Protocol for the fast chromatin immunoprecipitation (ChIP) method

Joel D Nelson[1,2], Oleg Denisenko[2] & Karol Bomsztyk[1,2]

[1]Molecular and Cellular Biology Program and [2]UW Medicine at Lake Union, University of Washington, Seattle, Washington 98109, USA. Correspondence should be addressed to K.B. (karolb@u.washington.edu).

Published online 27 June 2006; doi:10.1038/nprot.2006.27

Chromatin and transcriptional processes are among the most intensively studied fields of biology today. The introduction of chromatin immunoprecipitations (ChIP) represents a major advancement in this area. This powerful method allows researchers to probe specific protein-DNA interactions *in vivo* and to estimate the density of proteins at specific sites genome-wide. We have introduced several improvements to the traditional ChIP assay, which simplify the procedure, greatly reducing the time and labor required to complete the assay. The simplicity of the method yields highly reproducible results. Our improvements facilitate the probing of multiple proteins in a single experiment, which allows for the simultaneous monitoring of many genomic events. This method is particularly useful in kinetic studies where multiple samples are processed at the same time. Starting with sheared chromatin, PCR-ready DNA can be isolated from 16–24 ChIP samples in 4–6 h using the fast method.

INTRODUCTION

Chromatin is composed of DNA, proteins and RNA[1–3]. Chromatin structure is dynamic, responds to intra- and extracellular signals, and controls gene expression, DNA replication and repair[3–5]. ChIP has proven to be a powerful assay by which to follow the protein-DNA interactions involved in these processes[3,6–8]. The assay determines whether a protein-DNA interaction is present at a given location within the genome and allows estimation of the density of a given factor in that region[9,10]. Along with other techniques[11,12], ChIP assays are uncovering an extraordinarily rich and dynamic chromatin environment[3,12–16].

In the ChIP assay, protein-DNA complexes are fixed by cross-linking with formaldehyde[6,7,17], and the chromatin is sheared, by sonication[6,7] or enzymatic[18] methods, into DNA fragment sizes of 200–1,000 base pairs. Complexes containing the factor of interest are immunoprecipitated using an antibody specific to that protein. DNA is purified from the isolated chromatin, and specific genomic regions are typically detected using PCR (**Fig. 1**). Appropriate numbers of PCR cycles are used to ensure that the specific and mock-precipitated DNAs are within the linear range of amplification. Enrichment of a specific DNA locus in immunoprecipitated material, as compared to mock precipitation, indicates that the factor of interest interacts with this genomic region, and the intensity of the PCR signal is proportional to occupancy at the binding site. Alternatively, an interaction of the factor with multiple genomic locations can be detected simultaneously by applying the isolated DNA to a DNA microarray (ChIP on chip)[14,19–21].

In the traditional ChIP assay, the reversal of cross-linking and DNA isolation are the most time-consuming and labor-intensive steps[6,22]. The ChIP protocol described here uses a Chelex 100–based DNA isolation procedure that reduces the total time of reversal of cross-linking and DNA isolation from 1–2 d down to approximately 1 h (ref. 9). The traditional assay uses a 5-h reversal of cross-linking (though this is typically done overnight), followed by proteinase K digestion, phenol:chloroform extraction and ethanol precipitation, requiring several tube transfers. Our protocol yields PCR-ready DNA with two short boiling steps and a short proteinase K digestion, a procedure done in one tube (**Fig. 1** and ref. 9).

In addition, we introduce the use of an ultrasonic bath to increase the rate of protein-antibody binding[23] so that for the antibodies that we tested, incubation time was decreased from $\geq$1 h down to 15 min (refs. 9,10 and **Fig. 2**).

One of the advantages of the fast ChIP method is that the reduced amount of labor per tube allows for more samples to be processed simultaneously. Starting with sheared chromatin, 16–24 samples can be easily processed in about 4–6 h (6–8 h when including the time required for real-time PCR with one or two primers). The ability to handle multiple samples in one ChIP experiment greatly facilitates studies in which the binding of several factors are profiled at several time points (**Fig. 3**). Another important advantage of our protocol is that it saves time when optimizing conditions for new studies or antibodies. Finally, the method is easy to learn even for an inexperienced researcher.

