RNA ChIP-IT®

Magnetic Chromatin Immunoprecipitation Kit

(version A6)

Catalog No. 53024

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Introduction

Chromatin Immunoprecipitation (ChIP) is a very powerful, established technique for studying the interactions between specific proteins and loci within the genome. Active Motif has a long history of developing innovative kits to serve the ChIP needs of researchers. Recent developments in chromatin research have made clear the importance of non-coding RNA molecules in regulating genome function (see Overview). As a consequence, there is a need for methodology to study the association of specific proteins and individual RNA molecules in the context of chromatin.

Active Motif's RNA ChIP-IT® Kit is the first kit of its kind, suitable for studying RNA-protein interactions in a chromatin context. In this method, intact cells are fixed using formaldehyde, which cross-links and preserves protein/RNA interactions. The RNA is then sheared into small, uniform fragments using sonication and, after a DNase treatment to remove residual DNA, specific protein/RNA complexes are immunoprecipitated using an antibody directed against the RNA-binding protein of interest. Following immunoprecipitation, cross-linking is reversed, RNA is extracted (by Trizol extraction), DNase I treated again (to remove residual DNA) and analyzed by RT-PCR to determine which RNA fragments were bound by the protein of interest.[1] Active Motif's RNA ChIP-IT Kit is innovative because all existing technologies on the market (RIP kits) use unfixed nuclear extracts that do not take into account the dynamic chromatin environment of the RNA-protein complexes of interest. The Active Motif method is fast and has been proven to be highly specific and very efficient for the study of long non-coding RNAs and pre-mRNA regulation in a chromatin context. All of the buffers, reagents and procedures in the RNA ChIP-IT Kit have been optimized for studying nuclear RNA-protein interactions *in vivo*.

The RNA ChIP-IT Control Kit – Human contains reagents designed to be used in conjunction with the RNA ChIP-IT Kit. It contains human-specific reagents (Suz12 rabbit polyclonal antibody, control rabbit IgG and lincSFPQ primer mix) to verify that the chromatin samples prepared by the user perform as expected with positive and negative control samples.

product	**format**	**catalog no.**
RNA ChIP-IT®	25 rxns	53024
RNA ChIP-IT® Control Kit – Human	5 rxns	53025

Background

The field of Epigenetics concerns the contextual information that is superimposed on the relatively stable underlying genomic sequence through the modulation of chromatin structure. Control of chromatin structure is a major hallmark of eukaryotic cells, and is the mechanism by which gene regulation is stably regulated during multi-cellular development and differentiation.

Evidence is building rapidly that RNA-directed processes play a critical role in orchestrating chromatin architecture and epigenetic memory. It is known that RNA is an integral component of chromatin: a recent study established that nucleic acids purified from chromatin are 2-5% RNA. The identities of these Chromatin-Associated RNAs (CARs) have been analyzed, and most of them do not correspond to nascent transcripts or mature RNA pol II transcripts. Rather, CARs appear to correspond to non-coding RNAs (ncRNAs) that are specifically associated with chromatin.[2]

Non-coding RNAs can be classified into two types: long non-coding RNAs (lncRNAs) and small RNAs (< 200 nt in length). Both classes have established epigenetic functions. Small RNAs include microRNAs (miRNA), PIWI-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs), all of which have roles in chromatin structure and/or transcriptional gene silencing.[3] Likewise, long non-coding RNAs are involved in many epigenetic processes, with increasing reports that lncRNAs can direct and regulate both chromatin activator complexes (CACs) and chromatin repressor complexes (CRCs). Long ncRNAs in particular play an important role in pluripotency, differentiation and in the higher-order chromatin structure of pericentric heterochromatin by organizing the heterochromatin components.[4]

Flow Chart of Process

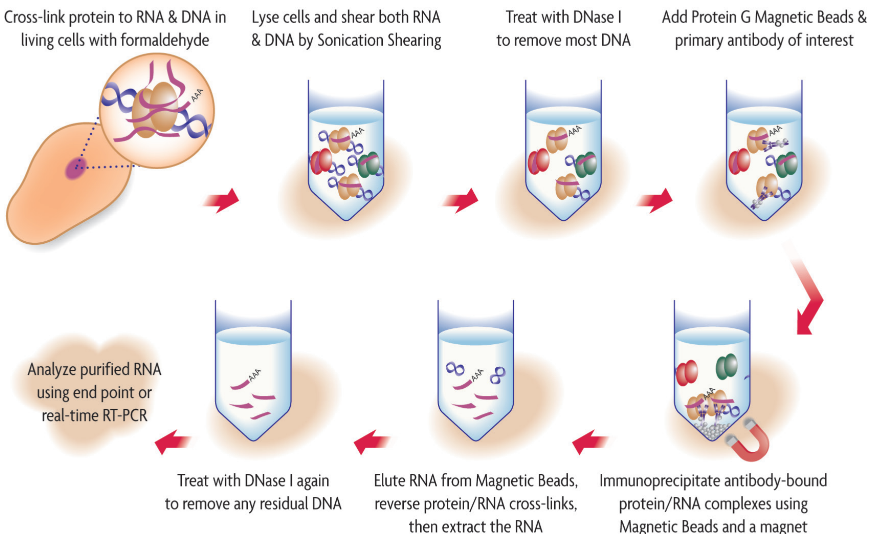


Figure 1: Flow chart of the RNA ChIP-IT process.

Kit Components and Storage
RNA ChIP-IT Kit (Catalog No. 53024)

Please store each component at the temperature indicated in the table below. **Do not re-freeze the Protein G Magnetic Beads after you have received this kit.**

Reagents	Quantity	Storage / Stability
10X PBS	120 ml	-20°C for 6 months
10X Glycine	30 ml	-20°C for 6 months
100 mM PMSF	400 µl	-20°C for 6 months
Protease Inhibitor Cocktail	400 µl	-20°C for 6 months
RNase Inhibitors	60 µl	-20°C for 6 months
DNase I	220 µl	-20°C for 6 months
DNA Digestion Buffer	150 µl	Room temperature
0.5M EDTA	200 µl	-20°C for 6 months
1X Lysis Buffer	12 ml	-20°C for 6 months
Shearing Buffer AM2	10 ml	-20°C for 6 months
5 M NaCl	200 µl	-20°C for 6 months
Protein G Magnetic Beads*	650 µl	4°C for 6 months
RNA-IP Buffer	2 ml	-20°C for 6 months
RNA-ChIP Wash Buffer 1	40 ml	4°C for 6 months
RNA-ChIP Wash Buffer 2	40 ml	4°C for 6 months
DEPC water	10 ml	Room temperature
RNA-ChIP Elution Buffer	4 ml	-20°C for 6 months
10X DNase I Reaction Buffer	100 µl	-20°C for 6 months
DNase I Stop Solution	100 µl	-20°C for 6 months
Mini Glue Dots	1 sheet	Room temperature
Bar Magnet	1	Room temperature

* The Protein G Magnetic Beads are shipped on dry ice, but **should not be re-frozen** by the customer. Upon receipt of this kit, the beads should be stored at 4ºC.

