Day-and-a-half and two-and-a-half day ChIP

Protocol Version 1.0

Jeremiah Faith

Bioinformatics Program
Boston University
Email: faith@bu.edu
http://www.jeremiahfaith.com/

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1 Introduction

The following document contains two chromatin immunoprecipitation (ChIP) protocols that were optimized using fractional factorial and response surface statistical optimization techniques. If you have any comments or questions, email faith@bu.edu or (preferably) add them to the following blog post: http://blog-di-j.blogspot.com/2007/12/optimized-chip-protocols.html
The blog post will also contain the most recent version of this protocol.

1.1 Protocol Change Log

- Thu Nov 29 16:36:15 EST 2007; Version 1.0 of this protocol released

2 Recipes

2.1 Dilution Buffer

Reagent	Concentration	Stock Conc.	Amount for 50ml
Triton X-100	1%	-	500 μl
EDTA	2 mM	0.5 M	200 μl
NaCl	150 mM	5 M	1.5 ml
Tris-HCl pH 8	20 mM	1 M	1 ml

Taken from www.abcam.com

2.2 High Salt Immune Complex Wash Buffer

Reagent	Concentration	Stock Conc.	Amount for 50ml
SDS	0.1%	-	250 μl (of 20%)
Triton X-100	1%	-	500 μl
EDTA	2 mM	0.5 M	200 μl
Tris-HCl pH 8	20 mM	1 M	1 ml
NaCl	500 mM	5 M	5 ml

2.3 Elution buffer

Reagent	Concentration
SDS	1%
Tris pH 8.0	50 mM
EDTA	10 mM

taken from Lee *et.al.*, Nature Protocols 2006.

2.4 Bead washing buffer

PBS + 0.5% BSA

3 Supplies

Many bio-supply companies sell the reagents listed in the recipes section above. Here we list the part numbers for the reagents we commonly use.

Item	**Supplier**	**Part Number**
BSA	Sigma	A7906-10G
EDTA pH 8.0	Ambion	AM9260G
NaCl 5 M	Ambion	AM9760G
PBS	Invitrogen	14200-075
protein G dynal beads	Invitrogen	100.04D
proteinase K	Ambion	AM2546
RNAse cocktail	Ambion	AM2286
SDS 20%	Fisher	BP1311-200
TE pH 8.0	Ambion	AM9849
Tris 1M pH 8.0	Ambion	AM9856
Triton X-100	MP Biomedicals	8074
15 ml centrifuge tubes	Corning	430053
LabQuake Rotisserie	Barnstead	415110
MPC-9600 magnet	Invitrogen	120.06D

4 Protocol

Steps specific to the day-and-a-half protocol have a red star ★. Steps specific to the two-and-a-half-day protocol have a blue star ★.

4.1 Cell Growth

Note that this growth subsection is specific to *E. coli* and the plasmid based tagged transcription factor we used.

1. Dilute an overnite culture of your strain(s) 1:100 in 50 ml of liquid media in a 250 ml flask with appropriate antibiotics
2. after 1 hr add IPTG or other inducer as needed for induction of any cloned constructs (if you have a specific non-cloned gene or tagged gene in the genome, this step is not necessary)
3. After your cells reach sufficient density (OD600 = 0.5) pipette a 15 ml sample into a 15 ml centrifuge tube

4.2 Crosslinking

1. ★for 1.5 day ChIP, add 40 μl of 37% formaldehyde[1] (0.1% final concentration)
   ★for 2.5 day ChIP, add 400 μl of formaldehyde (1% final concentration)
2. invert tube 10 times to mix
3. incubate 10 min at room temp. Do NOT crosslink too long!
4. quench the crosslinking reaction with 1/20th volume of 2.5 M glycine (750 μl for a 15 ml sample)
5. pellet cells by centrifugation for 15 min at 3200g
6. decant supernatant into a formaldehyde/media hazardous waste bin; remove any remaining drops of media by placing the centrifuge tubes upside down on a stack of paper towels for a few seconds

[1] formaldehyde is typically supplied as a 37% solution

7. wash the cells 1x in 10 ml of ice-cold PBS (spin 3200g for 10 min in between washes to pellet cells). From here on, keep samples on ice to limit the effect of proteases.

