diagenode

A Hologic Company

CHIL-SEQ Protocol

USER GUIDE

Version 1 03_2022

Please read this manual carefully
before starting your experiment

Contents

Introduction

Chromatin immunoprecipitation followed by sequencing (ChIP–seq) has been the standard technique for examining protein–DNA interactions across the whole genome. However, it is difficult to obtain epigenomic information from limited numbers of cells using ChIP–seq.

A new technology named **Ch**romatin **I**ntegration **L**abelling (**ChIL**) was described in 2019. ChIL-seq is a method for epigenomic profiling of low input samples and the simultaneous detection of the antibody target localization [1].

ChIL-seq allows the study of a wide range of histone modifications and transcription factors in a 96-well format with results comparable to ChIP-seq, CUT&Tag, or CUT&RUN. Moreover, the use of a fluorescent ChIL probe allows spatial localization under a microscope and the control of the immunostaining reaction. The main features of ChIL-seq are listed below:

- Sample requirement: **100 – 100K adherent cells**
- Visualization of the **spatial localization**
- **Chromatin fragmentation-free method**
- **Robust library preparation** – by applying an antibody-targeted controlled *in situ* transposition by Tn5 to link the adapters
- **Decreased PCR duplicates & increased unique reads** due to the use of a linear amplification by the T7 RNA polymerase
- 96-well format **allowing massively parallel DNA-sequencing**

As shown in the workflow (see Method Overview & Timetable), the ChIL protocol involves plating adherent cells in a 96-well plate, fixing, permeabilizing and staining cells with a primary antibody, as in a standard immunostaining procedure, before incubating with the ChIL probe. Then, an optional step can be applied to detect *in situ* localization by fluorescence microscopy.

The ChIL probe is the key reagent of the technology and is constituted by a secondary antibody (anti-mouse or anti-rabbit IgG) conjugated with a double-stranded DNA oligo (ChIL DNA). The latter contains a T7 promoter sequence for linear amplification, the sequence of mosaic end A (ME-A) for Tagmentase (Tn5 transposase) binding and a primer sequence for the library preparation before sequencing. Moreover, the ChIL probe is labelled with tetramethylrhodamine (TAMRA) for microscopic investigation for *in situ* localization by immunofluorescence (Figure 1).

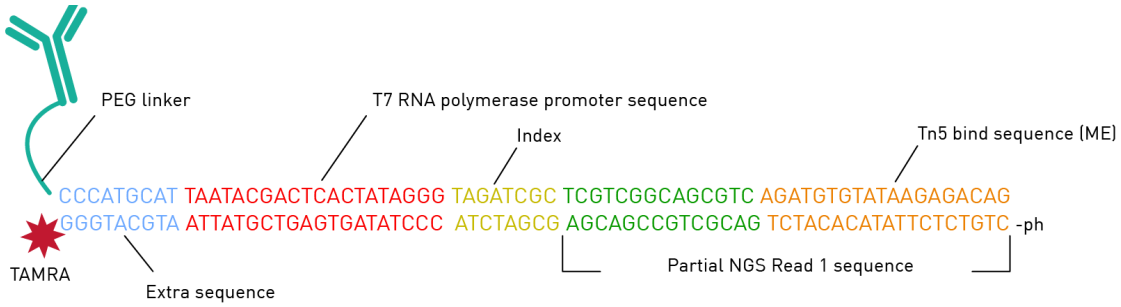


Figure 1. ChIL probe structure [1, 2]. ME, mosaic end; NGS Read 1, next-generation sequence for Read 1 sequencing; PEG, polyethylene glycol.

After staining the cells with the ChIL probe, the cells are incubated first with Tagmentase, which binds the mosaic end A of the ChIL DNA, and then incubated with mosaic end B (MEDS-B) to form a complete transposome. The Tagmentase (Tn5 transposase) is finally activated in the presence of magnesium ions and then the ChIL DNA is integrated into the genomic region of interest and the reaction is completed by gap fill-in enzymes. The ChIL DNA sequence is used for *in situ* transcription by the T7 RNA polymerase and linear amplification of the genomic region. The transcribed RNA molecules are later purified, used to prepare libraries for deep sequencing and the sequenced reads are mapped to the appropriate reference genome for further analysis [1,2].

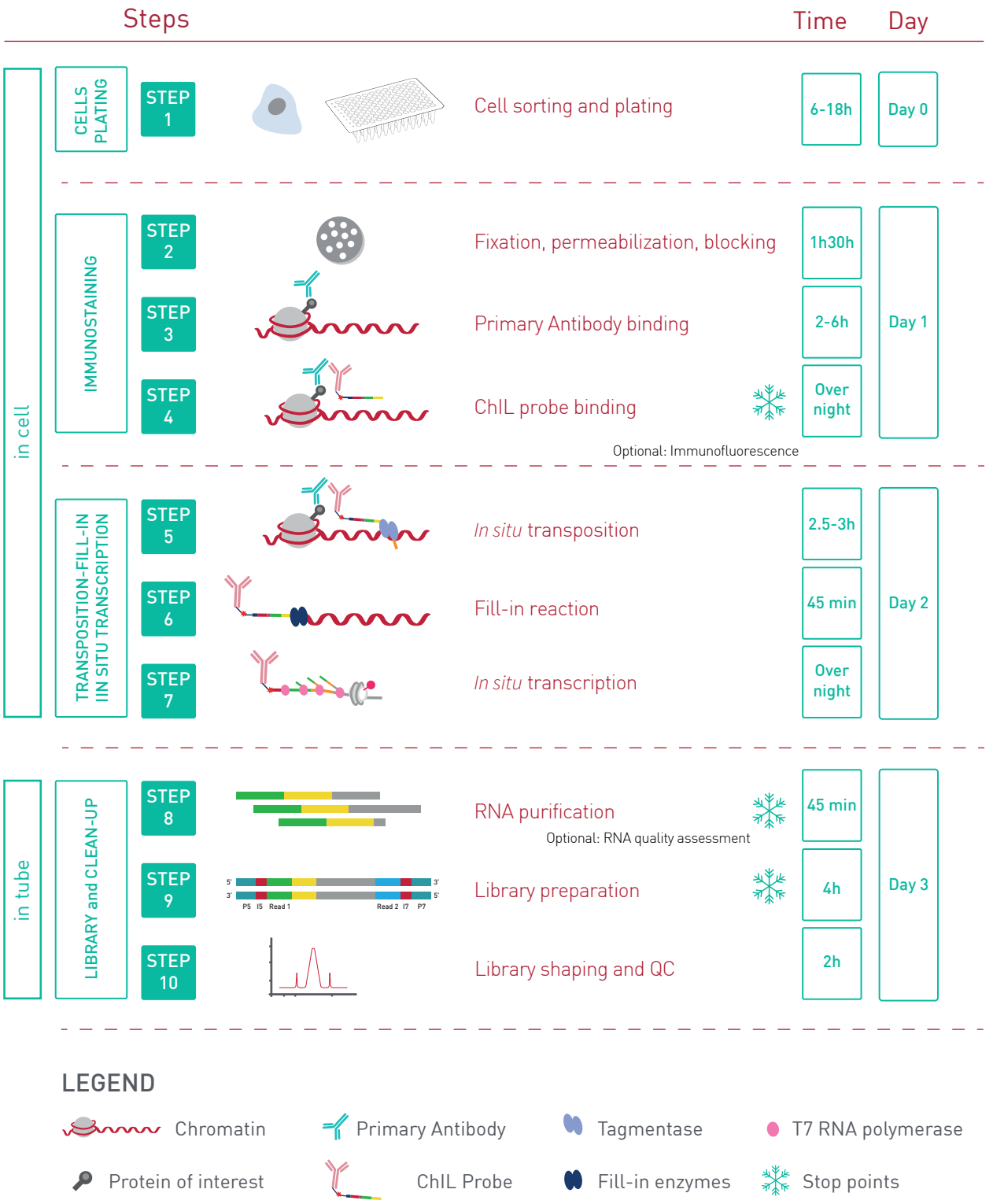
In this document we are describing the detailed ChIL-seq protocol and key reagents required for ChIL-seq (Table 1). Diagenode's ChIL-seq protocol has been adapted from the original ChIL-seq protocol described and published in *Nature Cell Biology* [1] and *Nature Protocols* [2]. More recently, the same research team has published an adaptation of ChIL-seq on tissue sections [3]. Please refer to original articles for optimizations and additional protocols.

