to the details of the instructions. Follow all the instructions carefully, especially with regard to incubation times and temperatures.

Related Products:

Catalog # 17-371	EZ-ChIP™ Chromatin Immunoprecipitation Kit
Catalog # 17-295	Chromatin Immunoprecipitation (ChIP) Assay Kit
Catalog # 17-245	Acetyl-Histone H3 Immunoprecipitation (ChIP) Assay Kit
Catalog # 17-229	Acetyl-Histone H4 Immunoprecipitation (ChIP) Assay Kit
Catalog # 16-157	Protein A agarose/Salmon Sperm DNA
Catalog # 16-201	Protein G agarose/Salmon Sperm DNA

Upstate's listing of ChIP qualified antibodies can be found at http://www/upstate.com/chipabs

II. EZ-ZYME™ KIT COMPONENTS

A. Provided Kit Components (Note Storage Temperatures)

Store at 4°C:

0.5M EDTA, Catalog # 20-158. One vial containing **250 µL** of 0.5M EDTA, pH 8.0.

5M NaCl, Catalog # 20-159. One vial containing **500 µL** of 5M NaCl.

1M Tris-HCl, pH 6.5, Catalog # 20-160. One vial containing **500 µL** of 1M Tris-HCl, pH 6.5.

10X Glycine, Catalog # 20-282. One vial containing **11 mL** of 1.25M Glycine.

10X PBS, Catalog # 20-281. One vial containing **24 mL** of 10X PBS.

EZ-Zyme™ Lysis Buffer, Catalog # 20-312. One vial containing **2.0 mL** of EZ-Zyme™ Lysis Buffer.

EZ-Zyme™ Digestion Buffer, Catalog # 20-313. One vial containing **1.65 mL** of EZ-Zyme™ Digestion Buffer.

EZ-Zyme™ Stop Buffer, Catalog # 20-315. One vial containing **1.65 mL** of EZ-Zyme™ Stop Buffer.

Store at -20°C:

Protease Inhibitor Cocktail II, Catalog # 20-283. One vial containing **110 µL** of Protease Inhibitor Cocktail II in DMSO.

RNase A, Catalog # 20-297. One vial containing **600 µg** of RNase A in 60 µL sterile water.

Proteinase K, Catalog # 20-298. One vial containing **600 µg** of Proteinase K in 60 µL of 50mM Tris-HCl, pH 8.0, 10mM $CaCl_2$.

EZ-Zyme™ Enzymatic Cocktail, Catalog # 20-314. One vial containing **76U** of Enzymatic Cocktail in 38 µL of a solution containing 50% glycerol.

B. Required Materials Not Provided

Reagents

- Cells, stimulated or treated as needed for the experimental system
- 37% Formaldehyde
- Liquid nitrogen (N_2) or dry ice/alcohol bath

  (Note: care should be exercised in handling liquid nitrogen as liquid nitrogen can damage skin or tissue on contact. Use appropriate personal protective equipment when working with liquid nitrogen or the dry ice/alcohol bath)

Equipment

- Vortex mixer
- Timer
- Variable volume (2-1000 µL) pipettes + tips
- Refrigerated Microcentrifuge
- Variable temperature water bath
- Cell scraper
- Microfuge tubes, 1.5 mL
- Filter-tip pipette tips

III. CHROMATIN PREP PROTOCOL

A. *In Vivo* Crosslinking and Cell Preparation

Prior to starting this section:

Optimal conditions required for enzymatically cleaving crosslinked (or non-crosslinked in Native ChIP) DNA to ~180-360 base pairs in length need to be determined empirically. See Appendix A for a detailed protocol. Once optimal cleaving conditions have been determined, proceed with the steps below. If performing Native ChIP, harvest and count cells and proceed with Step A.8 below.

- Stimulate or treat, if necessary, adherent mammalian cells in a 150mm culture dish containing 25 mL of growth media.
    - For HeLa cells, at ~80% confluency, this is approximately 4 x 10^7 cells. This will generate a preparation of chromatin that can be used for approximately 11 separate immunoprecipitations given a final concentration of ~3.3 X 10^6 cell equivalents of sheared chromatin per immunoprecipitation.
    - Include one extra dish to be used solely for estimation of cell number.
    - Alternate culture vessels can be utilized as long as ratios of reagents added per cell number are scaled accordingly. All volumes included in the protocol reflect preparation from 4 x 10^7 cells.
- Obtain ice for incubation of PBS (see Step 3) and for incubating culture dish (see Step 6).
- Prepare 40 mL of 1X PBS (4 mL 10X PBS and 36 mL water) for each 150mm culture dish and put on ice. This will be used for washes and needs to be ice cold.
- Thaw Protease Inhibitor Cocktail II at room temperature for use in Step 3 and 10. This product contains DMSO and will remain frozen below 18.4°C.
- Prepare a liquid N_2 or dry ice/alcohol bath.

