diagenode

Innovating Epigenetics Solutions

µChIPmentation Kit for Histones

Cat. No. C01011011 (24 rxns)
C01011012 (8 rxns)

USER GUIDE

Version 1 27_01_2021

Please read this manual carefully
before starting your experiment

Contents

Introduction

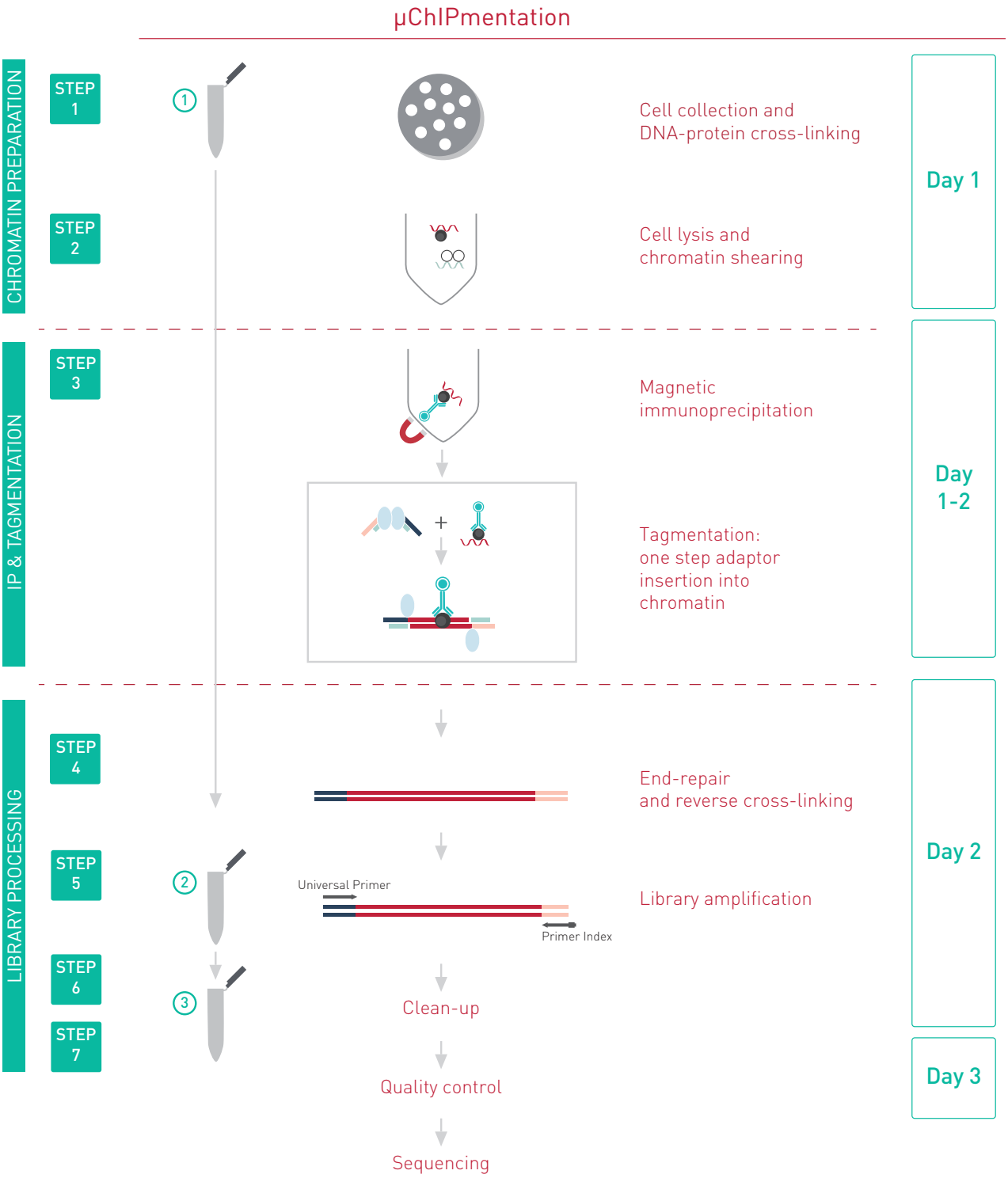
Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) is the method of choice to identify, from the whole genome, which specific regions are associated with proteins of interest, like chromatin remodelers or transcription factors.

Traditional ChIP-seq protocols require large amounts of cells, which makes their use to study limited material such as patients samples or embryonic tissues, inaccurate. In order to solve that, Diagenode has combined several high quality tools, to offer the new µChIPmentation for histones protocol for efficient ChIP-seq on 10,000 cells:

- An optimized chromatin preparation protocol based on Diagenode's True MicroChIP technology and shearing in 0.2 ml tubes with Bioruptor Pico
- The adaptation of the workflow to use only 3 tubes per sample for the whole process, from cell fixation to purified libraries, to reduce DNA lost
- The use of the ChIPmentation technology which enables the integration of the library preparation during the ChIP itself using transposase and sequencing-compatible adaptors for a reduced number of steps

ChIPmentation was developed in the collaboration with CeMM in Vienna. The improved protocol of µChIPmentation was developed in collaboration with Robert Månsson and Charlotte Gustafsson at Karolinska Institutet, Sweden.

