NOVUS BIOLOGICALS

ChromataChIP™ Kit

a simple ChIP by number and by color manual

Table of Contents

Introduction

The Novus ChromataChIP™ kit was designed to be the most complete kit available for chromatin immunoprecipitation. You will find that this manual and the kit components are separated into five color-coded phases. The color of each page matches the phase's components in your kit. We think that performing the ChIP experiments with this kit will be fun and easy! With the supplied materials, the ChromataChIP™ kit will allow you to perform 25 IPs, with 25 or more PCR reactions from each IP. An epigenetic positive control antibody and human primer set have also been included to help you analyze your results.

If at any point during the protocol you feel that you need a helping hand, please contact our elite technical support team:

Live Chat
www.novusbio.com

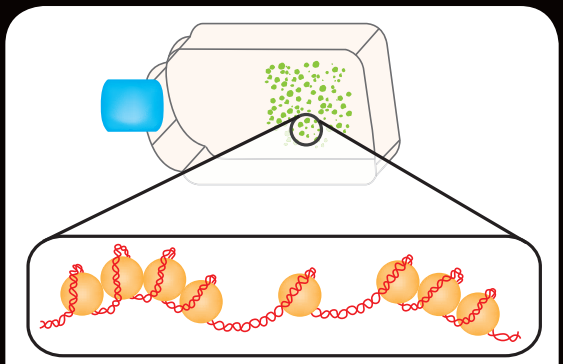
Email
technical@novusbio.com

Novus USA
1-888-50-NOVUS

Novus Canada
1-855-668-8722

Novus Europe
+44 (0)1223 426001

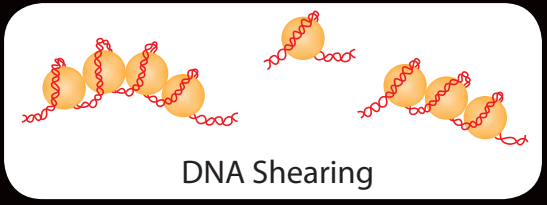
1



Cell Fixation and
Chromatin Cross-Linking

45 minutes

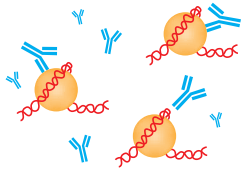
2



DNA Shearing

30 minutes

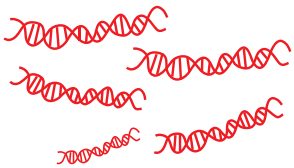
3



Immunoprecipitation

Overnight
incubation
+ 2 hours

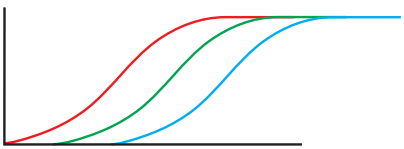
4



Reverse Cross-Linking
and DNA Purification

30 minutes

5



PCR

30 minutes
+ 2 hour PCR

ChromataChIP™ Kit Components

Component	Quantity	Storage
10x Glycine	30 mL	4° C
IP Dilution Buffer	10 mL	RT
H3 K4Me3 Positive Control Antibody	0.025 mg	-20° C
Protein A/G Magnetic Beads	0.8 mL	4° C
IP Wash Buffer 1	15 mL	4° C
IP Wash Buffer 2	15 mL	4° C
IP Wash Buffer 3	15 mL	4° C
IP Wash Buffer 4	15 mL	4° C
IP Elution Buffer	7 mL	RT
5M NaCl	0.5 mL	4° C
Proteinase K	0.05 mL	-20° C
Purification Columns	25 columns	RT
Purification Column Tubes	25 tubes	RT
DNA Binding Buffer	15 mL	RT
DNA Wash Buffer	4 mL (add 16 mL EtOH)	RT
DNA Elution Buffer	2 mL	RT
Human RPL30 Positive Control Primer Pair	0.5 mL @ 10 μM	-20° C

Additional Materials Required*

These reagents and equipment must be provided by the end user. All of these items will be marked with a * throughout this manual.