1. Add 694 µL of 37% formaldehyde (1.43 mL of fresh 18.5% formaldehyde) to 25 mL of growth media to crosslink and gently swirl or rock dish to disperse.
    - Final formaldehyde concentration is 1%.
    - Addition of formaldehyde may result in a color change.
    - Use high quality formaldehyde. Do not use if formaldehyde is past the expiration date as suggested by the manufacturer. To make fresh formaldehyde before each experiment, see Appendix B.
2. Incubate at room temperature for 10 minutes.
    - Agitation of cells is not necessary.
3. During the incubation in Step 2, remove 2.5 mL of ice cold 1X PBS to a separate tube for every dish and add 12.5 µL of Protease Inhibitor Cocktail II to each 2.5 mL of 1X PBS and put on ice. Set aside until Step 10.
4. Add 2.5 mL of 10X Glycine to each dish to quench unreacted formaldehyde.
    - Addition of glycine may result in a color change.
5. Swirl to mix and incubate at room temperature for 5 minutes.
6. Place dish on ice.
7. Aspirate medium, removing as much medium as possible, being careful not to disturb the cells.
8. Add 20 mL of cold 1X PBS to wash cells.

9. Remove PBS and repeat PBS washes, step 7 and 8.
10. Add 2.5 mL cold PBS containing Protease Inhibitor Cocktail II to dish (made in Step 3).
11. Scrape cells from each dish into one 15 mL conical tube to collect cells together.
12. Centrifuge the cells at 720 x g at 4°C for 10 minutes. Remove and discard supernatant.
    - Cell pellet can be snap frozen in a liquid N_2 or dry ice/alcohol bath and stored at -80°C at this step.

B. Nuclei Preparation and Enzymatic Digestion to Cleave DNA

Prior to starting this section:

- Thaw Protease Inhibitor Cocktail II at room temperature for use in Step 1 and 10. This product contains DMSO and will remain frozen below 18.4°C.
- Warm EZ-Zyme™ Stop Buffer to room temperature and mix gently to resuspend.
- Prepare a 37°C water bath.

1. It is recommended to prepare a master enzyme mix to perform digestion on bulk chromatin, and then aliquot digested chromatin for individual chromatin immunoprecipitations. Calculate the volume of EZ-Zyme™ Lysis Buffer needed according to the following formula: 80µl of ice-cold EZ-Zyme™ Lysis Buffer (supplemented with 0.8µl Protease Inhibitor Cocktail II) for every $4x10^6$ cell equivalents that will be processed. Store on ice until use.
    - Add Protease Inhibitor Cocktail II just prior to use.
2. If necessary, thaw the pellet of 4×10^7 cells from Section A on ice.
3. Resuspend cells in 800 µL ice-cold EZ-Zyme™ Lysis Buffer containing Protease Inhibitor Cocktail II.
4. Incubate on ice for 15-30 minutes.
5. Snap-freeze cell lysate by placing tubes in a liquid N_2 or dry-ice/alcohol bath. Make sure cell lysate is completely frozen.
    - Be sure to wear the appropriate personal protective equipment when handling liquid N_2, dry-ice and alcohol.
    - A solvent-resistant marker is recommended for labeling microfuge tubes.
6. Once cell lysate is completely frozen, place tubes in 37°C water bath until just completely thawed.
7. Repeat steps 5 and 6 two more times.
8. Incubate on ice for at least 5 minutes.
9. Centrifuge cell lysate at 2500 x g for 10 minutes at 4°C to pellet nuclei. Remove supernatant and discard.