Kit method overview & time table



LEGEND

- Protein of interest
- Other protein
- DNA
- Antibody
- Magnetic bead
- Magnet
- Tagmentation Enzyme loaded with adaptors
- Indicates the sample transfer to the next tube

Kit materials

The µChIPmentation Kit for Histones contains all reagents necessary for chromatin preparation, chromatin immunoprecipitation and library preparation for NGS as described in the Table1.

Please, note that indexes for multiplexing are not included in the kit. The indexes are available separately:

Single indexes:

24 SI for tagmented libraries, Cat. No. C01011032

8 SI for tagmented libraries, Cat. No. C01011033

Unique Dual Indexes:

24 UDI for tagmented libraries - Set I, Cat. No. C01011034

8 UDI for tagmented libraries - Set I, Cat. No. C01011035

24 UDI for Tagmented libraries - Set II, Cat. No. C01011036

Table 1. Number of reactions included in the kit µChIPmentation for Histones

Kit reference	Number of				
	Chromatin preparation	ChIP reactions	Inputs	Shearing controls	Library preparations
C01011011	30	24	6	6	24
C01011012	10	8	2	2	8

Table 2. Components supplied with the kit µChIPmentation for Histones (for 24 and 8 rxns)

Component	Cap color	Qty (24 rxns)	Qty (8 rxns)	Storage
Protease inhibitor cocktail 200x	black	74 µl	28 µl	-20°C
Rabbit IgG	white	3 µg	1 µg	-20°C
ChIP-seq grade antibody H3K4me3	white	3 µg	1 µg	-20°C
Tagmentase (loaded)	yellow	30 µl	10 µl	-20°C
2x High-Fidelity Mastermix	violet	900 µl	300 µl	-20°C
100 x SYBR	none	3 µl	1 µl	-20°C
Human GAPDH TSS primer pair	green	90 µl	30 µl	-20°C
Human Myoglobin exon 2 primer pair	red	90 µl	30 µl	-20°C

Component	Cap color	Qty (24 rxns)	Qty (8 rxns)	Storage
Glycine	white	1380 µl	460 µl	4°C
Lysis Buffer tL1	white	750 µl	250 µl	4°C
DiaMag protein A-coated magnetic beads	none	240 µl	80 µl	4°C
Bead Wash Buffer tBW1	n/a	7040 µl	2100 µl	4°C
ChIP Buffer tC1	n/a	2400 µl	800 µl	4°C
Wash Buffer tW1	n/a	5600 µl	1500 µl	4°C
Wash Buffer tW2	n/a	5600 µl	1500 µl	4°C
Wash Buffer tW3	n/a	5600 µl	1500 µl	4°C
Wash Buffer tagW1	n/a	11200 µl	4400 µl	4°C
Wash Buffer tagW2	n/a	7600 µl	3200 µl	4°C
Tagmentation Buffer	yellow	646 µl	228 µl	4°C
Nuclease-free water	none	825 µl	275 µl	4°C
Resuspension Buffer	none	1360 µl	400 µl	4°C
Primers Dilution Buffer	none	925 µl	310 µl	4°C
Elution Buffer tE1	clear	300 µl	100 µl	4°C
Elution Buffer tE2	clear	24 µl	8 µl	4°C
$MgCl_2$	blue	6 µl	6 µl	4 °C

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

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml tubes
- RNase/DNase-free 15 ml and 50 ml tubes
- RNase/DNase-free 0.2 ml tubes (or 8-tube strips)
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS) buffer
- 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
- Cell culture medium
- Trypsin-EDTA
- Hank's balanced salt solution (HBSS) buffer

Equipment

- Cell counter system
- Fume hood
- Bioruptor® Pico (Diagenode, Cat. No. B01060001) and 0.2 ml microtubes for Bioruptor® Pico (Cat. No. C30010020)
- Refrigerated centrifuge for 1.5 ml and 0.2ml tubes
- Centrifuge for 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)
- Tube holder for 0.2 ml tubes with a cap
- Magnetic rack for 0.2 ml tubes: DiaMag02 (Diagenode, Cat. No. B04000001)

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

Optional supplies

- Chromatin EasyShear Kit – Ultra Low SDS (Cat. No. C01020010)
- RNase cocktail (e.g. Ambion, AM2286A), for chromatin shearing assessment
- MicroChIP DiaPure Columns (Diagenode, Cat. No. C03040001), for chromatin shearing assessment
- qPCR SYBR® Green Mastermix, for quantitative PCR analysis

Remarks before starting

1. Cell number

This protocol has been optimized for the use of 10,000 cells per reaction. Two options are possible, either starting directly from 10,000 cells for fixation, or doing the fixation of a larger amount of cells, sorting them and continuing the protocol with 10,000 cells per tube.

