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Innovating Epigenetics Solutions

iDeal ChIP-seq Kit for Histones

MANUAL KIT

Cat. No. C01010050 (10 rxns)
C01010051 (24 rxns)
C01010059 (100 rxns)

AUTOMATED KIT

Cat. No. C01010057 (24 rxns)
C01010171 (100 rxns)

USER GUIDE

Version 2 06_2020

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

Please read this manual carefully before starting your experiment

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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-seq Kit for Histones (both manual and automated versions) provides a robust ChIP protocol suitable for the investigation of histone modifications within 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 histone modification. Protein A magnetic beads are used to isolate the protein-DNA complexes of interest. The immunoprecipitated DNA is eluted and purified using unique IPure magnetic beads. The eluted DNA is ideal for ChIP-seq library preparation. Additionally, the kit includes a negative (IgG) and a positive (H3K4me3) control antibody and primer pairs amplifying H3K4me3 positive and negative loci.

The iDeal ChIP-seq kit for Histones offers unique benefits:

- Thoroughly optimized protocol for ChIP-seq from cells and tissues validated with multiple histones marks
- Most complete kit available (covers all steps and includes control antibodies and primers)
- Magnetic beads make ChIP easy, fast, and more reproducible
- Provides high yields with excellent specificity and sensitivity in combination with Diagenode ChIP-seq antibodies
- Eluted DNA suitable for any downstream application
- Automated and validated on IP-Star Compact Automated System
- Easy-to-follow protocol

Kit method overview

		Time needed	Day
STEP 1	Cell or tissue collection and DNA-protein cross-linking	30 minutes to 1 hour	1
STEP 2	Cell lysis and chromatin shearing	1 to 2 hours	1
STEP 3	Magnetic immunoprecipitation	Overnight	1-2
STEP 4	Elution, decross-linking and DNA purification	5 hours	2
STEP 5	Quantitative PCR and data analysis prior to library preparation and Next-Generation Sequencing	2 to 3 hours	2

qPCR

LEGEND

- Protein of interest
- Other protein
- DNA
- Antibody
- Magnetic bead
- Magnet

Kit materials

The automated and manual iDeal ChIP-seq Kit for Histones contain enough reagents to perform the number of chromatin preparations, chromatin immunoprecipitations and DNA purifications as described in Table 1. Reagents for chromatin size assessment of each batch of chromatin are also included.

Table 1. Number of reactions included in the automated and manual versions of the iDeal ChIP-seq Kit for Histones

Kit reference	Number of chromatin preparations	Number of ChIP reactions	Number of DNA purifications
Manual kits			
C01010050	2	10	14
C01010051	4	24	32
C01010059	17	100	134
Auto kits			
C01010057	4	24	32
C01010171	17	100	134

Table 2. Components supplied with the iDeal ChIP-seq Kit for Histones (for 10, 24 and 100 rxns) and the Auto iDeal ChIP-seq Kit for Histones (for 24 and 100 rxns)

Component	Qty (x10)	Qty (x24)	Qty (x100)	Storage
Protease inhibitor cocktail	35 µl	76 µl	310 µl	-20°C
5% BSA (DNA free)	60 µl	144 µl	600 µl	-20°C
Rabbit IgG (1 µg/ µl)	8 µg	8 µg	35 µg	-20°C
ChIP-seq grade antibody H3K4me3 (1 µg/ µl)	8 µg	8 µg	35 µg	-20°C
Human GAPDH TSS primer pair	42 µl	96 µl	400 µl	-20°C
Human Myoglobin exon 2 primer pair	42 µl	96 µl	400 µl	-20°C
Carrier	28 µl	64 µl	300 µl	-20°C

Component	Qty (x10)	Qty (x24)	Qty (x100)	Storage
Glycine	200 µl	400 µl	1.7 ml	4°C
Shearing Buffer iS1	3.6 ml	7.2 ml	30.6 ml	4°C
DiaMag protein A-coated magnetic beads	200 µl	480 µl	2 ml	4°C Do not freeze
Wash Buffer iW1	3.5 ml	8.4 ml	35 ml	4°C
Wash Buffer iW2	3.5 ml	8.4 ml	35 ml	4°C
Wash Buffer iW3	3.5 ml	8.4 ml	35 ml	4°C
Wash Buffer iW4	3.5 ml	8.4 ml	35 ml	4°C
ChIP-seq grade water	3 ml	7 ml	30 ml	4°C
Elution Buffer iE2	64 µl	144 µl	600 µl	4°C
Lysis Buffer iL1	20 ml	40 ml	170 ml	4°C
Lysis Buffer iL2	20 ml	40 ml	170 ml	4°C
Wash Buffer 1 w/o iso-propanol	700 µl	2 ml	8 ml	4°C
Wash Buffer 2 w/o iso-propanol	700 µl	2 ml	8 ml	4°C
Buffer C	700 µl	1.6 ml	6.65 ml	4°C
IPure Beads v2	140 µl	320 µl	1.67 ml	4°C Do not freeze
Elution Buffer iE1	1.5 ml	3.4 ml	14.5 ml	4°C
5x ChIP Buffer iC1	950 µl	2.2 ml	9.2 ml	4°C

***NOTE**: Upon receipt, store the components at the indicated temperatures.*

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml and 15 ml tubes
- Formaldehyde, 37%, molecular grade
- Phosphate buffered saline (PBS) buffer
- Trypsin-EDTA
- Cell culture medium
- qPCR SYBR® Green Mastermix
- ChIP-seq grade antibodies – www.diagenode.com
- Fluorescence-based assay for DNA concentration measurement, e.g. the Qubit High Sensitivity assay (Life Technologies #Q32851)

Additional supplies for tissue protocol

- Equipment for tissue disruption and homogenization: Dounce homogenizer with loose and tight pestles (2 ml) and TissueLyser (Qiagen) with 2 ml tubes or stainless steel beads, 5 mm (Qiagen, Cat. No.69989)
- Scalpel blades
- Petri dishes
- Liquide nitrogene (required if the TissueLyser is used)

Equipment

- DiaMag 1.5ml magnetic rack (Diagenode, Cat. No. B04000003)
- Cell counter system
- Bioruptor® sonication device and the associated microtubes:
  - Bioruptor Pico (Diagenode, Cat. No. B01060001) and 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016)
  - Bioruptor Plus (Diagenode, Cat. No. B01020001) and 1.5 ml TPX Microtubes (Cat. No. C30010010)
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- DiaMag rotator (Rotating wheel) (Diagenode, Cat. No. B05000001)

- Vortex
- Thermomixer
- Qubit® Fluorometer (ThermoFisher Scientific)
- qPCR cycler

Optional supplies

- Chromatin Shearing Optimization kit - Low SDS (iDeal Kit for Histones) (Cat. No. C01020010)
- 1M Sodium butyrate (NaBu) (Diagenode, Cat. No. C12020010)
- RNase cocktail (e.g. Ambion, AM2286A), required for chromatin shearing assessment
- MicroPlex Library Preparation™ Kit v2 (Diagenode, Cat. No. C05010012, Cat. No. C05010013, Cat. No. C05010014) or
- iDeal Library Preparation Kit x24 (incl. Index Primer Set 1) (Diagenode, Cat. No. C05010020)

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)
- 96 well microplates (Diagenode, Cat. No. 30080030)
- DiaMag02 magnetic rack (Cat. No. B04000001)

Remarks before starting

Cell number

The protocol describes the preparation of a batch of chromatin from approximately 7 million cells which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. Approximately 1 million cells per IP reaction are used in this standard protocol. The protocol is optimized for the use of 100 µl of sheared chromatin in a total volume of ChIP reaction equal 200 µl (auto kit) or 300 µl (manual kit). It is crucial to keep these volumes consistent for optimal results.

Depending on the abundance of the target, the specificity of the antibody, and the number of cells available, it may be possible to scale down to 100.000 cells as described below. Scaling up is usually not necessary but if a higher yield of IP'd DNA is required, we recommend performing separate ChIPs and pool the IP'd DNA before purification. For using lower numbers of cells per IP:

A. You can start with a batch of 7 million of cells (as in a standard protocol) and follow the protocol up to the chromatin shearing step. Then simply dilute the sheared chromatin in the shearing buffer iS1 before adding it to the IP reaction. The final volume of diluted chromatin containing the desired number of cells should be 100 µl per IP reaction.

B. You can start with a smaller batch of cells. 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 down the volume of iL1 and iL2 buffers by 1 ml of iL1 and 1 ml of iL2 per 1 million cells. Define the volume of shearing buffer iS1 taking into account that you will need:

- 100 µl of sheared chromatin (containing the desired number of cells) per IP reaction
- 1 µl of sheared chromatin per input
- 50 µl of sheared chromatin for chromatin shearing assessment
- 5% excess of iS1

Resuspend the cells in the required volume of shearing buffer iS1 and follow the standard protocol.

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

Tissue amount

The protocol describes the preparation of a batch of chromatin from approximately 30-40 mg of tissue which is sufficient for 6 IP reactions, 1 input sample and 1 sample for chromatin shearing assessment. Approximately 5 mg of tissue per IP reaction are used in this standard protocol. The protocol is optimized for use of 100 µl of sheared chromatin in a total volume of ChIP reaction equal 200 µl (auto kit) or 300 µl (manual kit). It is crucial to keep these volumes constant for optimal results.

Tissue samples have to be homogenized mechanically before sonication. Soft tissues (e.g. liver or brain) can be successfully homogenized using a Dounce homogenizer while it is preferable to use the TissueLyser (Qiagen, or a similar system) for hard fibrous tissues (e.g. muscles). Please note that the TissueLyser workflow is only compatible with frozen tissues since the grinding of fresh tissue will not be efficient with this protocol.