1 - Harada, A., Maehara, K., Handa, T. et al. A chromatin integration labelling method enables epigenomic profiling with lower input. Nat Cell Biol 21, 287–296 (2019). https://doi.org/10.1038/s41556-018-0248-3

2 - Handa, T., Harada, A., Maehara, K. et al. Chromatin integration labeling for mapping DNA-binding proteins and modifications with low input. Nat Protoc 15, 3334–3360 (2020). https://doi.org/10.1038/s41596-020-0375-8

3 - Maehara K, Tomimatsu K, Harada A, Tanaka K, Sato S, Fukuoka M, Okada S, Handa T, Kurumizaka H, Saitoh N, Kimura H, Ohkawa Y. Modeling population size independent tissue epigenomes by ChIL-seq with single thin sections. Mol Syst Biol. 2021 Nov;17(11). https://doi.org/10.15252/msb.202110323

Method overview & timetable



Materials available at Diagenode

Table 1. Diagenode supplies required for ChIL-seq protocol.

Product	Cat. No.	Storage
ChIL probe (goat anti-rabbit) – 24 rxns	C01060020	-20°C
ChIL probe (rat anti-mouse) – 24 rxns	C01060021	-20°C
Tagmentase (Tn5 transposase) unloaded	C01070010	-20°C
Tagmentase Dilution Buffer	C01070011	-20°C
8 UDI for tagmented libraries	C01011035	-20°C
Or 24 UDI for tagmented libraries – Set I	C01011034	-20°C
Or 24 UDI for tagmented libraries – Set II	C01011036	-20°C
Or 24 UDI for tagmented libraries – Set III	C01011037	-20°C
Or 8 SI for tagmented libraries	C01011033	-20°C
Or 24 SI for tagmented libraries	C01011032	-20°C
Negative control Mouse IgG	C15400001	-20°C
Negative control Rabbit IgG	C15410206	-20°C
Positive control (H3K4me3 antibody)	C15410030	-20°C
Positive control (H3K27me3 antibody)	C15210017	-20°C
Antibody to an epitope of interest (ChIP-seq grade antibodies)	www.diagenode.com	-20°C
DiaMag 0.2ml magnetic rack compatible with 0.2 ml tubes	B04000001	/

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

Required Materials not available at Diagenode

Equipment

- Gloves to wear at all steps
- Cell culture standard consumables
- Cell counter
- Microscope
- Cell culture incubator
- Chemical hood
- µ-Plate 96-well TC (ibiTreat) (ibidi, ib89626)
- Plate sealing films
- Protein LoBind 1.5 ml tube (nuclease-free)
- 15 and 50 ml tubes
- 0.2 ml 8-strip PCR tubes
- Centrifuge
- Temperature controlled orbital shaker or microplate shaker
- Thermocycler
- Agilent Bioanalyzer 2100 or Fragment Analyzer or equivalent
- Qubit® Fluorometer (Thermo Fisher Scientific) or equivalent

Reagents

- Adherent cell line of interest
- Cell culture standard medium and reagents
- 1X DPBS (-)
- Ultra-pure DNase, RNase free water
- Formaldehyde 37 % solution (FA)
- Triton X-100 (100 %) solution
- Sodium dodecyl sulfate (SDS) solution 20 %
- Blocking One-P (Gentaure 05999-84)
- Tris solution 1M pH 7.4
- Tris solution 1M pH 8.0
- HEPES (Sigma H3375)
- TAPS (Sigma T5130)
- EDTA solution 0.5 M
- N,N-Dimethylformamide (DMF - Sigma D4551)
- NaCl solution 5M

- $MgCl_2$ solution 2M
- Glycerol solution 100 %
- Ethanol (96-100 %)
- Dithiothreitol (DTT) solution 1M
- Adenosine 5'-triphosphate disodium salt hydrate (ATP) solution 100 mM
- T4 DNA ligase and 10X T4 DNA ligase reaction buffer (NEB, M0202 and B0202S)
- T4 DNA polymerase (NEB, M0203)
- T7 RNA polymerase and 10X Transcription Buffer (Thermo Fisher Scientific, cat. no AM2085 or Toyobo, cat. no. TRL-252)
- 10 mM of each deoxyribonucleotide triphosphate (dNTPs) (NEB N0447S)
- 100 mM ribonucleoside triphosphates (NTPs) (Takara; cat. nos. 4041, 4042, 4043, and 4044)
- Recombinant RNase inhibitor (Takara, 2313A)
- Recombinant DNase I (RNase free) (Takara, 2270A)
- RNA purification kit (RNeasy MinElute Cleanup Kit (Qiagen, cat. no. 74204)) or Zymo RNA Quick Prep kit, cat no. R1050)
- SuperScript™ II Reverse Transcriptase components (Thermo Fisher Scientific, cat. no. 18064022)
- RNase A, DNase and protease-free (10 mg/mL) (Thermo Fisher Scientific, EN0531)
- NEBNext® High-Fidelity 2X PCR Master Mix (NEB, M0541)
- Agilent High Sensitivity DNA Kit (Agilent Technologies, cat. no. 5067-4626)
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851)
- RNase AWAY or equivalent
- AMPure XP beads (Beckman Coulter, Inc. #A63881)

NOTE: *The above references are given as examples and can be ordered from other suppliers however, we strongly encourage you to check the correct final concentration of each reagent in the protocol.*

Oligo DNAs

- Mosaic end Adapter B (MEDS-B forward strand) – order it lyophilized and HPLC purified

  5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'
- Mosaic end Adapter B (MEDS-B reverse strand) – order it lyophilized and HPLC purified

  5'-CTGTCTCTTATACACATCT-3'
- Read 2 primer for reverse transcription – order it lyophilized and desalted

  5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNN-3'

Remarks before starting

1. General recommendations

- Read the complete manual before first time use.
- Decontaminate the working area as well as all the tools with RNase AWAY™ reagent. All containers and storage areas must be free of contaminants and nucleases.
- Wear gloves at all steps to protect the sample from degradation by contaminants and nucleases.
- Add enzymes to reaction solutions last and thoroughly incorporate them by pipetting up and down the solution several times, and never vortex reagents mix.
- We strongly recommend using a positive and negative control (IgG)
- To process multiple wells efficiently, a multichannel pipette should be used for buffers addition and removal.
- Throughout the protocol, gentle shaking is needed. Orbital shaker should be set to 50-100 rpm maximum to avoid cell detachment.
- When handling the plate, do not shake it and always add or remove buffers gently and slowly with a pipette, avoiding bubbles formation to minimize cell detachment.
- Unless otherwise stated, the lid of the thermocycler used to carry the reactions should be set at 105°C.
- **In all steps of the protocol, all volumes of buffers are given for 1 reaction well and should be scaled up when using several wells.**

2. Starting material

This protocol has been optimized for the use of **100 to 100K adherent cells** per ChIL reaction in µ-Plate 96-well TC (ibiTreat). The protocol is not suitable for cell suspension.

Two options are possible for cells preparation:

- Cell sorting for less than 100 cells per well
- Manual preparation for more than 100 cells per well

We strongly recommend using µ-Plate 96-well TC (ibiTreat) from Ibidi as they are suitable for cell adherence and for fluorescence imaging.