> ***NOTE:*** *It is recommended to prepare one extra-tube per experiment and to use it for chromatin shearing assessment (see shearing optimization chapter).*

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 EasyShear kit - High SDS (Cat. No. C01020012) which contains all buffers needed for chromatin preparation compatible with the µChIPmentation Kit.

The reagents included in the kit µChIPmentation for Histones 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 6-8-10 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)

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 and to model the background of the ChIP experiment after sequencing. We recommend including one input per cell type.

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 sample type 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.

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, so 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 18, 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. 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 Bioanalyzer or Fragment Analyzer (Agilent). 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.

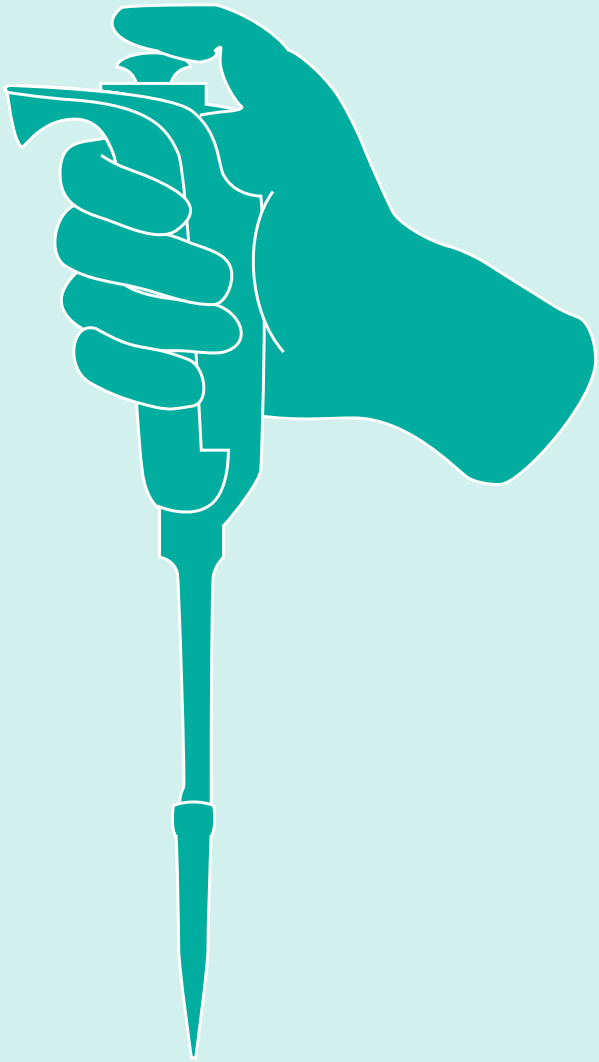
The Bioanalyzer system or Fragment Analyzer (Agilent) provide sizing and quantification information about the library analysed, but not about the clustering competency. Quantification can be done both on unpurified or purified samples. In a case of unpurified samples, a region corresponding to libraries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

UV absorption/fluorescence detection-based methods (i.e., Nanodrop® (Thermo Scientific), Qubit®2.0 Fluorometer (Life Technologies), or Quant-iT™ 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.

10. Quantitative PCR analysis

Prior to the sequencing, we recommend analysing the input and immunoprecipitated samples by SYBR® Green qPCR using 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.

PROTOCOL



MANUAL PROCESSING

STEP 1

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

1 Day 1 1.5 hours

***NOTE**: This protocol has been optimized for the use of 10,000 cells per reaction. Alternatively, fixation of a larger amount of cells, sorting them and continuing the protocol with 10,000 cells per tube can be performed. For the second option please refer to the "Protocol for fixation of a larger amount of cells - 100 000 cells" in the section "Additional protocols".*

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. 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 15 ml tube.

1.5 Rinse the flask by adding **10 ml** of **PBS**. Add this volume to your 15 ml tubes containing cells from point 1.4. Proceed immediately with step 1.6.

For suspension cells:

***NOTE**: Collect suspension cells in a 1.5 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 cell culture medium and count them.

1.8 Label 0.2 ml shearing tubes and distribute cell suspension in them in order to have **10,000 cells per tube**. Add medium to reach a final volume of 100 µl in each tube.

1.9 Under a fume hood, add **2.7 µl of 37% formaldehyde** to each tube containing 100 µl of cell suspension and mix gently.

1.10 Incubate 8 minutes at room temperature with occasional manual agitation to allow fixation to take place.

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

1.11 Add **11.5 µl of Glycine** (white cap) to the cells to stop the fixation. Mix gently. Incubate for 5 minutes at room temperature to stop the fixation. Keep everything at 4°C or on ice from now on.