Reagents	Equipment
Chromatin source: Cells or tissue	Siliconized centrifuge tubes
Formaldehyde or Formalin	PCR tubes
PBS	15 mL conical tubes
Protease inhibitors	Cell scraper
Lysis buffer	Magnetic tube separator rack (Cat# NBP1-72032)
95-100% EtOH	Vortex mixer
PCR master mix	Sonicator
PCR primers	Centrifuge
DNase free H_20	Rotating wheel/platform
	Thermal cycler

1 Cell Fixation and Chromatin Cross-Linking

1. In this protocol, we will be using one 150 cm^2 flask of HeLa cells (~1 x 10^7 cells) as an example. Start with cells* that are 80% confluent. Other cell types or tissue may require optimization.

2. Add formaldehyde* to a final concentration of 1% in growth medium. Incubate for 10 minutes at room temperature (Use precautions when working with formaldehyde, including use in the chemical hood and appropriate personal protective gear).

3. Add enough 10X Glycine to each flask to reach a final concentration of 1X in the medium, and thereby quench the formaldehyde and stop the cross-linking. Incubate for 5 minutes at room temperature.

4. Remove all medium and wash with 20 mL of ice cold 1X PBS*.

5. Repeat step 4.

6. Remove PBS and add 4 mL of ice cold 1X PBS with appropriate protease inhibitors*. Scrape cells and transfer to a 15 mL conical tube*.

7. Spin cells at 4° C for 5 minutes at 800 x g.

8. Discard supernatant and resuspend cell pellet in 400 μL of RIPA lysis buffer* (containing protease inhibitors) per 1 x 10^7 cells (enough for 10 IPs). Transfer resuspended cells to a microcentrifuge tube and incubate at 4° C for 15 minutes.

2 DNA Shearing by Sonication

1. Sonicate cross-linked DNA to fragment sizes of 100-1000 base pairs (*Fig. 1*).

    a. **Important:** Conditions for shearing must be empirically derived and optimized before proceeding to the following phases. Conditions vary depending on equipment, cell type, cell density, and cross-linking efficiency.

    b. Keep samples ice cold to prevent denaturing of chromatin.

    c. Keep sonicator tip close to the bottom of each tube and limit samples to a maximum of 400 µL to prevent foaming of samples, as foaming decreases efficiency.

    d. A time-course of multiple short sonications is preferable to fewer extended treatments due to heat buildup and subsequent denaturing of samples. For example, six 15 second pulses at 50% output with a 60 second ice rest in between pulses works well for us.

2. Centrifuge sheared samples at 4° C for 5 minutes at 12,500 x g to remove debris. Remove supernatant and transfer to a new tube. Discard pellet. Sample can now be moved into ten 40 µL aliquots, each of which is sufficient for a single IP. Set aside one 40 µL aliquot for the input control, which will not go through the IP process. Although it is preferable to proceed directly to the following Phases, sheared chromatin can now be frozen at -80° C for up to 1 month.

a. Optional: Test the efficiency of the shearing by preparing an aliquot to run on a 1.5% agarose gel.

i. Dilute a 40 µL aliquot with 160 µL of IP Elution Buffer and 8 µL of 5M NaCl.

ii. Incubate at 95° C for 15 minutes to reverse cross-linking.

iii. Optional: Cleanup DNA by following the procedure in the DNA purification phase (Phase Four).

iv. Run 10 µL of sample on the 1.5% agarose gel with loading buffer and DNA binding dye alongside a 100 bp ladder. Verify fragment size.