Depending on the abundance of the target, the specificity of the antibody, and the amount of tissue available, it may be possible to scale down to 1.5 mg of tissue per IP as described below. Scaling up is usually not necessary but if a higher yield of immunoprecipitated DNA is required, we recommend performing separate ChIPs and pool the immunoprecipitated DNA before purification.

For using lower amount of tissue per IP:

A. You can start with 30-40 mg of tissue (as in a standard protocol) and follow the protocol up to the chromatin shearing step. Then simply dilute the sheared chromatin in shearing buffer iS1 before adding it to the IP reaction. The final volume of diluted chromatin containing the desired starting amount should be 100 µl per IP reaction.

B. You can start with a smaller batch of tissue. First, determine the amount that you will use per IP and the total number of IPs. Follow the standard protocol for tissue fixation, collection and lysis. Do not scale lysis buffers iL1b and iL2. Define the volume of shearing buffer iS1 taking into account that you will need:

- 100 µl of sheared chromatin (containing the desired amount of tissue) per IP reaction
- 1 µl of sheared chromatin per input
- 50 µl of sheared chromatin for chromatin shearing assessment
- Add 5% excess of iS1

Resuspend the tissue in the required volume of shearing buffer iS1 and follow the standard protocol.

Please note that changing 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. Usually, the amount of chromatin required per IP with iDeal ChIP-seq Kit for Histones is 0.3-3 µg. We recommend performing a pilot experiment to determine the optimal amount of tissue. Once determined, it should be kept consistent between experiments.

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 additional optimization but usually a fixation of 8 to 10 minutes is suitable for most histone proteins. Please note that a longer fixation may lead to chromatin that is resistant to sonication.

Shearing optimization

Chromatin shearing is one of the most critical steps for a successful ChIP experiment. Chromatin fragments between 100-600 bp are ideal for the ChIP experiments. The optimal time of sonication depends on many factors such as 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 Histones) (Cat. No. C01020010) which contains all buffers needed for chromatin preparation compatible with the iDeal ChIP-seq Kit for Histones. The reagents included in this kit allow preparing chromatin using already optimized shearing settings only; it 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 (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 (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. Avoid over-sonication, as it may lead to a drop in efficiency in ChIP experiments.

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 homogenous 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 polyclonal antibodies, mouse IgG2a, IgG2b and IgA, guinea pig IgG, dog IgG, pig IgG.

ChIP-seq grade antibodies

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

Input

The input sample corresponds to whole DNA which went through the full ChIP procedure without any immunoselection. The input sample is used as a reference to calculate the recovery at the end of the ChIP procedure. It is also used by most of the bioinformatics tools for analysis of ChIP-seq data where it serves to determine the bias which may result from experimental conditions. We recommend including one input for each series of ChIP reactions.

Negative and positive controls

The kit contains a negative (IgG) and a positive (H3K4me3) control antibody to monitor the efficiency of the IP on the same chromatin as the one used with the antibody of interest. We recommend including one negative IgG control in each series of ChIP. We also recommend using the positive control ChIP-seq grade H3K4me3 antibody at least once.

Quantification

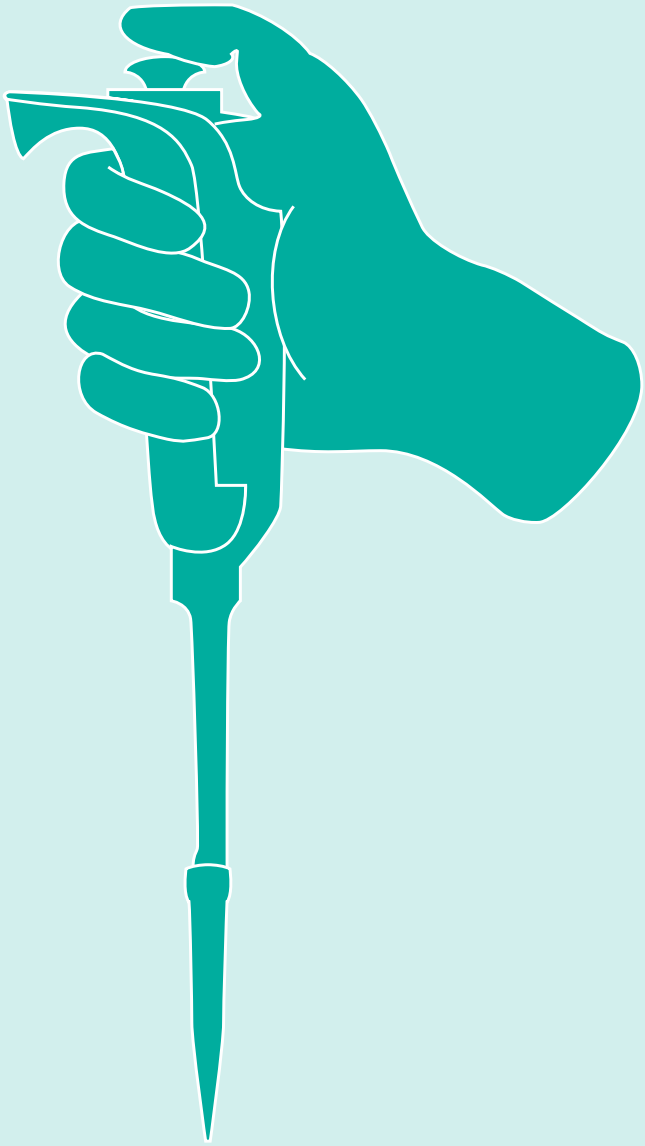
After the ChIP, determine the concentration of the immunoprecipitated DNA with a highly sensitive method such as the dsDNA HS Assay Kit on the Qubit® system from Thermo Fisher Scientific. PicoGreen® is also suitable but UV spectrophotometric methods such as the NanoDrop are usually not sufficiently sensitive. In most cases it is sufficient to use

approximately 10% of the immunoprecipitated material for quantification. The DNA yield will be dependent on different factors such as cell type, quality of the antibody used and antibody target. The expected DNA yield obtained with the positive control H3K4me3 antibody on 1,000,000 HeLa cells is approximately 10 ng.

Quantitative PCR analysis

Prior to the sequencing, 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. The kit contains two primer pairs targeting two regions which are positive (GAPDH TSS control region) and negative (Myoglobin Exon 2) for the control antibody provided in the kit (H3K4me3 ChIP-seq grade antibody). Each specific antibody will require specific control primers designed by the user. For each primer pair, run the input DNA alongside the immunoprecipitated samples. In order to have sufficient DNA left for sequencing, we recommend not using more than 10% of the total IP'd DNA for qPCR. You can dilute the DNA (1/10 or more) to perform sufficient PCR reactions. PCR reactions should be performed at least in duplicate although performing in triplicate is recommended to be able to identify potential outliers.

PROTOCOL



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MANUAL PROCESSING

Protocol

The iDeal ChIP-seq Kit for Histones is suitable for chromatin preparation and immunoprecipitation from cells and tissues. The protocol for Step 1 differs for cells and tissues. Please refer to the corresponding section.

The protocol below describes the chromatin preparation (formaldehyde cross-linking, cell collection, lysis and chromatin shearing) from a batch of approximately 7 million cells or 30-40 mg of tissue. This will be sufficient for 6 ChIP reactions (using approximately 1 million cells or 5 mg of tissue per IP), 1 input and 1 sample for chromatin shearing assessment.

If using a different amount of starting material, please refer to "Remarks before starting".

STEP 1

Cell collection and DNA-protein cross-linking

1 *Day 1* *1 hour*

FOR CULTURED CELLS

***NOTE**: PBS at different temperatures (ice-cold, room temperature and pre-warmed at 37 °C) will be required at this step.*

For adherent cells:

1.1 Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.

1.2 Remove the medium and rinse the cells with **pre-warmed PBS** (10 ml for a 75 cm^2 culture flask). Gently shake the flask for 2 minutes.

1.3 Remove the PBS and add sterile **trypsin-EDTA** to the culture flask to detach adherent cells from the bottom. Add enough trypsin-EDTA to completely cover the cells (e.g. 1 ml for a 75 cm^2 culture flask). Gently shake the culture flask for 1-2 minutes or until the cells start to detach.

***NOTE**: The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment may damage the cells. Regularly check if the cells start to detach.*

1.4 Immediately add **fresh culture medium** to the cells when they are detached. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA (e.g. 2 ml for a 75 cm2 culture flask). Transfer cell suspension to a 15 ml tube.

1.5 Rinse the flask by adding **10 ml** of **warm PBS**. Add this volume to your 15 ml tubes containing cells from above point. Proceed immediately with step 1.6.

For suspension cells:

Collect suspension cells in a 50 ml tube and go directly to point 1.6 of the protocol.

1.6 Centrifuge for 5 minutes at 500 x g at room temperature and remove the supernatant.

1.7 Resuspend the cells in **20 ml** of **warm PBS** and count them. Collect the cells (approximately 7 millions) by centrifugation for 5 minutes at 500 x g at room temperature.

1.8 Resuspend the cells (approximately 7 millions) in **500 µl** of PBS (room temperature) and add **13.5 µl** of **37% formaldehyde** (under a fume hood) to each tube. Mix by gentle vortexing and incubate 8 minutes at room temperature to allow fixation to take place.

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

1.9 Add **57 µl of Glycine** to the cells to stop the fixation. Incubate for 5 minutes at room temperature with gentle shaking. Keep everything at 4°C or on ice from now on.

1.10 Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.