1.12 Collect the cells by centrifugation at 300 x g for 10 minutes at 4°C.

NOTE: *We recommend the use of a swing-out rotor with soft settings for deceleration.*

1.13 Aspirate the supernatant slowly and leave approximately 30 µl of solution. Do not disturb the pellet. Take care to not remove these cross-linked cells.

1.14 Prepare **complete HBSS Buffer** by adding the **protease inhibitor cocktail 200x** (black cap) (e.g. add 7 µl of protease inhibitor cocktail 200x to 1.4 ml of HBSS). This complete HBSS Buffer will be used twice: 120 µl per tube - point 2.2 and 15 µl per tube - point 2.6.

1.15 Wash the cross-linked cells with **120 µl of ice-cold complete HBSS** as follows:

- Add **120 µl of complete HBSS**
- Gently mix to resuspend the cells
- Centrifuge at 300 x g for 10 minutes at 4°C (in a swing-out rotor with soft settings for deceleration). Discard 140 µl of supernatant in order to leave approximately 10 µl of solution.

STEP 2

Cell lysis and chromatin shearing from cells

1 Day 1 45 minutes

2.1 Prepare **complete Lysis Buffer tL1** by adding the **protease inhibitor cocktail 200x** (e.g. add 1.25 µl of protease inhibitor cocktail 200x (black cap) to 250 µl of Lysis Buffer tL1 (white cap)). 25 µl of complete Lysis Buffer tL1 will be needed for each tube. Keep the buffer at room temperature until use. Discard what is not used within a day.

Caution: *Make sure that there are no crystals in the Lysis Buffer tL1 before using. Gently heat and mix until crystals disappear.*

2.2 Add **25 µl of complete Lysis Buffer tL1** to **10,000 cells**. Agitate manually the bottom of the tube to resuspend the cells and allow bubbles to form.

2.3 Incubate for 5 minutes on ice to ensure complete cell lysis.

2.4 Add **15 µl of complete HBSS** to the cell lysate.

NOTE: *If the cells have been FACS-sorted in 25 µl of Lysis Buffer tL1 (white cap) add 25 µl of complete HBSS to reach a final volume of 50 µl.*

2.5 Shear the chromatin by sonication using the Bioruptor Pico:

- Shear for 6 to 10 cycles [30 seconds "ON", 30 seconds "OFF"]

NOTE: *We recommend performing pilot experiments for each new sample type using the Chromatin EasyShear Kit -High SDS, Diagenode, Cat. No. C01020012.*

2.6 Use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 4 months.

2.7 Take one tube 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 aliquots at -20°C until analysis.*

STEP 3

Magnetic immunoprecipitation and tagmentation

1 Day 1-2 1h overnight incubation, 1.5 hours

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. **10 µl of beads are required per IP**.

3.2 Wash the beads 4 times with **50 µl of ice-cold Beads Wash Buffer tBW1 per IP**. To wash the beads, add tBW1, 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.

3.3 After the last wash, resuspend the beads in Beads Wash Buffer tBW1 adding the original volume of beads (this means 10 µl per IP).

3.4 Prepare the Immunoprecipitation mix as described in the table below. Add **150 µl** of **Immunoprecipitation mix** to each chromatin sample.

Component	Volume per reaction
HBSS	50 µl
ChIP Buffer tC1	100 µl
200x Protease Inhibitor Cocktail (black cap)	0.75 µl

Set aside **4 µl** of each sample to be used as an **input sample** and keep at 4°C.

3.5 Add the specific antibody to each tube.

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 0.5 µg of the H3K4me3 positive control antibody (white cap). If a negative control IP is included, use 0.5 µg of Rabbit IgG (white cap).*

3.6 Add **10 µl of the washed magnetic beads** to each tube.

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

NOTE: *A tube holder for 0.2 ml tube with a cap can be attached to the DiaMag Rotator with an elastic band.*

3.8 Perform the washes as follows: briefly spin the tubes and place them in the DiaMag0.2 magnetic rack. Wait for 1 minute and remove the supernatant. Add **150 µl of Wash Buffer tW1**: 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 tW2, tW3 and tagW1, respectively.

3.9 Prepare the ChIPmentation mix as described in the table below for the desired number of reactions, including the inputs. Mix thoroughly with a pipette. Keep on ice until used.

Component	Volume per reaction
Tagmentation Buffer (yellow cap)	19 µl
Tagmentase (loaded) (yellow cap)	1 µl

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

3.11 Add **20 µl** of **ChIPmentation mix** to each IP tube and gently resuspend the beads by pipetting.

3.12 Add **20 µl** of **ChIPmentation mix** and **1 µl** of **MgCl2** (blue cap) to each input sample and gently mix by pipetting.