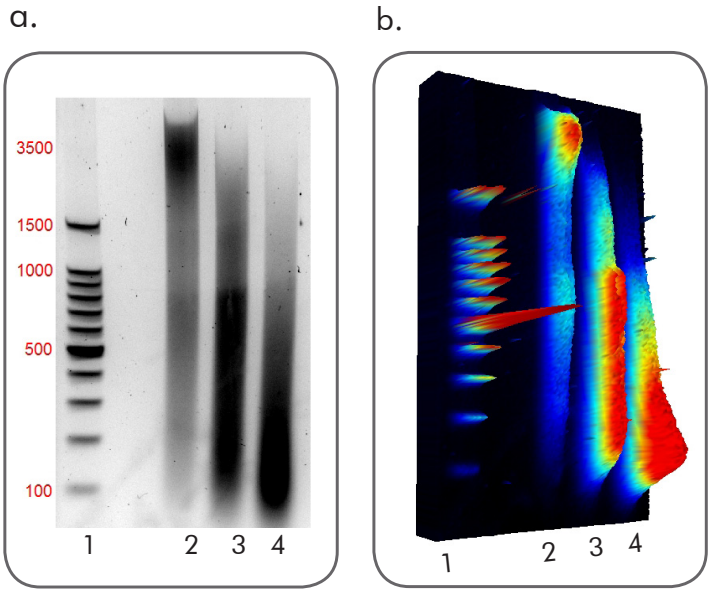


Figure 1. (a) A 1.5% agarose gel was loaded with a 100 bp ladder (lane 1), under-sheared chromatin concentrated at 3500 bp (lane 2), optimally sheared chromatin evenly distributed from 100-1000 bp (lane 3), or over-sheared chromatin concentrated between 100-200 bp (lane 4). (b) A spectral 3-D map of the same gel from (a) to emphasize the differences in shearing efficiency.

3 Chromatin Immunoprecipitation

1. Dilute each IP sample 1:10 by adding 40 µL of sheared chromatin to 360 µL IP Dilution Buffer, along with protease inhibitors. Save your undiluted input sample at 4° C for Phase Four.

2. Add your antibody of interest* to each of your samples. It is also recommended to run the separate following controls:

    a. H3 K4Me3 Positive Control: Add 2 µg of H3 K4Me3 antibody to a separate sample from step 1.

    b. No Antibody Negative Control: A separate sample from step 1 that does not have any antibody added.

3. Incubate tubes with rotation overnight at 4° C.

4. For each IP, you will use 25 µL of fully suspended vortexed Protein A/G Magnetic Bead slurry. Beads must first be washed by adding 25 µL of suspended beads to 1 mL of IP Dilution Buffer and vortexed. Pellet magnetic beads with a magnetic separator and remove the supernatant.
Important: Do not let the beads dry out in any step!

5. Add each sample to 25 µL of washed beads and incubate tubes with rotation for one hour at 4° C.

6. Pellet magnetic beads with a magnetic separator and remove the supernatant. Add 500 µL cold IP Wash Buffer 1 and wash for 5 minutes with rotation at 4° C. Pellet magentic beads with magnetic separator and discard supernatant.

7. Add 500 µL cold IP Wash Buffer 2 and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

8. Add 500 µL cold IP Wash Buffer 3 and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

9. Add 500 µL cold IP Wash Buffer 4 and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

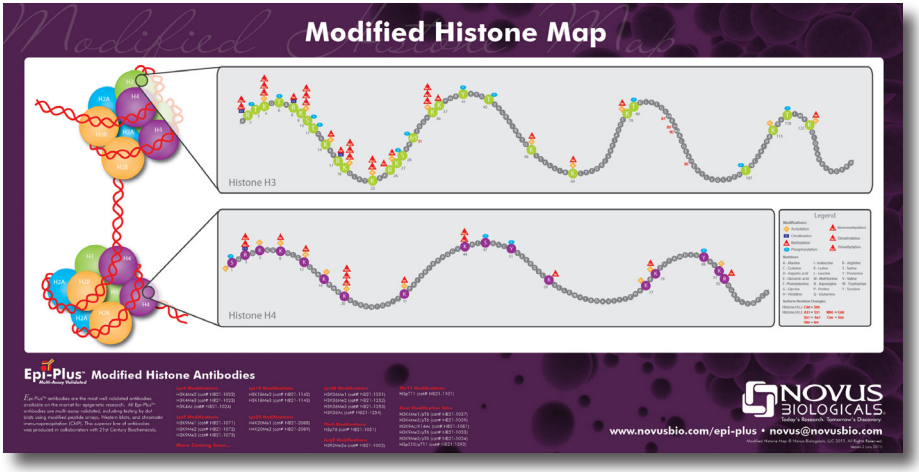
10. Elute complex by adding 200 µL IP Elution Buffer and rotate at room temperature for 15 minutes. Pellet beads with separator and discard beads, keeping the supernatant.

