Magna ChIP™ G Tissue Kit

Catalog No. 17-20000

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NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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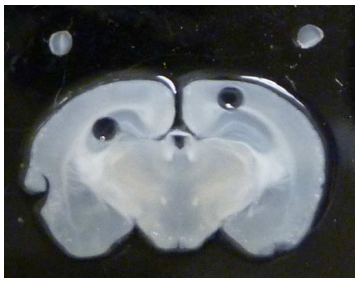
Tissue Chromatin Immunoprecipitation (ChIP) Assay Introduction

Chromatin Immunoprecipitation (ChIP) is a powerful technique for mapping the *in vivo* distribution of proteins associated with chromosomal DNA. These proteins can be histone subunits and post-translational modifications thereof or other chromatin associated proteins such as transcription factors, chromatin regulators, etc. Additionally, ChIP can be used to identify regions of the genome associated with these proteins, or conversely, to identify proteins associated with a particular region of the genome. Genome-wide maps of protein-DNA interactions or histone modifications using ChIP can provide insight into gene regulatory networks.

ChIP protocols are geared towards homogenous populations of cell culture. In contrast, tissue samples are more complex and the cells are heterogeneous thus making ChIP analysis less amenable. The **Magna ChIP™ G Tissue Kit** provides the tools necessary to obtain ***repeatable, reliable, and site specific*** tissue biopsies. Microdissected functionally related populations of cells within a heterogeneous tissue can now be analyzed easily using this ChIP assay.

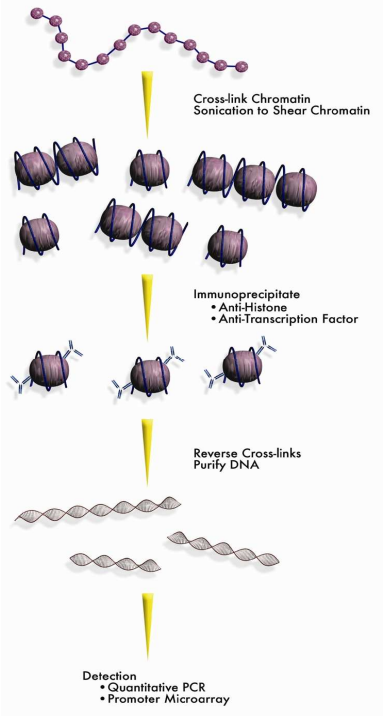
Region Specific Tissue Isolation

Cryosection tissue and isolate region specific tissue sample with provided 1mm micro-dissection punch. An example of region specific tissue isolation is shown in the image at right. A 300µm coronal mouse brain section was obtained and two microdissections were carried out (hippocampus on the left and cortex on the right). The purified tissue is then dispersed with Tissue Stabilizing Solution followed by formaldehyde treatment. Formaldehyde cross-links proteins to DNA to ensure co-precipitation.



Chromatin Sample Prep

Lyse then sonicate the tissue. Cells are broken open and sonication is performed to shear the chromatin to a manageable size. Generally, it is recommended to generate fragments of 200-1000 bp of DNA because it is small enough to achieve a high degree of resolution during the detection step. It is critical that average fragment size be confirmed empirically by gel electrophoresis.



Immunoselection

ChIP is very similar to a standard immunoprecipitation using a primary antibody bound to protein G-conjugated magnetic beads. The immunoselection step enriches for the specific DNA-protein complex of interest.

DNA Purification and Detection

Protein-DNA crosslinks are reversed and DNA is purified to remove the chromatin proteins and to prepare the DNA for the detection step.

Detection

A variety of detection methods can be employed at this step. However, the most meaningful results will be obtained with quantitative PCR. Real Time Quantitative PCR (qPCR) is ideal but this method requires a specialized PCR machine that may not be available.

Kit Overview

Quantity: Two boxes containing all necessary reagents to perform 22 individual chromatin immunoprecipitation (ChIP) reactions.

Use: The Magna ChIP™ G Tissue Kit contains reagents optimized for immunoprecipitation of chromatin from mouse tissue. ChIP validated antibodies and PCR controls should be used to ensure successful optimization of the assay. Detection of the DNA region, gene or promoter of interest in immunoprecipitated chromatin must be empirically determined by the researcher. PCR using promoter-specific primers is recommended for detection and analysis of enriched DNA.