1.11 Wash the cells twice with **1 ml** of **cold PBS**: resuspend the cell pellet in **1 ml** of **cold PBS**. Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 1

Tissue disaggregation and DNA-protein cross-linking

1 *Day 1* *30 minutes*

FOR TISSUES

***NOTE**: PBS at different temperatures (cie-cold and room temperature) will be required at this step*

1.1 Weigh 30-40 mg of fresh or frozen tissue in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

1.2 Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade. Disaggregate sample using a Dounce homogenizer or TissueLyzer.

For Dounce homogenizer:

Add 5 µl of protease inhibitors cocktail to 1 ml of ice-cold PBS and resuspend the tissue pieces. Transfer it to the Douce homogenizer and disaggregate using a loose pestle to get a homogeneous suspension.

Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 850 x g for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet. Proceed with the fixation (step 1.3)

For TissueLyzer:

Transfer tissue pieces to 2 ml tubes, supplemented with 2 stainless

steel beads (5 mm) and immerse in liquid nitrogen for a few minutes. Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 8 hours at -80°c) and operate the TissueLyser for 2-4 minutes at 25-30 Hz. Proceed with the fixation on the grinded sample (step 1.3).

1.3 Add **27 µl of 37% formaldehyde** (under a fume hood) to **1 ml of PBS** (room temperature) , add 1 ml of the cross-linking solution directly to the tissue lysate and resuspend the tissue suspension in diluted formaldehyde.

1.4 Mix by vortexing and incubate for 8 minutes at room temperature with gentle rotation on a DiaMag Rotator to allow fixation to take place.

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

1.5 Add **100 µl of Glycine** to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing on a DiaMag rotator.

1.6 Centrifuge samples at 850 x g for 5 minutes at 4°C. Discard the supernatant without disturbing the pellet. Keep everything at 4°C or on ice from now on.

1.7 Wash the pellet twice with **1 ml of cold PBS**: resuspend the cell pellet in **1 ml** of **cold PBS**. Collect the cells by centrifugation at 850 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 2

Cell lysis and chromatin shearing

1 *Day 1* *1-2 hours*

FOR CELLS AND TISSUES

2.1 Add **1 ml of ice-cold Lysis Buffer iL1** to the 1.5 ml tube containing cells or tissue suspension. Resuspend the samples by pipetting up and down several times and transfer them to a 15 ml tube. Add **6 ml of Lysis Buffer iL1** and incubate for 10 minutes at 4°C with gentle mixing.

2.2 Pellet samples by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.3 Resuspend the cell pellet in **1 ml of ice-cold Lysis Buffer iL2** by pipetting up and down several times. Add another **6 ml of Lysis Buffer iL2** and incubate for 10 minutes at 4°C with gentle mixing.

2.4 Pellet the cells again by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.5 Prepare **complete Shearing Buffer** by adding **3.5 µl of 200x protease inhibitor cocktail** to **700 µl of Shearing Buffer iS1**. Keep on ice.

2.6 Add **700 µl of complete Shearing Buffer iS1** to the cells (7 million cells) or tissue sample (30-40 mg of tissue). The final cell concentration in the Shearing Buffer should be 1 million of cells or ~5 mg tissue per 100 µl of iS1. Resuspend by pipetting up and down several times and incubate for 10 minutes in ice.

NOTE - *optional, for tissue samples:*

If the suspension is not homogeneous after pipetting, an additional homogenization using a Dounce homogeniser or TissueLyser could be performed.

*For **Dounce homogeniser**, transfer the suspension to the homogeniser, perform several strokes to get a homogeneous suspension and proceed with the sonication as described below.*

*For **TissueLyser**, transfer the suspension to 2 ml tubes, supplemented with 2 stainless steel beads (5 mm). Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 2 hours at 4°c) and operate the TissueLyser for 2-4 minutes at 25 Hz. Proceed with the sonication as described below.*

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

- When using the Bioruptor Pico shear for 5 to 15 cycles [30 seconds "ON", 30 seconds "OFF"].
- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.

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.*

NOTE*: We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization kit - Low SDS (iDeal Kit for Histones) (Cat. No. C01020010).*

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

2.9 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol is described in the "Additional Protocols" section.

***NOTE**: We recommend analysing the shearing for each batch of chromatin. This step can be omitted when sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. Store the chromatin aliquot at -20°C until analysis.*

STEP 3

Magnetic immunoprecipitation for cells and tissues

Day 1-2 *Overnight + 30 minutes*

FOR CELLS AND TISSUES

3.1 Determine the number of IP reactions to be run including the negative and positive control IPs. Take the required amount of DiaMag Protein A-coated magnetic beads and transfer it to a clean 1.5 ml tube. **20 µl of beads** are required per IP.

3.2 Dilute **300 µl of 5x ChIP Buffer iC1** with **1.2 ml of ChIP-seq grade water** to obtain 1x ChIP Buffer iC1. Place the diluted ChIP Buffer iC1 on ice.

3.3 Wash the beads 4 times with **320 µl of ice-cold 1x ChIP Buffer iC1**. To wash the beads, add 1x ChIP buffer iC1, resuspend the beads by pipetting up and down several times and place the tubes in the DiaMag 1.5ml magnetic rack (Cat. No. B04000003). Wait for 1 minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

3.4 After the last wash, resuspend the beads in 1x ChIP Buffer iC1 adding the original volume of beads (this means 20 µl per IP).

3.5 Set aside **1 µl of the sheared chromatin** to use as **INPUT** sample starting from the step 4.2.

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

- 6 µl of BSA
- 1.5 µl of 200x protease inhibitor cocktail
- 56 µl of 5x ChIP buffer iC1
- 100 µl of sheared chromatin
- 20 µl of DiaMag Protein A-coated magnetic beads
- (116.5 µl –x µl) ChIP-seq grade water
- Add x µl of ChIP-seq grade antibody
  x - amount of ChIP grade antibody

The total volume of the ChIP reaction mix per IP is 300 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 H3K4me positive control antibody. If required, NaBu (20 mM final concentration) or other inhibitors can be added.*

3.7 Incubate overnight at 4°C on a DiaMag Rotator.

3.8 Perform the washes as follows: briefly spin the tubes and place them in the DiaMag 1.5ml magnetic rack (Cat. No. B04000003). 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 DiaMag rotator at 4°C.

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

STEP 4

Elution, decross-linking and DNA purification

2 *Day 2* *5 hours*

FOR CELLS AND TISSUES

***NOTE**: Before the first use of the kit, prepare Wash Buffer 1 and Wash Buffer 2 by adding an equal volume of isopropanol. Wash Buffers 1 and 2 should be stored at 4°C. Never leave the bottle open during storage to avoid evaporation.*

4.1 After removing the last wash buffer, add **100 µl of Elution Buffer iE1** to the beads, resuspend the beads pellet and incubate for 30 minutes on the DiaMag rotator at room temperature.

***NOTE**: If a precipitation is observed in Elution Buffer iE1, warm it at 37°C until it becomes clear. This will not impair the reaction.*

4.2 Briefly spin the tubes and place them into the DiaMag1.5ml magnetic rack. Wait for 1 minute, transfer the supernatant to a new 1.5 ml tube and add **4 µl of Elution Buffer iE2**. At the same time, add **99 µl of Elution Buffer iE1** and **4 µl of Elution Buffer iE2** to the 1 µl INPUT sample. Incubate for 4 hours or overnight at 65°C with shaking.

***NOTE**: If desired, include a chromatin sample for shearing assessment (from step 2.9) at this step. Perform decross-linking and DNA purification in parallel with the IP and input samples. Please follow the instructions described in a separate section "Protocol for chromatin shearing analysis"*

4.3 Add **2 µl of carrier** to each IP and INPUT sample.

4.4 Add **100 µl of 100% isopropanol** to each IP and INPUT sample.

***NOTE**: Following the addition of isopropanol the solution may become cloudy.*

This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

4.5 Resuspend the **IPure beads v2** by vortexing and transfer **10 µl** to each IP and INPUT sample.

4.6 Incubate the IP and INPUT samples for 10 minutes at room temperature on the DiaMag Rotator.

4.7 Briefly spin the tubes, place in the DiaMag 1.5ml magnetic rack, wait 1 minute and discard the buffer.

4.8 Add **100 µl of Wash Buffer 1** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes, place in the DiaMag 1.5ml magnetic rack, wait 1 minute and discard the buffer.

4.9 Add **100 µl of Wash Buffer 2** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the DiaMag 1.5ml magnetic rack, wait 1 minute and discard the buffer.

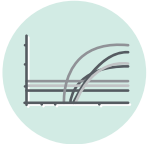
4.10 Spin the tubes again and place them on the DiaMag 1.5ml magnetic rack. Discard the remaining Wash Buffer 2 if necessary. Resuspend the beads pellet in **25 µl of Buffer C**. Incubate at room temperature for 15 minutes on the DiaMag Rotator.

4.11 Spin the tubes and place them into the DiaMag 1.5ml magnetic rack, wait 1 minute and transfer the supernatant containing the immunoprecipitated DNA into a new 1.5 ml tubes. Discard the beads.

4.12 Determine the total number of regions to be analyzed by qPCR for each sample. Take the required volume of Input and immunoprecipitated samples for qPCR analysis. Take into account that these samples will be diluted 1/10 and 5 µl will be used per PCR.

