ChIP-Adem-Kit

AutoMag Solution

(Cat #04643)

Instruction manual for automation protocol

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General overview

1. Overview

Biomagnetic separation technology is a simple technique based on the separation of **superparamagnetic beads** using a magnetic field. When added to a complex medium, the magnetic particles will bind to their target. This interaction is based on the specific affinity of the ligand to the surface of the beads. The resulting target-bead complex can be removed from the suspension using a magnet. The inherent benefits of magnetic handling allow for easy washing, separation and concentration of the target without any need of centrifugation or columns.

Superparamagnetic beads exhibit magnetic properties only when placed within a magnetic field and show no residual magnetism when removed from this field.

2. Product principle

A central element of genome function is the expression of the individual genome elements and the factors that regulate those events. Transcription factors, histone proteins that interact with DNA are critical players within the regulation of gene expression events.

Chromatin Immunoprecipitation (**ChIP**) assay, is an experimental method used in molecular biology to determine whether proteins including transcription factors or histones bind to a particular region on the endogenous chromatin. In outline, the method consists of the following steps. The protein under study is crosslinked to DNA which is subsequently extracted and sheared into approximately 0.2-1kb fragments. Whole Protein-DNA complexes can be immunoprecipitated using an antibody specific for the targeted protein. The DNA from the isolated Protein / DNA fraction can then be purified. The identity of the DNA fragments isolated in complex with the protein of interest can then be determined by PCR or Real-Time PCR using specific primers for the DNA regions that the protein in question is hypothesized to bind.

ChIP Adem-Kit Protocol

1. Product description

1.1 General description:

The ChIP-Adem-Kit is an innovative system especially designed for monitoring transcription factors or histones / DNA interactions.

MasterIP are uniform sized superparamagnetic particles (500nm) conjugated with protein AG. Protein AG is a genetically-engineered protein that combines the IgG binding domains of both Protein A and G. Recombinant fusion protein AG contains five Ig-binding regions of protein A and three Ig-binding. Protein AG binds to all IgG subclasses from various mammalian species, including all IgGs that bind both Protein A and Protein G, making the ideal choice for purification of all kinds of polyclonal and monoclonal IgG antibodies. The protein AG lacks albumin, cell wall binding regions and others non-specific binding regions to ensure maximum specific IgG binding. MasterIP after incubation with the Blocking Buffer have a specific surface designed for efficient Protein / DNA complex immunoprecipitation and to minimize non-specific enrichment. Proteins and other contaminants are eliminated in the washing steps. The ChIP protocol is greatly simplified by using blocked Protein recombinant A/G-coated nanoparticles. Some steps have been completely eliminated. Unlike traditional agarose beads, chromatin pre-clearing is not required. Master IP combined to especially designed buffers reduce background and maximize the amount of templates available for the reaction, achieving greater sensitivity where maximum DNA recovery is critical. The isolated DNA can be used in downstream applications : PCR and Real-Time PCR.

The ChIP-Adem-Kit contains all the buffers and reagents necessary to perform 24 chromatin immunoprecipitations. The protocol is optimized for use with mammalian cells

1.2 Reagents provided with the kit:

ChIP-Adem-Kit for AutoMag Solution (cat=04643)			
	Amount	**Component**	**Storage**
R1	13 ml	Lysis Buffer I	+ 4°C
R2	25 ml	Glycine Buffer	+ 4°C
R3	13 ml	Lysis Buffer II	+ 4°C
R4	3 ml	Lysis Buffer III	+ 4°C
R5	27ml	IP Buffer	+ 4°C
R6	1.2 ml	Master IP	+ 4°C
R10	60ml	Washing Buffer I	+ 4°C
R11&R12	3,8ml	Elution Buffer	+ 4°C
R13	250µl	Proteinase K (10mg/ml)	+ 4°C
R14	12,5µl	Protease Inhibitor Cocktail	+ 4°C
R15	6ml	**Blocking Buffer**	**+ 20°C**

The blocking Buffer must be stored at room temperature.

Properly stored kits are guaranteed until the expiry date. Note that the shipping is realized at room temperature which will not affect its stability.