Millipore's complete list of ChIP qualified antibodies can be found at http://www.millipore.com**/epigenetics.**

Kit Components

Magna ChIP™ G Tissue Kit Contents (Cat. No. 17-20000)		
Magna ChIP G Tissue Kit (2° to 8°C) MAGNA0020		
Magna ChIP G Tissue Kit (-20°C) MAGNA0021		
Magna ChIP™ G Tissue Kit (2° to 8 °C) MAGNA0020		
Component	**Catalog #**	**Quantity**
Magnetic Protein G beads	CS200638	450 µL
ChIP Dilution Buffer	CS205298	50 mL
Low Salt Wash Buffer	CS200625	12.5 mL
High Salt Wash Buffer	CS200626	12.5 mL
LiCl Wash Buffer	CS200627	12.5 mL
TE Buffer	CS200628	12.5 mL
Tissue Lysis Buffer	CS205299	15 mL
10X Tissue Stabililizing Solution	CS205300	10 mL
ChIP Elution buffer (w/o Proteinase K)	CS200629	5 mL
10X Glycine	20-282	11 mL
10X PBS	20-281	24 mL
Store the Following at Room Temperature Upon Receipt		
1mm Micro-dissection punch	**N/A**	5 EA
Spin Filters	20-290	22 Filters
Collection Tubes	20-291	22 Tubes
Bind Reagent A	20-292	25 mL
Wash Reagent B	20-293	12.5 mL
Elution Reagent C	20-294	1.5 mL
Magna ChIP™ G Tissue Kit (-20°C) MAGNA0021		
Protease Inhibitor Cocktail II, 200X	CS205297	750 µL ****Contains DMSO**
Proteinase K	20-298	600 µg K in 60 µL

Materials Required But Not Supplied	
Reagents	**Equipment**
• Antibody of interest for chromatin immunoprecipitation • 37% Formaldehyde • *Taq* DNA polymerase for standard PCR • dNTPs, 2.5 mM each • SYBR®-Green Master Mix for qPCR • DNase and RNase-free sterile H_2O • O.C.T. – Optimal Cutting Temperature compound • RNase A	• Cryostat-microtome • Cryomold • Magnetic Separator (Magna Grip Rack (8 Well), Catalog #20-400) • Vortex mixer • Rotating wheel/platform • Microfuge • Sonicator • Thermomixer or Hybridization Oven • Variable temperature water bath or incubator • Variable volume (5-1000 µl) pipettors + tips • Microfuge & PCR tubes • Thermal cycler • Filter-tip pipette tips

Hazards:

- **Caution: Eye, hand, face, and clothing protection should be worn when handling this material. If direct contact occurs, wash areas of contact immediately with water.**
- All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Storage and Stability

Upon receipt, store components at the temperatures indicated on the labels. Kit components are stable for 6 months from date of shipment when stored as directed.

Section I: Detailed Magna ChIP™ G Tissue Kit Protocol

For advanced users, an abbreviated protocol flow chart is provided at the end of this section and may be used in place of the detailed protocol.

A. Preparation of Tissue: **Region Specific Tissue Isolation**
Researchers should optimize the tissue extraction procedure for their own applications. The following protocol has been successfully employed for several different tissue types including brain, heart, lung, liver, and kidney. For non-region-specific tissue preparation see appendix B.

1. Isolate non-fixed fresh tissue as desired. Transfer into a 50 ml tube and wash twice with ice cold PBS.

   ***Note**: Specimens should be handled carefully and promptly to avoid compromising specimen integrity.*

2. Position specimen in a cryomold, cover with O.C.T and freeze using dry ice. Transfer specimen to -80˚C until ready to section.

   ***Note**: <u>DO NOT</u> cryo-preserve specimen with sucrose.*

3. When ready to section, place specimen mold, microfuge tubes, and microdissection punch inside cryostat set at -20˚C, for 30 minutes.

4. Follow standard protocol and section the specimen at 300 microns.

   ***Note**: We recommend 300 µm sections. However, the section thickness may need to be adjusted based upon the researchers' desired volume or punch depth*.

5. Adhere the flattened section onto a microscope slide in the cryostat.

Note: *The slides should be kept at room temperature so the cryo-sectioned tissue will slightly melt onto the slide. At this point do not take the slide out of the cryostat but maintain the slide in the cryostat. This will re-freeze the section onto the slide. While this section is equilibrating, you can continue to generate additional sections.*

6. Using the 1 mm microdissection punch, make a vertical incision that passes completely through the frozen slice. Slightly rotate the punch with your fingers back and forth. Expel the sample into a labeled microfuge tube in the cryostat by pressing the plunger on top of the microdissection punch. The samples can now be stored at -80°C until ready for ChIP analysis.

