Miltenyi Biotec

Chromatin immunoprecipitation (ChIP) using the MACSflex™ MicroBeads

Contents

1. Description

1.1 Background information

Chromatin immunoprecipitation (ChIP) is a method used to characterize proteins that are associated with specific regions of genomic DNA. It offers a versatile solution by combining the specificity of immunoprecipitation and the sensitivity of polymerase chain reaction (PCR). However, ChIP can be technically challenging and difficult to validate without well-proven reagents. In ChIP, intact cells are fixed using formaldehyde, which cross-links and therefore stabilizes protein/DNA interactions. DNA is then sheared into small uniform fragments and the DNA/protein complexes are immunoprecipitated using an antibody directed against the DNA-binding protein of interest. Following immunoprecipitation, the DNA is washed, cross-linking is reversed, and the proteins are removed by Proteinase K treatment. The DNA is then purified before a PCR reaction is performed to identify the presence of a DNA fragment of interest. If the fragment is enriched, then the DNA-binding protein had bound this fragment. Current ChIP protocols are largely based on an indirect immunoprecipitation using DNA-binding protein specific antibodies and Protein A or G immobilised to agarose matrices. MACS® Technology offers multiple advantages of rapid binding kinetics, extremely efficient wash steps and reduced centrifugation requirements to improve and streamline ChIP performance and enable both the ChIP procedure and sample analysis to be carried out in one day. The use of MACSflex™ MicroBeads further improves performance by reducing background binding.

Downstream assays include PCR, quantitative polymerase chain reaction (qPCR), and microarray analysis (ChIP-on-ChIP).

1.2 Applications

- Detection of transcriptionally active genes.
- Identification of transcription factor binding sites.
- Identification of transcription or DNA replication complexes.

1.3 Reagent and instrument requirements

- **Antibody-coupled MACSflex™ MicroBeads (# 130-105-806, # 130-105-805)**
- **µ Columns (# 130-042-701)**
- **25× protease inhibitors (PI), EDTA free, 2 mL** (e.g. Complete, EDTA-free, Roche # 11873580001)
  ▲ After dissolving, dispense in 0.25 mL aliquots, store at –20 °C.
- **10 mg/mL herring sperm DNA** (HSDNA, e.g. Sigma # D6898 1G)
  ▲ Store at 4 °C.
  ▲ **Note:** Never vortex solution otherwise DNA will become fragmented.
- **2 M glycine, pH 7.5, 100 mL**
- **SDS-lysisbuffer, 100 mL**
  1% SDS, 10 mM EDTA, 50 mM Tris (pH 8.1)
  ▲ Store at room temperature.
- **ChIP dilution buffer, 100 mL**
  0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl
  ▲ Store at 4 °C.
- **Low salt immune complex wash buffer, 100 mL**
  0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl
  ▲ Store at room temperature.
- **High salt immune complex wash buffer, 100 mL**
  0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl
  ▲ Store at room temperature.
- **LiCl immune complex wash buffer, 100 mL**
  0.25 M LiCl, 1% IGEPAL®-CA630 (NP-40), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)
  ▲ Store at room temperature.
- **TE buffer, 100 mL**
  1 mM EDTA (pH 8.0), 10 mM Tris-HCl
  ▲ Store at room temperature.
- **Elution buffer, 10 mL**
  1% SDS, 0.1 M $NaHCO_3$
  ▲ **Note:** The buffer has to be freshly prepared. Add 1 mL 10% SDS and 84.01 mg $NaHCO_3$ to 9 mL sterile autoclaved double-distilled water. Microwave the solution until boiling. Care should be taken as SDS induces rapid foam generation on boiling.

SP006401

Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

Miltenyi Biotec Inc.
2303 Lindbergh Street, Auburn, CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871, Fax +1 530 888 8925
macs@miltenyibiotec.com

- **16% formaldehyde, without methanol** (e.g. Polysciences, Inc., USA)
- **10% SDS stock solution**
- **5 M NaCl**
- **Proteinase K**
- **Ultra pure distilled water**
- **DNA purification kit** (e.g. NucleoSpin® Extract II Kit, Macherey-Nagel)
- **Thermomixer, heated orbital shaker**
- **Sonicator**

2. Protocols

2.1 Cell fixation

1. Harvest cells while growing in the logarithmic phase.
2. Place cells in a suitable tube.
3. Centrifuge at 300×g for 10 minutes at 4 °C. Aspirate supernatant completely.
4. Resuspend cell pellet with 5 mL 4 °C PBS per 10^7 cells.
5. For formaldehyde fixation, add 156 µL formaldehyde (16% stock) per 5 mL PBS to a final concentration of 0.5% formaldehyde and vortex.
6. Incubate for 4 minutes at room temperature on an orbital shaker.
7. To stop the fixation, add 312.5 µL glycine (2 M, pH 7.5) per 5 mL PBS to a final concentration of 125 mM glycine and vortex.
8. Incubate for 5 minutes at room temperature on an orbital shaker.
9. Centrifuge at 300×g for 10 minutes at 4 °C. Aspirate supernatant completely.
10. Resuspend cell pellet in 20 mL 4 °C PBS and vortex.
11. Centrifuge at 300×g for 10 minutes at 4 °C. Aspirate supernatant completely.
12. Resuspend cell pellet in 50 µL SDS-lysisbuffer per 10^6 cells.
13. Add 2 µL of 25× protease inhibitors (PI) per 50 µL SDS-lysisbuffer and vortex.
14. Incubate for 10 minutes on ice.
15. Divide the solution into aliquots (50 µL per 10^6 cells each).