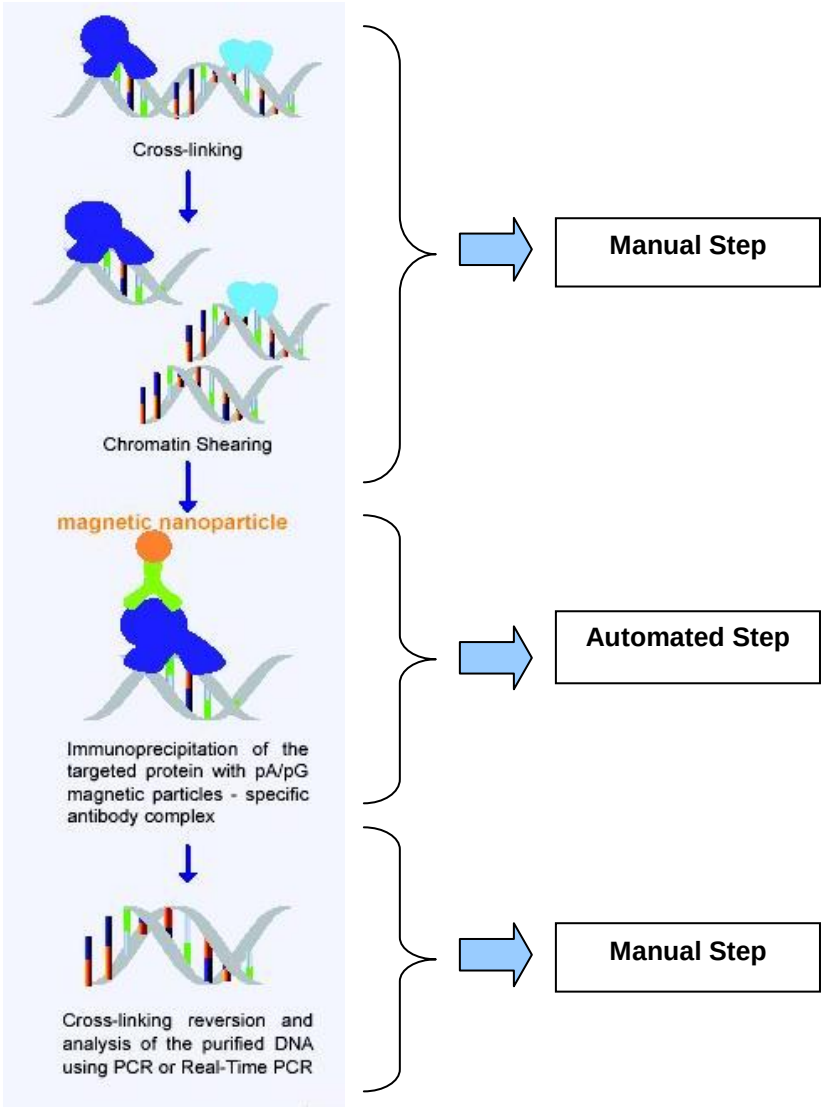
1.3 Required equipment to be supplied by the user:

- Instrument for AutoMag Solution (Cat.# 21101)
- Antibodies of interest for chromatin immunoprecipitation
- 37% Formaldehyde
- Sonicator Thermal shaker or Heat block
- Disposable gloves
- Shaking platform
- Thermomixer (MixHeat Thermo Shaker # 21200)

Instrument for AutoMag Solution (Cat.# 21101)

2. ChIP protocol

2.1 Magnetic Immunoprecipitation Chromatin Procedure:



2.2 General Guidelines:

- The ChIP-Adem-Kit is suitable for studying the protein / DNA interactions and combines the specificity of the immunoprecipitation with qualitative or quantitative PCR.

- The method requires high quality antibodies capable of recognizing fixed protein that is bound to DNA and/or complexed with other proteins.

- Before starting, you must optimize sonication conditions to shear your DNA (200-1000bp), carefully read the paragraph 2.3.2.

- Blocking Buffer must be stored at room temperature. Lysis Buffer III and Elution Buffer contain SDS, warm these buffers to room temperature to ensure SDS is in solution before processing.

- Formaldehyde is defined as toxic and should be used in a ventilated fume hood. Appropriate safety precautions (gloves, safety glasses…) should also be taken.