8. prior to shearing remove any remaining drops of PBS by placing the centrifuge tubes upside down on a stack of paper towels

4.3 Shearing

For this protocol, the cells are not lysed chemically. Instead, we found that sonication is itself sufficient to lyse the cells.

1. add 2 μl of RNAse Cocktail to each milliliter of dilution buffer used in the following step

2. resuspend the PBS-washed, crosslinked sample in 1 ml of the RNAse containing dilution buffer

3. transfer 1 ml crosslinked sample to a 1.5 ml tube and place samples on ice

4. sonicate samples using a Branson 250 Sonifier for 30 secs at 20% power[2] (Figure 1)

5. keep samples on ice for at least 1 min between sonications

6. ★for 1.5 day ChIP, sonicate each sample *two* times for a shearing range of between 1000bp and 100bp with an average around 500bp (Figure 2A)

   ★for 2.5 day ChIP, sonicate each sample *four* times for a shearing range of between 700bp and 100bp with an average around 350bp (Figure 2B)

18 samples takes around an hour for the 2.5 day protocol and 30 minutes for the 1.5 day protocol; dreadfully slow and boring; I play my ipod under the protective sonicator earphones. After half-an-hour this becomes painful if you have the ipod earbuds, so adjust them (in between sonications) until they feel comfortable again. Shearing is much less tedious with some tunes.

[2] more consistent results can be obtained by using a digital Branson where the exact time can be set. also, it helps to rig up the stand holding the sonifier tip to also hold the eppy tube so the tip is used in the tube location for each sample

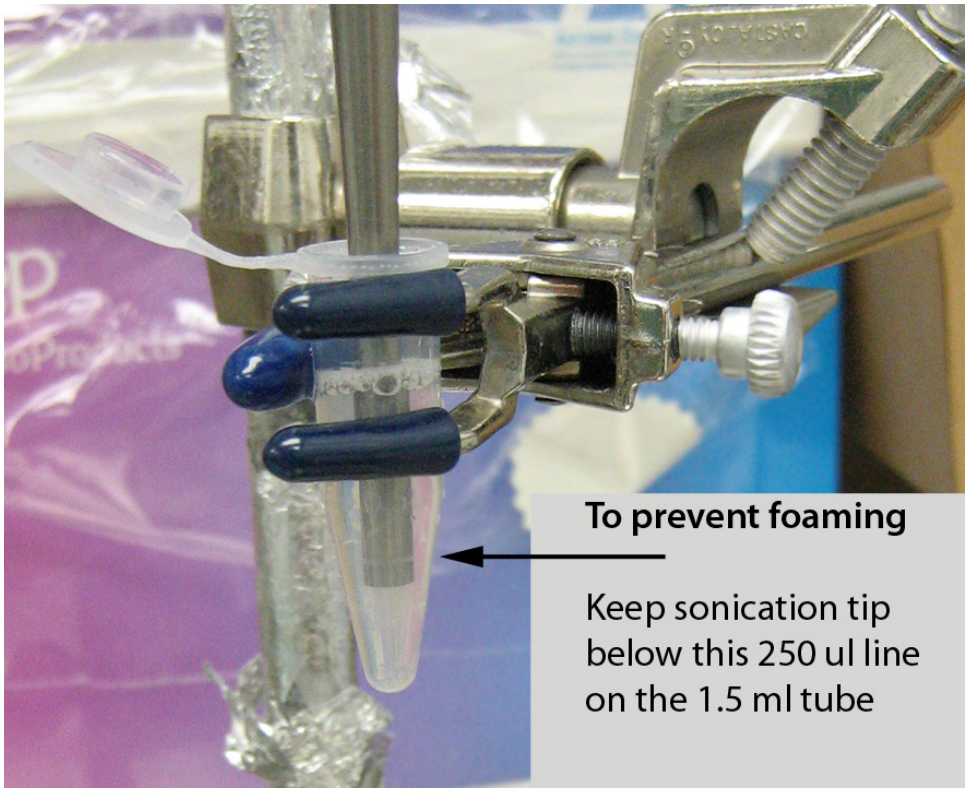

Figure 1: Samples sonicated with the Branson 250 sonicator should be held in place using a stand to ensure consistent shearing from sample to sample. It is important to place the tip of the sonicator just below the 250 μl marking on the 1.5 ml tube to prevent foaming, which leads to inconsistent shearing. Do not place the sonication tip too close to the bottom as it alters the sonication power.