10. During the centrifugation in Step 9, prepare the diluted EZ-Zyme™ Enzymatic Cocktail containing Protease Inhibitor Cocktail II for each sample by:
    - Adding 0.6 µL of Protease Inhibitor Cocktail II to 60 µL of ice-cold EZ-Zyme™ Digestion Buffer per $4x10^6$ cells. Store on ice.
    - Adding 0.4 µL (0.8U) of EZ-Zyme™ Enzymatic Cocktail to 60 µL of EZ-Zyme™ Digestion Buffer containing Protease Inhibitor Cocktail II per $4x10^6$ cells.
        - It is recommended to use a P2 or P10 pipetteman to remove the EZ-Zyme™ Enzymatic Cocktail.
        - If desired, prepare a control solution of 60 µL of ice-cold EZ-Zyme™ Digestion Buffer containing Protease Inhibitor Cocktail II with no EZ-Zyme™ Enzymatic Cocktail for analysis of uncleaved DNA.
11. Pre-warm the diluted EZ-Zyme™ Enzymatic Cocktail at 37°C for 5 minutes.
12. Add 600 µL of the prewarmed diluted EZ-Zyme™ Enzymatic Cocktail, to the tube of pelleted nuclei (containing $4x10^7$ cell equivalents, from Step 9). Resuspend nuclei by gently pipetting up and down.
13. Incubate at 37°C for 10 minutes.
    - Mix 3-4 times by gently flicking the tube during the incubation to resuspend nuclei.
14. Place the tube on ice and add 600 µL of EZ-Zyme™ Stop Buffer (or 60 µL of EZ-Zyme™ Stop Buffer per $4x10^6$ cells) .
15. Incubate on ice for 10 minutes.
16. Centrifuge samples at 12,000-15,000 x g for 15 minutes at 4°C to remove insoluble material.
17. Collect supernatant containing cleaved chromatin and aliquot 100 µL each ($3.3x10^6$ cell equivalents) into the new tubes on ice.
    - The cleaved chromatin can be used for chromatin immunoprecipitation immediately or frozen at -80°C for up to a few months prior to use.
    - The amount of chromatin used for chromatin immunoprecipitation may need to be empirically determined by the user.
    - For users who intend to use Upstate's EZ ChIP™ kit, Cat# 17-371, for chromatin immunoprecipitation; join the chromatin immunoprecipitation protocol at Section C.
    - For users who intend to use Upstate's Chromatin Immunoprecipitation (ChIP) Assay Kit, Cat #17-295; join the chromatin immunoprecipitation protocol at Part B, Step 2.

Figure A: Chromatin Digestion

Chromatin from formaldehyde-crosslinked Hela cells was prepared and digested by following Section A, Section B and Appendix A (steps 12-17). 20 μL of digested chromatin (lane 2) was electrophoresed through a 2% agarose gel and stained with ethidium bromide. Lane 2 shows that the majority of the chromatin has been digested to lengths of mono- or dinucleosomes.

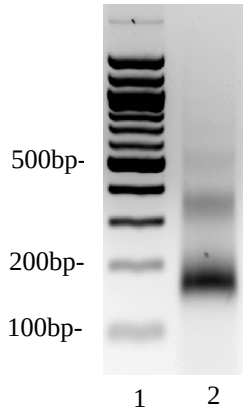
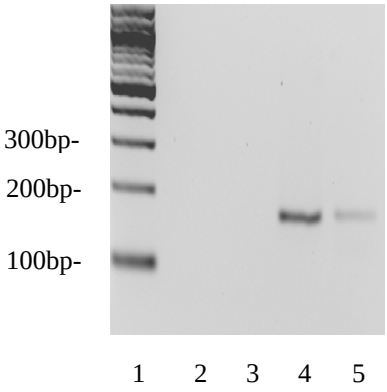


Figure B: PCR Analysis of Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed using digested chromatin from HeLa cells and either anti-Acetylated Histone H3 (Catalog # 06-599) or Normal Rabbit IgG (Catalog # 12-370) as the immunoprecipitating antibody. Purified DNA was then analyzed by PCR using Control Primers specific for the β-actin promoter. PCR product was observed in the anti-Acetylated Histone H3 ChIP (lane 4) and not in the Normal Rabbit IgG ChIP (lane 3). β-actin promoter primer specificity was also observed in the input (lane 5) and not in the "No DNA" PCR control (lane 2). GAPDH primers (Catalog #22-004) from EZ-ChIP (Catalog 17-371) produced similar results (data not shown).



IV. APPENDIX A: Optimization of DNA Cleavage

Optimal conditions required for cleaving cross-linked (or non-crosslinked) DNA to 180-360 base pairs in length depend on the cell type and cell concentration as well as on the concentration of EZ-Zyme™ Enzymatic Cocktail. Below is an optimization protocol to determine the optimal units of EZ-Zyme™ Enzymatic Cocktail required for a given cell type and concentration.

