Yeast Chromatin Immunoprecipitation (ChIP) Protocol: Mechanical Breakage & FA Lysis Buffer

Updates: DVG 5/24/02; PBM 3/13/06 & 1/8/08.

This protocol is based on:

Dedon PC, Soults JA, Allis CD, Gorovsky MA. (1991). A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. Anal. Biochem. 197, 83–90.

Meluh PB, Koshland D. (1997). Budding yeast centromere composition and assembly as revealed by in vivo cross-linking. Genes Dev. 11(24):3401-3412.

Meluh PB, Broach JR. (1999). Immunological analysis of yeast chromatin. Methods Enzymol. 304:414-30.

Hecht A, Grunstein M. (1999). Mapping DNA interaction sites of chromosomal proteins using immunoprecipitation and polymerase chain reaction. Methods Enzymol. 304:399-414.

Two Days Before:

- Start a 5 ml overnight culture from a single colony.

One Day Before:

- Start a larger overnight culture using part of the 5 ml culture (sub-culture).

Day One – Part One:

- Grow culture until it is in log phase: OD_{600} = 0.7 to 1.2.
- For each sample you collect, put the appropriate amount of 37% formaldehyde in a 50 ml tube and label. The calculation for the correct amount of 37% formaldehyde to use is: **volume of culture/36**. This gives a **1% final concentration of formaldehyde**. For 50 ml samples, use 1.4 ml 37% formaldehyde. Alternatively formaldehyde can be added directly to the culture flask.
- When you are ready to fix a sample, fill one of the tubes with culture to the line right under the 50 ml marking. Mark the time on the tube and put it on a flat shaker.
- At 1 hr and 50 minutes after the fixing start time:

1. Spin tubes @ 2K for 5 min, RT.
2. Decant and blot on paper towel.
3. Vortex pellet.
4. Resuspend pellet in about **25 ml of 1X Buffer A** (Tris Buffered Saline).

5. Repeat steps 1) to 4) *twice*.
6. Resuspend in **1 ml of 1X Buffer A** (TBS).
7. Transfer to 1.5 ml microfuge tube.
8. Spin @ 10K in microcentrifuge, 1 min, RT, aspirate supernatant.
9. Put tube with cell pellet on ice.
10. For long term storage, place tube in dry ice to flash freeze sample, then store at –80˚C until ready for the next step.

Dave's Notes:

1. *This protocol was written assuming pellets contain 50-OD_{600} equivalents. Volumes can be adjusted as required for the specific application.*
2. *We use Fisherbrand 50 ml disposable, sterile, conical tubes to harvest. It's not important that the harvesting is done exactly like this.* ***What is important is the fixation time!***
3. ***About fixation:*** *The amount of fixation time used depends upon the protein of interest. For histone proteins, short fixation times (10-20 minutes) are required; however, for MIF2, the amount of DNA in the IP increases with increasing fixation time. When testing a new protein, we usually harvest two pellets-one fixed for 20 minutes, the other for 2 hours and compare. Also, TBS (tris-buffered saline) helps to quench the fixation reaction; therefore, the fixation time is considered the amount of time between the addition of the formaldehyde and the first resuspension of the cells in TBS. This is why we begin the spins early.*

Day One – Part Two:

- Thaw pellets on ice for 10-30 min (as needed).

- **Bead Break Cells** – Mechanical Breakage:

  1. Resuspend pellets in **250 µl of FA lysis buffer w/protease inhibitors** by pipeting up and down. (NOTE: You might want to use **SDS Lysis Buffer** if you are having background with anti-HA. See Pam's older protocol and/or alternative recipes at end for SDS Lysis Buffer and IP Dilution Buffer.)
  2. Add **acid-washed, sterilized glass beads**, so there is a 1 to 2 mm layer of liquid above the beads.
  3. Vortex vigorously in the TOMY Mixer. Set it to level 10, mix **8 times** for 1 minute each, and put on ice for one minute between mixes. O O O O O O O O

- Spin @ 500 rpm for 15 seconds to collect liquid at bottom of tube.

- Add **250 µl of FA lysis buffer w/protease inhibitors** and vortex to resuspend any material that pelleted during the brief spin.

- **Stacked Transfer** – Transfer lysate to new tube.

