Farnham Lab MicroChIP Protocol
(Acevedo et al. 2007; In Press, Biotechniques)

Note: This protocol should be used for $1x10^4$ to $1x10^5$ cells.

Day 0: Summary: Staph A cells, which are used to precipitate the antibody-chromatin complexes, are prepared in large batches, aliquoted, and stored in liquid nitrogen prior to the pre-treatment (blocking) required for ChIP assays (see below). Staph A cells are heat-killed, formalin-fixed Staphylococcus aureus cells that bear a high cell-surface density of protein A. They are useful as a solid-phase IgG-binding reagent due to the high affinity interaction of protein A with the Fc domain of IgG. These cells work best when the antibody is human (IgG1, IgG2, IgG4), rabbit IgG (all isotypes), or mouse (IgG2a, IgG2b, IgG3). If other isotypes are used, a bridging secondary antibody can be employed.

Preparation of Staph A Cells

Resuspend 1 gram of lyophilized Staph A cells (Pansorbin, CalBiochem 507862) in 10 mL of 1X dialysis buffer. Centifuge at 9,000xg for 5 minutes at 4°C. Repeat. Resuspend in 3 mL of 1X PBS plus 3% SDS and 10% BME. Boil for 30 minutes. Centifuge at 9,000xg for 5 minutes. Wash in 1X dialysis buffer and centrifuge at 10,000 rpm for 5 minutes. Repeat. Resuspend in 4 mL of 1X dialysis buffer. Divide into 200 µL aliquots, snap freeze and store in liquid nitrogen.

Day 1: **Summary:** On Day1, the cells are crosslinked and the chromatin is prepared for use in ChIP assays. The crosslinked cells can be used immediately or stored at -80°C prior to use in the chromatin preparation step. The chromatin can also be prepared and used immediately or stored at -80 prior to use in the ChIP assay. A portion of the chromatin should be purified by QIAquick column and quantified by Nanodrop before starting the immunoprecipitations. Also, Staph A cells, which are used to precipitate the antibody-chromatin complexes, are treated overnight with non-specific protein (BSA) and DNA (herring sperm) to reduce background in the ChIP assay.

Preparation of cross-linked cells

1. In a fume hood, add formaldehyde (37% stock) directly to tissue culture media to a final concentration of 1%. Incubate adherent cells on a shaking platform and suspension cells on a stir plate for 10' at RT (cross-linking for longer periods of ≥ 30' may cause cells to form aggregates that do not sonicate efficiently).
2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125M continuing to rock/spin for 5' at RT (for adherent cells use a 10X stock solution but for suspension cells add powdered glycine directly to flask).
3. For adherent cells, pour off media and rinse plates twice with 1X PBS; scrape adherent cells from culture dish to 50 mL tube; rinse the culture dish with 30 mL PBS, add the rinse solution to the 50 mL tube; centrifuge adherent cells at 200xg for 10' at 4°C. Carefully aspirate supernatant and wash by resuspending the pellet in 50 mL of 1X PBS. Pellet again and aspirate the supernatant. Optional: For adherent cultures add an appropriate volume of trypsin (e.g. 10 mL per 500 cm^2 dish) and incubate for 10' at 37°C (this step is useful for cells that are difficult to swell)
4. For suspension cells, wash twice by centrifuging and resuspend in 1X PBS.

5. At this point, the cells can be used immediately for ChIP assays or aliquoted, snap frozen in liquid nitrogen, and stored in liquid nitrogen or a -80°C freezer indefinitely.

Chromatin Preparation

For 10^5 cells per ChIP:

1. Resuspend cell pellet in 500 µL of cell lysis buffer plus protease inhibitors PMSF (10 µL/ mL), aprotinin (1 µL/ mL) and leupeptin (1 µL/ mL). The final volume of cell lysis buffer should be sufficient so that there are no clumps of cells. Incubate on ice for 10-15 minutes.
2. Lyse cells using a B dounce homogenizer several times to aid in nuclei release.
3. Microfuge nuclei at 3000xg at 4ºC. Discard supernatant.
4. Resuspend nuclei in 300 µL of nuclei lysis buffer plus the same protease inhibitors as for the cell lysis buffer. Incubate on ice for 10 minutes.

For 10^4 cells per ChIP: Proceed directly to the nuclei lysis step by adding 100 µL of nuclei lysis buffer plus protease inhibitors. Incubate on ice for 10 minutes.

