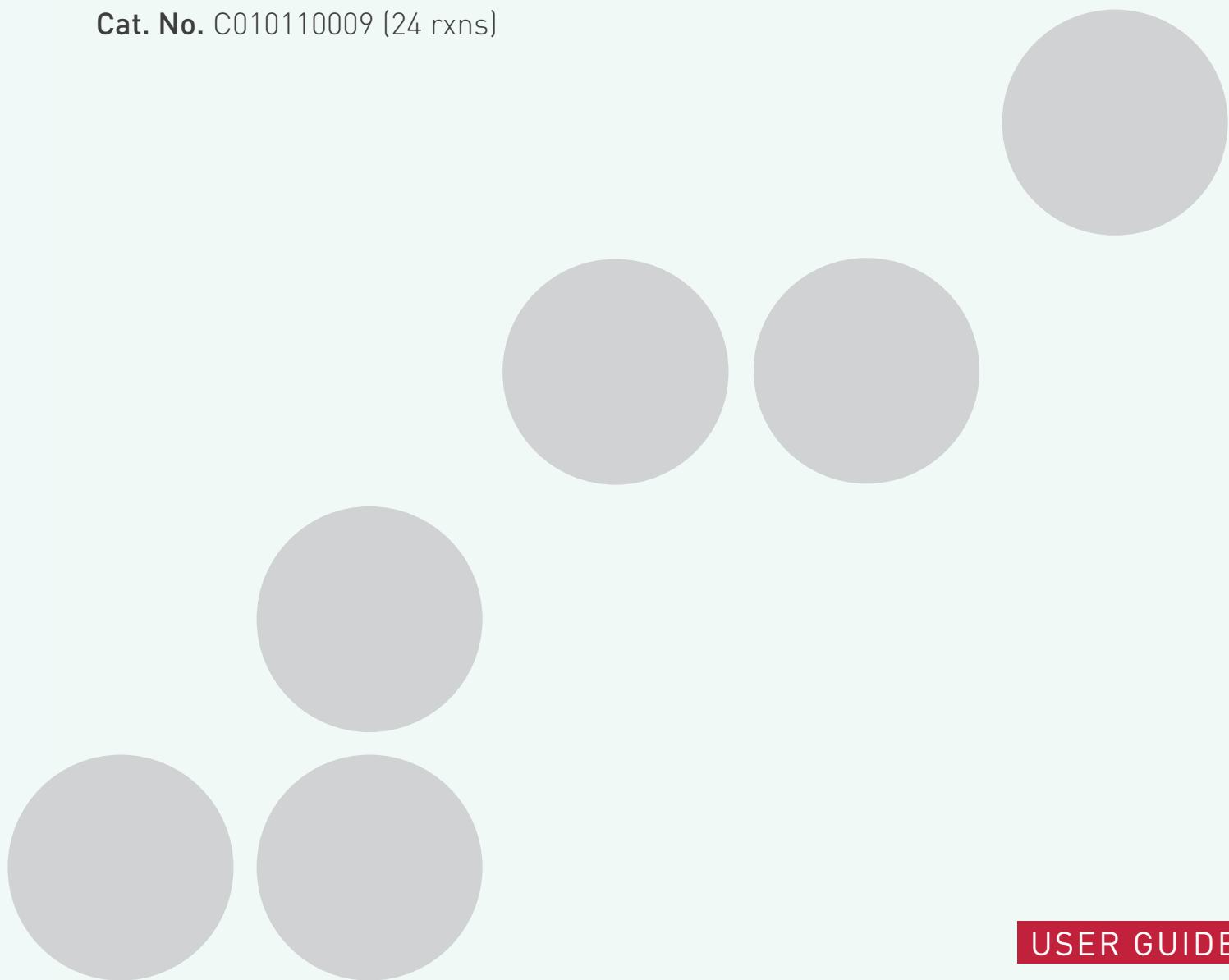




Innovating Epigenetics Solutions

ChIPmentation Kit for Histones

Cat. No. C010110009 (24 rxns)



USER GUIDE

Version 1 21_01_2021

The ChIPmentation Kit for Histones has been validated manually and on IP-Star® Compact Automated System. The corresponding protocols are described in this document.



Please read this manual carefully
before starting your experiment

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Introduction

Association between proteins and DNA influences various vital cellular functions such as cellular proliferation and epigenetic regulation. It is therefore crucial to understand these interactions and the mechanisms by which they control and guide gene expression. Chromatin Immunoprecipitation (ChIP) is a technique to analyze the association of proteins with specific genomic regions in intact cells, by using a specific antibody that targets the protein of interest. ChIP can be used to study changes in epigenetic signatures, chromatin remodeling and transcription regulator recruitment to specific genomic sites.

In addition, Next-Generation Sequencing (NGS) makes it possible to analyze DNA-protein interactions at a genome-wide level, but requires many additional processing steps of the samples in order to generate libraries with sequencing adaptors.

In traditional ChIP-seq library preparation, the ligation of adaptors is performed after chromatin immunoprecipitation and prior to library preparation. This process requires three additional steps that include the adaptor ligation that may introduce artifacts and the need for greater hands-on time.

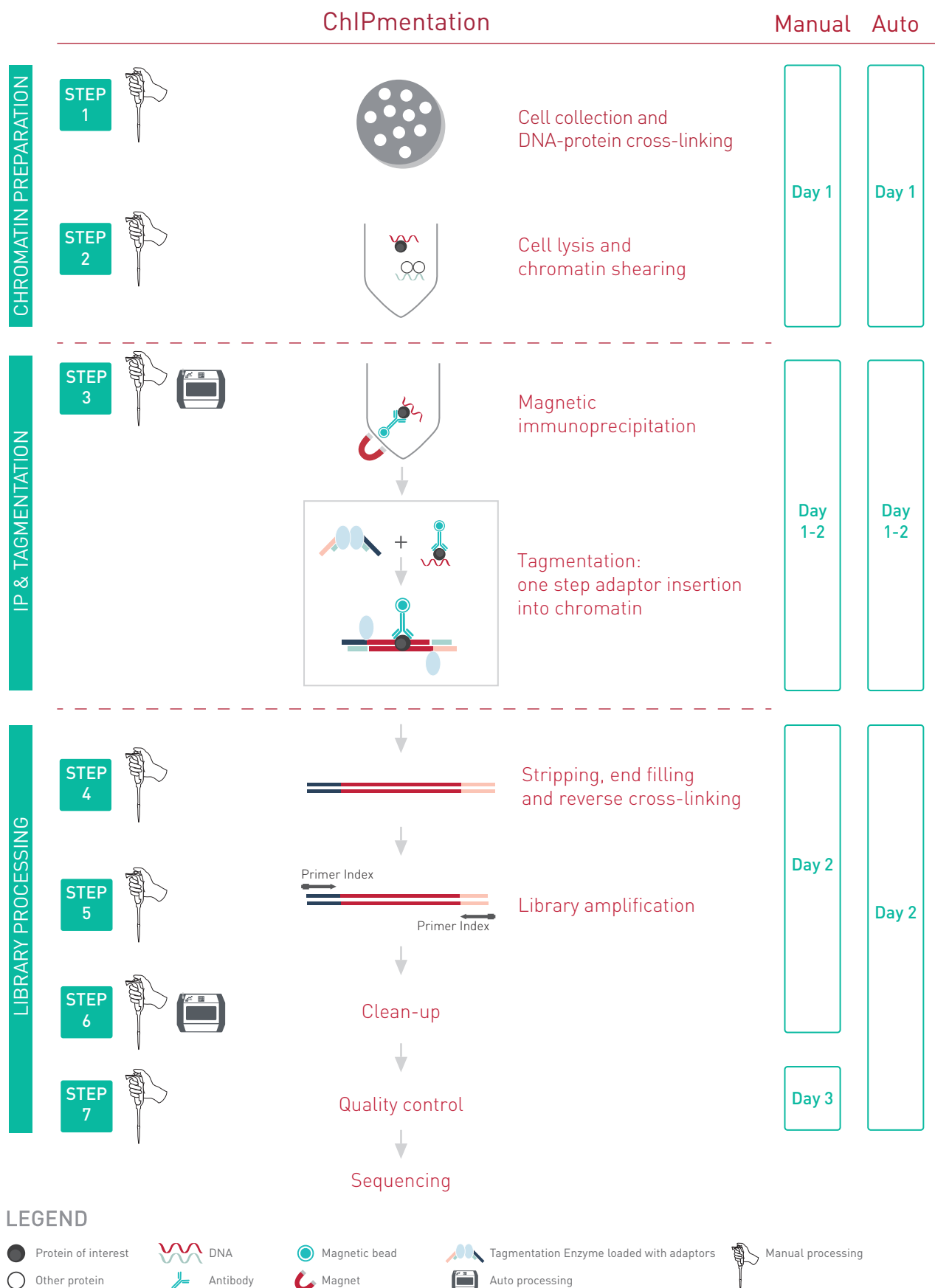
The new Diagenode ChIPmentation technology solves this problem. ChIPmentation is based on tagmentation which enables the integration of the library preparation during the ChIP itself using transposase and sequencing-compatible adaptors. Unlike standard library preparation techniques that require multi-step ligation, ChIPmentation incorporates a much easier and shorter protocol. Two versions of protocol have been validated: manual and an automated protocol on the IP-Star Compact Automated System. The combination of direct adaptor incorporation and automation allows for higher sensitivity from low cell numbers and reproducibility of results.

This kit was developed in collaboration with CeMM in Vienna.

Benefits of the ChIPmentation system for optimal ChIP-seq

- Ensure high quality data on low cell numbers and rare cell types
- Save one full day from standard protocols in generating ChIP-seq libraries
- Benefit from the elimination of sequencing adaptor dimers
- Enjoy an easier protocol
- Automate on the IP-Star to support standardization and reproducibility

Kit method overview & time table



Kit materials

The ChIPmentation Kit for Histones contains reagents necessary for chromatin preparation, chromatin immunoprecipitation and library preparation for NGS. The primer indexes for multiplexing are not included in the kit and must be purchased separately. The list of available primer indexes kits can be found in the section: Required materials not provided.

