ab500

ChIP Kit

Instructions for Use

For the mapping of modifications in the genome

This product is for research use only and is not intended for diagnostic use.

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1. Overview

ab500 ChIP Kit allows the mapping of target proteins/histone modifications to specific loci in the genome. Antibodies and protein A beads are used to immunoprecipitate chromatin/DNA complexes. The DNA region of interest is then quantified using quantitative PCR.

2. Background

ChIP (Chromatin immunoprecipitation) is a powerful technique for studying protein-DNA interaction *in vivo*. The principle of ChIP is simple: the selective enrichment of a chromatin fraction containing a specific protein, which can be used to determine the relative abundance of that protein at one or more locations in the genome. ChIP is therefore a convenient tool to identify modifications in the genome of interest. Moreover, ChIP can be coupled with microarrays for further profiling or mapping binding patterns.

3. Components and Storage

A. Kit Components

Item	Quantity (24 assays)	Storage temperature
Buffer A	10 mL	4 °C
1.25M Glycine	10 mL	4 °C
Buffer B (Lysis)	30 mL	4 °C
Buffer C (Lysis)	30 mL	4 °C
Buffer D (Chromatin Shearing)	3 mL	4 °C
5x ChIP Buffer	84 mL	RT
Protease Inhibitor	1 tablet	4 °C
DNA purifying slurry	3 mL	4 °C
Proteinase K	30 µL	-20 °C
unblocked Protein A beads	960 µL	4 °C
PCR-grade H_20	10.2 mL	4 °C
Positive control (ab1791 Histone H3 antibody)	25 µg	-20 °C

B. Additional Materials Required

- Trypsin (pre-warm to 37°C before use)
- Cell Culture media (pre-warm to 37°C before use)
- PBS (ice cold before use)
- Formaldehyde (pre-warm to 37°C before use)
- Heating block
- 1.5% agarose gel
- Desktop centrifuge (up to 14,000 rpm)
- Sonicator
- Rotary mixer
- Pipettes and pipette tips
- 1.5 ml microcentrifuge tubes
- 15 ml conical tubes

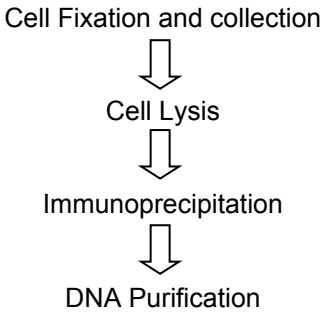
NOTE: No primers are included in this ChIP kit.

C. Storage

- Upon receipt store the following components at -20°C:
  - Proteinase K
  - Positive control (ab1791 Histone H3 antibody)
- Store 5x ChIP buffer at room temperature (RT).
- Store all remaining kit components at 4°C.

4. Protocol

Protocol Summary



Before starting each step ensure you have reagents for each step ready:

- Buffer C: ice-cold
- Buffer D: room temperature (RT) to ensure there is no SDS precipitate.
- Protein A Sepharose beads: beads need to be swollen before use. Wash beads twice in 1 ml cold PBS and briefly spin down by pulse in a microcentrifuge (14,000rpm, 5 seconds) and resuspend in 1ml of 1x ChIP buffer. Resuspended beads can be used on the day or can be stored for up to one week at 4C.

A. Cell Fixation and Collection:

The minimum number of cells to be processed is $3x10^6$ cells for 3 ChIPs. The sample is split into 3 at the immunoprecipitation stage (**Step D**). In order to process more cells, the volumes need to be altered accordingly. Tables have been included for this purpose.

1. Trypsinize cells e.g. wash cells with 10ml PBS, add 1 ml of trypsin to a 92 cm^2 dish, incubate at 37°C for 5 min, and stop reaction by adding 4 ml media by pipetting

repeatedly to generate a unicellular suspension. Cells can be counted at this stage. Aliquot a minimum of **3x10^6 cells** into a tube.

2. Pellet cells by centrifugation, 10 min, 500 g, RT. Discard the supernatant.
3. Wash cells in 1 ml PBS and pellet cells by centrifugation, 10 min, 500 g, RT.
4. Resuspend cells in Buffer A/Formaldehyde/PBS mix as indicated in table below. Please note the final concentration of formaldehyde is ~ 1.1%.