The DNA yield from fast ChIP is as good as or better than that from the traditional method[9]. We have used this protocol with chromatin from mammalian tissue culture as well as from yeast[9], and we believe that it should be easy to adapt for use with other chromatin sources. A formaldehyde cross-linking and whole-cell extract preparation protocol for budding yeast can be found in ref. 6 and a protocol for animal tissues is described in ref. 24. Immunoprecipitation and DNA purification steps (Steps 10–22 in PROCEDURE) are identical for all chromatin sources. With an additional DNA purification (Qiagen MinElute Kit), the fast ChIP method has been used by others in combination with microarrays (B. Bernstein, Harvard University, Boston; personal communication). We believe that the fast ChIP protocol detailed below might improve most ChIP applications.

To learn the procedure, we suggest beginning with antibodies to RNA pol II and histone H3, because densities of these factors are high and these antibodies work well in ChIP (refs. 9,25). For mock IP (background), we recommend either an antibody blocked with a specific peptide (available for RNA pol II antibody but not for H3), rabbit IgG fraction or no added antibodies. Extracts from cells with

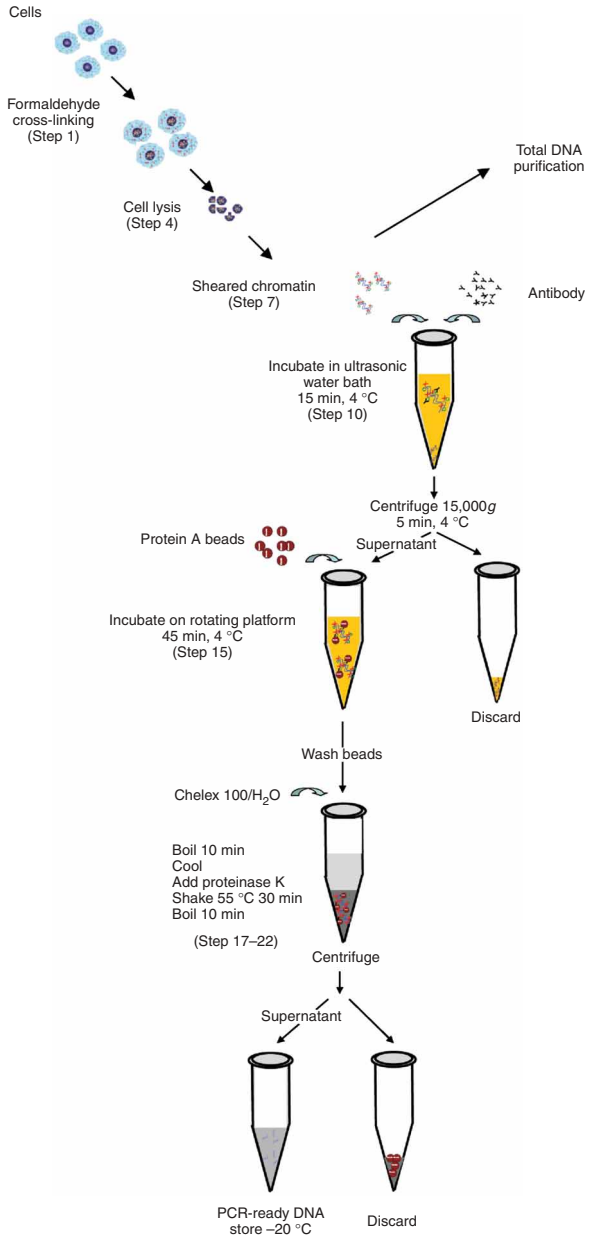

Figure 1 | General outline of the fast ChIP procedure. After cross-linking with formaldehyde, the cells are lysed, the fraction containing nuclear pellets is isolated and chromatin is sheared. Chromatin samples are incubated with the antibodies in an ultrasonic bath (15 min, 4 °C); after centrifugation, the precleared samples are mixed with protein A beads (45 min, 4 °C). After several washes, Chelex 100 suspension is added to the beads; the suspension is boiled (10 min), the tubes are allowed to cool and then proteinase K is added. The mix is incubated in a shaker (30 min, 55 °C at 1,400 rpm) and the beads are then boiled again (10 min). After centrifugation, the PCR-ready DNA is collected. Samples are stored at –20 °C and can be thawed and frozen repeatedly.

the factor of interest knocked out and a specific antibody could also be used as a mock control[26]. Others have also used whole DNA (input) as a reference to estimate the enrichment of proteins at a genomic site[27], but this method of normalization has been questioned[20].