If you use other type of cell culture plates, we recommend ensuring good cell adherence. Moreover, make sure they are compatible with inverted microscope imaging (see optional protocol A).

3. Cell fixation

Formaldehyde is the most used cross-linking reagent ideal for two molecules which interact directly. The standard protocol described below uses 1% formaldehyde for 5 min. This condition works for varieties of cell types, but an additional optimization might be required because fixation time can depend on your cell type or target of interest.

4. Immunostaining

The Diagenode's ChIL-seq protocol is described for the use of a primary antibody combined with a ChIL probe as secondary antibody.

The quality of primary antibody used in ChIL-seq is essential for success. We recommend using only validated antibody that specifically recognizes 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**

When using the protocol for the first time, for a definite type of sample and target of interest, we strongly recommend using a positive and abundant histone mark (Diagenode H3K4me3 antibody, C15410030) and negative controls (IgG) following the recommendations of the manufacturer. Moreover, we recommend performing fluorescence microscopy to evaluate the specificity of the primary antibody binding before completing the complete ChIL reaction (see optional protocol A).

5. SDS washes

We recommend carefully adding/removing the buffers because SDS loosens cell attachment and using microscope to check for cell adherence after SDS washes.

6. *In situ* transcription

We strongly recommend adding the components at room temperature as they are listed in the protocol to avoid precipitation of nucleotides in solution. During this step, cover the wells tightly with a sealing film to prevent evaporation and incubate at 37 °C overnight with gentle agitation (using a temperature-controlled orbital or microplate shaker).

7. ChIL RNA purification and assessment of quality

Different methods can be used to purify the ChIL RNA, using for example RNeasy MinElute Cleanup Kit (Qiagen, cat. no. 74204)) or Zymo RNA Quick Prep kit (cat no. R1050).

As the quantity of recovered RNA can be low, we do not recommend quantifying it. We recommend performing a RT-qPCR to evaluate the quality of RNA and the enrichment of ChIL assay (see additional protocol B). We recommend proceeding to sequencing when > 4-fold difference from the negative control sample (IgG) is seen for active histone marks (example of H3K4me3 abundant histone mark).

8. Number of amplification cycles

The number of PCR cycles affects the library complexity and therefore, should be optimized depending on the cell number and the target abundance.

Use the following Amplification Guide for selecting the number of PCR cycles:

Number of cells	Number of PCR cycles
100 or 1K	17
10K	14
100K	12

Please note that the indicated number of cycles might need an additional optimization, depending on target abundance. Avoid an over-amplification that could lead to low complexities of reads and decreased uniquely mapped reads.

9. Primers indexes

Diagenode's Primer indexes for tagmented libraries, compatible with the ChIL-seq protocol, are available in several formats allowing a choice between single or unique dual-indexing and several numbers of different indexes. For more details about the differences between the kits and pooling guidelines for the indexes, please check the Primer index for tagmented libraries manual: https://www.diagenode.com/en/documents/primer-indexes-for-tagmented-libraries_manual

10. Library purification or size selection

Purification using Agencourt AMPure® XP (Beckman Coulter) is the preferred method because sequence complexity is conserved. Do not use silica-based filters for purification. The final library size corresponds to the initial size of

ChIL RNA fragments plus approximately 150 bp due to the tagmented adapters and PCR primers and should be in the range of 150-650 bp. To get rid of PCR primers, adapters peaks (around 150 bp), we recommend the size selection described in the section "Library shaping and QC".

11. Quantification of libaries

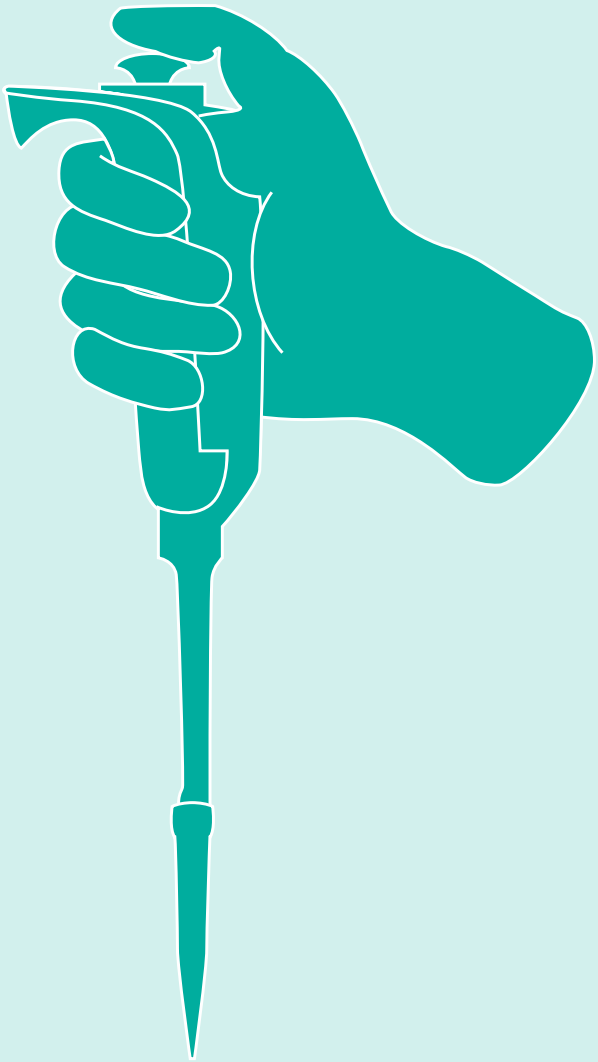
After ChIL library preparation, determine the concentration of the libraries with a highly sensitive method such as the dsDNA HS Assay Kit on the Qubit® system from ThermoFisher Scientific. PicoGreen® is also suitable but UV spectrophotometric methods such as the NanoDrop are usually not sufficiently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

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 and purified samples. In a case of unpurified samples, a region corresponding to libraries should be limited to discriminate between primers/ adapters 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 adapters 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.



PROTOCOL

Buffers preparation

A. STOCK BUFFERS

NOTE: *To ensure a minimal handling time during ChIL protocol, all buffers should be prepared in advance and stored accordingly to the recommendations below.*

Buffers	Preparation	Storage
1 M HEPES-KOH (pH 7.2)	Dissolve 11.925 g of HEPES in 40 ml of ultra-pure dH2O, add potassium hydroxide until the pH is 7.2, make up to a final volume of 50 ml with dH2O and sterilize by filtration (0.2µm)	RT (23-26°C) for ≥ 1 year (in the dark)
1 M TAPS-NaOH (pH 8.5)	Dissolve 12.5 g of TAPS in 40 ml of ultra-pure dH2O, add sodium hydroxide until the pH is 8.5, make up to a final volume of 51.5 ml with dH2O and sterilize by filtration (0.2µm)	RT (23-26°C) for ≥ 1 year (in the dark)
10% (vol/vol) Triton X-100 in water	Add 1 ml of Triton X-100 with ultra-pure dH2O to a final volume of 10 ml	RT (23-26°C) for ≥ 1 year
1% (vol/vol) Triton X-100 in DPBS (-)	Add 1 ml of 10% (vol/vol) Triton X-100 in 9 ml of DPBS (-)	RT (23-26°C) for ≥ 1 year
10% (vol/vol) SDS in water	Add 5 ml of SDS 20 % solution in 5 ml ultra-pure dH2O	RT (23-26°C) for ≥ 1 year
0.2% (vol/vol) SDS in water	Add 0.2 ml of 10 % SDS in 9.8 ml of ultra-pure dH2O	RT (23-26°C) for ≥ 1 year
1 M DTT	Dissolve 1.54 g of DTT in nuclease-free water, make up to a final volume of 10 ml	Store 1-ml aliquots at -20°C for at least 1 year
25 mM mix of each NTP	Mix 100 µl of each of the 100 mM NTPs	Store 50-100 µl aliquots at -80 °C for at least 1 year
Annealing Buffer (40mM Tris-HCl (pH8.0), 50mM NaCl)	Mix 400 µl of 1 M Tris-HCl (pH 8), 100 µl of 5 M NaCl. Make up to a final volume of 10 ml with nuclease-free water	Store 1-ml aliquots at -20°C for at least 1 year
1X Tn5 transposase dialysis buffer (50 mM HEPES-KOH (pH 7.2), 100 mM NaCl, 0.1 mM EDTA, 10% (vol/vol) glycerol, 0.1% (vol/vol) Triton X-100 and 1 mM DTT)	Mix 0.5 ml of 1 M HEPES-KOH (pH 7.2), 0.2 ml of 5 M NaCl, 2 µl of 0.5 M EDTA, 1 ml of glycerol, 0.1 ml of 10% (vol/vol) Triton X-100, 10 µl of 1 M DTT. Make up to a final volume of 10 ml with nuclease-free water	Store 1-2 ml aliquots at -20°C for at least 6 months