5. Flash freeze nuclei in liquid nitrogen and thaw at room temperature to aid in nuclear lysis.
6. Sonicate the chromatin. The pulse duration, intensity, and number of pulses required for each sonicator and cell type will vary. Ideally, the least amount of input energy that gives the desired chromatin size should be used. As a starting point, we suggest the following: 100 uL of chromatin in a 500 µL Eppendorf tube using a Bioruptor (Diagenode, Belgium) at maximum power; set for 30 seconds ON followed by 1 min OFF for a total time of 10 minutes. Test sonications, followed by the determination of the size of the chromatin, can be performed prior to the ChIP assays if sufficient sample is available.
7. Centrifuge sonicated chromatin at 16,000xg for 10 minutes at 4ºC. Collect supernatant.

Chromatin Quantitation

1. Take 10 µL of the chromatin and add 90 uL of H_2O, then add 10 µL of 5M NaCl. Boil for 15 minutes. Add 1 µL DNase-free RNase (Fermentas Inc. EN0531). Incubate 15 minutes at 37 ºC. Add 1 µL Proteinase K (Roche 3115828). Incubate 15 minutes at 67 ºC. Purify chromatin using a QIAquick column (Qiagen 28104). Elute in 50 µL of water.
2. Measure DNA concentration by NanoDrop.
3. The chromatin can be flash frozen in liquid nitrogen and stored at -80ºC prior to use in ChIP assays.

Determining the size of the chromatin

Although one could determine the size of the sonicated chromatin at this step, prior to proceeding to ChIP analysis, this would require using the equivalent amount of chromatin as needed for several ChIP assays. Because this MicroChIP protocol has been developed for the analysis of limiting amounts of starting material, we recommend that the size of the chromatin be determined using the supernatants from the ChIP samples (see **Day 3**).

Pre-treatment (blocking) of Staph A cells.

1. Thaw 1 tube (200 µL) of prepared Staph A cells (Pansorbin, CalBiochem 507862). One tube should be enough for 10-15 ChIP experiments.
2. Add 25 µL of herring sperm DNA (Promega D1815, 10 mg/ml; previously boiled for 5 minutes and quickly chilled in ice).
3. Add 25 µL of BSA (NEB B9001S, 10 mg/ml).
4. Incubate on the rotating platform at 4 ºC overnight.
5. Microfuge for 5 minutes at 16,000xg at 4 ºC.
6. Remove supernatant and wash pellet twice with 1.4 mL dialysis buffer.
7. Resuspend cells in a volume of dialysis buffer (without sarkosyl) that is equal to the original starting volume (200 µL for 1 tube). Add PMSF at a 1:100 dilution (see below).

Day 2. Summary: Antibodies are added to the chromatin and allowed to form complexes**.**

Formation of antibody/chromatin complexes

1. Preclear chromatin by adding 30 µL of blocked Staph A cells.
2. Incubate on a rotating platform at 4ºC for 15 minutes.
3. Microfuge at 16,000xg for 10 minutes.
4. Transfer supernatant to a clean tube and measure the volume.
5. Calculate the volume of chromatin required for each ChIP assay (using 200-500 ng/ChIP assay). Remember to set aside an aliquot for Input DNA (see step Preparation of the INPUT DNA on **Day 3**). For each ChIP assay, transfer the required volume of chromatin to a 0.6 mL low retention siliconized tube (Fisher 02-681-330). Be sure to include an IgG control. You can also include a "mock" sample that contains 1X dialysis buffer instead of chromatin (IgG and mock are critical to control for nonspecific interactions and DNA contamination of IP and wash solutions, respectively).
6. Adjust the final volume of each ChIP sample with IP dilution buffer plus protease inhibitors to 500 µL volume.
7. Add between 0.5-1 µg of antibody to each sample.
8. Incubate on a rotating platform at 4ºC overnight.

Day 3: Summary: The antibody/chromatin complexes are purified, the Input DNA sample is prepared, and the size of the chromatin is determined.

Purification of the antibody/chromatin complexes.