  1. Label and open new tubes.

2. Clean the outside of original tube first with sterile dH_2O, then with 70% ethanol. This can be accomplished by dipping the bottom 2/3 of each tube into small beakers. Pat dry with sterile Kimwipe or paper towel.
3. Turn tube upside down (don't touch the bottom half) and "flick" tube to get all liquid to top of tube.
4. Using a Bunsen burner, heat the tip of an 18-gauge needle until red.
5. Make a small hole in the bottom of the tube. The beveled tip of the needle should go half way into the tube.
6. When the plastic has hardened, pull out the needle.
7. Nest the full tube into the empty tube.
8. Spin all of the nested tubes @ 3K, 1 min, 4˚C. All of the liquid and cell debris – without beads – should now be in the bottom tube.
9. Resuspend cell debris.

- **Sonication –** Breaking DNA into 500 bp fragments.

Repeat **6 times** for each sample:

1. Rinse the sonicator tip with water, then ethanol and dry with Kimwipe.
2. Set the sonicator to Power = 1.5 and Duty Cycle = constant.
3. Sonicate leftover FA lysis buffer w/supplements for a moment.
4. Put open sample tube in round, cold rack (from freezer) and sonicate for 10 seconds. Avoid aerating the sample.
5. Let sample sit on ice for 5 minutes. O O O O O O

Dave's Notes:

1. *We use **Branson Sonifier 250 with a 3/16" tapered microtip.***
2. *Time on ice can be decreased. I usually only wait 1-2 minutes.*

- **Clarify Sonicate**

1. Spin in microcentrifuge @ 15K, 20 min, 4˚C (clarifying spin). Transfer supernatant to Falcon snap cap tube, discard pellet.

Dave's Note: *We use 15 ml snap cap, round bottom tubes from Falcon (cat#35-2059)*

2. Dilute supernatant 1:9 with ice-cold **FA Lysis Buffer w/Protease Inhibitors**.
3. At this point, it's best to let the diluted extract sit on ice for 30 min to allow time for non-specific precipitation of material.
4. Spin in high speed centrifuge @ 8250 rpm, 10 min, 4˚C.
5. Transfer supernatant by carefully decanting into a new 15 ml conical tube – this is the **Chromatin Solution**.

- **Set-up ChIP's**

1. We do our IP's in microfuge tubes, using about 1 ml of Chromatin Solution per IP.
2. Add antibodies to the appropriate dilution. Remember to include a no antibody control for each yeast strain used. Store remaining Chromatin Solution at 4˚C; at least 300 μl are needed for preparation of Total DNA sample.

3. Nutate the IP's overnight at 4°C.

Day Two

- **Harvest Immune Complexes**

1. To each tube, add:
   4 µl sonicated lambda DNA (Gibco BRL Cat#25250-010, must be sonicated like the ChIP extracts & checked on gel to verify small size – add sodium azide to 0.1%)
   40 µl Protein A Sepharose Beads (prepared as 50% slurry in TE/0.1% BSA/0.1% azide)
2. Incubate 2 hr. @ RT on Nutator.

Pam's Note: *If using monoclonal antibodies, make sure they are of an isotype recognized by Protein A – otherwise, might need to use Protein G beads instead (e.g. for anti-myc 9E10).*

- **Wash IP's** – Try to get through these as quickly as possible.

1. Spin down beads @ 10 K, 1min, RT.
2. Aspirate supernatant.
3. Wash beads with 1 ml of the following buffers and mix by inverting 3 or 4 times. Only aspirate supernatant to 0.1 ml marking so no beads are lost:
   - **FA Lysis Buffer (no supplements)**
   - **FA Wash Buffer**
   - **LiCl/Detergent Buffer**
   - **TE**
4. Wash beads a second time with TE:
   - Wash with 500 µl TE.
   - Transfer beads and wash to second tube.
   - Wash the old tube with 500 µl TE and transfer to second tube.