<table>
<tr><th>Buffers</th><th>Preparation</th><th>Storage</th></tr>
<tr><td>5X TAPS-DMF buffer
(50 mM TAPS-NaOH (pH 8.5), 25 mM $MgCl_2$ and 50% (vol/vol) DMF)</td><td>Mix 0.5 ml of 1M TAPS-NaOH (pH 8.5), 0.25 ml of 1 M $MgCl_2$, 5 ml of DMF 100%. Make up to a final volume of 10 ml with nuclease-free water</td><td>Store 1 ml aliquots at -20°C for up to 6 months</td></tr>
<tr><td>Read 2 primer for reverse transcription 100 µM</td><td>Dissolve the primer in nuclease-free water to a final concentration of 100 µM for stock solutions</td><td>Store 100 µM primer at -20°C for at least 1 year</td></tr>
<tr><td>Read 2 primer for reverse transcription 12 µM</td><td>Dilute in separate aliquot in nuclease-free water to a final concentration of 12 µM for working solution</td><td>Store 12 µM primer at -20 °C for at least 1 year</td></tr>
<tr><td>MEDS-B oligos 100 µM (forward and reverse strands)</td><td>Resuspend the two separate MEDS-B oligos in Annealing Buffer to a final stock concentration of 100 µM.</td><td>Store 100 µM oligos at -20°C for at least 1 year.</td></tr>
<tr><td>Annealed MEDS-B oligo 10 µM</td><td>Prepare double stranded MEDS-B oligo by annealing the forward and reverse MEDS-B oligos. In a PCR tube, mix 10 µl of each 100 µM stock oligos (forward and reverse strands MEDS-B) with 80 µl of nuclease-free water. Separate in 2 PCR tubes and run the following program:
<table>
<tr><th>Temperature</th><th>Time</th></tr>
<tr><td>95°C</td><td>5 minutes</td></tr>
<tr><td>Cool to 65°C</td><td>-0.1°C/second</td></tr>
<tr><td>65°C</td><td>5 minutes</td></tr>
<tr><td>Cool to 4°C</td><td>-0.1°C/second</td></tr>
</table></td><td>Store 10 µM annealed MEDS-B oligo at -20°C for at least 1 year.</td></tr>
</table>

B. FRESH BUFFERS

NOTE: *All buffers should be prepared freshly and stored accordingly to the recommendations below until use. All volumes of buffers are sufficient for eight wells (volumes in excess included), when using several samples, prepare the buffers for desired number of reactions.*

Buffers	Preparation (for 8 wells)	Storage
1% Formaldehyde in culture medium	Add 27 µl of 37% FA in 973 µl of culture medium	Under chemical hood, wear gloves, RT until use
0.1X Blocking One-P in DPBS(-)	Mix 0.1 ml of 1X Blocking One-P with 0.9 ml of DPBS (-)	+4°C until use
0.1X Blocking One-P in DPBS(-) containing 0.5 M NaCl	Mix 0.1 ml of 1X Blocking One-P, 0.8 ml of DPBS (-) and 0.1 ml of 5 M NaCl	+4°C until use

Buffers	Preparation (for 8 wells)	Storage
1X TAPS-DMF buffer	Mix 0.2 ml of 5X TAPS-DMF buffer with 0.8 ml of nuclease-free water	RT until use
1X T4 DNA Ligase reaction buffer	Mix 0.1 ml of 10X T4 DNA Ligase reaction buffer with 0.9 ml of nuclease-free water	RT until use
1X Transcription buffer	Mix 0.1 ml of 10X Transcription buffer with 0.9 ml of nuclease-free water	RT until use
20 nM annealed MEDS-B in 1X Tn5 transposase dialysis buffer	Mix 1 µl of 10 µM annealed MEDS-B oligo with 499 µl of 1X Tn5 transposase dialysis buffer	On ice until use

DAY 0

CELLS PLATING

STEP 1: CELL SORTING AND PLATING - 6 to 18h

1.1 Plate **100 to 100k adherent cells** per well in **100 µl** of the corresponding cell culture medium on a 96-well ibidi plate (triplicates per condition).

1.2 Incubate at 37°C in a humidified atmosphere of 5% CO_2 overnight.

NOTE: *This protocol is described for the use of 100 to 100k. For <100 cell per well or for single cell, sort the cells by cell sorter in the morning of day 1 and incubate ~6h incubation before fixation to allow sufficient cell adherence.*

DAY 1

IMMUNOSTAINING

STEP 2: FIXATION, PERMEABILIZATION AND BLOCKING – 1h30

Prepare the following fresh buffer (see Buffers preparation)

- 1% Formaldehyde in culture medium

2.1 Take the 96-well plate out of the incubator and check for cell adherence under the microscope. Remove the cell culture medium, add **100 µl of freshly prepared 1% formaldehyde in culture medium**, cover the plate and incubate at room temperature for 5 min, **without shaking** (under a chemical hood).

2.2 Remove formaldehyde solution and gently wash the cells with **200 µl of DPBS (-)**.

NOTE: *The fixed cells can be stored at +4°C for at least 1 week. However, it is recommended to proceed with the following steps when a low number of cells is used.*

2.3 Remove DPBS (-), add **150 µl of 1% Triton X-100 in DPBS (-)**, cover the plate and incubate at room temperature for 20 minutes on an orbital shaker.

2.4 Remove the solution and gently wash the cells with **200 µl of DPBS (-)**.

2.5 Remove DPBS (-), add **100 µl of 1X Blocking One-P**, cover the plate and incubate at room temperature for 20 min on an orbital shaker.

2.6 Remove the blocking solution and gently wash the cells with **200 µl of DPBS (-)**. Proceed directly to step 3.

STEP 3: PRIMARY ANTIBODY BINDING – 2 to 6h

Prepare the following fresh buffers (see Buffers preparation)

- 0.1X Blocking One-P in DPBS (-)
- Primary antibody dilution (1-2 µg/ml) in 0.1X Blocking One-P in DPBS (-): 100 µl of this dilution is needed per well.

3.1 Remove DPBS (-) from step 2.6 and add **100 µl of primary antibody dilution**. Cover the plate and incubate at room temperature for 2 to 6h on an orbital shaker.

NOTE: *Follow the manufacturer recommendations for the concentration and incubation time of the primary antibody. We recommend processing a control sample with a non-specific IgG in parallel to check the unspecific binding of ChIL probe.*

3.2 Remove the primary antibody solution and gently wash the cells with **200 µl of DPBS (-)**. Gently shaking on an orbital shaker at room temperature for 5 min. Repeat this step for a total of three times.

3.3 Place the plate on ice and proceed directly to step 4.

STEP 4: CHIL PROBE BINDING – Overnight

Prepare the following fresh buffers (see Buffers preparation)

- 0.1X Blocking One-P in DPBS (-) containing 0.5 M NaCl, ice cold
- ChIL probe 1/200 dilution in 0.1X Blocking One-P in DPBS (-) containing 0.5 M NaCl, ice cold: 100 µl of this dilution is needed per well.