Additional materials required

- An antibody directed against the protein of interest (preferably ChIP validated)
- Pipettors and sterile RNase-free filter pipette tips
- 0.2 ml 8-strip PCR tubes and caps (DNase and RNase free). We recommend Standard PCR strip tubes (Sigma Cat. No. T9692) and caps for 0.2 ml strip tubes (Sigma Cat. No. Z377910, or tubes with caps (Thermo Fisher Cat. No. AB-0451).
- 200 µl multi-channel pipettor to make the wash steps easier
- Reservoir (RNase free) for multi-channel pipetting of wash buffers
- Thermal cycler
- 37% formaldehyde solution (formalin) with 10-15% methyl alcohol to prevent polymerization (*e.g.* Sigma Cat. No. F8775). We do not recommend paraformaldehyde.
- Magnetic stand. You can assemble a magnetic stand using the provided bar magnet (see Appendix – Section D) or use commercially available stands (*e.g.* the Promega Magne-Sphere® Technology twelve-position Magnetic Separation Stand).
- Spectrophotometer or equivalent to quantify RNA
- Sonicator (*e.g.* Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051))
- Minimal cell culture media
- Agarose gel electrophoresis apparatus
- Cell scraper (rubber policeman) if using adherent cells
- Rocking platform for culture plates
- Microcentrifuge and microcentrifuge tubes
- Apparatus to rotate tubes end-to-end at 4°C (*e.g.* Labquake from Barnstead/Thermolyne)
- Trizol reagent (*e.g.* Trizol LS, Life Technologies Cat. Nos. 10296-010 or 10296-028)
- Chloroform (RNase free)
- Isopropanol (RNase free)
- GlycoBlue (15 mg/ml, blue co-precipitating agent). (Applied Biosystems (Ambion) Cat. No. AM9515). Glycogen is an alternative, but the pellet is easier to visualize with GlycoBlue.
- 75% ethanol
- dH_2O
- Reagents for Reverse Transcriptase PCR (RT-PCR)

References

1. Selth, L.A. *et al.* (2009) *Cold Spring Harb. Protoc.* doi:10.1101/pdb.prot5234.
2. Rodriguez-Campos, A. and Azorín, F. (2007) *PLoS ONE* 2(11): e1182.
3. Dinger, M.E. *et al.* (2008) *PLoS Computational Biology* 4(11): e1000176.
4. Mattick, J.S *et al.* (2009) *BioEssays* 31(1): 51-59.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Points to consider:

- RNA is highly susceptible to RNase degradation. Ensure all ancillary reagents are RNase free.
- Wear gloves at every step of the protocol to avoid RNase contamination from hands.
- **Cell growth and chromatin preparation.** When planning an experiment, calculate the number of chromatin preparations you will require and determine the number of RNA-ChIP reactions you plan to perform on each chromatin preparation. Be sure to include the appropriate control RNA-ChIP reactions in your calculations. Also, note that if you wish to analyze the effect of particular compounds or culturing conditions on protein/RNA interactions, you should prepare chromatin from control (untreated) cells as a reference sample.
- **Protein G-coated magnetic beads.** The supplied magnetic beads must be washed two times with DEPC water before use in RNA-ChIP. They also must be resuspended to form a homogeneous slurry. There is no need to pre-block the beads or pre-clear the sample. For best results, gently shake and roll the tube before and after washes. The beads settle quickly, and therefore should be resuspended just before pipetting. **Protein G Magnetic Beads are shipped on dry ice, but should not be re-frozen by the customer. Upon receipt, the beads should be stored at 4°C.** The ChIP-IT Protein G Magnetic Beads are also sold separately (Catalog No. 53014).
- **Minimize the number of freeze/thaw cycles**. While it is possible to stop the procedure and store the sample at the end of Steps A, B, C, F, G and H, it is not recommended to stop at the end of every step. Every freeze/thaw cycle degrades some of the RNA. **To minimize RNA degradation, plan your experiment with the fewest number of freeze/thaw cycles possible. We recommend stopping the experiment after step C, and G or H (2 stops total).**
- **Antibodies must be suitable for ChIP.** At present, very few antibodies have been validated for RNA-ChIP. RNA-ChIP validated antibodies must recognize fixed, native protein that is bound to RNA and/or complexed with other proteins. Many antibodies that perform well in other applications do not perform in RNA-ChIP. Thus, RNA-ChIP performed with an antibody that has not been RNA-ChIP validated (or at least ChIP-validated) must include appropriate controls (positive control antibody and negative control IgG) to validate the chromatin preparation and the RNA-ChIP methodology. Unfortunately, there is no real universal control antibody as RNA expression is cell dependent.
- **8-well PCR strips.** Perform RNA ChIP-IT in 8-well PCR strips. Do not use 1.5 ml or 1.7 ml microcentrifuge tubes for the immunoprecipitation, as the background will be higher.
- **Bar magnet.** The provided bar magnet can be used with 8-well PCR strips (see Appendix – Section D for detailed instructions).
- **Resuspend solutions completely.** Thaw the PMSF at room temperature until fully dissolved. Vortex gently and spin down briefly before use. Before using, the RNA-ChIP Elution Buffer must be thawed at room temperature, as it will remain frozen when stored on ice.

- **RNase Inhibitors in buffers.** Every buffer that is supplemented with RNase Inhibitors must be used immediately and cannot be stored after the addition of the RNase Inhibitors.
- **Maximum volume of chromatin.** Chromatin shearing (sonication) buffers usually contain detergents (*e.g.* 0.1% SDS and 0.5% sodium deoxycholate is typical). If you plan to use more than 60 µl sonicated chromatin in a RNA-ChIP assay, use the 150 µl reaction volume as described on page 10. This will ensure that the detergent in the shearing buffer does not interfere with antibody binding. Do not use more than 60 µl of sheared chromatin in a 100 µl reaction.
- **Quantity of antibody.** Optimal results are typically achieved with 1-3 µg of antibody. However, this will vary according to the affinity of the antibody and the quality of the chromatin, and you may need to use more of a particular antibody.
- **Safety precautions.** Formaldehyde and PMSF are highly toxic chemicals. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used. Also, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood. Finally, chromatin sonication should be performed in a biosafety hood if the chromatin is extracted from biohazardous or infectious materials.

Protocols

Important: While it is possible to stop the procedure and store the sample after each of Steps A, B, C, F, G and H, this is not recommended because each freeze/thaw cycle will cause some RNA degradation. To minimize RNA degradation, plan your experiment with the fewest number of stops possible, as this eliminates freeze/thaw cycles. We recommend no more than 2 stops total.

A. Cell Fixation

This protocol describes fixation of cells from one 15 cm plate (approximately 1.5×10^7 cells). The fixation and shearing protocols assume that you have already optimized shearing conditions for your specific cell line and treatment. Protocols are provided below for fixation of either adherent cells or suspension cells.

Note: Several of the buffers used below require addition of PMSF and protease inhibitors (PIC). Thaw these reagents before starting the chromatin preparation (*i.e.* 30 minutes at room temperature), then add to the buffers immediately before use.

Fixation of Adherent Cells

1. Grow cells to 80-90% confluency in one 15 cm plate. If applicable, stimulate cells as desired to activate the pathway of interest.
2. When cells are ready to harvest, freshly prepare the following solutions. The volumes below are calibrated to one 15 cm plate:
   a. **Fixation Solution:** Add 540 µl 37% formaldehyde to 20 ml minimal cell culture medium (no serum) and mix thoroughly. Leave at room temperature.
   b. **1X PBS Solution**: Add 2.33 ml 10X PBS to 21 ml dH_2O, mix and place on ice.
   c. **Glycine Stop-Fix Solution:** Combine 1 ml 10X Glycine Buffer, 1 ml 10X PBS and

8 ml dH_2O. Mix well and leave at room temperature.

d. **Cell Scraping Solution:** Add 500 µl 10X PBS to 4.5 ml dH_2O, mix and place on ice.