***NOTE**: The dilution of samples and the volume per PCR may vary depending on a sensitivity of a master mix and qPCR cycler used.*

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



STEP 5

Quantitative PCR analysis

Day 2 *2 to 3 hours*

***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 4.12) and dilute them 1/10 using ChIP-seq grade water.

5.2 Prepare the qPCR mix as follows (20 µl reaction volume using the provided control primer pairs):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of water
- 5 µl of diluted immunoprecipitated 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		

**Carefully check 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 immunoprecipitated 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:

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

- 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 (1 µl INPUT vs 100 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:

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

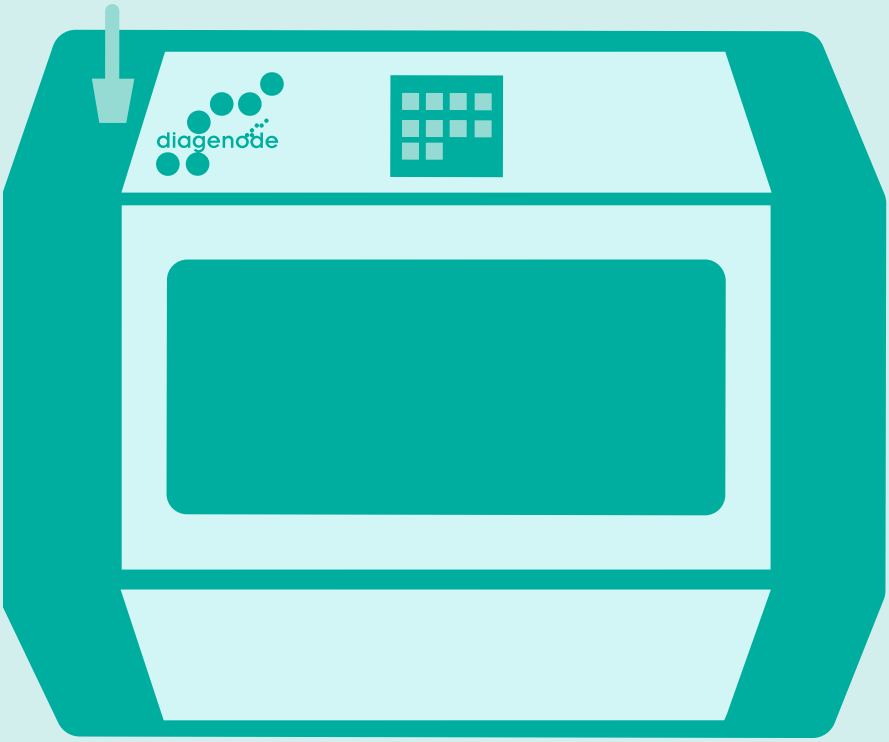
Where: log2(x) accounts for the INPUT dilution

***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:*

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

5.6 If an expected enrichment over positive loci with a low background is observed, proceed to the library preparation using an appropriate protocol (not supplied with iDeal ChIP-seq Kit for Histones).

AUTO PROTOCOL



CELLS	**STEP 1** - Cell collection and DNA-protein cross-linking	33
TISSUES	**STEP 1** - Tissue disaggregation and DNA-protein cross-linking	35
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AUTOMATED PROCESSING

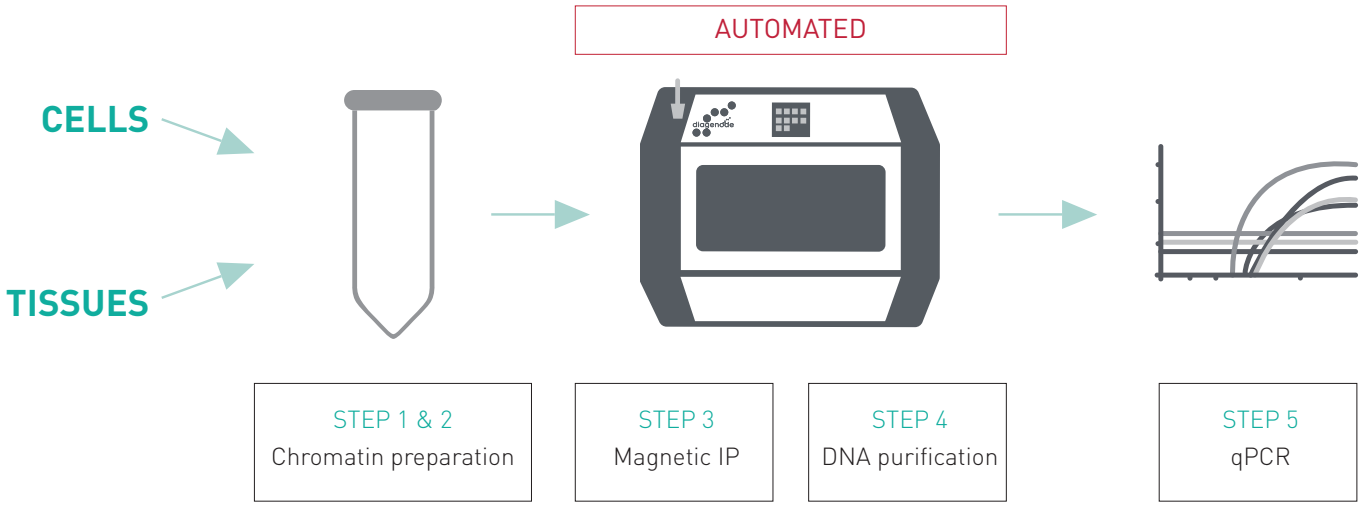
Protocol for ChIP using the IP-Star Compact Automated System

The iDeal ChIP-seq Kit for Histones 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 purification (STEP 4) from cells and tissues. Please refer to the corresponding section.

The chromatin is prepared from a batch of approximately 7 million cells or 30-40 mg of tissue. This will be sufficient for 6 ChIP reactions (using approximately 1 million cells or 5 mg of tissue per IP), 1 input and 1 sample for chromatin shearing assessment. If using a different amount of starting material, please refer to "Remarks before starting".

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

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



At the end, you recover eluted dsDNA in ultrapure solution ready for any desired downstream applications. In case you wish to sequence your samples, the library can be prepared on the IP-Star Compact with the MicroPlex Library Preparation kit V2 (Diagenode, Cat. No. C05010012, C05010014).

STEP 1

Cell collection and DNA-protein cross-linking

1 *Day 1* *1 hour*

FOR CULTURED CELLS

***NOTE**: PBS at different temperatures (ice-cold, room temperature and pre-warmed at 37 °C) will be required at this step.*

For adherent cells:

1.1 Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.

1.2 Remove the medium and rinse the cells with **pre-warmed PBS** (10 ml for a 75 cm2 culture flask). Gently shake the flask for 2 minutes.

1.3 Remove the PBS and add sterile **trypsin-EDTA** to the culture flask to detach adherent cells from the bottom. Add enough trypsin-EDTA to completely cover the cells (e.g. 1 ml for a 75 cm2 culture flask). Gently shake the culture flask for 1-2 minutes or until the cells start to detach.

***NOTE**: The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment may damage the cells. Regularly check if the cells start to detach.*

1.4 Immediately add **fresh culture medium** to the cells when they are detached. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA (e.g. 2 ml for a 75 cm2 culture flask). Transfer cell suspension to a 15 ml tube.

1.5 Rinse the flask by adding **10 ml** of **warm PBS**. Add this volume to your 15 ml tubes containing cells from above point. Proceed immediately with step 1.6.

For suspension cells:

Collect suspension cells in a 50 ml tube and go directly to point 1.6 of the protocol.

1.6 Centrifuge for 5 minutes at 500 x g at room temperature and remove the supernatant.

1.7 Resuspend the cells in **20 ml** of **warm PBS** and count them. Collect the cells (approximately 7 millions) by centrifugation for 5 minutes at 500 x g at room temperature.

1.8 Resuspend the cells (approximately 7 millions) in **500 µl** of PBS (room temperature) and add **13.5 µl** of **37% formaldehyde** (under a fume hood) to each tube. Mix by gentle vortexing and incubate 8 minutes at room temperature to allow fixation to take place.

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

1.9 Add **57 µl of Glycine** to the cells to stop the fixation. Incubate for 5 minutes at room temperature with gentle shaking. Keep everything at 4°C or on ice from now on.

1.10 Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.

1.11 Wash the cells twice with **1 ml** of **cold PBS**: resuspend the cell pellet in **1 ml** of **cold PBS**. Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 1

Tissue disaggregation and DNA-protein cross-linking

1 *Day 1* *30 minutes*

FOR TISSUES

***NOTE**: PBS at different temperatures (cie-cold and room temperature) will be required at this step*

1.1 Weigh 30-40 mg of fresh or frozen tissue in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

1.2 Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade. Disaggregate sample using a Dounce homogenizer or TissueLyzer.

For Dounce homogenizer:

Add 5 µl of protease inhibitors cocktail to 1 ml of ice-cold PBS and resuspend the tissue pieces. Transfer it to the Douce homogenizer and disaggregate using a loose pestle to get a homogeneous suspension.

Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 850 x g for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet. Proceed with the fixation (step 1.3)

For TissueLyzer:

Transfer tissue pieces to 2 ml tubes, supplemented with 2 stainless

steel beads (5 mm) and immerse in liquid nitrogen for a few minutes. Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 8 hours at -80°c) and operate the TissueLyser for 2-4 minutes at 25-30 Hz. Proceed with the fixation on the grinded sample (step 1.3).

1.3 Add **27 µl of 37% formaldehyde** (under a fume hood) to **1 ml of PBS** (room temperature) , add 1 ml of the cross-linking solution directly to the tissue lysate and resuspend the tissue suspension in diluted formaldehyde.

1.4 Mix by vortexing and incubate for 8 minutes at room temperature with gentle rotation on a DiaMag Rotator to allow fixation to take place.

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

1.5 Add **100 µl of Glycine** to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing on a DiaMag rotator.

1.6 Centrifuge samples at 850 x g for 5 minutes at 4°C. Discard the supernatant without disturbing the pellet. Keep everything at 4°C or on ice from now on.