4.1 Remove DPBS (-) from step 3.3 and add **100 µl of ChIL probe dilution**.

NOTE: *During manipulation of the ChIL probe, always keep it on ice and pay attention to light exposure.*

4.2 Cover the plate and incubate overnight at +4° C on an orbital shaker, in the dark.

DAY 2

TRANSPOSITION – FILL-IN *IN SITU* TRANSCRIPTION

STEP 5: *IN SITU* TRANSPOSITION – 4 to 5h

5.1 Remove the ChIL probe solution from step 4.2 and gently wash the cells with 200 µl of DPBS (-) (ice-cold). Gently shake at +4°C for 20 min on an orbital shaker in the dark. Repeat this step for a total of three times.

PAUSE POINT: *Cells stained with ChIL probe can be stored at +4°C for at least 1 week in the dark and used for fluorescence microscopy. However, it is recommended to proceed directly with the following steps of ChIL-seq protocol when low number of cells are used.*

OPTIONAL: FLUORESCENCE MICROSCOPY. If you want to perform fluorescence microscopy, please refer to optional protocol A.

Thaw and prepare the following buffers (see Buffers preparation)

- 1X Tn5 transposase dialysis buffer (stored at -20°C)
- 20 nM annealed MEDS-B in 1X Tn5 transposase dialysis buffer
- 1x TAPS-DMF buffer
- Dilution of Tagmentase (Tn5 transposase) unloaded to have a working solution of 75 ng/µl: In a 1.5 mL nuclease-free tube, take 1 µl of Tagmentase (C01070010, stock concentration at 2 mg/ml) and add 25.67 µl of Tagmentase Dilution Buffer (C01070011).

NOTE: *This volume of working solution of Tagmentase (75ng/µl) is sufficient for 24 wells (volumes in excess included), when using several samples, prepare the working solution for desired number of reactions.*

5.2 Remove DPBS (-) from step 5.1 and immediately add **1 µl of working solution of Tagmentase (75 ng/µl)** with **49 µl of 1X Tn5 transposase dialysis buffer** per well. Cover the plate and incubate at room temperature for 10 min on an orbital shaker.

5.3 **Without removing** the Tagmentase solution, add **50 µl of freshly prepared 20 nM annealed MEDS-B in 1X Tn5 transposase dialysis buffer**.

5.4 Cover the plate and incubate at room temperature for 1h on an orbital shaker.

5.5 Remove the supernatant and gently wash the cells with **100 µl of DPBS (-)**. Cover the plate and incubate at room temperature for 5 min on an orbital shaker. Repeat this step for a total of three times.

5.6 Remove DPBS (-) and gently wash the cells with **100 µl of 1X Tn5 transposase dialysis buffer**.

5.7 Remove the buffer and add **100 µl of 1X TAPS-DMF buffer**. Cover the plate with a sealing film and incubate at 37°C for 1h on an orbital shaker.

5.8 Remove the buffer and slowly add **100 µl of 0.2% SDS solution**. Cover the plate and incubate at room temperature for 10 min, **without shaking**, paying attention to not disturb the cells.

5.9 Remove SDS and gently rinse the cells with **100 µl of DPBS (-)** twice, without interval. Proceed directly to step 6.

STEP 6: FILL-IN REACTION – 45 min

Prepare the following fresh buffer (see Buffers preparation)

- 1x T4 DNA Ligase reaction buffer

6.1 Remove DPBS (-) from step 5.9 and add **100 µl of 1X T4 DNA Ligase reaction buffer**.

6.2 Prepare the fill-in mix (according to the number of reactions), following the order:

Reagent	Amount per well (µl)	Final concentration
Nuclease-free water	88	
10X T4 DNA Ligase reaction buffer	10	1X
dNTP mix (10 mM)	1	0.1 mM each
T4 DNA Ligase	0.5	2 U/µl
T4 DNA Polymerase	0.5	0.015 U/µl
Total	**100**	

6.3 Remove the buffer and add **100 µl of fill-in mix**. Cover the plate and incubate at room temperature for 30 min on an orbital shaker.

STEP 7: *IN SITU* TRANSCRIPTION – Overnight

Prepare the following fresh buffer (see Buffers preparation)

- 1X Transcription buffer

7.1 Take out of the freezer all reagents for *in situ* transcription (except the enzyme and the RNAse inhibitor), let them thaw at room temperature for 15 min. Put back the nucleotides on ice after thawing.

7.2 Remove the buffer from step 6.3 and slowly add **100 µl of 0.2% SDS solution**. Cover the plate and incubate at room temperature for 10 min, **without shaking**, paying attention to not disturb the cells.

7.3 Remove SDS and gently rinse the cells with **100 µl of DPBS (-)** twice, without interval.

7.4 Remove DPBS (-) and add **100 µl of 1X Transcription buffer** at room temperature.

7.5 Prepare the *in situ* transcription mix (according to the number of reaction) **at room temperature, following the order:**

Reagent	Amount per well (µl)	Final concentration
Nuclease-free water	80	
NTP mix (25 mM each)	8	2 mM each
10X Transcription buffer	10	1X
Recombinant RNase Inhibitor 40U/µl	1	0.4 U/µl
T7 RNA Polymerase (200U)	1	2 U/µl
Total	**100**	

7.6 Remove the buffer and add **100 µl of *in situ* transcription mix**. Cover the plate with a sealing film and incubate overnight at 37°C on an orbital shaker.

NOTE: *We recommend sealing the 96-well plate correctly to avoid evaporation and to keep a correct in situ transcription reaction volume.*

DAY 3

LIBRARY and CLEAN-UP

STEP 8: RNA PURIFICATION – 45 min

8.1 Add **0.5 µl of Recombinant DNase I** per well. Cover the plate with a sealing film and incubate at 37° C for 30 min on an orbital shaker.

NOTE: *Below we present the protocol with Zymo RNA Quick Prep kit (cat no. R1050), but the ChIL RNA can be purified by other column-based methods (for example, RNeasy MinElute Cleanup Kit) or by RNAClean XP beads (Beckman Coulter cat no. A63987).*

8.2 Recover the **100 µl** supernatant of each well into a 1.5 ml nuclease-free tube.

8.3 Add **300 µl of RNA Lysis Buffer** (3:1) and mix well.

8.4 Add **1 volume of ethanol (96-100%) (400 µl)** to 1 volume of sample lysed in RNA Lysis Buffer (1:1) and mix well.

8.5 Transfer the mixture into a Zymo-Spin IC column in a 2 ml collection tube and centrifuge 30 sec at 16,000g. Discard the flow-through.

8.6 Add **400 µl of RNA Prep Buffer** to the column and centrifuge. Discard the flow-through.

8.7 Add **700 µl of RNA Wash Buffer** to the column and centrifuge. Discard the flow-through.

8.8 Add **400 µl of RNA Wash Buffer** to the column and centrifuge. Discard the flow-through.

8.9 Centrifuge again at 16,000g for 1 min to ensure complete removal of the wash buffer.

8.10 Carefully transfer the column into a 1.5 ml nuclease-free tube.

8.11 Add **10 µl of Nuclease-free water** directly to the membrane. After waiting for 1 min, centrifuge at 16,000g for 1 min.

8.12 **Recover the flow through and add it again to the membrane**. After waiting for 1 min, centrifuge again at 16,000g for 1 min to recover the purified ChIL RNA.

PAUSE POINT: *the ChIL RNA can be stored at -80°C for at least 1 month.*

OPTIONAL: ChIL RNA QUALITY ASSESSMENT. If you want to evaluate the quality of ChIL RNA before library preparation, please refer to optional protocol B.