Cell No.	No. ChIPs	Buffer A	Formaldehyde	PBS
3 x 10^6	3	45 µL	20 µL	0. 6 mL
6 x 10^6	6	90 µL	40 µL	1.2 mL
10 x 10^6	10	150 µL	67 µL	2.0 mL
15 x 10^6	15	225 µL	100 µL	3.0 mL
24 x 10^6	24	360 µL	161 µL	4.8 mL

5. Mix gently and incubate for 10 min at RT.
6. Add glycine to quench the formaldehyde as indicated in the table below and mix.

Cell No.	Glycine
3 x 10^6	65 µL
6 x 10^6	130 µL
10 x 10^6	210 µL

15 x 10^6	315 µL
24 x 10^6	504 µL

7. Pellet cells by centrifugation, 5 min, 500 g, 4°C.
8. Wash cells with 1 ml of ice cold PBS. Pellet cells by centrifugation, 5 min, 500 g, 4°C.

B. Cell Lysis and sonication:

1. Prepare 25x Protease Inhibitor (PI) solution by dissolving Protease Inhibitor tablet in 450 µl of water. Store in aliquots at -20°C to avoid multiple freeze-thaw cycles.
2. Resuspend cells in Buffer B as indicated in table below.

Cell No.	**Buffer B**
3 x 10^6	1.0 mL
6 x 10^6	2.0 mL
10 x 10^6	3.3 mL
15 x 10^6	5.0 mL
24 x 10^6	7.9 mL

3. Mix gently and incubate, 10 min, RT.
4. Pellet samples by centrifugation, 5 min, 500 g, 4°C. Discard the supernatant.
5. Resuspend the pellet in ice cold Buffer C in appropriate volume as indicated in table below.

Cell No.	**Buffer C**

3 x 10^6	1.0 mL
6 x 10^6	2.0 mL
10 x 10^6	3.3 mL
15 x 10^6	5.0 mL
24 x 10^6	7.9 mL

6. Mix gently and incubate, 10 min, 4°C.
7. Pellet samples by centrifugation, 5 min, 500 g, 4°C. Discard the supernatant.
8. Prepare Buffer D/PI mix (25x) as indicated in table below.

Cell No.	**No. ChIPs**	**Buffer D**	**PI mix (25x)**	**Total (µL)**
3 x 10^6	3	96 µL	4 µL	100 µL
6 x 10^6	6	192 µL	8 µL	200 µL
10 x 10^6	10	320 µL	13 µL	333 µL
15 x 10^6	15	480 µL	20 µL	500 µL
24 x 10^6	24	768 µL	31 µL	800 µL

9. Add 100 µl of the Buffer D/PI mix to the pellet from step B7.
10. Shear DNA using a sonicator to an optimal DNA fragment size of 200-1000 bp. Ensure that samples are kept ice cold throughout the sonication.
11. Pellet cell debris by centrifugation, 5 min, 14000 g, 4°C. The supernatant contains the sheared chromatin and should be kept on ice.

12. Optimal conditions to obtain the desired fragment size should be determined prior to the immunoprecipitation (IP) by performing a sonication time course.

C. Reverse Cross-link:

13. Once chromatin is sonicated, reverse the cross-link using the procedure below.
    a Add 100 µl of PCR-grade H_2O to 20 µl of sonicated chromatin.
    b. Mix the DNA purifying slurry by inversion, and add 100 µl of slurry to the sonicated chromatin/ H2O mix.
    c. Mix the samples by inversion and incubate for 10 min, 98°C.
    d. After incubation, leave at RT for 20 min to cool.
    e. Centrifuge briefly to remove condensation from the tube lid 10 sec, 10000 g.
    f. Add 1 µl of proteinase K. Vortex for 5 sec at medium power.
    g. Incubate samples for 30 min at 55°C.
    h. Incubate samples for 10 min, 98°C.
    i. Pellet DNA purifying slurry by centrifugation, 1 min, 14000 g, RT.
    j. Transfer the supernatant to a 1.5 ml tube without disturbing the DNA purifying slurry pellet.

k. Load 10 µl on a 1.5 % agarose gel to analyze DNA fragment size. Analysis of DNA fragment size is shown below.

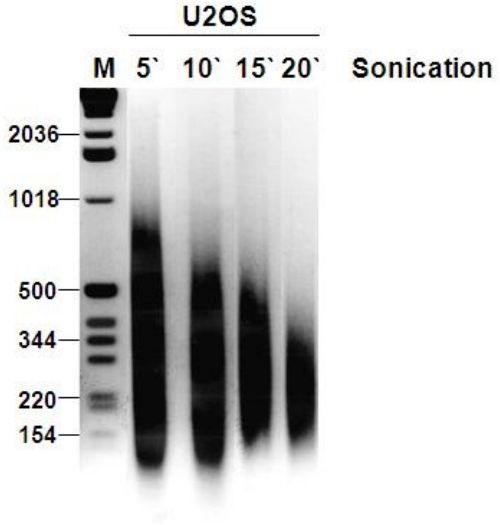


Figure 1: U2OS cells were sonicated for 5, 10, 15 and 20 min. The fragment size decreases during the time course. The optimal fragment size is observed at 15 min.

