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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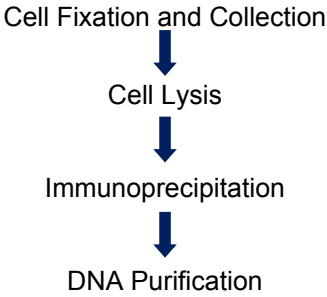
1. BACKGROUND

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.

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. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

5. MATERIALS SUPPLIED

Item	24 Tests	Storage Condition (Before Preparation)
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

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

NOTE: No primers are included in this ChIP kit.

- 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

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 **Buffer C**

Ice cold.

9.2 **Buffer D**

Room temperature to ensure there is no SDS precipitate.

9.3 **1X ChIP Buffer**

Dilute the 5x ChIP buffer to 1x using PCR grade H_2O.

9.4 **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 4°C.

9.5 **25X Protease Inhibitor Solution**

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.

10. SAMPLE PREPARATION

10.1 **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 (Section 11.1.3). In order to process more cells, the volumes need to be altered accordingly. Tables have been included for this purpose.

10.1.1 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 3 x 10^6 cells into a tube.

10.1.2 Pellet cells by centrifugation, 10 min, 500 g, RT. Discard the supernatant.

10.1.3 Wash cells in 1 ml PBS and pellet cells by centrifugation, 10 min, 500 g, RT.

10.1.4 Resuspend cells in Buffer A/Formaldehyde/PBS mix as indicated in table below. Please note the final concentration of formaldehyde is ~ 1.1%.

Cell Number	No. ChIP reactions	Buffer A (µL)	Formaldehyde (µL)	PBS (mL)
3 x 10^6	3	45	20	0.6
6 x 10^6	6	90	40	1.2
10 x 10^6	10	150	67	2.0
15 x 10^6	15	225	100	3.0
24 x 10^6	24	360	161	4.8

10.1.5 Mix gently and incubate for 10 min at RT.

10.1.6 Add glycine to quench the formaldehyde as indicated in the table below and mix.

Cell Number	Glycine (µL)
3 x 10^6	65
6 x 10^6	130
10 x 10^6	210
15 x 10^6	315
24 x 10^6	504

10.1.7 Pellet cells by centrifugation, 5 min, 500 g, 4°C.

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

10.2 Cell Lysis and Sonication

10.2.1 Resuspend cells in Buffer B as indicated in table below.

Cell Number	Buffer B (mL)
3 x 10^6	1.0
6 x 10^6	2.0
10 x 10^6	3.3
15 x 10^6	5.0
24 x 10^6	7.9

10.2.2 Mix gently and incubate, 10 min, RT.

10.2.3 Pellet samples by centrifugation, 5 min, 500 g, 4°C. Discard the supernatant.

10.2.4 Resuspend the pellet in ice cold Buffer C in appropriate volume as indicated in table below.

Cell Number	Buffer C (mL)
3 x 10^6	1.0
6 x 10^6	2.0
10 x 10^6	3.3
15 x 10^6	5.0
24 x 10^6	7.9

10.2.5 Mix gently and incubate, 10 min, 4°C.

10.2.6 Pellet samples by centrifugation, 5 min, 500 g, 4°C. Discard the supernatant.

10.2.7 Prepare Buffer D/PI mix (25x) as indicated in the next table.

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

10.2.8 Add 100 µL of the Buffer D/PI mix to the pellet from step 10.2.6.

10.2.9 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.

10.2.10 Pellet cell debris by centrifugation, 5 min, 14000 g, 4°C. The supernatant contains the sheared chromatin and should be kept on ice.

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

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

10.3 Measure DNA fragment length

10.3.1 Add 100 µL of PCR-grade H_2O to 20 µL of sonicated chromatin.

10.3.2 Mix the DNA purifying slurry by inversion, and add 100 µL of slurry to the sonicated chromatin/ H_2O mix.

10.3.3 Mix the samples by inversion and incubate for 10 min, 98°C.

10.3.4 After incubation, leave at RT for 20 min to cool.

10.3.5 Centrifuge briefly to remove condensation from the tube lid 10 sec, 10000 g.

10.3.6 Add 1 µL of Proteinase K. Vortex for 5 sec at medium power.

10.3.7 Incubate samples for 30 min at 55°C.

10.3.8 Incubate samples for 10 min, 98°C.

10.3.9 Pellet DNA purifying slurry by centrifugation, 1 min, 14000 g, Room Temperature.

10.3.10 Transfer the supernatant to a 1.5 mL tube without disturbing the DNA purifying slurry pellet.

10.3.11 Load 10 µL on a 1.5 % agarose gel to analyze DNA fragment size.

11. ASSAY PROCEDURE

11.1 Immunoprecipitation

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

Cell Number	No. ChIP reactions	5x ChIP Buffer (µL)	H_2O (mL)	25X PI Mix (µL)
3 x 10^6	3	170	0.65	34
6 x 10^6	6	340	1.29	68
10 x 10^6	10	567	2.15	113
15 x 10^6	15	850	3.23	170
24 x 10^6	24	1361	5.16	271

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

Cell Number	No. ChIP reactions	1x ChIP Buffer/PI (mL)	Sheared Chromatin (µL)
3 x 10^6	3	0.83	100
6 x 10^6	6	1.65	200
10 x 10^6	10	2.75	333
15 x 10^6	15	4.12	500
24 x 10^6	24	6.6	799

11.1.3 For each ChIP, aliquot 280 µL of diluted chromatin from Step 11.1.2 into a 1.5 mL tube. Freeze the remaining chromatin which will be used in step 11.3.2 for the INPUT preparation. All volumes indicated from this point of the protocol are for 1x10^6 cells/ 1 ChIP unless otherwise stated.

