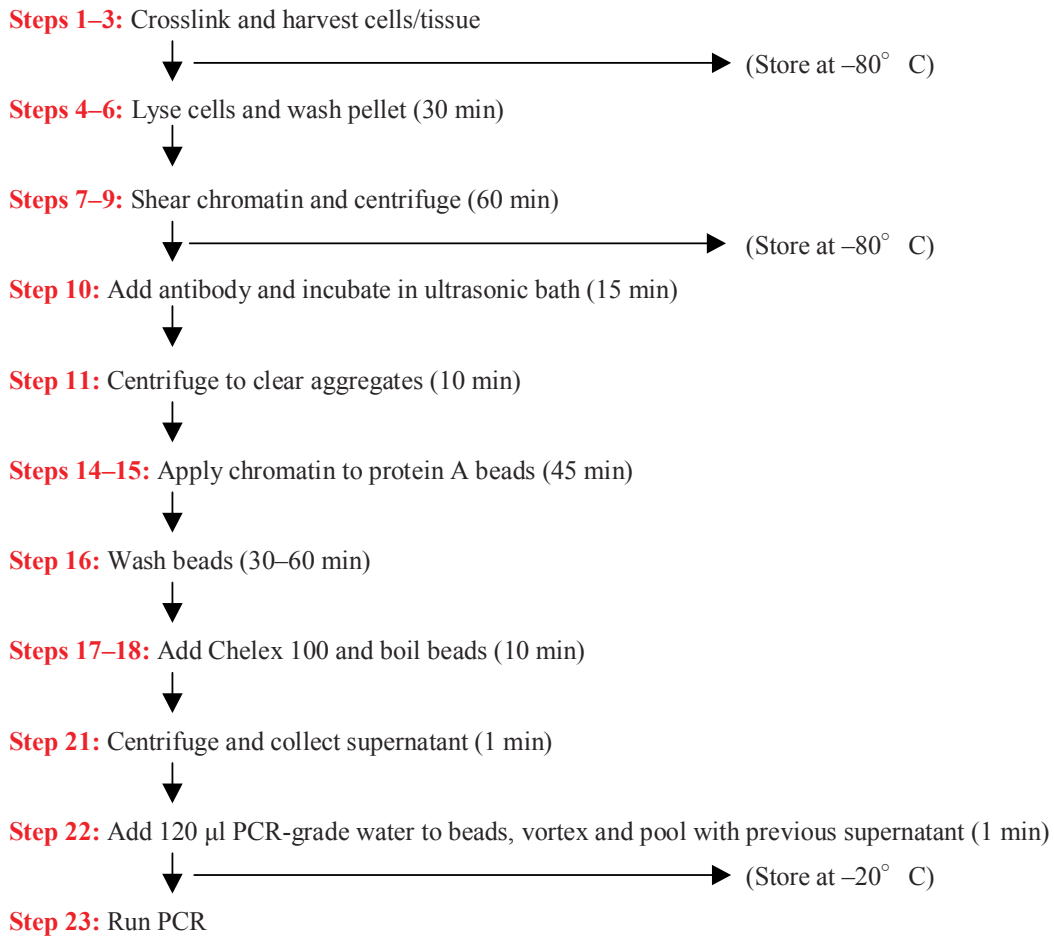




Chromatin Immunoprecipitation (ChIPs) Protocol (Karol Bomsztyk Lab)

27 June 2006

TIMELINE





MATERIALS

REAGENTS

- **Chelex 100** (Bio-Rad, cat. no. 142-1253)
- Proteinase K (Invitrogen, cat. no. 25530-015)
- Protein A–Sepharose (Amersham, cat. no. 17-5280-01)
- Formaldehyde (J.T. Baker, cat. no. 2106-02) ! CAUTION Very toxic if inhaled, ingested or absorbed through skin.
- PMSF (Sigma, cat. no. P-7626) CAUTION Can form flammable gases when reacting with water. Flush trap well after disposal down a drain. Toxic if absorbed through skin or ingested.
- Leupeptin (Sigma, cat. no. L-2884)
- Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) (Sigma, cat. no. S-6646) CAUTION Harmful if inhaled or ingested.
- beta-Glycerophosphate (Sigma, cat. no. G-6251)
- Sodium fluoride (NaF) (Sigma, cat. no. S-1504) CAUTION Very toxic if inhaled or ingested.
- Sodium orthovanadate (Na_3VO_4) (Sigma, cat. no. S-6508)
- p-Nitrophenylphosphate di(tris) salt (Sigma, cat. no. N-3254)
- SYBR Green PCR Master Mix (ABI Biotechnology, cat. no. 4309155)
- Anti–RNA polymerase II (Santa Cruz Biotechnology, cat no. sc899)
- Anti–histone H3 (Abcam, cat. no. ab1791)
- Nonimmune IgG fraction (Vector Labs, cat. no. I-1000)