3. Pour medium off the cells and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

   Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation times.

4. Pour Fixation Solution off and wash by adding 10 ml ice-cold 1X PBS to each plate. Rock the plate for 5 seconds, then pour off the PBS.
5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix Solution to each of the plates. Swirl to cover, and then rock at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix Solution, then adding 10 ml ice-cold 1X PBS. Rock the plate for 5 seconds, then pour off the PBS.
7. Just before use, add 25 µl 100 mM PMSF to 5 ml Cell Scraping Solution. Add 5 ml of this ice-cold Cell Scraping Solution to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 5 ml pipette to transfer the cells to a 15 ml conical tube on ice.
8. Pellet the cells from step 7 by centrifugation for 10 minutes at 3,500 rpm (820 x *g*) at 4°C.
9. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.

Fixation of Suspension Cells

1. Add 37% formaldehyde to the cell culture to reach a 1% final concentration.
2. Incubate on a shaking platform at room temperature for 10 minutes.

   Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation times.

3. Stop the fixation reaction by adding the appropriate volume (1:10) of 10X Glycine Buffer directly to the medium.
4. Incubate for 5 minutes on a shaking platform at room temperature.
5. Transfer the cells to a 15 ml conical tube, then centrifuge the cells for 10 minutes at 3,500 rpm (820 x *g*) at 4°C.
6. Remove the supernatant and discard. Wash the cell pellet by adding 10 ml 1X PBS. Pipet up and down several times to mix.
7. Centrifuge for 10 minutes at 3,500 rpm (820 x *g*) at 4°C.
8. Repeat Steps 6 and 7 one more time.
9. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing, add 1 µl 100 mM PMSF and 1 µl PIC and freeze pellet at -80°C.

B. Lysis & Shearing of Chromatin by Sonication

This protocol describes sonication shearing of cells from one 15 cm plate (approximately 1.5 x 10^7 cells). It assumes you have already optimized shearing conditions for your specific cell line and treatment to prepare chromatin for RNA-ChIP. (We have found the optimal shearing conditions to prepare chromatin for RNA-ChIP to be slightly different than those used when preparing chromatin for normal ChIP.) If you have not optimized conditions for RNA-ChIP, do not use this protocol now as it does not prepare enough chromatin to test for optimal conditions. Instead, use the protocols in Appendix – Sections A-C, which use 3 plates of cells to test multiple shearing conditions for your cell type (and treatment) to determine the optimal conditions. The optimized conditions are then used below.

Shearing tips: RNA-ChIP experiments usually require chromatin that has been sheared to a size of 100-1000 bp. In general, shearing efficiency is improved through the use of a small shearing volume and a V-bottom tube rather than a round-bottom tube. Also, note that shearing is inefficient if the chromatin sample becomes emulsified with air bubbles. This can be avoided by using lower shearing power and by turning the power up gradually. If a shearing reaction becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm (or 5,000 x *g*) in a 4ºC microcentrifuge to remove trapped air. Finally, to prevent overheating and denaturation of chromatin, samples should be kept on ice as much as possible during shearing, and shearing should be performed discontinuously (*i.e.* sonicate for 20 seconds, then place on ice/water for 30 seconds, sonicate again for 20 seconds, *etc.*).

1. Just before beginning, freshly prepare the following buffers. The volumes below are calibrated to one 15 cm plate:
    a. **Complete Lysis Buffer:** Add 5 µl PIC, 5 µl PMSF and 1 µl RNase Inhibitors to 1 ml 1X Lysis Buffer. Mix well and place on ice.
    b. **Complete Shearing Buffer AM2:** Add 1.75 µl PIC and 0.5 µl RNase Inhibitors to 350 µl Shearing Buffer AM2. Mix well and place on ice.
2. Thaw pellet (if necessary) on ice. Resuspend cells in 1 ml ice-cold Complete Lysis Buffer Incubate on ice for 30 minutes.
3. Transfer the cells to a 1.7 ml microcentrifuge tube and centrifuge for 10 minutes at 5,000 rpm (2,400 x *g*) in a 4°C microcentrifuge to pellet the nuclei.
4. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 350 µl Complete Shearing Buffer and place on ice.
5. Shear the DNA with your sonicator using the conditions that were previously determined to provide optimally sheared chromatin for your cell line. Let sit at least 30 seconds on ice/water between each sonication. (If you do not have optimized chromatin shearing conditions for RNA-ChIP, you should first use the protocols in Appendix – Sections A-C.)
6. Centrifuge the sheared chromatin samples for 10 minutes at 15,000 rpm (18,000 x *g*) in a 4°C microcentrifuge. Carefully transfer supernatant to a fresh 1.7 ml microcentrifuge tube. This is the sheared chromatin. It can be used right away in Step C or stored at -80°C.

C. DNase I Treatment of Chromatin (optional; recommended)

For a more efficient RNA-ChIP, treat the chromatin with DNase I before performing the IP.

1. Add 10 µl DNA Digestion Buffer to the 350 µl of chromatin prepared in Step B, then add 10 µl DNase I. Incubate for 20 minutes at 37°C.

   Note: Chromatin becomes cloudy (some white precipitate) after addition of the DNA Digestion Buffer. Mix well by inverting the tube after DNase I addition.

2. Stop the reaction by adding 10 µl 0.5M EDTA. Mix well by inverting the tube.
3. Centrifuge for 10 minutes at 15,000 rpm (18,000 x *g*) in a 4°C microcentrifuge, then transfer the supernatant to a fresh tube. This is the DNase I-treated chromatin.

   Note: The centrifugation step eliminates the precipitate.

Before using this chromatin in an IP, check the quality/shearing efficiency on an agarose gel. The RNA concentration in the chromatin must also be determined at this stage. Remove 50 µl of chromatin, then proceed to Appendix – Section C. The remaining chromatin should be stored at -80°C while assessing the chromatin quality. However, before freezing, the chromatin should be aliquoted to minimize freeze-thaw cycles.

D. Immunoprecipitation

Before use, wash the magnetic beads twice with DEPC water as follows:

a. Resuspend the protein G magnetic beads by inverting and/or vortexing the bottle.

b. Take the desired volume of beads for your IP reactions plus one extra reaction and put it in a fresh RNase-free microcentrifuge tube. (For example, if you plan to perform 8 IPs,
pipet 9 x 25 µl of beads.)

c. Put the tube on the magnet.

d. Remove the supernatant and using your pipette, measure the volume removed.

e. Add 800 µl DEPC water, vortex, put the tube on the magnet and remove the water.

f. Add 800 µl DEPC water, vortex, put the tube on the magnet and remove the water.

g. Add the same volume of DEPC water as was removed in the Step d., before washing.

