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iDeal ChIP-qPCR kit

MANUAL KIT

Cat. No. C01010180 (24 rxns)

AUTOMATED KIT

Cat. No. C01010181 (24 rxns)

USER GUIDE

Version 1 08_2023

The iDeal ChIP-qPCR Kit has been validated on the IP-Star® Compact Automated System. Two versions of the protocol (manual and automated) are described in this document.

⚠ Please read this manual carefully before starting your experiment

Contents

Introduction

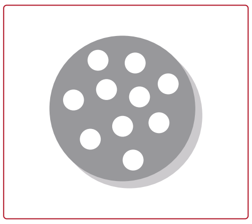
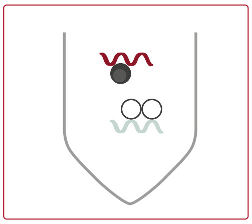
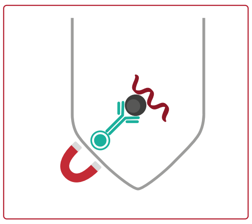
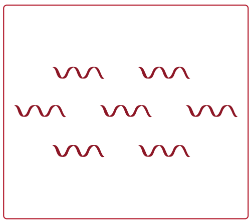
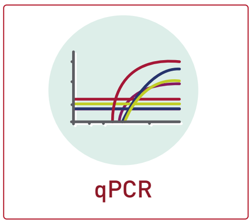
Association between proteins and DNA has a major influence on gene transcription, epigenetic silencing and other nuclear processes which control many vital cellular functions. It is crucial to understand these interactions and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation. Chromatin immunoprecipitation (ChIP) is a technique to analyze the association of proteins with specific genomic regions in intact cells. ChIP can be used to study changes in epigenetic signatures, chromatin remodeling and transcription regulator recruitment to specific genomic sites.

The iDeal ChIP-qPCR Kit provides a robust ChIP protocol suitable for investigation of histones and other proteins such as transcriptional factors associated with chromatin from cells and tissues. The protocol involves protein-DNA cross-linking with formaldehyde, followed by cell lysis and fragmentation of the cross-linked chromatin. The subsequent immunoprecipitation of chromatin is performed with an antibody (user supplied) specific to a target protein. Protein A magnetic beads are used to isolate the protein-DNA complexes of interest, after which the immunoprecipitated DNA is eluted. The eluted DNA is ideal for qPCR analysis.

The iDeal ChIP-qPCR Kit offers unique benefits:

- Fast and highly optimized protocol for ChIP-qPCR from cells and tissues
- Easy ChIP made faster and more reproducible with magnetic beads
- High yields with excellent specificity and sensitivity due to combination of Diagenode ChIP-grade antibodies
- Eluted DNA suitable for qPCR analysis
- Automated and validated on IP-Star Compact Automated System

Kit method overview & time table

			Time needed	Day
STEP 1		Cell or tissue collection and DNA-protein cross-linking	30 minutes	1
STEP 2		Cell lysis and chromatin shearing	1 to 2 hours	1
STEP 3		Magnetic immunoprecipitation	Overnight	1-2
STEP 4		DNA isolation and decross-linking	30 minutes	2
STEP 5		Quantitative PCR and data analysis	2 to 3 hours	2

LEGEND

● Protein of interest
○ Other protein
DNA
Antibody
◉ Magnetic bead
Magnet

Kit materials

The automated and manual iDeal ChIP-qPCR Kit contains enough reagents to perform 4 chromatin preparations, 24 chromatin immunoprecipitations and 32 DNA isolations.

The kit content is described in Table 1. Upon receipt, store the components at the indicated temperatures.

Table 1. Components supplied with the iDeal ChIP-qPCR Kit

Description	Quantity	Storage
Protease inhibitor cocktail	80 µl	-20°C
5% BSA (DNA free)	784 µl	-20°C
Proteinase K	32 µl	-20°C
Rabbit IgG (1µg/µl)	8 µg	-20°C
Glycine	8.8 ml	4°C
Shearing Buffer iS1b	9 ml	4°C
DiaMag protein A-coated magnetic beads	720 µl	4°C, DO NOT FREEZE
Wash Buffer iW1	8.4 ml	4°C
Wash Buffer iW2	8.4 ml	4°C
Wash Buffer iW3	8.4 ml	4°C
Wash Buffer iW4	8.4 ml	4°C
ChIP-seq grade water	26.6 ml	4°C
Fixation Buffer	8 ml	4°C
DNA isolation Buffer (DIB)	3,2 ml	4°C
5x ChIP Buffer iC1b	6.9 ml	4°C
Lysis Buffer iL1b	100 ml	4°C
Lysis Buffer iL2	60 ml	4°C
Elution Buffer iE1	220 µl	4°C
Elution Buffer iE2	20 µl	4°C

Required materials not provided

Materials and Reagents

- Gloves to wear during all steps
- RNase/DNase-free 1.5 ml tubes
- Formaldehyde, 37%, molecular grade
- Phosphate buffered saline (PBS)
- qPCR SYBR® Green Mastermix
- ChIP-grade antibodies – www.diagenode.com
- ChIP-grade primers - www.diagenode.com
- Cell culture scraper (for adherent cells)
- Reagents for DNA purification, required for chromatin shearing assessment (eg. IPure Kit v.2, Diagenode, Cat. No. C03010014 or MicroChIP DiaPure columns, Diagenode Cat. No. C03040001)

Additional supplies for tissue protocol:

- Protease inhibitor cocktail (Diagenode, Cat. No. C12010011 or C12010012) (100 µl per chromatin preparation)
- Scalpel blades
- Petri dishes

Equipment

- Magnetic rack for 1.5 ml tubes
- Bioruptor® sonication device and the associated microtubes:
    - Bioruptor® Plus (Diagenode, Cat. No. B01020001) and 1.5 ml TPX Microtubes (Cat. No. C30010010) or
    - Bioruptor® Pico (Diagenode, Cat. No. B01080010) and 1.5 ml Bioruptor® Microtubes with Caps (Cat. No. C30010016)
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- Rotating wheel
- Vortex
- Thermomixer
- qPCR cycler

Optional supplies

- Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Diagenode, Cat. No. C01020013)
- 1M Sodium butyrate (NaBu) (Diagenode, Cat. No. C12020010)
- ChIP Cross-link Gold (Diagenode, Cat. No. C01019027)
- RNase cocktail (e.g. Ambion, AM2286A), required for chromatin shearing assessment
- Fluorescence-based assay for DNA concentration measurement, e.g. Qubit dsDNA BR Assay Kit (Life Technologies #Q32853)
- Qubit® Fluorometer (ThermoFisher Scientific)

Additional supplies if working with IP-Star Compact

- IP-Star Compact Automated System (Diagenode, Cat. No. B03000002)
- 200 µl tube strips (8 tubes/strip) + cap strips (Diagenode, Cat. No. C30020002)
- Tips (box) (Diagenode, Cat. No. C30040021)
- Tips (bulk) (Diagenode, Cat. No. C30040020)
- 2 ml microtube (Diagenode, Cat. No. C30010014)
- Medium reagent container (Diagenode, Cat. No. C30020003)
- DiaMag02 magnetic rack (Cat. No. B04000001)

Remarks before starting

1. Cell number

The protocol describes the preparation of a batch of chromatin which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. The starting number of cells per batch depends on the target to be studied: histone marks require less starting material than transcriptional factors. Depending on the target we recommend to work with the following numbers of cells:

Target	Cells/batch	Cells/IP	Number of IPs
Transcription factors	25,000,000	~ 4,000,000	6 IPs + 1 INPUT + 1 shearing assessment
Histones	7,000,000	1,000,000	6 IPs + 1 INPUT + 1 shearing assessment

Scale the amount of cells per batch accordingly to the experimental plan.

Please note that the described protocol uses a prompt direct fixation in the cell culture plate. Fixed and scraped cells can not be accurately counted. This means that for adherent cells you need to use an approximate estimation of cell number per plate. Alternatively, an additional parallel plate for counting can be prepared. Suspension cells can be counted before the fixation.

Depending on the abundance of the target, the specificity of the antibody, and the number of cells available, it may be possible to scale up and down the number of cells per IP and/or start with a smaller or a bigger batch of cells.

For using lower numbers of cells per IP, you can start with a recommended batch and follow the protocol up to the chromatin shearing step. Then simply dilute the sheared chromatin in Shearing Buffer iS1b before adding it to the IP reaction. The final volume of diluted chromatin containing the desired amount of cells should be 250 µl (manual kit) or 200 µl (automated kit) per IP reaction.

If starting with an amount of cells different from the standard protocol or if you want to use a higher amount of cells per IP, first determine the number of cells that you will use per IP and the total number of IPs. Fix cells as described in the standard protocol. For cell collection and lysis, scale up or down the volume of iL1b and iL2 buffers using 1 ml of iL1b and 0.6 ml of iL2b per 1 million cells. Define the volume of Shearing Buffer iS1b taking into account that you will need:

	Manual kit	**Automated kit**
Sheared chromatin (containing the desired amount of cells) per IP reaction	250 µl	200 µl
Sheared chromatin per input	2.5 µl	2 µl
Sheared chromatin for chromatin shearing assessment	50 µl	50 µl
Add 5% excess of iS1b		

Resuspend the cells in the required volume of Shearing Buffer iS1b and follow the standard protocol.

Please note that an increased or decreased cell concentration in the Shearing Buffer may impact the shearing efficiency and an additional optimization of the shearing conditions may be required.

If you are studying both histones and transcription factors from the same batch, please, refer to our recommendations for TFs. Then for histone marks simply dilute the sample(s) in Shearing Buffer iS1b to obtain 1,000,000 cells per IP.

The protocol is optimized for use of 250 µl (manual kit) or 200 µl (automated kit) of sheared chromatin in a total volume of ChIP reaction equal to 350 µl (manual kit) or 200 µl (automated kit). It is crucial to keep these volumes constant for optimal results.

2. Tissue amount

The protocol describes the preparation of a batch of chromatin which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. The starting amount of tissue per batch depends on a target to be studied: histone marks require less starting material than transcriptional factors. Depending on the target we recommend to work with the following amounts of cells:

Target	Tissue/batch	Tissue/IP	Number of IPs
Transcription factors	200 mg	~30 mg	6 IPs + 1 INPUT + 1 shearing assessment
Histones	40 mg	~7 mg	6 IPs + 1 INPUT + 1 shearing assessment

Scale the amount of tissue per batch accordingly to the experimental plan.

Depending on the abundance of the target, the specificity of the antibody and the amount of tissue available, it may be possible to scale up or scale down the amount of tissue per IP and/or start with a smaller or a bigger batch of tissue.

