# University of Groningen

## Protocol of the Double-Click Seq Method to Evaluate the Deposition Bias of Chromatin Proteins

van der Wouden, Petra E.; Zwinderman, Martijn R. H.; Chen, Deng; Borghesan, Michela; Dekker, Frank J.

[Link to publication in University of Groningen/UMCG research database](#)

# Exosome Production, Isolation and Applications: From Cancer Research to Theraputic Targets

## A New Article Collection.
## Download for free.
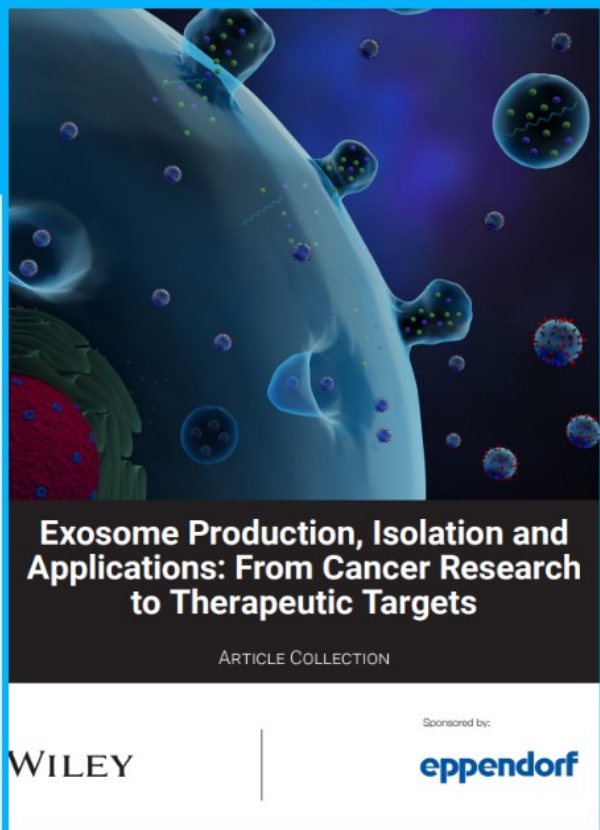
Exosomes play a crucial role in intercellular communication, including in cancer. Here we explore their potential in novel exosome production modulators, plant isolation and purification, in tumor immunology, and bioprocessing for diagnosis and treatment. Isolating exosomes is vital for developing tools and therapies.

This article collection presents innovative methods for exosome isolation and purification, emphasizing their role in developing new diagnostic tools and therapies for diverse diseases, with the goal of enabling researchers to explore their potential.

**Key topics include:**
- Isolation techniques of exosomes
- Biomarkers for cancer diagnosis/prognosis
- Engineered exosomes

eppendorf

WILEY

# Protocol of the Double-Click Seq Method to Evaluate the Deposition Bias of Chromatin Proteins

Petra E. van der Wouden,[1] Martijn R. H. Zwinderman,[1] Deng Chen,[1] Michela Borghesan,[1] and Frank J. Dekker[1,2]

[1]Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands
[2]Corresponding author: *f.j.dekker@rug.nl*

Published in the Molecular Biology section

Symmetrical deposition of parental and newly synthesized chromatin proteins over both sister chromatids is important for the maintenance of epigenetic integrity. However, the mechanisms to maintain equal distribution of parental and newly synthesized chromatid proteins over sister chromatids remains largely unknown. Here, we describe the protocol for the recently developed double-click seq method that enables mapping of asymmetry in the deposition of parental and newly synthesized chromatin proteins on both sister chromatids in DNA replication. The method involved metabolic labeling of new chromatin proteins with l-Azidohomoalanine (AHA) and newly synthesized DNA with Ethynyl-2′-deoxyuridine (EdU) followed by two subsequent click reactions for biotinylation and subsequently by corresponding separation steps. This enables isolation of parental DNA that was bound to nucleosomes containing new chromatin proteins. Sequencing of these DNA samples and mapping around origins of replication in the cellular DNA enables estimation of the asymmetry in deposition of chromatin proteins over the leading and lagging strand in DNA replication. Altogether, this method contributes to the toolbox to understand histone deposition in DNA replication. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

**Basic Protocol 1:** Metabolic labeling with AHA and EdU and isolation of nuclei
**Basic Protocol 2:** First click reaction, MNase digestion and streptavidin enrichment of labeled nucleosomes
**Basic Protocol 3:** Second click reaction, Replication-Enriched Nucleosome Sequencing (RENS) Protocol

Keywords: chromatin proteins • DNA replication • double-click seq • Ethynyl-2′-deoxyuridine (EdU) • histone deposition • l-Azidohomoalanine (AHA)

## INTRODUCTION

Post-translational modifications of histone (PTMs) are crucial for regulating gene transcription and nuclear organization (Allis & Jenuwein, 2016). Some modifications are stable and can be inherited during DNA replication (Audergon et al., 2015; Ragunathan et al., 2015), but they differ between parental and newly synthesized histones (Reverón-Gómez et al., 2018), making it important to maintain balanced deposition during replication to ensure similar chromatin states for the sister chromatids after cell division. The preservation of repressed chromatin domains by the redeposition of parental histones highlights the functional importance of this process (Escobar et al., 2019). Accurate mechanisms for deposition of old and new histones are therefore critical (Alabert et al., 2015).

Recent studies have shed light on how histones are deposited during DNA replication by using immunoprecipitation of PTMs that are characteristic of either parental histones (H4K20me2) or new histones (H4K5ac) (Petryk et al., 2018; Yu et al., 2018). When this approach was coupled with the labeling of new DNA using the thymidine analog 5-ethynyl-2′deoxyuridine (Edu) to separate parental from newly synthesized DNA, a slight deposition bias of new histones to the lagging strand was revealed in mouse embryonic stem cells (Petryk et al., 2018) and a slight bias of new histones deposition onto the leading strand in budding yeast cells treated with hydroxyureas (HU) (Yu et al., 2018). Of note, both studies concluded that the structural integrity of the replisome is essential to maintain near-symmetrical histone inheritance. Expanding further the technology to differentiate parental versus new chromatin proteins would be certainly of benefit for the research field.

Despite recent advances in understanding how histones are deposited during DNA replication, there are several limitations to existing approaches. Firstly, the current methods rely on specific post-translational modifications (PTMs) of histones, such as H4K20me2, H5K5ac, and H4K5ac, to distinguish between parental and newly synthesized histones. However, these PTMs may not be entirely reliable markers, as they can also be present in other contexts or exhibit dynamic changes during replication or other cellular processes. Secondly, histone deposition during DNA replication may vary depending on the cell type, tissue context, and disease state, and it is important to consider these factors in future research. Thirdly, while the current approaches have provided insights into the biased deposition of new histones to the leading or lagging strand during replication, the underlying molecular mechanisms are not fully understood. The role of replisome integrity in maintaining near-symmetrical histone inheritance needs further investigation, as well as the potential involvement of other factors or regulatory pathways.