1. Thaw chromatin. Transfer 10 µl to a microcentrifuge tube, then add 88 µl RNA IP Buffer, 0.5 µl RNase Inhibitors and 2 µl 5M NaCl. This tube is the "input sample" that will be processed in Step F4. It will then be used as a control in RT-PCR analysis. Store this reserved chromatin at -80°C until then.
2. Set up the RNA-ChIP reactions by adding the components shown in the table below to 8-well PCR strips. You may create a master mix with all the components except the beads and the antibody if using the same chromatin for the all the samples in the experiment. Keep the reagents and reaction tubes on ice during mix preparation. **The antibody should be the final component added to the reaction.**

Table 1

Reagent	One RNA-ChIP reaction (if using less than 60 µl of chromatin)	One RNA-ChIP reaction (if using more than 60 µl of chromatin)
Pre-washed Protein G Magnetic Beads	25 µl	25 µl
RNA IP Buffer	10 µl	15 µl
Sheared Chromatin	10 µg*	10 µg*
RNase Inhibitors	0.1 µl	0.1 µl
Protease Inhibitor Cocktail (PIC)	1 µl	1 µl
DEPC H_20	Add enough so that the final reaction volume will be 100 µl	Add enough so that the final reaction volume will be 150 µl
Antibody (added last)	1-4 µg	1-4 µg
Total Volume	**100 µl**	**150 µl**

***Note:** The 10 µg corresponds to the RNA amount in chromatin quantified after DNase I treatment (see Appendix – Section C for the method to quantify RNA). If you followed our chromatin preparation protocol, but did not quantify the chromatin, 35-50 µl of chromatin from a 15 cm plate will contain around 10 µg of RNA. Depending on the application, RNA-ChIP can be performed using anywhere from 5-50 µg of chromatin. An important factor is the volume of chromatin added, as the detergents used during sonication will impact RNA-ChIP. Use the 150 µl RNA-ChIP in the right column above if the volume of chromatin is greater than 60 µl.

3. Cap tube and incubate on an end-to-end rotator for 4 hours to overnight at 4°C. In some cases, sensitivity may be improved if the incubation is performed overnight.
4. Spin tube briefly to collect liquid from the inside of the cap.
5. Place tube on a magnetic stand to pellet beads on the tube side.
6. Carefully remove and discard the supernatant.

E. Wash Magnetic Beads

Note: During the washes, it is highly recommended to leave the tubes on ice as much as possible. Do not allow the beads to “dry out”. Allow no more than 1 minute to elapse between removing buffer and then adding the next wash or the elution buffer. For suggestions regarding bead washing, see Appendix – Section E.

1. Prepare Complete RNA-ChIP Wash Buffer 1 and Complete RNA-ChIP Wash Buffer 2 for the required number of reactions only; they must be used immediately after addition of RNase Inhibitors, and cannot be stored. Add 0.25 µl RNase Inhibitors per ml of RNA-ChIP wash buffer The charts below give some examples to prepare the Complete RNA-ChIP wash buffers:

Complete RNA-ChIP Wash Buffer 1	4 rxns	8 rxns	16 rxns	20 rxns	25 rxns
RNA-ChIP Wash Buffer 1	4.5 ml	9 ml	16 ml	20 ml	25 ml
RNase Inhibitors	1.13 µl	2.25 µl	4 µl	5 µl	6.25 µl

Complete RNA-ChIP Wash Buffer 2	4 rxns	8 rxns	16 rxns	20 rxns	25 rxns
RNA-ChIP Wash Buffer 2	3 ml	5 ml	9 ml	11 ml	13 ml
RNase Inhibitors	0.75 µl	1.25 µl	2.25 µl	2.75 µl	3.25 µl

2. Wash beads four times with 200 µl Complete RNA-ChIP Wash Buffer 1.
3. Wash beads two times with 200 µl Complete RNA-ChIP Wash Buffer 2.
4. After the final wash, remove as much supernatant as possible without disturbing the beads.

F. Elution and Reversal of Cross-links

Note: The RNA-ChIP Elution Buffer must be thawed at room temperature. Do not keep it on ice, as it will remain frozen.

1. Prepare Complete RNA-ChIP Elution Buffer for the required number of reactions; use 100 µl Complete RNA-ChIP Elution Buffer for each elution. To prepare 1 ml Complete RNA-ChIP Elution Buffer (enough to elute 10 IP reactions), add 1 µl RNase Inhibitors to 1 ml RNA-ChIP Elution Buffer.

   Note: Prepare only enough Complete RNA-ChIP Elution Buffer as is needed as it must be used immediately; it cannot be stored.

2. Add 100 µl Complete RNA-ChIP Elution Buffer to the bead pellet and resuspend thoroughly by pipetting up and down several times. Rotate for 15 minutes on the end-to-end rotor at room temperature. Spin tube briefly to collect liquid from the inside of the cap, if needed.
3. Use the magnet to pellet the beads, then transfer the supernatants into fresh microcentrifuge tubes.
4. Add 2 µl 5M NaCl and 2 µl Proteinase K to each sample. Add 2 µl Proteinase K to the input sample (from Step D1) also. Incubate the samples and input at 42°C for 1 hour to digest the proteins. Then, incubate for 1.5 hours at 65°C to reverse the cross-links.

G. RNA Purification with Trizol

Note: Steps 1, 2 and 4 must be performed in a fume hood because of reagent toxicity. For more information, refer to the technical data sheet and MSDS for Trizol.

1. Return tubes (samples and input) to room temperature and add ~150 µl RNase-free water (or DEPC water) to each tube to reach 250 µl. Add 0.75 ml Trizol LS (Life Technologies Cat. No. 10296). Pipet up and down several times to mix. Incubate for 5 minutes at room temperature.

2. Add 200 µl chloroform and mix vigorously without vortexing for 15 seconds, then incubate for 15 minutes at room temperature.
3. Centrifuge the samples for 10 minutes at 12,000 x *g* (max) at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless, upper aqueous phase. The RNA is exclusively in the upper aqueous phase.
4. Take the upper phase (aqueous phase that contains the RNA) and transfer it to a fresh microcentrifuge tube. Take the same volume of upper phase for each of your samples; it should be around 550 µl.
5. To precipitate RNA, add 500 µl isopropanol (RNase free) and 1 µl GlycoBlue (to help the precipitation and pellet visualization), then mix well. Incubate 15 minutes at room temperature.
6. Centrifuge for 10 minutes at 12,000 x *g* (max) at 4°C.
7. Remove and discard the supernatant without disturbing the pellet. Wash the pellet by adding 1 ml ice-cold 75% ethanol (RNase free) and centrifuge for 5 minutes at 7,500 x *g* at 4°C.
8. Carefully remove and discard the supernatant. Try to remove as much liquid as possible, but do not disturb the pellet. Allow pellet to air dry briefly (too long of an exposure to dry air should be avoided) and resuspend in 20 µl DEPC water. Proceed directly to Step H, or store the samples at -80°C.

H. DNase I Treatment

DNase I treatment removes any residual DNA within the samples.

1. Incubate the samples for 15 minutes at 37°C.
2. Add 2 µl 10X DNase I Reaction Buffer and 2 µl DNase I (4 Units) to the 20 µl samples. Dilute input sample 10 fold by mixing 2 µl input stock with 18 µl DEPC water. Then, add 2 µl 10X DNase I Reaction Buffer and 4 µl DNase I (8 Units).
3. Incubate all samples for 25 minutes at 22°C.
4. Add 2 µl Stop Solution to each reaction, mix, spin down and incubate for 10 minutes at 65°C to heat inactivate the DNase I.

I. Analysis of Samples by RT-PCR

RNA precipitated from the immune complex can be analyzed by Reverse Transcriptase-PCR (RT-PCR). This section explains the procedure for end point RT-PCR and real-time RT-PCR analysis. There are several RT-PCR kits on the market. For end point PCR, we recommend the One-Step RT-PCR Kit (USB Affymetrix Cat. No. 78350). The Two-Step RT-PCR Kit (USB Affymetrix Cat. No. 78355) can also be used, but only if the RNA to amplify is poly-adenylated RNA (mRNA); it may not be suitable for amplifying most long non-coding RNAs (not containing poly-A).