EQUIPMENT

- Misonix Sonicator 3000 with micro tip (Misonix, cat. no. S3000; brand and model not critical)
- **Ultrasonic bath** (Branson, cat. no. B3510-MT CPN-952-316)
- Shaking heatblock (Eppendorf, cat. no. 022670000; model not critical)
- Refrigerated microcentrifuge
- Means for quantitative PCR (e.g., real-time PCR ABI 7900 system, ABI Biotechnology, or thermocycler with means of determining band intensity either with ^{32}P incorporation or EtBr staining)
- Tube rotator or tumbler at 4°C



REAGENT SETUP

- **IP buffer:** 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1.0% vol/vol). For 500 ml, add 4.383 g NaCl, 25 ml of 100 mM EDTA (pH 8.0), 25 ml of 1 M Tris-HCl (pH 7.5), 25 ml of 10% (vol/vol) NP-40 and 50 ml of 10% (vol/vol) Triton X-100.

CRITICAL: Per 1 ml IP buffer, add the following immediately before use and keep on ice: 5 ml of 0.1 M PMSF in isopropanol (-20°C) and 1 ml of 10 $\mu\text{g}/\mu\text{L}$ leupeptin (aliquots at -20°C). Add the following phosphatase inhibitors if necessary: 10 ml of 10 mM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (4°C), 10 ml of 1M beta-glycerophosphate (4°C), 10 ml of 1 M NaF (4°C), 1 ml of 100 mM Na_3VO_4 (aliquots at -20°C) and 13.84 mg of p-nitrophenylphosphate (-20°C).

- **1 M glycine:** Dissolve 18.8 g glycine in ddH₂O (may require gentle heating) and bring up to 250 ml with ddH₂O.
- **10% (wt/vol) Chelex:** 100 Add 1 g Chelex 100 resin to water (MilliQ or NANOpure) and bring up to a final volume of 10 ml. Store at room temperature, 20–25°C.
- **20 $\mu\text{g}/\mu\text{L}$ proteinase K:** Dissolve 100 mg in 5 ml water (MilliQ or NANOpure), aliquot, and store at -20°C .



PROCEDURE

Cross-linking and harvesting cells

- 1) Add 40 μ l of 37 % (wt/vol) formaldehyde per 1 ml of overlaying medium to obtain a final concentration of 1.42 %; incubate for 15 min at room temperature.

CRITICAL STEP: The cross-linking time and formaldehyde concentration can affect both the efficiency of chromatin shearing and the efficiency of precipitating a specific antigen. Shorter cross-linking times (5–10 min), lower formaldehyde concentrations (1%, wt/vol) or both may improve shearing efficiency; however, for some proteins, especially those that do not directly bind DNA, this might reduce the efficiency of cross-linking and thus the yield of precipitated chromatin.

- 2) Quench formaldehyde with 125 mM glycine for 5 min at room temperature (141 μ l of 1 M glycine per 1 ml of medium).
- 3) Scrape cells and collect by centrifugation (2,000g for 5 min at 4°C), then wash twice with cold PBS.

PAUSE POINT Cell pellets can be stored at –80°C for at least 1 year.

Lysis *CRITICAL STEP Steps 4–16 must be performed on ice or at 4°C.*

- 4) Lyse cells from one plate (10–15 cm in diameter) with 1 ml IP buffer containing protease inhibitors (and phosphatase inhibitors if needed), by resuspending the pellet and pipetting up and down several times in a microcentrifuge tube (there will be a lot of insoluble material).
- 5) Centrifuge at 12,000g for 1 min at 4°C and aspirate the supernatant.
- 6) Wash the nuclear pellet with 1 ml IP buffer containing inhibitors, by resuspending the pellet, followed by centrifugation.

Sonication

- 7) To shear the chromatin, sonicate the washed pellet resuspended in 1 ml of IP buffer (with inhibitors) per 10-million cells (do not sonicate in volumes above 1 ml as this can decrease sonication efficiency).



CRITICAL STEP: Sonication conditions must be determined empirically for each cell or tissue type, and sonicator model; optimal average DNA fragment sizes are 0.5–1 kb. Details of sonication procedure are given in “ADVICE ON SONICATION”.

- 8) Clear the lysate by centrifuging at 12,000g for 10 min at 4°C. Retain the supernatant.

- 9) Transfer an aliquot of sheared chromatin (equivalent to 0.2 million cells) to a new microcentrifuge tube; this will be used for isolation of total DNA, to determine shearing efficiency and as a control for the amount of input DNA used in precipitations (to extract total DNA from this aliquot, skip to Step 19). The lysate can be aliquoted for use with multiple antibodies at this point. **For best results, each aliquot should contain chromatin from 2 million cells.**

PAUSE POINT The chromatin can be stored at –80°C for months.

Immunoprecipitation

- 10) Add antibody to samples and incubate in **an ultrasonic water bath for 15 min at 4°C**. For IP, use the desired antibody; for mock IP, use the same antibody preincubated with saturating amounts of its epitope-specific peptide for 30 min at room temperature. Alternatively, for mock IP, use the nonimmune IgG fraction from the same species in which the antibodies were produced. Incubation with beads without antibodies could also be used as a mock IP. If multiple antibodies are to be used with the same chromatin preparation, a single mock IP is sufficient as a control for all the antibodies used.