Despite these limitations, improving our understanding of histone deposition during DNA replication could greatly advance our knowledge of epigenetic regulation and nuclear organization, and have significant implications for biology and disease research. For example, a better understanding of how histones are accurately deposited during replication could shed light on how epigenetic information is faithfully transmitted during cell division and inherited across generations. Additionally, dysregulation of histone deposition has been implicated in various diseases, including cancer and neurodegenerative disorders, highlighting the importance of studying this process for therapeutic development. Therefore, further advancements in technology and approaches to accurately differentiate parental from new chromatin proteins could provide valuable insights into the mechanisms underlying histone deposition during DNA replication and its functional significance in biology.

Here, we present the double-click-seq method, which is a novel approach complementary to previously established immunoprecipitation-based methods for enriching parental
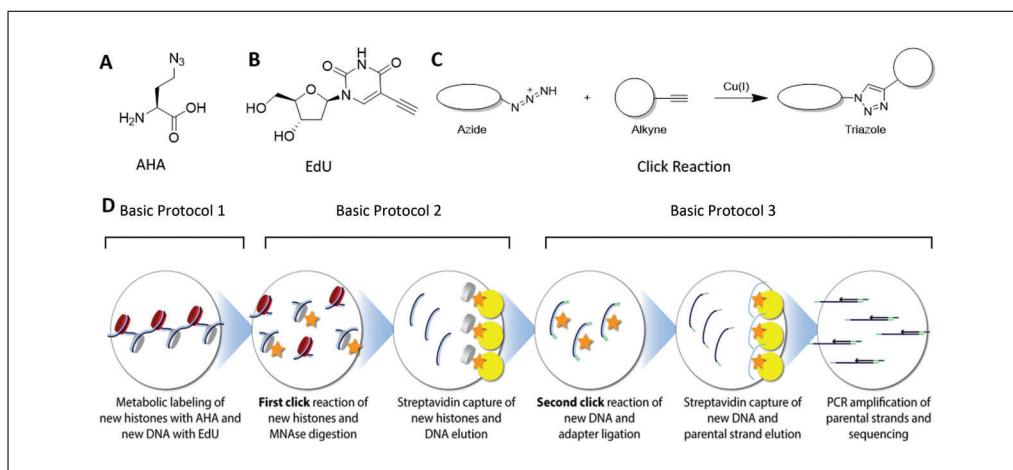
**van der Wouden et al.**

**Figure 1** Overview of the steps for the double-click-seq protocol. Image credit: Zwinderman et al., 2021. ACS Chemical Biology 2021 16(11) 2193-2201.

DNA from new chromatin proteins. The double-click-seq technique utilizes stable labeling of de novo synthesized chromatin proteins by co-translational incorporation of the methionine surrogate azidohomoalanine (AHA), which can be covalently attached to a biotin affinity tag via copper-catalyzed alkyne to azide cycloaddition reaction, commonly referred to as the "click" reaction (Kolb et al., 2001). This enables enrichment of nucleosomes containing new histones by conducting a click reaction with a biotin affinity tag. The incorporation of this technology into the double-click-seq protocol offers a powerful method to investigate the distribution of parental versus new histones over the leading and lagging strand during DNA replication. The double-click-seq method provides several advantages compared to other methods for enriching parental DNA from newly synthesized chromatin proteins:

1. Complementary approach: The double-click-seq method complements existing immunoprecipitation-based methods, offering an additional tool for investigating chromatin proteins and allowing for a more comprehensive understanding of parental versus new histone distribution during DNA replication.
2. Stable labeling: The double-click-seq method utilizes stable labeling of de novo synthesized chromatin proteins with azidohomoalanine (AHA) during translation, ensuring reliable capture and enrichment of labeled chromatin proteins for accurate assessment.
3. Covalent attachment with biotin affinity tag: The double-click-seq method employs a specific and stable covalent attachment of a biotin affinity tag to AHA-labeled chromatin proteins using a "click" reaction, minimizing nonspecific binding and background noise.
4. High sensitivity and specificity: The use of biotin affinity tag in the double-click-seq method enables efficient and specific enrichment of nucleosomes containing new histones, resulting in a highly sensitive and specific approach for studying chromatin protein dynamics during DNA replication.
5. Powerful and versatile: The incorporation of double-click-seq technology into the protocol provides a powerful and versatile method for investigating the distribution of parental versus new histones in various biological contexts, offering insights into chromatin dynamics and epigenetic regulation.

The double-click-seq method involves three basic protocols (Fig. 1). In Basic Protocol 1, newly synthesized proteins in the cell are labeled with AHA, and newly synthesized DNA is labeled with 5-ethynyl-2′deoxyuridine (EdU). After cell lysis and isolation of the nuclei, Basic Protocol 2 involves subjecting the isolated nuclei to the first click reaction

Current Protocols

using alkyne-labeled biotin to biotinylate AHA-labeled proteins. It is important to carefully design and optimize the click reactions to minimize any potential cross-reactivity or competition between different components. In Basic Protocol 2, where the first click reaction is performed to biotinylate AHA-labeled proteins using alkyne-labeled biotin, it is crucial to ensure that the click reaction is selective and specific for the intended targets (AHA-labeled proteins) and does not cross-react with other components, such as the DNA alkyne or azide groups.

To minimize the potential for cross-reactivity between the DNA alkyne and the protein azide, it is important to choose appropriate click reaction conditions, such as optimizing the concentration and ratio of the reactants, reaction time, and temperature. Additionally, using highly selective click chemistry reactions, such as copper-catalyzed azide-alkyne cycloaddition (CuAAC) or strain-promoted azide-alkyne cycloaddition (SPAAC), can help minimize cross-reactivity and maximize specificity.

It is recommended to carefully validate and optimize the click reactions in your specific experimental setup to ensure that the biotinylation of AHA-labeled proteins is efficient and specific, while minimizing any potential cross-reactivity with DNA alkyne or azide groups. This may involve conducting control experiments and adjusting reaction conditions as needed to achieve the desired specificity and efficiency in the click reaction.

Mono-nucleosomes are then prepared by DNA digestion with MNase treatment, and biotinylated nucleosomes are isolated using streptavidin-coated magnetic beads. The double-stranded DNA is eluted from the beads by Proteinase to digest the nucleosomes. In Basic Protocol 3, the second click reaction is then performed using azide-labeled biotin to biotinylate Edu that is incorporated into newly synthesized DNA. This step is also used for aptamer ligation. Subsequently, the biotinylated DNA is captured using streptavidin-coated magnetic beads, and the complementary strand is eluted to provide the parental DNA strand. Finally, the resulting DNA library is amplified by PCR and prepared for next-generation sequencing (NGS).