For real-time RT-PCR, we recommend the RNA-to-CT 1-Step Kit or the RNA-to-CT 2-Step Kit from Applied Biosystems. They both exist in SYBR Green and TaqMan versions.

Important: When designing your RT-PCR experiments, it is important to include points corresponding to PCR without the RT reaction. Likewise, you can determine if there is some DNA remaining that contaminates samples.

A. End point RT-PCR analysis

Below is an example of a one-step RT-PCR reaction. Please follow the instructions provided with your RT-PCR kit.

1. RNA/primer mix preparation

In fresh PCR tubes, combine the RNA from Step H and the oligonucleotide primers to a final volume of 10 µl on ice:

Reagent	1 rxn
RNA	5 µl
Forward primer (10 µM)	2 µl
Reverse primer (10 µM)	2 µl
Water (RNase free)	1 µl
Total volume	**10 µl**

Mix well and centrifuge briefly to collect the liquid in the bottom of the tubes.

2. Heat treatment of RNA/primer

Heat the RNA/primer mixture for 5 minutes at 75°C. Then, cool immediately on ice for at least 5 minutes and store on ice.

3. Preparation of reaction master mix.

In a separate microcentrifuge tube on ice, prepare a master mix as follows:

Reagent	1 rxn
Water (RNase free)	27 µl
5X RT-PCR Buffer	10 µl
PCR nucleotide mix (10 mM)	1 µl
RNase Inhibitors (4 Units/µl)	1 µl
50X RT-PCR Enzyme mix	1 µl
Total volume	**40 µl**

Mix well and centrifuge briefly to collect the liquid in the bottom of the tubes.

4. Complete reaction mix

Add 40 µl of the reaction master mix to the RNA/primer samples. Mix well by vortexing, then centrifuge briefly to collect liquid in the bottom of the tubes. Keep on ice.

5. RT-PCR reaction

Design a thermal cycler program appropriate to RT-PCR amplification of your specific target. The following program is generally useful, but is only a guideline.

Stage	Step	Temperature	Time
Holding	Reverse Transcription	42°C	30 minutes
	Activation of *Taq* DNA polymerase	95°C	3 minutes
Cycling (40 cycles)	Denature	95°C	30 seconds
	Annealing	55°C (depending on the sequence of your primer pair)	30 seconds
	Extension	72°C	1 minute
Holding	Additional extension	72°C	3 minutes

Start the thermal cycler program. Upon completion of the program, transfer the tubes to ice.

6. Analysis

Analyze samples (10 µl aliquots + 2 µl loading dye 6X) by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining. Visualize PCR product in gel with a UV transilluminator.

Note: Primers should be designed to generate products of approximately 100-200 bp.

B. Real-time RT-PCR analysis

This section describes 1-step quantitative RT-PCR on your RNA samples using SYBR Green detection. Please follow the instructions provided by the suppliers of your real time PCR instrument and your RT-PCR reagents.

Quantitative RT-PCR primers are chosen according to the classic rules of qPCR primer design:

- Ideally, the amplicon should be 50-100-bp in length.
- The optimal primer length is 20 bases.
- Keep the GC content of the primers in the range of 30-80%.
- Avoid stretches of identical nucleotides. If repeats are unavoidable, avoid 4 or more consecutive G residues.
- Keep the T_m at 58-60°C.
- Make sure that the last 5 nucleotides at the 3´ end contain no more than 2 G and/or C bases.

We recommend you use *Primer Express version 3.0* software to help design efficient qPCR primers. After design, primers need to be validated for qPCR. Therefore, a standard curve must be generated from genomic DNA for reference. Practically, every primer set in qPCR should be tested using a 5- or 10-fold serial dilution of genomic DNA. The PCR efficiency (depending on the

assay, the master mix performance and sample quality) can be calculated using the following formula:
Amplification Efficiency (AE) = $10^{(-1/slope)}$, where "slope" is the slope of your standard curve. A slope of -3.3 gives a PCR efficiency of 100%. Generally, an efficiency between 90-110% is acceptable. Most qPCR programs offer automatic calculation of PCR efficiency, as well as calculation of the DNA quantity in the samples by comparing with the Ct and known quantities of DNA standards. Once these preliminary requirements are successfully optimized, the RNA-ChIP samples can be analyzed by qRT-PCR.

1. Prepare the RT-PCR reactions

In a microcentrifuge tube on ice, prepare a mix as follows:

Reagent	1 rxn
Power SYBR Green RT-PCR Mix (2X)	10.0 µl
Forward primer (5 µM)	0.6 µl
Reverse primer (5 µM)	0.6 µl
RT Enzyme Mix (125X)	0.16 µl
RNA template*	5 µl
RNase-free H_2O	3.64 µl
Total volume	**20.0 µl**

Note: The primers and RNA template volumes can be further optimized if needed (see instructions from the kit manufacturer).

*If preparing a master mix, do not add RNA to the mix. It should be added to the plate or strip.

Pipette the reactions into the wells of a reaction plate or strip appropriate for your real-time PCR system. Seal the reaction plate with optical adhesive films or optical caps, then centrifuge the reaction plate briefly.

2. Run the experiment

Stage	Step	Temperature	Time
Holding	Reverse Transcription	48°C	30 minutes
	Activation of *Taq* DNA polymerase	95°C	10 minutes
Cycling (40 cycles)	Denature	95°C	15 seconds
	Annealing/Extension	60°C	1 minute
Holding	Additional extension	72°C	3 minutes
Melt curve (optional)	Denature	95°C	15 seconds
	Anneal	60°C	15 seconds
	Denature	95°C	15 seconds

Start the thermal cycler program. Upon completion of the program, transfer the tubes to ice.

3. Analyze the results

The efficiency of an RNA-chromatin immunoprecipitation assay to detect protein binding to a particular RNA locus can be calculated using different mathematical models (absolute versus relative quantitation). Please follow the manufacturer's recommendations to choose the most appropriate model for your experiment.

In an RNA-ChIP assay where you must compare input RNA with RNA-ChIP samples, the following formula may be used:

$$\textbf{\% of input (recovery)} = AE^{(\text{Ct input - Ct sample})} * \ Fd \ \times \ 100\%$$

Here, AE is the amplification efficiency as calculated above = $10^{(-1/slope)}$; Ct input and Ct sample are threshold values obtained from exponential phase of qPCR for a particular sample of chromatin; Fd is a dilution factor of the input RNA to balance the difference in the amounts of RNA-ChIP samples and input RNA used for qPCR.

Use this formula to calculate the % of input (recovery) of the RNA-ChIP for your RNA-ChIP samples, then graph the results.

In the following example, Suz12 antibody (Active Motif Cat. No. 39357) was used to immunoprecipitate RNA associated with Polycomb Repressor Complex 2 in chromatin prepared from HeLa cells. Primers amplifying SFPQ long intergenic non-coding RNA were used for qRT-PCR.

38 µl of HeLa chromatin (corresponding to 10 µg of RNA) were used in each RNA-ChIP reaction performed in duplicate. For input sample controls, 10 µl of chromatin were used and diluted 10 fold as recommended in the protocol. Following that, a dilution factor was calculated:

Samples: 38 µl used   Input: 10 µl diluted 10 fold = 1 µl used   Fd = 1/38

The amplification efficiency (AE) was determined through use of a standard curve using lincSFPQ primers. Using the curve's slope of -3.204, the AE value calculates as 2.05 (AE = $10^{(-1/-3.204)}$ = 2.05).