3.13 Incubate IP and input samples for 10 minutes at 37°C in the preheated 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.*

3.14 Put the samples on ice and immediately add **150 µl of cold Wash Buffer tagW2** to each IP sample, while setting aside the input samples. Gently shake the IP samples to resuspend the beads and incubate for 5 minutes on the DiaMag Rotator at 4°C.

3.15 Briefly spin the IP samples and place them in the DiaMag02, wait until supernatant is clear and discard the supernatant.

3.16 Add **150 µl of cold Wash Buffer tagW1** to each IP samples, gently shake the tubes to resuspend the beads and incubate for 5 minutes on the DiaMag Rotator at 4°C.

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

STEP 4

End repair, reverse cross-linking

 Day 2  30 minutes

4.1 Remove the strip from magnetic rack and add **25 µl of nuclease-free water** to each IP samples.

4.2 Add **25 µl of 2x High-Fidelity Mastermix** (violet cap) and to each IP and input sample, mix by pipeting 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)	

4.3 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 IP or input sample is 50 µl.*

STEP 5

Library amplification

2 Day 2 2.5 hours

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 Dilute 5x the primers with the Primers Dilution Buffer before using them.

5.2 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.

Component	Volume per reaction
Primer Pair S1 (diluted)	0.4 µl
2x High-Fidelity Mastermix (violet cap)	5 µl
100x SYBR	0.1 µl
Nuclease-free water	2.5 µl

5.3 Dispense 8 µl of the Quantification Mix into 0.2 ml tubes or strips according to the number of libraries.

5.4 Add 2 µl of IP'ed or input DNA to each tube and mix by pipetting.

5.5 Briefly spin the tubes and run the PCR 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 and input DNA on ice during the qPCR.

5.6 Analyse the Ct values. The optimal cycle number for the amplification of the rest of the ChIPmentation DNA is typically Ct +1.

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+1 rule applies well in your conditions.*

5.7 Add **2 µl** of the **diluted Primer Pair** with the appropriate index in each tube from step 4.3 and mix by pipetting.

NOTE: *The tubes already contain the mastermix as it was added at step 4.2.*

5.8 Briefly spin the tubes and run the PCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
X (Ct rounded up +1)	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 sizing device 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

Clean-up

2 Day 2 45 minutes

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 µl, add 90 µl 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 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.

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 µl** of **Resuspension Buffer**.

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

2 Day 3

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).*

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. 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 µl of a 10 nM library to 5 µl of a 20 nM library, you have 10 µl 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.*

? ASK THE EXPERTS

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.

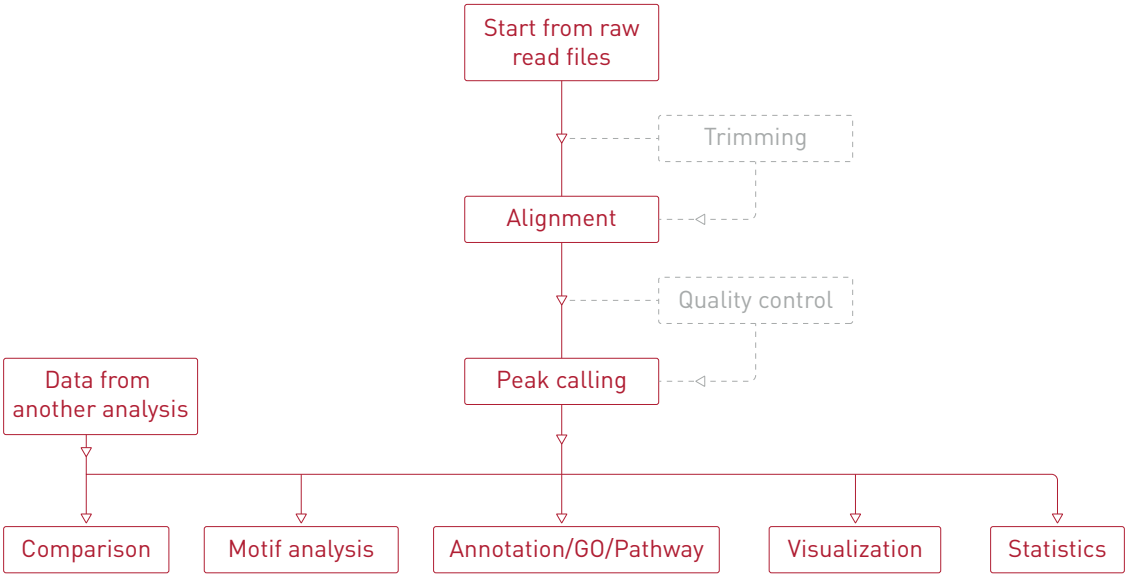


Figure 1. ChIP-seq data analysis workflow

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. Pyicoteo
    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. USCS 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

Chromatin shearing assessment

After chromatin preparation (step 2), one tube of chromatin from 10,000 human K562 cells has been used to check the shearing quality, following the "Protocol for chromatin shearing analysis" provided in the "Additionnal protocols".