<table>
<tr><td>BASIC PROTOCOL 1</td><td>

## METABOLIC LABELING WITH AHA AND EDU AND ISOLATION OF NUCLEI

</td></tr>
</table>

This protocol describes the method for *de novo* labeling of newly synthesized histones and DNA. Cells are grown in appropriate medium containing the methionine analog azidohomoalanine (AHA) and 5-ethynyl-2′deoxyuridine (EdU). Dividing cells incorporate AHA in their newly synthesized proteins including histones. EdU will be incorporated in newly synthesized DNA during the S-phase. In our method we used the hTERT RPE-1 cell line. This cell line was chosen because it is an immortalized nearly diploid cell line from human retinal pigment epithelial cell with an average chromosome number of 46. This feature makes sequencing analysis feasible. The nuclei are being isolated through a simple and fast method using 0.1% of NP-40 in PBS and a few short centrifugation steps.

### Materials

RPE-1 cells (hTERT RPE-1, ATCC®, CRL-4000™)
Standard medium (see recipe)
GlutaMAX (Gibco, cat. no. 35050-038)
Penicillin-streptomycin (10,000 U penicillin and 10 mg streptomycin/ml) (Gibco, cat. no. 11548876)
Fetal bovine serum (Serana, cat. no. S-FBS-SA-0150)
AHA (Azidohomoalanine) medium
5-ethynyl-2′deoxyuridine (EdU) (stock solution of 67 mM in DMSO, stored at −20°C) (Bio-Connect cat. no. CLK-N001)
Phosphate-buffered saline (PBS; Gibco, cat. no. 10010056)

van der Wouden et al.

Trypsin-EDTA (Gibco, cat. no. 15400-054)
0.1% NP-40 in PBS with protease inhibitor (see recipe)

175-cm$^2$ cell culture flask (Greiner, cat. no. 660175)
Inverted microscope (Leica)
50-ml centrifuge tubes (Sarstedt, cat. no. 62547254)
Swing-out centrifuge (Eppendorf 5810)
ULT Freezer
Benchtop centrifuge (Eppendorf 5415R)
1.5-ml centrifuge tubes (Sarstedt, cat. no. 72690001)
Vortex mixer

### *Harvest cells*

1. Seed four 175-cm$^2$ flasks each with $3 \times 10^6$ RPE-1 cells per flask (12 million cells in total) in appropriate (standard) medium. Incubate overnight at 37°C with 5% $CO_2$.

   *We used RPE-1 cells for their nearly diploidy. RPE-1 cells were cultured in DMEM, high glucose, pyruvate, GlutaMAX$^{TM}$, containing 10% fetal bovine serum and 1% penicillin/streptomycin.*

   *It is important that cells are seeded in such a concentration so that they are still actively dividing ($\pm$60% confluency). Check cells for mycoplasma regularly.*

2. Prepare 95% AHA medium by mixing 28.5 ml AHA medium with 1.5 ml standard medium per 175-cm$^2$ flask.

   *It is important to add 5% standard medium (which already contains methionine) to ensure the continuation of cell division.*

3. Aspirate the medium from the cells.

   *There is no need to wash the cells.*

4. Add 9 μl EdU (67 mM stock) to 30 ml of the 95% AHA medium (this results in 20 μM final concentration of EdU) to the cells and incubate for 24 hr at 37°C with 5% $CO_2$.

   *The 24 hr of incubation is based on the doubling time of the cells. In this step, it is possible to add compounds for treatment to your cells. It is advised to pre-mix the medium with the EdU before adding it to the cells. Turn off the light in the flow-cabinet because EdU is light sensitive.*

5. Incubate the cells for 24 hr at 37°C with 5% $CO_2$.

   *The time for one cell division is 24 hr for RPE-1 cells cultured in AHA medium. If you use other cells in your experiment, please check the division time beforehand. Be aware that the division time can change when adding treatment compound.*

6. Aspirate the medium and wash the cells twice, each time with 10 ml PBS.

7. Add 3 ml Trypsin-EDTA and incubate at 37°C until all the cells are detached from the bottom of the flask.

   *Trypsinization takes about 5 min. You can check under the microscope whether all the cells have detached.*

8. Quench the trypsin by adding 3 ml standard medium with FBS.

   *The FBS in the medium quenches the activity of the trypsin*

9. Collect the cells in 50-ml tubes.

   *The cells from four 175-cm$^2$ flasks are collected in one 50-ml centrifuge tube.*

10. Centrifuge the cells for 5 min at 200 $\times$ g at room temperature.

**van der Wouden et al.**

**5 of 17**

11. Wash the cells with 5 ml ice-cold PBS and centrifuge again for 5 min at $200 \times g$ at room temperature.

12. Remove the supernatant and store the pellets at $-80°C$ until further use or continue directly to step 13 to extract the nuclei.

    *Pellets can be stored at least 4 months at $-80°C$.*

### Nuclei extraction

13. To extract the nuclei, the thawed pellet is resuspended in 1 ml of ice-cold PBS containing 0.1% NP-40 supplemented with $1\times$ protease inhibitor cocktail.

14. Transfer the cell suspension into a 1.5-ml centrifuge tube.

15. Vortex the cells suspension for 5 sec at maximum speed.

    *Vortexing at maximum speed will lyse all the cells.*

16. Centrifuge the lysed cell suspension for 5-10 sec at $9000 \times g$, $4°C$, in a benchtop centrifuge.

17. Discard the supernatant.

    *The pellet contains most nuclei.*

18. Resuspend the nuclei in 1 ml ice-cold PBS containing 0.1% NP-40 and supplemented with $1\times$ protease inhibitor cocktail.

    *In this step, the nuclei are being washed.*

19. Centrifuge the washed nuclei for 5-10 sec at $9000 \times g$, $4°C$, in a benchtop centrifuge and discard the supernatant.

20. Store the nuclei at $-80°C$ until further use.

    *The nuclei can be stored at $-80°C$ for at least 4 months.*

**BASIC PROTOCOL 2**

## FIRST CLICK REACTION, MNASE DIGESTION AND STREPTAVIDIN ENRICHMENT OF LABELED NUCLEOSOMES

In this second protocol we describe the first click reaction performed on isolated nucleosomes. During the incubation with AHA in Basic Protocol 1, newly synthesized proteins have incorporated the unnatural methionine analog. This molecule contains an azide moiety, which is employed in a click reaction to covalently link to a biotinylated alkyne. After the click reaction, the protocol proceeds with an MNase digestion of the nuclei to obtain mononucleosomes. It is important to optimize the MNase concentration per cell type and per cell density in order to avoid over digestion or under digestion (Fig. 2). After digestion, the mono-nucleosomes can diffuse out of the nuclei where they are captured in a streptavidin pull-down with magnetic beads, which enables enrichment of intact newly synthesized nucleosomes.