Formaldehyde		Toxic
Risk Statements		23/24/25-34-39/23/24/25-40-43
Safety Statements		26-36/37/39-45-51

Please read the entire protocol before starting

2.3 Prococol for ChIP:

2.3.1 Cell collection and *In Vivo* crosslinking

 Crosslinking is a critical step that must be optimized.

Advice on crosslinking
- It is important to optimize the fixation step by testing different formaldehyde concentrations (1-3%) and different incubation times (5-30 min). - Efficiencies of chromatin shearing and immunoprecipitation of the targeted protein are linked to the crosslinking process. - Longer crosslinking times and / or higher formaldehyde concentrations may decrease shearing efficiency but could improve immunoprecipitation of proteins that are not directly bound to DNA. - Use high quality formaldehyde. - Histones are tightly linked to DNA, crosslinking step is optional. - To obtain a good reproducibility, we recommend to culture a constant and well known number of cells 24 h before your experiment.

The protocol from point 1 to 5 is done for a single culture plate = one ChIP test.

1. Cells (treated or untreated) are grown to 60%-80% confluence on a 100mm plate (2-4x10^6 cells/plate).
2. Remove as much cell medium as possible. Cross link proteins to DNA by adding 10 ml PBS 1X and 270 µl of 37% formaldehyde to the plate and incubate on a shaking platform (gently shaking) for 10min at room temperature.
3. Stop crosslinking reaction by adding 1 ml ***Glycine Buffer*** to the plate. Incubate on a shaking platform (gently shaking) for 5min at room temperature.
4. Remove medium and wash cells twice using 10 ml ice cold PBS 1X to the plate.
5. Remove medium and add 5 ml ice cold PBS 1X. Scrape cells from the plate and collect them into a 15 ml or 50 ml conical tube.
6. Centrifuge at 800 x g at room temperature for 5 min to pellet cells.
7. Remove supernatant and resuspend cell pellet in 0.5 ml ***Lysis Buffer I***.
   Note : We recommend using 0.5ml of Lysis Buffer I and II for one plate containing 2.5x10^6 cells.
8. Centrifuge at 800 x g at room temperature for 6 min.
9. Remove supernatant and resuspend cell pellet in 0.5 ml ***Lysis Buffer II***.
10. Centrifuge at 800 x g at room temperature for 6min.
11. During centrifugation, prepare a Lysis Buffer III supplemented with Protease Inhibitors Cocktail.
12. Remove supernatant and resuspend cell pellet in 0.1 ml ***Lysis Buffer III containing Protease Inhibitors Cocktail***.

2.3.2 Chromatin Shearing

Sonication is a critical step that must be optimized.

Advice on sonication
- Sonicate chromatin to an average length of about 500 bp is recommended. - In order to monitor the sonication process, extract total DNA from sheared chromatin and analyse it on 1% agarose gel for each sonication cycle. - In order to avoid any delay in gel migration, due to proteins fixed onto DNA, we strongly recommend to reverse sheared chromatin before agarose gel analysis.

- Power, time and number of sonication cycles have to be tuned depending on sonicator, cell type, crosslinking degree and protein of interest.
- To avoid excessive heating that denatures the DNA, the total sonication time is usually divided into cycles of sonication (~5 cycles each of 15s, with a pause point between cycles). Be sure to keep samples on ice to prevent chromatin from degradation.
- The chromatin can be stored at -80°C for months, depending on the targeted protein.

1. If desired, remove 5 µl of cell lysate for agarose analysis of unsheared chromatin.
2. Sonicate cell lysate on wet ice.
3. Centrifuge at 13.000 x g at room temperature for 10 min to remove insoluble material. Collect the supernatant.
4. If desired remove 5 µl aliquot for agarose gel analysis of the sheared chromatin.
5. 100 µl aliquot is recommended for one immunoprecipitation.

Freshly sheared chromatin is recommended for high quality ChIP.

2.3.3 Immunoprecipitation of crosslinked protein / DNA

The follow protocol is done for the *automate-ChIP-Flash* procedure.

Please follow the manual instruction of the instrument to optimize the automated step.