The kit allows the preparation of 4 chromatin preparations (up to 7 M cells per batch), 24 ChIP reactions, 4 inputs and 24 libraries. For IP-Star users : the kit includes enough reagents for 24 ChIP reactions, which can be performed in up to 4 runs on IP-Star.

Components supplied with the ChIPmentation for histones

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

Table 1. Components stored at +4°C

Component	Cap color	Volume	Storage
Glycine	blue	400 µl	4°C
Shearing Buffer iS1	na	7.2 ml	4°C
DiaMag protein A-coated magnetic beads	none	480 µl	4°C
Wash buffer iW1	na	8.4 ml	4°C
Wash buffer iW2	na	8.4 ml	4°C
Wash buffer iW3	na	8.4 ml	4°C
ChIP-seq grade water	na	7 ml	4°C
Lysis Buffer iL1	na	40 ml	4°C
Lysis Buffer iL2	na	40 ml	4°C
5x ChIP Buffer iC1	na	2.2 ml	4°C
Tagmentation Buffer	blue	870 µl	4°C
Wash Buffer tagW1	na	15,6 ml	4°C
Wash Buffer tagW2	na	9,6 ml	4°C
Stripping Reagent	blue	300 µl	4°C
MgCl ₂	blue	300 µl	4°C
Resuspension Buffer	blue	500 µl	4°C

Table 2. Components stored at -20°C

Component	Cap color	Volume	Storage
Protease inhibitor cocktail	black	72 µl	-20°C
5% BSA (DNA free)	black	144 µl	-20°C
Rabbit IgG (1 µg/µl)	white	8 µg	-20°C
ChIP-seq grade antibody H3K4me3 (1µg/µl)	white	8 µg	-20°C
ChIP-seq grade GAPDH TSS primer pair (human)	green	96 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair (human)	red	96 µl	-20°C
Tagmentase (loaded)	yellow	24 µl	-20°C
2x High-Fidelity Mastermix	violet	820 µl	-20°C
100 x SYBR	brown	3 µl	-20°C

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml tubes
- RNase/DNase-free 0.2 ml tubes (or 8-tube strips)
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS) buffer
- 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)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- 100% Ethanol, Molecular Grade
- One primer index for tagmented libraries kit among the following:
 - Single-indexes for Tagmented libraries (Diagenode, 8 SI - Cat. No. C01011033, 24 SI - Cat. No. C01011032)
 - Unique Dual-Indexes for tagmented libraries (Diagenode, 8 UDI - Cat. No. C01011035, 24 UDI - Set I - Cat. No. C01011034, 24 UDI - Set II - Cat. No. C01011036)

Equipment

- 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) or
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- DiaMag Rotator (Rotating wheel) (Diagenode, Cat. No. B05000001)
- Magnetic rack for 1.5 ml tubes: DiaMag1.5 (Diagenode, Cat. No. B04000003)
- Magnetic rack for 0.2 ml tubes: DiaMag02 (Diagenode, Cat. No. B04000001)

- Vortex
- Heating block
- Qubit® Fluorometer (ThermoFisher Scientific)
- qPCR cycler
- Thermocycler
- Sizing equipment such as BioAnalyzer (Agilent) or Fragment Analyzer (Advanced Analytical) and their associated high sensitivity kits.

Additional supplies if working with IP-Star Compact

- IP-Star® Compact Automated System (Diagenode, Cat. No. B03000002)
- 200 µl strips (8 tubes/strip) + cap strips for IP-Star Compact Automated System (Cat. No. C30020002)
- 96 well microplates for IP-Star Compact Automated System (Cat. No. C30080030)
- Tips (box) (Cat. No. C30040021)
- Tips (bulk) (Cat. No. C30040020)
- 2 ml microtube for IP-Star Compact Automated System (Cat. No. C30010014)
- Medium reagent container for IP-Star Compact Automated System (Cat. No. C30020003)

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
- IPure kit v2 (Diagenode, Cat. No. C03010014) – for chromatin shearing assessment

Remarks before starting

1. 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 and it is crucial to keep these volumes consistent for optimal results.

ChIPmentation enables working with a low number of cells. Depending on the abundance of the target, the specificity of the antibody, and the number of cells available, the protocol can be adjusted, following one of those two options:

- a. For using lower numbers of cells per IP, 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 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. If starting with a number of cells that is different from the standard protocol, 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 iL1 and iL2 buffers using 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 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.

2. 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 (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.

3. 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 ChIPmentation 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 (<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. As the DNA is also fragmented during the tagmentation, ChIPmentation is less sensitive to the presence of large fragments than classical ChIP-seq. Avoid over-sonication, as it may lead to a drop in efficiency in ChIP experiments.

4. 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.

5. ChIP-seq grade antibodies

The quality of antibodies used in ChIP-seq is essential for success. It is recommended using 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

6. Input

The input sample corresponds to entirety of the DNA that undergoes the full ChIP procedure without any immunoselection. The input sample is used as a reference to calculate the recovery by qPCR at the end of the ChIP procedure. We recommend including one input for each series of ChIP reactions. The input is a good control for qPCR, however it is not incubated with the Tagmentation enzyme and therefore it cannot be sequenced.

7. IP controls and normalization

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 using the positive control ChIP-seq grade H3K4me3 antibody and the negative control IgG at least once per experiment. It is necessary to include one IgG control in each series of ChIP reactions and to sequence it because it will be used by most of the bioinformatics tools for analysis of ChIP-seq data. The IgG sequencing profile serves to model the background of the ChIP experiment and to determine the bias which may result from experimental conditions.

8. Tagmentation time

The optimal tagmentation time can vary between 1 and 30 minutes depending on several factors like the abundance of the target, the cell number and the affinity of the antibody for the target. The recommended 10 minutes have been validated on multiple histone marks and cell numbers, therefore it should be suitable for a large range of conditions. Nevertheless if the Ct values obtained at step 5.6 are too high, resulting in a needed number of amplification cycles superior to 17, the tagmentation time may be increased in order to improve the library preparation efficiency. At the opposite, if the percentage of recovery after the immunoprecipitation is not satisfying the tagmentation time may be decreased in order to increase signal-to-noise ratio.

9. Primer indexes

Diagenode provides several kits containing primer indexes compatible with the ChIPmentation kit, in order to give flexibility. Diagenode primer indexes for tagmented libraries are available in several formats allowing to choose between single or dual-indexing, and several numbers of different indexes. For more details about the differences between the kits and pooling guidelines for the indexes, please check the Primer index for tagmented libraries manual : https://www.diagenode.com/en/documents/primer-indexes-for-tagmented-libraries_manual

10. Quantification

After ChIPmentation, determine the concentration of the libraries with a highly sensitive method such as the dsDNA HS Assay Kit on the Qubit® system from ThermoFisher Scientific. PicoGreen® is also suitable but UV spectrophotometric methods such as the NanoDrop are usually not sufficiently sensitive.

There are several approaches available for library quantification including real-time PCR, UV absorption, fluorescence detection, or sizing and quantification using the Agilent Bioanalyzer or Fragment Analyzer (Advanced Analytical). It is important to understand the benefits and limitations of each approach.

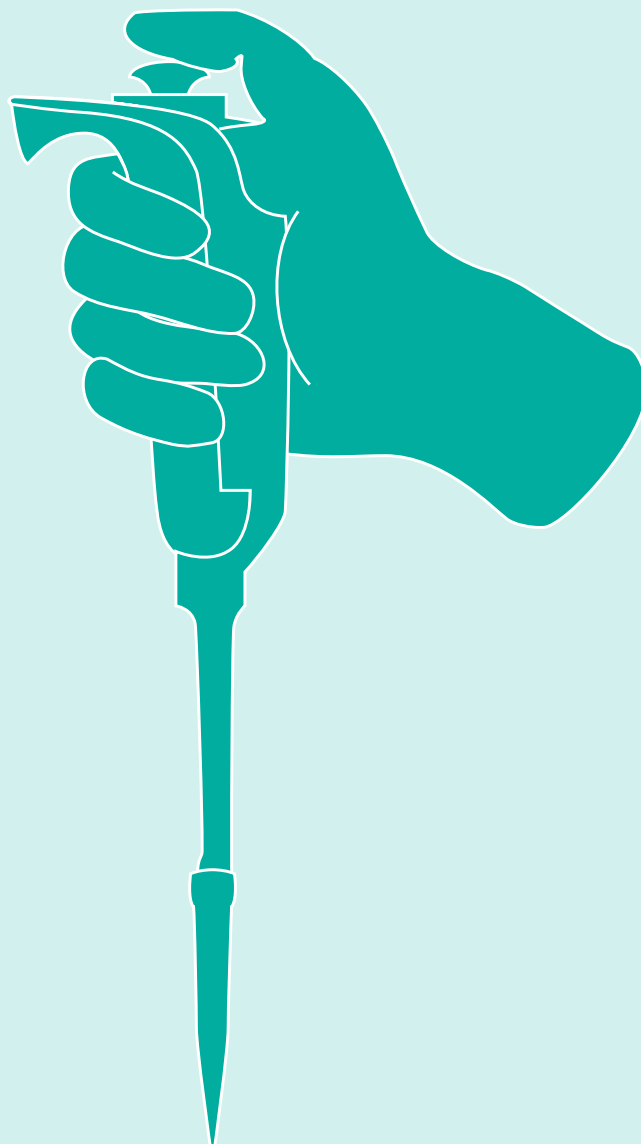
Real-time PCR-based approaches such as the KAPA Library Quantification Kit from Kapa Biosystems) quantify the library molecules that carry the Illumina adapter sequences on both ends and, therefore reflect the quantity of the clustering competent library molecules. This approach assumes a relatively uniform size of sheared or fragmented starting gDNA inserts used for library construction. Quantification by PCR can be done on unpurified libraries.

The Agilent Bioanalyzer system or Fragment Analyzer (Advanced Analytical) provide sizing and quantification information about the library analysed, but not about the clustering competency. Quantification can be done both on unpurified or purified samples. In a case of unpurified samples, a region corresponding to libraries should be limited in order to discriminate between primers/adaptors and a library itself.