Choose genes for which you can measure mRNA levels (RT-PCR). At silent loci or intergenic untranscribed regions, the signal/background ratio for the histone H3 should be 80–120, whereas for RNA pol II it should be 1.0 (**Fig. 3**). At very active genes, the signal/background ratio for RNA pol II could be as high as 50–100, whereas, depending on the gene, for the histone H3 it could be as low as 20. In general, the level of RNA pol II recruited to a gene should correspond to the level of the transcript, particularly for transcripts with short half-lives[9,28].

The major limitation of the ChIP assay is the quality of the antibody, as some antibodies work poorly or not at all in ChIP. This limitation could be circumvented by using tagged protein constructs (hemagglutinin (HA), Flag or other) expressed from plasmids[26,29].

The ability of some antibodies to recognize their targets may depend on the phosphorylation state of the target protein. Thus, in our assays we use phosphatase inhibitors during extraction in order to maintain these phosphorylated states[9,30]. Phosphatase inhibitors may not be necessary for all antibodies.

Note: timing considerations for this procedure are listed in Figure 4.

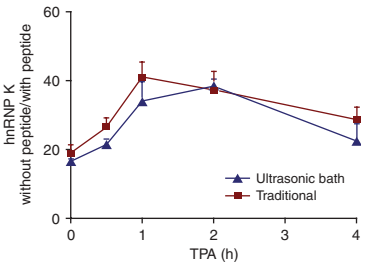

Figure 2 | Short incubation of chromatin with antibodies in ultrasonic bath is equivalent to overnight binding. Antibody to heterogeneous nuclear ribonucleoprotein K (hnRNP K) was preincubated with or without blocking peptide for 30 min (refs. 9,30). Equal aliquots of sheared chromatin from rat mesagial cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[10] were incubated either in the ultrasonic bath (15 min, 4 °C) or rotated overnight (12 h, 4 °C, 'traditional'). Purified DNA was analyzed by real-time PCR with primers to the *egr-1* gene. The graph shows the ratio of mean PCR signal from chromatin IP without blocking peptide to that with the peptide (mock). Error bars represent s.d. of the PCR (done in triplicate).

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 ($Na_2MoO_4 \bullet 2H_2O$) (Sigma, cat. no. S-6646) **! CAUTION** Harmful if inhaled or ingested.
- β-Glycerophosphate (Sigma, cat. no. G-6251)
- Sodium fluoride (NaF) (Sigma, cat. no. S-1504) **! CAUTION** Very toxic if inhaled or ingested.
- Sodium orthovanidate (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 µl of 0.1 M PMSF in isopropanol (–20 °C) and 1 µl of 10 µg µl^{-1} leupeptin (aliquots at –20 °C). Add the following phosphatase inhibitors if necessary: 10 µl of 10 mM $Na_2MoO_4 \cdot 2H_2O$ (4 °C), 10 µl of 1 M β-glycerophosphate (4 °C), 10 µl of 1 M NaF (4 °C), 1 µl 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 ddH_2O (may require gentle heating) and bring up to 250 ml with ddH_2O.
- **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 µg µl^{-1} 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 µl 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.

? TROUBLESHOOTING

2| Quench formaldehyde with 125 mM glycine for 5 min at room temperature (141 µl of 1 M glycine per 1 ml of medium).

3| Scrape cells and collect by centrifugation (2,000*g* 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).