### Materials

Isolated nuclei (Basic Protocol 1)
Nuclei buffer (see recipe)
100 mM Alkyne-PEG-Biotin in Methanol (Merck, cat. no. 764213)
$CuSO_4$ (50 mM)
THPTA (Merck, cat. no. 762342)
Sodium ascorbate (100 mM)
MNase enzyme (New England Biolabs cat. no. M0247S) containing:
    MNase reaction buffer
Bovine serum albumin (BSA; 20 mg/ml) (New England Biolabs cat. no. B9000)
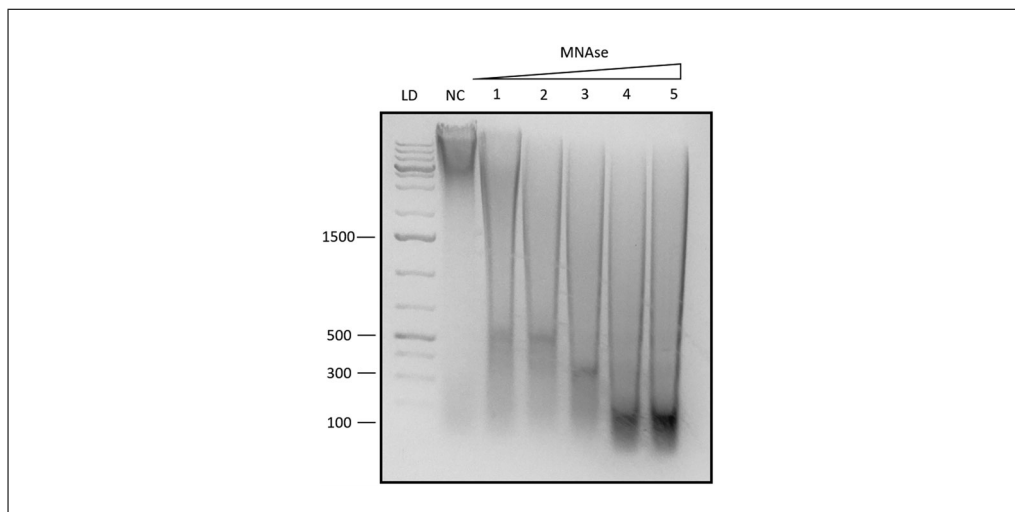    (provided with the MNase)

**Figure 2** Representative image of nucleosome digestion by the MNase enzyme. Increasing concentration of MNase results in different DNA fragments size. In lanes 1-2 are shown fragments of 450 bp corresponding to trinucleosomes. In lane 3, the fragments of 300 bp in size correspond to dinucleosome. In lanes 4-5 the fragments of 147-150 bp correspond to mononucleosome. Abbreviations: LD = ladder, NC = negative control, bp = base pairs.

0.5 M EDTA (Gibco, cat. no. 15575-038)
Wash buffer (see recipe)
Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen™, cat. no. 65001)
Phosphate-buffered saline (PBS; Gibco, cat. no. 10010056)
Proteinase K (Fisher Scientific, cat. no. 10181030)
NaCl (5 M)
Ampure XP beads (Beckman, cat. no. A63881)
Nuclease-free water
Generuler 1 kb+ marker (Thermo Scientific cat. no. SM1333)

End-over-end rotator (VWR, cat. no. 444-500)
1.5-ml centrifuge tubes (Sarstedt, cat. no. 72690001)
Heated water bath (Grant Sub 28 or similar)
Benchtop centrifuge (Eppendorf 5415R)
Magnet rack (Dynal MPC-S or similar)

### *First click reaction: Biotinylation of proteins metabolically labeled with AHA*

1. Resuspend the nuclei in 250 μl nuclei buffer.

2. Add 10 μl of 100 mM alkyne-PEG-biotin to click histones.

3. Pre-mix 5 μl 50 mM $CuSO_4$ with 5 μl 250 mM THPTA and 8 μl of this mix to the sample.

4. Add 10 μl freshly prepared 100 mM sodium ascorbate.

5. Place the nuclei in an end-over-end rotator for 30 min at room temperature.

6. Repeat the cycloaddition reaction with freshly prepared ingredients (steps 2 to 5).

    *There is no need to centrifuge your sample.*

7. Pellet the nuclei for 5-10 sec at 10,000 rpm.

    *In this step the excess of Alkyne-PEG-Biotin is being removed. An excess of biotin would interfere with the streptavidin pull-down.*

8. Discard the supernatant.

### MNase digestion to obtain mono-nucleosomes

9. Resuspend the nuclei in 100 μl MNase buffer + BSA (100 μg/ml final concentration) and warm in a water bath for to 37°C for about 5 min.

   *MNase is not active in PBS because of a salt concentration >100 mM.*

10. Add 2 μl MNase enzyme to 398 μl MNase buffer to obtain a concentration of 10 gel Units of enzyme per μl in MNase buffer. Store on ice.

    *Upon arrival, store MNase in 5-μl aliquots at −20°C to avoid freeze-thaw cycles.*

11. Add 60 μl of the diluted MNase to the nuclei and incubate for 5 to 10 min at 37°C.

12. Add 10 μl 100 mM EDTA and cool the sample on ice.

    *The MNase reaction is quenched by the addition of EDTA and cooling on ice.*

13. Add 100 μl wash buffer.

14. Spin down 5-10 sec at 10,000 rpm to pellet the nuclei and store the supernatant in a new 1.5-ml microtube. Discard the pellet.

    *The supernatant will contain the mono-nucleosomes, which diffuse out of the nuclei. The pellet contains nuclear debris.*

### First streptavidin pull-down: Enrichment of AHA labelled nucleosomes

15. Resuspend the tube containing the Dynabeads™ MyOne™ Streptavidin C1 for at least 30 s.

    *Make sure that the Dynabeads are well resuspended.*

16. Transfer 30 μl of the resuspended Dynabeads™ MyOne™ Streptavidin C1 to a new 1.5 ml centrifuge tube.

17. Add 1 ml PBS.

    *Dynabeads are washed with 1 ml PBS.*

18. Place the tube on a magnet rack for at least 1 min and discard the supernatant.

19. Remove the tube containing the beads from the magnet rack and resuspend the washed beads in 30 μl PBS.

20. Repeat the washings for a total of 3 washes (3 × 30 μl).

### Capturing and washing of biotinylated mononucleosomes and release of associated DNA

21. Add the mononucleosomes from step 14 to the Dynabeads™ MyOne™ Streptavidin C1 and incubate for 1 hr at room temperature in an end-over-end rotator.

22. Place the sample on a magnet for 3-4 min and remove the supernatant.

23. Wash the beads five times, each time with 200 μl wash buffer.

    *Incubate the sample for 1 min on the rotator during each wash.*

24. Add 100 μl PBS containing 1 mg/ml proteinase K and incubate for 1 hr at 50°C.

    *Pipet the mixture up and down every 15 min to ensure homogeneous mixing.*

    *Proteinase K is added to digest the captured histones and other proteins, while leaving the DNA intact.*

25. Add 100 μl 5 M NaCl and incubate for 15 min on an end-over-end rotator.

    *The Ampure beads used in the next step also contain high salt. The DNA is now compatible for purification with the Ampure beads.*

26. Transfer 200 µl of elution sample (containing DNA) into a new 1.5-ml tube.

27. Add 360 µl (1.8× sample volume) Ampure beads and purify the DNA according to the manual.

    *Warming of the Ampure beads at room temperature for at least 1 hr before use is important.*

28. Elute the sample in 80 µl nuclease-free water.

## REPLICATION-ENRICHED NUCLEOSOME SEQUENCING (RENS) PROTOCOL

This protocol describes all the steps necessary to obtain a library from parental DNA originating from newly synthesized histones.