UV absorption/fluorescence detection-based methods (i.e., Nanodrop® (Thermo Scientific), Qubit®2.0 Fluorometer (Life Technologies), or QuantiT™ PicoGreen® dsDNA Assay Kit (Life Technologies) simply quantify total nucleic acid concentration. These methods do not discriminate adapter presence and offer no information about the size of the library molecules. They can be used only on purified libraries. We better recommend fluorescence-based assays than spectrophotometric measurements (e.g. NanoDrop) due to higher specificity and sensitivity.

11. 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 human 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. You can dilute the DNA 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



STEP 1



Day 1



30 minutes

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

For adherent cells:

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

***NOTE:** Cold PBS will be needed at step 1.12.*

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. Gently shake the culture flask for **1-2 minutes** or until the cells start to detach. The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment with trypsin 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. Transfer cell suspension to a 50 ml tube.

1.5 Rinse the flask by adding **10 ml of PBS**. Add this volume to your 50 ml tubes containing cells from point 1.4. 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 PBS and count them. Collect the cells by centrifugation for 5 minutes at 500 x g (at room temperature) and remove supernatant.
- 1.8 Resuspend the cells in PBS to obtain a concentration of 7 million cells per 500 µl of PBS. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.

NOTE: If desired, the cell concentration can be adjusted from 1 to 10 million cells per 500 µl.

- 1.9 Under a fume hood, add 13.5 µl of 37% formaldehyde to each tube containing 500 µl of cell suspension. 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".

- 1.10 Add 57 µl of Glycine (blue cap) 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.
- 1.11 Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.
- 1.12 Wash the cells twice with 1 ml of cold PBS as follows:
Add cold PBS
Invert the tube 2-3 times
Centrifuge at 500 x g 5 minutes at 4°C
Discard supernatant

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

1 Day 1  1 to 2 h

Cell lysis and chromatin shearing from cells

- 2.1** Add **1 ml of ice-cold Lysis buffer iL1** to the 1.5 ml tube containing 7 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 6 ml of buffer iL1 and incubate for **10 minutes** at 4°C with gentle mixing.

***NOTE:** For scaling up or down, use 1 ml of buffer iL1 per 1 million of cells.*

- 2.2** Pellet the cells by centrifugation at 500 x g and 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 buffer iL2** and incubate for **10 minutes** at 4°C with gentle mixing.

***NOTE:** For scaling up or down, use 1 ml of buffer iL2 per 1 million of cells.*

- 2.4** Pellet the cells again by centrifugation for **5 minutes** at 500 x g and 4°C and discard the supernatant.

- 2.5** Prepare complete Shearing buffer by adding 3.5 µl of **200x protease inhibitor cocktail** (*black cap*) to **700 µl of Shearing buffer iS1**. Keep on ice.

***NOTE:** For scaling up or down, prepare 100 µl of complete Shearing Buffer per 1 million cells.*

- 2.6** Add **700 µl of complete Shearing buffer iS1** to 7 million cells. The final cell concentration in the shearing buffer should be 1 million of cells per 100 µl of iS1. Resuspend the cells by pipetting up and down several times. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes:

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

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

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 12 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 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 μ l 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 optimal 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



1-2 Day 1-2

1 h, overnight incubation, 1.5 h

Magnetic immunoprecipitation and tagmentation

- 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 DiaMag1.5 magnetic rack. 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).

Set aside **1 µl of the sheared chromatin** to use as **INPUT** sample starting from the step 4.1.
- 3.5 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). Use 1 µg of Rabbit IgG for the negative control IP.

Component	Volume per reaction
BSA (<i>black cap</i>)	6 μ l
200x protease inhibitor cocktail (<i>black cap</i>)	1.5 μ l
5x ChIP Buffer iC1	56 μ l
sheared chromatin	100 μ l
DiaMag Protein A-coated magnetic beads	20 μ l
ChIP-seq grade water	116.5 μ l –x μ l
ChIP-seq grade antibody	x μ l

The total volume of the ChIP reaction mix per IP is 300 μ l.

NOTE: The required amount of antibody per IP varies. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. If a positive control IP is included, use 1 μ g of the H3K4me positive control antibody. If required, NaBu (20 mM final concentration) or other inhibitors can be added.

- 3.6** Incubate **overnight** at 4°C on a DiaMag Rotator.
- 3.7** Perform the washes as follows: briefly spin the tubes and place them in the DiaMag1.5 magnetic rack. Wait for **1 minute** and remove the supernatant. Add **350 μ l of Wash Buffer iW1**: gently shake the tubes to resuspend the beads and incubate for **5 minutes** on the DiaMag rotator at 4°C.

Repeat the washing step as described above once with **Wash Buffer iW2, iW3** and **tagW1**, respectively.

- 3.8** After removing the last wash buffer, add **150 μ l of Wash Buffer tagW1** to the beads, resuspend the beads pellet and transfer to a new 0.2 ml tube.
- 3.9** Prepare the ChIPmentation mix as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Component	Volume per reaction
Tagmentation Buffer (<i>blue cap</i>)	29 μ l
Tagmentase (<i>yellow cap</i>)	1 μ l

3.10 Put the tubes from step 3.8 on the DiaMag02. Wait until supernatant is clear and discard the supernatant.

3.11 Add **30 μ l** of **ChIPmentation mix** to each tube and gently resuspend the beads by pipetting.

3.12 Incubate for **10 minutes** at 37°C in the pre-heated thermocycler. After **5 minutes** of incubation, briefly mix the tubes to resuspend the beads.

***NOTE:** The recommended tagmentation time is 10 minutes, but the optimal time can vary depending on the cell number and the antibody used. See “Remarks before starting” section for more details.*

3.13 Put the samples on ice and immediately add **150 μ l of cold Wash Buffer tagW2** to each tube, gently shake the tubes to resuspend the beads and incubate for **5 minutes** on the DiaMag Rotator at 4°C.

3.14 Briefly spin the tubes and place them in the DiaMag02, wait until supernatant is clear and discard the supernatant.

3.15 Add **150 μ l of cold Wash Buffer tagW1** to each tube, gently shake the tubes to resuspend the beads and incubate for **5 minutes** on the DiaMag Rotator at 4°C.

3.16 Briefly spin the tubes and place them in the DiaMag02, wait until supernatant is clear and discard the supernatant.

STEP 4

2 Day 2  1-1.5 h

Stripping, end filling, reverse cross-linking

- 4.1** Remove the strip from magnetic rack, add **10.5 µl of Stripping Reagent** (*blue cap*) to the beads and resuspend by pipetting. Add **9.5 µl of Stripping Reagent** (*blue cap*) to the 1µl INPUT.
- 4.2** Heat the immunoprecipitated and input samples **30 minutes** at 50°C using a thermocycler.
- 4.3** Add **10.5 µl MgCl₂** (*blue cap*) and **25 µl of 2x High-Fidelity Mastermix** (*violet cap*) to each IP'ed and input samples and incubate as follows:

Step	Temperature	Time
End repair	72°C	5 minutes
Reverse cross-linking	95 °C	10 minutes
	Cooling at 4°C (or ice)	

- 4.4** Magnetize beads from the immunoprecipitated samples and transfer the supernatant to a new 0.2 ml tube. Keep the samples at 4°C (or on ice).

NOTE: The total volume of each sample or INPUT is 46 µl.

STEP 5

 2 Day 2  2.5 h

Library amplification

Optional: Take 3 µl of each immunoprecipitated and input sample, dilute it 8x with water and use it to perform a qPCR to check the % of recovery of one positive and one negative control region. The protocol for qPCR analysis is described in a separate section “Protocol for quantitative PCR analysis”.

Determination of the optimal cycle number for the enrichment PCR

NOTE: for this step only 2 µl of each library will be used.

- 5.1** Prepare the **Quantification Mix** as described in the table below for the number of desired reactions. Mix by pipetting and keep on ice until use.

NOTE: Primer indexes for tagmented libraries are not included in this kit and should be ordered separately.

NOTE: Starting from this step the input samples are not processed anymore.

Component	Volume per reaction
Primer pair SI 1 or UDI 1 (<i>transparent cap</i>)	0.3 µl
2x High-Fidelity Mastermix (<i>violet cap</i>)	5 µl
100x SYBR (<i>brown tube</i>)	0.1 µl
ChIP-seq grade water	2.6 µl

NOTE: we recommend using primer pair 1 for more reproducibility, but if needed it is possible to use any other primer pair from primer indexes for tagmented libraries kit.

- 5.2** Dispense **8 µl of the Quantification Mix** into 0.2 ml tubes or strips according to the number of libraries.
- 5.3** Add **2 µl of IP'ed DNA** to each tube and mix by pipetting.
- 5.4** Briefly spin the tubes and run the qPCR program described below. Keep the IP'ed DNA on ice during the qPCR.

Cycles	Temperature	Time
1	98°C	30 seconds
25	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

- 5.5** Analyse the Ct values. The optimal cycle number for the amplification of the rest of the ChIPmentation DNA is typically Ct (rounded up) +2.

NOTE: The Ct value is highly dependent on the thermocycler you use, as well as the way you analyze the qPCR results. Thus when using the kit for the first time you may need to verify that the Ct+2 rule applies well in your conditions.

- 5.6** Add **1.5 µl of the appropriate primer index pair** to each tube from step 4.4. and mix by pipetting.

NOTES: The tubes already contain the mastermix as it was added at step 4.3. Use different indexes for samples that you want to sequence in the same lane (see index sequences and pooling recommendations in the Manual of Primer indexes for tagmented libraries kit).

- 5.7** Briefly spin the tubes and run the PCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
X (Ct rounded up +2)	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

NOTE: After amplification it is possible to use 1µl of library to run on a 2100 BioAnalyzer (Agilent) while keeping the samples on ice. It permits to check that enough material was generated. If needed additional amplification cycles can then be performed. However higher yields may come at the expense of reduced sequencing quality. Therefore we recommend not using more than 18 cycles in order to avoid an over-amplification.