4 Reverse Cross-Linking

1. For each IP sample, add 8 µL of 5M NaCl. For the 40 µL input control that did not go through the preceeding IP steps, add 160 µL of IP Elution Buffer and 8 µL of 5M NaCl.
2. Incubate at 95° C for 15 minutes.
    a. Optional: Some lysates may need extended reverse cross-linking and a protein degradation step. If you find your total DNA yield to be low, perform the following:
        i. After completion of Step 1, add 2 µL of the provided Proteinase K and incubate at 62° C for at least 2 hours (or overnight).
        ii. Incubate at 95° C for 10 minutes to deactivate the Proteinase K.

DNA Purification

1. Add 600 µL of DNA Binding Buffer to each sample and vortex.
2. Place a Purification Column into the supplied tube and add binding buffer/sample mix. Spin at 15,000 x g for 1 minute. Discard flow-through.
3. Add 700 µL of DNA Wash Buffer into the column and spin at 15,000 x g for 1 minute. Discard flow-through.
    a. Before using the wash buffer for the first time, add 16 mL of 95-100% EtOH* to the bottle.
4. Spin the empty column at 15,000 x g for 2 minutes. Discard flow-through and tube.
5. Place the Purification Column into a new clean tube* and add 50 µl of DNA Elution Buffer directly to the membrane. Let sit for 1 minute to adsorb onto the membrane and then spin at 15,000 x g for 1 minute. Purified DNA can be stored at -20° C until ready for Phase Five.



Want a full-size copy of this modified histone poster?
Request your free copy at **www.novusbio.com/mailing-list**

Need help? Call, email or chat with our elite technical support team for one-on-one guidance

Live Chat
www.novusbio.com

Email
technical@novusbio.com

Novus USA
1-888-50-NOVUS

Novus Canada
1-855-668-8722

Novus Europe
+44 (0)1223 426001

Innovator's Reward™

Want to try one of our histone antibodies in new species or applications? The Novus Innovator's Reward™ program is designed to support your innovative research with minimal financial risk to you. Should you decide to use one of our products in an application or species for which it has not been tested, Novus will provide you with a 50% refund on the purchased product as well as a 50% discount on a future product of equal or lesser value.

Learn more at www.novusbio.com/guarantee

5 DNA PCR Amplification

Purified DNA can now be measured by PCR. Quantitative real-time PCR is the preferable method of amplification due to its sensitivity. The method described below uses a 2X SYBR green reaction mix containing all necessary components (dNTPs, DNA polymerase, buffers). It is recommended to run each PCR reaction in triplicate for each sample. Samples to be assayed include: immunoprecipitated sample from the antibody of interest, the H3 K4Me3 positive control sample, the no antibody negative control sample, and your purified input control. A mix of forward and reverse human RPL30 primers have been included in your kit to measure activity of the H3 K4Me3 positive control sample and relative background of the no antibody negative control. Each sample will use 2 µL of purified DNA for its template. This will yield 25 PCR reactions from each sample.

1. For the input control fraction **only**, dilute the template to 1% of the original concentration (1:100 dilution). All other samples are left undiluted.