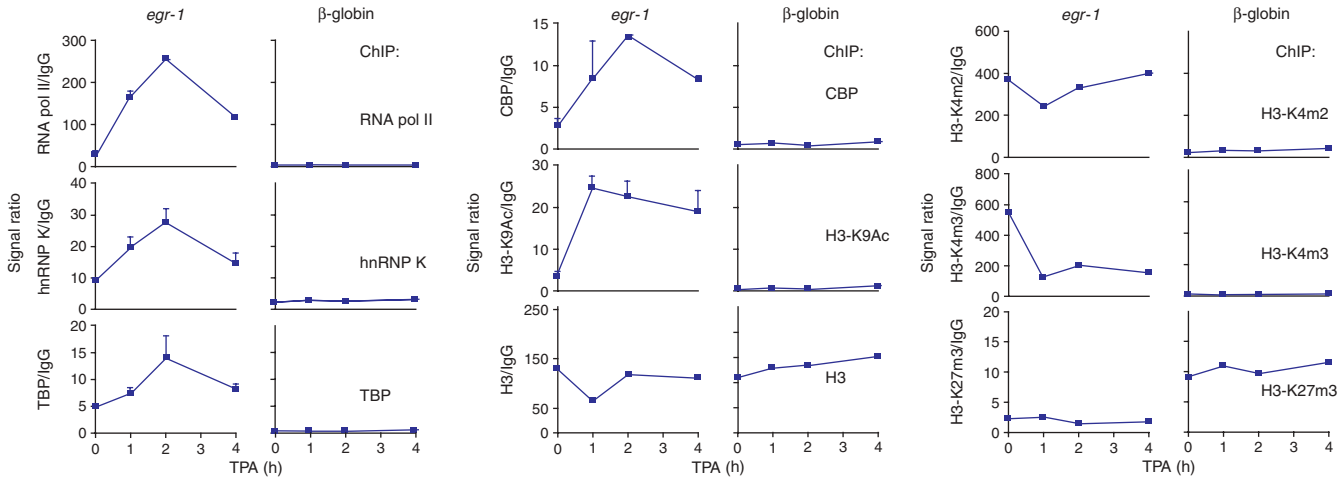

Figure 3 | Example of fast ChIP assay done with multiple antibodies in a single experiment. Rat mesagial cells grown in 15-cm plates (per time point) were treated with 10^{-7} M TPA at the indicated time points. Equal aliquots of sheared chromatin from a single plate were used in fast ChIP with the following antibodies: anti–RNA polymerase II, anti–hnRNP K (4 µg)[30], anti–TATA-binding protein (anti-TBP, 4 µg, Santa Cruz Biotechnology, cat. no. sc273), anti–CREB binding protein (anti-CBP, 4 µg, Santa Cruz Biotechnology, cat. no. sc369), anti–acetylated histone H3 (anti–H3-K9Ac, 2 µl Cell Signaling, cat. no. 9671S), anti–histone H3, anti–dimethyl H3 (anti–H3-K4m2,1 µl, Upstate Biotechnology, cat. no. 07-030), anti–trimethyl H3 (anti–H3-K4m3,1 µg, Abcam, cat. no. ab8580) and anti–H3 lysine 27 trimethylation (anti–H3-K27m3,1 µg, Abcam, cat. no. Ab6002). Nonimmune IgG fraction was used as a mock control. Purified DNA was used in real-time PCR using either a pair of primers to the promoter of the TPA-inducible *egr-1* or the second intron of the silent β-*globin* gene[9]. Results are expressed as ratios of the real-time PCR signals obtained for IP with specific antibodies to those obtained for mock IP with IgG. The results represent mean ± s.d. signal ratios of PCR done in triplicate.

5| Centrifuge at 12,000*g* 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 **Box 1**.

8| Clear the lysate by centrifuging at 12,000*g* 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.
? TROUBLESHOOTING

11| Clear the chromatin by centrifugation at 12,000*g* for 10 min at 4 °C.
? TROUBLESHOOTING

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 (1,000–2,000*g*) for a few seconds at 20–25 °C, and aspirating the supernatant.

BOX 1 | 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.