1.7 Wash the pellet twice with **1 ml of cold PBS**: resuspend the cell pellet in **1 ml** of **cold PBS**. Collect the cells by centrifugation at 850 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 2

Cell lysis and chromatin shearing

1 *Day 1* *1 to 2 hours*

FOR CELLS AND TISSUES

2.1 Add **1 ml of ice-cold Lysis Buffer iL1** to the 1.5 ml tube containing cells or tissue suspension. Resuspend the samples by pipetting up and down several times and transfer them to a 15 ml tube. Add **6 ml of Lysis Buffer iL1** and incubate for 10 minutes at 4°C with gentle mixing.

2.2 Pellet samples by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.3 Resuspend the cell pellet in **1 ml of ice-cold Lysis Buffer iL2** by pipetting up and down several times. Add another **6 ml of Lysis Buffer iL2** and incubate for 10 minutes at 4°C with gentle mixing.

2.4 Pellet the cells again by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.5 Prepare **complete Shearing Buffer** by adding **3.5 µl of 200x protease inhibitor cocktail** to **700 µl of Shearing Buffer iS1**. Keep on ice.

2.6 Add **700 µl of complete Shearing Buffer iS1** to the cells (7 million cells) or tissue sample (30-40 mg of tissue). The final cell concentration in the Shearing Buffer should be 1 million of cells or ~5 mg tissue per 100 µl of iS1. Resuspend by pipetting up and down several times and incubate for 10 minutes in ice.

NOTE - *optional, for tissue samples:*

If the suspension is not homogeneous after pipetting, an additional homogenization using a Dounce homogeniser or TissueLyser could be performed.

*For **Dounce homogeniser**, transfer the suspension to the homogeniser, perform several strokes to get a homogeneous suspension and proceed with the sonication as described below.*

*For **TissueLyser**, transfer the suspension to 2 ml tubes, supplemented with 2 stainless steel beads (5 mm). Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 2 hours at 4°c) and operate the TissueLyser for 2-4 minutes at 25 Hz. Proceed with the sonication as described below.*

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

- When using the Bioruptor Pico shear for 5 to 15 cycles [30 seconds "ON", 30 seconds "OFF"].
- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.

***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.*

***NOTE**: We recommend performing pilot experiments for each new sample type using the Chromatin Shearing Optimization kit - Low SDS (iDeal Kit for Histones) (Cat. No. C01020010).*

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

2.9 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol is described in the "Additional Protocols" section.protocol which is adapted to your device:

- When using the Bioruptor Pico shear for 5 to 15 cycles [30 seconds "ON", 30 seconds "OFF"].
- When using the Bioruptor Standard or Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.

***NOTE**: We recommend analysing the shearing for each batch of chromatin. This step can be omitted when sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. Store the chromatin aliquot at -20°C until analysis.*

STEP 3

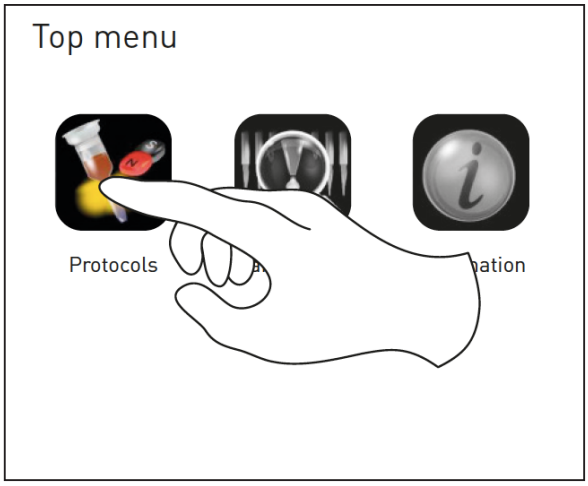
Magnetic immunoprecipitation

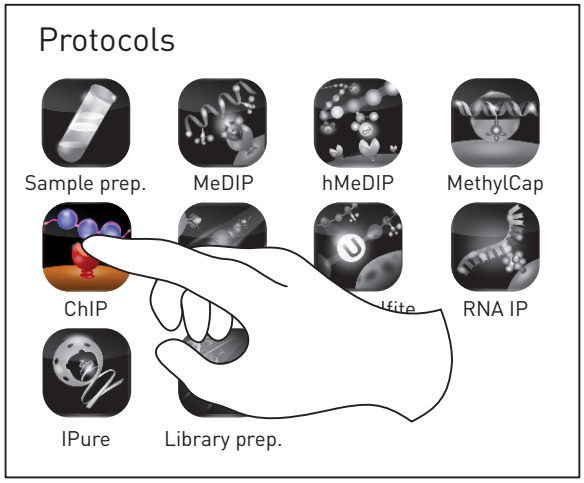
1-2 *Day 1-2* *1.5 hours hands on time and overnight run*

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:

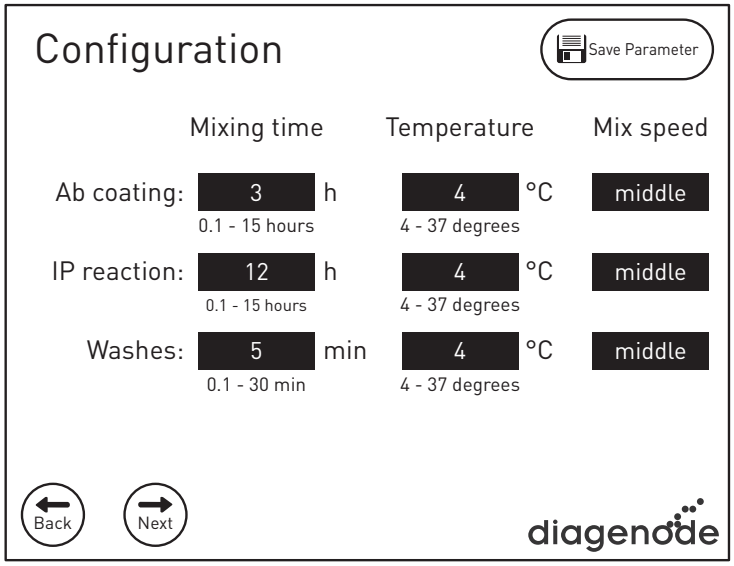
"**ChIP_08_IPure_200_D**" if you plan to run between **1** and **8** samples
"**ChIP_16_IPure_200_D**" if you plan to run between **9** and **16** samples

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.

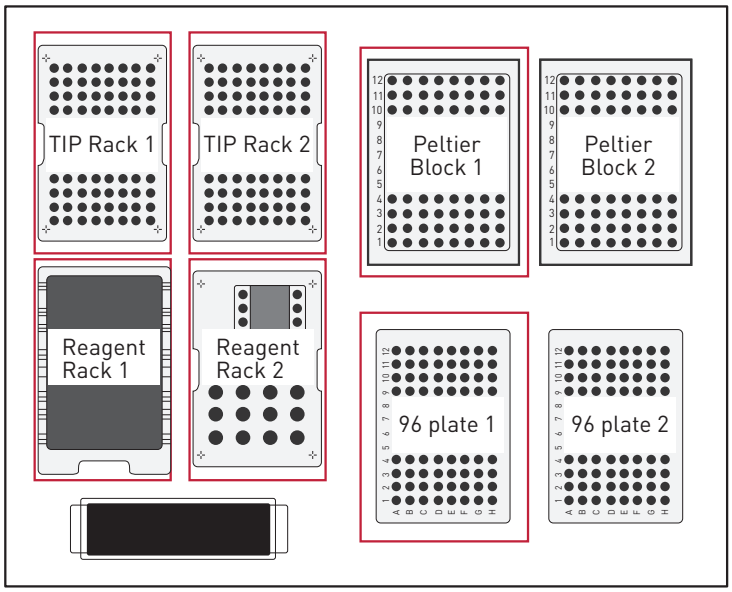
***NOTE**: The Peltier Block is now cooling down to 4°C to keep your samples cold.*

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

Recommended parameters:



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 iC1	80 µl	240 µl	320 µl	400 µl	480 µl	560 µl	640 µl	720 µl
ChIP-seq grade Water	320 µl	960 µl	1280 µl	1600 µl	1920 µl	2240 µl	2560 µl	2880 µl
TOTAL ChIP Buffer	400 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 iC1	960 µl	1040 µl	1120 µl	1200 µl	1280 µl	1360 µl	1440 µl	1520 µl
ChIP-seq grade Water	3840 µl	4160 µl	4480 µl	4800 µl	5120 µl	5440 µl	5760 µl	6080 µl
TOTAL ChIP Buffer	4800 µl	5200 µl	5600 µl	6000 µl	6400 µl	6800 µl	7200 µl	7600 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
5% BSA (DNA free)	2 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	100 µl
ChIP Buffer	100 µl
BSA 5%	4 µl
200x Protease Inhibitor Cocktail	1 µl

- Keep aside **1 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

Elution, decross-linking and DNA purification

2 *Day 2* *30 minutes hands on time and 5 hours run*

Decross-linking

10 minutes hands on time and 4 hours incubation.

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 Briefly spin the samples and place them on DiaMag02 magnetic rack (Cat. No. B04000001).