For using lower amounts of tissue per IP, start with a recommended amount of tissue and follow the protocol up to the chromatin shearing. Then simply dilute the sheared chromatin in Shearing Buffer iS1b before adding it to the IP reaction. The final volume of diluted chromatin containing a desired amount of tissue should be 250 µl (manual kit) or 200 µl (automated kit) per IP reaction.

If starting with a tissue amount different from the standard protocol or if a starting higher amount of tissue per IP is desired, first determine the amount of tissue that you will use per IP and the total number of IPs.

Fix the tissue as described in the standard protocol. Follow the standard protocol for tissue fixation, collection and lysis. Do not scale Lysis Buffers iL1b and iL2. Define the volume of Shearing Buffer iS1b taking into account that you will need:

	Manual kit	Automated kit
Sheared chromatin (containing the desired amount of tissue) on per IP reaction	250 µl	200 µl
Sheared chromatin per input	2.5 µl	2 µl
Sheared chromatin for chromatin shearing assessment	50 µl	50 µl
Add 5% excess of iS1b		

Resuspend the tissue in the required volume of Shearing Buffer iS1b and follow the standard protocol.

Please note that the increased cell concentration in the Shearing Buffer may impact the shearing efficiency and an additional optimization of the shearing conditions may be required.

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. The amount of chromatin per IP for histone marks should be in the range 0.5-3 µg, while a higher amount (3-10 µg) is required for transcription factors. We recommend performing a pilot experiment to determine the optimal amount of tissue. Once determined, it should be kept constant between experiments.

The protocol is optimized for use of 250 µl (manual kit) or 200 µl (automated kit) of sheared chromatin in a total volume of ChIP reaction equal to 350 µl (manual kit) or 200 µl (automated kit). It is crucial to keep these volumes constant for optimal results.

3. Fixation optimization

Formaldehyde is the most commonly used cross-linking reagent ideal for two molecules which interact directly. The fixation time can depend on your target of interest and might require an additional optimization. Generally, a shorter fixation is required for histone marks (8-10 min) than for transcription factors (10-20 min) using a standard formaldehyde single step fixation protocol. Please note that longer fixation times may lead to chromatin resistant to sonication.

However, for higher order and/or dynamic interactions, other cross-linkers should be considered for efficient protein-protein fixation, e.g. Diagenode ChIP Cross-link Gold, an innovative dual cross-linking ChIP fixation reagent (Diagenode, Cat. No. C01019027).

4. Shearing optimization

Chromatin shearing is one of the most critical steps in the ChIP procedure. Chromatin fragments between 100-600 bp are ideal for ChIP experiments. The optimal sonication time depends on many factors, like cell type, cell density, sample volume, fixation time, etc. Hence it is important to optimize the sonication conditions for each new ChIP project. For optimization of the shearing conditions, we recommend using the Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Cat. No. C01020013) which contains all buffers needed for chromatin preparation compatible with the iDeal ChIP-qPCR Kit. The reagents included in this kit allow to prepare chromatin using optimized shearing settings; the kit does not contain sufficient reagents for optimization of chromatin shearing.

When using the Bioruptor Pico, an initial time-course experiment of 5-10-15 sonication cycles 30'' ON/30'' OFF is recommended. Please refer to the Guide for Successful Chromatin Preparation using the Bioruptor Pico (https://www.diagenode.com/files/protocols/bioruptor-pico-chromatin-preparation-guide.pdf)

When using the Bioruptor Plus, an initial time-course experiment of 10-20-30 sonication cycles 30'' ON/30'' OFF at High Power is recommended. Please refer to The Ultimate Guide for Chromatin Shearing Optimization with Bioruptor Standard and Plus (https://www.diagenode.com/files/protocols/The_Ultimate_Guide_for_Chromatin_Shearing_Optimization_with_Bioruptor_protocol.pdf)

Choose the shortest sonication time resulting in an efficient chromatin shearing. Over-sonication may lead to a drop in ChIP efficiency.

5. Magnetic beads

This kit includes DiaMag Protein A-coated magnetic beads. Make sure the beads do not dry out during the procedure as this will result in reduced performance. Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will decrease reproducibility. Do not freeze the beads.

DiaMag Protein A-coated magnetic beads are suitable for immunoprecipitation of rabbit, guinea pig, dog and pig polyclonal and monoclonal Abs, mouse IgG2a, IgG2b and IgA and human IgG1, IgG2 and IgG4.

6. ChIP-grade antibodies

The quality of antibodies used in ChIP is essential for success. It is recommended to use only validated antibodies, specifically recognizing the target. Diagenode offers extensively validated and high-performance antibodies, confirmed for their specificity in ChIP/ChIP-seq. Each batch is validated, and batch-specific data are available on the website www.diagenode.com

7. Input

The input sample corresponds to complete genomic DNA which went through the full ChIP procedure without immunoselection. The input sample is used as a reference to calculate the recovery at the end of the ChIP procedure. We recommend including one input for each ChIP experiment.

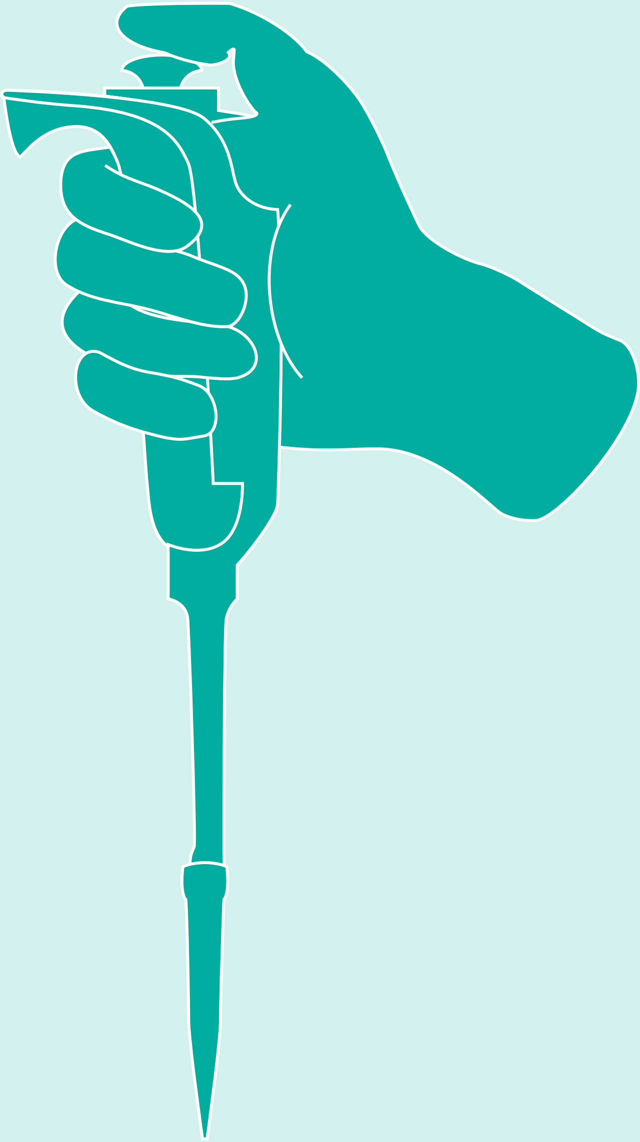
8. Negative control

The kit contains a negative IgG antibody. We recommend including one negative IgG control in each series of ChIP reactions.

9. Quantitative PCR analysis

We recommend analysing the input and immunoprecipitated samples by SYBR® Green qPCR using at least 1 positive and 1 negative control region to determine the enrichment. Each specific antibody will require specific control primers designed by the user. For each primer pair, run the input DNA alongside the immunoprecipitated samples. PCR reactions should be performed at least in duplicate although performing in triplicate is recommended to be able to identify potential outliers.

PROTOCOL



CELLS

TISSUES

CELLS & TISSUES

MANUAL PROCESSING

Protocol

The iDeal ChIP-qPCR Kit is suitable for chromatin preparation and immunoprecipitation from cells and tissues for histones and transcriptional factors. The protocol for Step 1 “DNA-protein cross-linking” and Step 2 “Cell lysis and chromatin shearing” differs for cells and tissues. Please refer to the corresponding section. Starting from Step 3 “Magnetic immunoprecipitation”, the protocol is identical for both sample types.

The protocol describes the preparation of a batch of chromatin which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. The exact amount of starting material depends on the target protein. In the below tables we recommend the amount of cells or tissue per batch and per IP for histones and non-histone proteins. Scale the amount of cells/tissue per batch accordingly to the experimental plan.

Table 2. Recommendations for cells

Target	Cells/batch	Cells/IP	Number of IPs
Transcription factors	25,000,000	~ 4.000,000	6 IPs + 1 INPUT + 1 shearing assessment
Histones	7,000,000	1,000,000	6 IPs + 1 INPUT + 1 shearing assessment

Table 3. Recommendations for tissues

Target	Tissue/batch	Tissue/IP	Number of IPs
Transcription factors	200 mg	~30 mg	6 IPs + 1 INPUT + 1 shearing assessment
Histones	40 mg	~7 mg	6 IPs + 1 INPUT + 1 shearing assessment

STEP 1

Cell collection and DNA-protein cross-linking from cultured cells

1 *Day 1* ⌛ *30 minutes*

FOR CULTURED CELLS

1.1 Equilibrate the Fixation Buffer to room temperature before use.

1.2 Prepare the cross-linking solution in a fume hood by adding **formaldehyde** to the **Fixation Buffer** to a final concentration of **11%** (e.g. add 0.6 ml of 37% formaldehyde to 1.4 ml of Fixation Buffer). Add the **diluted formaldehyde** directly to the cell culture medium in a proportion of 1:10. For a 20 ml cell culture you will need 2 ml of cross-linking solution.

1.3 Incubate the cells for 10 minutes at room temperature with gentle shaking.

***NOTE**: The fixation time might require an additional optimization. Please refer to the "Remarks before starting".*

1.4 Add **Glycine** to the cell culture medium in a proportion of 1:10 to stop the fixation (e.g. add 2.2 ml of glycine when you started with a 20 ml cell culture volume). Incubate for 5 minutes at room temperature with gentle shaking. Proceed to the next step immediately.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 4 months. To freeze the cells, remove the medium, wash the cells once with 20 ml of PBS. Add another 5 ml of PBS, collect the cells by scrapping and/or centrifugation at 500 x g for 5 minutes and 4°C and discard the supernatant. Store the cell pellet at -80°C.*

STEP 2

Cell lysis and chromatin shearing from cells

Day 1 *1 to 2 hours*

FOR CULTURED CELLS

For adherent cells:

2.1 Remove the medium and wash the cells once with **20 ml of PBS**. Discard the PBS. Keep everything at 4°C or on ice from now on.

2.2 Add **5 ml of cold Lysis buffer iL1b** to the plate. Collect the cells by scraping and transfer them into a 50 ml tube.

2.3 Rinse the flask with the amount of **Lysis Buffer iL1b** recommended in the below table and add this to the 50 ml tube.