4.3.1 Quantification of starting DNA

We prefer to start with roughly the same amount of chromatin for each precipitation. We estimate the starting amount by cleaning up and quantifying a portion of the sheared chromatin. We can then start all of our precipitations with equal micrograms of DNA.

1. remove 100 μl from each sample to quantify the amount of DNA in each lysate and to verify the shearing range

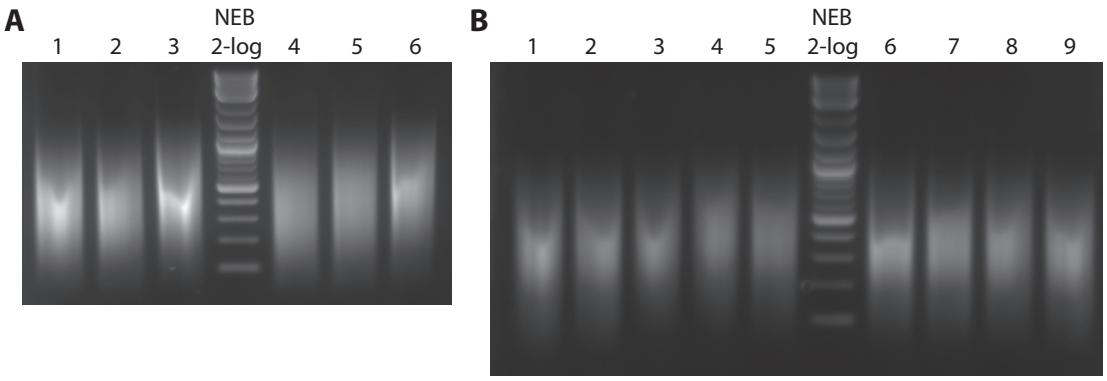

Figure 2: Chromatin samples sheared using a Branson 250 Sonifier. A) 2x30 sec at 20% power have a shearing range of 100-1000bp with average length of around 400bp B) 4x30 sec at 20% power have a range from 700bp-100bp with an average length of around 350bp.

2. place the remaining 900 μl at 4C or on ice for use in the immunoprecipitation
3. add 5 μl of 20mg/ml proteinase K and 25 μl of water to each 100 μl sample
4. incubate the 125 μl sample at 65C for 1 hr
5. clean up the DNA using a Qiagen PCR purification column
6. resuspend the DNA in 50 μl of EB buffer
7. quantify the DNA using spectrophotometer (e.g. a Nanodrop)

The cleaned up DNA should be frozen at -20C and saved. It can serve as the positive control for downstream qPCR reactions. 500-600ng of each sheared

DNA sample should be run an a 1.5% agarose gel to verify shearing range (Figure 2).

4.4 Immunoprecipitation

Immunoprecipitations are begun with equal starting DNA (2 μg)[3]. The following steps can be done in 1.5 ml tubes, in PCR strips, or in a 96-well plate. For 1.5 ml, tubes use the Dynal MPC-S magnet from Invitrogen. For PCR strips or a 96-well plate, use the Dynal MPC-9600. For rotating the samples, I use a LabQuake Rotisserie.

4.4.1 Add primary antibody

1. take two 2 μg samples from the 900 μl remaining sheared chromatin. Label these **A**, **B**[4]
2. add 3.3 μg of the primary antibody to sample **A** (e.g. if your tag is myc put anti-myc here); sample **B** is a negative control with no primary antibody
3. dilute all samples with dilution buffer to bring the total volume to 80 μl
4. ★ for 1.5 day ChIP, rotate all samples for 40 minutes[5]
   ★ for 2.5 day ChIP, rotate all samples overnite
5. ★ for 1.5 day ChIP, begin at this step 10-20 minutes after you begin rotating your samples above
   ★ for 2.5 day ChIP, begin at this step the following morning

   while the samples are incubating, prepare the dynal beads by washing them one time with 180 μl of 0.5% BSA in PBS
6. resuspend the beads in 100 μl of 0.5% BSA in PBS

[3] As long as the quantified samples have roughly the same concentrations (e.g. no sample more than 2-fold different from the others), you can use the mean DNA concentration to determine the amount of sheared chromatin to be used for all samples. For example if you have 3 samples at 100 ng/μl , 150 ng/μl , and 120 ng/μl respectively, your average concentration is 123 ng/μl , and you can use 32.5 μl of chromatin (2 μg) for all three immunoprecipitations.