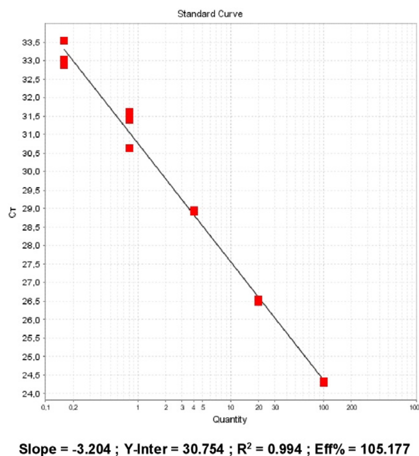


Figure 2: Standard curve linear regression plot for lincSFPQ primers.

Human genomic DNA was tested in triplicate and plotted against the Ct value.

The next table shows the raw data from the RNA-ChIP assays:

	Ct values (+RT)
Suz12 antibody	27.25
Rabbit IgG	34.15
Input RNA	26.75

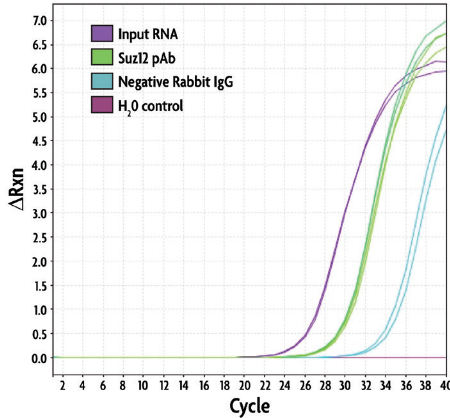


Figure 3: Real-time RT-PCR analysis of Suz12/rabbit IgG RNA-ChIP samples.

The RNA ChIP-IT Kit was used on 10 µg samples of DNase I-treated HeLa chromatin with 10 µl of Suz12 antibody (Cat. No. 39357) and 2 µg of rabbit IgG. The RNA-IP was performed overnight at 4°C. Real-time RT-PCR was performed using primers for the lincRNA SFPQ locus. The amplification plot is shown.

The percentages of input recovery of the Suz12 antibody and rabbit IgG were then calculated using the values in the table above and the equation on the previous page (with AE = 2.05 and Fd = 1/38). Dividing the input recovery of the Suz12 antibody by that of the rabbit IgG indicates a 141-fold enrichment of the lincSFPQ region with the Suz12 antibody.

	% input recovery
Suz12 antibody	1.825
Rabbit IgG	0.01293

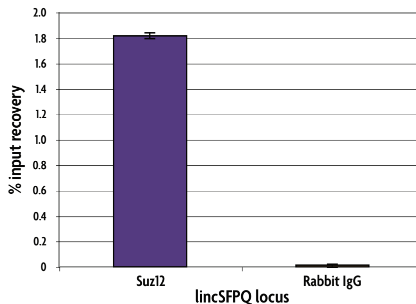


Figure 4: The % input recoveries of the RNA-ChIP reactions illustrates the enrichment by Suz12 antibody.

Optimizing the Shearing Conditions

Optimum chromatin shearing conditions can vary significantly depending on the cell type and, occasionally, the cell culture & cell stimulation conditions. However, after shearing has been optimized for a given cell type, those conditions usually give consistent results with that cell type. For this reason, we recommend use of the following protocol to determine optimal shearing conditions the first time you make chromatin from a particular cell line.

Section A. Cell Fixation to Optimize Shearing Conditions

In the protocols below, chromatin is prepared from cells grown in three 15 cm plates (approximately 4.5x 10^7 cells) and the chromatin is sheared using 3 different conditions. Generally, at least one of these conditions yields chromatin suitable for use in RNA-ChIP. Because only 50 µl of each sample is used for shearing analysis, one or more of them (usually ~300 µl for each shearing condition) may be used for approximately 6 RNA-ChIP experiments, provided that RNase inhibitors, protease inhibitor cocktail (PIC) and PMSF are included in the buffers. Use of RNase inhibitors, PIC and PMSF during optimization will, however, reduce the number of shearing and RNA-ChIP reactions that can be performed once you have established optimal shearing conditions and are ready to perform RNA-ChIP. If you are performing optimization simply to identify shearing parameters, do not add PIC and PMSF to the buffers. Nevertheless, RNase inhibitors should be added in order to prevent RNA degradation. This will conserve PIC and PMSF so that you can prepare 10 samples of sheared chromatin using your optimized conditions, then perform 25 RNA-ChIP assays.

1. Grow cells to 80-90% confluency on three 15 cm plates. If applicable, treat all three plates equally to influence the pathway of interest, as they will be combined into a single sample.
2. When cells are ready to harvest, prepare fresh Fixation Solution, ice-cold 1X PBS, Glycine Stop-Fix Solution and Cell Scraping Solution as follows:
    a. **Fixation Solution:** Add 1.62 ml of 37% formaldehyde to 60 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.
    b. **1X PBS:** Add 7 ml 10X PBS to 63 ml dH_2O, mix and place on ice.
    c. **Glycine Stop-Fix Solution:** Combine 3 ml 10X Glycine Buffer, 3 ml 10X PBS and 24 ml dH_2O. Mix well and leave at room temperature.
    d. **Cell Scraping Solution:** Add 1.8 ml 10X PBS to 16.2 ml dH_2O, mix and place on ice.
3. Pour medium off the three plates and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

    Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation times.

4. Pour Fixation Solution off the plates and wash by adding 10 ml ice-cold 1X PBS to each plate. Rock the plate for 5 seconds, then pour off the PBS.

5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix Solution to each plate. Swirl to cover the cells and then rock at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix Solution, then adding 10 ml ice-cold 1X PBS. Rock the plate for 5 seconds, then pour off the PBS.
7. Just before use, add 90 µl 100 mM PMSF to Cell Scraping Solution. Add 5 ml of this ice-cold Cell Scraping Solution to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a
5 ml pipette to transfer the cells to a 50 ml conical tube on ice. Do the same for the other two plates, pooling the cells from all three plates in one tube.
8. Pellet the pooled cells by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.
9. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C. When you are ready, continue with Section B below.

Section B. Optimization of Chromatin Shearing by Sonication

In the protocol below, chromatin is sheared for 5, 10 and 15 pulses. Generally, at least one of these conditions yields chromatin suitable for use in RNA-ChIP. Because only a fraction of each preparation is used to analyze the shearing efficiency, all remaining optimal preparation(s) can be used for RNA-ChIP experiments, provided that RNase inhibitors, PIC and PMSF are used in the buffers below, and those above for cell fixation.

Our sonication optimization protocol was developed using Active Motif's EpiShear Sonicator with a 1/8" probe at 40% power in a volume of approximately 300 µl.

1. Thaw pellet (if necessary) on ice and resuspend cells in 3 ml ice-cold Lysis Buffer (supplemented with 15 µl PIC, 15 µl PMSF, 3 µl RNase inhibitors). Incubate on ice for 30 minutes.
2. Centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
3. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 1.0 ml Shearing Buffer (supplemented with 5 µl PIC, 5 µl PMSF, and 3 µl RNase inhibitors), aliquot into equal volumes into three 1.7 ml microcentrifuge tubes, then place on ice. Each aliquot should be approximately 300 µl.
4. Shear the three aliquots of fixed chromatin at 40% power using three different conditions:
   a. Five pulses of 20 seconds each, with a 30-second rest on ice between each pulse.
   b. Ten pulses of 20 seconds each, with a 30-second rest on ice between each pulse.
   c. Twenty (or fifteen) pulses of 20 seconds each, with a 30-second rest on ice between each pulse.