CRITICAL STEP: The amount of antibody added should be in excess of the factor being precipitated and thus should be determined empirically for each factor/antibody. For abundant proteins, like histones, we typically used 1–2 µg of affinity-purified antibody or 2 µL of whole serum per IP. In addition, whereas the incubation time (15 min) has worked for many of the antibodies. We have used, the kinetics for reaching the equilibrium of antibody binding may differ for each antibody. The incubation time may need to be increased for some antibodies. If an ultrasonic bath is not available, a long incubation at 4 °C should be used. In the traditional method, the times of incubation range from 1 to 12 h and should be determined empirically for each antibody.

- 11) Clear the chromatin by centrifugation at 12,000g for 10 min at 4°C.

- 12) During Steps 10 and 11, wash protein A–agarose beads (20 µl per IP sample) three times with IP buffer to remove ethanol. A wash consists of resuspending the beads with 1 ml of IP buffer, centrifuging (1,000–2,000g) for a few seconds at 20–25°C, and aspirating the supernatant.



ADVICE ON SONICATION

To determine fragment size, extract total DNA from an aliquot of sheared chromatin (Steps 19–22) and run on 1% (wt/vol) agarose gel (stain with EtBr). Use a sonicator with a microtip, and sonicate in a 1.5-ml tube. When sonicating, do not allow the sample to foam up, as this decreases the efficiency of DNA shearing. To avoid foaming, keep the tip of the sonicator probe no more than a few millimeters from the bottom of the tube. If foaming does occur, stop sonication and wait until the bubbles rise to the surface before continuing sonication. Sonication leads to heating of the sample; therefore, hold the sample in an ice-water bath during sonication. There are two main variables to test when determining optimum sonication conditions: the length of sonication and the power output. To avoid excessive heating, the total sonication time is usually broken up into a number of ‘rounds’ of sonication, with a rest on ice between rounds (for instance, 4 rounds, each with 15 s of sonication, with a 2-min rest between rounds). Also, sonication using a series of short pulses is more efficient than that with a single long pulse (i.e., 15 1-s-long pulses versus 1 15-s-long pulse), as the power output of the sonicator tip decreases from the beginning to the end of a pulse. To start optimization, set the power output to 50 % of the maximum output for the microtip, and sonicate with 10 to 15 1-s-long pulses per round, for two, four, six or eight rounds (with 2-min rest between rounds). Examine the shearing efficiency by means of gel analysis of the sizes of DNA fragments, and if increasing the sonication time (number of rounds) does not give the desired average fragment size, try increasing the power output. When using very high power outputs, the total time for each round of sonication may need to be decreased if the samples become excessively heated. An example of sonication conditions: we used three rounds of 15 pulses each at 50 % power output and 90 % duty cycle using a Branson Sonifier 200. We also used a Misonix 3000 with 4–6 rounds of 15 1-s pulses at 50 % power output.



- 22) Add 120 μ l of water (MilliQ or NANOpure) to beads, vortex for 10 s, centrifuge contents down at 12,000g for 1 min at 4°C, collect 120 μ l of supernatant and pool with the previous supernatant. Mix before using.

PAUSE POINT: Store at –20°C. We have thawed and frozen the samples repeatedly (more than 20 times over months) without loss of PCR signal.

Data analysis

- 23) Purified DNA can be used in PCR at up to 25 % of the reaction volume. We used SYBR Green Master Mix in a 10 ml reaction (2.5 ml DNA template, 0.3 ml primer pair (10 mM each), 5 ml Master Mix and 2.2 ml H₂O) in 384-well plates on an ABI 7900 (default three-step method, 40 cycles). Use ROX dye to correct for loading. Acquire data using the SDS 2.2.1 program (ABI Biotechnology). For each primer pair, set the readout in the middle of the linear range of amplification signals. The data can be exported to Excel spreadsheets. The relative occupancy of the immunoprecipitated factor at a locus is estimated using the following equation:

$2^{-(Ct \text{ mock} - Ct \text{ specific})}$, where Ct mock and Ct specific are mean threshold cycles of PCR done in triplicate on DNA samples from mock and specific immunoprecipitations. If gel electrophoresis is used to estimate the intensities of PCR products, then the relative occupancy of a factor at a locus is estimated as the ratio of the intensity of the specific IP band to that of the mock IP band¹⁰.

ANTICIPATED RESULTS

We found that four 15-cm plates of primary rat mesangial cells (80% confluence, approximately $2.5\text{--}3.0 \times 10^7$ cells) yield 2 ml of sheared chromatin, of which 150 μ l is sufficient for ChIP with one antibody. This allowed us to probe 12 different factors from one chromatin preparation. Each chromatin IP yields sufficient amounts of DNA for 80 10- μ l real-time PCRs, which allowed us to monitor as many as 20–25 genomic sites.