	Buffer iL1b
TF (25,000,000 cells)	**20 ml**
Histones (7,000,000 cells)	**2 ml**

(For up or down scaling use 1 ml of buffer iL1b per million of cells)

Proceed immediately with step 2.4.

For suspension cells:

2.1 Transfer cells into a 50 ml tube. Pellet the cells by centrifugation at 500 x g and 4°C for 5 minutes. Discard the cell culture medium.

2.2 Resuspend the cells in **20 ml of ice-cold PBS**, centrifuge at 500 x g and 4°C for 5 minutes and gently discard the supernatant. Keep everything at 4°C or on ice from now on.

2.3 Resuspend the cells in **1 ml of ice-cold Lysis Buffer iL1b** by pipetting up and down several times. Add the amount of buffer iL1b from the table below:

	Buffer iL1b
TF (25,000,000 cells)	**24 ml**
Histones (7,000,000 cells)	**6 ml**

(For up or down scaling use 1 ml of iL1b per 1 million cells)

Proceed immediately with step 2.4.

2.4 Incubate at 4°C for 20 minutes with gentle mixing on a rotating wheel. Pellet the cells by centrifugation at 500 x g and 4°C for 5 minutes and discard the supernatant.

2.5 Resuspend the cell pellet in **1 ml of ice-cold Lysis Buffer iL2** by pipetting up and down several times. Add the amount of buffer iL2 from the below table:

	Buffer iL2
TF (25,000,000 cells)	**14 ml**
Histones (7,000,000 cells)	**3.2 ml**

(For scaling up or down, use 600 µl of buffer iL2 per 1 million of cells)

2.6 Incubate for 10 minutes at 4°C with gentle mixing on a rotating wheel. Pellet the cells again by centrifugation for 5 minutes at 500 x g and 4°C and discard the supernatant.

2.7 Add **8.4 µl of 200x protease inhibitor cocktail** to **1.67 ml of Shearing Buffer iS1b**. Keep on ice.

2.8 Add the **complete Shearing Buffer iS1b** to the cell pellet. The cell concentration in the Shearing Buffer should be as following:

TF	**1.5 million cells** per 100 µl iS1b
Histones	**420,000 cells** per 100 µl iS1b

2.9 Resuspend the cells by pipetting up and down several times and incubate on ice for 10 minutes. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes:

- When using the Bioruptor Plus use 1.5 ml TPX Microtubes (Cat. No. C30010010)
- When using the Bioruptor Pico use 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016)

NOTE*: The maximum volume for shearing with the Bioruptor is 300 µl per1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.*

2.10 Shear the chromatin by sonication using the Bioruptor. Choose the protocol which is adapted to your device:

- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.
- When using the Bioruptor Pico shear for 5 to 12 cycles [30 seconds "ON", 30 seconds "OFF"].

NOTE*: We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Cat. No. C01020013).*

2.11 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 x g and 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin.

2.12 Take an aliquot of 50 µl of the sheared chromatin for shearing assessment as described in a separate section "Protocol for chromatin shearing analysis".

NOTE*: We recommend analysing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been established previously.*

2.13 Use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

STEP 1

Tissue disaggregation and DNA-protein cross-linking

1 *Day 1* *30 minutes*

FOR TISSUES

1.1 Equilibrate the Fixation Buffer to room temperature before use.

1.2 Prepare the cross-linking solution in a fume hood by adding **54 µl** of **37% formaldehyde** to **2 ml of Fixation Buffer** to a final concentration of 1%. Use 2 ml of Fixation Buffer for one chromatin preparation.

1.3 Put the required amount of fresh or frozen tissue (up to 200 mg) in a petri dish on ice. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

1.4 Chop the tissue into small pieces (between 1-3 mm^3) using a scalpel blade and transfer it into a Dounce homogenizer. Add **1 ml of formaldehyde diluted in Fixation Buffer**.

1.5 Immediately disaggregate the tissue using a Dounce homogenizer (loose fitting pestle) to get a homogeneous suspension. Set the timer for a total fixation time of 10 minutes. Start deducting the fixation time from this point.

__NOTE__: The fixation time might require an additional optimization. Please refer to the "Remarks before starting".

1.6 Transfer the tissue suspension into a 15 ml tube. Rinse the Dounce homogenizer with an additional **1 ml of diluted formaldehyde** and pool with the sample in the same 15 ml tube.

1.7 Incubate for a total fixation time of 10 minutes (starting at step 1.5) at room temperature with gentle rotation on a rotating wheel.

1.8 Add **200 µl** of **Glycine** to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing on a rotating wheel. Proceed to the next step immediately.

***NOTE**: We strongly recommend using freshly fixed tissue for preparation of sheared chromatin prior to ChIP. If not possible, the fixed tissue can be stored at -80°C for up to 4 months. To freeze the tissue, remove the medium and wash the tissue once with 2 ml of PBS. Store the tissue pellet at -80°C.*

STEP 2

Cell lysis and chromatin shearing derived from tissue samples

Day 1 *1 to 2 hours*

FOR TISSUES

***NOTE**: An additional 100 µl of protease inhibitors cocktail is required per chromatin preparation – available separately from Diagenode*

2.1 Centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet.

2.2 Wash the pellet with **10 ml of ice-cold PBS** and centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant. Keep everything at 4°C or on ice from now on.

2.3 Add **50 µl of 200x protease inhibitor cocktail** to **10 ml of ice-cold Lysis Buffer iL1b**.

2.4 Add **1 ml of ice-cold complete Lysis Buffer iL1b** to the pellet and resuspend by pipetting up and down several times. Add **the remaining amount of complete Buffer iL1b.**

2.5 Incubate at 4°C for 20 minutes with gentle mixing on a rotating wheel.

2.6 Pellet the cells by centrifugation at 850 x g for 5 minutes at 4°C and discard the supernatant.

2.7 Add **50 µl of 200x protease inhibitor cocktail** to **10 ml of ice-cold Lysis Buffer iL2**. Add **1 ml of ice-cold complete Lysis Buffer iL2** to the cell pellet and resuspend the cells by pipetting up and down several times. Add **the remaining amount of complete Buffer iL2**.

2.8 Incubate at 4°C with gentle mixing on a rotating wheel for 10 minutes.

2.9 Pellet the cells again by centrifugation at 850 x g for 5 minutes at 4°C and discard supernatant.

2.10 Add **8.4 µl of 200x protease inhibitor cocktail** to **1.67 ml of Shearing Buffer iS1b**. Keep on ice.

2.11 Add **the complete Shearing Buffer iS1b** to the pellet. Resuspend the cells by pipetting up and down several times and homogenize using a bounce homogenizer (tight pestle). Incubate for 10 minutes on ice.

2.12 Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate 1.5 ml microtubes:

a. When using the Bioruptor Plus use 1.5 ml TPX Microtubes (Cat. No. C30010010)

b. When using the Bioruptor Pico use 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016)

NOTE: *The maximum volume for shearing with the Bioruptor is 300 µl per 1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.*

2.13 Shear the chromatin by sonication using the Bioruptor. Choose the protocol which is adapted to your device:

- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.
- When using the Bioruptor Pico shear for 5 to 12 cycles [30 seconds "ON", 30 seconds "OFF"].

NOTE: *We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Cat. No. C01020013).*

2.14 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 x g at 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin.

2.15 (Optional) DNA concentration in the supernatant can be quantified at this step. We recommend using fluorometric quantitation (eg. Qubit assay, ThermoFischer Scientific).

2.16 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol for chromatin shearing analysis is described in a separate section "Protocol for chromatin shearing analysis".

NOTE*: We recommend analysing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimised previously.*

2.17 Use the chromatin in immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

STEP 3

Magnetic immunoprecipitation

1-2 *Day 1-2* *2-4 hours, overnight, 30 minutes*

FOR CELLS AND TISSUES

3.1 Determine the amount of IP reactions to perform including the negative control IP (IgG). Take **30 µl** of DiaMag Protein A-coated magnetic beads per IP and transfer it to a clean 1.5 ml tube.

3.2 Prepare **4 ml of 1x ChIP Buffer iC1b** by mixing the following reagents:

- 3.2 ml ChIP-seq grade water
- 0.8 ml 5x ChIP Buffer iC1b
- 80 µl of 5% BSA

Keep the diluted ChIP Buffer iC1b on ice.

NOTE*: 4 ml of 1x ChIP Buffer iC1b is enough to wash the beads for up to 6 IPs*

3.3 Wash the beads 3 times with **1 ml of ice-cold 1x ChIP Buffer iC1b**. To wash the beads, add 1 ml of 1x ChIP Buffer iC1b directly to the bead suspension, resuspend the beads by pipetting up and down several times and incubate at 4°C with gentle shaking for 5 minutes. Spin the tubes and place them in the magnetic rack. Wait for one minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 2 times.

3.4 After the last wash, resuspend the beads in **1x ChIP Buffer iC1b** by adding the original volume of beads (30 µl per IP).

3.5 Take the required number of 1.5 ml tubes (one tube per IP) and add **30 µl of the washed beads** to each tube.

3.6 Prepare the **ChIP reaction mix** for 1 IP as described below. Scale according to the number of IPs including a small excess (0.5 extra reaction).

NOTE: *Prepare separate reaction mixes if you use different antibodies or different amounts of antibody*

- 6 µl of BSA
- 1.8 µl 200x protease inhibitor cocktail
- 20 µl 5x iC1b Buffer
- (42.2 µl –x µl) water
- Add x µl of ChIP-grade antibody

The total volume of the ChIP reaction mix per IP is 70 µl.

NOTE: *The required amount of antibody per IP may vary. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. Use 1 µg of IgG (negative control antibody) for the negative control IP.*

If required, NaBu (20 mM final concentration) or other inhibitors can be added.

3.7 Add **70 µl of ChIP reaction mix** to the individual tubes containing **30 µl** of washed **Protein A-coated magnetic beads**. Incubate the tubes for 2-4 hours at 4°C under constant rotation on the rotating wheel.

3.8 Briefly spin the tubes containing the ChIP reaction mix and add **250 µl of sheared chromatin**. Keep aside **2.5 µl of the sheared chromatin** at 4°C to be used as an **INPUT** starting from step 4.3. Incubate the tubes overnight at 4°C under constant rotation on the rotating wheel.

3.9 Perform the washes as follows: briefly spin the tubes and place them in the magnetic rack. Wait for 1 minute and remove the supernatant. Add **350 µl of Wash Buffer iW1**: gently shake the tubes to resuspend the beads and incubate for 5 minutes on the rotating wheel at 4°C.

