APPLICATION NOTE

ChIP-Seq Using Nanogram Amounts of Immunoprecipitated DNA

I. Introduction

The Ovation® Ultralow Library Systems provide a simple, fast and scalable solution for producing NGS libraries from as little as 1.0 ng of DNA. Researchers have successfully used this library construction system for a wide range of applications, including target capture, RNA-Seq, whole genome sequencing, and ChIP-Seq.

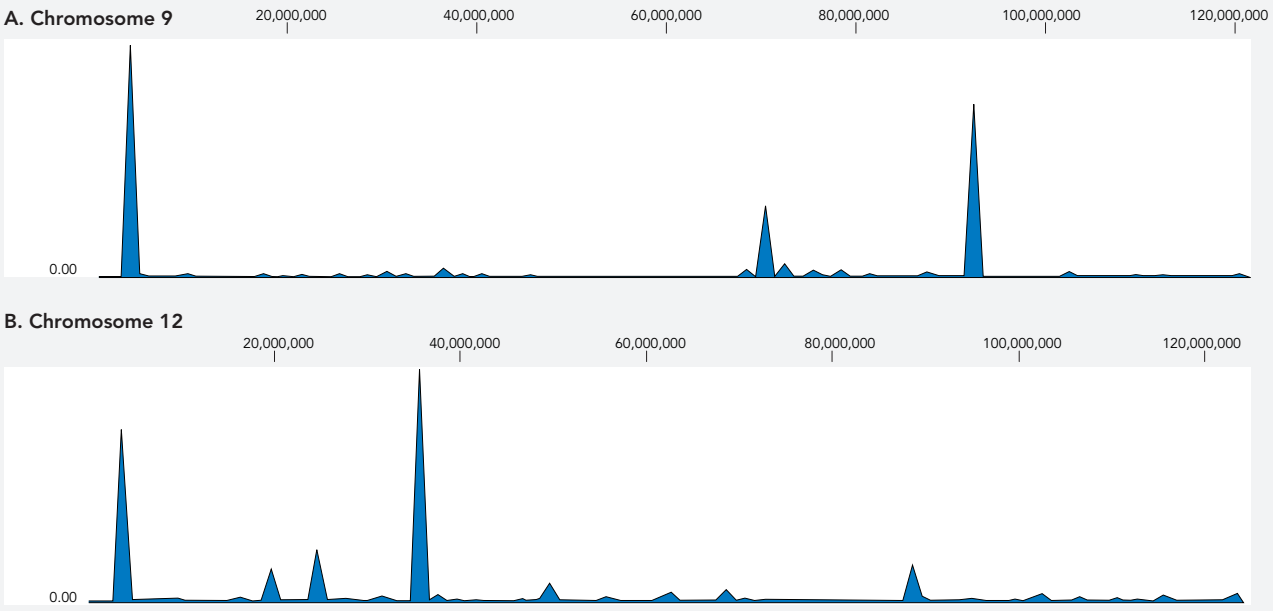
For ChIP-Seq in particular, the low input requirements of the Ovation Ultralow Library System help conserve precious sample materials, enabling researchers to ask more specific biological questions by targeting particular cell populations without compromising results.

This application note provides a validated chromatin immunoprecipitation protocol that has been used in conjunction with the Ovation Ultralow Library System for ChIP-Seq. The multiplexing capability of the Ovation Ultralow Library System used in this protocol provided an efficient and cost effective means for identification of protein binding domains using as little as 0.5 ng to 10 ng of ChIP DNA.

Low-Input ChIP-Seq

Figure 1 shows sequencing reads specifically aligned to transcription factor binding sites found on chromosomes 9 and 12 in the mouse genome. This representative data illustrates the high quality of data generated using as little as 10 ng of starting DNA from chromatin immunoprecipitation as input into the Ovation Ultralow DR Multiplex System 1-8 (Part No. 0330-32).

FIGURE 1. **Mouse ChIP results identify transcription factor binding sites with 10 ng ChIP DNA**



Mouse chromatin was immunoprecipitated using an antibody specific to a mouse transcription factor with binding sites on chromosomes 9 (A) and 12 (B). Of the recovered DNA, 10 ng was used as input into the Ovation Ultralow DR Multiplex System 1-8 and the libraries sequenced on an Illumina MiSeq with 50 bp single end reads. Approximately 17 million reads were obtained for each sample. *Data provided by researchers at the University of California, San Francisco.*

NuGEN®
A Tecan Group company

II. Chromatin Immunoprecipitation Protocol

Based on the protocol cited in O'Green et. al., referenced below.

Required Materials

Equipment

- Cell scraper
- Shaker
- Diagenode Biorupter or Covaris Fragmentor
- Centrifuge set at 4°C
- Water bath
- Nanodrop or Qubit for quantification of double-stranded DNA samples
- Glass dounce homogenizer (type B)

Reagents

- Ice
- Ice-cold 1X PBS
- 16% formaldehyde (methanol-free ampules)
- 2.5 M glycine
- Halt Protease Inhibitor cocktail (Thermo Scientific; 100X)
- DNase-free RNase A (Fermentas; 10 mg/mL)
- 5 M NaCl (autoclaved)
- IGEPAL CA-630

Buffers

Note: Add protease inhibitors after filter sterilization and just prior to use.