STEP 6



Day 2



45 minutes

Clean-up

- 6.1 Carefully resuspend the room temperature AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.
- 6.2 Estimate the library volume and add 1.8x volume of AMPure XP beads (e.g. for a sample volume of 50 μ l, add 90 μ l of beads). Mix by pipette 8 – 10 times until the mixture is homogeneous.
- 6.3 Incubate at room temperature for 10 minutes.
- 6.4 Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (~2 minutes).
- 6.5 Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
- 6.6 Wash the beads pellet 2 times as follows:
 - With the tubes on the magnet, add **100 μ l** of freshly prepared **80% ethanol** without disturbing the bead pellet and wait for 5 seconds.
 - Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
- 6.7 Leaving the tube open, let the beads dry on the DiaMag02 for 5 minutes.
- 6.8 Remove tubes from DiaMag02 and elute DNA by resuspending the beads in **20 μ l of Resuspension Buffer** (*blue cap*).
- 6.9 Incubate for 10 minutes at room temperature.
- 6.10 Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (~2 minutes).
- 6.11 Without disturbing the pellet, carefully aspirate and transfer the supernatant containing purified libraries to a new tube.

STEP 7

Quality control

- 7.1 Determine the concentrations of your samples by the use of a fluorescence-based assay such as the Qubit High Sensitivity assay (ThermoFischer Scientific).
- 7.2 Run a part of each library on a High Sensitivity chip for BioAnalyzer (Agilent) or on Fragment Analyzer (Advanced Analytical) according to the manufacturer's instructions.

NOTE: In some cases, for example when large fragments are still present, a size selection can be performed (see the "Additional protocols" section, page 55).

- 7.3 Your libraries are now ready for pooling and sequencing.

NOTE: Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to the manual of Primer indexes for tagged libraries kit). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries' molarities in the final volume, e.g. if you add 5 μ l of a 10 nM library to 5 μ l of a 20 nM library, you have 10 μ l of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.



Please, do not hesitate to contact our customer support team if you have any questions about the design of your ChIPmentation experiment or the bioinformatics data analysis.

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AUTO PROTOCOL

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AUTOMATED PROCESSING



STEP 1

1

Day 1



30 minutes

Cell collection and DNA-protein cross-linking from 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.

NOTE: Cold PBS will be needed at step 1.12.

- 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. Gently shake the culture flask for **1-2 minutes** or until the cells start to detach. The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment with trypsin 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. Transfer cell suspension to a 50 ml tube.
- 1.5 Rinse the flask by adding **10 ml of warm PBS**. Add this volume to your 50 ml tubes containing cells from point 1.4. 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 by centrifugation for 5 minutes at 500 x g (at room temperature) and remove the supernatant.
- 1.8 Resuspend the cells in **PBS** to obtain a concentration of **7 million cells per 500 µl of PBS**. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.

***NOTE:** If desired, the cell concentration can be adjusted from 1 to 10 million cells per 500 µl.*

- 1.9 Under a fume hood, add **13.5 µl of 37% formaldehyde** to each tube containing 500 µl of cell suspension. 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".*

- 1.10 Add **57 µl of Glycine** (blue cap) 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.
- 1.11 Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.
- 1.12 Wash the cells twice with **1 ml of cold PBS** as follows:

Add cold PBS

Invert the tube 2-3 times

Centrifuge at 500 x g 5 minutes at 4°C

Discard supernatant

***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

1 Day 1  1 to 2 h

Cell lysis and chromatin shearing from cells

- 2.1** Add **1 ml of ice-cold Lysis buffer iL1** to the 1.5 ml tube containing 7 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 6 ml of buffer iL1 and incubate for **10 minutes** at 4°C with gentle mixing.

***NOTE:** For scaling up or down, use 1 ml of buffer iL1 per 1 million of cells.*

- 2.2** Pellet the cells by centrifugation at 500 x g and 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 buffer iL2** and incubate for **10 minutes** at 4°C with gentle mixing.

***NOTE:** For scaling up or down, use 1 ml of buffer iL2 per 1 million of cells.*

- 2.4** Pellet the cells again by centrifugation for **5 minutes** at 500 x g and 4°C and discard supernatant.

- 2.5** Prepare complete Shearing buffer by adding 3.5 µl of **200x protease inhibitor cocktail (black cap)** to **700 µl of Shearing buffer iS1**. Keep on ice.

***NOTE:** For scaling up or down, prepare 100 µl of complete Shearing Buffer per 1 million cells.*

- 2.6** Add **700 µl of complete Shearing buffer iS1** to 7 million cells. The final cell concentration in the shearing buffer should be 1 million of cells per 100 µl of iS1. Resuspend the cells by pipetting up and down several times. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes:

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

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

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.

2.7 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 12 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 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 optimal 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.



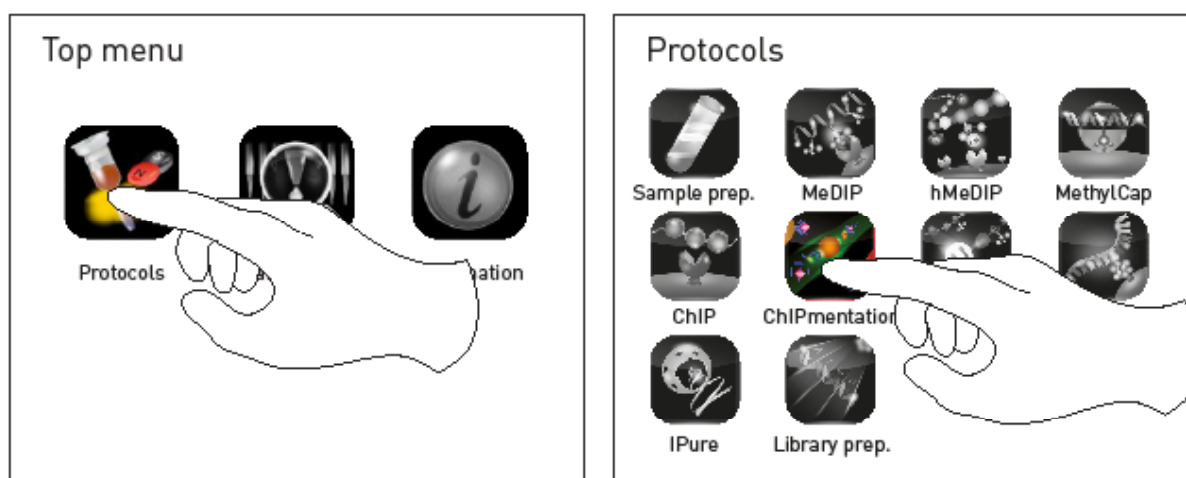
STEP 3



Day 1-2
1.5 h hands on time
and overnight run

Magnetic immunoprecipitation and tagmentation

- 3.1 Switch ON the IP-Star Compact.
- 3.2 Select “**Protocols**” icon and then “**ChIPmentation**” category.



- 3.3 Select “**ChIPmentation_08_D**” if you plan to run between 1 and 8 samples or “**ChIPmentation_16_D**” if you plan to run between 9 and 16 samples.
- 3.4 Setup 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 ChIPmentation experiment and press “Next”

Recommended parameters:

	Mixing time	Temperature	Mix speed
Ab coating:	3 h 0.1 – 16 hours	4 °C 4 – 27 degrees	middle
IP reaction:	13 h 0.1 – 16 hours	4 °C 4 – 27 degrees	middle
Washes:	5 min 0.1 – 20 min	4 °C 4 – 27 degrees	middle
Tagmentation:	10 min 1 – 30 min		

Back Next

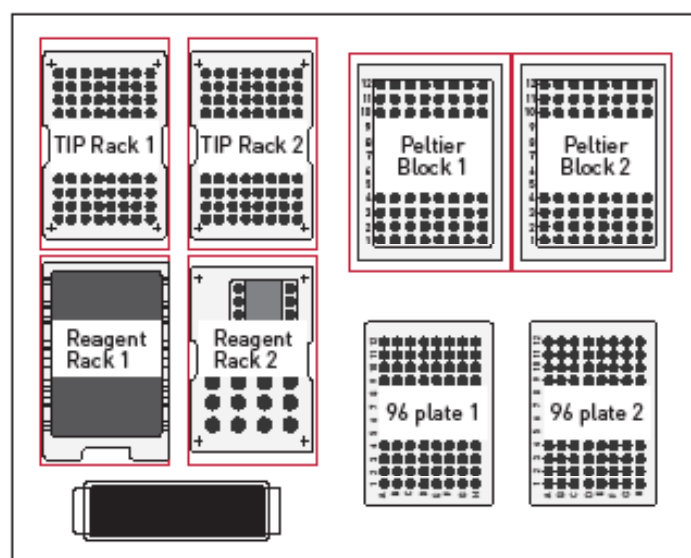
diagenode

NOTE: The recommended tagmentation time is 10 minutes, but the optimal time can vary depending on the cell number and the antibody used. See “Remarks before starting” section for more detail

3.6 Setup 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 **20 µl of DiaMag Protein A-coated magnetic beads** in each well of row 3.
- Prepare **ChIP Buffer** as described in the table below. The volumes are in µl and 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. Use 1 µg of Rabbit IgG for the negative control IP.

	Per IP
Antibody	x µl
ChIP Buffer	100 – x µl
5% BSA (<i>black cap</i>)	2 µl
200x Protease Inhibitor cocktail (<i>black cap</i>)	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. 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.

	Per IP
Sheared chromatin	100 µl
ChIP Buffer	100 µl
5% BSA (<i>black cap</i>)	4 µl
200x Protease Inhibitor Cocktail (<i>black cap</i>)	1 µl

- Keep aside **1 µl of the sheared chromatin** at 4°C to be used as an **INPUT** starting from step 4 point 4.4.

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

- Distribute **1 µl of Tagmentase** (*yellow cap*) in each well of row 2.

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

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 ChIPmentation is running. The “Remaining time” calculation will give you an estimation of the processing time of your experiment.