STEP 9: LIBRARY PREPARATION – 4h

9.1 Put **5 µl of ChIL RNA** in a 0.2 ml PCR tube add **1 µl of Read 2 primer for reverse transcription (12 µM)** and mix well.

9.2 Incubate at 65°C for 5 min in a thermocycler, then put the tube to ice directly to stop the reaction.

9.3 Prepare the reverse transcription mix (according to the number of reaction) **at room temperature, following the order:**

Reagent	Amount per rxn (µl)	Final concentration
5X first-strand buffer	2	
DTT 100 mM	1	22.2 mM
dNTP mix (10 mM)	0.5	1.11 mM
Recombinant RNase Inhibitor 40U/µl	0.5	2.22 U/µl
SuperScript II Reverse Transcriptase	0.5	11.1 U/µl
Total	**4.5**	

9.4 Add **4 µl** of the following mix at room temperature to the 6 µl of ChIL RNA and Read 2 primer.

9.5 Incubate in a thermocycler for 10 min at 25°C, then 1h at 42°C (lid at 50°C) and hold at 4°C.

9.6 Add **0.5 µl of RNAse A (5 µg)** and incubate at 37°C for 15 min in a thermocycler.

9.7 Prepare the library preparation mix (according to the number of reaction) **at room temperature, following the order:**

Reagent	Amount per rxn (µl)	Final Concentration
NEBNext® High-Fidelity 2X PCR Master Mix	25	1x
SI/UDI primer pair	2	0.5 mM
Nuclease-free water	13	
Total	**40**	

NOTE: *Primer pairs provided in the Primer index for tagmented libraries kits include indexes for the sequencing. Use different indexes for samples that you want to sequence in the same lane.*

9.8 Add **40 µl of library preparation mix** to each sample and run the following PCR program:

Step	Temperature	Time & Cycles	
Initial denaturation	98°C	30 seconds	
Denaturation	98°C	10 seconds	Number of cycles
Annealing	63°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	10 minutes	
Hold	4°C	∞	

NOTE: *The number of PCR cycles should be optimized depending on the sample characteristics (for example, cell number and the target abundance). We recommend avoiding an over-amplification, please refer to the amplification guide below.*

Amplification guide	
Number of starting cells	**Number of cycles**
Single – 100 – 1K	17
1K-10K	16
10K	14
10K-100K	12

PAUSE POINT: *The ChIL Library can be stored at -20°C for at least 1 month.*

STEP 10: LIBRARY SHAPING AND QC – 2h

Prepare the following:

- Freshly prepared 80% (v/v) ethanol
- DiaMag 0.2 magnetic rack
- Nuclease-free water

10.1 Take the AMPure® XP beads out of the fridge and resuspend them gently on a rotating wheel or orbital shaker at room temperature before use.

10.2 Add **40 µl of beads (0.8 X)** to the 50 µL of amplified ChIL library from step 9.8. Mix thoroughly by pipetting up and down several times or by vortexing a few seconds.

10.3 Incubate for 10 minutes at room temperature under mild agitation.

10.4 Spin down the sample in a table top centrifuge and place the tube on a magnetic rack.

10.5 When the solution is clear, **carefully aspirate and discard the supernatant without disturbing the pellet**.

10.6 Wash the beads pellet 2 times as follows:

- With the tubes on the magnet, add **200 µl of freshly prepared 80% ethanol** without disturbing the bead pellet and wait for 30 seconds.
- Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

10.7 Spin down the sample in a table top centrifuge, place the tube back on the magnetic rack and remove remaining ethanol.

10.8 Leave the tube open for 2-3 minutes to let the beads pellet dry (do not over-dry the beads as it may result in low recovery, over-dried beads become light brown with cracks) and then remove it from the magnetic rack.

10.9 Add **50 µl of nuclease-free water**. Mix slowly by pipetting up and down to resuspend the beads.

10.10 Incubate the beads for 5 minutes at room temperature.

10.11 Spin down the sample in a table top centrifuge and place the tube on a magnetic rack.

10.12 When the solution is clear and without disturbing the beads, carefully aspirate and transfer the **50 µl** of supernatant to a new tube.

10.13 Repeat the complete procedure (from steps 10.2 to 10.12) with **40 µl AMPure® XP Beads (0.8X)** but elute the purified library in **20 µl of nuclease-free water**. If more concentrated library is needed, the elution can be done in 10-15 µl of nuclease-free water.

ChIL Library QC

10.14 Run **1 µl** of each library on a High Sensitivity DNA chip for BioAnalyzer (Agilent) or **2 µl** on Fragment Analyzer (Agilent) according to the manufacturer's instructions. A successful library amplification and purification should show a smear ranging from 250 to 650 bp.

10.15 It is preferred to quantify the individual ChIL library before the pooling. Determine the concentrations of your samples using a fluorescence-based assay such as the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851) or using Real-time PCR-based approaches such as the KAPA Library Quantification Kit (Kapa Biosystems).

10.16 Your libraries are now ready for pooling and sequencing. Pool the same amount of DNA for each library. The library fragment sizes should be similar.

NOTE: *Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to the manual: Primer indexes for tagmented libraries. 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.*

Sequencing recommendations

We recommend sequencing in Single-End 50 base pair or 100 base pair as Read1 is contained in ChIL DNA, which allows to detect the closest position on the genomic region of interest. We recommend counting 10-20 million of reads per sample and PhiX % should be applied as recommended by the sequencer.

Following the use of single or unique dual indexes, the construct will bear one or two separated indexes. The "i5" index can be read in a reverse complement workflow or in a forward strand way depending on the sequencer.

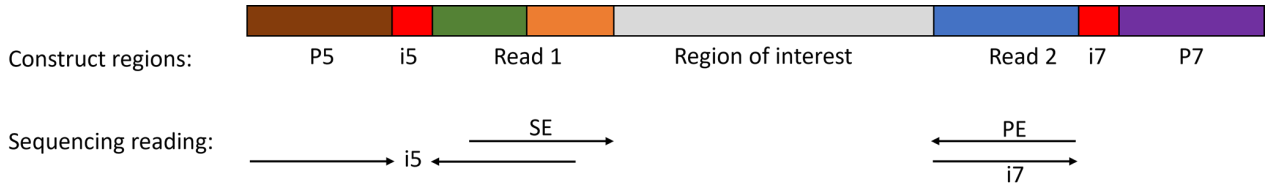


Figure 2. ChIL library construct. SE (single-end sequencing), PE (paire-end sequencing), i5/i7 (SI or UDI indexes).

Data analysis recommendations

In the following section we will guide you through the basics of ChIL-seq data analysis. We will also recommend some software tools suitable for each step.