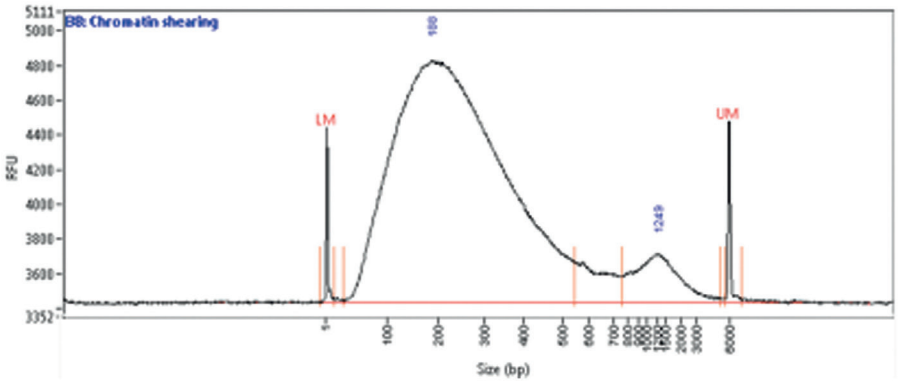


Figure 2. *Size distribution of purified DNA from decross-linked chromatin. The profile has been generated by running 2 µl of DNA on a Fragment Analyzer (Agilent).*

Quality control of the libraries before sequencing (Step 7)

µChIPmentation was performed using sheared chromatin from 10,000 cells, in combination with 0.5 µg of the positive control antibody H3K4me3 (Cat. no. C15410003) or 0.5 µg of the negative IgG control. Tagmentation was applied for 10 minutes. After amplification and purification, H3K4me3 µChIPmentation library was analyzed on Fragment Analyzer (Agilent).

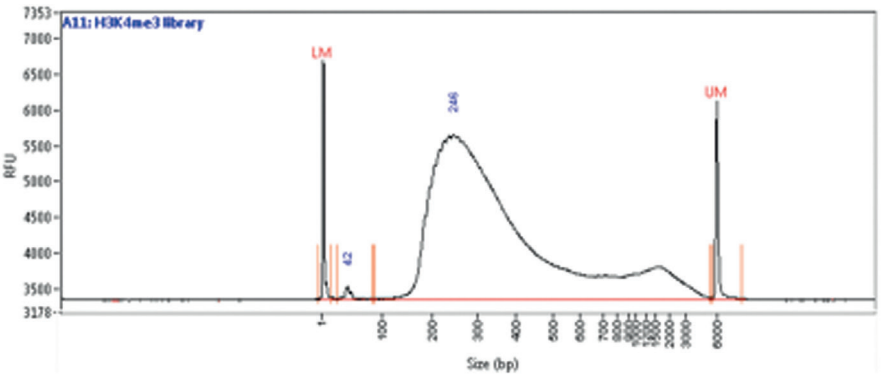


Figure 3. *Library quality control. The distribution of the fragments sizes was assessed by loading 6 ng of library on Fragment Analyzer (Agilent).*

Sequencing

µChIPmentation libraries were finally sequenced on Illumina's HiSeq3000/4000 and the data analyzed as described in the section "ChIP-seq data analysis recommendations".

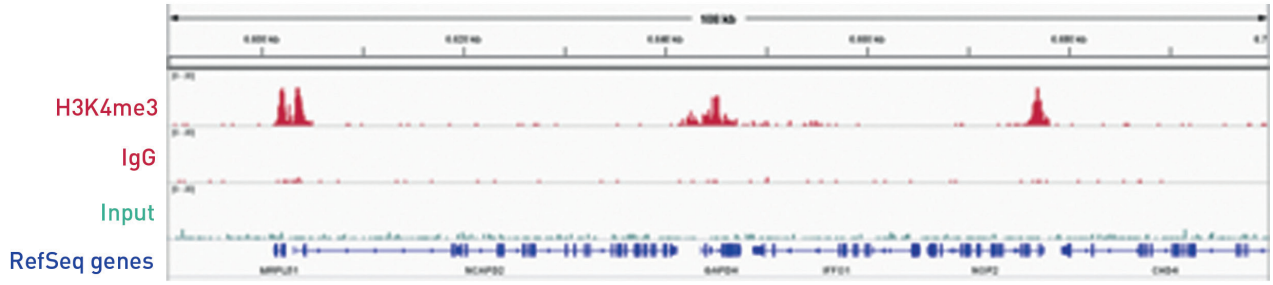


Figure 4. *Distribution of the µChIPmentation dataset for H3K4me3, in a representative region of the genome.*

ADDITIONAL PROTOCOLS

Protocol for fixation of a larger amount of cells - 100,000 cells

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. 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 15 ml tube.