1. Add 5 µL of blocked Staph A cells to each sample. (Important note: If you are using monoclonal antibodies or antibodies that have low reactivity with Staph A cells, you should add 1 µg of an appropriate secondary antibody and incubate for an additional 1 hour prior to adding the blocked Staph A cells).
2. Incubate on the rotating platform at room temp for 15 minutes.
3. Microfuge samples at 14,000xg at 4ºC.
4. Remove the supernatant from each ChIP sample using a 200 µL pipette, combine the supernatants, and save to determine size of the chromatin.
5. Wash pellets twice with 0.5 mL of 1X dialysis buffer (if using a monoclonal antibody, use buffer without the sarkosyl). For each wash, dissolve the pellet in 250 µL of buffer. Use a 200 µL pipette tip set to 100 µL to resuspend the pellet. Then add the remaining 250 µL of buffer. For each wash, incubate the samples on a rotating platform for 3 minutes, then

microfuge at 16,000xg for 3 minutes at 4ºC. Using a 2 µL pipette tip, remove as much buffer as possible after each wash without aspirating the Staph A cells.

6. Wash 3 times (as described in step 5) with 0.5 mL of IP wash buffer (pH 8.0 for monoclonal antibodies or pH 9 for polyclonal antibodies).
7. After the last wash in IP wash buffer, resuspend pellet in 250 µL IP wash buffer and transfer to a new tube. Add the remaining 250 µL of buffer, incubate samples on a rotating platform for 3 minutes, microfuge at 16,000xg for 3 minutes at 4ºC and remove as much buffer as possible without aspirating the Staph A cells. Repeat centrifugation step to remove the last traces of buffer with a 2 µL pipette tip (be sure to orient the pellets in the microfuge).
8. Elute antibody/chromatin complexes by adding 50 µL of IP elution buffer.
9. Shake on vortexer for at least 15 minutes at setting "vortex 3".
10. Microfuge at 16,000xg for 5 minutes at 4ºC.
11. Transfer supernatant to a clean tube.
12. Repeat elution step and combine both elutions in the same tube.
13. Microfuge samples at 16,000xg for 5 minutes at 4ºC to pellet any traces of Staph A cells. Transfer supernatants to clean tubes.
14. Add 10 µL of 5M NaCl to a final concentration of 0.45 M.
15. Boil samples in a water bath for 15 minutes to reverse formaldehyde crosslinks.
16. Purify ChIP DNA using a QIAquick MINelute column; elute in 10 µL of H_2O.

Preparation of the INPUT DNA

1. Add H_2O to each INPUT DNA sample (saved **on Day 2**) to a final volume of 100 µL.
2. Add 10 µL of 5M NaCl.
3. Boil samples in the water bath for 15 minutes to reverse formaldehyde crosslinks.
4. Add 1 µL of DNase-free RNase.
5. Incubate 15 minutes at 37ºC.
6. Add 1 µL of Proteinase K.
7. Incubate 15 minutes at 67ºC.
8. Purify DNA in a QIAquick MINelute column. Elute in 10 µL of water. Measure concentration by NanoDrop.

Determining the size of the chromatin

1. Collect all the supernatants from the ChIPs (**Day 3, step 4**) and combine in one tube. Add 5M NaCl to a final concentration of 0.45M.
2. Boil samples in the water bath for 15 minutes to reverse formaldehyde crosslinks.
3. Add DNase-free RNase to a final concentration of 25 µg enzyme/mL.
4. Incubate 15 minutes at 37 ºC.
5. Add 3 µL of Proteinase K per mL of solution.
6. Incubate 15 minutes at 67 ºC.
7. If the volume is larger than 1 mL, concentrate sample in Microcon YM-30 (Amicon 42410) to a final volume of 300 µL or less.
8. Purify DNA using a QIAquick column. Measure concentration by NanoDrop. Run between 1-2 µg of chromatin in a 1.5% agarose gel to check chromatin size.

Day 4. Summary: To obtain enough DNA to analyze by PCR or to hybridize to an array, the ChIP sample must be amplified. It is critical that precisely the right amount of sample be used in the amplification protocol. Therefore, the ChIP sample must be quantitated. Because a MicroChIP starts with such a low number of cells, half of the ChIP sample is used for quantitation. A single round of amplification provides enough sample for many PCR reactions or to probe one or two arrays. To probe a set of arrays that represent the entire genome, a second round of amplification must be performed.