Pam's Notes:

1. *Outfit aspirator with very fine gel loading tips. I recommend changing the tip for each sample when removing the IP supernatant and the first FA Lysis Buffer wash. After that it's probably okay to use the same tip for the entire set.*
2. *The transfer of beads to a second clean microfuge tube during the TE washes is critical to avoid background that comes from non-specific sticking of chromatin to the walls of the original tube.*

- **Elute**

1. Aspirate after final wash (very carefully, get as close to beads as possible without loosing any).
2. Add **250 µl 1% SDS/0.1 M $NaHCO_3$**.
3. Incubate ≥20 minutes at room temperature on Nutator.

Pam's Note: *It's OK to incubate longer or stop here for a while.*

4. Spin @ 10 K, 1 min, RT.
5. Transfer eluate to new tube using gel loading pipet tips, making sure no beads are transferred. Be patient and let the liquid drain from the pipet tip. Quantitative transfer is critical for reproducible (and meaningful) results!
6. **Repeat** Steps 2-5.
7. Transfer second eluate to same new tube, using the method in step 6, but when most of it is transferred, get the last bit by pressing the tip against the bottom of the tube while pushing some air out (so no beads enter) and pipet up as much of the remaining liquid as possible.
8. Add **20 µl 5 M NaCl** to the combined eluates.
9. Vortex. Spin down briefly. Make sure lids are closed completely.
10. Heat at 65˚C to 70°C for 6 hours to overnight to reverse the formaldehyde cross-links.

- **Totals**

1. Aliquot 300 µl of Chromatin Solution (no antibodies).
2. Add **3.2 µl 5 M NaCl**.
3. Vortex.
4. Heat at 65˚C to 70°C from 6 hours to overnight to reverse cross-links.

Dave's Note: *I usually prepare 2 Total samples. That way, if something goes wrong during extractions (see below), you don't have to start over from the beginning.*

Pam's note: *You might want to use lid locks or put a heavy weight on top of all tubes to prevent evaporation.*

Day Three

- **Proteinase K Treatment**

1. To **ChIP's**: Add **20 µl 1 M Tris-HCl pH 6.8** and **10 µl 0.5 M EDTA**. Best to make up cocktail and add 30 µl to each tube.
2. To **Totals**: Add **33.3 µl 10% SDS** and **5.5 µl 0.5 M EDTA**.
3. Vortex.
4. Add **2 µl Proteinase K Solution** (Boehringer Mannheim, 1413 783, ~18.6 mg/ml)
5. Invert 3-4 times to mix.
6. Incubate @ 42˚C for 2 hours.

- **Extractions** – To remove SDS.

***For all extractions:

- Mix on TOMY mixer (max speed) for 2 minutes.
- Spin @ 10 K, 5 min, room temperature. ***
- Transfer aqueous phases as quantitatively as possible.

1. Prepare **fresh Phenol/Chloroform/Iso-amyl Alcohol** (PCI; 25:24:1). Spin PCI @ 2K, 5 min, RT, to make sure there is a water layer on top. Balance by weight (PCI is denser than water).

2. Extraction of **ChIP's**:
   - Extract once with an equal volume of PCI (500 µl).
   - Extract once with an equal volume of Chloroform (500 µl).
   - YYH recommends extracting with an equal volume of Chloroform (500 µl) a second time.
   - Final volume should be ~500 µl.

3. Extraction of **Totals**:
   - Add 60 µl TE to bring volume up to ~400 µl.
   - Extract twice with an equal volume of PCI (400 µl).
   - Extract twice with an equal volume of Chloroform (400 µl).
   - Back extract organic phases with 100 µl TE.
   - Final volume should be ~500 µl.

   For each Total sample, it is convenient to set up five tubes in a row:

   Tube 1 Original tube plus first PCI
   Tube 2 Second PCI
   Tube 3 First $CHCl_3$
   Tube 4 Second $CHCl_3$
   Tube 5 Empty tube for final sample

   After vortexing and spinning the sample, transfer the aqueous layer from Tube 1 (first PCI) to Tube 2 (second PCI). Then add 100 µl TE to the residual organic phase left behind in Tube 1. Vortex & spin Tubes 1 and 2 at the same time. Transfer the aqueous layer from Tube 2 (second PCI) to Tube 3 (first $CHCl_3$), then *using the same pipet tip*, transfer the back-extracted aqueous layer from Tube 1 to Tube 2. Vortex & spin Tubes 2 and 3 at the same time. Transfer the aqueous layer from Tube 3 (first $CHCl_3$) to Tube 4 (second $CHCl_3$), then *using the same pipet tip*, transfer the back-extracted aqueous layer from Tube 2 to Tube 3. Vortex & spin Tubes 3 and 4 at the same time. Transfer the aqueous layer from Tube 4 (second $CHCl_3$) to Tube 5 (final tube), then *using the same pipet tip*, transfer the back-extracted aqueous layer from Tube 3 to Tube 4. Vortex & spin Tube 4. Transfer the back-extracted aqueous layer from Tube 4 to Tube 5.