3.10 Repeat the washing step as described above once with **Wash Buffer iW2, iW3 and iW4**, respectively.

STEP 4

DNA isolation and decross-linking

2 *Day 2* *30 minutes*

FOR CELLS AND TISSUES

4.1 Prepare **complete DIB Buffer** by adding **1 µl of Proteinase K** to 1**00 µl** of **DIB Buffer**. Scale according to the number of samples including the input sample (you will need 100 µl per IP sample and 97.5 µl for the input).

4.2 Briefly spin the tubes containing the IP samples and place them in the magnetic rack. Wait for 1 minute and remove the supernatant.

4.3 Briefly centrifuge the input sample and treat it in parallel with the IP samples from this point onwards.

4.4 Remove the tubes from the Magnetic Rack and add **100 µl of complete DIB** to the IP samples. Resuspend the beads by pipetting up and down a few times. Add **97.5 µl complete DIB** to the input sample.

4.5 Incubate at 55°C for 15 minutes followed by 15 minutes at 100°C.

4.6 Briefly spin down the tubes to bring down the liquid caught in the lid.

4.7 Place the tubes in the magnetic rack and wait for 1 minute.

4.8 Transfer the supernatant which contains the DNA to new labeled tubes. Determine the total number of regions to be analyzed by qPCR for each sample and take the required volume for qPCR analysis.

NOTE*: Depending on a sensitivity of a Master Mix and qPCR cycler used the samples can be diluted before PCR and the volume per PCR may vary*

4.9 Store the remaining DNA at -20°C until further use.

STEP 5

Quantitative PCR analysis

2 *Day 2* *2 to 3 hours*

FOR CELLS AND TISSUES

NOTE: *For each primer pair, run the INPUT DNA alongside the immuno-precipitated samples and negative IgG control.*

5.1 Take an aliquot of immunoprecipitated DNA and a corresponding INPUT (step 4.8).

5.2 Prepare the **qPCR mix** as follows (20 µl reaction volume using the user supplied primer pairs:

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of PCR-grade water
- 5 µl of IP'd or INPUT DNA

5.3 Use the following PCR program:

NOTE: *These conditions may require optimization depending on the type of Master Mix, qPCR system used and user provided primer pair.*

Step	Time/cycles		Temperature
1. Denaturation	3-10 min*		95°C
2. Amplification	30 seconds	40 cycles	95°C
	30 seconds		60°C
	30 seconds		72°C (acquire fluorescence data)
3. Melting curve**	Follow qPCR instrument manufacturer recommendations		

**Please check carefully supplier's recommendations about Taq polymerase* *activation time*

***Include and inspect the melting curves based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single specific product*

5.4 Record the threshold cycles (Ct values) from the exponential phase of the qPCR for the IP'd DNA sample and input for each primer pair.

5.5 Calculate the relative amount of immunoprecipitated DNA compared to INPUT DNA for the control regions (% of recovery) using the following formula:

$$\% \text{ recovery} = 2\hat{}((Ct_{input} - 6.64) - Ct_{sample}) * 100\%$$

- Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and INPUT, respectively.
- 2 is the amplification efficiency
- 6.64 is a compensatory factor to correct the input dilution

***NOTE**: This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency with given primer pair has to be close to 100% meaning that for each cycle the amount of product is doubles (E=2). The real amplification efficiency, if known, should be used. The formula takes into account that 1% of INPUT was used as suggested in the protocol (2.5 µl INPUT vs 250 µl of chromatin per IP). If the amount of INPUT used is different from 1%, an introduction of a compensatory factor in the formula is required to correct the input dilution (x) as follows:*

$$\%\text{recovery} = 2\hat{}\ [(Ct_{input} - \log2\ (X) - Ct_{sample})] \times 100\%$$

Where: log2 (X) accounts for the INPUT dilution

***Example**: If you use an INPUT of 5 µl from 250 µl of chromatin used per IP, it corresponds to 50 X dilution. The compensatory factor is equal to log2(50)=5.64 and the formula to calculate the recovery will be as follows:*

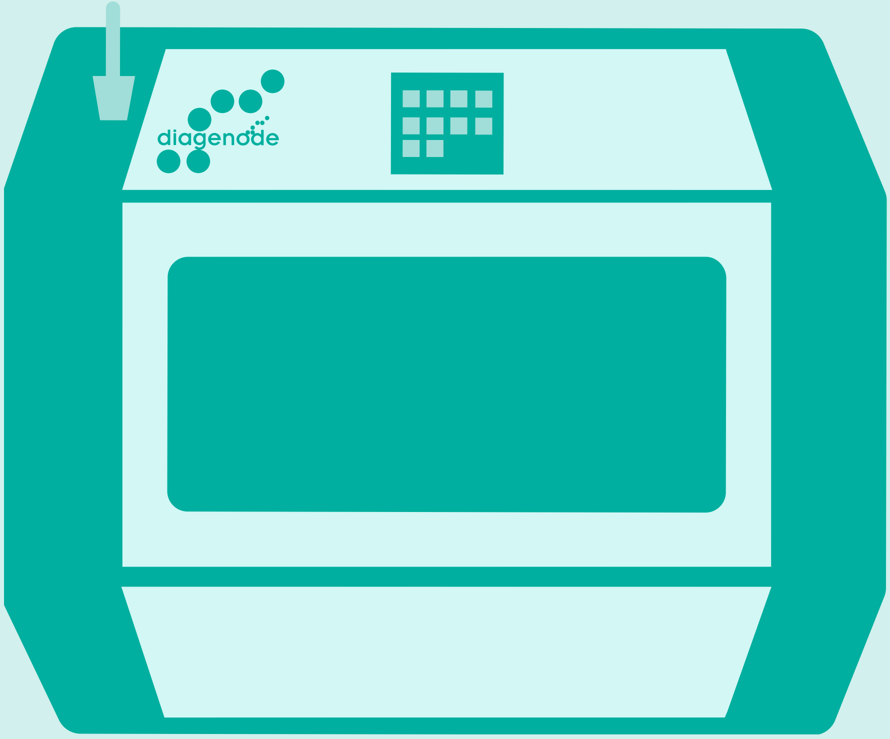
$$\%\text{recovery} = 2\hat{}\ [(Ct_{\ input} - 5.64\ Ct_{\ sample})] \times 100\%.$$

ASK THE EXPERTS

Please do not hesitate to contact our customer support team if you have any questions about the design of your ChIP experiment.

customer.support@diagenode.com

AUTO PROTOCOL



CELLS

TISSUES

CELLS & TISSUES

AUTOMATED PROCESSING

Protocol for ChIP using the IP-Star Compact Automated System

The iDeal ChIP-qPCR Kit has been optimized on the IP-Star Compact Automated System (Diagenode, Cat. No. B03000002) for higher reproducibility. The below protocol is suitable for chromatin preparation (STEPS 1 & 2), immunoprecipitation (STEP 3), and the DNA isolation (STEP 4) from cells and tissues for histones and transcriptional factors. Please refer to the corresponding section. Starting from Step 3 "Magnetic immunoprecipitation", the protocol is identical for both sample types.

The protocol describes the preparation of a batch of chromatin which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. The exact amount of starting material depends on the target protein. In the below tables we recommend the amount of cells or tissue per batch and per IP for histones and non-histone proteins. Scale the amount of cells/tissue per batch accordingly to the experimental plan.

Table 2. Recommendations for cells

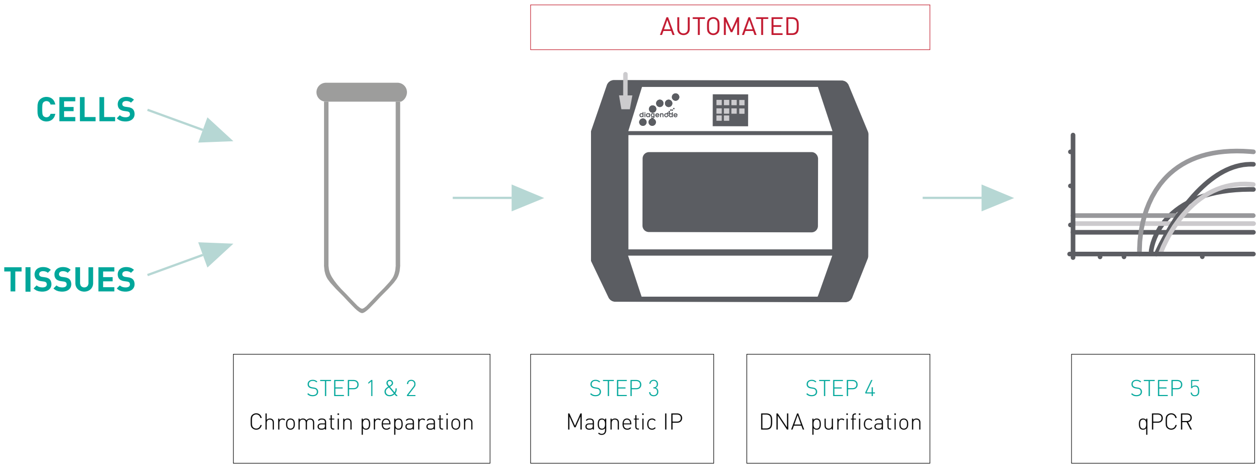
Target	Cells/batch	Cells/IP	Number of IPs
Transcription factors	25,000,000	~ 4.000,000	6 IPs + 1 INPUT + 1 shearing assessment
Histones	7,000,000	1,000,000	6 IPs + 1 INPUT + 1 shearing assessment

Table 3. Recommendations for tissues

Target	Tissue/batch	Tissue/IP	Number of IPs
Transcription factors	200 mg	~30 mg	6 IPs + 1 INPUT + 1 shearing assessment
Histones	40 mg	~7 mg	6 IPs + 1 INPUT + 1 shearing assessment

The immunoprecipitation and the DNA isolation steps are simple push-button protocols that provide flexibility to run 1 to 16 samples with reduced hands on time.

iDeal ChIP-qPCR workflow using IP-Star Compact Automated System



STEP 1

Cell collection and DNA-protein cross-linking from cultured cells

1 *Day 1* *30 minutes*

FOR CULTURED CELLS

1.1 Equilibrate the Fixation Buffer to room temperature before use.

1.2 Prepare the cross-linking solution in a fume hood by adding **formaldehyde** to the **Fixation Buffer** to a final concentration of **11%** (e.g. add 0.6 ml of 37% formaldehyde to 1.4 ml of Fixation Buffer). Add the **diluted formaldehyde** directly to the cell culture medium in a proportion of 1:10. For a 20 ml cell culture you will need 2 ml of cross-linking solution.

1.3 Incubate the cells for 10 minutes at room temperature with gentle shaking.

NOTE*: The fixation time might require an additional optimization. Please refer to the "Remarks before starting".*

1.4 Add **Glycine** to the cell culture medium in a proportion of 1:10 to stop the fixation (e.g. add 2.2 ml of glycine when you started with a 20 ml cell culture volume). Incubate for 5 minutes at room temperature with gentle shaking. Proceed to the next step immediately.