13| Dilute beads 1:1 with IP buffer and aliquot 40 µl of the slurry to clean tubes.
▲ **CRITICAL STEP** Because agarose beads bind DNA nonspecifically, variation in the amount of added beads can affect the specific signal/background ratio. Make sure to keep the slurry suspended while distributing and cut off the tips of small-bore pipette tips.
? TROUBLESHOOTING

14| Transfer the top 90% of cleared chromatin (Step 11) to a tube with protein A–agarose slurry.
▲ **CRITICAL STEP** It is very important to avoid carrying over any precipitated material, as this contains aggregated unspecific DNA complexes that can contaminate immunoprecipitated material.
? TROUBLESHOOTING

15| Rotate tubes at 4 °C for 45 min on a rotating platform (20–30 rotations per min).

16| Washing the beads. Centrifuge the slurry at 1,000–2,000*g* for few seconds of 4–25 °C and remove the supernatant. Wash the beads 5–6 times with 1 ml cold IP buffer without inhibitors. A wash consists of resuspending the beads, centrifuging and aspirating the supernatant.

DNA isolation
Steps 17–22 are performed at room temperature.
17| Add 100 µl 10% (wt/vol) Chelex 100 slurry directly to the washed beads. Keep Chelex beads in suspension while pipetting. Also, make sure to cut off the tips of the fine-bore pipette tips before pipetting the Chelex slurry.

18| Briefly (10 s) vortex samples to mix the slurry, and boil for 10 min.

19| Precipitate the total DNA aliquot taken in Step 9 with 2.5–3 volumes of ethanol, and wash with 70% (vol/vol) ethanol. Then dissolve the dried pellet in 100 µl 10% (wt/vol) Chelex 100 suspension, boil for 10 min and continue processing in the same way as the IP samples.

20| Optional. Proteinase K treatment. Wait for the samples to cool after boiling and add 1 µl of 20 µg μl^{-1} proteinase K to each sample. Vortex, and then shake samples at 55 °C for 30 min on a thermal mixer (1,000 rpm). Boil again for 10 min. This boiling step is important to inactivate proteinase K, which may interfere with subsequent PCR.

21| Centrifuge condensate to the bottom of the tube at 12,000*g* for 1 min of 4 °C. Transfer supernatant (80 µl) to a new tube. Be careful to avoid transferring any Chelex resin as it can lead to a loss of PCR signal.

22| Add 120 µl of water (MilliQ or NANOpure) to beads, vortex for 10 s, centrifuge contents down at 12,000*g* for 1 min of 4 °C, collect 120 µl 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 µl reaction (2.5 µl DNA template, 0.3 µl primer pair (10 µM each), 5 µl Master Mix and 2.2 µl H_2O) 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: $2\text{^}(Ct^{mock} - Ct^{specific})$, where Ct^{mock} and $Ct^{specific}$ are mean threshold cycles of PCR done in triplicate on DNA samples from mock and specific immunoprecipitations (ref. 9 and **Figs. 2** and **3**). 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 band[10].
? TROUBLESHOOTING

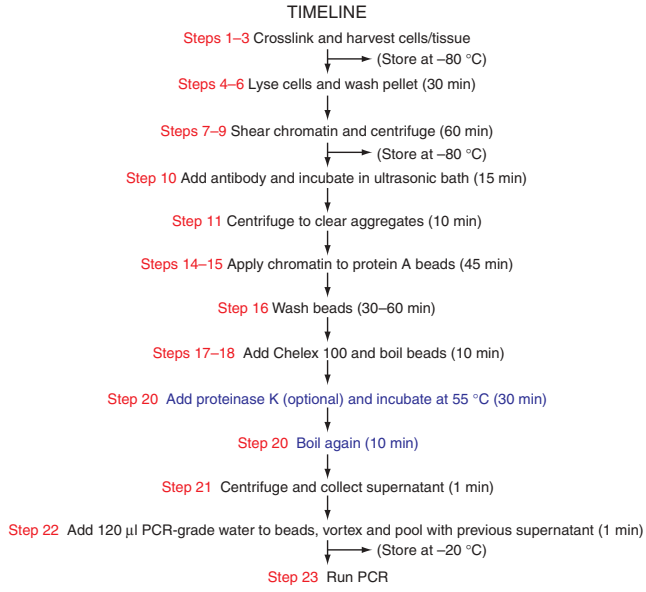

Figure 4 | Timeline of the fast ChIP protocol.