### Materials

Sample: Double-stranded DNA (Basic Protocol 2)
100 mM Azide-PEG3-Biotin (in methanol) (Merck, cat. no. 762024)
50 mM CuSO$_4$
THPTA (Merck, cat. no. 762342)
Sodium Ascorbate (100 mM)
Ampure beads (Beckman)
Nuclease-free water
NEB next Ultra$^{TM}$ DNA library kit for Illumina® (New England Biolabs, cat. no. E7645)
Dynabeads$^{TM}$ MyOne$^{TM}$ Streptavidin T1 (C1 (Invitrogen$^{TM}$, cat. no. 65601)
2× B&W buffer (10 mM Tris·Cl, pH 7.5, 1 mM EDTA, 2 M NaCl) (see recipe)
1× B&W buffer (diluted 2× B&W buffer with an equal amount of water)
1× B&WT buffer (see recipe)
Alkaline buffer (see recipe)
Acetic acid
20 mM EDTA, pH 8.0 (Gibco)
NEBNext® multiplex oligos for Illumina® index primer set 1 or 2 (New England Biolabs, cat. no. E7335 or cat. no. E7500)
Qubit$^{TM}$ dsDNA high-sensitivity assay kit (Thermo Scientific, cat. no. Q32854)

End-over-end rotator
Magnet rack for 1.5-mltubes
1.5-ml centrifuge tubes
Vivaspin® 2 5000 Da MW cut-off centrifugal concentrator
Benchtop centrifuge
Swing bucket centrifuge for 15-ml centrifuge tubes
Thermal cycler
PCR tubes (strips or single tubes)
Vortex mixer
Qubit Fluorometer Tapestation System (Agilent)
High-Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)

### Second click reaction: Biotinylation of DNA metabolically labeled with EdU

1. Add 1 µl 100 mM Azide-PEG3-Biotin to the eluted DNA from the previous step.

    *DNA is labeled with EdU. In this step, the EdU-labeled DNA is clicked to the Azide-PEG4-Biotin.*

2. Pre-mix 5 µl of 50 mM CuSO$_4$ with 5 µl mM of 250 mM THPTA and add 8 µl of this mix to the sample.

3. Add 10 μl freshly prepared 100 mM sodium ascorbate to the sample.

4. Place the sample on an end-over-end rotator for 45 min at room temperature.

5. Add 0.7× sample volume of Ampure beads and incubate for 5 min at room temperature.

   *In this step, DNA fragments larger than 200 bp are discarded. The DNA wrapped on a mono-nucleosome is approximately 147 bp in size.*

   *It is advised to measure the exact sample volume with a pipette before adding the Ampure beads.*

6. Place the sample on a magnet rack and wait until the supernatant is clear.

   *It takes usually about 2 min until the sample is clear.*

7. Discard the beads and pipet the supernatant into a new 1.5-ml microcentrifuge tube.

8. Add 1.8× sample volume Ampure beads to the sample and perform the clean-up according to the manufacture's protocol.

9. Elute the sample in 55.5 μl nuclease-free water.

### End prep and adaptor ligation

10. Perform end prep and adaptor ligation according to the manual of the NEB next Ultra™ DNA library kit for Illumina®.

    *Use the protocol without performing the size selection*

11. Elute the DNA in 50 μl nuclease-free water.

12. Add 25 μl Dynabeads™ MyOne™ Streptavidin T1 into a 1.5-ml centrifuge tube.

13. Place on a magnet until the supernatant is clear.

14. Remove the supernatant.

15. Add 1 ml of 1× B&W buffer and resuspend the beads.

16. Place the tube on the magnet rack for about 1 min and discard the supernatant.

17. Repeat the washing steps for a total of three washes.

18. Add the sample obtained in step 11 to 25 μl washed Dynabeads™ MyOne™ Streptavidin T1 magnetic beads.

### Isolation of replicated double-stranded DNA fragments

19. Incubate the sample with the beads for 15 min at room temperature in an end-over-end rotator.

20. Place the tube with the beads on the magnet rack and wait until the supernatant is clear.

    *Spin down the tube shortly if necessary to collect all the beads from the side of the tube.*

21. Wash the beads four times, each time with 200 μl of 1× B&WT buffer.

    *Discard the supernatant after each wash step. Make sure to keep the beads.*

22. Wash the beads once with 200 μl of 2× concentrated B&W buffer.

### Isolation of replicated parental strands

23. Add 100 μl alkaline buffer to the beads and incubate for 1 min at room temperature to elute the parental strand.

*Addition of the alkaline buffer separates double-stranded DNA strands. In this case it results in biotinylated single-stranded DNA (ssDNA) to remain bound by the Dynabeads and the biotinylated ssDNA to enter into the solution.*

24. Place the tube with the beads on a magnet and wait until the supernatant is clear.

25. Collect the supernatant and pipet into a clean 1.5-ml centrifuge tube.

26. Repeat the elution for a total of three times. Collect the supernatant in the same tube. The final volume will be 300 μl.

27. Neutralize the combined sample to a final concentration of 0.1 M acetic acid and 2 mM EDTA pH = 8.0.

28. Dilute the sample to a final volume of 1 ml with nuclease-free water.

29. Add the sample to a Vivaspin® 2 (5 kDa cut-off) centrifugal concentrator and concentrate to approximately 20 μl final volume by centrifuging for 15 min at $4000 \times g$, room temperature, in a swinging bucket.

30. Perform a reversed spin according to the manufacturer's instruction. Centrifuge for 2 min at $3000 \times g$, room temperature, in a swinging bucket to collect the sample.

### Library amplification

31. Amplify libraries using the NEBNext Ultra DNA library prep kit for Illumina using 13 PCR cycles.

    *We used the NEB Next multiplex oligos for Illumina. Index primer set 1 or 2 can be used for this.*

    | | |
    |---|---|
    | Adapter ligated DNA Fragments | 15 μl |
    | NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 μl |
    | Index primer/i7 primer | 5 μl |
    | Universal PCR primer/i5 primer | 5 μl |
    | Total volume | 50 μl |

    | Cycle step | Temperature °C | Time | Cycles |
    |---|---|---|---|
    | Initial denaturation | 98°C | 30 sec | 1 |
    | Denaturation | 98°C | 10 sec | 13 |
    | Annealing/extension | 65°C | 75 sec | |
    | Final extension | 65°C | 5 min | 1 |
    | Hold | 4°C | Indefinitely | |

32. Purify the libraries according to the manual with $0.9 \times$ Ampure Beads (45 μl).

33. Elute the sample in 30 μl nuclease-free water.

34. Check the quantity and the quality of the DNA library using Qubit and Tapestation analysis.

    *It is advised to check the quantity of DNA by using a fluorometric method. In our experiments we used the Qubit assay for dsDNA. The TapeStation (Agilent) electropherogram can be used to determine the quality and the quantity of the sample, as well as to assess the degree of sample fragmentation.*