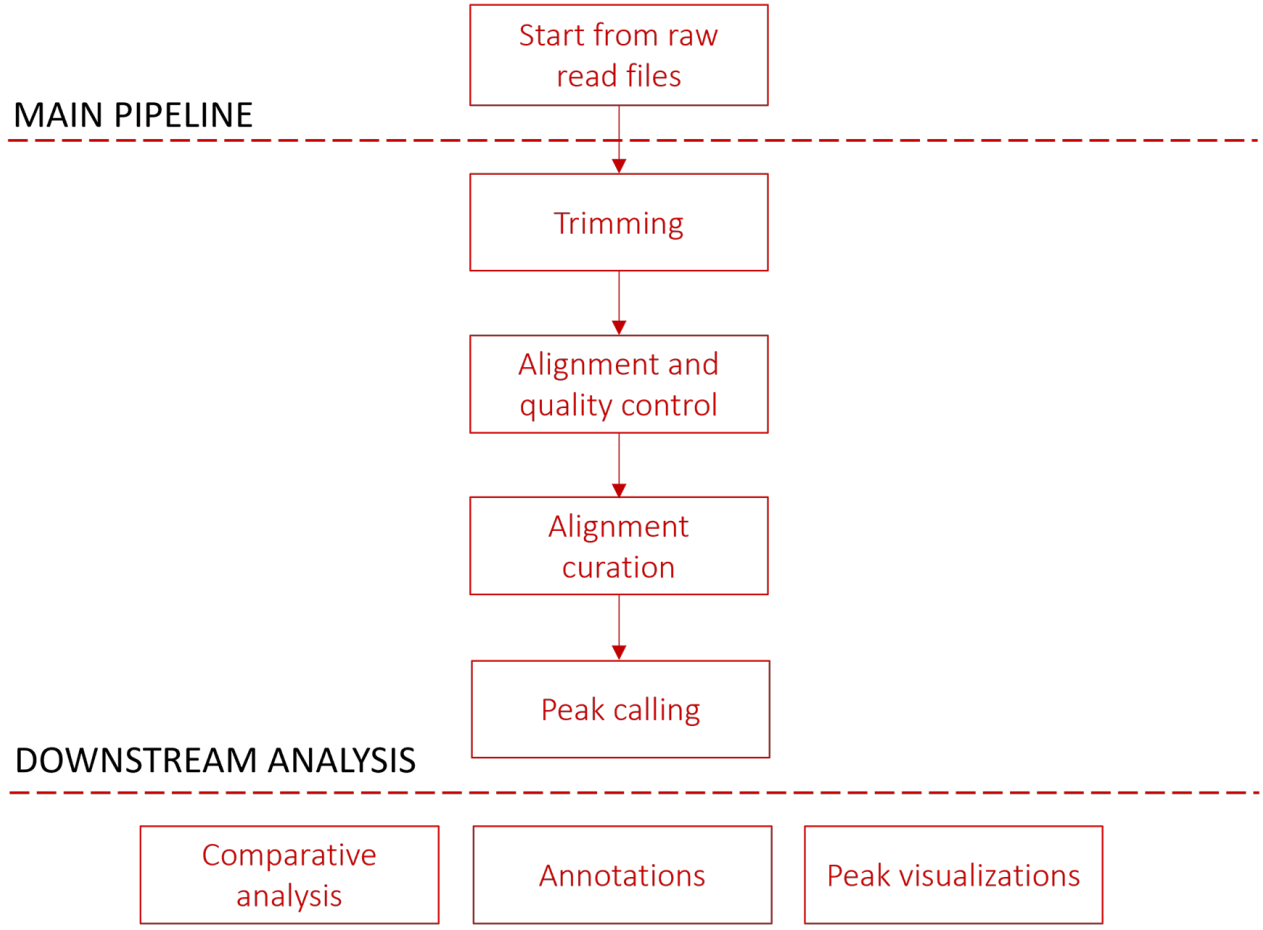


Figure 3. ChIL data analysis workflow. General view of the data analysis recommendations.

MAIN PIPELINE

1. **Trimming:** use trimming to get rid of low-quality bases and artefacts in the readset, such as adapter contaminations.

   We recommend using Trim_galore software [1]. One might also want to consider the --2color option of Trim_Galore if libraries were sequenced on NextSeq or NovaSeq to ignore qualities of G bases, which are potentially confounded with basecalls without any signal.

2. **Alignment:** in this step, reads (single-end or paired-end) will be mapped against an indexed reference genome. Quality of the alignment can be assessed by several metrics obtained, for example, by using Qualimap [2]. Among others, any artifact in the fragment length distribution could indicate some improper alignments or problems at the wet-lab level.

   We recommend aligning trimmed reads to the reference genome (obtained from the UCSC genome browser) [3] using BWA software [4].

(Optional step) Quality control: The general quality of the sequencing and the aligned reads can be checked by running FastQC tool [5] on the trimmed fastq files and on the bam alignment file, respectively.

3. **Alignment curation:** To get more reproducible results, removing reads aligned with low mapping score and technical artifacts must be performed.

   - Removal of PCR duplicates and multi-mapping reads using samtools [6].
   - ENCODE blacklisted regions [7]: those genomic regions, mainly due to their nucleotide content, have a probability of mapping reads that is artefactually high and are therefore highly unreliable to study enrichments. ENCODE blacklisted regions are available for common species (human, mouse, drosophila) and any reads aligned to these regions are usually filtered out before peak calling. Note that "greylists" can be generated if official ENCODE blacklist is not referenced for your species of interest.

4. **Peak calling:** during peak calling the software will detect sites of enrichment (reads accumulation) along the genome. Special care should be taken to ensure using proper parameters to call the peaks.

DOWNSTREAM ANALYSIS

After this general analysis, the peaks can be analyzed further to get answers to our biological questions. There are countless ways and tools for further analyses (comparative analysis, annotations, peaks visualization), the project's goals determine which ones to 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. Recommended software and R packages include deeptools [8], HOMER suite [9], Diffbind [10] and annotatr [11].

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Optional protocols

A. FLUORESCENCE MICROSCOPY

EVALUATION OF THE PRIMARY ANTIBODY SPECIFIC BINDING

Equipment

- µ-Plate 96-well TC (ibiTreat) (ibidi, ib89626)
- Plate sealing films
- Inverted Fluorescence microscope (imaging from the plate bottom) with 40x objective

Reagents

- Counterstain agent (examples Hoechst 33342 or NucBlue™ Fixed Cell Ready Probes™ Reagent (DAPI)
- Immersive oil (refer to Fluorescence Microscope manufacturer)
- Alexa Fluor 488 secondary antibody (optional)

Remarks before starting

We recommend the following conditions of primary antibody to test the specificity of the binding:

- A positive control antibody for an abundant epitope (H3K27me3 for example)
- A negative control antibody (IgG)
- A primary antibody of your choice (target of interest)

A titration of the primary antibody can be necessary to optimize the reaction and check the specificity. During this optimization step and to avoid using too much ChIL probe, we recommend using fluorescent dye-conjugated secondary antibody instead of ChIL probe (example using an Alexa Fluor 488 secondary antibody, following the recommendations of manufacturer).

Cells stained with primary and secondary antibodies can be stored at +4°C for at least 1 week in the dark and used for fluorescence microscopy. We strongly recommend making 96-well plates in parallel as the plate used for fluorescence microscopy cannot be proceed for the rest of ChIL-seq protocol.

Protocol

1. Follow the described ChIL-seq protocol up to step 5.1 (including).

2. After the washes, recover the plate from step 5.1.

3. To perform fluorescence microscopy, we recommend to counterstain the nuclei using one of the two examples below:

    - Hoechst 33342 diluted at 0.1 µg/ml in PBS, distribute 100 µl of this solution per well and incubate for 1h at +4 °C, in the dark.
    - NucBlue™ Fixed Cell Ready Probes™ Reagent (DAPI), add 2 drops for 1 ml of PBS and distribute 100 µl of this solution per well and incubate for 30 minutes at room temperature, in the dark.

4. After counterstaining, cover the plate with sealing film and keep it at +4°C in the dark until the imaging under inverted fluorescence microscope.

5. Follow the recommendations of manufacturer to use the inverted fluorescence microscope and use immersive oil if necessary, taking pictures from the plate bottom.

6. Set the microscope parameters for DAPI detection (all cell nuclei) and TAMRA- tetramethylrhodamine (target stained with ChIL probe): Excitation length: 552 nm - Emission length 578 nm.

B. ChIL RNA QUALITY ASSESSMENT

Equipment

- Real-time qPCR instrument
- 1.5 ml tube (nuclease-free)
- 96-well qPCR plate (depending on the Real-time qPCR instrument)
- Optically transparent plate sealing film

Reagents

- Nuclease-free water
- Luna Universal One-Step RT-qPCR kit (NEB E3005)
- Forward primer of genomic region of interest (to design)
- Reverse primer of genomic region of interest (to design)

Remarks before starting

When using the protocol for the first time, for a definite type of sample and target of interest, we strongly recommend using a positive and abundant histone mark (Diagenode H3K4me3 antibody, C15410030) and negative controls (IgG) following the recommendations of the manufacturer.