- **Cell Lysis Buffer** (store at room temp): 5 mM PIPES pH 8, 85 mM KCl. Add IGEPAL (NP-40) fresh each time to give a final concentration of 1% (10 µL/mL). Warm buffer in 37°C water bath and vortex briefly to mix. After mixing, place buffer with IGEPAL on ice to cool and then add protease inhibitors
- **Nuclei Lysis Buffer** (store at room temp): 50 mM Tris-HCl pH 8, 10 mM EDTA, 1% (w/v) SDS. Place buffer on ice right before use to avoid precipitation of SDS and add protease inhibitors just prior to use
- Elution Buffer (store at room temp): 50 mM $NaHCO_3$, 1% (w/v) SDS
- **IP Dilution Buffer** (store at 4C): 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) IGEPAL, 0.25% (w/v) deoxycholic acid, 1 mM EDTA pH 8. Add protease inhibitors just prior to use. This buffer is used to adjust the salt and SDS concentrations for the immune-precipitation step
- **IP Wash Buffer 1** (store at 4°C): same as IP Dilution Buffer, but without protease inhibitors
- **IP Wash Buffer 2** (store at room temp): 100 mM Tris–HCl pH 9, 500 mM LiCl, 1% (v/v) IGEPAL, 1% (w/v) deoxycholic acid
- **IP Wash Buffer 3** (store at room temp): 100 mM Tris–HCl pH 9, 500 mM LiCl, 150 mM NaCl, 1% (v/v) IGEPAL, 1% (w/v) deoxycholic acid
- **Elution Buffer** (store at room temp): 50 mM $NaHCO_3$, 1% (w/v) SDS

Supplies and Labware

- 15 mL Falcon tubes (resistant to cracking in liquid nitrogen)
- Liquid nitrogen
- Agencourt® Ampure XP DNA purification beads (and magnet)
- Magnetic protein G beads (e.g., Dynabeads)

Crosslinking

1. Grow the cells to desired confluency in 10-cm plates.
2. Add 625 µL of 16% formaldehyde (final concentration = 1%) directly to the plates that contain cells to fix.
3. Shake at room temperature for 10–15 minutes.
4. Quench with 125 mM glycine (530 µL of 2.5 M glycine) to stop the formaldehyde fixing.
5. Shake at room temperature for ~5 minutes.
6. Pour the media/formaldehyde/glycine solution into a formaldehyde waste container.
7. Wash twice with 1X cold PBS; dump wash into waste container each time.
8. Pipette ~2 mL cold PBS onto each plate, scrape the cells and collect in a liquid nitrogen-resistant 15 mL conical tube. If desired, wash the plates one more time with ~2 mL cold PBS to collect any remaining cells.
9. Centrifuge at 430 rcf for 5 minutes at 4°C.
10. Aspirate the supernatant completely. Proceed to cell lysis step or snap-freeze the cells in liquid nitrogen.

Cell Lysis and Solubilization of Chromatin

If using frozen cells, thaw on ice. Keep all cells and chromatin samples on ice at all times.

1. Prepare 1 mL cell lysis buffer per 1 x 10^7 cells.
    a. Add 10 µL IGEPAL per mL of Cell Lysis Buffer. Agitate or incubate without stirring for 5 minutes at 37°C to dissolve.

b. Cool on ice, then add 10 µL of 100X Halt Protease Inhibitor cocktail.

2. Add the buffer to the cells and resuspend. No clumping should be visible.
3. Incubate on ice for 15 minutes.
4. Homogenize the cells using a glass dounce homogenizer (type B) to release the nuclei (20 total strokes on ice).
5. Centrifuge cells at 430 rcf for 5 minutes at 4°C.
6. Start cooling the Nuclei Lysis Buffer (1 mL + 10 µL Halt Protease Inhibitor cocktail).