Quantitation of the ChIP sample:

1. Follow the PicoGreen (Invitrogen P7589) manufacturer instructions to quantify DNA **EXCEPT** do not use the lambda DNA standard that is provided. Instead, use the Input DNA as your standard. Prepare a standard curve with the Input DNA at the following concentrations: 10 ng/µL, 1 ng/µL, 100 pg/µL and 10 pg/µL. Use 10 µL of each standard.
2. Use 5 µL of the ChIP sample (half the sample) and add 5 µL of TE buffer (remember to adjust calculations for quantitation by 2 fold).
3. Add 100 µL of the PicoGreen working solution to the standards and the samples.
4. Read standards and sample fluorescence on a spectrofluorometer at standard fluorescein wavelengths (exitation 480 nm, emission 520 nm).
5. Calculate ChIP concentration and yield by correlating with the standard curve. A typical yield from 10^5 cells is approximately 300 picograms of DNA.

Amplicon Preparation and Confirmation

ChIPs samples can be amplified using 2 different protocols, SIGMA WGA2 or WGA4, depending on the amount of DNA obtained in the immunoprecipitate. Recommendations are:

WGA2: Use between 10-100 ng of ChIP or Total Input per amplification reaction.
WGA4: Use between 10-100 pg of ChIP or Total Input per amplification reaction.

Step 1: WGA4. Due to the small amount of DNA obtained from a MicroChIP, the WGA4 protocol must be used. Follow WGA4 kit instructions starting from Step 6 of the Library Preparation section; omit Steps 1-5. Add 5 µL of ChIP DNA and bring the volume up to 11 µL with water. After the Amplification step, purify DNA in a QIAquick column and elute in 50 µL of water. Typically, the yield should be between 5-8 µg of amplicon.

Step 2: Confirmation of the WGA4 amplicons. It is essential that a positive and negative control PCR be performed. Take a small aliquot of the Round 1 WGA4 amplicons, prepare dilutions (5 ng/µL), and test 2 µL by PCR with positive and negative control primer sets. Experimental sample enrichment should be present when comparing the ChIP versus the Total Input and IgG control. These samples can be applied to a microarray or tested in further PCR reactions.

Step 3: WGA3. To obtain enough amplicon to analyze the entire genome, a portion of the amplicons prepared by WGA4 are re-amplified by the WGA3 method. Take 10 ng of the WGA4 amplicons and amplify with SIGMA WGA3 kit. Follow manufacturer's instructions. Important note: There is no library preparation step, instead proceed directly to the amplification step for 14 cycles. Typically, the yield should be between 5-8 µg of Round 2 amplicons for every 10 ng of Round 1 used. To obtain enough product to hybridize to multiple arrays, perform several

WGA3 re-amplifications in parallel, using the original WGA4 amplicons. Purify DNA in a QIAquick column and elute in 50 µL of H_2O.

Step 4: Confirmation of the WGA3 amplicons. Take a small aliquot of the Round 2 amplicons prepare dilutions (5 ng/µL) and test by PCR using positive and negative control primer sets. Sample enrichment should be present when comparing the ChIP versus the Total Input and IgG control. The ChIP amplicons can be applied to a microarray using either the Total Input sample or the IgG sample as a hybridization control.

Solutions

Cell Lysis buffer
5 mM PIPES pH 8.0
85 mM KCL
0.5% Igepal (SIGMA I3021; add fresh)
protease inhibitors (add fresh)

Nuclei Lysis buffer
50 mM Tris-Cl pH 8.1
10 mM EDTA
1% SDS
protease inhibitors (add fresh)

IP Dilution buffer
0.01% SDS
1.1% Trition X 100
1.2 mM EDTA
16.7 mM Tris-Cl pH 8.1
167 mM NaCl

1X Dialysis buffer
2 mM EDTA
50 mM Tris-Cl pH 8.0
0.2 % Sarkosyl (omit for monoclonal antibodies)

IP Wash buffer
100 mM Tris-Cl pH 9.0 (8.0 for monoclonal antibodies)
500 mM LiCl
1% Igepal
1% deoxycholic acid (Fisher Scientific BP349-100)

Elution buffer
50 mM NaHCO3
1% SDS

Protease Inhibitors
100 mM PMSF (Sigma P-7626); make in ethanol, use at 1:100
10 mg per ml aprotinin (Sigma A-1153); make in 0.01 M HEPES pH 8.0, use at 1:1,000
10 mg per ml leupeptin (Sigma L-2884); make in water, use at 1:1,000