2. It is best to first create a PCR master mix for each primer set and dispense the mix into each reaction well first, adding the template last. In the case of your positive control primer set master mix, each reaction will contain the following:

   i. 7 µL of DNase free water*
   ii. 1 µL of 10 µM RPL30 primers
   iii. 10 µL of 2x SYBR reaction mix*
   } **Master Mix**
   iv. 2 µL of purified DNA template (added directly to wells)

3. Perform PCR according to manufacturer's recommendations for the SYBR reaction mix*.

Data Analysis

Data can now be analyzed by taking the cycle threshold (Ct) values from the qRT-PCR assay. All samples will be adjusted to a signal relative to the total input fraction.

1. Example
    a. First, find out what the 1% input control fraction would be at 100%. Ex. The raw Ct of the 1% input fraction=30. Adjust Ct from 1% to 100% by subtracting 6.64 cycles (log_2 of 100); 30 - 6.64 = 23.4 = 100% input Ct (*Fig. 2*).
    b. Next, look at the Ct from the antibody of interest sample. Let's say this value is a Ct=28.
    c. Now, normalize the signal from the antibody of interest to the total input. In this example, signal relative to input would be 2 ^ (23.4-28) = 0.04 = 4%. From this, it can be shown that 4% of all the available DNA in the sample (specific to your primer sequence) was bound to the protein of interest.
    d. Finally, look at the background signal levels to make sure that they are significantly less than the antibody precipitated samples. Measure the signal relative to input of the no antibody control. If the raw Ct from this reaction was 36, the signal relative to input would be 2 ^ (23.4-36) = 0.01%. From this it can be said the background is significantly less compared to the antibody of interest (4% vs 0.01%).

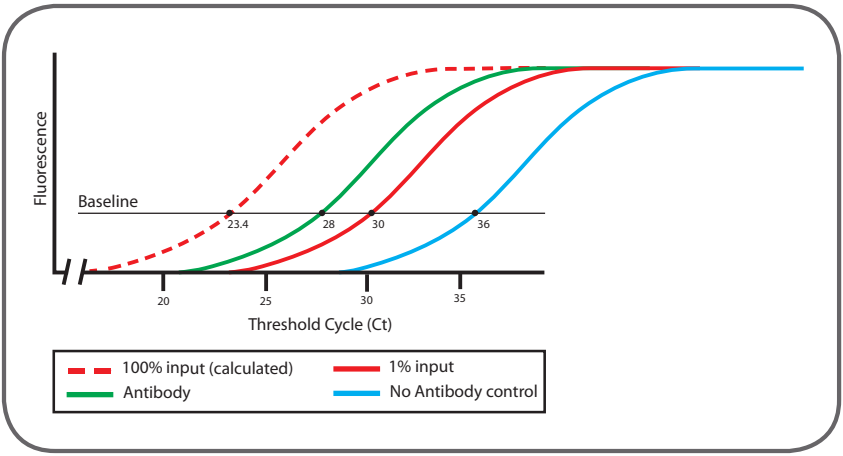


Figure 2. qRT-PCR graph representing the example from above.

Troubleshooting

Phase	Issue	Recommendations and Comments
1. Cross-Linking	Too much/little cross-linking	Under cross-linking can prevent the disassociation of protein-DNA complexes in the following steps and result in poor yield. Over cross-linking can mask epitope sites crucial for antibody binding, prevent proper chromatin shearing, and inhibit the successful uncross-linking of the complex in subsequent steps. If using paraformaldehyde, ensure that it is freshly prepared.
2. Chromatin Shearing	Foaming	Use siliconized 1.7 mL microcentrifuge tubes with no more than 400 µL of sample. Keep sonicator tip very close to the bottom of the tube.
	Under-sheared Chromatin	Perform more shearing replications, turn up the sonication power, cross-link less, or use fewer cells.
	Over-sheared Chromatin	Perform fewer shearing replications, turn down the sonication power, cross-link more, or use more cells.
	Chromatin degradation	Samples must be placed on ice between sonications. If the sonication is too long or powerful, unwanted denaturing will take place.
3. Chromatin IP	Magnetic beads	Always fully resuspend beads by vortexing before pipetting. Always store at 4° C and never allow beads to dry out. Check that the subclass of the antibody is compatible with Protein A/G.
	Antibody	Verify that the antibody of interest is ChIP validated. Specificity of antibody can be verified by western blot after IP. Too little antibody can result in too little material for successful PCR. Too much can increase PCR background. Some antibodies may allow short room temperature incubations with lysate but in general an overnight incubation at 4° C will increase signal and specificity.
4. Reverse Cross-Linking	Inefficiency	For most complexes, a 15 minute incubation at 95° C will be sufficient. However, with some samples Proteinase K treatment for 2 or more hours at 62° C may be necessary. Initial cross-linking time may also need to be reduced.