NOTE*: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 4 months. To freeze the cells, remove the medium, wash the cells once with 20 ml of PBS. Add another 5 ml of PBS, collect the cells by scrapping and/or centrifugation at 500 x g for 5 minutes and 4°C and discard the supernatant. Store the cell pellet at -80°C.*

STEP 2

Cell lysis and chromatin shearing from cells

1 *Day 1* *1 to 2 hours*

FOR CULTURED CELLS

For adherent cells:

2.1 Remove the medium and wash the cells once with **20 ml of PBS**. Discard the PBS. Keep everything at 4°C or on ice from now on.

2.2 Add **5 ml of cold Lysis buffer iL1b** to the plate. Collect the cells by scraping and transfer them into a 50 ml tube.

2.3 Rinse the flask with the amount of **Lysis Buffer iL1b** recommended in the below table and add this to the 50 ml tube.

	Buffer iL1b
TF (25,000,000 cells)	**20 ml**
Histones (7,000,000 cells)	**2 ml**

(For up or down scaling use 1 ml of buffer iL1b per million of cells)

Proceed immediately with step 2.4.

For suspension cells:

2.1 Transfer cells into a 50 ml tube. Pellet the cells by centrifugation at 500 x g and 4°C for 5 minutes. Discard the cell culture medium.

2.2 Resuspend the cells in **20 ml of ice-cold PBS**, centrifuge at 500 x g and 4°C for 5 minutes and gently discard the supernatant. Keep everything at 4°C or on ice from now on.

2.3 Resuspend the cells in **1 ml of ice-cold Lysis Buffer iL1b** by pipetting up and down several times. Add the amount of buffer iL1b from the table below:

	Buffer iL1b
TF (25,000,000 cells)	**24 ml**
Histones (7,000,000 cells)	**6 ml**

(For up or down scaling use 1 ml of iL1b per 1 million cells)

Proceed immediately with step 2.4.

2.4 Incubate at 4°C for 20 minutes with gentle mixing on a rotating wheel. Pellet the cells by centrifugation at 500 x g and 4°C for 5 minutes and discard the supernatant.

2.5 Resuspend the cell pellet in **1 ml of ice-cold Lysis Buffer iL2** by pipetting up and down several times. Add the amount of buffer iL2 from the below table:

	Buffer iL2
TF (25,000,000 cells)	**14 ml**
Histones (7,000,000 cells)	**3.2 ml**

(For scaling up or down, use 600 µl of buffer iL2 per 1 million of cells)

2.6 Incubate for 10 minutes at 4°C with gentle mixing on a rotating wheel. Pellet the cells again by centrifugation for 5 minutes at 500 x g and 4°C and discard the supernatant.

2.7 Add **8.4 µl of 200x protease inhibitor cocktail** to **1.67 ml of Shearing Buffer iS1b**. Keep on ice.

2.8 Add the **complete Shearing Buffer iS1b** to the cell pellet. The cell concentration in the Shearing Buffer should be as following:

TF	**2 million cells** per 100 µl iS1b
Histones	**500,000 cells** per 100 µl iS1b

2.9 Resuspend the cells by pipetting up and down several times and incubate on ice for 10 minutes. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes:

- When using the Bioruptor Plus use 1.5 ml TPX Microtubes (Cat. No. C30010010)
- When using the Bioruptor Pico use 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016)

NOTE: *The maximum volume for shearing with the Bioruptor is 300 µl per1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.*

2.10 Shear the chromatin by sonication using the Bioruptor. Choose the protocol which is adapted to your device:

- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.
- When using the Bioruptor Pico shear for 5 to 12 cycles [30 seconds "ON", 30 seconds "OFF"].

NOTE: *We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Cat. No. C01020013).*

2.11 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 x g and 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin.

2.12 Take an aliquot of 50 µl of the sheared chromatin for shearing assessment as described in a separate section "Protocol for chromatin shearing analysis".

NOTE: *We recommend analysing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been established previously.*

2.13 Use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

STEP 1

Tissue disaggregation and DNA-protein cross-linking

Day 1 *30 minutes*

FOR TISSUES

1.1 Equilibrate the Fixation Buffer to room temperature before use.

1.2 Prepare the cross-linking solution in a fume hood by adding **54 µl** of **37% formaldehyde** to **2 ml of Fixation Buffer** to a final concentration of 1%. Use 2 ml of Fixation Buffer for one chromatin preparation.

1.3 Put the required amount of fresh or frozen tissue (up to 200 mg) in a petri dish on ice. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

1.4 Chop the tissue into small pieces (between 1-3 mm3) using a scalpel blade and transfer it into a Dounce homogenizer. Add **1 ml of formaldehyde diluted in Fixation Buffer**.

1.5 Immediately disaggregate the tissue using a Dounce homogenizer (loose fitting pestle) to get a homogeneous suspension. Set the timer for a total fixation time of 10 minutes. Start deducting the fixation time from this point.

NOTE*: The fixation time might require an additional optimization. Please refer to the "Remarks before starting".*

1.6 Transfer the tissue suspension into a 15 ml tube. Rinse the Dounce homogenizer with an additional **1 ml of diluted formaldehyde** and pool with the sample in the same 15 ml tube.

1.7 Incubate for a total fixation time of 10 minutes (starting at step 1.5) at room temperature with gentle rotation on a rotating wheel.

1.8 Add **200 µl** of **Glycine** to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing on a rotating wheel. Proceed to the next step immediately.

NOTE*: We strongly recommend using freshly fixed tissue for preparation of sheared chromatin prior to ChIP. If not possible, the fixed tissue can be stored at -80°C for up to 4 months. To freeze the tissue, remove the medium and wash the tissue once with 2 ml of PBS. Store the tissue pellet at -80°C.*

STEP 2

Cell lysis and chromatin shearing derived from tissue samples

Day 1 *1 to 2 hours*

FOR TISSUES

***NOTE**: An additional 100 µl of protease inhibitors cocktail is required per chromatin preparation – available separately from Diagenode.*

2.1 Centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet.

2.2 Wash the pellet with **10 ml of ice-cold PBS** and centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant. Keep everything at 4°C or on ice from now on.

2.3 Add **50 µl of 200x protease inhibitor cocktail** to **10 ml of ice-cold Lysis Buffer iL1b**.

2.4 Add **1 ml of ice-cold complete Lysis Buffer iL1b** to the pellet and resuspend by pipetting up and down several times. Add **the remaining amount of complete Buffer iL1b.**

2.5 Incubate at 4°C for 20 minutes with gentle mixing on a rotating wheel.

2.6 Pellet the cells by centrifugation at 850 x g for 5 minutes at 4°C and discard the supernatant.

2.7 Add **50 µl of 200x protease inhibitor cocktail** to **10 ml of ice-cold Lysis Buffer iL2**. Add **1 ml of ice-cold complete Lysis Buffer iL2** to the cell pellet and resuspend the cells by pipetting up and down several times. Add **the remaining amount of complete Buffer iL2**.

2.8 Incubate at 4°C with gentle mixing on a rotating wheel for 10 minutes.

2.9 Pellet the cells again by centrifugation at 850 x g for 5 minutes at 4°C and discard supernatant.

2.10 Add **8.4 µl of 200x protease inhibitor cocktail** to **1.67 ml of Shearing Buffer iS1b**. Keep on ice.

2.11 Add **the complete Shearing Buffer iS1b** to the pellet. Resuspend the cells by pipetting up and down several times and homogenize using a bounce homogenizer (tight pestle). Incubate for 10 minutes on ice.

2.12 Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate 1.5 ml microtubes:

c. When using the Bioruptor Plus use 1.5 ml TPX Microtubes (Cat. No. C30010010)

d. When using the Bioruptor Pico use 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016)

NOTE: *The maximum volume for shearing with the Bioruptor is 300 µl per 1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.*

2.13 Shear the chromatin by sonication using the Bioruptor. Choose the protocol which is adapted to your device:

- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds “ON”, 30 seconds “OFF”] each at High power setting.
- When using the Bioruptor Pico shear for 5 to 12 cycles [30 seconds “ON”, 30 seconds “OFF”].

NOTE: *We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization Kit - Low SDS (iDeal Kit for TFs) (Cat. No. C01020013).*

2.14 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 x g at 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin.

2.15 (Optional) DNA concentration in the supernatant can be quantified at this step. We recommend using fluorometric quantitation (eg. Qubit assay, ThermoFischer Scientific).

2.16 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol for chromatin shearing analysis is described in a separate section "Protocol for chromatin shearing analysis".

***NOTE**: We recommend analysing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimised previously.*

2.17 Use the chromatin in immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

STEP 3

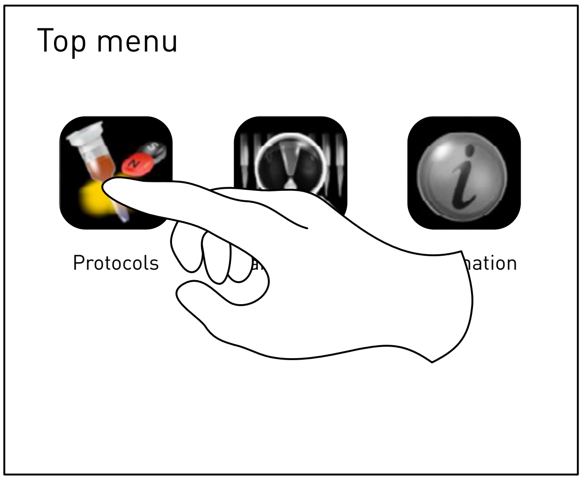
Magnetic immunoprecipitation

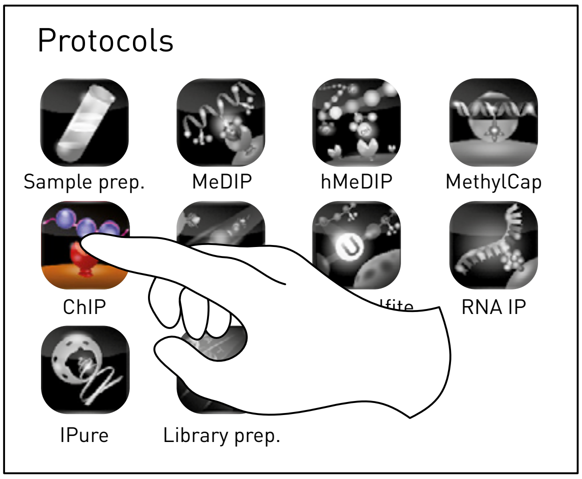
1-2 *Day 1-2* *1.5 hours hands on time and overnight run*