Pam's note: *Be as quantitative as possible with each transfer step, taking care not to leave residual liquid in the pipet tips.*

4. After ChIP and Total extractions are complete, add to each sample:
   5 µg **Glycogen** (from 20 µg/µl stock – 0.25 µl per tube)
   50 µl **3 M NaOAc**
   1 ml **"100%" EtOH** (the good stuff!)

It's easiest to prepare a cocktail of glycogen plus NaOAc, aliquot 50.25 µl to each sample, then add the EtOH separately.

5. Store @ -20˚C overnight.

Day Four

- Spin down all EtOH precipitates (ChIP and totals) @ 15K, 20 min, 4˚C.
- Decant immediately after spin stops. Be careful because ChIP DNA pellets should be VERY, VERY TINY, almost invisible. If the pellets are easy to see (i.e. large), it probably indicates residual SDS. In this case, the samples should be re-extracted with $CHCl_3$ at least one more time and re-precipitated.
- Wash with **500 µl 70% EtOH** (DNA is not soluble in 70% EtOH).
- Spin @ 15K, 10 min, 4˚C.
- Decant wash.
- Dry in speed-vac.
- **Resuspend in TE:**

1. For **ChIP** DNA pellets, we usually add 1 µl TE per 10 µl chromatin solution used for IP. NOTE: For MIF2 and CSE4-HA under wild type conditions, you can resuspend in 5 µl TE per 10 µl chromatin solution used for IP.

Amt used _____

2. For **Totals**, use 1µl/1 µl chromatin solution

Amt used _____

Recipes

FA Lysis Buffer (500 ml)

25 ml	1 M HEPES-KOH, pH 7.5
14 ml	5 M NaCl
1 ml	0.5 M EDTA, pH 7.6
25 ml	20% Triton-X-100 (v/v)
10 ml	5% DOC (deoxycholic acid; w/v)
425 ml	Sterile water

Add the following supplements to **10 ml FA Lysis Buffer**, as required:

10 µl	1 mg/ml Leupeptin (Leu)
10 µl	1 mg/ml Pepstatin (Pep)
50 µl	0.2 M PMSF (in ethanol or methanol)

Vortex buffer immediately after adding PMSF.

FA Wash Buffer (500 ml) – Same as Lysis Buffer but w/o supplements.

25 ml	1 M HEPES-KOH, pH 7.5

50 ml	5 M NaCl
1 ml	0.5 M EDTA, pH 7.6
25 ml	20% Triton-X-100 (v/v)
10 ml	5% DOC (deoxycholic acid)
389 ml	Sterile water

LiCl/Detergent Wash (250 ml)

2.65 g	LiCl
25 ml	10% NP-40
50 ml	5% DOC
0.5 ml	0.5 M EDTA
2.5 ml	1 M Tris-HCl, pH 8.1

1X TE (100 ml)

1 ml	1 M Tris-HCl, pH 8.0
200 µl	0.5 M EDTA, pH 7.6

Bring to 100 ml with sterile water.

Alternative Breakage Buffers – probably less background with anti-HA.

SDS Lysis Buffer	*100 mL*:
1% SDS	10 mL 10% SDS (UltraPure)
10 mM EDTA	2 mL 0.5 M EDTA
50 mM TRIS, pH 8.1	5 mL 1 M TRIS

IP Dilution Buffer (for 1:9 dilution)	*250 mL*:
1.1% Triton X 100	13.8 mL 20% Triton X 100
1.2 mM EDTA	0.6 mL 0.5 M EDTA
16.7 mM TRIS, pH 8.1	4.2 mL 1 M TRIS, 8.1
167 mM NaCl	8.35 mL 5 M NaCl