AQUA-FAANG – Standard Operating Protocol **uChIPmentation protocol for turbot tissues – Day 1: Tissue disruption, crosslinking and sonication of turbot muscle and gonad**

OVERVIEW

This protocol is based on the UNIPD uChIPmentation modified protocol for seabass frozen tissues (not yet uploaded to the repository), which has been adapted for turbot. This protocol describes a method for the disruption of different turbot tissues, crosslinking and sonication for uChIPmentation procedures.

EQUIPMENT

- Microscope, cell counting chamber, coverslips and Trypan Blue
- 1,5ml Eppendorf tubes
- 1.5/5ml LoBind tubes
- 130µl microTUBE Snap-Cap sonication tubes
- Tweezers
- A 15ml douncer with pestles; one loose (A) and one tight (B)
- Sonication device (Covaris S2)
- Rotatory system (HulaMixer)
- Ice Bucket
- 1,5/2ml centrifuge set-up at 4ºC
- An Incubator (Thermomixer) set-up at 68ºC
- Easy Shear Kit
- A ceramic mortar and pestle (**muscle only**)
- A metallic spoon or spatula (**muscle only**)
- Liquid nitrogen (preferred) or dry ice (**muscle only**)
- Qubit device and reagents (High Sensitivity)
- Bioanalyzer, chips and reagents (High Sensitivity)
- MinElute PCR Purification Kit Qiagen

SOLUTIONS TO PREPARE FRESH:

- PBS with PIC (2ml/sample): 1 PIC tablet in 10ml PBS or 10µl PIC solution (kit) in 2ml PBS
- Complete tL1 (80ul/sample): 1µl PIC solution (kit) and 200µl tL1
- Complete HBSS (1ml/sample + 75µl/lysis): 1µl PIC solution (kit) + 200µl HBSS
- 37% formaldehyde (27ul/sample)
- HBSS (Hanks Balanced Salt Solution)
- NaCl 5M
- RNase (100mg/ml)
- Proteinase K (20mg/ml)

TISSUE DISRUPTION

1. **Muscle only:** Transfer the piece of muscle into a refrigerated cold mortar (ice box with liquid nitrogen or dry ice) and grind the pieces until obtaining powder
2. Transfer the piece of tissue (or grinded muscle powder) into a douncer containing 1.2ml of PBS with PIC in ice

Tissue	Recommended fragment size (mg)
Muscle	100
Female Gonad	60
Male Gonad	60

3. Use pestles A and B for the homogenization until obtaining a homogenized solution or feel no more strong resistance (recommended starting point is 5 strokes with pestle A and 10 with pestle B. Gonads might require more strokes).

   NOTE: We recommend to take an aliquot of 5µl and mix with trypan blue (1:1). Assess the level of homogenization. If there are many big clumps of tissue, collect and filter the homogenate on a 70um cell strainer. If not, got to step 4.
4. Transfer the homogenate directly into a 2ml Eppendorf tube.
5. Rinse the douncer and each pestle with 400µl for muscle and 200µl for gonads of PBS with PIC and add the volume to the previous solution.
6. Mix well to make the cell suspension homogeneous.
7. Take an aliquot of 10µl to assess the number nuclei. We recommend assessing it with a 10x and 1x dilution. Add trypan blue in a 1:1 proportion for microscopy observation and estimate the nuclei amount in the total volume (Should be ~1.4ml).
8. Transfer 600-800µl to a 1.5ml LoBind tube.

CROSSLINKING

9. Add 27µl (per ml of sample) of 37% stock formaldehyde to each tube containing the cell suspension and mix gently.
10. Incubate under constant agitation at RT, 8 minutes (HulaMixer, 50 rpm)

11. Quench the reaction with 115µl of Glycine to the sample to stop crosslinking. Mix gently and incubate under constant agitation at RT, 5 min (HulaMixer, 50 rpm)
12. Centrifuge 10 min, 300g, 4ºC (if possible, use soft deceleration)
13. Remove and eliminate in appropriate trash the supernatant containing formaldehyde, leaving ~30µl of PBS + PIC
14. Add 1ml of ice cold complete HBSS and gently vortex to resuspend the cells
15. Centrifuge 10min, 300g, 4ºC (if possible, use soft deceleration)
16. Remove the supernatant and keep the cell pellet on ice.

CELL LYSIS AND CHROMATIN SHEARING

NOTE: For each ~3 million nuclei, double the volume of reagents indicated below. For our samples, the number of nuclei ranged from 4 to 7 million in muscle, and 3 to 8 million in gonad

17. Add 25 µl of complete HBSS and gently resuspend the pellet
18. Add 70 µl of complete tL1, agitate manually the bottom of the tube to resuspend cells. Avoid bubbles.
19. Incubate 8 min on ice to ensure complete lysis
20. Add 40 µl of complete HBSS and mix gently.
21. Transfer 130µl of cell suspension to a microTUBE Snap-Cap (130 µl), or more tubes if the number of nuclei was above 3 million
22. Shear the chromatin by sonication using the following parameters (for Covaris S2):

Tissue	Sonication parameters (Covaris S2)	Treatment time (minutes)
Muscle	Duty Cycle: 2% Intensity: 3 Cycles/Burst 200	6
Female Gonad		7
Male Gonad		6
Immature Gonad		7

23. Transfer the sonicated solution into a LoBind 1.5 ml Eppendorf tube and note the volume. If more than one tube were used for a sample, pool all of them together in the same LoBind 1.5ml Eppendorf tube
24. Take out an aliquot of 20µl for sonication control for each sample
    OPTIONAL STOP POINT: Place the tubes of sonicated chromatin and aliquots for sonication control for storage in -80ºC

TEST OF THE SONICATION

25. To the 20µl for sonication control, add 67µl of elution buffer, 2µl of RNase A, 5µl of proteinase K and 6µl of NaCl 5M
26. Incubate 1h30m, 68ºC under agitation (Thermomixer, 500rpm)
27. Purify with a Qiagen DNA Minelute PCR purification kit. Follow the kits protocol but elute with 20µl of elution buffer instead of the kit's buffer at the final step.
28. Quantify by HSQubit the chromatin concentration of 1µl of eluate and report the value (ng/ul)
29. Run 1µl of the eluate in the Bioanalyzer (High Sensitivity) to assess the profile

NOTE: To be qualified as good for uChIPmentation, minimum 60% of the sonicated chromatin should have a size distribution of 200-700bp, centered around 350-400bp.

AQUA-FAANG – Standard Operating Protocol **uChIPmentation protocol for turbot tissues – Day 1: Cell lysis and chromatin shearing of crosslinked turbot embryos**

OVERVIEW

This protocol is based on the Roslin institute uChIPmentation modified protocol for Atlantic salmon and rainbow trout crosslinked embryos (not yet uploaded to the repository), which has been adapted for turbot embryos. This protocol describes a method for the shearing of chromatin fragments.

EQUIPMENT

- Sonication device (Covaris S2)
- Rotatory system (HulaMixer)
- 1,5/2ml centrifuge set-up at 4ºC
- A 1.5ml plastic pestle
- 130µl microTUBE Snap-Cap sonication tubes
- 1,5/2ml Eppendorf tubes
- MinElute PCR Purification Kit Qiagen
- Ice Bucket
- An Incubator set-up at 68ºC
- Easy Shear Kit
- Qubit device and reagents (High Sensitivity)
- Bioanalyzer, chips and reagents (DNA1000)
- Microscope, cell counting chamber, coverslips Trypan Blue
- 1.5/2ml LoBind tubes

SOLUTIONS TO PREPARE FRESH:

- PBS with PIC (2ml/sample): 1 PIC tablet in 10ml PBS, or 10µl PIC solution (kit) and 2ml PBS
- Complete tL1 (80ul/sample): 1µl PIC solution (kit) and 200µl tL1
- Complete HBSS (1ml/sample + 75ul/lysis): 1µlPIC solution (kit) + 200µl HBSS
- 37% formaldehyde (27ul/sample)
- HBSS (Hanks Balanced Salt Solution)

- NaCl 5M
- RNase (100mg/ml)
- Proteinase K (20mg/ml)

EMBRYO DISRUPTION

1. Take out the crosslinked samples (~50 crosslinked embryos in 1 ml PBS) and thaw them at RT by hand
2. Add 5µl of 200x PIC (easy shear kit) directly and transfer the samples to a 2ml LoBind tube. The resulting solution will be PBS + PIC 1X
3. Centrifuge 2500g, 5 min 4ºC
4. Discard the supernatant without disturbing the pellet, leaving ~100µl of PBS + PIC
5. Keeping the sample on ice, homogenize using a P200 pipette (early stages) or plastic pestle (late stages) until sample looks milky and most big chunks disappear (20-40 strokes)
   OPTIONAL STEP: Pass through 70um filter if big chunks of tissue remain
6. Bring up the volume to 500ml with PBS + PIC 1X
7. Check and count in a haemocytometer using a 1:1 with Trypan Blue
8. Centrifuge 2500g, 5 min 4ºC
9. Discard supernatant

For late stages (Late segmentation and prehatching)

10. Lyse in 189µl of complete Lysis Buffer tL1 (easy shear kit) and 0.945 µl PIC (kit), mix well by pipetting and keep on ice for up to 30min
11. Add 111 µl complete HBSS

For early stages (Blastula, Gastrula, Early and Mid segmentation)

12. Lyse in 81.9µl of complete Lysis Buffer tL1 (easy shear kit) and 0.4µl PIC (kit), mix well and keep on ice for up to 30 min
13. Add 48.1µl complete HBSS to bring up the volume to 130ul

SONICATION

14. Transfer the suspension to a 130µl sonication tube, and keep on ice until sonication
15. Sonicate the chromatin with the following parameters (Covaris S2):

Sample	Sonication parameters	Treatment time
Pools of embryos	Duty Cycle: 2% Intensity: 3 Cycles/Burst 200	7-8 min

16. Transfer the sonicated solution into a LoBind 1.5 ml Eppendorf tube and note the volume
17. Take out an aliquot of 20µl for sonication control for each sample
18. OPTIONAL STOP POINT: Place the tubes of diluted sonicated chromatin and aliquots for sonication control for storage in -80ºC

TEST OF THE SONICATION

19. To the 20µl for sonication control, add 67µl of elution buffer, 2µl of RNase A, 5µl of proteinase K and 6µl of NaCl 5M
20. Incubate 1h30m, 68ºC under agitation (Thermomixer, 500rpm)
21. Purify with a Qiagen DNA Minelute PCR purification kit and elute with 20µl of elution buffer instead of the kit's buffer.
22. Quantify by HSQubit the chromatin concentration of 1µl µl of eluate and report the value (ng/ul)
23. Run 1µl of the eluate in the Bioanalyzer (High Sensitivity) to assess the profile

To be qualified as good for uChIPmentation, minimum 60% of the sonicated chromatin should have a size distribution of 200-700bp, centered around 350-400bp.