5. Centrifuge the sheared chromatin samples for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Transfer the supernatants to fresh 1.7 ml microcentrifuge tubes and save 50 µl aliquots from each, which will be used to determine shearing efficiency. The sheared chromatin and the aliquots can be stored at -80°C. Or, use the 50 µl aliquots immediately in Section C.

Section C. RNA Clean Up to Assess Shearing Efficiency and RNA Concentration

Note: It is not necessary to treat samples with DNase I for checking shearing efficiency and chromatin quality.

1. If necessary, thaw the 50 µl aliquots of your chromatin samples from Step C.
2. Add 2 µl 5M NaCl, 48 µl DEPC water, 0.5 µl RNase Inhibitors and 2 µl Proteinase K to each tube.
3. Incubate the chromatin samples for 1 hour at 42°C to digest the proteins, followed by an additional 1.5 hours at 65°C to reverse the cross-links.
4. Return the tubes to room temperature and add ~150 µl RNase-free water, enough to reach a total volume 250 µl. Add 750 µl Trizol LS (from Life Technologies) under a fume hood. Pipet up and down several times to mix. Incubate for 5 minutes at room temperature.
5. Under the fume hood, add 200 µl chloroform and mix vigorously without vortexing for 15 seconds, then incubate for 15 minutes at 15-30°C.
6. Centrifuge the tube for 10 minutes at 12,000 x *g* (max) at 4°C.
7. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless, upper aqueous phase. The RNA is exclusively in the upper aqueous phase.
8. Take the upper phase (aqueous phase which contains the RNA) and transfer it to a new microcentrifuge tube. The volume of the aqueous phase should be around 500 µl.
9. For precipitation, add 500 µl isopropanol (RNase free) to the RNA and 1 µl GlycoBlue (to help aid precipitation) and mix well. Incubate for 15 minutes at room temperature.
10. Centrifuge for 10 minutes at 12,000 x *g* (max) at 4°C.
11. Remove and discard the supernatant without disturbing the pellet. Wash the pellet by adding 1 ml ice-cold 75% ethanol (RNase free) and centrifuge for 5 minutes at 7,500 x *g* at 4°C.
12. Carefully remove and discard the supernatant. Try to remove as much liquid as possible, but do not disturb the pellet. Allow pellet to air dry briefly (too long of an exposure to dry air should be avoided) and resuspend in 50 µl DEPC water.
13. Use a spectrophotometer to measure the OD at 260 nm to determine the RNA concentration of chromatin. (1.0 A260 unit = 40 µg/ml)

14. Check the RNA fragment sizes on an agarose gel; it is recommended to load each sample on the gel in two different amounts to avoid over- or under-loading. Add 4 µl 6X Loading Buffer to 16 µl sample, then load 5 µl and 10 µl of each sample on a 1-2% TAE agarose gel. Run the gel at 100V for 45 minutes to 1 hour, until the loading dye reaches ¾ of the way to the end of the gel rack.
15. Optimal sonication shearing should results in a 100-1000 bp smear.

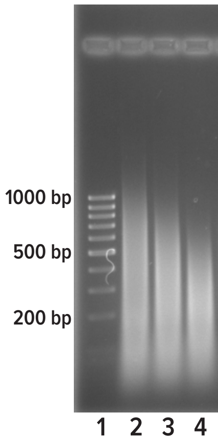


Figure 5: Gel analysis of sonication shearing (RNA ChIP-IT).
HeLa cells were fixed for 10 min with 1% formaldehyde and then chromatin was prepared using the RNA-ChIP-IT Express Kit. Chromatin was sheared with 5, 10 and 15 pulses at 40% power using Active Motif's EpiShear Sonicator with a 1/8" probe in a volume of approximately 300 µl. Each pulse consisted of a 20-second sonication followed by a 30-second rest on ice. The sheared chromatin samples were treated with Proteinase K and subjected to cross-link reversal. RNA was extracted with Trizol and then precipitated with isopropanol as described. Samples were separated by electrophoresis through a 2% agarose gel. Optimally sheared chromatin will yield a smear between 100-1000bp.

Lane 1: 100 to 1000 bp ladder.
Lane 2: HeLa RNA sheared for 5 pulses (optimal).
Lane 3: HeLa RNA sheared for 10 pulses (optimal).
Lane 4: HeLa RNA sheared for 15 pulses.
Note: From this experiment, the RNA sonicated for both 5 and 10 pulses are suitable for use in RNA-ChIP. Nonetheless, the RNA sonicated for 15 pulses would also be acceptable.

Section D. Use of Magnetic Beads and Included Bar Magnet

- The magnet should be stored in the provided tube.
- Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
- Use the provided Mini Glue Dots to attach the bar magnet to an empty pipette tip box to create an effective magnetic stand for use with either PCR strips or microcentrifuge tubes.
- If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of the surface. The magnet may break if you attempt to pull one end or pry it away from the metal.

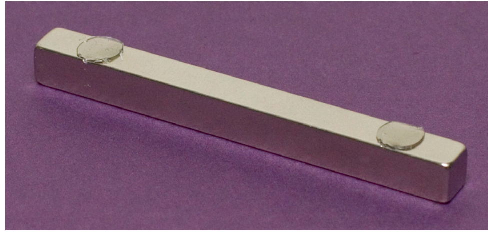
Caution: The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

Creating a magnetic stand for 8-well PCR strips:

Note: 8-well strip tubes for use with standard 96-well PCR cyclers are recommended (*e.g.* Thermo Fisher AB-0451).

1. Place a strip of PCR tubes in the wells of an empty tip box (200 µl tips) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
2. Remove the covering tape from one side of two glue dots and attach the glue dots on the

bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.

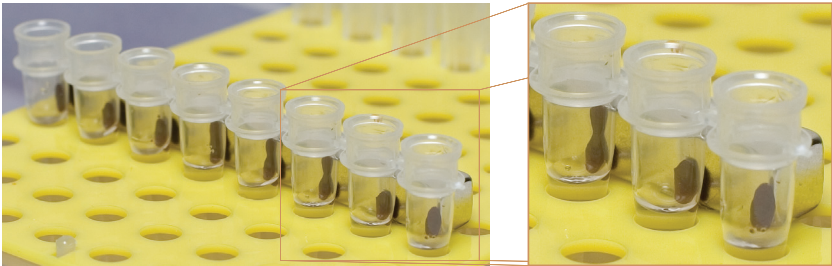


3. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

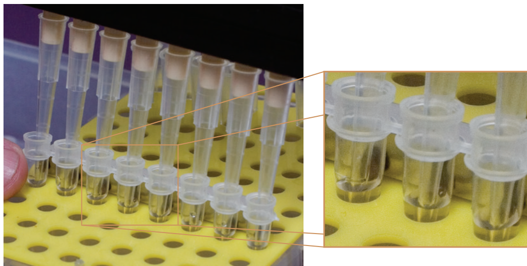
Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl ChIP Buffer 1 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is best to move the tubes away from the magnet for resuspension steps.