   Note*: The microdissection punch and the microfuge tube should be kept at -20°C. Otherwise the tissue will thaw and it will not come out of the metal tip cleanly. Depending on the experiment and the quantity of chromatin needed, the researcher can pool multiple dissected samples into a single microfuge tube. If cross contamination is a concern, clean the metal tip between punches.*

B. Preparation of Reagents

Tissue Stablizing Solution: Tissue Stabilizing Solution (TSS) is provided as a 10X stock. To make a working solution of TSS, dilute the 10X stock to 1X using Milli-Q™ water. You can store the 1X TSS solution for 2 weeks at 4°C. ***TSS/PI*****:** When ready to use, make by freshly adding the 200X protease inhibitors (PI). For example add 5 µl of 200X PI to 995 µl of 1X TSS.

1% Formaldehyde: Make fresh 1% formaldehyde before each experiment by adding 270 µl of 37% formaldehyde to 10 ml of PBS. Use high quality formaldehyde. Do not use formaldehyde if past its expiration date.

1X PBS: PBS is provided as a 10X stock. To make a working solution of PBS, dilute the 10X stock to 1X using Milli-Q™ water. For example, to make 240 ml of 1X PBS, add 216 ml of Milli-Q™ water to 24 ml of 10X PBS. ***PBS/PI*****:** When ready to use, make by adding the 200X protease inhibitors (PI). For example add 5 µl of 200X PI to 995 µl of 1X PBS and use immediately.

Tissue Lysis Buffer/PI: When ready to use, make by adding the 200X protease inhibitors (PI). For example add 2.5 µl of 200X PI to 497.5 µl of Tissue Lysis Buffer and use immediately.

Dilution Buffer/PI: When ready to use, make by adding the 200X protease inhibitors (PI). For example add 2.5 µl of 200X PI to 497.5 µl of Dilution Buffer and use immediately.

ChIP Elution Buffer/ProtK: When ready to use, make by adding 1 µl Proteinase K to 100 µl of ChIP Elution Buffer and use immediately**.**

C. Chromatin Stabilization and Fixation

Depending on the experiment and the quantity of chromatin needed, the researcher can pool multiple region specific dissected samples into a single microfuge tube for chromatin preparation. Researchers should optimize the following procedure for their own applications. We have successfully pooled as many as 40 dissected samples in the following protocol.

1. Thaw the dissected samples on ice. Add ice cold TSS/PI (500 µl to 1000 µl). Carefully pipette up and down ten times using a 1 ml pipette to shear the tissue.

   Note*: Not all tissue types will disperse evenly. Total cell disruption will occur during the sonication step. Be careful that the tissue does not stick to the pipette tip.*

   TSS is a proprietary solution designed to enhance chromatin stability. Specimens should be handled carefully and promptly to avoid compromising specimen integrity.

2. Spin at 800 x g at 4°C for 5 min to pellet tissue. Remove the supernatant and resuspend pellet in 900 µl of freshly diluted 1% formaldehyde and incubate for 10 min at room temperature.
3. Add 100 µl of 10X Glycine (the final concentration will be 1X). Mix and incubate for 5 min at room temperature.
4. Spin at 800 x g at 4°C for 5 min to pellet tissue. Remove the supernatant and resuspend pellet in 1 ml of ice cold PBS/PI and incubate for 5min on ice.
5. Repeat step 4 two additional times.

D. Chromatin Sample Prep

To attain a high degree of resolution during the detection step, samples must be sonicated sufficiently to produce DNA fragments that are 200 -1000 bps in size.

1. Take the cell pellet from the chromatin fixation step above and resuspend in 500 µl of Tissue Lysis Buffer/PI. Incubate on ice for 15 min. Vortex the suspension briefly every 5 minutes.
2. Spin the cell suspension at 800 x g at 4°C for 5 min.
3. Carefully remove supernatant and resuspend pellet in 0.5 ml of ice cold Dilution Buffer/PI.

   Note*: For every microdissection sample, 0.125 ml of Dilution Buffer/PI is recommended for this protocol. Adjust accordingly as the ratio of Dilution Buffer/PI to number of* dissected sample *is important for reliable cell lysis.*
4. Sonicate lysate on wet ice.

   Note*: Keep lysate ice-cold. Sonication produces heat, which can denature the chromatin. Allow time between cycles of sonication to prevent sample overheating. Sonication conditions must be empirically determined using methods described in Appendix A. The efficiency of sonication depends upon tissue type, cell equivalents and instrumentation. Where possible, consult your instrument manufacturer's guidelines for instrument operation. As a guideline, using a Misonix 3000, at power setting 6, with a microtip, we sonicated multiple tissue types for 15 sec followed by a rest period of 50 sec, for total of 8 cycles.*
5. Spin at a minimum of 10,000 x g, but not exceeding 15,000 x g, at 4°C for 10min to remove insoluble material.
6. Remove supernatant to fresh microfuge tubes in 125 µl aliquots. Sheared crosslinked chromatin can be stored at -80°C for up to 3 months.