    *The samples are now ready for next-generation sequencing.*

## REAGENTS AND SOLUTIONS

### Alkaline buffer

0.1 M NaOH (Acros Organics, cat. no. 424330010)
0.05% (v/v) Tween 20 (Promega, cat. no. H5151)
Store up to 1 month at 4°C

**van der Wouden et al.**

### *Azidohomoalanine (AHA) medium*

DMEM high glucose, no glutamine, no methionine, no cysteine (Gibco, cat. no. 11570546)

10% Fetal bovine serum (FBS; Serana, cat. no. S-FBS-SA-0150)

Penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin/ml) (Gibco, cat. no. 11548876)

4 mM GlutaMAX (Gibco, cat. no. 35050-038)

0.2 mM ʟ Cystine 2HCl (Sigma-Aldrich, cat. no. C8755)

1 mM Sodium pyruvate (Gibco cat. no. 11360070)

0.2 mM AHA (Santa Cruz Biotechnology, cat. no. sc285947)

20 µM EdU (Bio-Connect, cat. no. CLK-N001)

Store up to 2 months at 4°C

### *B&W buffer, 2×*

10 mM Tris·Cl, pH 7.5 (Merck, cat. no. T5941)

1 mM EDTA (Fisher Scientific, cat. no. 15575-020)

2 M NaCl (Duchefa Biochemie, cat. no. S0520)

Store up to 1 month at 4°C

### *B&WT buffer, 1×*

5 mM Tris·Cl, pH 7.5 (Merck, cat. no. T5941)

0.5 mM EDTA (Fisher Scientific, cat. no. 15575-020)

1 M NaCl (Duchefa Biochemie, cat. no. S0520)

0.05% (v/v) Tween 20 (Promega, cat. no. H5151)

Store up to 1 month at 4°C

### *NP-40 in PBS with protease inhibitor, 0.1%*

Phosphate-buffered saline (PBS; Fisher Scientific, cat. no. 11540486)

0.1% (v/v) NP-40 (Sigma, cat. no. 74385)

Protease inhibitor cocktail (cOmplete™, EDTA-free Protease Inhibitor Cocktail; Roche, cat. no. 11873580001)

NP-40 in PBS can be stored up to 1 month at 4°C. Add Protease inhibitor cocktail just before use.

### *Nuclei buffer*

320 mM Sucrose (Duchefa Biochemie, cat. no. S0809.0025)

5 mM MgCl$_2$ (Merck, cat. no. 1.05833.1000)

10 mM HEPES, pH 7.4 (Duchefa Biochemie, cat. no. H1504)

1× cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche, cat. no. 11873580001)

Prepare a 50× concentrated stock solution of the Protease inhibitor cocktail (store at −20°C)

Add just before use

Store up to 1 month at 4°C

### *Standard medium*

DMEM, high glucose, pyruvate, GlutaMAX™ (Gibco, cat. no. 31966047)

10% Fetal bovine serum (FBS; Serana, cat. no. S-FBS-SA-0150)

1% Penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin/ml; Gibco, cat. no. 11548876)

Store up to 6 months at 4°C

### *Wash buffer*

Phosphate-buffered saline (PBS; Fisher Scientific, cat. no. 11540486)

0.1% (w/v) bovine serum albumin (BSA; VWR, cat. no. 1.12018.0100)

**van der Wouden et al.**

0.01% Tween 20 (Promega, cat. no. H5151)
Store up to 1 month at 4°C

## COMMENTARY

### Background Information

The advancement in the study of histone deposition during DNA replication has been achieved through the application of immunoprecipitation techniques on histone post-translational modifications (PTMs) like H4K20me2 and H4K5ac (Petryk et al., 2018; Yu et al., 2018). Research in mouse embryonic stem cells and budding yeast cells using labelling of new DNA with a thymidine analogue (EdU) in the presence of hydroxyurea (HU) showed a slight bias of new histone deposition on the lagging strand in mouse cells and leading strand in yeast cells. Several methods are currently considered state of the art in unravelling the deposition bias in histones in DNA replication.

The sequencing-based techniques, such as ChOR-seq, SCAR-seq, and eSPAN, enable the investigation of various chromatin components during replication in a time-sensitive and locus-specific manner. These methods use labeling of newly synthesized DNA strands with various nucleotide analogs (EdU, CldU, IdU), followed by chromatin immunoprecipitation (ChIP) of histones and DNA sequencing. By analyzing the distribution of labeled DNA strands in relation to histone modifications, the directionality and magnitude of histone deposition bias during DNA replication can be revealed. Specifically, these techniques have demonstrated that histones H3-H4 are symmetrically recycled to both daughter strands during replication. Replication factors with chaperone activity, including MCM2, POLE3/4, and POLA1, have been found to recycle H3-H4 to either the leading or the lagging strand (Petryk et al., 2018; Yu et al., 2018). In a recent study (Flury et al., 2023) the ChOR-seq and SCAR-seq methods were used to track the occupancy of H2AK119ub1, H2BK120ub1, and H2A.Z on newly replicated DNA in mouse embryonic stem cells. Based on these technologies it was found that the histones H2A-H2B are efficiently and accurately recycled during replication, through a distinct mechanism independent of H3-H4 recycling, involving the lagging strand polymerase POLA1. Furthermore, the H2A-H2B PTMs landscapes were found to be rapidly restored post-replication, with H2AK119ub1 guiding the timely and accurate restoration of H3K27me3, which plays an important role in propagating the epigenome.

Other advanced techniques are available to analyze the epigenome landscape, including labeling DNA with nucleotide analogues in pulse-chase experiments, followed by CHIP-seq of histones and DNA sequencing; amino acid labeling in cell culture using isotopes couple with mass spectrometry (pSILAC) (Nakamura et al., 2022), and DNA labeling and isolation by 5-ethynyl-deoxyuridine for mass spectrometry (iDEMS) (Stewart-Morgan et al., 2023). In addition to histone deposition bias, these methods reveal details about the regulation of PTMs during replication, such as the dynamics of H3K56 acetylation, H4K20 methylation, and hydroxymethylation.

Generally the methods described above all rely on histone PTMs for separation of parental versus newly synthesized histones. Application of a metabolic labelling provides a complementary method that does not rely on histone PTMs but provides an unambiguous labelling of newly synthesized proteins. Concomitantly, the metabolic labelling method does not unambiguously allow for the isolation of only histones and might also include other tightly associated chromatin proteins. Ideally, metabolic labelling methods can be combined with a histone chromatin immunoprecipitation followed by separation of the parental and novel DNA strand by EdU labelling. However, such a triple-separation protocol would not deliver sufficient sample material for amplification and sequencing with the current state of technology.