STEP 4

 Day 2  1-1.5 h

Stripping, end filling, reverse cross-linking

- 4.1 The next morning, after the overnight incubation, recover the sample tubes in row 12. The volume in each tube is 150 µl. Press “OK” and “Back” until the homepage appears on the screen.
- 4.2 Briefly spin the strip and place it on DiaMag02 magnetic rack.
- 4.3 Wait until supernatant is clear and discard the supernatant.
- 4.4 Remove the strip from magnetic rack, add **10.5 µl of Stripping Reagent** (*blue cap*) to the beads and resuspend by pipetting. Add **9.5 µl of Stripping Reagent** (*blue cap*) to the 1 µl INPUT.
- 4.5 Heat the immunoprecipitated and input samples **30 minutes** at 50°C using a thermocycler.

NOTE: During incubation time 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.

- 4.6 Add **10.5 µl MgCl₂** (*blue cap*) and **25 µl of 2x High-Fidelity Mastermix** (*violet cap*) to each IP'ed and input samples and incubate as follows:

Step	Temperature	Time
End repair	72°C	5 minutes
Reverse cross-linking	95 °C	10 minutes
	Cooling at 4 °C (or ice)	

- 4.7 Magnetize beads from the immunoprecipitated samples and transfer the supernatant to a new IP-Star strip (Cat. No. C30020002).

NOTE: The total volume of each sample or INPUT is 46 µl.

STEP 5

 Day 2  2.5 h

Library amplification

Optional: Take 3 μl of each immunoprecipitated and input sample, dilute it 8x with water and use it to perform a qPCR to check the % of recovery of one positive and one negative control region. The protocol for qPCR analysis is described in a separate section “Protocol for quantitative PCR analysis”.

Determination of the optimal cycle number for the enrichment PCR

NOTE: for this step only 2 μl of each library will be used.

- 5.1** Prepare the **Quantification Mix** as described in the table below for the number of desired reactions. Mix by pipetting and keep on ice until use.

NOTE: Primer indexes for tagmented libraries are not included in this kit and should be ordered separately.

NOTE: Starting from this step the input samples are not processed anymore.

Component	Volume per reaction
Primer pair SI 1 or UDI 1 (<i>transparent cap</i>)	0.3 μl
2x High-Fidelity Mastermix (<i>violet cap</i>)	5 μl
100x SYBR (<i>brown tube</i>)	0.1 μl
ChIP-seq grade water	2.6 μl

NOTE: we recommend using primer pair 1 for more reproducibility, but if needed it is possible to use any other primer pair from primer indexes for tagmented libraries kit.

- 5.2** Dispense **8 μl of the Quantification Mix** into 0.2 ml tubes or strips according to the number of libraries.
- 5.3** Add **2 μl of IP'ed DNA** to each tube and mix by pipetting.
- 5.4** Briefly spin the tubes and run the qPCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
25	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

Keep the IP'ed DNA on ice during qPCR.

- 5.5** Analyse the Ct values. The optimal cycle number for the amplification of the rest of the ChIPmentation DNA is typically Ct (rounded up) +2 (e.g. for a Ct of 8.76 use 11 amplification cycles).

NOTE: The Ct value is highly dependent on the thermocycler you use, as well as the way you analyze the qPCR results. Thus when using the kit for the first time you may need to verify that the Ct+2 rule applies well in your conditions. With a correct number of cycles you should obtain a DNA concentration which is between 10 and 30 ng/μl.

- 5.6** Add **1.5 μl of the appropriate primer index pair** to each tube from step 4.7. and mix by pipetting.

NOTES: The tubes already contain the mastermix as it was added at step 4.6. Use different indexes for samples that you want to sequence in the same lane (see index sequences and pooling recommendations in the Manual of Primer indexes for tagmented libraries kit).

- 5.7** Briefly spin the tubes and run the PCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
X (Ct rounded up +2)	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

NOTE: After amplification it is possible to use 1μl of library to run on a 2100 BioAnalyzer (Agilent) when keeping the samples on ice. It permits to check that enough material was generated. If needed additional amplification cycles can then be performed. However higher yields may come at the expense of reduced sequencing quality. Therefore we recommend not using more than 18 cycles in order to avoid an over-amplification.

STEP 6

Clean-up

2

Day 2

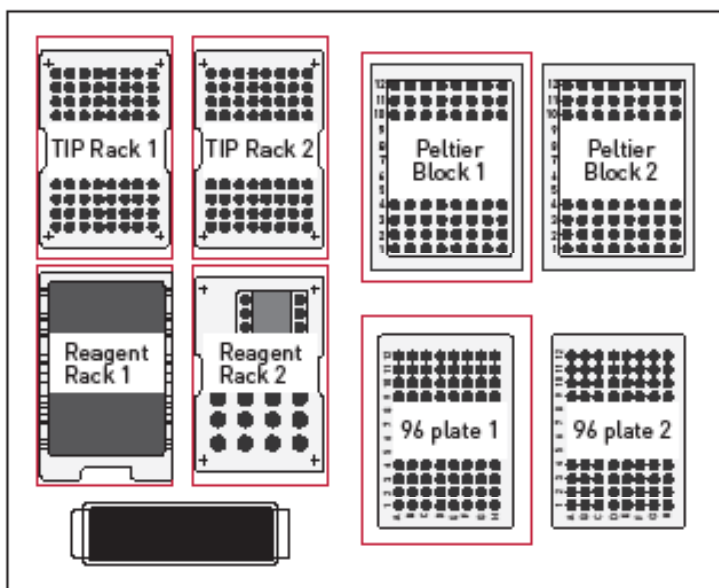


20 min hands on time
40 min run for each
series of 8 samples
1h10 min run for each
series of 16 samples

NOTE: Use the IP-Star Compact and room temperature AMPure XP beads for the clean-up.

- 6.1 Select “**Protocols**” icon and then “**ChIPmentation**” category.
- 6.2 Select “**ChIPmentation_Purification_08**” if you plan to run between 1 and 8 samples, or “**ChIPmentation_Purification_16**” if you plan to run between 9 and 16 samples.
- 6.3 Setup the exact number of samples that you want to process by pressing the black box.

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



- 6.4 Setup 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 Rack 1 & 2** with reagent containers according to the screen.
 - Fill **96 plate 1** with a 96 well microplate.
 - Fill **Peltier Block 1** with 200 µl tube strips according to the screen.

6.5 Fill the robot with all reagents.

- Put your samples in lane 1 (and 2 if processing more than 8 samples) of the **Peltier Block 1**.
- Distribute room temperature AMPure XP Beads in row 1 (and 5 if processing more than 8 samples) of the **96-well Microplate**. The amount of beads must be 1.8x the volume of sample (e.g. for 45 µl of samples use 81 µl of beads).

NOTE: Resuspend the beads with pipetting up and down several times before dispense them.

- Fill the container of the **Reagent Rack 1** with freshly prepared 80% Ethanol according to the screen.
- Fill the container of **Reagent Rack 2** with Resuspension Buffer according to the screen.
- Check the proper insertion of the racks and the consumables.

6.6 Close the door and press “Run” to start.

6.7 After the run, recover your samples on the upper row of the **Peltier Block 1**. The final volume is 20 µl per each sample.

6.8 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.

NOTE: 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 7

Quality control

2 Day 2

- 7.1 Determine the concentrations of your samples by the use of a fluorescence-based assay such as the Qubit High Sensitivity assay (ThermoFischer Scientific).
- 7.2 Run a part of each library on a High Sensitivity chip for BioAnalyzer (Agilent) or on Fragment Analyzer (Advanced Analytical) according to the manufacturer's instructions.

NOTE: In some cases, for example when large fragments are still present, a size selection can be performed (see the "Additional protocols" section, page 55).

- 7.3 Your libraries are now ready for pooling and sequencing.

NOTE: Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to the manual of Primer indexes for tagmented libraries kit). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries' molarities in the final volume, e.g. if you add 5 μ l of a 10 nM library to 5 μ l of a 20 nM library, you have 10 μ l of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.



Please, do not hesitate to contact our customer support team if you have any questions about the design of your ChIPmentation experiment or the bioinformatics data analysis.

Contact for Europe, Asia, Oceania and Africa:

custsupport@diagenode.com

Contact for North and South America:

custsupport.na@diagenode.com

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.

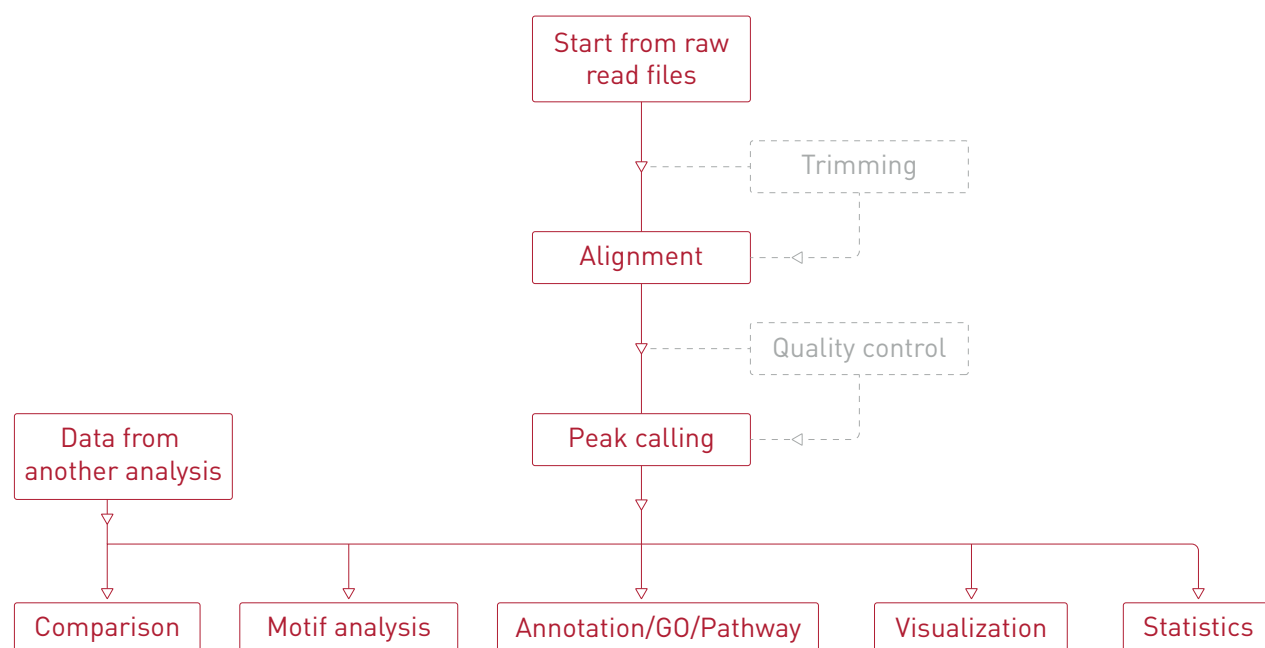


Figure 1. ChIP-seq data analysis workflow

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. Pycoteo f. MUSIC g. SPP h. hiddenDomains