NOTE; Sonicating for too long will disrupt nucleosome-DNA interactions therefore the band size should not be smaller than 200bp.

D. Immunoprecipitation:

1. Dilute the 5x ChIP buffer to 1x using PCR grade H_2O, and add PI mix (25x) as indicated in table below.

Cell No.	No. of ChIPs	5x ChIP buffer	H_2O	PI mix(25x)
3 x 10^6	3	170 µL	0. 65 mL	34 µL
6 x 10^6	6	340 µL	1.29 mL	68 µL
10 x 10^6	10	567 µL	2.15 mL	113 µL
15 x 10^6	15	850 µL	3.23 mL	170 µL
24 x 10^6	24	1361 µL	5.16 mL	271 µL

2. Add 1x ChIP buffer/PI mix to the sheared chromatin as indicated in table below. Vortex for 5 sec.

Cell No.	No. of ChIPs	1x ChIP buffer/PI	Sheared Chromatin
3 x 10^6	3	0. 83 mL	100 µL
6 x 10^6	6	1.65 mL	200 µL
10 x 10^6	10	2.75 mL	333 µL
15 x 10^6	15	4.12 mL	500 µL
24 x 10^6	24	6.6 mL	799 µL

3. For each ChIP, aliquot 280 µl of diluted chromatin from step D2 into a 1.5 ml tube. Freeze the remaining chromatin which will be used in step 4 for the INPUT preparation. All volumes indicated from this point of the protocol are for 1x10^6 cells/ 1 ChIP unless otherwise stated.

4. The minimum number of 3 samples correspond to the following:
    (1) Antibody of interest
    (2) Positive control (ab1791)
    (3) Negative control (Beads only).

    Additional antibodies can be included but the number of cells needs to be increased accordingly. See Appendix 1 for additional positive control antibodies).
5. Add antibodies to the appropriate sample. The amount of antibody can vary but 2-5 µg is a good starting point. Incubate overnight with rotation at 4°C.
6. Next day, prepare the antibody binding beads as described below. Make sure beads are resuspended into a uniform suspension before each use.
    - Take your beads washed and resuspended in 1xChIP buffer (see page 7).
    - Cut off the tip of a 1000 µl pipette-tip to pipet the bead suspension.
    - Determine how many IPs you want to do (see table).
    - Keep the beads in suspension when pipetting aliquots out.

    The following volumes are for 3 ChIPs/ $3x10^6$ (see table below for other volume sizes and amount of 1x ChIP buffer needed for all washing steps).

Please note that PI mix is not required anymore from this stage.

Cell No.	No. of ChIPs	Bead Mix	1x ChIP buffer (for washes)
1 x 10^6	1	40 µL	5.3 mL
3 x 10^6	3	120 µL	16 mL
6 x 10^6	6	240 µL	32 mL
10 x 10^6	10	400 µL	53 mL
15 x 10^6	15	600 µL	80 mL
24 x 10^6	24	960 µL	127 mL

a. Transfer 120 µl of protein A bead suspension mix into 10 ml of 1x ChIP buffer. Mix by inversion and pellet beads by centrifugation, 3 min, 500 g, 4°C. Discard the supernatant. b. Add 1 ml of 1x ChIP buffer and mix by inversion.
c. Aliquot 300 µl of the bead/ChIP buffer mix into 3 x 1.5 ml tubes.
d. Pellet beads by centrifugation, 2 min, 500 g, 4°C. Carefully discard the supernatant.

7. Pellet the antibody/chromatin samples obtained from step C5 by centrifugation to remove insoluble material, 10 min, 14000 g, 4°C.

8. Remove 250 µl of the supernatant and transfer to the aliquoted beads from step D6.
9. Rotate for 30-60 min at 4°C.
10. After incubation, add 1 ml of 1x ChIP buffer.
11. Mix gently by inversion.
12. Pellet beads by centrifugation, 3 min, 500 g, 4°C. Carefully discard the supernatant.
13. Repeat washing step 3 times more with 1x ChIP buffer. Carefully discard the supernatant.