4.3 Prepare a new strip for the samples and the INPUT.

4.4 Fill the new strip with the INPUT as described in the table below.

	1 INPUT
Sheared chromatin	1 µl
Elution Buffer iE1	95 µl

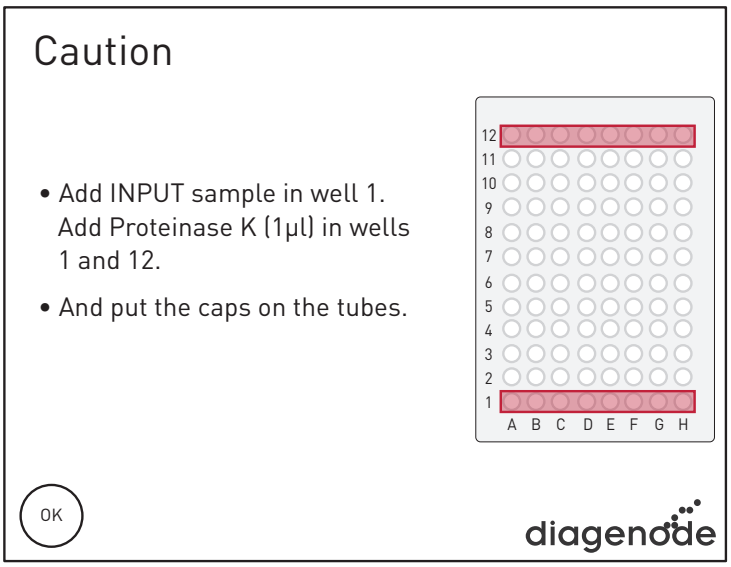
4.5 Regarding the samples, make sure the supernatant is clear. Transfer the supernatant to the new strip and discard the beads.

***NOTE**: To recover all supernatant set 110 µl on your pipette.*

4.6 Add **4 µl of Elution Buffer iE2** (5 M NaCl) in all samples and the INPUT.

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

***NOTE**: The Peltier Block 1 heats evenly, so the strips with samples and INPUT(s) can be placed in every row of this block.*



4.8 Close the door and press "OK" to start the incubation (4 hours, 65°C).

4.9 After the incubation, recover the samples and the INPUT (keep at 4°C).

4.10 Press "OK", then "YES" and "BACK" until the homepage appears on the screen.

You are now ready for purification.

DNA purification

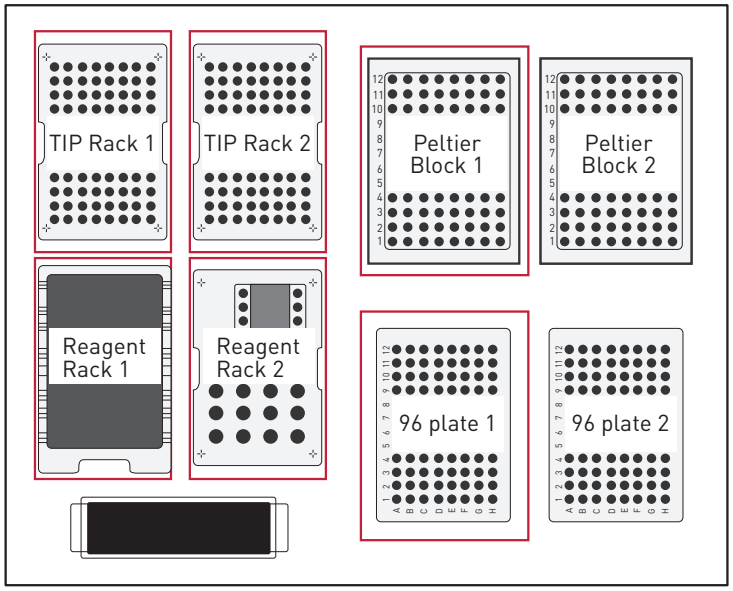
20 minutes hands on time and 40 minutes run for each series of 8 samples

4.11 Select "**Protocols**" icon and then "**IPure**" category.

4.12 Select "**IPure**" protocol for an elution in **50 µl** (if going to perform a **qPCR**) and "**IPure-seq**" protocol for an elution in **25 µl** (if going to **sequence**).

***NOTE**: If you plan to run between:*
1 and 8 samples, choose IPure_08 or IPure-seq_08.
9 and 16 samples, choose IPure_16 or IPure-seq_16.
17 and 24 samples, choose IPure_24 or IPure-seq_24.

4.13 Set up the exact number of samples for your experiment by pressing the black box. Each IP and INPUT has to be counted as a sample.



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

4.14 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 **96 plate 1** with a 96-well Microplate.
- Fill **Peltier Block 1** with 8-tube strips according to the screen.

4.15 Add **2 µl of carrier** to each IP and INPUT sample and place them on the **Peltier Block 1** according to the screen instructions.

4.16 Resuspend and dispense **10 µl of IPure v2 beads** for each sample in the 96-well Microplate as shown on the IP-Star screen.

***NOTE**: Keep the IPure v2 beads in liquid suspension and at 4°C during storage and all handling steps to ensure a high efficiency.*

Make sure the beads are homogeneously in suspension at all the time during pipetting steps because the beads are precipitating rapidly.

4.17 At the first use of the kit, dilute **Wash Buffers 1 and 2** with isopropanol (ratio 1:1) as described in the table below.

	24 rxns kit (C01010057)	100 rxns kit (C01010171)
Wash Buffer w/o isopropanol	2 ml	4 ml
Isopropanol (100%)	2 ml	4 ml
Total volume	4 ml	8 ml

4.18 Dispense **Wash Buffers 1 & 2** with isopropanol in the appropriate container in the IP-Star.

4.19 Dispense **Buffer C** in the appropriate container in the IP-Star.

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

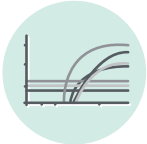
4.21 Close the door, and press "Run" to start.

4.22 IPure protocol is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

4.23 After the run, recover your samples on the upper row of the **Peltier Block 1** (at 4°C). You can now proceed to any desired downstream applications, or store the samples at -20°C or -80°C until further use.

4.24 Press "OK" and "Back" until the homepage appears on the screen. Press "Shutdown" and wait until the screen is black before switching off the IP-Star.

4.25 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

Day 2 *2-3 hours*

***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 4.23) and dilute them 1/10 using ChIP-seq grade water.

5.2 Prepare the qPCR mix as follows (20 µl reaction volume using the provided control primer pairs):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of water
- 5 µl of diluted immunoprecipitated 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		

**Carefully check 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 immunoprecipitated 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:

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

- 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 (1 µl INPUT vs 100 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:

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

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:*

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

5.6 If an expected enrichment over positive loci with a low background is observed, proceed to the library preparation using an appropriate protocol (not supplied with iDeal ChIP-seq Kit for Histones).

Library preparation and sequencing recommendations

The automated and manual version of the iDeal ChIP-seq for Histones protocol has been validated for ChIP-seq on Illumina®HiSeq Next-Gen sequencers.

For library preparation compatible with Illumina® sequencers, we highly recommend using Diagenode kits: MicroPlex Library Preparation Kit v2 (Cat. No. C05010012, 12 indices; C05010014, 48 indices) or iDeal Library Preparation Kit x24 (incl. Index Primer Set 1) (Cat. No. C05010020).

ChIP-seq data analysis recommendations

ChIP-seq data analysis workflow

In the following chapter we will guide you through the basics of ChIP-seq data analysis. We will also provide some examples of software tools suitable for each step. We cite numerous analysis tools, including free and commercial softwares.

1. (Optional step) Trimming: use trimming to get rid of low quality bases and artefacts in the readset, such as adapter contaminations
   a. Cutadapt
   b. Trim Galore!
   c. Trimmomatic
2. Alignment: in this step you will map the reads against a known reference sequence
   a. ELAND
   b. Tmap
   c. BWA
   d. Bowtie2
3. (Optional step) Quality control: you can check the general quality of the sequencing and the alignment
   a. FastQC
   b. Picard Tools
4. Peak calling: during peak calling the software will detect sites of enrichment along the genome
   a. MACS2
   b. SICER
   c. ZINBA
   d. PeakRanger

e. Pyicoteo
f. MUSIC
g. SPP
h. hiddenDomains

After above described basic analysis, the peaks can be analyzed further to get answers to our biological questions. There are countless ways and tools for further analyses, the project goals determine which ones we should pick. Just as in the case of basic analysis, we recommend to thoroughly study the manual of the chosen software tool to understand its purpose and its function. Below we will list a few common analysis types to further process the peaks, along with example tools again.

5. Visualization: the peaks, the reads, and other data (e.g. gene positions) can be displayed in a suitable genome browser

a. IGV
b. IGB
c. USCS Genome Browser

6. Descriptive statistics: the peaks can be described in various useful ways, like how many reads fall into them, what is their number, mean size and significance etc.; these figures are also very useful when you compare datasets

a. Peak callers usually provide per peak and/or summary statistics after peak detection
b. HOMER
c. GREAT
d. BEDTools

7. Motif search: in the case of TF data peaks frequently occur at specific motifs, though some HM peaks can also show preference to certain sequence patterns; therefore identifying these motifs and checking enrichments over them is a good practice for TF data analysis, and also applicable for HM data.