The double-click-seq protocol recently developed by us (Zwinderman et al., 2021) is a complementary method that enriches parental DNA from new histones. It uses stable labelling of newly synthesized histones through co-translational incorporation of azidohomoalanine (AHA). A copper catalyzed reaction, known as the "click" reaction, is used to attach a biotin affinity tag to the labelled histones. This allows for enrichment of nucleosomes containing new histones for analysis. This protocol provides a novel approach for understanding the distribution of parental versus new histones on the leading and lagging strands in DNA replication, without the risk of ambiguous results due to the dynamic nature of PTMs.

**van der Wouden et al.**

Current Protocols

It is important to consider the potential limitations and caveats of using azidohomoalanine (AHA) or ethynyluridine as labeling methods in the study of histone deposition during DNA replication.

AHA and ethynyluridine are analogs that are incorporated into newly synthesized proteins or DNA, respectively, during replication. However, these analogs may not perfectly mimic the native molecules, and their incorporation could potentially disrupt normal biological interactions. For example, AHA incorporation could affect protein folding, stability, or interactions with other cellular components, which may in turn affect histone deposition or other cellular processes. The "click" reaction used in the AHA labeling protocol involves copper-catalyzed chemistry, which may have potential limitations. Copper ions can generate reactive oxygen species that could potentially cause oxidative damage to cellular components, including DNA and proteins. Additionally, copper ions can also interact with other cellular components, potentially leading to off-target effects or interference with normal cellular processes.

The AHA and ethynyluridine labeling methods may require specialized techniques and expertise for their implementation, as such it is important to be aware of their potential limitations and consider appropriate controls and validation measures to ensure the accuracy and reliability of the results.

## Critical Parameters

### Cell culture conditions

The exact number of cells needed for optimal amounts of DNA to use for library amplification and sequencing steps will depend on several factors, such as the type of sequencing technology, the complexity of the genome, and the desired depth of sequencing. As a general rule, most next-generation sequencing platforms require between 1 and 10 nanograms (ng) of DNA for library preparation. However, this amount can vary widely depending on the platform and the sample type.

To obtain sufficient amounts of DNA for library amplification and sequencing, it is recommended to start with a concentration of cells above 20 million, although this can vary depending on the type of cell and the experimental conditions. It is important to note that the yield and quality of DNA obtained from cells will depend on the extraction method used, the age and health of the cells, and the presence of contaminants in the samples. It is important to routinely test cell cultures once a month for mycoplasma contamination to ensure that samples are suitable for use in experiments.

To ensure optimal yields and quality of DNA, it is recommended to culture cells under appropriate conditions. For example, cells should be dividing at the time of harvesting, as this ensures that DNA replication has occurred, and there is sufficient genomic material for extraction. Additionally, the confluency of the cells in culture should not exceed 70%, as overcrowding can lead to stress and changes in gene expression that may affect the yield and quality of DNA. Finally, the cell's culture medium should contain 3% to 5% methionine to allow proper cell growth and AHA incorporation.

### MNase enzyme

The concentration of the MNase enzyme should be optimized according to the number of cells seeded. The MNase should be promptly aliquoted upon arrival to avoid freeze-thaw cycles.

To ensure that the amount of MNase is optimal, we recommend performing a titration experiment to test the range of enzyme concentrations to determine the optimal amount required for efficient chromatin digestion. In the titration experiment, fixed chromatin samples must be incubated with different concentrations of MNase (e.g., 0.5U, 1U, 2U, 5U and 10U of enzyme per microgram of chromatin) and then check the chromatin digestion efficiency by running the samples on a 2% agarose gel. The optimal concentration of MNase is the one that generates a chromatin digestion pattern with a range of DNA fragments from mononucleosomes to higher-order oligonucleosomes, without over-digestion or under-digestion. Over-digestion may lead to the loss of some regions of interest, while under-digestion may result in incomplete chromatin fragmentation.

### DNA measurements

To determine the amount of DNA obtained for sequencing and downstream analysis it is recommended to measure the absorbance of the DNA samples at 260 nm by using a spectrophotometer or a Qubit fluorometer (ThermoFisher Scientific).

The absorbance value or the fluorescence signal emitted by the samples is then used to calculate the concentration of DNA. One advantage of using the fluorometry method over traditional spectrophotometric methods is the ability to selectively detect only the

target molecule (DNA in this case) and no other contaminants, such as salts or organic compounds that may be present in the sample. For the click-seq protocol a minimum of 5-10 ng/µl of DNA is required for sequencing applications.

The TapeStation (Agilent) electropherogram can be used to determine the quality and the quantity of the sample, as well as to assess the degree of sample fragmentation, identify the presence of contaminants, and confirm the success of a particular step in the library preparation for sequencing. The electropherogram displays peaks representing the DNA fragments of a specific size, the height of each peak represents the relative concentration of the corresponding fragment. Multiple peaks can be indicative of different issues including: adapter dimers due to insufficient removal of unligated adaptor (peak at 60 bp), insufficient fragmentation, overloaded sample and degraded DNA.

Adapter dimers can be removed by using gel purification, while to improve fragmentation it is possible to increase the MNase shearing time and or conditions. To prevent DNA degradation it is recommended to store the samples at −20°C and to use fresh reagents. Multiple peaks can also be indicative of low library complexity, which can result from inefficient adapter ligation or low input DNA. To improve library complexity, it is recommended to optimize the adapter ligation conditions and increasing the amount of input DNA.

## Troubleshooting

The frequently encountered problems and their possible causes and solutions are listed in Table 1.

## Understanding Results

### MNase enzyme

MNase (Micrococcal Nuclease) is an enzyme commonly used in chromatin research to digest chromatin into mono-nucleosomes, di-nucleosomes, and higher-order nucleosome fragments. It cleaves the linker DNA regions between nucleosomes, resulting in characteristic banding patterns on agarose gels that can be used to assess the efficiency of chromatin digestion. In this protocol, the optimal concentration of MNase enzyme was determined by testing different concentrations and analyzing the resulting nucleosome fragments on an agarose gel. Figure 2 shows a representative example of the gel with increasing concentrations of MNase enzyme and the corresponding banding pattern. Mono-nucleosomes (150 bp), di-nucleosomes (300 bp), and tri-nucleosomes (450 bp) bands are clearly observed. The concentration of MNase enzyme that provides the highest yield of mono-nucleosomes is considered optimal for further experiments (Fig. 2, lane 5).

In our experiments we achieved a final concentration of DNA between 5 and 100 ng/µl measured by Qubit in a total volume of 30 µl. After DNA quality control analysis that was performed with the TapeStation system (Agilent) a peak of approximately 320 bp was observed (Fig. 3). The expected peak size is around 270 bp (150 bp DNA from mononucleosomes and two adapters, which are each 60 bp in size). The higher average peak size of 320 bp can be explained by the fact that the DNA wrapped around the histones is flanked by linker DNA.