1. Generate a nuclei pellet from $4x10^6$ cell equivalents by following Sections A1-B8. Prepare enough individual tubes of nuclei for the number of conditions tested.
2. Be sure to keep the samples on wet ice at all times.
3. For each nuclei pellet to be tested, prepare ice-cold EZ-Zyme™ Digestion Buffer containing Protease Inhibitor Cocktail II.
   - Add 0.6 μL of Protease Inhibitor Cocktail II to 60 μL of ice-cold EZ-Zyme™ Digestion Buffer for each nuclei pellet to be tested.
4. Vary the amount of EZ-Zyme™ Enzymatic Cocktail by adding different units (e.g., 0U, 0.4U, 1U, 2U, 4U) to each tube containing 60 μL EZ-Zyme™ Digestion Buffer containing Protease Inhibitor Cocktail II prepared in the previous step.
   - It may be necessary to dilute the EZ-Zyme™ Enzymatic Cocktail for use in this step. If so, use EZ-Zyme™ Digestion Buffer containing Protease Inhibitor Cocktail II.
   - It is recommended to have a zero (0) U enzyme for analysis of uncleaved DNA.
5. Warm the tubes containing the diluted EZ-Zyme™ Enzymatic Cocktail for 5 minutes at 37°C.
6. Resuspend each nuclei pellet with the prewarmed diluted EZ-Zyme™ Enzymatic Cocktail containing the appropriate amount of EZ-Zyme™ Enzymatic Cocktail.
7. Incubate at 37°C for 10 minutes.
   - Mix 3-4 times by gently flicking the tube during the incubation to resuspend nuclei
8. Place tubes on ice and add 60 μL EZ-Zyme™ Stop Buffer.
9. Incubate on ice for at least 10 minutes.
10. Centrifuge samples at 12,000-15,000 x g for 15 minutes at 4°C to remove insoluble material.
11. Collect supernatant containing cleaved chromatin and place in new tube.
12. Remove 12 μL of the uncleaved and cleaved chromatin from each condition to a fresh tube.
    - The remaining cleaved chromatin can be stored at -80°C if the analysis in the steps below needs to be repeated or for chromatin immunoprecipitation, if desired.
13. To all the 12 μL samples (uncleaved and cleaved), add 35 μL ddH_2O and 2 μL 5M NaCl.
14. Incubate at least 4-5 hours to overnight at 65°C to reverse the DNA – protein crosslinks.
15. Add 1 μL of RNase A and incubate for 30 minutes at 37°C.
    - Alternatively, the RNase A can be added at Step 16 to save time.
16. Add 1 μL 0.5M EDTA, 2 μL 1M Tris-HCl and 1 μL Proteinase K and incubate at 45°C for 1-2 hours.

17. Load 10 μL and 20 μL on a 2-4% agarose gel with a 100bp DNA marker for each sample.
    - Loading different amounts helps to avoid under- or over-loading.
18. Observe which of the cleaving conditions gives a smear of DNA in the range of 180bp-360bp. See Figure A for an example.
19. Repeat optimization of the cleaving conditions if the results indicate that the resulting DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that the user does not alter the cell concentration or volume of lysate per microfuge tube for subsequent chromatin preparations.

V. APPENDIX B: Preparation of Fresh 18.5% Formaldehyde

This recipe is for making fresh 18.5% formaldehyde from powdered paraformaldehyde. Use appropriate safety precautions when performing this procedure.

1. Add 4.8 mL of distilled water to a 50ml conical plastic tube.
2. Add 0.925g paraformaldehyde.
3. Add 35 μL of 1N KOH.
4. Cap tube tightly and place in a 400-600 mL glass beaker filled with approximately 200 mL of water.
5. Microwave beaker with tube until water in beaker begins boiling.
6. Remove beaker and vortex tube until paraformaldehyde begins dissolving.
7. Repeat steps 5 & 6 until paraformaldehyde is completely in solution. This step may need to be repeated several times.
8. Store on ice until cool.
9. Use immediately.

References:

Kouskouti A, Talianidis I. (2005). *EMBO J.* **24**: 347-357.
Ladenburger EM *et al.* (2002). *Mol Cell Biol* **22**:1036-1048.
O'Neill LP and Turner BM. (1995). *EMBO J*. **14**: 3946-3957.

VI. TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Cell Lysis	Inefficient disruption of cells	It is important to have enough lysis buffer per cell concentration. Monitor the cell lysis using trypan blue under the microscope. Nuclei release can be further augmented by dounce homogenization.
Enzymatic Digestion	Not enough soluble chromatin after digestion	Perform optimization of digestion by varying the amount of enzyme used and the duration of digestion. Modify the crosslinking condition. Efficiency of enzymatic digestion decreases as the degree of crosslinking increases. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.
Immunoprecipitation and PCR	No PCR product or No difference in quantity between PCR product from Negative control and Target Protein IPs	In addition to referring to the TROUBLESHOOTING section in the instruction manual of EZ ChIP (17-371), please consider the following possibilities: • Whereas majority of the digested chromatin are mono- and/or di-nucleosome, primers are designed too far apart to amplify target DNA sequence from mono- or di-nucleosome chromatin templates. Solution: design primers to produce a shorter amplicon, preferably below 150 bps. Note that primers producing an amplicon below 150 bps may also be used directly in Quantitative PCR. • Occasionally, in order to design primers that provide the highest resolution, the positions of the nucleosomes in the target region need to be determined. This can be accomplished by using techniques such as indirect end-labeling of chromatin partially digested with micrococcal nuclease. • Factors that are recruited to the nucleosomal free regions are not the suitable targets for ChIP with enzymatic shearing.