4. DNA Purification	Poor yield	Increase initial cell quantity. Verify that the column is completely dry after the wash step purification as any leftover wash will inhibit elution. Ensure the elution buffer is placed directly onto the silica membrane and allowed to adsorb for at least 1 minute.
5. PCR	High background (High amplification of no antibody control)	Keep IP buffers cold and increase wash stringency. DNA improperly sheared. Too much antibody or template DNA.
	No amplification of product	Not enough antibody. Verify that your primers are properly designed and that your thermal cycler protocol is agreeable with your Taq master mix. Use more template DNA.

Have a different question? Call, email or chat with our elite technical support team for one-on-one guidance

Live Chat
www.novusbio.com

Email
technical@novusbio.com

Novus USA
Phone: 303-730-1950
Toll Free: 1-888-50-NOVUS
Fax: 303-730-1966
novus@novusbio.com

Novus Canada
Phone: 905-827-6400
Toll Free: 855-668-8722
Fax: 905-827-6402
canada@novusbio.com

Novus Europe
Phone: +44 (0)1223 426001
Fax: +44 (0)871 971 1635
europe@novusbio.com

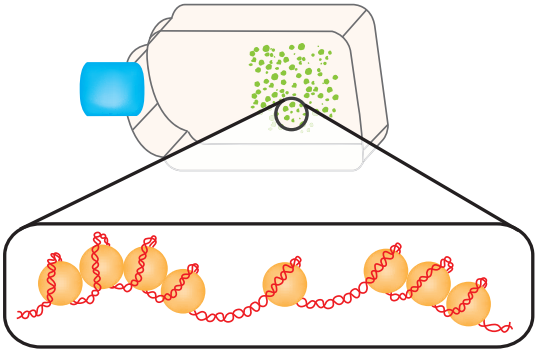
Novus Germany
Phone: +49-6922-22340-60
Fax: +49-0800-58926-79
germany@novusbio.com

Novus Italy
Phone: +39 02 4032 6786
Fax: +39 02 4032 6340
italy@novusbio.com

NOVUS BIOLOGICALS

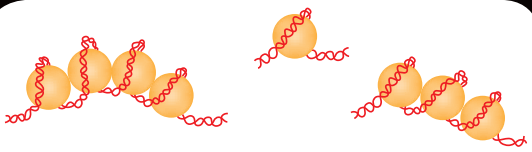
www.novusbio.com/ChromataChIP

1



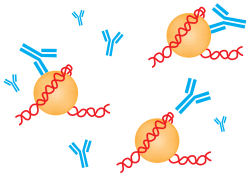
Cell Fixation and
Chromatin Cross-Linking

2



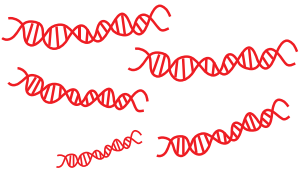
DNA Shearing

3



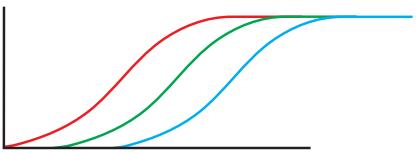
Immunoprecipitation

4



Reverse Cross-Linking
and DNA Purification

5



PCR