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

11.1.5 Additional antibodies can be included but the number of cells needs to be increased accordingly. See Section 11.2 for details of Positive control antibodies.

11.1.6 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.

11.1.7 Next day, prepare the antibody binding beads as described below. Make sure beads are resuspended into a uniform suspension before each use.

11.1.7.1 Take your beads washed and resuspended in 1xChIP buffer (Section 11.1.1).

11.1.7.2 Cut off the tip of a 1000 µL pipette-tip to pipet the bead suspension.

11.1.7.3 Determine how many IPs you want to do (see table).

11.1.7.4 Keep the beads in suspension when pipetting aliquots out.

11.1.8 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).

Note: *PI mix is not required anymore from this stage*

Cell Number	No. ChIP reactions	Bead Mix (µL)	1x ChIP Buffer (mL)
1 x 10^6	1	40	5.3
3 x 10^6	3	120	16.0
6 x 10^6	6	240	32.0
10 x 10^6	10	400	53.0
15 x 10^6	15	600	80.0
24 x 10^6	24	960	127.0

11.1.8.1 Transfer 120 µL of protein A bead suspension mix into 1 mL of 1x ChIP buffer. Mix by inversion and pellet beads by centrifugation, 3 min, 500 g, 4°C. Discard the supernatant.

11.1.8.2 Add 1 mL of 1x ChIP buffer and mix by inversion.

11.1.8.3 Aliquot 300 µL of the Bead/ChIP buffer mix into 3 x 1.5 mL tubes.

11.1.8.4 Pellet beads by centrifugation, 2 min, 500 g, 4°C. Carefully discard the supernatant.

11.1.9 Pellet the antibody/chromatin samples obtained from Section11.1.6 by centrifugation to remove insoluble material, 10 min, 14000 g, 4°C.

11.1.10 Remove 250 µL of the supernatant and transfer to the aliquoted beads from step 11.1.7 and 11.1.8.

11.1.11 Rotate for 30-60 min at 4°C.

11.1.12 After incubation, add 1 mL of 1x ChIP buffer.

11.1.13 Mix gently by inversion.

11.1.14 Pellet beads by centrifugation, 3 min, 500 g, 4°C. Carefully discard the supernatant.

11.1.15 Repeat washing step 3 times more with 1x ChIP buffer. Carefully discard the supernatant.

11.2 Details of 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:

11.2.1 **Enriched at actively transcribed genes:**
ab1012/ ab12209/ ab8580 Histone H3 (tri methyl K4)
ab5131 RNA polymerase II CTD repeat YSPTSPS (phosphor S5)

11.2.2 **Enriched at promoters of actively transcribed genes:**
ab51841 TATA binding protein TBP

11.2.3 **Enriched at inactive loci:**
ab8898 Histone H3 (tri methyl K9)
ab6002 Histone H3 (tri methyl K27)

11.3 DNA Purification

11.3.1 Add 100 µL of DNA purifying slurry to the samples (Protein A beads).

11.3.2 Take 50 µL of the frozen chromatin from step 11.1.3 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.

11.3.3 Mix the samples by inversion and incubate for 10 min, 98°C.

11.3.4 After incubation, leave at RT for 20 min to cool.

11.3.5 Centrifuge briefly to remove condensation from the tube lid, 10 sec, 10000g

11.3.6 Add 1 µL of Proteinase K. Vortex for 5 sec at medium power.

11.3.7 Incubate samples for 30 min at 55°C.

11.3.8 Incubate samples for 10 min, 98°C.

11.3.9 Pellet DNA slurry by centrifugation, 1 min, 14000 g, RT.

11.3.10 Transfer 70 µL of the supernatant to a 1.5 mL tube.

11.3.11 Add 130 µL of PCR-grade H_2O to the DNA slurry. Vortex for 10 sec at medium power.

11.3.12 Pellet DNA slurry by centrifugation, 1 min, 14000 g, 4°C.

11.3.13 Transfer 130 µL of supernatant to the supernatant from step 11.3.10 (total: 200 µL).

11.3.14 The samples can now be used for quantitative PCR or stored at -20°C for analysis later.

11.4 Details of Affinity Beads

Species	Ig Isotype	Protein A	Protein G
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	-	++
Rabbit	All Isotypes	+++	++

12. ANALYSIS

Example of Analysis of DNA Fragment for Section 10.3

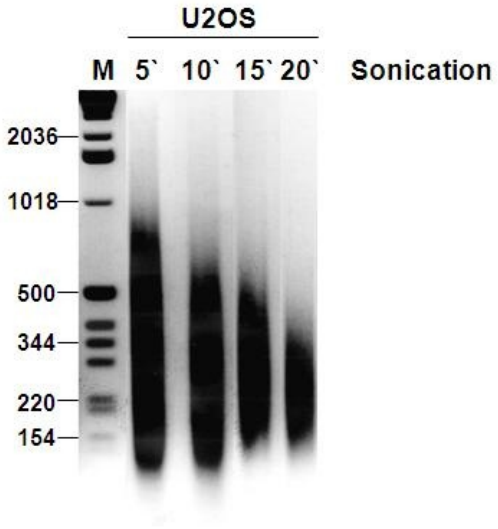


Figure 1: U2OS cells were sonicated for 5, 10, 15 and 20 min. The fragment size decreases during the time course. The optimal fragment size is observed at 15 min. 10µl on a 1.5 % agarose gel.

13. 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
	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

Problem	Cause	Solution
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

14. NOTES

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