The ChIL RNA can be analyzed by RT-qPCR to evaluate the enrichment of ChIL assay. For each mark, we recommend using primers for positive and negative regions to correctly evaluate the recovery. Moreover, the RT-qPCR primers should be designed within < 100 bp in the promoter region of the target and should not exceed 20-22 nucleotides long.

Although it is difficult to set a clear criterion to judge the quality of ChIL RNA from RT-qPCR because the enrichment can depend on the PCR primers target sequence, we recommend proceeding to sequencing when > 4-fold difference from the negative control sample (IgG) is seen for active histone marks (example of H3K4me3 abundant histone mark).

Please read completely the manual of Luna Universal One-Step RT-qPCR kit (NEB E3005).

Protocol

1. Follow the described ChIL-seq protocol up to step 8 (including).

2. After the recovery of ChIL RNA, dilute 1 µl of ChIL RNA with 19 µl of nuclease-free water (20x dilution), keep it on ice until use for RT-qPCR.

3. Thaw all reagents of Luna Universal One-Step RT-qPCR kit at room temperature, then place them on ice. After thawing completely, briefly mix each component by pipetting or gentle vortexing.

4. Determine the total volume for the appropriate number of reactions, adding 10% overage, and prepare the following reaction mix for each target primers pair.

Components	Amount for 1 reaction	Final concentration
Luna Universal One-Step Reaction Mix (2x)	10 µL	1X
Luna WarmStart RT Enzyme Mix (20x)	1 µL	1X
Forward Primer (10 µM)	0.8 µL	0.4 µM
Reverse Primer (10 µM)	0.8 µL	0.4 µM
Nuclease-free water	6.4 µL	
Total	**19 µl**	

5. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.

6. Distribute 19 µl of reaction mix into each qPCR well. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.

7. Add 1 µl of RNA template to each qPCR well. Seal plate with optically transparent plate sealing film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.

8. Spin plate briefly to remove bubbles and collect liquid to the bottom (1 minute at 2,500–3,000 rpm).

9. Program real-time qPCR instrument with indicated thermocycling protocol (see table below). Ensure that a plate read is included at the end of the extension step. Use the SYBR or SYBR/FAM scan mode setting on the real-time qPCR instrument.

Cycle Step	Temperature	Time	Cycles
Reverse transcription	55°C	10 minutes	**1**
Initial Denaturation	95°C	1 minute	**1**
Denaturation	95°C	10 seconds	**40-45**
Extension	60°C	30 seconds* + plate read	
Melting Curve	60-95°C**	various + plate read	**1**

NOTE: ** Depending on real-time qPCR instrument, 60 seconds of extension can be used (please refer to the manual of Luna Universal One-Step RT-qPCR kit (NEB E3005).*
*** Follow real-time qPCR instrument recommendations for melting curve step.*

Troubleshooting guide

In this section, we present some advice to overcome potential troubleshooting. Moreover, we recommend reading the complete manual before first time use, specially the "Remarks before starting" section.

DAY 1: IMMUNOSTAINING

To perform fluorescence microscopy, we recommend following the optional protocol A.

Problem	Possible causes	Suggestions
No/fuzzy signal	Wrong cell culture plate	Check for plate compatibility with microscopy. We recommend using µ-Plate 96-well TC (ibiTreat) from Ibidi as they are suitable for cell adherence and for fluorescence imaging.
Weak signal	Poor activity of the primary antibody	Increase the concentration of the primary antibody and incubation time in step 3. A titration of the primary antibody can be necessary using fluorescent dye-conjugated antibody instead of ChIL probes (example using an Alexa Fluor 488 secondary antibody).
High background signal, non-specific signal	Primary antibody concentration is too high	The quality of primary antibody used in ChIL-seq is essential for success and specificity of the reaction. We recommend using only validated antibody that specifically recognize the target. Decrease the concentration of the primary antibody and incubation time in step 3. Use a negative control sample (IgG) and check for primary antibody signal specificity.

DAY 2: TRANSPOSITION / FILL-IN / *IN SITU* TRANSCRIPTION

The Tn5-mediated transposition can be monitored by genomic PCR using the Tn5 evaluation protocol (described in Box 1, Handa T. et al., Nature Protocols 2020). Pay attention that plate used for this optional protocol cannot be proceed for the rest of ChIL-seq protocol (make plates/wells in parallel). We recommend monitoring the Tn5-mediated transposition if optimizations of fixation are necessary.

Problem	Possible causes	Suggestions
Sample loss after SDS wash	Poor attachment of the cells	The SDS wash step is critical for cell adherence/ loss (sample loss can be assessed under a microscope). Do not shake the plate during SDS incubation. Use an extracellular matrix to further support cell adhesion. Add/remove the buffers carefully and avoid over drying the cells between each buffer addition. A longer fixation time prevents the detachment of cells during buffer exchanges but lowers the enzymes efficiency. If optimization of the fixation condition is needed, we recommend checking cell adherence after SDS washes under microscope and evaluating the *in situ* transposition efficiency (described in Box 1, Handa T. et al., Nature Protocols 2020).
Evaporation of liquid into wells	Wells not covered or not controlled temperature incubation	When recommended, cover the wells tightly with a sealing film to prevent evaporation and incubate at 37°C overnight with gentle agitation (using a temperature-controlled orbital or microplate shaker).
Precipitation during *in situ* transcription	Cold reagents Too much enzyme	We recommend adding the components at room temperature as they are listed in the protocol to avoid precipitation of nucleotides in solution. Check the amount of T7 RNA polymerase per well (too much enzyme may lead to precipitation and inhibition of reaction).

DAY 3: RNA PURIFICATION / LIBRARY PREPARATION AND CLEAN-UP

As the quantity of recovered RNA can be low, we do not recommend quantifying it. We recommend performing a RT-qPCR to evaluate the quality of RNA and the enrichment of ChIL assay (see additional protocol B).

Problem	Possible causes	Suggestions
No enrichment at RT-qPCR level No library amplification	Primary antibody specificity	The quality of primary antibody used in ChIL-seq is essential for success and specificity of the reaction. We recommend performing fluorescence microscopy to evaluate the specificity of the primary antibody binding before completing the complete ChIL reaction. We recommend to always make a negative control condition (IgG) for measurement of enrichment.
	Sample loss, poor attachment of the cells	Do not shake the plate during SDS incubation. Use an extracellular matrix to further support cell adhesion. Add/remove the buffers carefully and avoid over drying the cells between each buffer addition. A longer fixation time prevents the detachment of cells during buffer exchanges but lowers the enzymes efficiency. If optimization of the fixation condition is needed, we recommend checking cell adherence after SDS washes under microscope and evaluating the *in situ* transposition efficiency.
	Problem with a reagent	Evaluate the *in situ* transposition (described in Box 1, Handa T. et al., Nature Protocols 2020). Evaluate the quality of RNA after *in situ* transcription (see optional protocol B). Run a positive control sample for an abundant epitope (H3K27me3 for example). Use fresh buffers and reagents in the ChIL reaction and library preparation.
< 150 bp DNA peak	Insufficient purification	Purify again with AMPure XP beads as described in step 10.
Different library concentrations measured by Bioanalyzer and Qubit, qPCR	This is typical for quantifying NGS libraries	qPCR or Qubit (when enough material) show higher accuracy and reproducibility. We recommend using a Bioanalyzer to evaluate only the size of libraries.
Low number of reads mapped to the genome (<70% of total reads)	This is a typical issue for low input or low expressed target	At Diagenode, we use to have >95% of mapped reads for abundant target. Start with an increased number of cells, if available. Avoid an over-amplification that could lead to low complexities of reads and decreased uniquely mapped reads. Run a positive control sample for an abundant epitope (H3K27me3 for example).

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