AQUA-FAANG – Standard Operating Protocol **uChIPmentation protocol for cell cultures – Day 1: Crosslinking and sonication of turbot *in vitro* leucocytes**

OVERVIEW

This protocol is based on the UNIPD uChIPmentation modified protocol for seabass frozen tissues (not yet uploaded to the repository), which has been adapted for turbot. This protocol describes a method for the crosslinking of primary leukocyte cultures from turbot and their sonication for uChIPmentation procedures.

EQUIPMENT

- Sonication device (Covaris S2)
- Rotatory system (HulaMixer)
- 1,5/2ml centrifuge set-up at 4ºC
- A 1.5ml plastic pestle
- 130µl microTUBE Snap-Cap sonication tubes
- 1,5/2ml Eppendorf tubes
- MinElute PCR Purification Kit Qiagen
- Ice Bucket
- An Incubator set-up at 68ºC
- Easy Shear Kit
- Qubit device and reagents (High Sensitivity)
- Bioanalyzer, chips and reagents (DNA1000)
- Microscope, cell counting chamber, coverslips Trypan Blue
- 1.5/2ml LoBind tubes

SOLUTIONS TO PREPARE FRESH:

- PBS with PIC (2ml/sample): 1 PIC tablet in 10ml PBS, or 10µl PIC solution (kit) and 2ml PBS
- Complete tL1 (80ul/sample): 1µl PIC solution (kit) and 200µl tL1

- Complete HBSS (1ml/sample + 75ul/lysis): 1µlPIC solution (kit) + 200µl HBSS
- 37% formaldehyde (27ul/sample)
- HBSS (Hanks Balanced Salt Solution)
- NaCl 5M
- RNase (100mg/ml)
- Proteinase K (20mg/ml)

TISSUE DISRUPTION

1. Thaw the pelleted cells on ice and resuspend in 1.5ml of PBS with PIC, until the cell suspension is homogeneous.
2. Take an aliquot of 10µl to assess the number nuclei. We recommend assessing it with a 10x and 1x dilution. Add trypan blue in a 1:1 proportion for microscopy observation and estimate the nuclei amount in the total volume (Should be ~1ml).

CROSSLINKING

3. Add µl 40µl (per ml of sample) of 37% stock formaldehyde to each tube containing the cell suspension and mix gently.
4. Incubate under constant agitation at RT, 8 min (HulaMixer 50 rpm)
5. Quench the reaction with µl 172.5µl of Glycine to the sample to stop crosslinking. Mix gently and incubate under constant agitation at RT, 5 minutes (HulaMixer 50 rpm)

Centrifuge 10 min, 300g, 4ºC (if possible, use soft deceleration)

6.
7. Remove and eliminate in appropriate trash the supernatant containing formaldehyde, leaving ~30µl of PBS + PIC
8. Add 1ml of ice cold complete HBSS and gently vortex to resuspend the cells
9. Centrifuge 10min, 300, 4ºC (if possible, use soft deceleration)

10. Remove the supernatant and keep the cell pellet on ice.

CELL LYSIS AND CHROMATIN SHEARING

NOTE: For each ~3 million nuclei, double the volume of reagents indicated below. For our samples, the number of nuclei ranged from 1.5 to 3 million.

11. Add 25 µl of complete HBSS and gently resuspend the pellet
12. Add 70 µl of complete tL1, agitate manually the bottom of the tube to resuspend cells. Avoid bubbles.
13. Incubate 8 min on ice to ensure complete lysis
14. Add 40 µl of complete HBSS and mix gently.
15. Transfer 130µl of cell suspension to a microTUBE Snap-Cap (130ul), or more tubes if the number of nuclei was above 3 million
16. Shear the chromatin by sonication using the following parameters (for Covaris S2):

Sample	Sonication parameters	Treatment time
Leukocyte cell cultures	Duty Cycle: 2% Intensity: 3 Cycles/Burst 200	6 min

17. Transfer the sonicated solution into a LoBind 1.5 ml Eppendorf tube and note the volume. If more than one tube were used for a sample, pool all of them together in the same LoBind 1.5ml Eppendorf tube
18. Take out an aliquot of 20µl for sonication control for each sample

OPTIONAL STOP POINT: Place the tubes of sonicated chromatin and aliquots for sonication control for storage in -80ºC

TEST OF THE SONICATION

19. To the 20µl for sonication control, add 67µl of elution buffer, 2µl of RNase A, 5µl of proteinase K and 6µl of NaCl 5M
20. Incubate 1h30m, 68ºC under agitation (Thermomixer, 500rpm)
21. Purify with a Qiagen DNA Minelute PCR purification kit. Follow the kit protocol but elute with 20µl of elution buffer instead of the kit's buffer at the final step.
22. Quantify by HSQubit the chromatin concentration of 1µl of eluate and report the value (ng/ul)
23. Run 1µl of the eluate in the Bioanalyzer (High Sensitivity) to assess the profile

NOTE: To be qualified as good for uChIPmentation, minimum 60% of the sonicated chromatin should have a size distribution of 200-700bp, centered around 350-400bp.

AQUA-FAANG – Standard Operating Protocol **uChIPmentation protocol for turbot tissues – Day 2: Beads preparation and immunoprecipitation**

Following the uChIPmentation Kit for Histones (Version 4 02_2022) start from “STEP 3 – Magnetic immunoprecipitation and tagmentation” with the following modifications to these steps:

3.5. Instead of 0.5ug, add 0.8ug of antibodies
3.13. Incubate input samples for 20 minutes and IP samples for 30 minutes

Be aware that some of the reagents and material mentioned in the kit’s protocol are not included in it, such as:

- Antibodies for the desired marks (H3K4me1, H3K4me3, H3K27ac, H3K27me3, CTCF)
- Hanks Balanced Salt Solution (HBSS)
- Magnetic rack
- Rotatory system (MX-RD-E)
- LoBind tubes (1.5/2ml)

AQUA-FAANG – Standard Operating Protocol **uChIPmentation protocol for turbot tissues – Day 3: Bead washing, decrosslinking and DNA recovery**

Following the uChIPmentation Kit for Histones (Version 4 02_2022) start from “STEP 4 – Stripping, end repair, reverse cross-linking” all the way to “STEP 7”, with the following modifications:

Be aware that some of the reagents and material mentioned in the kit’s protocol is not included with said kit, such as:

- Ampure XP beads
- Magnetic rack
- 80% ethanol (freshly prepared)
- LoBind tubes (1.5/2ml)

For “STEP 7 – Quality control” two additional kits are required:

- Qubit High Sensitivity assay kit (ThermoFisher Scientific) or similar
- High Sensitivity chip and reagents for Bioanalyzer (Agilent) or similar

NOTE: To be qualified as good for uChIPmentation, minimum 60% of the sonicated chromatin should have a size distribution of 200-700bp, centered around 350-400bp. If the size distribution is lower than 60% for the 200-700bp range, consider following Diagenodes “Protocol for manual size selection”.

diagenode

A Hologic Company

Chromatin EasyShear Kit - High SDS

Previous name: Chromatin Shearing Optimization Kit – High SDS (True MicroChIP kit)

Cat. No. C01020012

Compatible with:

True MicroChIP Kit

µChIPmentation Kit for Histones

USER GUIDE

Version 6 03_2021

Please read this manual carefully
before starting your experiment

Contents

Introduction

Chromatin shearing is a crucial step for the success of ChIP experiments. It is important to establish optimal conditions to shear cross-linked chromatin to get the correct fragment sizes needed for ChIP. A successful chromatin preparation relies on the optimization of **cross-linking**, **cell lysis** and **sonication** itself. All 3 steps are interconnected and need to be optimized individually for different experimental settings. Our **Chromatin EasyShear Kits (previous name: Chromatin Shearing Optimization Kits)** together with the **Bioruptor®** combine **efficient cell lysis** and **chromatin shearing** leading to **consistent results**.

The Chromatin EasyShear Kits are recommended for:

- The optimization of the chromatin shearing of a new cell line/ new sample type prior to ChIP using Diagenode's ChIP kits
- The optimization of the chromatin shearing and/or chromatin preparation prior to ChIP using other protocols

Each Chromatin EasyShear Kit provides optimized reagents and a thoroughly validated protocol according to your specific experimental needs. SDS concentration is adapted to each workflow taking into account target-specific requirements.

Choose an appropriate kit for your specific experimental needs and get consistent results.

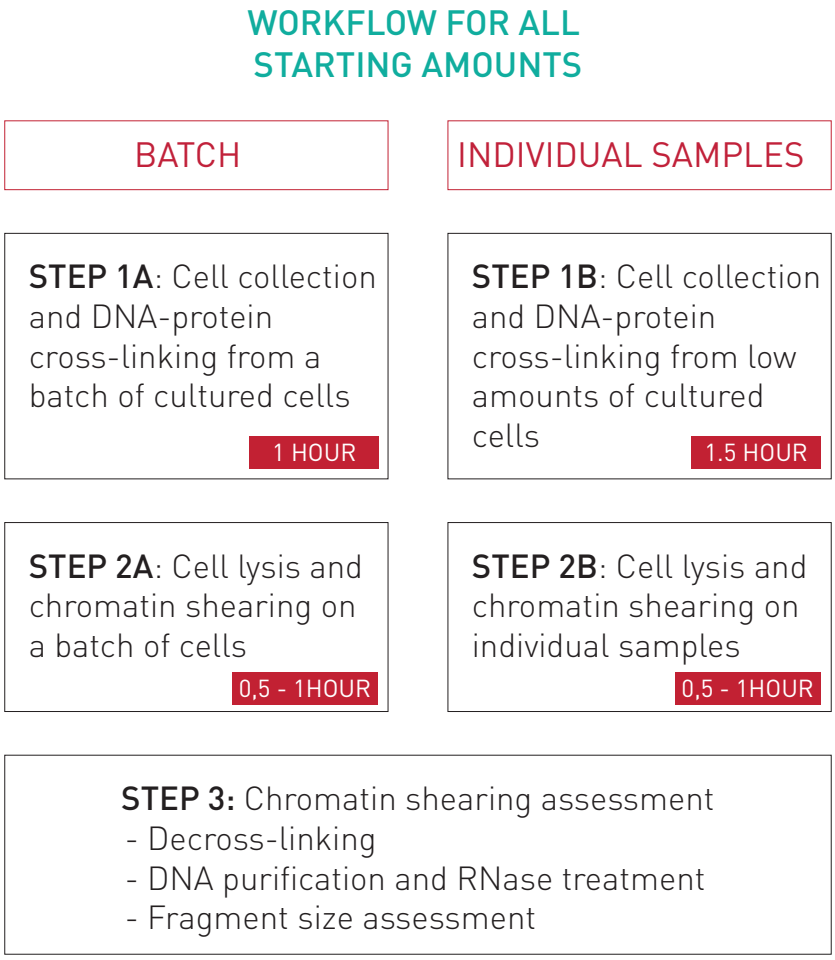
	Chromatin EasyShear Kit Ultra Low SDS	**Chromatin EasyShear Kit** Low SDS	**Chromatin EasyShear Kit** for Plant	**Chromatin EasyShear Kit** High SDS
Cat. No.	C01020010	C01020013	C01020014	C01020012
Sample type	Cells, tissue	Cells, tissue	Plant tissue	Cells - low amount
Target	Histones	Transcription Factors and histones	Histones	Histones
Nuclei isolation	Yes	Yes	Yes	No
SDS concentration	< 0.1%	0.2%	0.5%	1%
Corresponding to shearing buffers from	iDeal ChIP-seq Kit for Histones ChIPmentation Kit for Histones	iDeal ChIP-seq Kit for Transcription Factors iDeal ChIP qPCR Kit iDeal ChIP-FFPE Kit	Universal Plant ChIP-seq Kit	True MicroChIP Kit µChIPmentation for Histones

Kit Method Overview

The Chromatin EasyShear Kit – High SDS is validated for the chromatin preparation from a **limited number of cells** and can be used for the optimization of chromatin preparation prior to ChIP on histones performed with the following Diagenode kits:

ChIP kit	Manual version	Automated version
TrueMicro ChIP kit	C01010130	C01010140
µChIPmentation Kit for Histones	C01011011 C01011012	N/A

If non-Diagenode protocol will be used for immunoprecipitation step, sheared chromatin should be diluted at least 5 times per immunoprecipitation reaction with a detergent-free buffer (no SDS, sodium deoxycholate etc...).