Washing should be performed as follows:

a. Place the tubes in the rack against the magnet and allow the beads to be pinned to the side of the tube, as shown below.



b. Remove supernatant with a 200 µl pipetteman or a 200 µl eight-channel pipetteman.



c. Move the tube strip into a row that is not adjacent to the magnet.

d. Add wash buffer and pipet up and down to fully re-suspend the beads. Ensure that a minimal amount of beads cling to the tips when the re-suspension is complete.

e. Repeat steps a-d until desired washing steps are complete.

Section E. Troubleshooting Guide

Problem/question	Recommendation
At what points in the protocol can I stop?	The protocol may be stopped and samples stored at the times and temperatures below: 1. After cell fixation, -80°C. 2. After lysis and shearing of chromatin by sonication, -80°C. 3. After DNase I treatment of chromatin, -80°C. 4. After the cross-link reversal, -80°C. 5. After Trizol purification of RNA, -80°C. 6. After DNase I treatment of samples, -80°C. Although the protocol may be stopped at all these points, it is recommended to minimize freeze/thaw cycles of chromatin and samples. Two or three cycles are acceptable.
Poor yield of sheared chromatin.	Nuclei not released. In this case, it is highly recommended to perform dounce homogenization. Use a dounce homogenizer with a small clearance pestle (*e.g.* Wheaton part no.357542 or Kimble-Kontes part no. 885302-002 with the tight-fitting “B” pestle), and monitor cell lysis under a microscope. (Take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells.) Generally the more cells that are lysed, the higher the sheared chromatin yield.
	Decrease the fixation time. Over-fixed cells are often very resistant to lysis and shearing. Cross-linking for longer periods of time tends to cause cells to form into a giant cross-linked aggregate that is not sheared efficiently. Decrease the incubation time of the formaldehyde fixation step to 5 minutes.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
	Use fresh formaldehyde when preparing Fixation Solution.
	Sample degraded. Multiple freeze/thaw cycles can destroy RNA and RNA-protein interactions. Samples must be kept on an ice during the sonication process. Sonication can cause excessive heat, which can damage the sample.
Shearing efficiency is not clear from gel analysis.	Material is stuck in the wells, and smears or streaks are seen from the top to bottom of the lane. The sheared chromatin needs to have the cross-links reversed, protein removed (Proteinase K and Trizol treatment) and purified (Trizol). Follow the protocol in Appendix – Section C.
	RNA was lost during the purification step. When performing isopropanol precipitation after Trizol treatment, be sure to add GlycoBlue (or glycogen) to improve precipitation and help visualize the RNA pellet. After the ethanol wash, the pellet may not stick to the wall of the tube: avoid disturbing the pellet when removing the supernatant. Perform an additional 5-minute spin if needed.
	High molecular weight products. Decrease the size of the fragments by re-sonicating the sample.
After sonication shearing and centrifugation, a viscous or cloudy layer is visible in the chromatin.	Depending upon the cell type, lipid or glycogen layers may be seen after centrifugation. For example, liver tissue may have a glycogen layer and a milky appearance, while fatty tissues may have a lipid layer. Avoid such layers when you remove the supernatant. However, if the whole supernatant is cloudy, it should not interfere with the IP reaction.

Problem/question	Recommendation
Poor or no enrichment with target antibody.	Too little chromatin. Generally, we recommend using 10 µg of chromatin for a “regular” RNA-ChIP. For low to moderately abundant proteins, 10-50 µg of chromatin is recommended for each IP reaction. Be sure to quantitate the concentration of RNA of the input to ensure both that adequate chromatin is used per sample, and that equal mass quantities of chromatin are used in each ChIP. Note that when quantifying chromatin, remaining DNA can interfere with absorbance reading at 260 nm. So RNA quantities are very often overestimated.
	Sample degradation. RNA can be degraded during sample washes and additional treatment. To avoid that, keep samples on ice during washes and be sure to keep your samples at the specified temperatures for the specified times during all other steps. Plan your experiment so that you minimize the number of freeze and thaw cycles needed.
	Antibody is not ChIP (or RNA-ChIP) validated. The antibody does not efficiently recognize fixed proteins, either because the epitope is destroyed by fixation or because the epitope is masked by other proteins in a larger complex. To assist in RNA-ChIP validating an antibody, it is very useful to use the RNA ChIP-IT Control Kit as it contains positive control antibody, negative IgG and PCR primers that have been proven to work in RNA-ChIP.
	Low-affinity antibody. Increase the incubation time of the RNA-ChIP reaction to overnight at 4°C on an end-to-end rotator.
	Antibody affinity to protein G is weak. Individual monoclonals have variable binding affinities to protein G, which are pH dependent; the optimal pH may vary for each Ig, For those with low to medium affinity, capture efficiency by protein G can be dramatically improved through use of our Bridging Antibody (Catalog No. 53017). This antibody is a rabbit anti-mouse pAb that recognizes all subclasses of mouse immunoglobulins. If your IgG has a weak/medium affinity to protein A or G, the Bridging Antibody will increase antibody capture by the beads without increasing background.
	RT-PCR or primer issues. Confirm the species specificity of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in real-time PCR. For other problems, please refer to your RT-PCR kit provider.
Performing RNA-ChIP with a large volume of chromatin.	This is not recommended. It is better to set up several small RNA-ChIP reactions (150 µl each) and pool the samples at the end, rather than trying to RNA-ChIP a single large sample. Do not perform a single scaled-up reaction, as the capture efficiency is lower.
The RT-PCR products are the correct size, but are very faint.	Load more RT-PCR product, and/or use smaller wells for the agarose gel if doing end point RT-PCR. You can also perform more PCR cycles after RT the reaction.
No RT-PCR products for the RNA-ChIP samples (but the input yields the correct RT-PCR product)	Increase the amount of chromatin used in the RNA-ChIP reaction, the amount of antibody used, or both.
	Use a different antibody.
No RT-PCR products for the input (but the RNA-ChIP samples yield the correct RT-PCR product)	Use more input RNA in the DNase I treatment (Step H); dilute the input 5 fold instead of the usual 10 fold.
No RT-PCR products	Optimize your RT-PCR conditions. Be sure that the IP’d RNA of interest is present in your cell line or tissue. If it is, choose another primer pair.
Control reactions without Reverse Transcriptase yield PCR products.	DNA remains and is contaminating the samples. Ensure that you have performed the DNase I treatment of the samples correctly (proper volumes of DNase I and 10X DNase I Reaction Buffer, correct incubation temperature, *etc.*). If everything has been performed properly, double the DNase I volume for each sample and for the input reaction.

Problem/question	Recommendation
High background.	Chromatin issue. The optional yet recommended DNase I treatment of chromatin before IP (Section C) can be very important to reduce background. Do not skip this step unless your protein-RNA interaction is through a DNA molecule.
	Chromatin not sheared enough. Shearing should produce RNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 100-1000 bp fragments. If the RNA fragments are too large, the background is increased. Consider increasing the number of pulses for sonication. Check the fragment size on a gel to assess your shearing efficiency.
	Antibody issue. Too much antibody relative to the amount of chromatin in the ChIP reaction. Excess antibody will result in more non-specific binding, which will be detected as increased background
	Too much template RNA. Reduce the amount of RNA in the RT-PCR reaction.
	Increase washes. In most cases, the washing protocol in this manual is appropriate. However, if the background is high you can increase washing stringency in several ways: 1. After adding Complete RNA-ChIP Buffer 1 and/or Complete RNA- ChIP Buffer 2 during the wash steps, gently agitate the samples for several minutes before removing the buffer. 2. Perform additional washes. Sufficient RNA-ChIP Buffer 1 and 2 is provided for two “extra” washes per sample.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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