FOR CELLS AND TISSUES

3.1 Switch ON the IP-Star Compact.

3.2 Select "**Protocols**" icon and then "**ChIP**" category





3.3 Select "**Direct method**" and then:

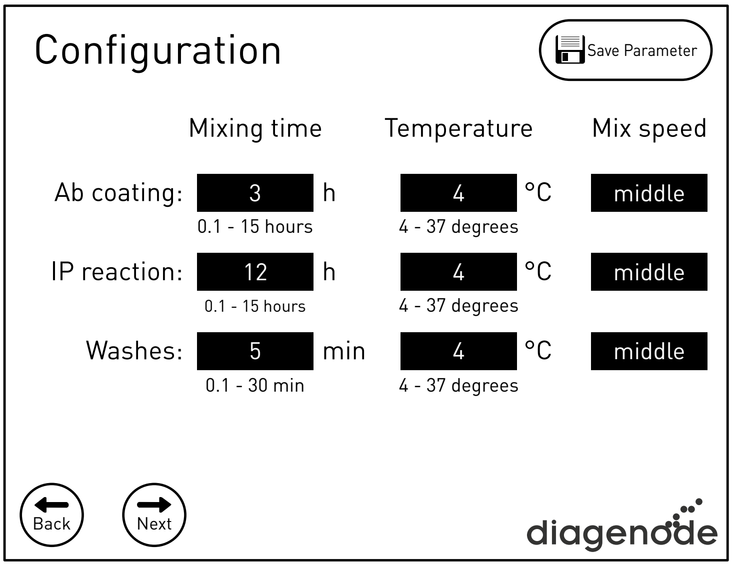
"**ChIP_08_qPCR_D**" if you plan to run between **1** and **8** samples
"**ChIP_16_qPCR_D**" if you plan to run between **9** and **16** samples

3.4 Set up the exact number of samples for your experiment by pressing the black box. This number includes the positive and negative control IPs and each IP has to be counted as a sample. Input will not undergo immunoprecipitation in the IP-Star and is therefore not considered as a sample.

***NOTE**: The Peltier Block is now cooling down to 4°C to keep your samples cold.*

3.5 Setup the parameters for your ChIP experiment and press “Next”.

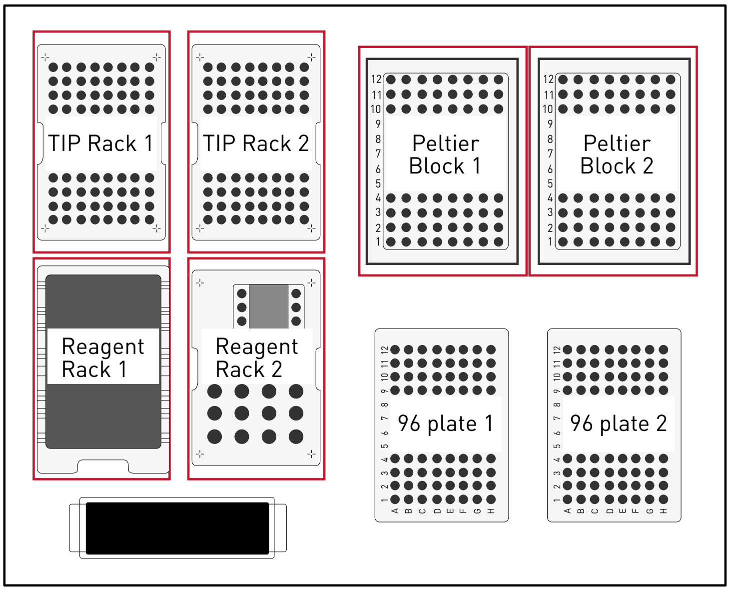
Recommended parameters:



Configuration

	Mixing time		Temperature		Mix speed
Ab coating:	3	h	4	°C	middle
	0.1 - 15 hours		4 - 37 degrees		
IP reaction:	12	h	4	°C	middle
	0.1 - 15 hours		4 - 37 degrees		
Washes:	5	min	4	°C	middle
	0.1 - 30 min		4 - 37 degrees		

3.6 Set up all the plastics on the platform according to the screen layout.



- Fill **TIP Rack 1** (and **2** if processing more than 8 samples) with tips according to the screen.
- Fill **Reagent Racks 1 & 2** with reagent containers according to the screen.
- Fill **Peltier Block 1** (and **2** if processing more than 8 samples) with 8-tube strips according to the screen.

***NOTE**: All the rows of the Peltier(s) Block(s) must be filled with a strip.*

3.7 Fill the strips with your samples and the reagents from the kit as described below and make sure that the liquid is at the bottom of each well.

- Distribute **10-30 µl of DiaMag Protein A-coated magnetic beads** in each well of row 3.

NOTE: *The binding capacity of 10 µl of magnetic beads is ~3µg of antibody. If you plan to use more than 3 µg of antibody per IP, we recommend that the quantity of beads is adjusted accordingly.*

NOTE: *Keep the magnetic beads (DiaMag Protein A-coated magnetic beads) in liquid suspension and at 4°C during storage and all handling steps to ensure a high efficiency. Resuspend the beads with pipetting up and down instead of vortexing to keep the proteins coated on the beads.*

- Prepare **ChIP Buffer** (also named Beads wash buffer) as described in the table below. The calculated volumes contain an excess.

	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs
5x ChIP Buffer iC1b	60 µl	240 µl	320 µl	400 µl	480 µl	560 µl	640 µl	720 µl
ChIP-seq grade Water	234 µl	960 µl	1280 µl	1600 µl	1920 µl	2240 µl	2560 µl	2880 µl
5% BSA (DNA free)	6 µl	18 µl	24 µl	30 µl	36 µl	42 µl	48 µl	54 µl
TOTAL ChIP Buffer	300 µl	1200 µl	1600 µl	2000 µl	2400 µl	2800 µl	3200 µl	3600 µl

	9 IPs	10 IPs	11 IPs	12 IPs	13 IPs	14 IPs	15 IPs	16 IPs
5x ChIP Buffer iC1b	1040 µl	1120 µl	1200 µl	1280 µl	1360 µl	1440 µl	1520 µl	1600 µl
ChIP-seq grade Water	4160 µl	4480 µl	4800 µl	5120 µl	5440 µl	5760 µl	6080 µl	6400 µl
5% BSA (DNA free)	78 µl	84 µl	90 µl	96 µl	102 µl	108 µl	114 µl	120 µl
TOTAL ChIP Buffer	5200 µl	5600 µl	6000 µl	6400 µl	6800 µl	7200 µl	7600 µl	8000 µl

- Prepare the **Ab coating mix** as described in the table below and distribute **100 µl** in each well of **row 6**.

	1 IP
Antibody	x µl
ChIP Buffer	100 – x µl
200x Protease Inhibitor cocktail	0.5 µl

NOTE*: The required amount of antibody per IP varies. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. Use 1 µg of IgG (negative control antibody) for the negative control IP. If a positive control IP is included, use 1 µg of the H3K4me3 positive control antibody.*

- Prepare the **immunoprecipitation mix** as described in the table below and distribute **200 µl** in each well of **row 7**.

	1 IP
Sheared chromatin	200 µl
BSA 5%	4 µl
200x Protease Inhibitor Cocktail	1 µl

- Keep aside **2 µl of the sheared chromatin** at 4°C to be used as an **INPUT** starting from step 4.

NOTE*: If required, NaBu (HDAC inhibitor, 20 mM final concentration) or other inhibitors can also be added to the chromatin sample.*

3.8 Fill **Reagent Racks 1 & 2** with reagents according to the screen instructions.

NOTE*: Beads wash buffer = ChIP Buffer*

3.9 Check the proper insertion of the racks and the consumables, and press "Next".

3.10 Check the selected parameters, close the door, and press "Run" to start.

3.11 ChIP is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

STEP 4

DNA isolation and decross-linking

Day 2 *10 minutes hands on time and 40 minutes run*

FOR CELLS AND TISSUES

4.1 The next morning, after the overnight run, recover the sample tubes from row 12 (at 4°C).

NOTE: *Remove all the plastics from the IP-Star platform.*

4.2 Prepare the INPUT as described in the table below.

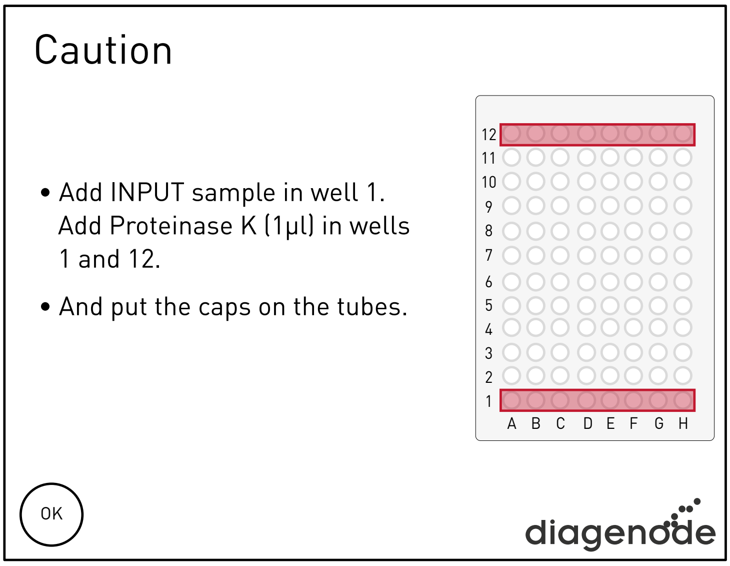
	1 INPUT
Sheared chromatin	2 µl
DNA Isolation Buffer (DIB)	98 µl

From now onwards treat the input DNA samples and IP samples in parallel.

4.3 Add **1 µl of Proteinase K** in all samples and the INPUT.

4.4 Close the tubes with caps and insert them on the Peltier Block 1.

NOTE: *The Peltier Block 1 heats evenly, so the strips can be placed in every row of this block.*



Caution

- Add INPUT sample in well 1. Add Proteinase K (1µl) in wells 1 and 12.
- And put the caps on the tubes.

4.5 Close the door and press “OK” to start the incubations.

4.6 The incubation is now starting. The “Remaining time” calculation will give you an estimation of the processing time.

4.7 When the protocol is completed, a screen appears telling you the run is over. Recover the sample tubes, spin down the tubes to bring down the liquid caught in the lid.

4.8 Place the tubes in the DiaMag02 magnetic rack (Cat. No. B04000001) and wait for 1 minute.

4.9 Transfer the supernatants which contains the DNA to new labeled tubes and discard the magnetic beads.

4.10 Now you have your DNA ready for qPCR analysis. Determine the total number of regions to be analyzed by qPCR for each sample and take the required volume for qPCR analysis.

__NOTE__: Depending on a sensitivity of a Master Mix and qPCR cycler used the samples can be diluted before PCR and the volume per PCR may vary.

4.11 Store the remaining DNA at -20°C until further use.

4.12 Now back to the IP-Star screen, press “OK” and then “Yes” to start a new run or press “Back” until the homepage appears on the screen. Press “Shutdown” and wait until the screen is black before switching off the IP-Star.