Kit materials

The Chromatin EasyShear Kit – High SDS allows a nice flexibility in the cell number and experimental design. It contains enough reagents to perform 150 chromatin preparations in most of the experimental plans.

This kit does not contain reagents for DNA purification. We highly recommend using MicroChIP DiaPure columns (Diagenode, Cat. No. C03040001), which can be purchased separately.

The kit content is described in Table 2. Upon receipt, store the components at the indicated temperatures.

Table 2. Components supplied with the Chromatin EasyShear Kit - High SDS

Description	Quantity	Storage
Glycine	17.25 ml	4°C
Lysis Buffer tL1	3.75 ml	4°C
Elution Buffer tE1	15 ml	4°C
Elution Buffer tE2	1200 µl	4°C
TE Buffer	7.5 ml	4°C
Protease inhibitors cocktail	160 µl	-20°C

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 and 15 ml tubes
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS)
- Cell culture medium
- Hank's balanced salt solution (HBSS – e.g. ThermoFisher Scientific, 14175095)
- MicroChIP DiaPure columns (Diagenode, Cat. No. C03040001)
- RNase cocktail (e.g. Ambion, AM2286)

Equipment

- Fume hood
- Bioruptor® sonication device and the associated microtubes:
  - **Bioruptor Pico (Diagenode, Cat. No. B01060010)**,
  - 0.2 ml microtubes (Cat. No. C30010020) or
  - 0.65ml microtubes (Cat. No. C30010011) or
  - 1.5 ml microtubes with caps (Cat. No. C30010016)
  - **Bioruptor Plus (Diagenode, Cat. No. B01020003)**,
  - 0.5ml microtubes (Cat. No. C30010013) or
  - 1.5 ml TPX microtubes (Cat. No. C30010010-300)
- Refrigerated centrifuge for 1.5 ml tubes
- Centrifuge for 15 ml tubes
- Thermomixer
- Cell counter
- Vortex
- DNA sizing equipment (an automated capillary electrophoresis instrument, e.g. Fragment Analyzer or Bioanalyzer (Agilent) and High Sensitivity Kits or agarose gel electrophoresis)

Remarks before starting

A high-quality chromatin sample combines two main features: a suitable fragment size range and the availability of cross-linked epitopes for ChIP. A perfect sonication profile is a profile from which the highest specific signal and the lowest background are generated.

There is not one universal size range generally applicable for ChIP. As a rule, fragments between **100-600 bp** are suitable for the majority of ChIP experiments and can be used as a starting point. This range can be fine-tuned for particular experimental requirements depending on the specificity of the target (histones or non-histone proteins) and the required down-stream analysis (ChIP-qPCR or ChIP-seq). Generally, a tighter fragment distribution is recommended for ChIP-seq than for ChIP-qPCR. Note that **100-300 bp** is compatible (but not absolutely necessary) with **histone ChIP-seq** while a **broader fragment range** is more suitable for **non-histone ChIP-seq** (transcriptional factors and proteins that are not bound directly to DNA and for long-distance interacting proteins). With the recent evolution of sequencing technologies, it is common to perform ChIP-seq experiments from chromatin with a distribution in the 100–800 bp range. Moreover, ChIPmentation is less sensitive to the presence of large fragments because the DNA is fragmented during the tagmentation step.

Before starting the ChIP, the chromatin should be sheared to the desired size. Sonication should be optimized for each ChIP project since samples are different in their resistance to sonication. The following parameters should be considered during the optimization of chromatin preparation:

- Starting amount of cells
- Fixation
- Shearing optimization (followed by shearing assessment)

Starting amount of cells

Several variations of the protocol are available depending on the starting material.

- For chromatin preparation from 20,000 to 700,000 cells use the BATCH protocol. When possible, this option is preferred in order to limit tube-to-tube variability.
- For chromatin preparation from 10,000 to 20,000 cells use the INDIVIDUAL SAMPLES protocol. The shearing volume has to be adapted depending on the sonication tubes used.

Please check table 3 for more details.

Table 3. Description of the two protocols

STEP		BATCH PROTOCOL	INDIVIDUAL SAMPLES PROTOCOL	
Fixation	*Cell number/tube*	**20k - 700k**	**10k - 20k**	**10k - 20k**
	Final volume/tube	**1 ml**	**100 µl**	**100 µl**
	Tube	**1.5 ml**	**0.5/0.65 ml (Bioruptor)**	**0.2 ml (Bioruptor)**
Glycine + HBSS wash				
Lysis	*Cell number/tube*	**20k - 700k**	**10k - 20k**	**10k - 20k**
	Final volume/tube	**Multiples of (25 µl + 75 µl) HBSS***	**25 µl + 75 µl HBSS**	**25 µl + 15 µl HBSS**
	Tube	**0.5/0.65 or 1.5 ml (Bioruptor)**	**0.5/0.65 ml (Bioruptor)**	**0.2 ml (Bioruptor)**
Shearing	*Cell number/tube*	**10k - 300k**	**10k - 20k**	**10k - 20k**
	Final volume/tube	**100 - 300 µl**	**100 µl**	**50 µl**
	Tube	**0.5/0.65 or 1.5 ml (Bioruptor)**	**0.5/0.65 ml (Bioruptor)**	**0.2 ml (Bioruptor)**
	Bioruptor compatibility	**Pico or Plus**	**Pico or Plus**	**Pico**
Immunoprecipitation (not included)	*Cell number/tube*	10k - 100k	10k - 20k	10k - 20k
	Final volume/tube	200 µl	200 µl	200 µl
	Tube	1.5 ml	1.5 ml	0.2 ml (Bioruptor)
True MicroChIP kit	*Kit compatibility*	**Yes**	**Yes, preferred**	**Yes**
µChIPmentation kit		**Yes**	**Yes**	**Yes, preferred**

** According to the number of cells chosen for each IP, and the number of IPs*

Option A – BATCH PROTOCOL

The protocol describes the preparation of a batch of chromatin. The starting number of cells for one batch depends on the experimental plan (availability of cells and number of reactions to run). First, determine the number of IP (or shearing) reactions you will perform and the number of cells to be used per reaction. Then, start with fixation of a unique batch of chromatin accordingly:

Number of cells per chromatin batch = Number of cells per IP (from 10,000 to 100,000) x number of IPs.

Notes:

- *The minimum recommended number of cells to start the chromatin preparation (at fixation step) is 20,000 cells. This is enough for 2 immunoprecipitation/sonication samples, each of 10,000 cells.*
- *The maximum recommended number of cells to start the chromatin preparation (at fixation step) is 700,000 cells. If more cells are needed, then proceed with a separate chromatin preparation.*

Option B – INDIVIDUAL SAMPLES PROTOCOL

The µChIPmentation kit for histones and the True MicroChIP kits allow successful ChIP on as low as 10,000 cells per reaction, starting from the fixation step. The fixation step is performed in a smaller volume and directly in the shearing tube.

Note: *The maximum recommended number of cells to start the chromatin preparation (at fixation step) is 20,000 cells. If more cells are needed, then proceed with the batch protocol.*

The choice of the shearing tube will have an impact on this protocol. Depending on the size of the shearing tubes used, the shearing volume will need to be adapted as described in the protocol.

The different options are:

- 0.2 ml microtubes (Cat. No. C30010020) they are only available for Bioruptor Pico. They are recommended when using µChIPmentation, where they allow a single-tube approach for

efficient ChIP. In this protocol the number of sample transfer is limited: chromatin preparation, immunoprecipitation and tagmentation are performed in one single microtube, in order to reduce sample loss.

- 0.5ml microtubes (Cat. No. C30010013): they are recommended when using Bioruptor Plus. They are easier to handle but are not compatible with a thermocycler for tagmentation step. Therefore they are not suited for µChIPmentation single-tube approach, but can be used in combination with True MicroChIP kit.
- 0.65ml microtubes (Cat. No. C30010011): They are easier to handle and associated with Bioruptor Pico. But they are not compatible with a thermocycler for tagmentation step. Therefore they are not suited for µChIPmentation single-tube approach, but can be used in combination with True MicroChIP kit.

Fixation optimization

The described protocols use a mild fixation sufficient for histone proteins. Cells should be re-suspended in PBS prior to the fixation to avoid cell clusters formation upon fixation to ensure a proper shearing efficiency.

Formaldehyde is the most commonly used cross-linking reagent ideal for two molecules which interact directly. The fixation time can depend on your target of interest and might require an additional optimization (usually between 8 and 10 minutes for histones). Please note that a longer fixation may lead to chromatin resistant to sonication. Adapt sonication time accordingly.

If different fixation times will be tested, we recommend starting with a corresponding number of chromatin preparations and testing different sonication settings for each preparation.

Shearing optimization

The length of sonication time depends on many factors (cell type, cell density, sample volume, fixation time). Hence it is important to optimize the sonication conditions for each new ChIP project.

During the sonication, the mean size of DNA fragments will decline progressively approaching a lower limit of 100-150 bp (mean size of the smear). It is recommended to choose a sonication time before reaching this lower limit. **As best practice, choose the shortest sonication time resulting in a satisfactory shearing and ChIP efficiency (highest recovery/ lowest background)**. Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments.

Note that the chromatin shearing from a limited number of cells is usually quite efficient and does not require a long sonication time. Ensure that only the recommended tubes are used for sonication. Please refer to the following guide:

https://www.diagenode.com/files/organigram/bioruptor-organigram-tubes.pdf

Caution: It is very important to carefully follow Diagenode's recommendations, because many parameters can influence the sonication efficiency, such as:

- The Bioruptor reference - sonication tubes recommended for the Bioruptor Pico are different from the tubes recommended for the Bioruptor Plus.
- The tube size - switching to another type of tubes (e.g. from 0.65 ml to 0.2 ml tubes) would require an additional optimization.
- The sample volume - the optimal volume is different for each tube reference. This sample volume should be kept consistent between experiments to ensure reproducible results.