● TIMING

See **Figure 4**.

? TROUBLESHOOTING

See **Table 1**.

TABLE 1 | Troubleshooting table

STEP	PROBLEM	REASON	SOLUTION
1, 10, 16	Lower than expected specific IP/mock IP signal ratio	Antibody not suitable for ChIP.	Step 10. Check to make sure that the antibody is suggested for use with ChIP. Try running an IP with uncrosslinked extracts, followed by a western blot to see if the antibody can precipitate the target protein. Also, try running a positive control antibody to make sure that all other aspects of the protocol are working. ChIP-grade antibody to RNA pol II is a good positive control as it works well and ChIP results can be compared to gene expression (transcript) levels.
		Insufficient time for antibody binding to the chromatin.	Step 10. Try increasing the time that the chromatin is incubated with the antibody.
		Cross-linking time is too long, masking the epitope.	Step 1. Try decreasing cross-linking time (or formaldehyde concentration).
		Insufficient amount of chromatin used. If the PCR signal is equally low for both specific and mock IPs, the input may be too low.	Step 10. Increase the amount of starting material until the specific/mock IP ratio stops improving. A good starting point: use 1–4 million cells per sample.
		Too few washes leaving high levels of nonspecific binding on the beads.	Step 16. Increase the number of washes, or add NaCl or LiCl (to a final concentration of 0.5 M or 250 mM, respectively) to the wash buffer during the IP, and see if the ratio increases.
10, 11, 13, 14	Variation in specific/mock IP ratios between experiments	Insoluble chromatin complexes were not cleared from soluble chromatin before incubation with protein A beads.	Steps 11 and 14. Centrifuge chromatin after incubation with antibody and only apply the top 80–90% to the protein A beads.
		Too little input leading to high Ct values for real-time PCR or high cycle numbers for regular PCR.	Step 10. Increase the amount of input chromatin until results for repeated PCR experiments are reproducible.
		Large variation in the amount of protein A–agarose beads incubated with the chromatin, as the beads bind chromatin nonspecifically and contribute to the background.	Step 13. Keep protein A–agarose beads suspended and use pipette tip with cut-off tip when distributing to tubes.
23	No PCR signal	PCR reaction did not work.	Step 23. Run a PCR with genomic DNA from the same organism, as a positive control for primers and master mix.
		Insufficient number of PCR cycles used (if not using real-time PCR).	Step 23. Run a few different reactions with increasing numbers of cycles until a band is obtained.

ANTICIPATED RESULTS

We found that four 15-cm plates of primary rat mesangial cells (80% confluence, approximately 2.5–3.0 $\times$ 10^7 cells) yield 2 ml of sheared chromatin, of which 150 µl is sufficient for ChIP with one antibody. This allowed us to probe 12 different factors from one chromatin preparation (**Fig. 3**). Each chromatin IP yields sufficient amounts of DNA for 80 10-µl real-time PCRs, which allowed us to monitor as many as 20–25 genomic sites.

ACKNOWLEDGMENTS We thank members of the K.B. lab for valuable discussions of the method. This work was supported by the US National Institutes of Health (DK45978 and GM45134) and the Juvenile Diabetes Research Foundation (K.B.).

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Bernstein, E. & Allis, C.D. RNA meets chromatin. *Genes Dev.* **19**, 1635–1655 (2005).
2. Schubeler, D. & Elgin, S.C. Defining epigenetic states through chromatin and RNA. *Nat. Genet.* **37**, 917–918 (2005).
3. Felsenfeld, G. & Groudine, M. Controlling the double helix. *Nature* **421**, 448–453 (2003).
4. Sims, R.J. III, Mandal, S.S. & Reinberg, D. Recent highlights of RNA-polymerase-II-mediated transcription. *Curr. Opin. Cell Biol.* **16**, 263–271 (2004).
5. Thiriet, C. & Hayes, J.J. Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. *Mol. Cell* **18**, 617–622 (2005).