After above described basic analysis, the peaks can be analyzed further to get answers to your biological questions. Several options and tools are available for further analyses. Your project goals will determine which ones you 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. UCSC Genome Browser
6. Descriptive statistics: the peaks can be described in various useful ways, like how many reads are in them, the number of peaks, mean size, significance, etc. These figures are also very useful for comparing datasets.
 - a. Peak callers usually provide per peak and/or summary statistics after peak detection
 - b. HOMER c. GREAT d. BEDTools
7. Motif search: For transcription factors, data peaks frequently occur at specific motifs, though some HM peaks can also lean toward certain sequence patterns. Therefore identifying these motifs and checking their enrichments over them is a good practice for TF data analysis, which is 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 with 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 optimal 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. DiffBin

Example of results

Recovery analysis (after Step 4)

ChIPmentation was performed on human K562 cells in duplicates. Sheared chromatin from 1 million cells was used per IP, in combination with 1 μ g of the positive control antibody H3K4me3 (Cat. no. C15410003) or 1 μ g of the negative IgG control. Tagmentation was applied for 10 minutes. The IP efficiency was checked by qPCR.

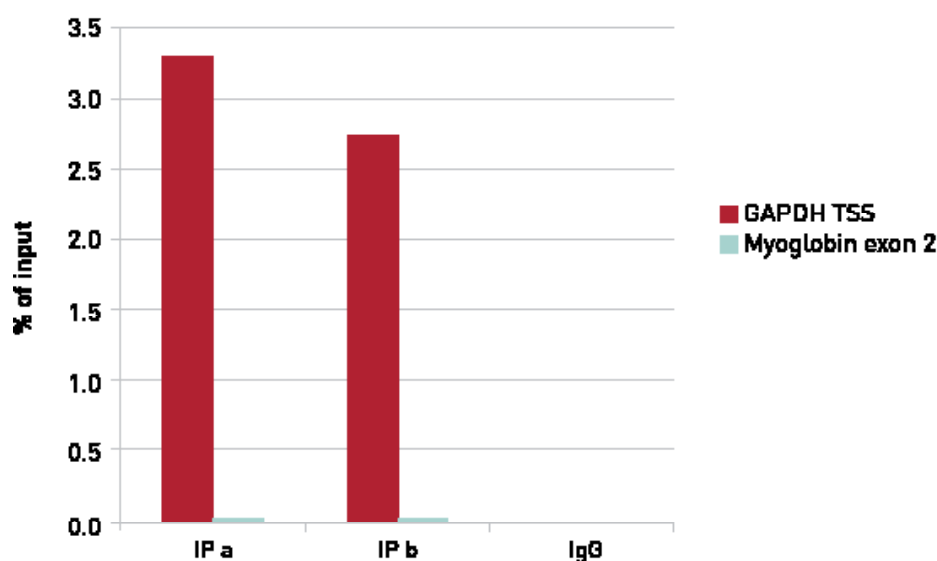


Figure 2. Recovery analysis. Quantitative PCR was performed with the positive control GAPDH-TSS and the negative control Myoglobin exon 2 primer sets from the kit. The recovery is expressed as a % of input and is the relative amount of immunoprecipitated DNA compared to input DNA.

Quality control of the libraries before sequencing (Step 7)

After amplification and purification, the H3K4me3 ChIPmentation libraries were analyzed on BioAnalyzer (Agilent).

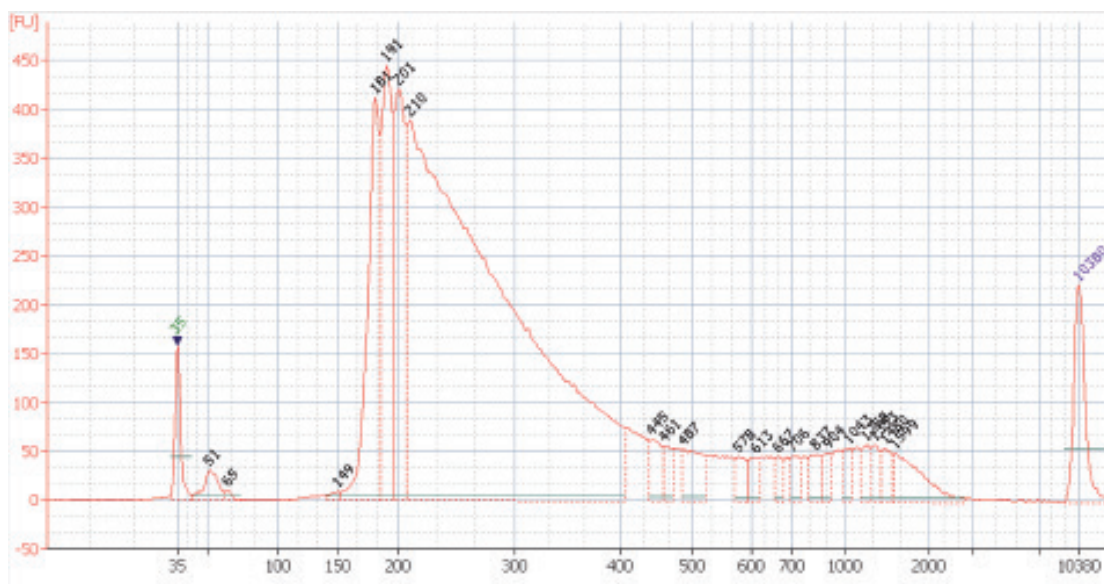


Figure 3. Library quality control. The distribution of the fragments sizes was assessed by loading 6 ng of library on BioAnalyzer (Agilent).

Sequencing

ChIPmentation libraries were finally sequenced on Illumina's HiSeq3000/4000 and the data analyzed as described page 50.

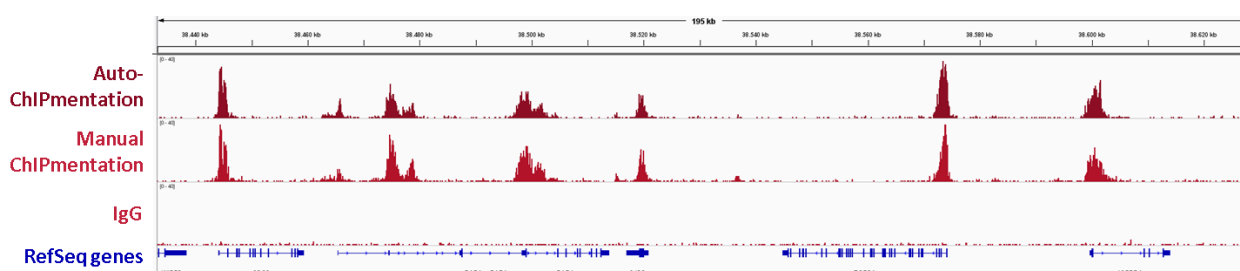
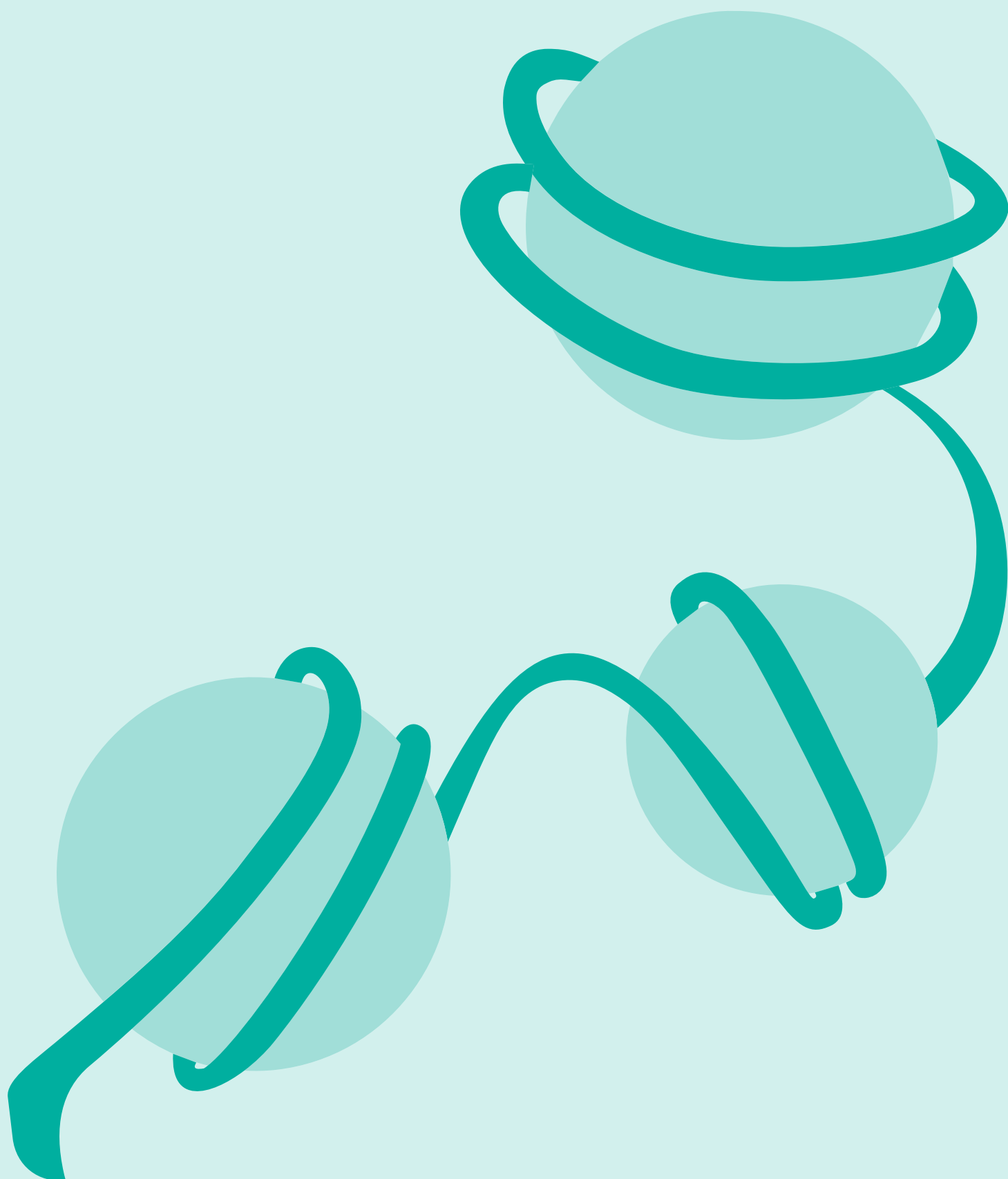