Any change in one of those parameters can lead to inefficient shearing and lack of reproducibility.

Chromatin shearing assessment

The analysis of shearing efficiency is not obvious when working with 10,000 cells due to the low amount of DNA recovered.

We recommend using the Fragment Analyzer or Bioanalyzer (Agilent) for the size assessment due to a high sensitivity of these systems comparing to a conventional agarose gel electrophoresis.

We highly recommend using MicroChIP DiaPure columns (not included in the kit) for DNA purification. The eluted DNA is enough concentrated for accurate sizing from 1,000-2,000 cells equivalent (Figure 1). The minimum DNA concentration allowing an appropriate visualization of sheared DNA on Fragment Analyze and Bioanalyzer is within 2-10 ng/µl.

However, some inconsistencies between Fragment Analyze and Bioanalyzer might be observed. The Bioanalyzer traces are sometimes biased towards the high-molecular weight fragments (Figure 2).

The conventional agarose gel is not sensitive enough to visualize low amounts of DNA. If chromatin shearing assessment is performed using agarose gel electrophoresis, a minimum input of 40,000-50,000 cells is required for reliable assessment (Figure 1). Therefore, several replicates, equivalent to 40,000-50,000 cells, should be pooled before loading onto agarose gel. We recommend using a thin agarose gel for better signal visualization. Both the pre- and post-staining of the agarose gel with ethidium bromide or SybrSafe dye can be used for visualization of sheared fragments. Some slight differences might be observed between post- and pre-stained gels. Post-staining eliminates any possibility that the dye interferes with the migration and ensures an even background noise. However, pictures are usually less clear and bright with some background noise. If pre-stained agarose gels are used, it is advised that the electrophoresis buffer contains the stain in the same concentration as in the gel. If the stain is present in the gel but not in the buffer, the gel will result in uneven staining because the free dye migrates towards the top of the gel leaving the bottom part with no stain. Therefore, the background noise becomes non-uniform.

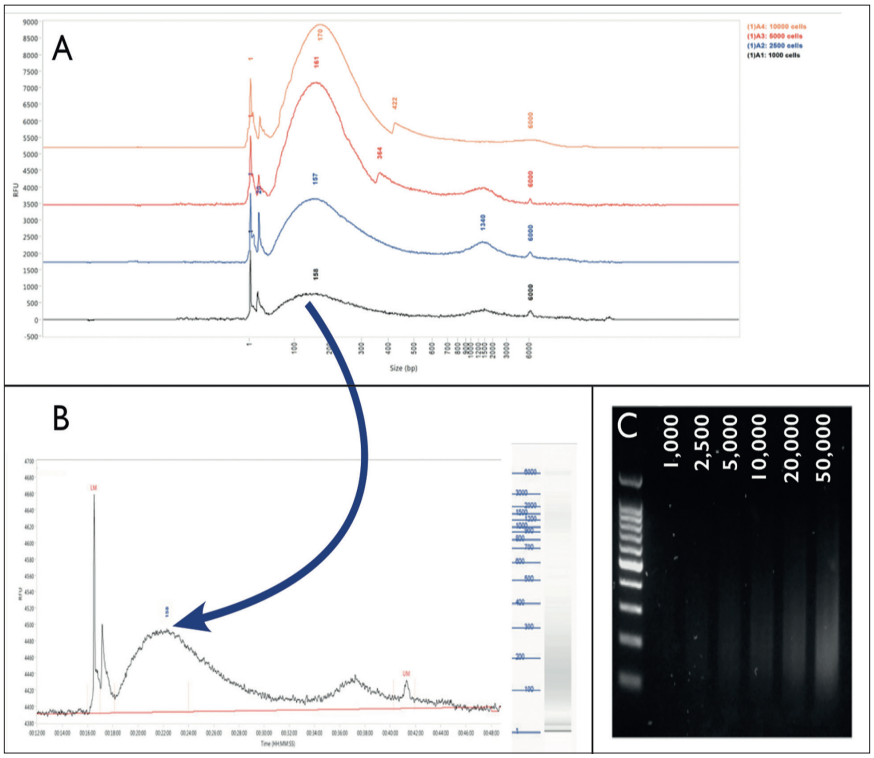


Figure 1. The sensitivity of chromatin shearing analysis using the Fragment Analyzer and agarose gel.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Chromatin EasyShear - High SDS kit (Cat. No. C01020012) using the Bioruptor Pico for 8 cycles. After reversal of cross-linking and purification, samples equivalent to 1.000 up to 50.000 cells (as indicated) were separated using the Fragment Analyzer with the HS NGS Fragment Kit (A&B) or agarose gel electrophoresis (C).

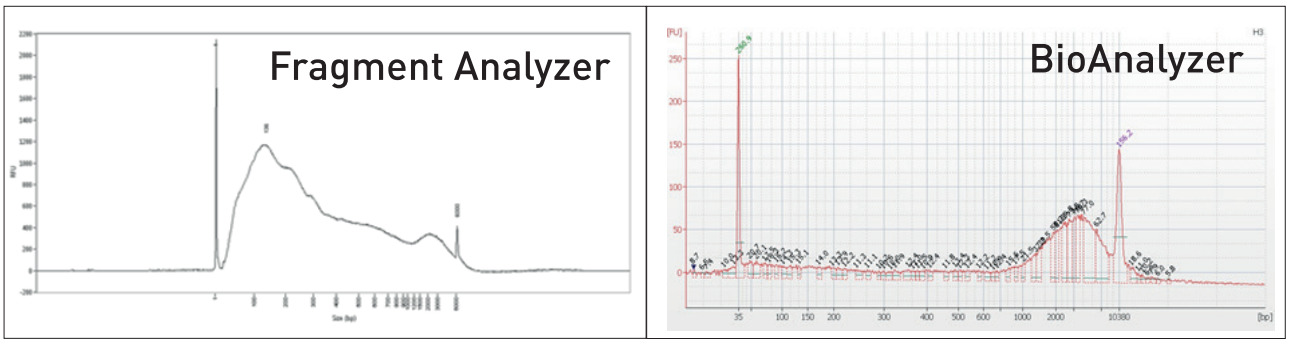


Figure 2. Inconsistencies between Fragment Analyzer and Bioanalyzer: the Bioanalyzer trace is biased towards the high-molecular- weight fragments.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Chromatin EasyShear - High SDS kit (Cat. No. C01020012) using the Bioruptor Pico for 8 cycles. After reversal of cross-linking and purification, an equivalent of 2.000 cells was analyze either using the Fragment Analyzer or BioAnalyzer.

When using Fragment Analyzer or Bioanalyzer (Agilent), please follow the manufacturer's instructions. Please keep in mind that traces are log-based, so a large distribution of higher molecular weight fragments are compacted into a much smaller area of the trace as compared to the smaller size fragments leading to a visual misinterpretation of fragment distribution. If high molecular weight fragments are present, it is recommended estimating a molar ratio between fragments in a

desired range and higher molecular weight fraction. The molarity allows estimating a number of molecules in a particular range. The presence of high molecular weight fragments up to 15% (molar ratio) is acceptable for the majority of ChIP-seq projects (Figure 3).

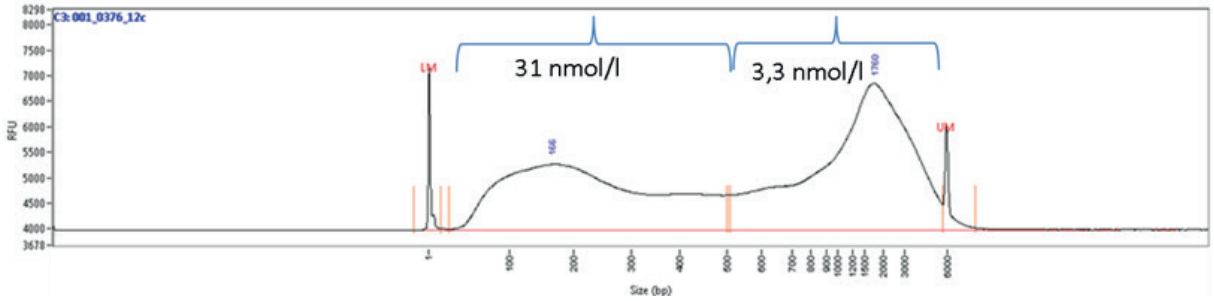
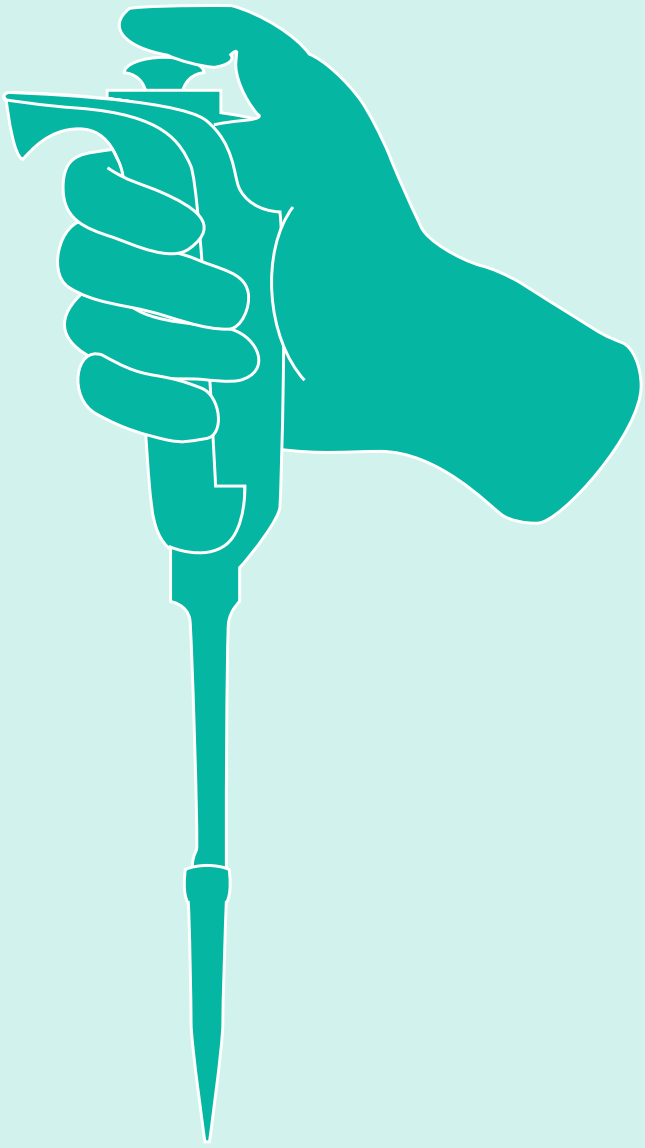


Figure 3. Estimation of a molar ratio between fragments in a desired range higher molecular weight fraction.

HeLa cells were fixed with formaldehyde for 10 min and chromatin was prepared according to Diagenode's protocol (Cat.No. C01020013). Samples were sonicated for 12 cycles of 30" ON/30" OFF with Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016) followed by de- crosslinking and DNA purification. The fragment size was assessed using Fragment Analyzer. The molar content of fragments in the range 100-500 bp and 500-5.000 bp was estimate d showing that large fragments do not exceed 15%.