   Note*: Each 125 µl aliquot contains 1* dissected sample *equivalent of lysate which is enough for one immunoprecipitation reaction.*

   If desired, remove one 5 µl aliquot for agarose gel analysis of the sheared DNA. To prepare an aliquot for agarose gel analysis, see Appendix A.

E. Immunoprecipitation (IP) of Crosslinked Protein/DNA

1. Prepare enough Dilution Buffer/PI containing protease inhibitors for the number of desired immunoprecipitations and store on ice. Each IP reaction requires the addition of 375 µl of Dilution Buffer/PI. It is recommended that the user include a negative control IgG of the same species as the antibody of interest.
2. Add 375 µl of Dilution Buffer/PI into each tube containing 125 µl of chromatin. One for the antibody of interest and another for its negative control.
3. Remove 5µl (1%) of the supernatant from each tube as "Input" and save at 4°C (until Section F, step 1 below).

   Note*: If multiple chromatin preparations are being carried together through this protocol, remove 1% of the chromatin as Input from each prep.*
4. Add 20 µl of fully re-suspended protein G magnetic beads.
5. Add *1-10 µg* of immunoprecipitating antibody or negative control IgG of the same species as the antibody of interest. Incubate for 2 hours to overnight at 4°C with rotation.

   Note*: The appropriate amount of antibody may need to be determined empirically and will depend upon antibody titre, purity and specificity. One µg of several different Millipore validated ChipAb+ antibodies have been successfully employed for several different tissue types.*
6. Pellet Protein G magnetic beads with the magnetic separator (Magna Grip Rack (8 Well), Cat.# 20-400, sold separately) and remove the supernatant completely.

7. Wash the Protein G bead-antibody/chromatin complex by resuspending the beads in 0.5 ml each of the cold buffers in the order listed below and incubating for 3-5 minutes on a rotating platform followed by magnetic clearance and careful removal of the supernatant fractions

    a. Low Salt Immune Complex Wash Buffer (Cat.# 20-154), one wash
    b. High Salt Immune Complex Wash Buffer (Cat.# 20-155), one wash
    c. LiCl Immune Complex Wash Buffer (Cat.# 20-156), one wash
    d. TE Buffer (Cat.# 20-157), one wash

8. When ready for the next step remove the TE Buffer

F. Protein-DNA Crosslink Reversal

In this step the protein-DNA crosslinks are reversed and the DNA is purified to remove chromatin proteins. The purified DNA is then ready for the detection step.

1. Prepare the final elution buffer for all IP tubes as well as all Input tubes (see Section E, step 3). For each tube, prepare 100 µl ChIP Elution Buffer/ProtK.
2. Add 100 µl ChIP Elution Buffer/ProtK into each tube containing protein G magnetic beads with the TE Buffer previously removed.
3. Incubate at 62°C for 2 hours with shaking.
4. Incubate at 95 ℃ for 10 minutes.
5. Allow the samples to cool to room temperature.
6. Separate beads using a magnet and keep supernatant by transferring into a new tube.

    Note*: The Input does not have any magnetic Protein G beads. For these samples skip step 6.*

G. DNA Purification Using Spin Columns

1. Remove one Spin Filter and Collection Tube for each sample tube from Section F.

    Add 0.5 ml of Bind Reagent "A" to each 100 µl DNA sample tube (Immunoprecipitations and Inputs) and mix well.

    Note*: 5 volumes of Bind Reagent "A" should be used for every 1 volume of sample. A precipitate may be observed but it will not interfere with this procedure.*

2. Transfer the sample/Bind Reagent "A" mixture to the Spin Filter that is held within a Collection Tube. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
3. Remove the Spin Filter from the Collection Tube, and then discard the liquid from the Collection Tube. Put the Spin Filter back into the same Collection Tube.
4. Add 500 µl of the Wash Reagent "B" to the Spin Filter in Collection Tube. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
5. Remove the Spin Filter from the Collection Tube, and then discard the liquid from the Collection Tube. Put the Spin Filter back into the same Collection Tube.
6. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g and then discard the Collection Tube and liquid.
7. Place the Spin Filter into a clean microfuge tube. Add 50 µl of Elution Buffer "C" directly onto the center of the white Spin Filter membrane and incubate for 30 seconds.
8. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g. Remove and discard Spin Filter. Eluate is now purified DNA. It can be analyzed immediately or stored frozen at -20°C.