[4] You can place the remaining sheared chromatin at -20C for later use.

[5] I've had similar success with 10 minute incubations. However, I find it takes me around 40 minutes to prepare the beads in 1.5 ml tubes.

4.4.2 Add beads to capture primary antibody

1. add the 100 μl of BSA/PBS washed dynal beads to all samples [6]

   ★ for the 1.5 day protocol, rotate at 4C for 40 minutes

   ★ for the 2.5 day protocol, rotate at 4C for 120 minutes

4.4.3 Bead/Immunoprecipitation Washing

1. wash beads 1x with 180 μl high salt wash at 4C; rotate for 5 minutes with the high salt wash
2. wash beads 2x with 180 μl TE at room temperature (perform steps at room temp from now on); don't rotate when you wash your samples if you use the MPC-9600, just use the magnet to mix them – it's faster (instructions come with the magnet)
3. elute by adding 180 μl elution buffer and incubating at 65C for 15 minutes (for PCR strips or a plate, you can use a PCR machine for this incubation)
4. pellet the beads with the magnet and transfer the supernatant to a new tube

4.5 Crosslink reversal

1. place the samples overnight at 65C for 12-15 hours

4.6 Final DNA cleanup

1. add 4 μl proteinase K and incubate the samples at 55C for 1 hr
2. clean up the DNA with a Qiagen PCR purification kit
3. resuspend DNA in 100 μl of EB buffer

Your enriched DNA is now ready.

[6] since the beads are very concentrated and tend to stick to the wall of the tube it is much better if you transfer your antibody/chromatin/dilution buffer sample into the tube containing the beads rather than pipetting the beads into the antibody/chromatin/dilution buffer samples.

5 Tips and Tricks

5.1 timing

It should take 7-10 hrs for the first day of the 2.5 day protocol and 9-12 hours for the first day of the 1.5 day protocol, so make sure to start early in the day[7]. A decent stopping point on the first day is after the samples have been sheared – just place them at -20C until you want to do the immunoprecipitation. The first time you try the procedure, you should assume it will take at least an extra day.

5.2 working efficiently

Depending on the details of how you perform the protocol above, you may need to transfer between PCR strips, plates, and 1.5 ml tubes. The Matrix Impact2 Equalizer Multichannel Pipettor (Part No: 2032) is extremely useful for this type of transfer, because it allows you to move up to eight samples at a time between plates, PCR strips, or 1.5 ml tubes.

6 Further increasing the throughput

Since the immunoprecipitation steps are all in 96-well format, moving to 384 samples or more in a day wouldn't be too hard – magnet-based protocols are also quite easily adapted to robotics (just browse Agencourt's website for some nice examples). So the immunoprecipitation potential is pretty much limitless. The major bottleneck in the protocol is cell growth and shearing.

The growth bottleneck could be solved pretty easily for most conditions by growing samples in a plate (e.g. a deep-well 96-well plate). However, the shearing bottleneck is more difficult. As far as I know, there is no way to shear in 96-well plates or PCR strips. However, the Biorupter sonicators from diagenode allow up to 48 samples at a time in single tubes (though I don't have one to try, so I don't know how well it works). With the lower formaldehyde concentration used in the day-and-a-half protocol, it's also possible that you can just cut the DNA with a restriction enzyme or two rather than sonicating. I have tested that DNA cuts quite readily with 0.1% formaldehyde crosslinks; I have not tested if the ChIP procedure works well

[7] The exact time depends on how many breaks you take and how many samples you are processing.

with digested rather than sonicated DNA. If the sonication was replaced by digestion, the chemical lysis step would also have to be added back in.

On a more ambitious level if you had a monoclonal antibody library with a specific antibody for each native-untagged transcription factor of interest, you would only need to grow a single culture per environmental condition, and the sheared DNA would just be aliquoted (e.g. with a plate-filler) into tubes containing the different antibodies. For a bacteria like, *E. coli* with around 300 known or predicted transcription factors, it would be possible to screen one or two conditions a day for *all* of the species' transcription factors if you had such an antibody library.