a. HOMER
b. MEME Suit

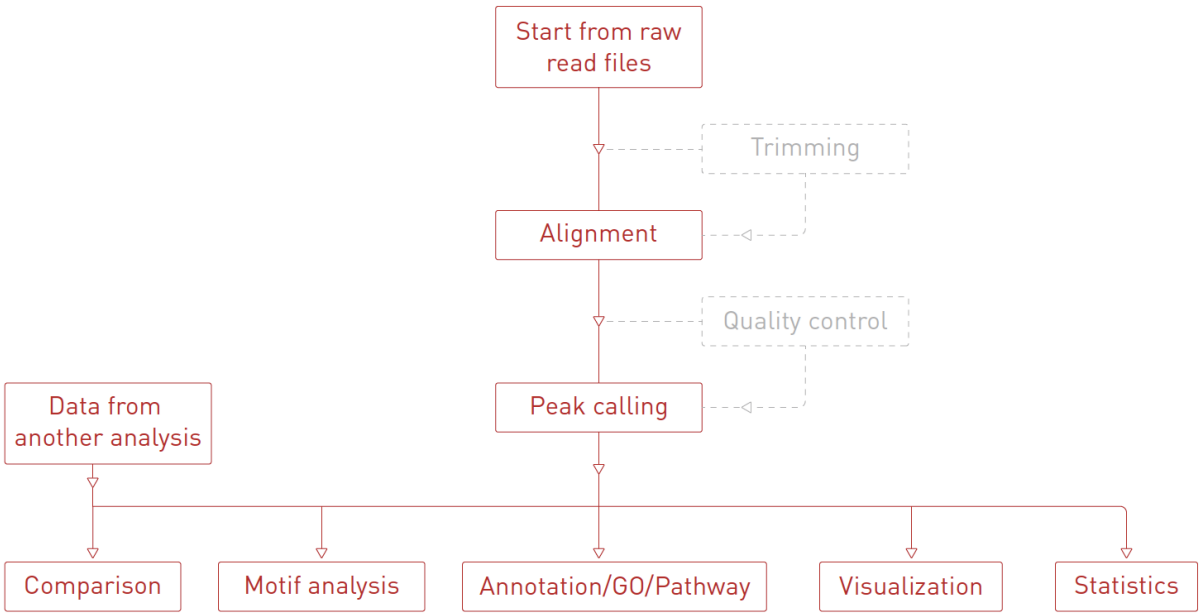
8. Annotation, Gene Ontology, Pathway analysis: after annotation/GO/Pathway analysis you will get a clear picture about which genomic features or pathways your peaks are associated to, providing an important information about disease mechanisms, the role of DNA binding proteins, or treatment effects

   a. HOMER
   b. GREAT
   c. BEDTools
   d. ReactomePA

9. Comparative analysis: this type of analysis is the obvious choice when you have several datasets from comparable conditions (e.g. treated and untreated cells) or when you want to check the performance of your ChIP-seq by comparing it to a reference; there are many different ways to compare peaks, including checking the overlaps, the correlation of enrichment sizes and performing statistical tests on the peaksets

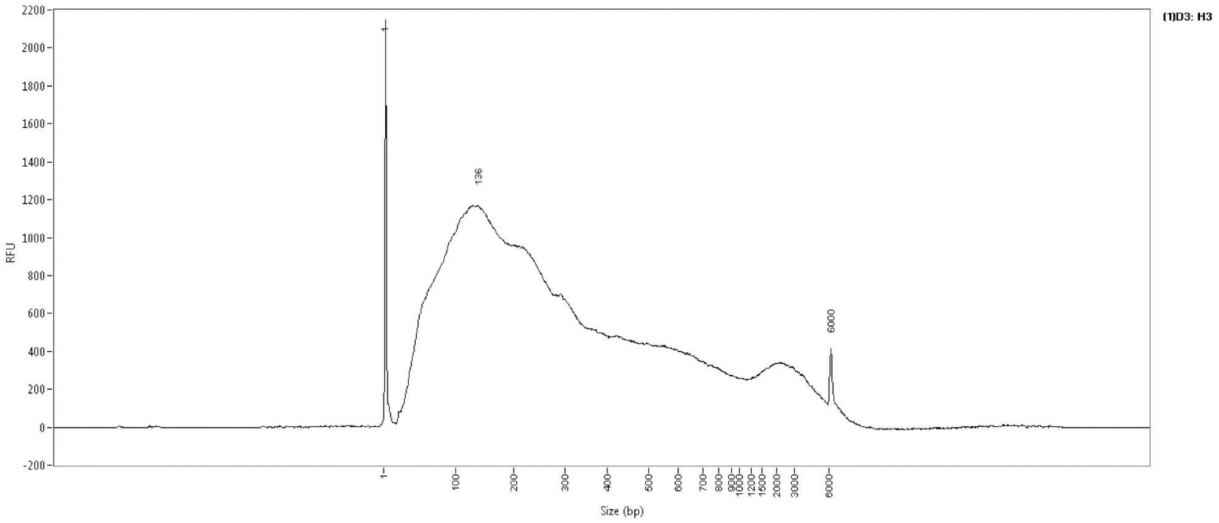
   a. HOMER
   b. BEDTools
   c. DiffBind

ChIP-seq data analysis workflow

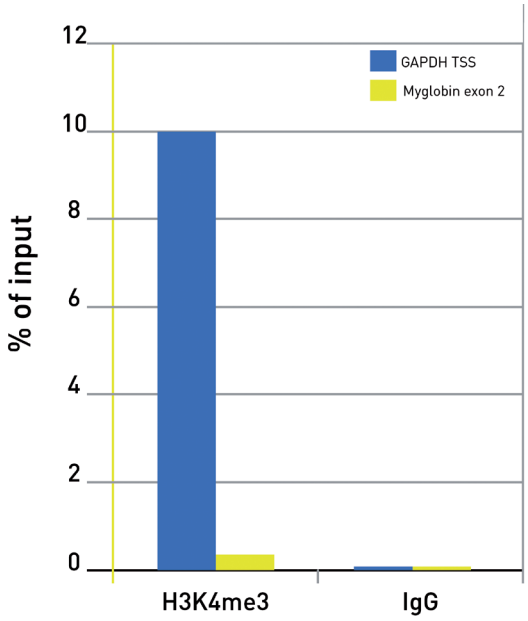


Example of results

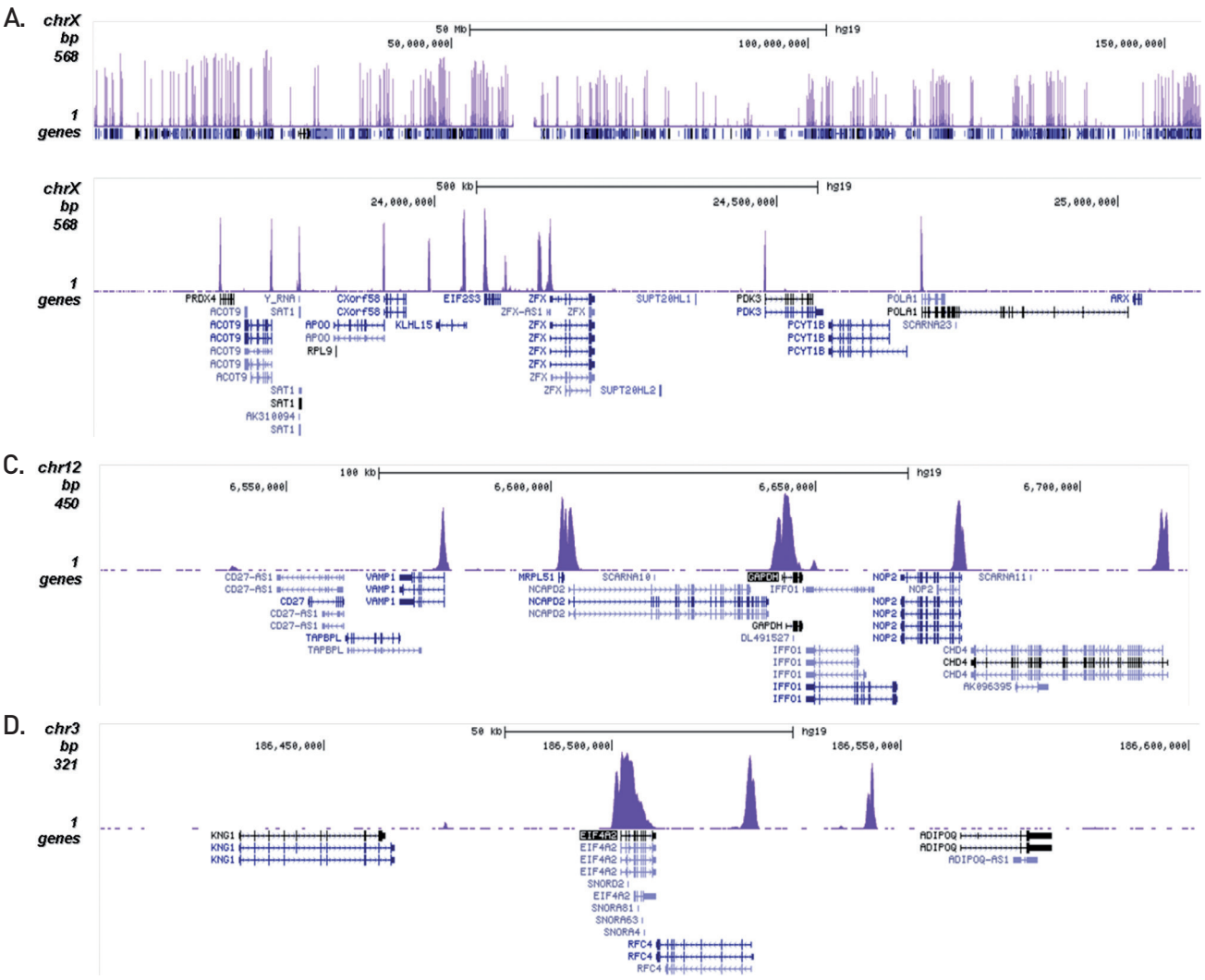
Chromatin preparation and immunoprecipitation analysis from cells



Successful chromatin shearing from HeLa cells using the Bioruptor Pico and the iDeal ChIP-seq Kit for Histones. Chromatin from HeLa was sheared using the Bioruptor Pico for 10 cycles (30'' On/30''Off). Chromatin was decrosslinked and purified accordingly to the protocol for chromatin shearing analysis and fragment size was assessed using the Fragment Analyzer (Advanced Analytical).