E. DNA Purification:

1. Add 100 µl of DNA purifying slurry to the samples (Protein A beads).
2. Take 50 µl of the frozen chromatin from step D3 and add 100 µl of DNA purifying slurry. This will be labelled as **INPUT**. Both the washed beads samples and the INPUT should be treated the same from this step onwards.
3. Mix the samples by inversion and incubate for 10 min, 98°C.
4. After incubation, leave at RT for 20 min to cool.
5. Centrifuge briefly to remove condensation from the tube lid, 10 sec, 10000g
6. Add 1 µl of proteinase K. Vortex for 5 sec at medium power.
7. Incubate samples for 30 min at 55°C.
8. Incubate samples for 10 min, 98°C.
9. Pellet DNA slurry by centrifugation, 1 min, 14000 g, RT.
10. Transfer 70 µl of the supernatant to a 1.5 ml tube.

11. Add 130 µl of PCR-grade H_2O to the DNA slurry. Vortex for 10 sec at medium power.
12. Pellet DNA slurry by centrifugation, 1 min, 14000 g, 4°C.
13. Transfer 130 µl of supernatant to the supernatant from step D10 (total: 200 µl).
14. The samples can now be used for quantitative PCR or stored at -20°C for analysis later.

5. Appendix 1- Positive Controls

Positive control targets should be present at one of the loci being analysed. The following antibodies could be used as additional positive controls:

Enriched at actively transcribed genes:

- ab1012/ ab12209/ ab8580 Histone H3 (tri methyl K4)
- ab5131: RNA polymerase II CTD repeat YSPTSPS (phospho S5)

Enriched at promoters of actively transcribed genes:

- ab51841 TATA binding protein TBP

Enriched at inactive loci:

- ab8898 Histone H3 (tri methyl K9)
- ab6002 Histone H3 (tri methyl K27)

6. Appendix 2- Affinity of beads

Species	Ig Isotype	Protein A	Protein G
Rabbit	All Isotypes	+++	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++
	IgM	Use anti Human IgM	
	IgE	-	+
	IgA	-	+
Mouse	IgG1	+	+++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+
	IgM	Use anti Mouse IgM	

Species	Ig Isotype	Protein A	Protein G
Rat	IgG1	-	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Chicken	All Isotypes	-	++
Cow	All Isotypes	++	+++
Goat	All Isotypes	-	++
Guinea Pig	All Isotypes	+++	++
Hamster	All Isotypes	+	++
Horse	All Isotypes	++	+++
Pig	All Isotypes	+	++
Sheep	All Isotypes	-	++

7. Troubleshooting

Problem	Cause	Solution
Little or No PCR Products	Insufficient tissues.	Increase tissue amount (ex: >10 mg tissues/per reaction).
	Insufficient or too much cross-linking.	Check if the appropriate cross-link step is carried out according to the protocol.
	Insufficient/too much sonication.	Follow the protocol instructions for obtaining the appropriate sized DNA. Keep the sample on ice during the sonication.
	Incorrect temperature/insufficient time for DNA release and reversal of cross-linking	Follow the guidelines in the protocol for appropriate temperature and time.
	Incorrect PCR conditions.	Check if all PCR components are added. Increase amount of DNA added to PCR reaction. Increase the number of cycles for PCR reaction.

Problem	Cause	Solution
	Incorrect or bad primers.	Ensure the designed primers are specific to the target sequence.
	The column is not washed with 90% ethanol.	Ensure that wash solution is 90% ethanol.
Little or No Amplification Difference Between the Sample and the Negative Control	Insufficient wash at each wash step.	Follow the protocol for appropriate wash.
	Antibody is added into the well forthe negative control by mistake.	Ensure antibody is added into the correct well.
	Too many PCR cycles.	If using conventional PCR, decrease the cycles to appropriate cycle number. Differences between quantities of starting DNA can be measured generally within the linear PCR amplification phase.

For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select "*contact us*" on www.abcam.com for the phone number for your region).

UK, EU and ROW
Email:
technical@abcam.com
Tel: +44 (0)1223 696000
www.abcam.com

US, Canada and Latin America
Email: us.technical@abcam.com
Tel: 888-77-ABCAM (22226)
www.abcam.com

China and Asia Pacific
Email: hk.technical@abcam.com
Tel: 108008523689 (中國聯通)
www.abcam.cn

Japan
Email: technical@abcam.co.jp
Tel: +81-(0)3-6231-0940
www.abcam.co.jp