H. Detection

Although there are numerous detection methods, the most meaningful results will be obtained utilizing quantitative PCR. Real Time Quantitative PCR (qPCR) is ideal but this method requires a specialized PCR machine that may not be available. For standard end-point PCR, primer selection is critical and oligonucleotides must be designed with close adherence to the guidelines noted below. General protocols for both of these different detection methods are provided.

***Note**: Filter-tip pipette tips are recommended to minimize the risk of contamination.*

Standard End-Point PCR

1. Label the appropriate number of 0.2 ml PCR tubes for the number of samples to be analyzed and place on ice.

   ***Note**: At a minimum, there will be 4 DNA samples to undergo PCR using the protocol provided: positive and negative control antibody immunoprecipitations, Input and a "no DNA" tube as a control for DNA contamination.*

2. Millipore has ChIPAb+ kits which contain control primers that have been optimized and verified to work. If the user wishes to design their own primers we recommend they follow the guidelines noted below. However, keep in mind that the PCR reaction conditions must be determined empirically:

Primer Length:	24 nt
Amplicon size:	100-200 base pairs
Optimum Tm:	60°C
Optimum GC:	50%

3. Add the appropriate amount of reagents to each PCR reaction tube on ice, adding the H_2O first and the *Taq* polymerase last, as indicated in Table I.

   ***Note**: It is recommended that the user employ a Hot-Start™ Taq polymerase. If a Hot-Start Taq polymerase is not used, Taq must be added to each tube after the initial denaturation step. If a master reaction mix is desired, dispense enough reagents for at least one extra tube to account for loss of volume*.

Table I. PCR reagent volumes

Reagent	Volume for 1 reaction (µl)
DNA / Sample	2.0
H_2O	12.6
10X PCR Buffer (w/o $MgCl_2$)	2.0
$MgCl_2$ (50mM)	0.6
2.5 mM dNTP	1.6
Control Primers	0.8
Taq (5U/µl)	0.4

4. Place the PCR reaction tubes in a thermal cycler.
5. Start the following PCR reaction program:

Initial Denaturation	94°C	3 min	
Denature	94°C	20 sec	repeat for a total of 32 times
Anneal	59°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	2min	

6. Remove the PCR tubes. Reactions can be stored at -20°C.
7. Remove 10 µl of each PCR reaction for analysis by gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product is 166 base pairs.

Real-time Quantitative PCR

1. Add 2 µl of the sample to the PCR plate suitable for your real time instrument of choice (Performing triplicate of qPCR reactions per ChIP sample is recommended).
2. Prepare a master reaction mix as shown in Table II below. Dispense enough reagents for at least one extra tube to account for loss of volume.

Table II

qPCR reagent assembly for 1 reaction:

ddH_2O	9.5 µl
SYBR®-Green Master Mix	12.5 µl
Primer mix	1 µl
Total	23 µl

3. Add 23 µl of qPCR mix to the 2 µl of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.
5. Run the qPCR reaction following these parameters:

Initial Denaturation 94℃ 10 min

Denature 94℃ 20 sec

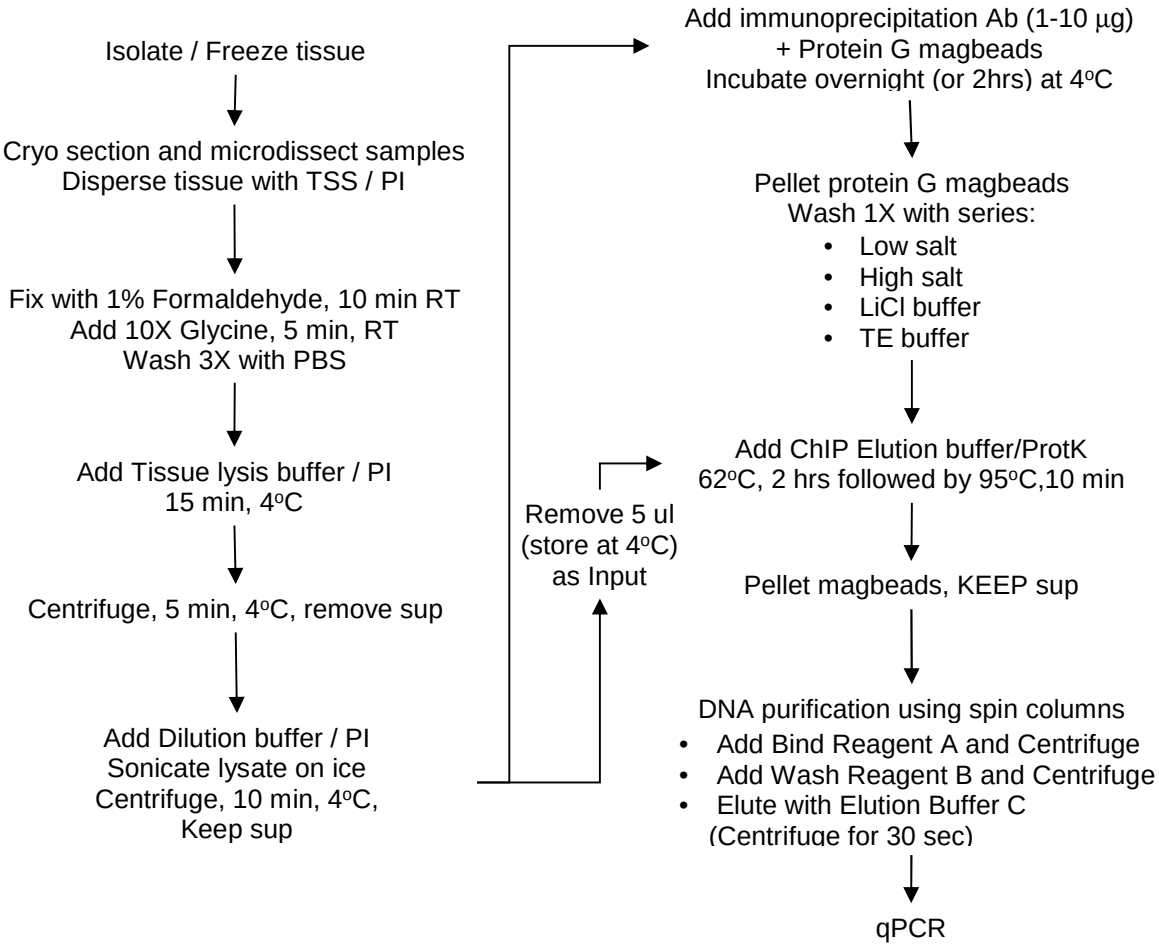
Anneal and Extension: 60℃ 1 min

} 50 times

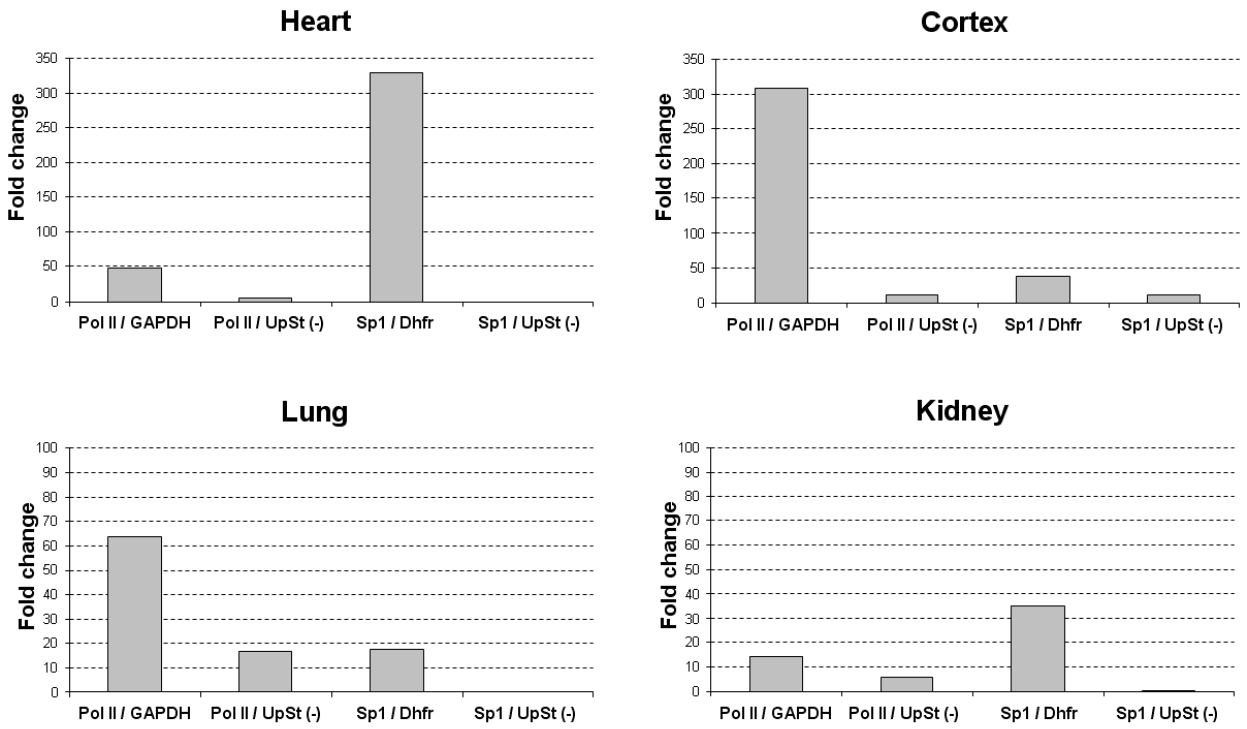
The polymerase chain reaction (PCR) is covered by one or more of the following U.S. patents: 4,683,202; 4,683,195; and 4,889,818 issue to Cetus Corporation and owned and licensed by Hoffman-LaRoche Molecular Systems, Inc. Purchase of the ***Magna ChIP™ G Tissue Kit*** *does not convey a license to use the PCR process covered by these patents. Purchasers of this product must obtain a license to use the PCR process before performing PCR.*