▲ Store at –20 °C or proceed directly to DNA shearing (refer to 2.2).

2.2 Cell sonication – DNA shearing

▲ Effective shearing of the genomic DNA is essential for a successful ChIP reaction. Refer to 4. Appendix for a detailed guide to optimization of the sonication procedure.

▲ Suggested sonication settings were optimised using a Bandelin Sonopuls with a Sonotrode MS73.

▲ Before starting, add HSDNA to the antibody-coupled MACSflex MicroBeads to block non-specific binding sites. Use 0.1 µL HSDNA (10 mg/mL) per 10 µL MicroBeads. Incubate on ice during the sonication steps, or for a minimum of 30 minutes. Do not vortex.

1. For each reaction, add 32 µL protease inhibitors (PI) and 8 µL HSDNA (c=10 mg/mL) to 800 µL ChIP dilution buffer.
2. Add 150 µL cold ChIP dilution buffer (including PI and HSDNA) carefully to 50 µL fixed cells (10^6 cells/50 µL) without producing bubbles. Do not vortex.
3. If using a water bath sonicator that is validated for shearing DNA, fill with ice-cold water. If using a probe sonicator, place the tube containing the fixed cells on a cool pack or in an ice bucket before sonicating.
4. Sonicate the cells at 30% power for approximately 10 seconds. Allow the cell lysate to cool on ice for 30 seconds. Repeat sonication and cooling step for a further 5–10 cycles.
5. Centrifuge at 13,000×g for 10 minutes at 4 °C.
6. Transfer supernatant to a fresh tube. Proceed to 2.3 ChIP.

2.3 ChIP

▲ Use 10–20 µL antibody-coupled MACSflex MicroBeads (containing 1–2 µg antibodies), pre-blocked with HSDNA (refer to 2.2) per ChIP reaction.

▲ Use room temperature wash buffers.

1. Adjust volume of the supernatant from 2.2, step 5, to 500 µL with ChIP dilution buffer, supplemented with PI and HSDNA, and mix. Remove 20 µL per reaction and store separately as original fraction.
2. Add the pre-incubated MicroBeads (10–20 µL of antibody-coupled MACSflex MicroBeads containing 1–2 µg antibodies) per reaction and mix.
3. Incubate for 2 hours at 4 °C.
4. Place µ Column in the magnetic field of the µMACS Separator and a suitable waste container under the µ Column. Prepare the µ Column by rinsing with 200 µL ChIP dilution buffer including PI and HSDNA. Columns are "flow stop" and do not run dry.
5. Apply the 0.5 mL suspension immediately to the µ Column. Let the liquid flow through. Magnetically labeled protein/DNA complexes are retained in the µ Column.
6. Wash the µ Column with 1 mL low salt immune complex wash buffer. Let the liquid flow through.
7. Wash the µ Column with 1 mL high salt immune complex wash buffer. Let the liquid flow through.
8. Wash the µ Column with 1 mL LiCl immune complex wash buffer. Let the liquid flow through.
9. Wash the µ Column with 1 mL TE buffer. Let the liquid flow through.
10. If a drop is present on the column tip, this should be removed by contacting the column tip with the waste tube or by using a fresh pipette tip.
11. Place a fresh tube under the µ Column.
12. Add 50 µL of freshly prepared, hot elution buffer to the µ Column and collect eluate. Incubate for 1 minute.
13. Repeat step 12 a further 3 times to collect a total, combined eluate of 200 µL.

SP009601

2.4 Defixation

1. Add 180 µL hot elution buffer to 20 µL of the original fraction (refer to 2.3, step 1).
2. Add 8 µL NaCl (5 M) and 1 µL proteinase K to both the original fraction and eluate (200 µL each).
   ▲ **Note:** Close cap of the tube firmly.
3. Incubate for 2 hours at 65 °C while shaking at 500 rpm on an orbital shaker.

2.5 DNA purification

For a detailed protocol for DNA purification please refer to the respective manufacturer user manual.

▲ If using NucleoSpin® Extract II Kit (Macherey-Nagel), binding buffer NT has to be replaced with NTB buffer.

▲ Eluat can be stored at –20 °C.