   Note: Do not cool too early — doing so may cause the the SDS to precipitate.
7. Discard the supernatant and resuspend the nuclear pellet in Nuclei Lysis Buffer plus protease inhibitors (~20 µL/10^6 cells).
8. Incubate on ice for 30 minutes.
9. **Optional:** Flash-freeze the cells to help break open the nuclei more efficiently (this step is critical if the homogenization step is not performed). After incubating the nuclei in Nuclei Lysis Buffer for 30 minutes, flash-freeze samples in liquid nitrogen and thaw at room temperature. Once thawed, transfer to ice; do not allow samples to warm up to room temperature. Proceed to sonication.
10. Sonicate using the Biorupter to achieve an average chromatin length of 150–500 bp. Sonication time varies depending on cell type, lysis buffer, etc. and will need to be optimized. TPX tubes from Diagenode may increase the efficiency of shearing.
11. Centrifuge at 10,000 rcf for 10 minutes at 4°C.
12. Carefully transfer the supernatant (sonicated chromatin) to new tubes, making sure to avoid transferring the cell debris.
13. Keep the sonicated chromatin on ice (4°C) while performing quantitation and determining shearing efficiency.
14. At this point the remaining chromatin can be flash frozen after pooling the samples and removing 20 µL (or the equivalent to 100,000–200,000 cells) for analysis.
15. Take an aliquot of chromatin sample and add ChIP elution buffer to a total volume of 100 µL.
16. Add 12 µL of 5 M NaCl to give a final salt concentration of 0.54 M.
17. Boil the samples in a water bath for 20 minutes to reverse crosslinks.
18. Allow the samples to cool, add 1 µL DNase-Free RNase (10 mg/mL) and incubate for 20 minutes at 37°C.
19. Purify the DNA using Ampure XP beads, elute in 28 µL nuclease-free water. Measure the chromatin concentration using a NanoDrop and calculate the chromatin yield.
20. Run the remaining chromatin on a 2% agarose gel for 1 hour at 110 V to visualize average chromatin size.

Chromatin Immunoprecipitation

1. Save 10% of the input (or a volume corresponding to 500 ng of chromatin). Store at –20°C or keep at 4°C until the next day.
2. Dilute chromatin 1:4 with ice-cold IP Dilution Buffer containing protease inhibitors (1 volume chromatin + 4 volumes IP Dilution Buffer – e.g., 200 µL chromatin + 800 µL IP Dilution Buffer)

   Note: For histone marks covering a small portion of the genome displaying sharp peaks (e.g., H3K4me3) 1 µg chromatin is typically used. For spreading histone marks that cover large portions of the genome (e.g., H3K9me3 or H3K36me3), use 5 µg of chromatin.
3. Wash the magnetic Protein G beads 2 times with IP Dilution Buffer (with an equal or greater volume of beads), and resuspend the beads in an equal volume of IP Dilution Buffer after washing.
4. Add 20 µL of the washed beads to the chromatin extract. Rotate for at least 2 hours at 4°C.
5. Remove the beads from the extract with a magnet and transfer the extract to tubes containing 1–5 µg of antibody.
6. Record the catalog and lot numbers of the antibodies used.
7. Incubate 8–16 hours (overnight) on a rotating platform at 4°C.

Next day:

1. Add 40 µL magnetic of pre-washed Protein G beads to each ChIP sample ranging from 1–5 µg chromatin starting material.
2. Incubate on a rotating platform for at least 2 hours at 4°C.
3. At room temperature, allow the beads to settle for 1 minute on a magnet separation rack. Carefully remove the supernatant and do not disturb the beads. The rest of the wash steps are performed at room temperature.

4. Wash the beads 2 times with IP Dilution Buffer. Put the samples on a rotator for 5 minutes each time to resuspend and wash.
5. Wash the beads 2 times with IP Wash Buffer 2. Remove the supernatant entirely between washes. Be careful not to cross-contaminate the samples.
6. Wash 1 time with the high-stringency IP Wash Buffer 3. Discard all wash solutions.
7. Elute the antibody/chromatin complexes by adding 100 µL elution buffer per ChIP sample.
8. Shake the samples on a vortexer for 30 minutes (a heated vortexer, set to 65°C, is preferred). Do the same for the "Input" sample. Add 80 µL of ChIP elution buffer to the input sample for 100 µL total.
9. After 30 minutes of heated vortexing, allow beads to settle onto the magnet. Carefully transfer the supernatant containing the antibody/chromatin complexes to a siliconized tube.
10. Add 12 µL of 5 M NaCl per 100 µL elution buffer mix to give a final concentration of 0.54 M NaCl (including the input sample).
11. Incubate all samples in a 67°C water bath overnight to reverse the formaldehyde crosslinks.

DNA Purification

1. Remove the samples from the 67°C water bath and allow to cool.
2. Add 1 µL of RNase A (10 mg/mL) to each sample. Incubate the samples at 37°C for 20–30 minutes.
3. Purify the DNA using Agencourt Ampure XP beads.
4. Elute each sample with 30 µL of EB Buffer (10 mM Tris).
5. Quantitate the samples using a NanoDrop or Qubit.
6. Use the quantified samples as inputs into the NuGEN Ovation Ultralow Library Systems.

References

O'Geen, H, Echipare, L, Farnham, PJ (2011). Using ChIP-seq Technology to Generate High-Resolution Profiles of Histone Modifications. *Methods in Molecular Biology.*

NuGEN®
A Tecan Group company

Tecan Genomics, Inc.

Headquarters USA
900 Chesapeake Drive
Redwood City, CA 94063 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
cservice-gn@tecan.com
techserv-gn@tecan.com

Europe
P.O. Box 109
9350 AC Leek
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe-gn@tecan.com

Worldwide
For our international distributors contact information, visit our website
www.nugen.com

M01348 v2.1