SYBR® is a registered trademark of Molecular Probes.

Flow Chart of Magna ChIP™ G Tissue Kit Preparation and IP Protocol



Section II: Performance of Various ChIP Antibodies using Magna ChIP™ G Tissue Kit Protocol



Chromatin from various cell lines was subjected to immunoprecipitation with the indicated antibodies using the **Magna ChIP™ G Tissue kit**. The IgG used for relative comparison was either Rabbit Purified IgG (Cat.# PP64B) or Mouse Purified IgG (Cat.# 12-371B), depending upon the ChIP antibody. Quantitative PCR data is presented as fold relative enrichment to IgG from independent experiments or as % input. For a biological negative control qPCR was assessed with primers upstream of the Dhfr gene (UpSt (-)): Forward: 5' CTT AAA CTG ATT TGC AAC TGC AG 3', Reverse: 5' CGT TTT ACT GTA CAG ATT TCC AG 3'. Antibodies and primers utilized were as follows:

Anti-RNA Polymerase II clone CTD4H8 (Cat.# 05-623B): 1 µg of mouse monoclonal affinity purified antibody immunoprecipitated with chromatin of various mouse tissue and qPCR assayed with primers specific for mouse GAPDH promoter: Forward: 5' CAC CAT CCG GGT TCC TAT AAA TAC 3', Reverse: 5' CAG CAT CCC TAG ACC CGT ACA 3'

Anti-SP1 (Cat.# 07-645): 1 µg of mouse monoclonal affinity purified antibody immunoprecipitated from chromatin of various mouse tissue and qPCR assayed with primers specific for mouse Dhfr promoter: Forward: 5' CAC GCC TCA ACC TGT GCG GGA 3', Reverse: 5' GCG GGG ATA AAA TCC TAC CAG CC 3'.

For a complete listing of Millipore's ChIPAb+ validated antibody/primer sets, visit www.millipore.com and search 'ChIPAb+'.

Section III: Appendices

APPENDIX A: Optimization of DNA Sonication

Optimal conditions for shearing crosslinked DNA to 200-1000 base pairs in length depend on the tissue type, tissue cell density, total volume and the specific sonication equipment, including the power settings and duration and number of pulses. Approaches for optimizing sonication may include changing microdissection sample number per ml in Dilution Buffer, number of cycles or power settings of sonication.

1. Resuspend lysed tissue in ice cold dilution buffer at 2, 4 and 8 micordissection samples, each in 500µl
2. For each dissected sample concentration, sonicate each tube on wet ice for a fixed number of cycles allowing rests between cycles according to the instrument manufacturer's guidelines. For example, using a Misonix 3000 instrument and a #419 microtip probe, use six cycles of 15sec pulse with 50 second rest in between pulses and power setting at 6. Keep tubes cool at all times.
3. Remove 5-10 µl from each sample into microfuge tubes and label.
4. Repeat one cycle (one 15 sec pulse) of sonication and remove 5-10 µl from each sample into microfuge tubes and label.
5. Repeat step 4 two more times.
6. Repeat steps 2-5 at a higher power setting.
7. To all the samples add ChIP elution buffer to a final volume of 50 µl.