6. Kuo, M.H. & Allis, C.D. *In vivo* cross-linking and immunoprecipitation for studying dynamic Protein: DNA associations in a chromatin environment. *Methods* **19**, 425–433 (1999).
7. Orlando, V., Strutt, H. & Paro, R. Analysis of chromatin structure by *in vivo* formaldehyde cross-linking. *Methods* **11**, 205–214 (1997).
8. Impey, S. *et al.* Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. *Cell* **119**, 1041–1054 (2004).
9. Nelson, J.D., Denisenko, O., Sova, P. & Bomsztyk, K. Fast chromatin immunoprecipitation assay. *Nucleic Acids Res.* **34**, e2 (2006).
10. Ostrowski, J., Kawata, Y., Schullery, D.S., Denisenko, O.N. & Bomsztyk, K. Transient recruitment of the hnRNP K protein to inducibly transcribed gene loci. *Nucleic Acids Res.* **31**, 3954–3962 (2003).
11. Dundr, M. *et al.* A kinetic framework for a mammalian RNA polymerase *in vivo*. *Science* **298**, 1623–1626 (2002).
12. Cheutin, T. *et al.* Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* **299**, 721–725 (2003).
13. Cosma, M.P., Tanaka, T. & Nasmyth, K. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* **97**, 299–311 (1999).
14. Liu, C.L. *et al.* Single-nucleosome mapping of histone modifications in *S. cerevisiae*.. *PLoS Biol.* **3**, e328 (2005).
15. Metivier, R. *et al.* Transcriptional complexes engaged by apo-estrogen receptor-alpha isoforms have divergent outcomes. *EMBO J.* **23**, 3653–3666 (2004).
16. Koyanagi, M. *et al.* EZH2 and histone 3 trimethyl lysine 27 associated with Il4 and Il13 gene silencing in Th1 cells. *J. Biol. Chem.* **280**, 31470–31477 (2005).
17. Solomon, M.J. & Varshavsky, A. Formaldehyde-mediated DNA-protein crosslinking: a probe for *in vivo* chromatin structures. *Proc. Natl. Acad. Sci. USA* **82**, 6470–6474 (1985).
18. Thorne, A.W., Myers, F.A. & Hebbes, T.R. Native chromatin immunoprecipitation. *Methods Mol. Biol.* **287**, 21–44 (2004).
19. Bernstein, B.E. *et al.* Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* **120**, 169–181 (2005).
20. Pokholok, D.K. *et al.* Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* **122**, 517–527 (2005).
21. Cawley, S. *et al.* Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* **116**, 499–509 (2004).
22. Orlando, V. Mapping chromosomal proteins *in vivo* by formaldehyde-crosslinked-chromatin immunoprecipitation. *Trends Biochem. Sci.* **25**, 99–104 (2000).
23. Chen, R. *et al.* Ultrasound-accelerated immunoassay, as exemplified by enzyme immunoassay of choriogonadotropin. *Clin. Chem.* **30**, 1446–1451 (1984).
24. Chaya, D. & Zaret, K.S. Sequential chromatin immunoprecipitation from animal tissues. *Methods Enzymol.* **376**, 361–372 (2004).
25. Schwabish, M.A. & Struhl, K. Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. *Mol. Cell. Biol.* **24**, 10111–10117 (2004).
26. Denisenko, O. & Bomsztyk, K. Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length. *Mol. Cell. Biol.* **22**, 286–297 (2002).
27. Kurdistani, S.K., Tavazoie, S. & Grunstein, M. Mapping global histone acetylation patterns to gene expression. *Cell* **117**, 721–733 (2004).
28. Sandoval, J. *et al.* RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res.* **32**, e88 (2004).
29. Waugh, D.S. Making the most of affinity tags. *Trends Biotechnol.* **23**, 316–320 (2005).
30. Van Seuningen, I., Ostrowski, J. & Bomsztyk, K. Description of an IL-1-responsive kinase that phosphorylates the K protein. Enhancement of phosphorylation by sequence-selective DNA and RNA motifs. *Biochemistry* **34**, 5644–5650 (1995).