3. Troubleshooting

General optimisation and control of ChIP procedure

To optimise the complete ChIP procedure, it is recommend using a constitutively active target such as the RNA polymerase II binding site upstream of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and an antibody specific for the RNA polymerase II. As negative control, an isotype control antibody against an irrelevant antigen should be used. Quantitative PCR analysis of the region around the RNA pol II binding site after ChIP and comparison with the original, non-enriched fraction can be used to assess efficiency of specific target enrichment. Background, non-specific enrichment of sequences can be assessed by analysing a region of genomic DNA that is at least 2000 bp distant from the target site. In the case of GAPDH, the 6000 bp distant chromosome condensation-related SMC-associated protein (CNAP1) locus can be analyzed in the same original and eluate fractions. Suggested primers for quantitative PCR analysis of human samples are:

GAPDH FWD 5′-TACTAGCGGTTTTACGGGCG-3′	
GAPDH REV 5’-GATGCGGCTGACTGTCGAAC-3’	180 bp fragment
CNAP1 FWD 5′-ATGGTTGCCACTGGGGATCT-3′	
CNAP1 REV 5′-TGCCAAAGCCTAGGGGAAGA-3′	174 bp fragment

▲ To improve the accuracy of downstream quantitative PCR analysis, it is recommend carrying out assays in duplicate or triplicate (starting from section 2.2).

Incorrect sonication

Excessive foam generation during sonication will decrease DNA shearing efficiency. Ensure that no air bubbles are present before sonication.
Do not touch tube wall with sonicator probe.
Damaged sonication probe. Check probe tip for the presence of scratches or pits. Replace if necessary.

Incorrect number of sonication cycles. Optimize sonication process (refer to 4. Appendix).

Lack of sample cooling during sonication process. To prevent sample overheating and possible protein denaturation, it is essential to cool the sample during the sonication process (use of ice bath or cool pack to cool tube) and to store samples on ice for at least 30 seconds between sonication pulses.

Incorrect fixation

If samples are not adequately fixed, the proteins may dissociate from the DNA during processing, leading to loss of target signal.

Only high quality, methanol-free formaldehyde that has been filled in an oxygen-free atmosphere should be used for reproducible fixation performance (e.g. 16% Formaldehyde, methanol fee, Ultra Pure, Polysciences Inc.). Aliquots can be stored at –20 °C for 2–3 months.

If samples are too strongly fixed, antibody epitopes may no longer be recognized resulting in poor IP performance. Overfixation of the chromatin may also lead to cross-linking of distant chromatin regions which could reduce sonication performance and increase background signal. In addition it may not be possible to completely reverse the fixation of the chromatin after the ChIP procedure resulting in loss of DNA during the DNA purification step.

4. Appendix

Optimisation of sonication process

For a successful ChIP reaction, it is essential that the genomic DNA is sheared into short fragments that are between 200–1000 bp in size. If the fragments are too large, ChIP is inefficient and there is a loss of resolution which may lead to false positive results. If the genomic DNA is too extensively sonicated, the nucleosome structure can be damaged and proteins denatured.

1. Prepare 2×10^7 fixed cells as described in section 2.1, resulting in 20 cell aliquots that can be stored at –20 °C.
2. Prepare samples for sonication as in section 2.2, steps 1–3 but using ChIP dilution buffer without supplements.
3. Sonicate 3 samples as in section 2.2, step 4 but with 5, 10, or 15 cycles, respectively.
4. Centrifuge at 13,000×g for 10 minutes at 4 °C.
5. Transfer supernatant to a fresh tube and add 8 µL NaCl (5 M) and 1 µL proteinase K.
   ▲ **Note:** Close cap of the tube firmly.
6. Incubate for 2 hours at 65 °C while shaking at 500 rpm on an orbital shaker.

SP006101

7. Purify DNA using DNA Purification Kit. If using NucleoSpin® Extract II Kit (Macherey-Nagel), binding buffer NT has to be replaced with NTB buffer.
8. Analyse DNA by 2% agarose gel electrophoresis. Use a 50bp DNA ladder. When DNA has been optimally sheared, it should have an even distribution of fragments from 200–1000 bp (figure 1).
9. If required, repeat optimisation on further cell aliquots using different power settings for the sonicator.

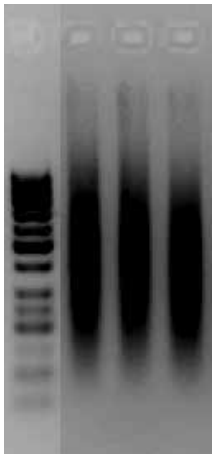

Figure 1: Example of optimally sheared chromatin prepared from 1×10^6 human embryonic kidney (HEK) cells according to the protocol in 4. Appendix.

5. References

1. Schiessl, K. *et al.* (2014) *Arabidopsis* JAGGED links floral organ patterning to tissue growth by repressing Kip-related cell cycle inhibitors. Proc. Natl. Acad. Sci. U.S.A. 111: 2830–2835.
2. Schlereth, A. *et al.* (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature 464: 913–917.
3. Cole, M. *et al.* (2009) *DORNRÖSCHEN* is a direct target of the auxin response factor MONOPTEROS in the *Arabidopsis* embryo. Development 136: 1643–1651.

All protocols and data sheets are available at **www.miltenyibiotec.com**.

SP006101