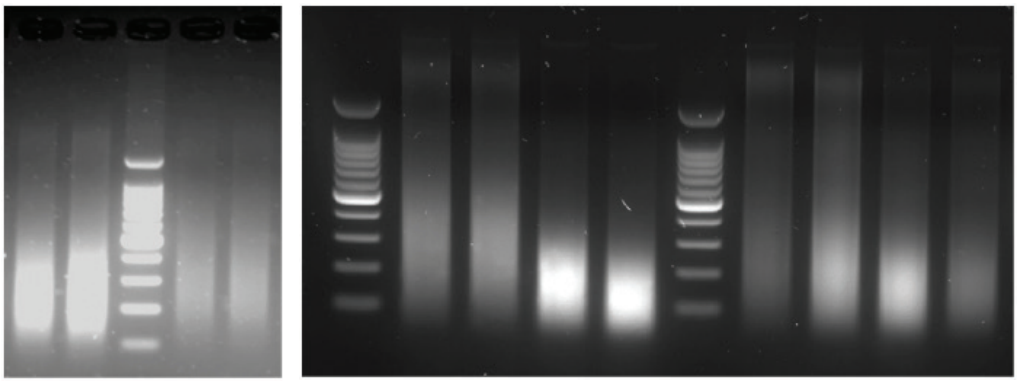


Chromatin preparation and immunoprecipitation analysis from HeLa cells using control H3K4me3 antibody. ChIP was performed on human HeLa using control H3K4me3 and negative IgG control antibodies from the iDeal ChIP-seq Kit for Histones. Quantitative PCR was performed with the positive control GAPDH TSS promoter and the negative control Myoglobin exon 2 primer sets. The recovery, expressed as a percent of input is shown.



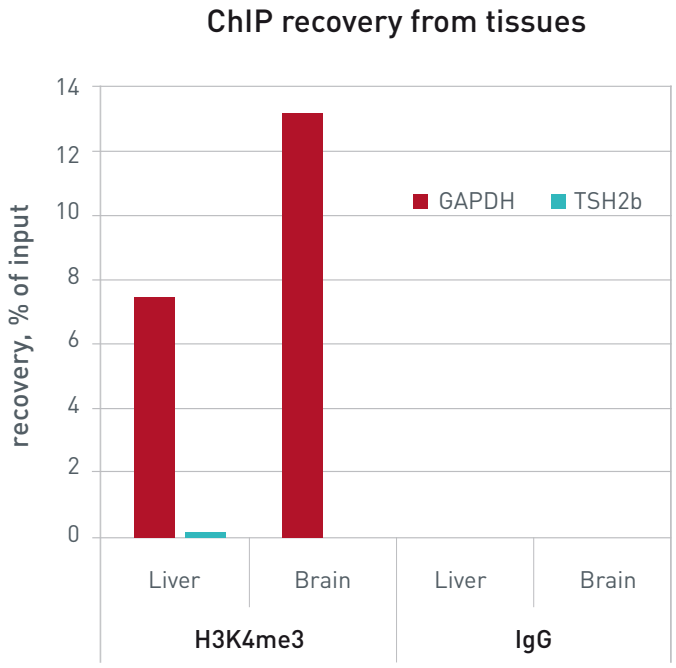
ChIP-seq results obtained with the Diagenode antibody directed against H3K4me3. ChIP was performed on sheared chromatin from 1 million HeLa cells using 1 µg of the Diagenode antibody against H3K4me3 (Cat. No. C15410003) and the iDeal ChIP-seq Kit for Histones. The IP'd DNA was subsequently analysed on an Illumina HiSeq 2000. Library preparation, cluster generation and sequencing were performed according to the manufacturer's instructions. The 50 bp tags were aligned to the human genome using the BWA algorithm. The figure shows the peak distribution along the complete sequence and a 1.6 Mb region of the human X-chromosome (figure A and B) and in two regions surrounding the GAPDH and EIF4A2 positive control genes, respectively (figure C and D).

Chromatin preparation and immunoprecipitation analysis from tissue



Liver *Brain* *Liver* *Brain*

Successful chromatin shearing from mouse liver and brain using the Bioruptor Plus or Pico and the iDeal ChIP-seq Kit for Histones. Left: chromatin from mouse liver and brain was sheared using the Bioruptor Plus for 3x10 cycles (30'' On/30''Off). Right: chromatin from mouse liver and brain was sheared using the Bioruptor Pico for 5 and 10 cycles (30'' On/30''Off). 100 bp ladder is loaded.



Chromatin immunoprecipitation analysis from mouse liver and brain using control H3K4me3 antibody. ChIP was performed on mouse brain and liver using control H3K4me3 and negative IgG control antibodies from iDeal Chip-seq Kit for Histones. Quantitative PCR was performed with the positive control mouse GAPDH promoter (Cat. No. C17021045-50) and the negative control mouse TSH2B (Cat. No. C17021042-50) primer sets (not included in the kit). The recovery, expressed as a percent of input is shown.

Protocol for chromatin shearing analysis

General remarks

We recommend using an agarose gel analysis or the Fragment Analyzer (Advanced Analytical) for the size assessment. Although the microfluidics technology (Agilent BioAnalyzer 2100, Agilent 2200 Tape Station System, Perkin Elmer Caliper LabChip GX Touch) is widely used for size assessment of DNA fragments before library preparation for NGS, this technology is less optimal for sheared chromatin resulting in a regular over-estimation of high molecular weight fragments.

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 (1 h, optional but highly recommended for an accurate size assessment)
- Reverse crosslinking (4 h or overnight)
- DNA purification using IPure beads v.2 (30 minutes)
- Fragment size assessment (agarose gel or Fragment Analyzer from Advanced Analytical) (1 h)

RNAse treatment

__NOTE__: RNase cocktail (e.g. Ambion, AM2286A) is not supplied with iDeal ChIP-seq Kit for Histones

1. Take **50 µl of sheared chromatin** (step 2.9 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-seq 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

1. Add **50 µl of Elution Buffer iE1** to the sample.

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

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

DNA purification

__NOTE__: The protocol below describes DNA purification using the IPure magnetic beads included in the kit. Other methods of DNA purification (columns-based DNA clean-up, e.g. DiaPure columns from Diagenode or a phenol–chloroform extraction followed by ethanol precipitation) can be used also.

1. Add **2 µl of carrier** to the sample.

2. Add **100 µl of 100% isopropanol** to the sample.

   __NOTE__: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

3. Resuspend by vortexing the **IPure beads v2** and add **20 µl** to the sample.

4. Incubate samples for 10 minutes at room temperature on the DiaMag rotator.

5. Briefly spin the tubes, place in the DiaMag1.5 magnetic rack, wait 1 minute and discard the buffer.

6. Add **100 µl of Wash Buffer 1** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes, place in the DiaMag 1.5ml magnetic rack, wait 1 minute and discard the buffer.

7. Add **100 µl of Wash Buffer 2** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the DiaMag 1.5ml magnetic rack, wait 1 minute and discard the buffer.

8. Spin the tubes again and place them on the DiaMag 1.5ml magnetic rack. Discard the remaining Wash Buffer 2 if necessary. Resuspend the beads pellet in **25 µl of Buffer C**. Incubate at room temperature for 15 minutes on the DiaMag rotator.

9. Spin the tubes and place them into the DiaMag 1.5ml magnetic rack. Wait 1 minute and transfer the supernatants into a new 1.5 ml tube. Discard the beads.

Fragment size assessment

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 from Advanced Analytical (Standard Sensitivity NGS Fragment Analysis Kit (DNF-473).

FAQs

Is the included control H3K4me3 antibody compatible with mouse?

Yes, the included control H3K4me3 antibody is compatible with mouse.

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-seq 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 selected from a source other than Diagenode, please refer to the corresponding technical data sheet. If no required amount of antibody is given, we suggest performing a titration experiment. For ChIP-seq 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?

20 µl of DiaMag Protein A-coated magnetic beads can bind 7 µg of antibody.

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

The Ideal ChIP Kit for Histones contains DiaMag Protein A-coated magnetic beads which 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 interests 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 (C03010021-220).

Can I use a monoclonal antibody in ChIP?

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.

What is the composition of buffers included in the kit?

The composition of the buffers is proprietary.

Can I use the Auto iDeal ChIP-seq Kit for Histones with the old version of IP-Star?

Yes, please, refer to the corresponding manual: https://www.diagenode.com/files/products/kits/MA_Auto-iDeal-ChIP-seq-kit-Histones-v2-07-15.pdf.

Regarding the questions related to the IP-Star Compact, please refer to the troubleshooting guide and the list of error codes pages 29-31 of the SX-8G IP-Star Compact manual.

Related products

Product	Cat. No.
Chromatin shearing optimization kit - Low SDS (iDeal Kit for Histones)	C01020010
iDeal ChIP-seq Kit for Transcription Factors	C01010055
Chromatin shearing optimization kit – Low SDS (iDeal Kit for TFs)	C01020013
MicroPlex Library Preparation Kit v2 (12 indices)	C05010012
MicroPlex Library Preparation Kit v2 (48 indices)	C05010014
iDeal Library Preparation Kit x24 (incl. Index Primer Set 1)	C05010020
DiaMag rotator	B05000001
DiaMag 1.5 magnetic rack	B04000003
Bioruptor Pico	B01060001
IP-Star Compact Automated System	B03000002

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

ChIP-seq grade antibody	Cat. No.
H2A.Z polyclonal antibody	C15410201
H2BK15ac polyclonal antibody	C15410220
H3K27ac polyclonal antibody	C15410196
H3K27me3 polyclonal antibody	C15410195
H3K36me3 polyclonal antibody	C15410192
H3K4me1 polyclonal antibody	C15410194
H3K4me3 polyclonal antibody	C15410003
H3K79me3 polyclonal antibody	C15410068
H3K9/14ac polyclonal antibody	C15410200
H3R17me2(asym)K18ac polyclonal antibody	C15410171
H4K20ac monoclonal antibody	C15210008
H4K20me3 polyclonal antibody	C15410207
macroH2A.1/H2A.2 monoclonal antibody	C15210003

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