4.13 Remove all the plastics from the IP-Star platform, empty the waste shuttle, and clean the inner side of the IP-Star with 70% ethanol.

STEP 5

Quantitative PCR analysis

2 *Day 2* *2 to 3 hours*

FOR CELLS AND TISSUES

***NOTE**: For each primer pair, run the INPUT DNA alongside the immunoprecipitated samples and negative IgG control.*

5.1 Take an aliquot of immunoprecipitated DNA and a corresponding INPUT (step 4.8) .

5.2 Prepare the **qPCR mix** as follows (20 µl reaction volume using the user supplied primer pairs:

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of PCR-grade water
- 5 µl of IP'd or INPUT DNA

5.3 Use the following PCR program:

***NOTE**: These conditions may require optimization depending on the type of Master Mix, qPCR system used and user provided primer pair.*

Step	Time/cycles		Temperature
1. Denaturation	3-10 min*		95°C
2. Amplification	30 seconds	40 cycles	95°C
	30 seconds		60°C
	30 seconds		72°C (acquire fluorescence data)
3. Melting curve**	Follow qPCR instrument manufacturer recommendations		

**Please check carefully supplier's recommendations about Taq polymerase activation time.*

***Include and inspect the melting curves based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single specific product*

5.4 Record the threshold cycles (Ct values) from the exponential phase of the qPCR for the IP'd DNA sample and input for each primer pair.

5.5 Calculate the relative amount of immunoprecipitated DNA compared to INPUT DNA for the control regions (% of recovery) using the following formula:

$$\% \text{ recovery} = 2\text{^}((Ct_{input} - 6.64) - Ct_{sample}) * 100\%$$

- Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and INPUT, respectively.
- 2 is the amplification efficiency
- 6.64 is a compensatory factor to correct the input dilution

NOTE*: This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency with given primer pair has to be close to 100% meaning that for each cycle the amount of product is doubles (E=2). The real amplification efficiency, if known, should be used. The formula takes into account that 1% of INPUT was used as suggested in the protocol (2.5 µl INPUT vs 250 µl of chromatin per IP). If the amount of INPUT used is different from 1%, an introduction of a compensatory factor in the formula is required to correct the input dilution (x) as follows:*

$$\%\text{recovery} = 2\text{^} [(Ct_{input} - \log2 (X) - Ct_{sample})] \times 100\%$$

Where: log2 (X) accounts for the INPUT dilution

Example*: If you use an INPUT of 5 µl from 250 µl of chromatin used per IP, it corresponds to 50 X dilution. The compensatory factor is equal to log2(50)=5.64 and the formula to calculate the recovery will be as follows:*

$$\%\text{recovery} = 2\text{^} [(Ct_{input} - 5.64\ Ct_{sample})] \times 100\%.$$

?

ASK THE EXPERTS

Please do not hesitate to contact our customer support team if you have any questions about the design of your ChIP experiment.

customer.support@diagenode.com

Example of results

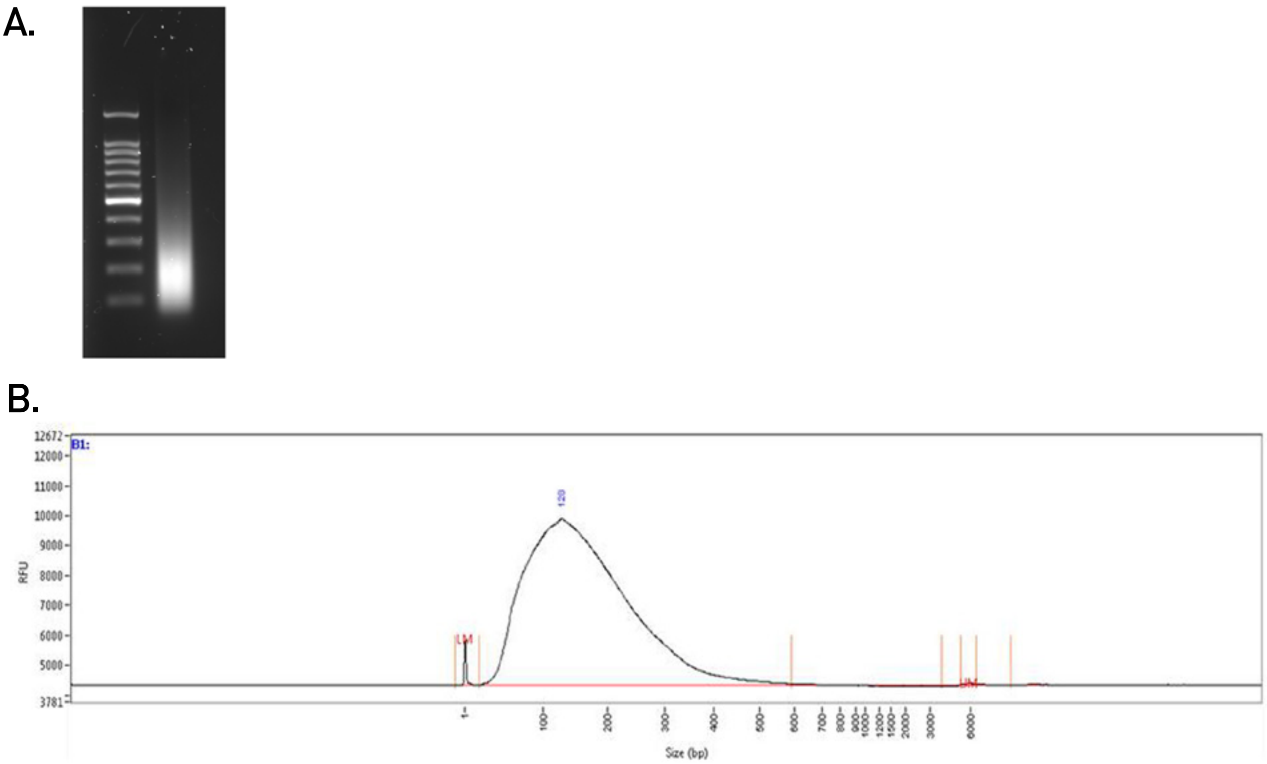


Figure 1. Successful chromatin shearing using the Bioruptor Pico and the iDeal ChIP-qPCR Kit.

HeLa cells were fixed with formaldehyde and chromatin was prepared according to the iDeal ChIP-qPCR protocol. Samples were sonicated for 8 cycles of 30" ON/30" OFF using the Bioruptor Pico. A 100 bp ladder was loaded as size standard. Panel A: Fragment size assessment by an agarose gel electrophoresis. Panel B: Fragment size assessment using a Fragment Analyzer (Advanced Analytical).

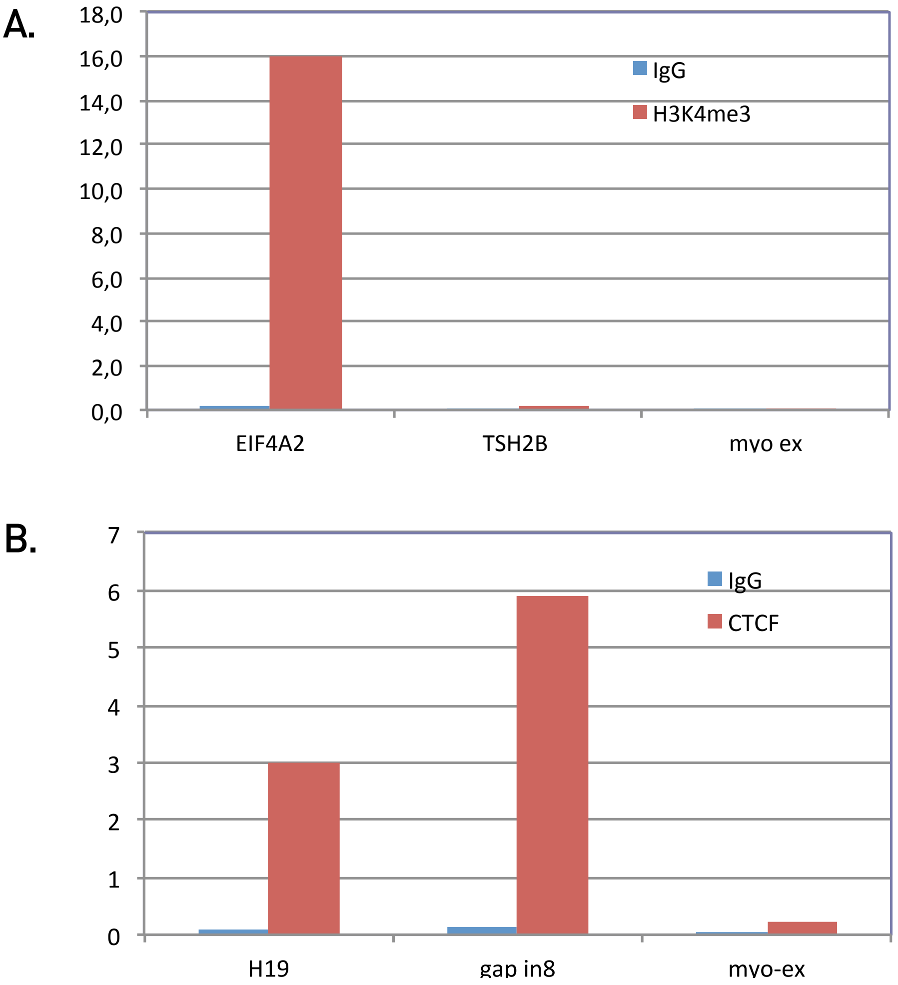


Figure 2. Chromatin immunoprecipitation analysis using H3K4me3 (A) and CTCF antibodies (B)

ChIP was performed on human HeLa cells using the H3K4me3 (Cat. No. C15410003) and CTCF (Cat. No. C15410210) antibodies. IgG was used as a negative control. The IP'd DNA was analyzed by qPCR with the following primer sets: EIF4A2, used as a positive control, and THS2B and Myoglobin exon 2, used as negative controls for H3K4me3. H19 imprinting control region and GAPDH intron 8, used as positive controls, and Myoglobin exon 2, used as a negative control for CTCF.

The figure shows the recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

Protocol for chromatin shearing analysis

General remarks

We recommend using an agarose gel analysis or the Fragment Analyzer (Advanced Analytical) for the size assessment.

For accurate size determination of the chromatin fragments, reverse crosslinking, including RNase treatment followed by DNA purification, is advised. Size estimation of chromatin fragments without reverse cross-linking is not precise. The presence of the crosslinks retards electrophoretic migration resulting in a misinterpretation of fragment size. RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing.