1.5 Rinse the flask by adding **10 ml** of PBS. Add this volume to your **15 ml** tubes containing cells from point 1.4. Proceed immediately with step 1.6.

For suspension cells:

Collect suspension cells in a 1.5 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 cell culture medium and count them.

1.8 Label 1.5 ml tubes and distribute cell suspension in them in order to have **100,000 cells per tube**. Add medium to reach a final volume of 1 ml in each tube.

1.9 Under a fume hood, add **27 µl of 37% formaldehyde** to each tube containing 1 ml of cell suspension and invert tubes immediately two to three times to ensure complete mixing.

1.10 Incubate 8 minutes at room temperature with occasional manual agitation to allow fixation to take place.

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

1.11 Add **115 µl of Glycine** (white cap) to the cells to stop the fixation. Mix by inversion of the tube four or five times. Incubate for 5 minutes at room temperature to stop the fixation. Keep everything at 4°C or on ice from now on.

1.12 Collect the cells by centrifugation at **300 x g** for 10 minutes at 4°C.

NOTE: *We recommend the use of a swing-out rotor with soft settings for deceleration.*

1.13 Aspirate the supernatant slowly and leave approximately 30 µl of solution. Do not disturb the pellet. Take care to not remove these cross-linked cells.

1.14 Prepare **complete HBSS Buffer** by adding the **protease inhibitor cocktail 200x** (black cap) (e.g. add 6 µl of protease inhibitor cocktail 200x to 1.2 ml of HBSS). This complete HBSS Buffer will be used twice: 1 ml per fixation tube - point 2.2 and 15 µl per 10,000 cells - point 2.6.

1.15 Wash the cross-linked cells with **1 ml of ice-cold complete HBSS** as follows:

- Add **1 ml of complete HBSS**
- Invert the tube 4-5 times to resuspend the cells
- Distribute the cell suspension in 0.2 ml shearing tubes to have 10,000 cells/tube (which is 100 µl of cell suspension).
- Centrifuge at 300 x g for 10 minutes at 4°C (in a swing-out rotor with soft settings for deceleration). Discard 90 µl of supernatant in order to leave approximately 10 µl solution.

NOTE: *Samples in complete HBSS could be FACS-sorted and collected directly in 0.2 ml shearing tubes, in Lysis Buffer tL1 (white cap). The concentration should be 10,000 cells per tube, in 25 µl of Lysis Buffer tL1.*

Proceed to the Step 2 in the section "Protocol".

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 validated solutions.

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

Workflow for analysis of sheared chromatin:

- Reverse crosslinking
- RNAse treatment using RNAse cocktail (e.g. Ambion, AM 2286A)
- DNA purification using MicroChIP DiaPure Columns (Diagenode, Cat. No. C03040001)
- Fragment size assessment (Fragment Analyzer, Agilent)

Reverse cross-linking (reagents included in the kit)

1. Add **50 µl** of **Elution Buffer tE1** (clear cap).

2. Add **4 µl** of **Elution Buffer tE2** (clear cap), mix thoroughly.

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

Loading on MicroChIP DiaPure columns

1. In a 1.5 ml microcentrifuge tube, add **5 volumes of ChIP DNA Binding Buffer** to each volume of sample (5:1). Mix briefly.

Example: Add 500 µl of ChIP DNA Binding buffer to 100 µl of cell lysate following DNA shearing, reverse cross-linking.

2. Transfer mixture to a provided spin column in a Collection tube.

3. Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.

RNAse treatment

1. Add **1 µl of RNase cocktail** to the column, directly on the membrane.

2. Incubate for 15 minutes at room temperature.

DNA purification

1. Add **200 µl of DNA Wash Buffer** to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.

2. Repeat centrifuge step to make sure that there is no ethanol left.

3. Add **6 µl of DNA Elution Buffer** directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube.

4. Centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.

Fragment size assessment

Use **2 µl** of sample for Qubit quantification and **1 or 2 µl** of sample for Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit (DNF-473 Agilent)).

Protocol for quantitative PCR analysis

NOTE: *For each primer pair, run the Input DNA alongside the immunoprecipitated samples and negative IgG control (white cap).*

1. Pick an aliquot of each library and dilute it to reach **0.4 ng/µl**.

2. Prepare the qPCR mix as follows (20 µl reaction volume):

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

3. Use the following PCR program:

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

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

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

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

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

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

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

• Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and input sample, respectively.

Protocol for manual size selection


1 hour

1. Add **30 µl** of Nuclease-free 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**.

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.