Microtiter Deep well 96 plate
(Cat.# 21102)

12-tip comb for Deep well 96 plate
(Cat.# 21103)

1. **Prepare the plate**

   Add the following reagents to the rows. Note that row B is reserved for the tip comb and should be left empty.

Plate name and type	Row	Row name	Content	Reagent/Sample volume per well
Microtiter Deep well 96 plate	A	Elution Step	Elution Buffer Proteinase K	200µl 10µl
	B	Tip	12-tip comb	Empty
	C	ChIP-Adembeads	ChIP-Adembeads	50µl
	D	Blocking Step	Blocking Buffer	250µl
	E	Binding Step	IP Buffer antibody	100µl xµl
	F	ChIP Step	IP Buffer Chromatin	900µl 100µl
	G	Washing Step I+II	Washing Buffer	500µl
	H	Washing Step III+IV	Washing Buffer	500µl

Note:

- **The amount of antibody added should be in excess and determined for each ChIP. Usually 1 to 3 µg of immunoprecipating antibody is recommended for each ChIP reaction.**
- **For mock IP, use the non-immune IgG fraction from the same species in which the specific antibodies were produced.**

2. **Place a 12-tip comb for 96 Deepwell plate into row B of the plate.**

12-tip comb for 96 Deepwell plate (Cat.# 21103)

3. **Load the plate and start the ChIP automated procedure.**

   Switch ON the Instrument and make sure that you are using the 12-pin magnet head and heating block. Start the ChIP automated protocol. Insert the plate into the instrument as indicated on the Instrument display and press **OK.**

Automated ChIP protocol	Duration	IP DNA	Sample for Blot	Which protocol to start?
ChIP Flash	1h30	1h	No	ChIP Flash to perform chromatin sonication optimization
ChIP Blot	1h30	1h	Yes 200µl sample to perform Western Blotting	ChIP Blot to validate antibody quality
ChIP O/N Step 1	30min	O/N TA or 4°C (outside AutoMAg)	No	For less protein expression overnight immunoprecipitation
ChIP O/N Step 2	30min	No	No	

4. **After the run is completed, remove the plate and store immunoprecipated DNA.**

   When the protocol is completed, remove the plate according to the instructions on the Instrument display and turn off the instrument.

   2.3.4 Western Blotting control

1- If desired, remove 200 µl of sample for blot into a microtube.

2- Place the tube on the magnet (AdemMag MODULO #20105) until supernatant clearing. Discard the supernatant.

3- Resuspend the beads in 25 µl of Elution Buffer

4- Incubate 15 min at 65°C

5- Place the tube on the magnet for at least 1min or until supernatant clearing. Collect the supernatant and use for western blot.

2.3.5 Crosslinking reversion and DNA purification

Incubate overnight the Elution fraction at 65°C under mixing (300 rpm).

Purify DNA for analysis using Phenol Chloroform extraction process or DNA purification columns.

1- Add an equal volume of ***phenol*** to DNA sample contained in 1.5ml microtube and vortex for 15-30 s.
2- Centrifuge the sample at 13.000 x g for 1 min at room temperature.
3- Remove about 95% of the upper aqueous layer to a clean microtube.
4- Add an equal volume of ***chloroform*** and vortex for 15-30 s.
5- Centrifuge the sample at 13.000 x g for 1 min at room temperature.
6- Repeat steps 3-5.
7- Add 500 µl of ***100% ethanol*** and 10 µl of ***NaCl 5M*** to the aqueous layer and invert to mix.
8- Incubate the sample at -20°C for 1 h to overnight.
9- Centrifuge the sample at 13.000 x g for 20 min at 4°C. Remove the supernatant.
10- Wash the pellet with 500 µl of ***75% ethanol***.
11- Centrifuge the sample at 13.000 x g for 10 min at 4°C. Remove the supernatant.
12- Repeat steps 10-12.
13- Air dry.
14- Resuspend dried DNA in 50µl ***DNAse free water***

2.3.6 PCR Controls

Note:

- **Optimize your PCR protocol: determine the optimal number of PCR cycles (no or low PCR product with the mock/ strong PCR product with the specific antibody).**
- **Starting with 30 cycles is recommended.**

PCR negative controls:

- PCR with DNA from sample immunoprecipitated with non-immune IgG fraction from the same species of the antibodies.
- PCR using DNA from ChIP samples, and specific primers for a DNA region where your antigen of interest is not bound.