For accurate size determination of the chromatin fragments, reverse crosslinking, including RNase treatment followed by DNA purification, is needed (Figure 4).

PROTOCOL



OPTION A - BATCH

Compatible with µChIPmentation kit for histones and with True MicroChIP kit

STEP 1

Cell collection and DNA-protein cross-linking from a batch of cultured cells

Day 1  *1 hour*

The protocol below describes the preparation of a batch of chromatin. It can be applied to a batch from 20,000 up to 700,000 cells, which can be further used for desired number of IPs, each IP being performed with the desired number of cells (from 10,000 to 100,000).

A1.1 Place PBS, cell culture medium and trypsin-EDTA (required for adherent cells) at 37°C. Ice-cold HBSS will be used at this step.

For adherent cells:

A1.2 Remove the cell culture medium and rinse the cells with pre-warmed PBS. Gently shake the flask for 2 minutes.

A1.3 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.

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

A1.4 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 and go directly to point A1.5 of the protocol.

For suspension cells:

A1.5 Centrifuge for 5 minutes at 500 x g at room temperature and remove the supernatant.

A1.6 Resuspend the cells in **1 ml of cell culture medium** (RT) and count the cells. You should have between 20,000 and 700,000 cells per ml of cell culture medium.

NOTE: *At this step it is possible to transfer the resuspended cells in a clean 1.5 ml tube for an easier handling.*

A1.7 Under a fume hood, add **27 µl of 37% formaldehyde** per **1 ml** of sample. The final concentration of formaldehyde should be 1%. Invert tubes immediately 2-3 times to ensure complete mixing.

NOTE: *Always use fresh formaldehyde.*

A1.8 Incubate for 8 minutes at room temperature to allow fixation to take place.

A1.9 Add **115 µl of Glycine** to the sample. Mix by gentle inversion of the tube 4-5 times. Incubate for 5 minutes at room temperature to stop the fixation. Keep everything at 4°C or on ice from now on.

A1.10 Centrifuge samples 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.*

A1.11 Aspirate slowly 1.1 ml of the supernatant to leave **30 µl** of solution. Do not disturb the pellet.

A1.12 Add **5 µl of protease inhibitor cocktail** to **1 ml of ice cold HBSS** and add it to the cell pellet. Invert tubes 4-5 times.

NOTE: *When working with 100,000 cells and more per batch, you should gently vortex to completely re-suspend the cells.*

A1.13 Centrifuge samples at 300 x g for 10 minutes at 4°C.

A1.14 Carefully discard the supernatant and keep the cell pellet on ice. Proceed directly to cell lysis.

NOTE: *We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 2 months.*

OPTION A - BATCH

STEP 2

Cell lysis and chromatin shearing of a batch of cells

1 *Day 1* *30 min. to1 hour*

CAUTION: *Make sure that there are no crystals in the Lysis Buffer tL1 before using it. Gently warm at room temperature and mix until crystals disappear.*

A2.1 **25 µl of complete Lysis Buffer tL1** will be needed for **each IP** reaction. Determine accordingly the total volume of Lysis Buffer tL1 needed.

NOTE: *It is possible to use from 10,000 to 100,000 cells per IP, therefore the volume of Lysis Buffer tL1 to prepare is 25 µl for 10,000 to 100,000 cells, depending on the experimental plan.*

A2.2 Prepare **complete Lysis Buffer tL1** by adding the protease inhibitor cocktail 200x (e.g. add 1 µl of protease inhibitor cocktail 200x to 200 µl of Lysis Buffer tL1). Keep the buffer at room temperature until use. Discard what is not used within a day.

A2.3 Add **25 µl of complete Lysis Buffer tL1** per **IP** reaction to the cells. Agitate manually the bottom of the tube to resuspend the cells and allow bubbles to form.

A2.4 Incubate for 5 minutes on ice to ensure complete cell lysis.

A2.5 **75 µl of complete HBSS** will be needed for **each IP** reaction. Determine accordingly the total volume of complete HBSS and prepare it by adding the protease inhibitor cocktail 200x (e.g. add 3 µl of protease inhibitor cocktail 200x to 600 µl of HBSS). Add 75 µl of complete HBSS per each IP reaction. The volume of each individual sample for IP should be 100 µl (25 µl tL1 and 75 µl HBSS).

NOTE: *Ensure that there are no crystals precipitates in samples. Otherwise, gently warm sample to room temperature until crystals disappear.*

A2.6 Transfer the cell suspension to sonication microtubes and if needed split it into aliquots.

The following tubes can be used for sonication:

Microtubes	Cat. No.	Bioruptor model	Sample volume
0.65 ml	C30010011	Pico	100 µl
1.5 ml with caps	C30010016	Pico	100-300 µl
0.5 ml	C30010013	Plus	100 µl
1.5 ml TPX	C30010010-300	Plus	100-300 µl

A2.7 Shear the chromatin by sonication using the Bioruptor. Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation. Choose the protocol which is adapted to your device:

- When using the **Bioruptor Pico**, an initial time-course experiment of **3-6-9** sonication cycles 30'' ON/30'' OFF is recommended.
- When using the **Bioruptor Plus**, an initial time-course experiment of **10-15-20** sonication cycles [30 seconds "ON", 30 seconds "OFF"] at High power is recommended.

A2.8 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. Collect the supernatant which contains the sheared chromatin.

A2.9 Use the chromatin for STEP 3 - Chromatin shearing assessment or store it at -80°C for up to 4 months. Avoid freeze/thaw cycles.

OPTION B - INDIVIDUAL SAMPLES

Compatible with µChIPmentation kit for histones
and with True MicroChIP kit

STEP 1

Cell collection and DNA-protein cross- linking from low amounts of cultured cells

Day 1

1h30

The protocol below describes the chromatin preparation from individual samples from 10,000 up to 20,000 cells. The chromatin can then be used directly for one immunoprecipitation reaction.

For adherent cells:

B1.1 Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.

B1.2 Remove the medium and rinse the cells with **pre-warmed PBS**. Gently shake the flask for 2 minutes.

B1.3 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.

B1.4 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 1.5 ml tube.

For suspension cells:

Collect suspension cells in a 1.5 ml tube and go directly to point B1.5 of the protocol.

B1.5 Centrifuge for 5 minutes at 500 x g (at room temperature) and remove the supernatant.

B1.6 Resuspend the cells in **cell culture medium** and count them.

B1.7 Distribute cell suspension containing the desired cell number to microtubes of choice:

- 0.2 ml microtubes (Cat. No. C30010020) for single tube workflow associated with the Bioruptor Pico
- 0.65 ml microtubes (Cat. No. C30010011) associated with Bioruptor Pico
- 0.5 ml microtubes (Cat. No. C30010013) associated with Bioruptor Plus

Add cell culture medium to reach a final volume of 100 µl in each tube.

NOTES:
The choice of the shearing tube will have an impact on the rest of the protocol. See remarks before starting to choose the best option.

The number of cells per IP should be from 10,000 to 20,000 cells.

B1.8 Under a fume hood, add **2.7 µl of 37% formaldehyde** to each tube containing **100 µl** of cell suspension and mix gently.

B1.9 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".*

B1.10 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.

B1.11 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.*

B1.12 Aspirate slowly 85 µl of supernatant slowly to **leave 30 µl** of solution. Do not disturb the pellet. Take care to not remove these cross-linked cells.

B1.13 Prepare **complete HBSS Buffer** by adding the protease inhibitor cocktail 200x (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 at point **B1.14**
- And 15 µl per tube at point **B2.5** if using 0.2 ml microtubes, or 65 µl per tube at point **B2.5** if using 0.5/0.65 ml microtubes

B1.14 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 10 µl** of solution.

OPTION B - INDIVIDUAL SAMPLES

STEP 2

Cell lysis and chromatin shearing of individual samples

Day 1

30 min. to 1 hour

CAUTION: *Make sure that there are no crystals in the Lysis Buffer tL1 before using it. Gently warm at room temperature and mix until crystals disappear.*

B2.1 **25 µl of Lysis Buffer tL1** will be needed for **each tube**. Determine accordingly the total volume of Lysis Buffer tL1 needed.

B2.2 Prepare **complete Lysis Buffer tL1** by adding the protease inhibitor cocktail 200x (e.g. add 1 µl of protease inhibitor cocktail 200x to 200 µl of Lysis Buffer tL1). Keep the buffer at room temperature until use. Discard what is not used within a day.

B2.3 **Add 25 µl of complete Lysis Buffer tL1** to **each tube**. Agitate manually the bottom of the tube to resuspend the cells and allow bubbles to form.

B2.4 Incubate for 5 minutes on ice to ensure complete cell lysis.

B2.5 Add **complete HBSS** to the cell lysate, according to the type of shearing tube as described in the table below:

Microtubes	Cat. No.	Bioruptor model	Complete HBSS volume
0.2 ml	C30010020	Pico	15 µl
0.65 ml	C30010011	Pico	65 µl
0.5 ml	C30010013	Plus	65 µl

B2.7 Shear the chromatin by sonication. Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation. Choose the protocol which is adapted to your device and tubes:

- When using the **Bioruptor Pico**, and 0.2 ml microtubes an initial time-course experiment of **3-6-9** sonication cycles 30'' ON/30'' OFF is recommended.
- When using the **Bioruptor Pico**, and 0.65 ml microtubes an initial time-course experiment of **3-6-9** sonication cycles 30'' ON/30'' OFF is recommended.
- When using the **Bioruptor Plus**, and 0.5 ml microtubes an initial time-course experiment of **10-15-20** sonication cycles [30 seconds "ON", 30 seconds "OFF"] at High power is recommended.

B2.8 Use the chromatin for STEP 3 - Chromatin shearing assessment or store it at -80°C for up to 4 months. Avoid freeze/thaw cycles.

STEP 3

Chromatin shearing assessment

Workflow for analysis of sheared chromatin:

- Reverse crosslinking
- RNAse treatment using RNAse cocktail
- DNA purification using MicroChIP DiaPure Columns (not provided, Diagenode, Cat. No. C03040001)
- Fragment size assessment

Reverse cross-linking (reagents included in the kit)

3.1 Take an aliquot, containing the required amount of sheared chromatin:

- If a microfluidic device (Fragment Analyzer or Bioanalyzer) will be used for size assessment, an equivalent of 1,000-2,000 cells (or higher) should be used
- If agarose gel electrophoresis will be used for size assessment, an equivalent of 40,000-50,000 cells, (or higher) should be used.If an individual sample does not contain enough cells, perform several reactions in parallel, and pool DNA obtained at the end of protocol before loading on agarose gel

3.2 Adjust, if needed, the volume of the sheared chromatin to **100 µl** using TE buffer

3.3 Add **100 µl of the Elution Buffer tE1** and **8 µl of Elution Buffer tE2** to each sample. Mix thoroughly and incubate samples at 65°C for 4 hours (or overnight) in a thermoshaker at 1300 rpm.