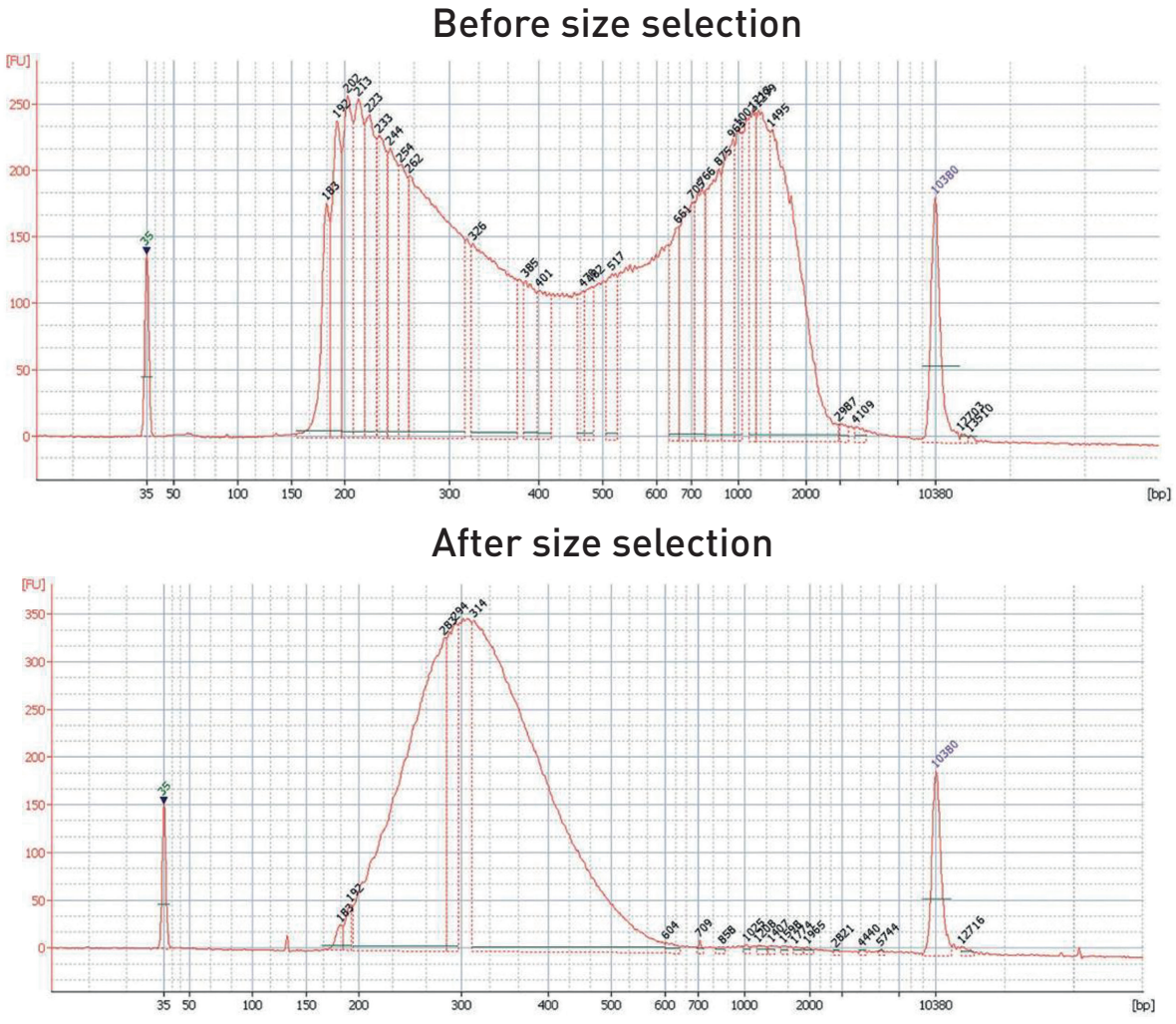


Figure 5. *Example of results of the size selection on a ChIPmentation library for H3K27me3. The library was run on a BioAnalyzer (Agilent) before and after the size selection.*

FAQs

Is the included control H3K4me3 antibody compatible with mouse?

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

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

10 µl of DiaMag Protein A-coated magnetic beads can bind 3 µg of antibody.

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

The µChIPmentation 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).

What are the expected concentration and size of µChIPmentation 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 2-4 ng/µl is enough for a quality control using the Qubit High Sensitivity assay (ThermoFischer Scientific) and the HS NGS Fragment Kit for Fragment Analyzer (Agilent), and for sequencing on Illumina NovaSeq6000.

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.

What is the composition of buffers included in the kit?

The composition of the buffers is proprietary.

Related products

Product	Cat. No.
24 SI for Tagmented libraries	C01011032
8 SI for Tagmented libraries	C01011033
24 UDI for tagmented libraries - Set I	C01011034
8 UDI for tagmented libraries - Set I	C01011035
24 UDI for Tagmented libraries - Set II	C01011036
Chromatin EasyShear kit – High SDS	C01020012
Bioruptor Pico	B01080010
Tube holder for 0.2 ml tubes	B01201144
0.2 ml Pico Microtubes	C30010020

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

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