PCR positive controls: PCR using input DNA

Troubleshooting

Observations	Comments and suggestions
Error message in instrument display	Refer to the user manual supplied with your AutoMag Instrument.
The tip comb holder lifting mechanism is out of position	Switch the instrument OFF and ON, and try again. If the error appears during initialization or is otherwise repeated, contact service.
The turntable rotating mechanism is out of position	Switch the instrument OFF and ON, and try again. If the error appears during initialization or is otherwise repeated, contact service.
The magnet head holder lifting mechanism is out of position	Switch the instrument OFF and ON, and try again. If the error appears during initialization or is otherwise repeated, contact service.
The plastic tip comb is not attached to the holder	Check if the tips are presents. If it looks all right, turn ON and OFF, and run the check protocol.

LOW OR NO PCR PRODUCTS		
STEP	**TROUBLES**	**SUGGESTION**
CROSSLINKING	Not enough or too much crosslinking	Efficient fixation of protein to chromatin is a crucial step. It is important to optimize the fixation step by testing different formaldehyde concentrations, different incubation times. Use high quality formaldehyde.
CELL LYSIS	Insufficient cell number	Increase cell number.
	Insufficient cell lysis	Do not use too many cells per amount of Lysis Buffer. Follow the protocol step 2.3.1.
	Protein degradation during lysis can occur	Perform cell lysis at 4°C or on ice. Keep samples and Buffers on ice. Add Protease Inhibitors to the Lysis Buffer III.

CHROMATIN SHEARING	Not enough or too much chromatin	Optimal shearing conditions are important for ChIP efficiency and must be optimized for each cell type, fixation protocol and sonicator apparatus. Follow advices on sonication to obtain the appropriate sized DNA. To analyze the shearing, reverse crosslink, purify DNA and do a 1% agarose gel.
	Denaturation of proteins during sonication	Keep the sample on ice during sonication.
IMMUNOPRECIPITATION	Antibody doesn't recognize protein in fixed chromatin	Choose ChIP or IP grade antibody directed to a different epitope of the protein.
		Decrease time of formaldehyde fixation or its concentration.
	Incorrect antibody class of isotype	Check if the subclass and the isotype of the antibody match well with pA /pG affinity.
	High background	Perform a preclearing step. -Incubate each chromatin with 25 µl blocked ChIP-Adembeads for 1 h at room temperature. -Place the tube on the magnet for at least 1 min or until supernatant clearing. -Collect the supernatant and use it in the step 9 / chapter 2.3.3.
	Beads agglutination	Beads agglutination may occur after immunoprecipitation step. This phenomenon corresponds to the capture of the antibody by the beads. During washing steps, resuspend thoroughly the beads by pipeting to avoid non specific binding.
REVERSION	Incorrect temperature, insufficient time for DNA release and reversion	Follow the protocol steps 2.3.5. Check if magnetic beads have been eliminated before incubation at 65°C.
PCR	Incorrect PCR conditions	Check if all PCR components are added. Ensure the designed primers are specific to the target sequence. Increase the number of cycles for PCR reaction. Increase or reduce amount of DNA added.

Warranty

This product is only for use in research. The purchaser is responsible to validate the performance of this product for any particular use, and to use the product in compliance with any applicable regulations.
The products are warranted to the original purchaser only to conform to the quality and contents stated on the vial and outer labels for duration of the stated shelf life. Ademtech's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Ademtech's expense, of any products which shall be defective in manufacture, and which shall be returned to Ademtech, transportation prepaid, or at Ademtech's option, refund of the purchase price. Claims for merchandise damaged in transit must be submitted to the carrier.

Ordering Information

- **Ademtech Kits**

CAT NO.	PRODUCT	PACKAGE SIZE
04643	ChIP Adem-Kit for AutoMag Solution	1 x 24

- **Instrument and consumables**

CAT NO.	PRODUCT	PACKAGE SIZE
21101	Instrument for AutoMag Solution	Each
21102	Microtiter Deepwell 96 plate	50 pcs
21103	12-tip comb for 96 Deepwell plate	50 pcs
21105	Combi pack for 96 Deepwell plate	Each