To verify sonication results follow protocol below:

1. Add 1 µl of RNase A (10 mg/ml, user provided) to all samples from above and incubate for 30 min at 37°C.
2. Add 1 µl Proteinase K and incubate at 62°C for 2 hour.

   Optional step: For improved gel electrophoresis results, it may be necessary to purify DNA after Proteinase K incubation by going through the DNA Purification Using Spin Columns provided in the protocol section (section G, page 10).
3. Load 10 µl and 20 µl on a 1-2% agarose gel with a 100 bp DNA marker.

   Note: *Loading different amounts helps to avoid under- or over-loading.*
4. Observe which of the shearing conditions gives a smear of DNA in the range of 200 bp-1000 bp.
5. If necessary, further optimization of the shearing conditions may be required if the results do not produce DNA at the desired size range. Once optimal conditions have been determined, it is advised that the user does not alter the dissected sample concentration or volume of lysate per microfuge tube for subsequent chromatin immunoprecipitation experiments.

APPENDIX B: Preparation of Tissue: Non-region-specific Tissue Preparation

1. Isolate the tissue sample and cut into a small piece (1-2 mm^3) with razor blade.
2. Transfer cut sample to a 15 ml conical tube and add 500 µl of TSS/PI buffer. Carefully pipette up and down ten times using a 1ml pipette to shear the tissue.
3. Proceed with step 2 of section C.

CHROMATIN IP OPTIMIZATION AND TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Crosslinking	Not enough or too much crosslinking	The appropriate amount of formaldehyde and time of crosslinking must be determined empirically. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time. Be sure to use fresh formaldehyde. Histones may not need to be crosslinked since they are tightly associated with DNA.
Tissue Lysis	Inefficient disruption of Tissue	It is important to have enough Tissue lysis buffer per cell concentration. Follow the guidelines in this protocol. Also, check the cell lysis by looking at a 10 µl portion of the cell lysate under the microscope for intact cells.
Chromatin Shearing	Not enough/too much sonication	Follow Appendix A to obtain DNA of desired size.
	Denaturation of proteins from overheating sample	Keep the sample on ice during the sonication. Shorten the time of each sonication and increase the number of cycles the sample is sonicated.
Addition of Primary Antibody	Antibody doesn't recognize protein in fixed chromatin	Choose an antibody directed to a different epitope of the antigen. Decrease the amount or time of formaldehyde fixation.
	Not enough or too much chromatin	Perform IP from a dilution series of antibody with a fixed amount of chromatin or vice versa.
	Poor Ab binding	• Incubate the antibody of interest with the chromatin at 4℃ overnight. • Select a different antibody with higher affinity. • Perform a Western blot of the immunoprecipitated protein to verify the antibody can precipitate the antigen of interest.
Addition of Secondary Reagent – Protein G	Not enough beads	The magnetic beads settle to the bottom of the tube over time. Make sure the Protein G magnetic beads are well mixed prior to removing the appropriate volume for IP.
	Incorrect Antibody Class or Isotype	Check that the subclass and isotype of the antibody can bind Protein G. Protein G is not recommended for IgM or chicken IgY.

Step	Potential Problems	Experimental Suggestions
Washing	High background	Increase the number of washes or incubation times during washes.
	Aspiration of the beads during buffer removal	Make sure that there are no beads in the supernatant prior to removing it.
Elution and Reversal of crosslinking	Incomplete elution	When performing elution, make sure that the temperature is near 60°C. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.
	Excessive Crosslinking	Although protocols exist, excessive crosslinking is innately difficult to reverse. To avoid and prevent from repeating, conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.
PCR	Incorrect Annealing Temperature or Amplification Conditions	• Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR on genomic DNA to confirm amplification conditions and ability of primers to generate a single DNA product of the expected size.
	Bad primers	Follow suggestions for primer design in section "Chromatin IP Assay Overview, section B".
	No PCR product	• Increase amount of DNA added to the PCR reaction. • Increase the number of cycles for the amplification reaction.
	PCR product is a smear	• Decrease amount of DNA added to the PCR reaction. • Use HotStart™ *Taq* polymerase to avoid non-specific annealing of primers.
	No difference in quantity between PCR product from RNA Polymerase II and Normal Mouse IgG IPs	• Ensure correct mass of antibody and the correct cell equivalents of chromatin are used for IP as indicated in protocol. Too much antibody and/or chromatin can result in increased non-specific binding. • Dilute DNA with water to decrease amount of DNA added to the PCR reaction. • Decrease the cycle number at which the DNA is analyzed. It is important that the PCR products are analyzed within the linear amplification phase of PCR, in which differences between quantities of starting DNA can be measured.

References

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