DNA purification using MicroChIP DiaPure Columns and RNase treatment

NOTE: *Before the first use of the DNA Wash Buffer, 24 ml of ethanol must be added to 6 ml of the Buffer. Never leave the bottle open during storage to avoid evaporation.*

3.4 Add **1 ml of ChIP DNA Binding Buffer** to each sample and mix briefly.

3.5 Transfer **0.6 ml** of the mixture to a provided spin column in a collection tube and centrifuge at ≥10,000 x g for 30 seconds. Discard the flow-through

3.6 Repeat step 3.5 again with the remaining 0.6 ml of the mixture.

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

3.8 Add **1 µl of RNase cocktail** directly to the center of the spin column membrane and incubate for 15 minutes at room temperature

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

3.10 Add **6 µl of DNA Elution Buffer** directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube and centrifuge at ≥10,000 x g for 30 seconds to elute the DNA.

NOTE: *Up to 50 µl of DNA Elution Buffer can be used if less concentrated DNA is required for analysis.*

Fragment size assessment

3.11 Analyze the purified DNA using the preferred option:

- Fragment Analyzer
- Bioanalyzer
- agarose gel electrophoresis

Use high sensitivity kits accordingly to the manufacture's recommendation.

3.12 Chose the shortest sonication time resulting in a satisfactory shearing profile. Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments.

FAQs

What is the composition of buffers included in the kit?

The composition of buffers is proprietary.

Is it possible to change the decross-linking protocol and use 5 minutes at 95°C to save time?

After heating at 95°C the DNA is single-stranded and therefore more difficult to analyze. It is not really compatible with agarose geland associated dyes, and Qubit or BioAnalyzer kits for ssDNA are not very accurate. Therefore in order to have shearing results that are trustable and easier to analyze you have to perform the decross-linking 4h at 65°C

Is RNase treatment mandatory for chromatin shearing assessment?

RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing. The presence of degraded RNA in the sample might lead to mis-interpretation of the shearing. Smear below 100 bp is due to degraded RNA but not over-sheared DNA.

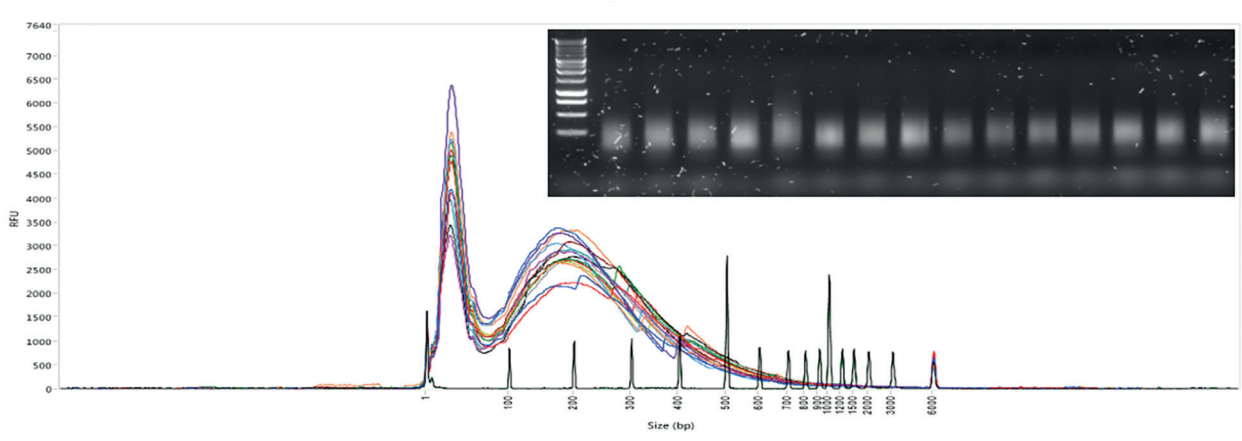


Figure 4. The presence of degraded RNA in the sample interfere with shearing assessment.

Chromatin from HeLa cells was prepared according to Diagenode's protocol. Samples were sonicated for 5 cycles of 30" ON/30" OFF Bioruptor Pico followed by de-crosslinking and DNA purification in the absence of RNase. The fragment size was assessed using agarose gel electrophoresis (100 bp ladder was loaded as the size standard) and Fragment Analyzer and High Sensitivity NGS Fragments kit (Agilent). A sharp peak below 100 bp correspond to degraded RNA.

Is reverse cross-linking mandatory for chromatin shearing assessment?

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 (Figure 5). Therefore it is important to follow the decross-linking protocol as described in "Step 3 – chromatin shearing assessment".

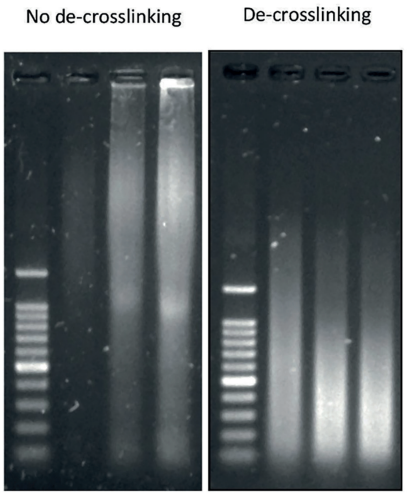


Figure 5. Reversing crosslinks is necessary for accurate size estimation.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's protocol. Samples were sonicated for 5, 10 and 15 cycles of 30" ON/30" OFF as indicated with the Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016). A 100 bp ladder was loaded as size standard. Left panel: non de-crosslinked chromatin. Right panel: de-crosslinked chromatin.

Related products

Product	Cat. No.
µChIPmentation kit for Histones	C01011011
True MicroChIP kit	C01010130
MicroChIP DiaPure columns	C03040001
Bioruptor Pico	B01060010
0.2 ml microtubes	C30010020
0.65 ml microtubes	C30010011
1.5 ml microtubes with caps	C30010016
Bioruptor Plus	B01020003
0.5 ml microtubes	C30010013
1.5 ml TPX microtubes	C30010010-300

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diagenode

A Hologic Company

µChIPmentation Kit for Histones

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

USER GUIDE

Version 4 02_2022

Please read this manual carefully
before starting your experiment

Contents

Introduction

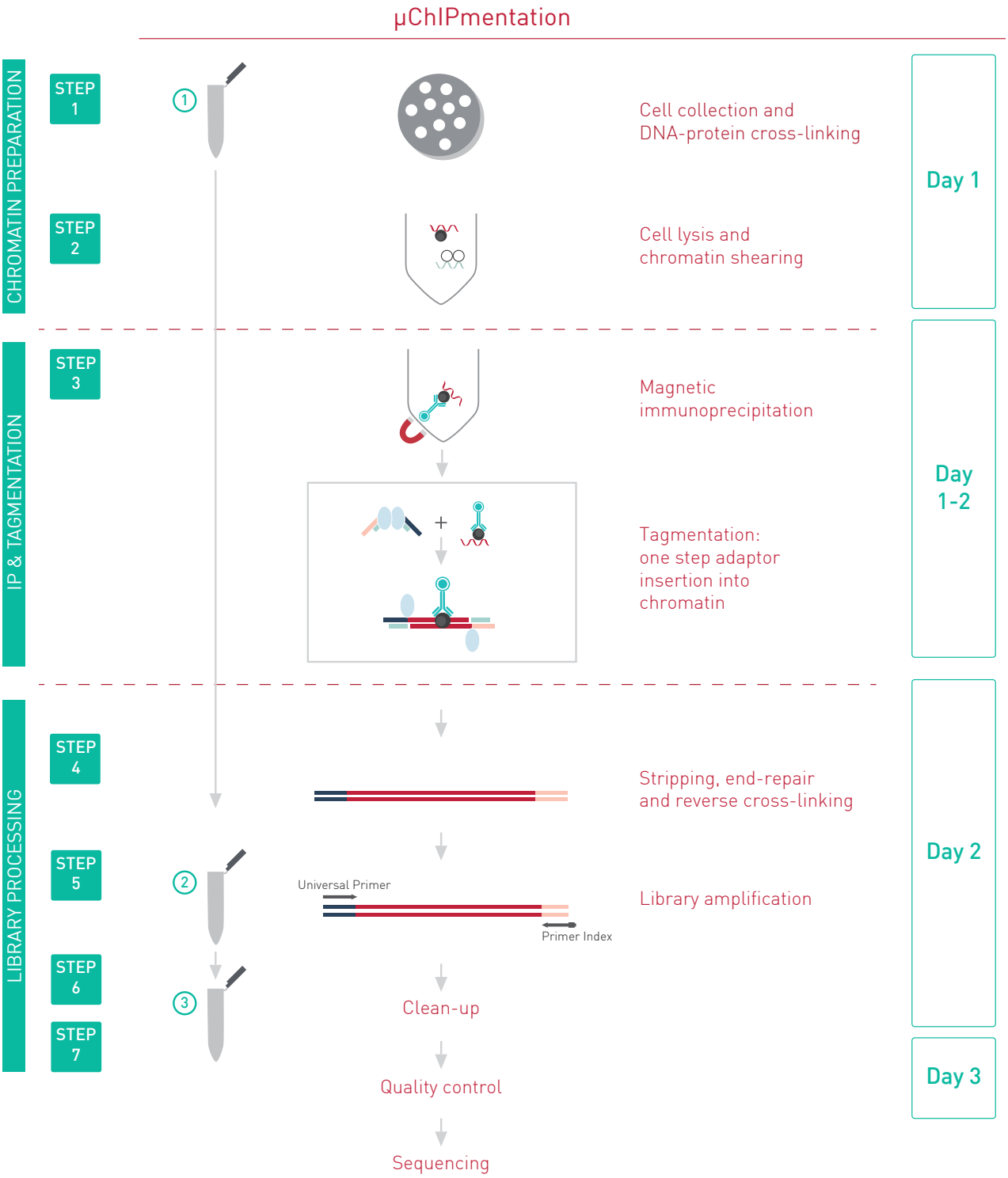
Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) is the method of choice to identify, from the whole genome, which specific regions are associated with proteins of interest, like chromatin remodelers or transcription factors.

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

24 UDI for tagmented libraries - Set III, Cat. No. C01011037

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	950 µl	315 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	325 µl	115 µl	4 °C
Stripping reagent	n/a	315 µl	105 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)
  Note: *Biorupotor Plus can be used per option A- chromatin preparation from the batch*
- 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
- 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 – High SDS (Cat. No. C01020012)
- 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 immunoprecipitation reaction. Three options are possible for chromatin preparation (fixation, cell lysis and chromatin shearing):

- Preparing chromatin from a **BATCH** (option A) of 20.000-100.000 cells. The prepared chromatin can be split into several immunoprecipitation reactions (from 2 to 10) containing 10,000 cells each. When possible, this option is preferred in order to limit tube-to-tube variability and cell loss.
- Starting directly from **INDIVIDUAL SAMPLES** (option B) of 10,000 cells. The prepared chromatin from each individual sample will be used per immunoprecipitation.
- Preparing chromatin from **FACS-sorted cells** (option C). Cells are fixed as per batch and 10,000 cells are sorted into individual tubes and proceed individually onwards (shearing and immunoprecipitation). The workflow describes the chromatin preparation from cells stained with LIVE/DEAD fixable stain and fixed prior to FACS-sorting. FACS sorting based on cell type–specific markers can be used but it is highly recommended to fix cells prior labelling and FACS to preserve the epigenetic signature. The cells should be sorted out into 25 µl of Lysis Buffer tL1.