Workflow for analysis of sheared chromatin:

- RNAse treatment (1h, optional but highly recommended for an accurate size assessment)
- Reverse cross-linking (4h or overnight)
- DNA purification using a method of choice (eg.: IPure Kit v.2, DiaPure columns or phenol-chloroform extraction). Reagents are not provided in the kit
- Fragment size assessment (agarose gel or Fragment Analyzer) (1h)

RNAse treatment

> ***NOTE***: *RNase cocktail (e.g. Ambion, AM2286A) is not supplied with iDeal ChIP-qPCR Kit.*

1. Take **50 µl of sheared chromatin** (step 2.12 for cells or step 2.16 for tissues in the protocol) and transfer to a 1.5 ml microtube.

2. Dilute **1 µl of RNase cocktail** (e.g. Ambion, AM2286A) in **150 µl of ChIP grade water**.

3. Add **2 µl of diluted RNase cocktail** to the aliquot of sheared chromatin.

4. Incubate for 1 hour at 37°C.

Reverse cross-linking

5. Add **50 µl of Elution Buffer iE1**.

6. Add **4 µl of Elution Buffer iE2**, mix thoroughly.

7. Incubate samples at 65°C for 4 hours (or overnight).

DNA purification

Purify DNA using a method of choice (eg. IPure Kit v.2, DiaPure columns or phenol-chloroform extraction) and analyze the purified DNA on a 1.5% agarose gel. Load around 300 ng of DNA for an optimal separation and visualization. Alternatively, you can use a Fragment Analyzer (Advanced Analytical, Standard Sensitivity NGS Fragment Analysis Kit (DNF-473)).

FAQs

What is the amount of DNA I can precipitate after IP?

The amount of DNA you precipitate largely depends on the antibody and cell type used. In general antibodies against histone modification precipitate larger amounts of DNA than antibodies against other targets. With the CTCF antibody the amount is ~20 ng from 4 million HeLa cells. A typical yield for a transcription factor will be in the range of 1-5 ng.

How much antibody is needed per IP?

The amount of antibody needed depends on different factors of which the antibody itself is the most important. Most of Diagenode ChIP-grade antibodies have been tested at different concentrations to determine the optimal amount and the suggested amount of antibody is given in the data sheet. Please note, however, that this can be assay-dependent and might need to be optimized for each experimental setting. If the antibodies are from other companies, please refer to the manufacturers' technical data sheets. If no required amount of antibody is given, we suggest performing a titration experiment. It is important to select the amount of antibody which gives the lowest background signal.

What is the binding capacity of DiaMag Protein A-coated magnetic beads?

30 µl of DiaMag Protein A-coated magnetic beads can bind up to 10 µg of antibody.

What is the specificity of protein A-coated magnetic beads?

DiaMag Protein A-coated magnetic beads included in the iDeal ChIP-qPCR allow an efficient capture of rabbit, guinea pig, dog and pig polyclonal and monoclonal Abs, mouse IgG2a, IgG2b and IgA and human IgG1, IgG2 and IgG4. If the antibody of interest belongs to a different class of immunoglobulins (mouse IgG1 and IgG3, rat or goat polyclonal Abs, and human IgG3), DiaMag Protein G-coated magnetic beads should be used instead of protein A coated beads. These beads are available separately (C03010021).

Can I use a monoclonal antibody in a ChIP experiment?

ChIP can be performed using either monoclonal or polyclonal antibodies. In general, polyclonal antibody populations will recognize a number of different epitopes, in contrast to monoclonal antibodies, which recognize a single epitope. Because monoclonals recognize a single epitope on a target protein, they often provide a high level of specificity, low non-specific binding, and low background signals. The major disadvantage of a monoclonal antibody is its recognition of only one epitope, which can be masked by cross-linking, decreasing the efficiency of immunoprecipitation.

Why do I observe a different DNA size on agarose gel and Bioanalyzer for sheared chromatin?

We recommend using an agarose gel or the Fragment Analyzer (Advanced Analytical) for an accurate size assessment of sheared chromatin.

Although the Agilent BioAnalyzer 2100 is widely used for size assessment of DNA fragments before library preparation for NGS, this technology is less optimal for analysis of sheared chromatin. For example, some inconsistencies between agarose gel and BioAnalyzer 2100 profiles have been documented. It may be linked to a higher sensitivity of microfluidics chips to residual contaminants (ions, SDS, proteins, carrier used for DNA precipitation, etc.), overloading and to conformation/spacial structure of DNA molecules which might be affected by fixation and not fully relieved by de-crosslinking.

Moreover, BioAnalyzer 2100 traces are log-based, so a large distribution of higher molecular weight fragments is compacted into a much smaller area of the trace as compared to the smaller size fragments leading to a visual misinterpretation of fragment distribution.

Another key point is about quantitation of BioAnalyzer 2100 peaks. If each region is calculated using molarity which represents the number of molecules in a particular range, a significantly higher quantity of molecules is found in low molecular weight region (fig. 3).

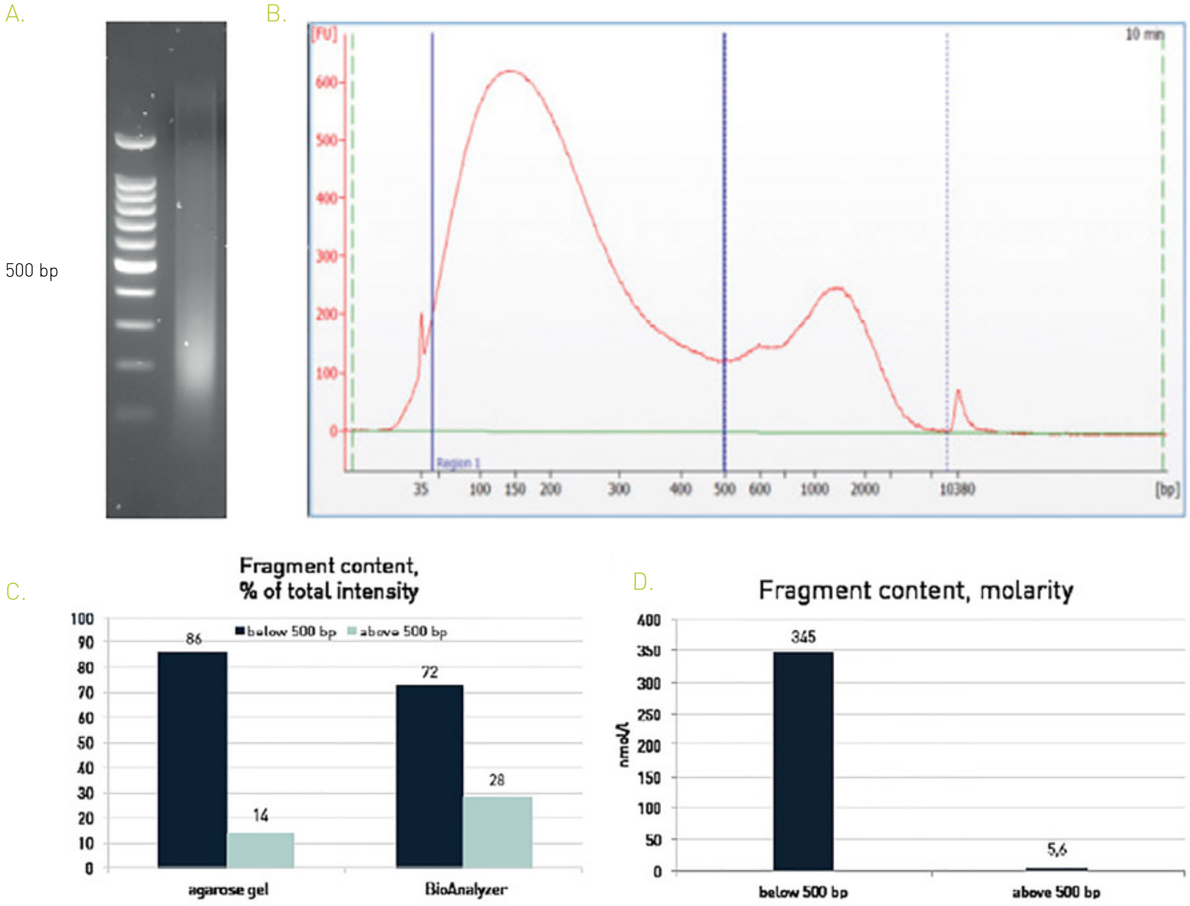


Figure 3. Size assessment of sheared chromatin using an agarose gel and the BioAnalyzer.

HeLa cells were fixed with formaldehyde and chromatin was prepared according to Diagenode's protocol. Samples were sonicated for 10 cycles of 30" ON/30" OFF with the Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (C30010016) and analyzed by an agarose gel (panel A) or by BioAnanlyzer, High Sensitivity Agilent DNA Kit (panel B). Fragment content below and above 500 bp was calculated as percentage of total surface (panel C). Panel D shows fragment content calculated as molarity (BioAnalyzer trace only).

What is the composition of buffers included in the kit?

The composition of the buffers is proprietary.

Can I use the Auto iDeal ChIP-qPCR Kit on the old version of the IP-Star?

Yes – for more information please contact Customer Support customer.support@diagenode.com

Related products

Product	Cat. No.
ChIP Cross-link Gold	C01019027
Chromatin Shearing Optimization Kit – Low SDS (iDeal Kit for TFs)	C01020013
iDeal ChIP-seq Kit for Transcription Factors	C01010055
Auto iDeal ChIP-seq Kit for Transcription Factors	C01010172
iDeal ChIP-seq Kit for Histones	C01010051
Auto iDeal ChIP-seq Kit for Histones	C01010171
ChIPmentation for Histones	C01011009
Auto ChIPmentation for Histones	C01011000
MicroChIP DiaPure columns	C03040001
IPure Kit v2	C03010014
IPure Kit v2	C03010015
Bioruptor Pico®	B01080010

Validated antibodies – check out the complete list at www.diagenode.com

ChIP-grade antibody	Cat. No.
AML1-ETO polyclonal antibody	C15310197
Pol II mAb	C1520000
CTCF pAb	C15410210
TBP mAb	C15200002
ER alpha mAb	C15100066
ETO pAb	C15310001
FOXA1 pAb	C15410231
FOXM1 pAb	C15410232
GR mAb	C15200010
GTF2E2 pAb	C15410264

ChIP-grade antibody	Cat. No.
H3K4me3 pAb	C15410003
H3K27me3 pAb	C15410069
H3pan mAb (clone 1B1B2)	C15200011
H3R8me2(asym) pAb	C15410286
H3K27ac pAb	C15410196
H3K9me3 pAb	C15410193
H3K4me1 pAb	C15410194
H4K20me3 pAb	C15410207
H2A.Z pAb	C15410201
H4K5ac pAb	C15410025

Revision history

Version	Date of modification	Description of modifications
V1 08_2023	August 2023	- Removal of obsolete references
V1 09_2019	September 2019	/

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