Figure 4. Distribution of the ChIPmentation datasets for H3K4me3 ChIP-seq, in a representative region of the genome.



ADDITIONAL PROTOCOLS

Protocol for chromatin shearing analysis

General remarks

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.

Here below we present the workflow for analysis of sheared chromatin using the validated solutions.

NOTE: *The recommended reagents are not included in this kit.*

Workflow for analysis of sheared chromatin:

- RNase treatment (1h, optional but highly recommended for accurate size assessment) using RNase cocktail (e.g. Ambion, AM 2286A)
- Reverse crosslinking (4h or overnight) using IPure Kit v2 (Diagenode, Cat. No. C03010014)
- DNA purification using IPure Kit v2 (30 minutes)
- Fragment size assessment (agarose gel or Fragment Analyzer)(1h)

RNase treatment

1. Take **50 µl of sheared chromatin** (end of step 2 in the protocol) and transfer to a 1.5 ml microtube.
2. Dilute **1 µl of RNase cocktail** 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 (IPure Kit v2)

5. Add **50 µl of Buffer A**.
6. Add **4 µl of Buffer B**, mix thoroughly.
7. Incubate samples at 65°C for **4 hours** (or **overnight**).

DNA purification

NOTE: The protocol below describes DNA purification using the IPure Kit v2. 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.

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.

8. Add **2 µl of carrier** to the sample.
9. Add **108 µl of 100% isopropanol** to the samples.

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.
10. Resuspend the **IPure beads v2** by vortexing and add **20 µl** to the sample.
11. Incubate samples for **10 minutes** at room temperature on the DiaMag Rotator.
12. Briefly spin the tubes, place in the DiaMag1.5 magnetic rack, wait **1 minute** and discard the buffer.
13. 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 DiaMag1.5 magnetic rack, wait **1 minute** and discard the buffer.
14. 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 1.5 magnetic rack, wait 1 minute and discard the buffer.

15. Spin the tubes again and place them on the DiaMag1.5. 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.
16. Spin the tubes and place them into the DiaMag 1.5, 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 (Standard Sensitivity NGS Fragment Analysis Kit (DNF-473)).

Protocol for quantitative PCR analysis

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

1. 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 IP'ed or INPUT DNA

2. 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 and temperature.

**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.

3. 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.

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

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

- 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

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:

$$\% \text{ recovery} = 2^{[(Ct_{\text{input}} - \log_2(X)) - Ct_{\text{sample}}]} \times 100$$

Where: $\log_2(X)$ 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 $\log_2(50)=5.64$ and the formula to calculate the recovery will be as follows:

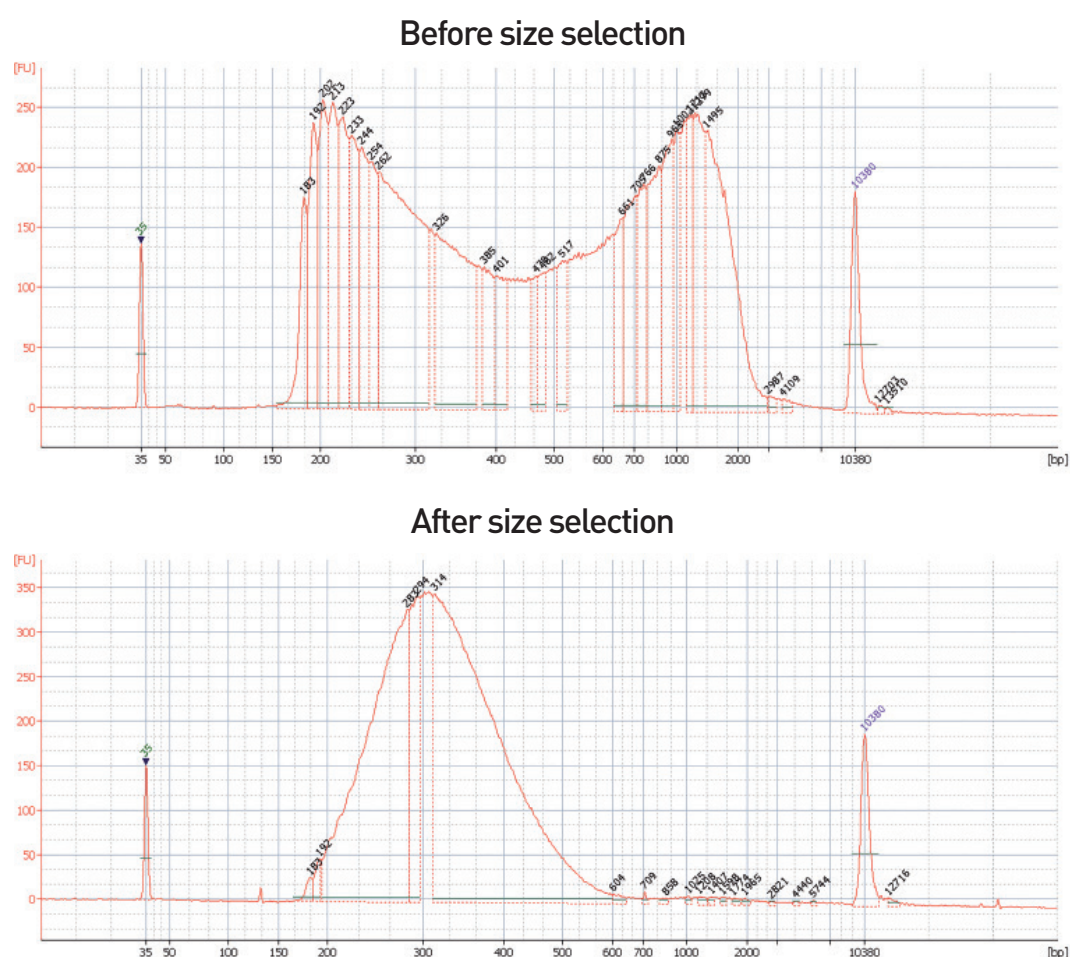
$$\% \text{ recovery} = 2^{[(Ct_{\text{input}} - 5.64) - Ct_{\text{sample}}]} \times 100$$

Protocol for manual size selection



1. Add **30 µl of ChIP-seq grade water** to each sample to have a final volume of 50 µl.
2. Carefully resuspend the room temperature AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.
3. Add **32.5 µl of AMPure XP beads** (corresponding to a 0.65x ratio). Mix by pipette 8 – 10 times until the mixture is homogeneous.
4. Incubate at room temperature for **10 minutes**.
5. Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (**~2 minutes**).
6. Without disturbing the pellet, carefully aspirate and transfer the supernatant to a new tube.
7. Add **12.5 µl of AMPure XP beads**. Mix by pipette 8 – 10 times until the mixture is homogeneous.
8. Incubate at room temperature for **10 minutes**.
9. Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (**~2 minutes**).
10. Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
11. Wash the beads pellet 2 times as follows:
 - With the tubes on the magnet, add **100 µl** of freshly prepared **80% ethanol** without disturbing the bead pellet and wait for **5 seconds**.
 - Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

12. Leaving the tube open, let dry the beads on the DiaMag02 for 3 minutes.
13. Remove tubes from DiaMag02 and elute DNA by resuspending the beads in **20 µl of Resuspension Buffer** (*blue cap*).
14. Incubate for 10 minutes at room temperature.
15. Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (~2 minutes).
16. Without disturbing the pellet, carefully aspirate and transfer the supernatant containing size selected libraries to a new tube.



Protocol for size selection on the IP-Star Compact



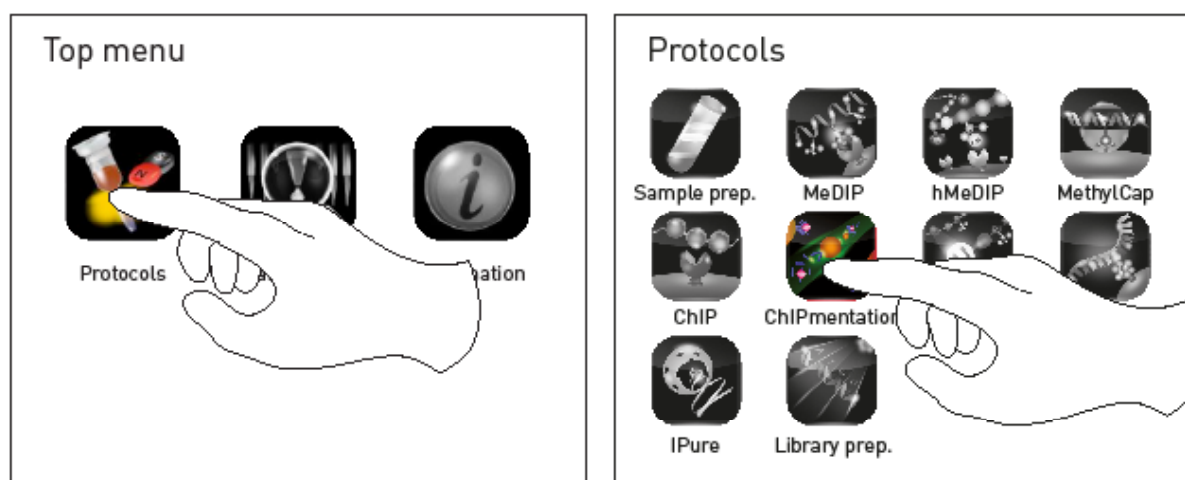
30 min hands on time



1h10 min run for each series of 8 samples

NOTE: Use the IP-Star and room temperature AMPure XP beads for the size selection.