  We highly recommend to check with FACS facilities for any specific requirements.

Please follow a corresponding protocol option.

> ***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

OPTION A - BATCH

STEP 1

Cell collection and DNA-protein cross-linking from a batch of cultured cells

Day 1

30 min to 1 hour

The protocol below describes the chromatin preparation starting from a **batch** containing **20,000** up to **100,000 cells**. The prepared chromatin can be split for a desired number of immunoprecipitation reactions, each IP being performed with 10,000 cells. The minimum recommended cell number per batch is 20,000. This is enough for 2 samples, each of 10,000 cells. The maximum recommended number of cells per batch is 100,000. This is enough for 10 samples, each of 100,000 cells. If more cells are needed, then proceed with a separate chromatin preparation.

Determine the number of immunoprecipitation reactions to be run including positive and negative controls and start with an appropriated number of cells per batch. An extra amount corresponding minimum to 2,000 cells (or higher) should be added per chromatin shearing assessment.

OPTION A

The final volume of sheared chromatin from 10,000 cells should be **50 µl** (25 µl of tL1 buffer + 25 µl of HBSS). PBS, cells culture medium and HBSS solution at different temperature will be used.

1.1 Place PBS, cell culture medium and trypsin-EDTA (required foradherent cells) at 37°C. Place HBSS solution on ice. Equilibrate a portion of cell culture medium to room temperature.

For adherent cells:

1.2 Remove the cell culture medium and rinse the cells with pre-warmed **PBS**. 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.

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

1.4 Immediately add fresh pre-warmed **culture medium** to the cells. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA. Transfer cell suspension to a 15 ml tube and go directly to point 1.5 of the protocol.

For suspension cells:

***NOTE**: Collect suspension cells in a 1.5 ml tube and go directly to point 1.5 of the protocol.*

1.5 Centrifuge for 5 minutes at 500 x g (at room temperature) and remove the supernatant.

1.6 Resuspend the cells in **1 ml of cell culture medium** (RT) and count the cells. Ensure that you have enough cells accordingly to your experimental design (between 20,000 and 100,000 cells per ml of cell culture medium). Transfer the resuspended cells in a clean 1.5 ml tube.

1.7 Under a fume hood, add **27 µl of 37% formaldehyde** per **1 ml** of **sample**. The final concentration of formaldehyde should be **1%**. Invert tubes immediately 2-3 times to ensure complete mixing.

1.8 Incubate for 8 minutes at room temperature to allow fixation to take place.

1.9 Add **115 µl of Glycine** to the sample. Mix by gentle inversion of the tube 4-5 times. Incubate for 5 minutes at room temperature to stop the fixation. Keep everything at 4°C or on ice from now on.

1.10 Centrifuge samples 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.*

1.11 Aspirate slowly the supernatant. Do not disturb the pellet.

1.12 Add **5 µl of Protease inhibitor cocktail** to **1 ml** of ice cold **HBSS** and add it to the cell pellet. Invert tubes 4-5 times.

NOTE: *When working with 100,000 cells and more per batch, you should gently vortex to completely re-suspend the cells.*

1.13 Centrifuge samples at 300 x g for 10 minutes at 4°C.

1.14 Carefully discard the supernatant and keep the cell pellet on ice. Proceed directly to cell lysis.

NOTE: *We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 2 months.*

STEP 2

Cell lysis and chromatin shearing of a batch of cells

1 Day 1 1 to 2 hours

CAUTION: *Make sure that there are no crystals in the Lysis Buffer tL1 before using it. Gently warm at room temperature and mix until crystals disappear.*

2.1 Prepare **complete Lysis Buffer tL1** and **complete HBSS** by adding the protease inhibitor cocktail 200x (e.g. add 1 µl of protease inhibitor cocktail 200x to 200 µl of Lysis Buffer tL1).

2.2 **25 µl of complete Lysis Buffer tL1** and **25 µl of complete HBSS** will be needed for each individual sample. Scale up the total volume of Lysis Buffer tL1 and HBSS accordingly to the number of immunoprecipitation reactions to be run from the batch. Keep the buffer at room temperature until use.

NOTE: *e.g., if 5 immunoprecipitation reactions will be run, 125 µl of complete Lysis Buffer tL1 and 125 µl of HBSS will be needed.*

2.3 Add **complete Lysis Buffer tL1** to the cell pellet and resuspend by pipetting.

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

2.5 Add **complete HBSS** to the solution and resuspend by pipetting.

NOTE: *The volume of each individual sample in the batch should be 50 µl (25 µl tL1 and 25 µl HBSS). The total batch volume should be 50 µl x number of individual samples (e.g., if 5 immunoprecipitation reactions will be run, batch volume should be 250 µl).*

Ensure that there are no crystals precipitates in samples. Otherwise, gently warm sample to room temperature until crystals disappear.

2.6 Transfer the cell suspension to sonication microtubes, split it into aliquots if needed.

The following tubes can be used for sonication:

Microtubes	Cat No.	Bioruptor Model	Sample Volume
0.65 ml	C30010011	Pico	100 µl
1.5 ml with caps	C30010016		100-300 µl
0.5 ml	C30010013	Plus	100 µl
1.5 ml TPX	C30010010-300		100-300 µl

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 3-9 sonication cycles 30'' ON/30'' OFF.
- When using the **Bioruptor Plus**, shear for of 10-20 sonication cycles [30 seconds "ON", 30 seconds "OFF"] at High power.

***NOTE**: We recommend performing pilot experiments for each new sample type using the Chromatin EasyShear kit – High SDS (Cat. No. C01020012)*

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. Collect the supernatant which contains the sheared chromatin.

2.9 Use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months. Avoid freeze/thaw cycles.

***NOTE**: An aliquot of sheared chromatin corresponding to minimum of 2,000 cell equivalent can be used per chromatin shearing assessment. The protocol is described in the "Additional Protocols" section. This step can be omitted when sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously.*

OPTION B - INDIVIDUAL SAMPLES

STEP 1

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

1 Day 1 1.5 hours

The protocol below describes the chromatin preparation from **individual samples** containing **10,000 cells** each. The chromatin can then be used directly for one immunoprecipitation reaction. If a chromatin shearing assessment will be performed, we recommend including one extra sample.

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: *Ensure that the final sample volume is exactly 50 µl.*

2.5 Shear the chromatin by sonication using the Bioruptor Pico:

- Shear for 3 to 9 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 2 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.*

OPTION C - FACS-SORTED CELLS

STEP 1

Cell collection and DNA-protein cross-linking from FACS-sorted cells

 Day 1  30 min to 1 hour

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The protocol below describes the preparation of **chromatin from FACS sorted cells**. Cells are fixed from a batch (up to 1 milion cells) and **10,000** are FACS-sorted directly to Lysis Buffer tL1 and proceed individually onwards. Cells can be (optionally) stained prior to the fixation with LIVE/DEAD fixable Aqua stain (or similar) to exclude dead cells during the sorting. We recommend checking the sheath fluid volume prior starting the experiment. Ensure that the final volume (sorted cells plus 25 µl of Lysis Buffer tL1) does not exceed **50 µl**. Determine the number of immunoprecipitation reactions to be run including positive and negative controls and start with an appropriated number of cells per batch. We recommend including one extra sample per chromatin shearing assessment.

Attention PBS, cells culture medium and HBSS solution at different temperature will be used.

1.1 Pre-warm PBS, cell culture medium and trypsin-EDTA (required for adherent cells) at 37°C. Place HBSS solution on ice. Equilibrate a portion of cell culture medium to room temperature.

For adherent cells:

1.2 Remove the cell culture medium and rinse the cells with pre-warmed **PBS**. 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.

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

1.4 Immediately add fresh **culture medium** to the cells when they are detached. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA. Transfer cell suspension to a 15 ml tube and go directly to point 1.5 of the protocol.

For suspension cells

NOTE: *Collect suspension cells in a 1.5 ml tube and go directly to point 1.5 of the protocol.*

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

1.6 Resuspend the cells in **cold PBS** in a clean 1.5 ml tube and count the cells. Adjust the cell density to have approximately **1 million cells per 1 ml PBS.**

1.7 (Optional) Add aqua dye (1 µl per 1 ml cell suspension) and stain for 30 minutes on ice.

1.8 Centrifuge samples at 300 x g for 10 minutes at 4°C and remove the supernatant. Do not disturb the pellet.

1.9 Resuspend cells in **1 ml of ice-cold PBS** and centrifuge samples at 300 x g for 10 minutes at 4°C and remove the supernatant.

1.10 Under a fume hood, add **27 µl of 37% formaldehyde** per **1 ml** of room-temperature **PBS**. The final concentration of formaldehyde should be **1%.**

1.11 Resuspend the cell pellet sample in this solution. Invert tubes immediately 2-3 times to ensure complete mixing.

1.12 Incubate for 8 minutes at room temperature to allow fixation to take place.

1.13 Add **115 µl of Glycine** to the sample. Mix by gentle inversion of the tube 4-5 times. Incubate for 5 minutes at room temperature to stop the fixation. Keep everything at 4°C or on ice from now on. Centrifuge samples 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.*

1.14 Add **200 µl of 5% BSA** to **1 ml of ice-cold PBS**. Aspirate slowly the supernatant and re-suspend cells in 1 ml of PBS/BSA

1.15 Filter the cell suspension using an appropriated cell strainer (<80 µm) accordingly to the supplier recommendations.

NOTE: *It is essential to filter all samples before reading them in a Flow Cytometer. During processing, cells will pass through a nozzle. Clumps and debris can clog the instrument fluidics and either distort the measurements or obstruct them completely.*

1.16 Sort 10.000 into **25 µl of complete Lysis Buffer tL1** supplemented with **protease inhibitors cocktail**. Ensure that the final volume (sorted cell and Lysis Buffer tL1) does not exceed **50 µl**. Adjust the final volume to 50 µl using complete HBSS if needed.

NOTE: *If the sheath fluid volume is not negligible, cells can be sorted into PBS, centrifuged at 300 x g for 10 minutes at 4°C and resuspended in 25 µl of Lysis Buffer tL1.*

STEP 2

Cell lysis and chromatin shearing - FACS-sorted cells

1 Day 1 1 hour

2.1 Transfer the cell suspension to **0.2 ml microtubes for Bioruptor® Pico (Cat. No. C30010020)**

2.2 Shear the chromatin by sonication using the Bioruptor Pico for 3-9 sonication cycles 30'' ON/30'' OFF.

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

2.3 Briefly spin down samples and use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months. Avoid freeze/thaw cycles.