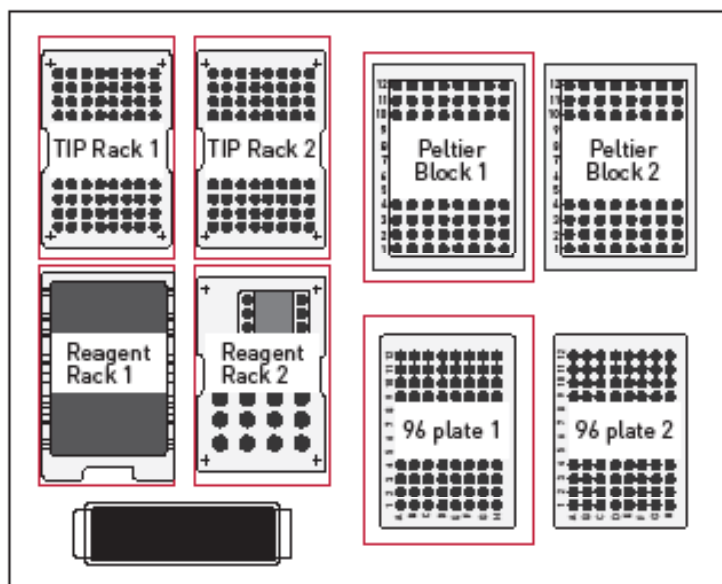
1. Switch on the IP-Star and select “**Protocols**” icon and then “**ChIPmentation**” category.



2. Select “**ChIPmentation_Size_Selection_08**” if you plan to run between 1 and 8 samples, or “**ChIPmentation_Size_Selection_16**” if you plan to run between 9 and 16 samples.
3. Setup the exact number of samples that you want to process by pressing the black box.

NOTE: The Peltier Block 1 is now cooling down to 4°C to keep your samples cold.

4. Setup 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 Rack 1 & 2** with reagent containers according to the screen.
 - Fill **96 plate 1** with a 96 well microplate.
 - Fill **Peltier Block 1** with 200 µl tube strips according to the screen.



5. Fill the robot with all reagents.

- Add **80 µl of ChIP-seq grade water** to each sample to have a final volume of 100 µl. Put your samples in lane 1 (and 2 if processing more than 8 samples) of the **Peltier Block 1**.
- Distribute **90 µl of room temperature AMPure XP beads** in row 1 (and 7 if processing more than 8 samples) of the 96-well Microplate.

NOTE: Resuspend the beads with pipetting up and down several times before dispense them.

- Fill the container of the **Reagent Rack 1** with freshly prepared 80% Ethanol according to the screen.
- Fill the container of **Reagent Rack 2** with Resuspension Buffer according to the screen.
- Check the proper insertion of the racks and the consumables.

6. Close the door and press “Run” to start.

7. After the run, recover your samples on the upper row of the Left Peltier Block. The final volume is 20 µl for each sample.

8. 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.

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

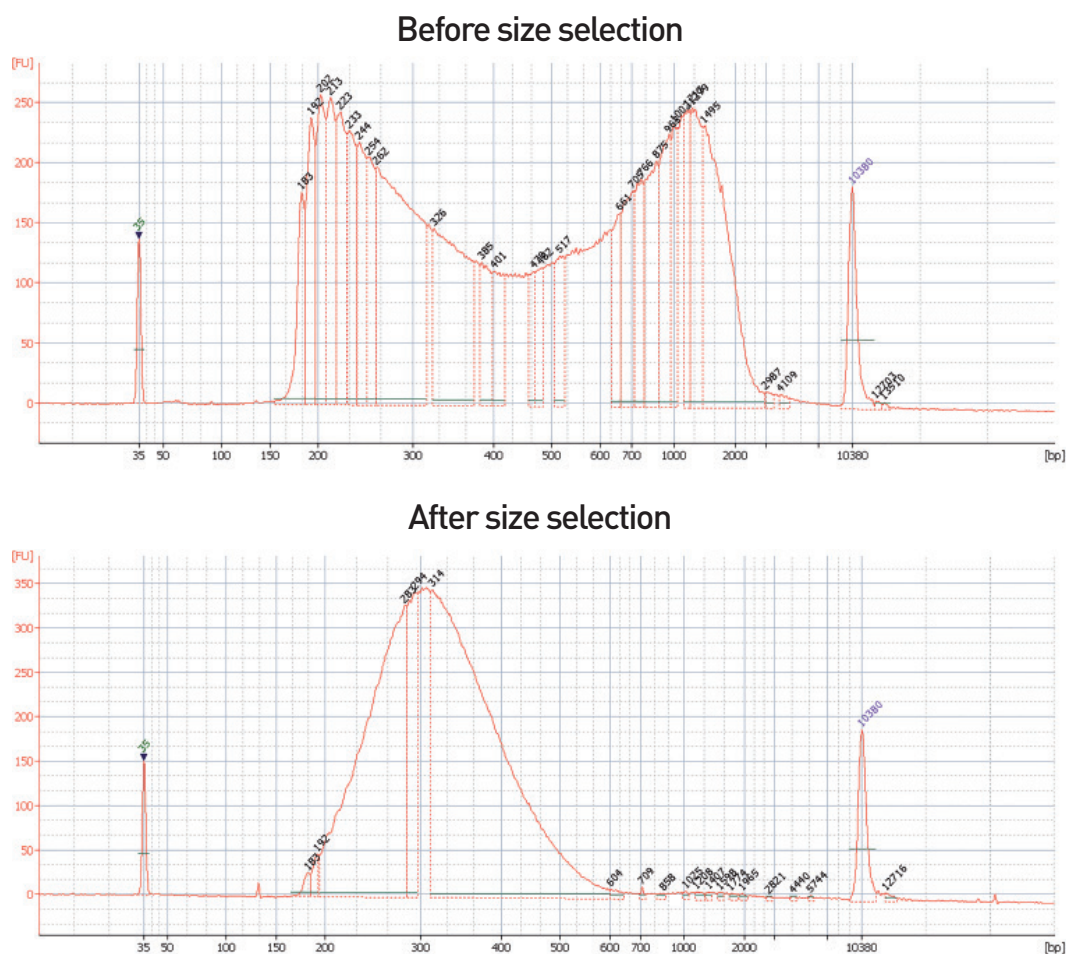


Figure 5. Example of results of the size selection on a library for H3K27me3 ChIP-seq. The library was run on a BioAnalyzer (Agilent) before and after the size selection.

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 come from other companies, 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?

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

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

The ChIPmentation 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 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.

What is the composition of buffers included in the kit?

The composition of the buffers is proprietary.

I want to do the optional qPCR step to check the immunoprecipitation efficiency, but I do not want to stop during the ChIPmentation experiment. Can I store the aliquotes and do the qPCR later?

Yes, definitely. It is possible to prepare and dilute aliquotes of samples at the end of step 4, and then to keep them at -20°C several weeks before doing the qPCR.

What is the difference between single and dual-indexing? What is the best for ChIPmentation libraries?

With single-indexing, during the PCR amplification, only one primer will contain a barcode, whereas the second primer is universal. This means that only one side of the library will bare a barcode. This is enough to demultiplex the pool of samples after sequencing.

Nevertheless with some sequencers, (e.g. the NovaSeq6000), a phenomenon called index hopping has been observed. This index hopping introduces mistakes in the reading of the index and can lead to a misattribution of some reads, to the wrong sample. To identify those mistakes you can use Unique Dual-Indexing. In this case, the two PCR primers are adding unique barcodes to each sample, on each side of the insert. This way, if one index is wrongly associated to one sample, the second index will allow to see it. Therefore when sequencing on NovaSeq, Unique Dual-Indexing is recommended, but not mandatory.

What are the expected concentration and size of ChIPmentation libraries?

The concentration of libraries that you need to reach will depend on the sensitivity of the machine and kits that you will use to perform the quality control and the sequencing of your libraries. Usually a concentration of 4-8 ng/μl is enough for a quality control using the Qubit High Sensitivity assay (ThermoFischer Scientific) and the High Sensitivity chip for BioAnalyzer (Agilent) and for sequencing on Illumina HiSeq3000/4000.

Regarding the size, ideally the library should show fragments around 150-500 bp. If some larger fragments are present the best would be to contact your sequencing provider to ask what are their requirements, because it can vary depending on the sequencer. If you want to remove the large fragments you can use the size selection protocol described in the manual.

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
8 SI for Tagmented libraries	C01011033
24 SI for tagmented libraries	C01011032
8 UDI for tagmented libraries	C01011035
24 UDI for tagmented libraries - Set I	C01011034
24 UDI for tagmented libraries - Set II	C01011036
DiaMag Rotator	B05000001
DiaMag 0.2ml magnetic rack	B04000001
DiaMag 1.5ml magnetic rack	B04000003
IPure Kit v2	C03010015
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
H4K20ac monoclonal antibody	C15210008
H4K20me3 polyclonal antibody	C15410207
macroH2A.1/H2A.2 monoclonal antibody	C15210003

Validated primer pairs - check out the complete list at www.diagenode.com

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