NOTE: *An aliquot of sheared chromatin corresponding to minimum of 2.000 cell equivalent can be used per chromatin shearing assessment. The protocol is described in the "Additional Protocols" section. This step can be omitted when sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously*

OPTION A, B and C

STEP 3

Magnetic immunoprecipitation and tagmentation

 Day 1-2  1h overnight incubation, 1.5 hours

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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 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 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 **$MgCl_2$** (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.

OPTION A, B and C

STEP 4

Stripping, end repair, reverse cross-linking

1 Day 2 30 minutes

4.1 Remove the strip from magnetic rack, add **10.5 µl of Stripping reagent** to the beads and resuspend by pipetting. Add **9.5 µl of Stripping reagent** 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 $MgCl_2$, 4 µl of ChIP-seq grade water** 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 IP or input sample is 50 µl.*

OPTION A, B and C

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.*

OPTION A, B and C

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.

OPTION A, B and C

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.

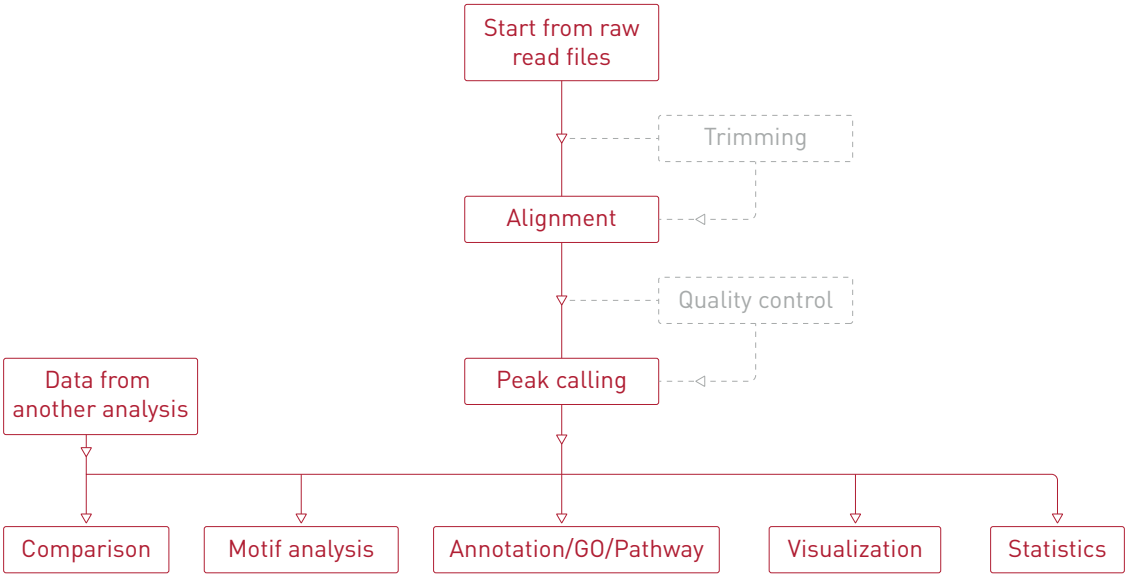

Figure 1. ChIP-seq data analysis workflow

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".

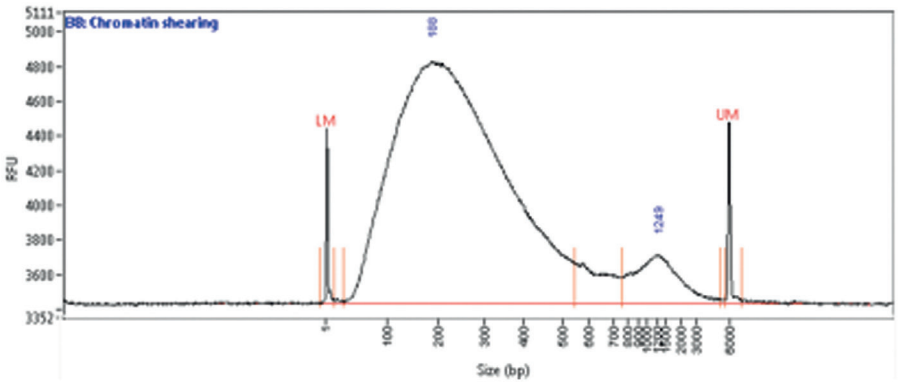

Figure 2. *Size distribution of purified DNA from decross-linked chromatin. The profile has been generated by running 2 µl of DNA on a Fragment Analyzer (Agilent).*

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).

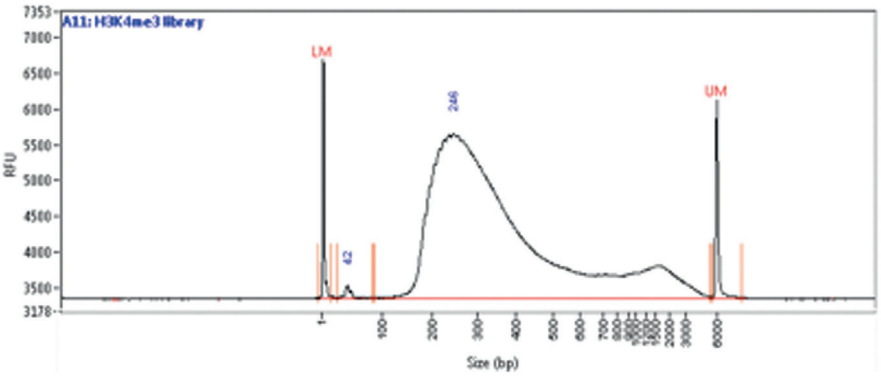

Figure 3. *Library quality control. The distribution of the fragments sizes was assessed by loading 6 ng of library on Fragment Analyzer (Agilent).*

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".

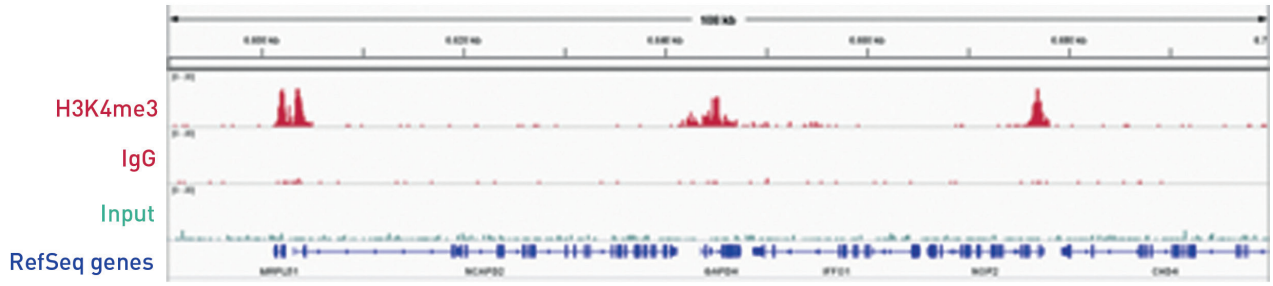

Figure 4. *Distribution of the µChIPmentation dataset for H3K4me3, in a representative region of the genome.*

ADDITIONAL PROTOCOLS

Protocol for 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.

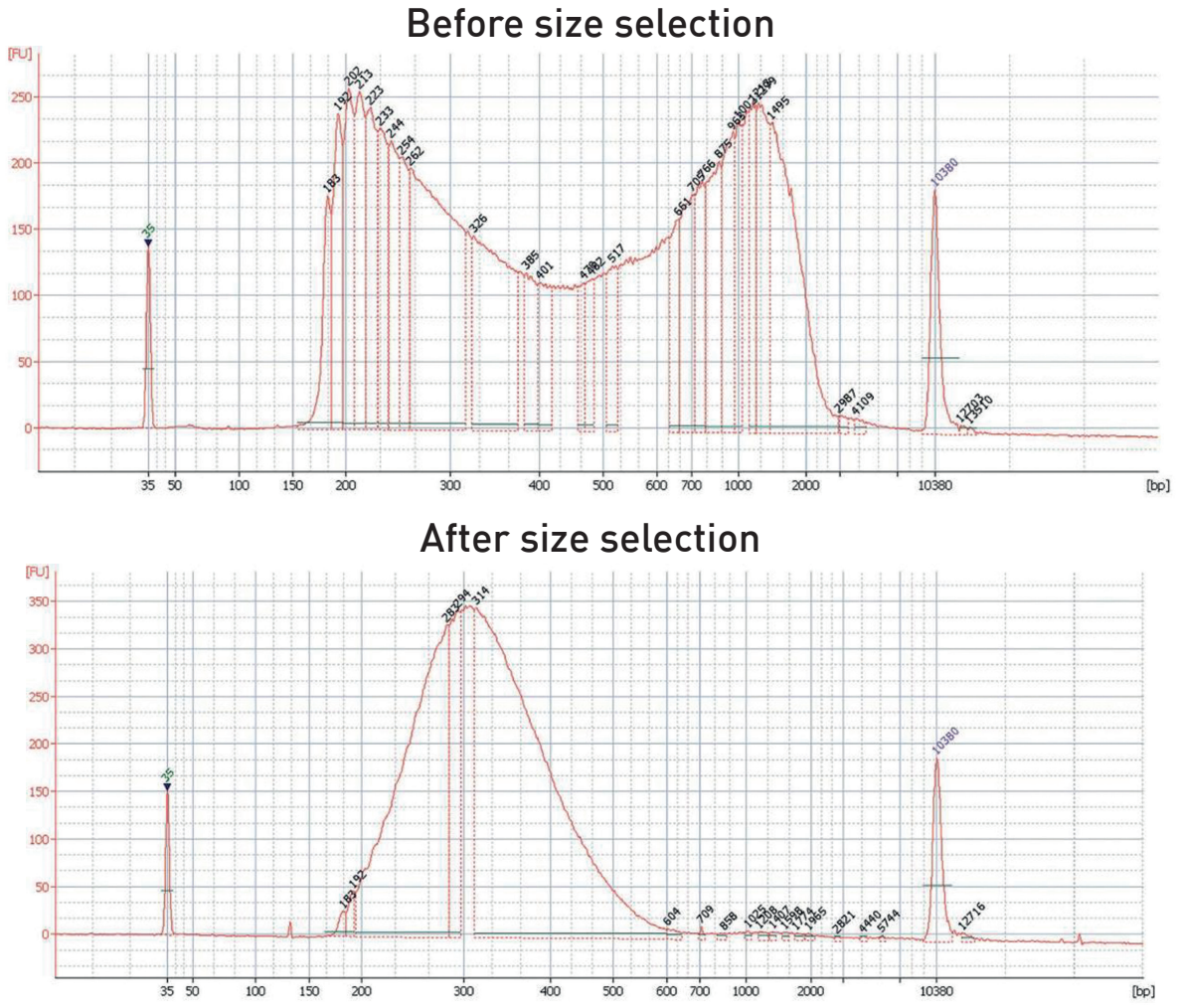

Figure 5. *Example of results of the size selection on a ChIPmentation library for H3K27me3. The library was run on a BioAnalyzer (Agilent) before and after the size selection.*

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	C01011035
24 UDI for Tagmented libraries - Set II	C01011036
24 UDI for tagmented libraries - Set III	C01011037
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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