**Table 1** Troubleshooting

| Problem | Possible cause | Solution |
|---|---|---|
| Low DNA yield | Insufficient amount of starting material | Increase amount of cells. Start with ≥12 million cells per experiment. |
| | Incomplete MNase digestion | Optimize MNase digestion/check activity of MNase |
| | Dried/temperature Ampure Beads | Before elution Ampure Beads should not be overdried. The beads should be at room temperature before using. |
| Multiple peaks at Tapestation | Insufficient removal of unligated adaptors (peak at 60 bp) | Perform an extra clean-up step using Ampure Beads Use recommended amount of DNA input in library preparation |
| | Bubble product (extra peak at the double size as expected bp) | Libraries with a bubble product can be sequenced without any problem |
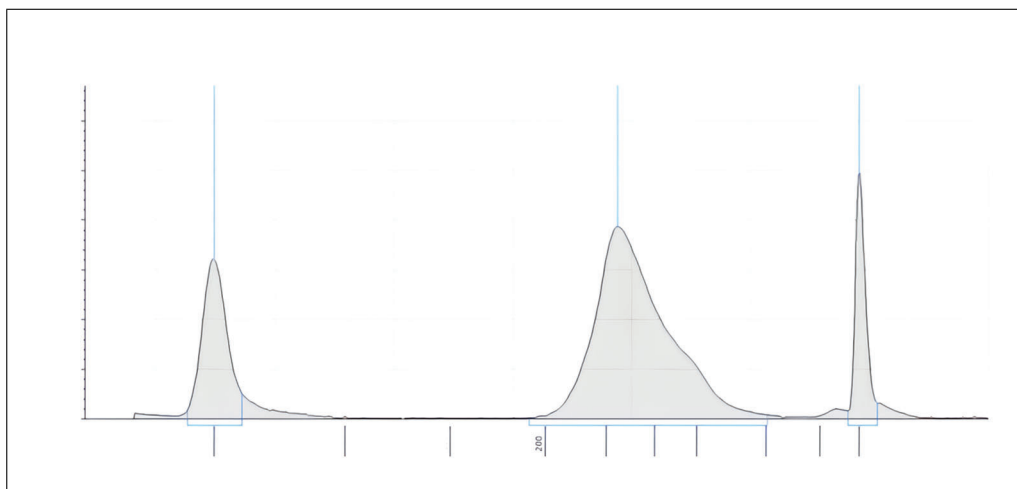
**Figure 3** Representative image of the TapeStation electropherogram for DNA peaks analysis. Following the steps of adaptor ligation and cDNA library amplification, the average DNA size is checked.

## Time Considerations

The complete double-click seq protocol, from cell to DNA libraries can be estimated to take 5-6 days. It is advised to have all the buffers and reagents prepared before the start of the protocols. Basic Protocol 1: the seeding, treatment, and harvesting of the cells may take 3 days up to a few weeks in order to obtain the optimal cell amount of 20 million. It is important to note that this is an estimate, and the actual time required may vary depending on the specific experimental condition. It is always recommended to monitor cell growth carefully, and to ensure that cells are healthy before harvesting. For example, some cell lines may double in number every 24 hr under optimal condition, while others may take several days or longer to reach the same number of cells. The amount of time it takes to reach 1 million cells can vary widely depending on several factors, including the type of cell, the growth condition, and the start density. The preparation of nuclei can be performed in as little time as 15 min. At this point it is safe to store the cell pellets and/or nuclei in the freezer at −80°C until further processing. It is possible to perform Basic Protocol 2 (5 hr) and Basic Protocol 3 (3 hr) without stopping. However, if necessary, it is possible to incorporate a stop after Basic Protocol 2. The sample can be stored safely overnight at −20°C. Basic Protocol 2 and Basic Protocol 3 can be performed in approximately 8 hr, although the time needed is dependent on the amount of samples that are being processed.

## Author Contributions

Petra van der Wouden did the: formal analysis, investigation, methodology development, validation, visualization and writing of the original draft. Martijn Zwinderman did the: conceptualization, formal analysis, investigation, methodology development, validation and visualization. Deng Chen did the: formal analysis, investigation, methodology development, validation and visualization. Michaela Borghesan did the: writing, review & editing. Frank Dekker did: conceptualization, funding acquisition, project administration, supervision, writing, review & editing.

## Conflict of Interest

The authors declare no conflict of interest. The funding sources had no involvement in collection, analysis and interpretation of the data.

## Data Availability Statement

As described in the main paper on the development of the double-click seq method (cite: ACS Chem. Biol. 2021, 16, 2193−2201) the data sets generated during this study are available at EBI ArrayExpress under accession number E-MTAB-8624. The code generated during this study is available at GitHub; *https://github.com/thamarlobo/histone_ deposition_analysis*. Further permission

related to the material excerpted should be directed to the ACS.

## Literature Cited

Alabert, C., Barth, T. K., Reverón-Gómez, N., Sidoli, S., Schmidt, A., Jensen, O. N., Imhof, A., & Groth, A. (2015). Two distinct modes for propagation of histone PTMs across the cell cycle. *Genes & Development*, *29*(6), 585–590. https://doi.org/10.1101/gad.256354.114

Allis, C. D., & Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. In *Nature reviews genetics* (Vol. 17, Issue 8, pp. 487–500). Nature Publishing Group. https://doi.org/10.1038/nrg.2016.59

Audergon, P. N. C. B., Catania, S., Kagansky, A., Tong, P., Shukla, M., Pidoux, A. L., & Allshire, R. C. (2015). Restricted epigenetic inheritance of H3K9 methylation. *Science*, *348*(6230), 132–135. https://doi.org/10.1126/science.1260638

Escobar, T. M., Oksuz, O., Saldaña-Meyer, R., Descostes, N., Bonasio, R., & Reinberg, D. (2019). Active and repressed chromatin domains exhibit distinct nucleosome segregation during DNA replication. *Cell*, *179*(4), 953–963.e11. https://doi.org/10.1016/j.cell.2019.10.009

Flury, V., Reveron-Gomez, N., Alcaraz, Stewart-Morgan, K. R., Wenger, A., Klose, R. J., & Groth, A. (2023). Recycling of modified H2A-HEB provides short-term memory of chromatin states. *Cell*, *186*, 1050–1065. https://doi.org/10.1016/j.cell.2023.01.007

Nakamura, K., Groth, A., & Alabert, C. (2022). Investigating mitotic inheritance of histone post-translational modifications by triple pSILAC coupled to nascent chromatin capture. *Methods in Molecular Biology*, *2529*, 407–417. https://doi.org/10.1007/978-1-0716-2481-4_17

Petryk, N., Dalby, M., Wenger, A., Stromme, C. B., Strandsby, A., Andersson, R., & Groth, A. (2018). MCM2 promotes symmetric inheritance of modified histones during DNA replication. *Science*, *361*(6409), 1389–1392. https://doi.org/10.1126/science.aau0294

Ragunathan, K., Jih, G., & Moazed, D. (2015). Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science*, *348*(6230), https://doi.org/10.1126/science.1258699

Reverón-Gómez, N., González-Aguilera, C., Stewart-Morgan, K. R., Petryk, N., Flury, V., Graziano, S., Johansen, J. V., Jakobsen, J. S., Alabert, C., & Groth, A. (2018). Accurate recycling of parental histones reproduces the histone modification landscape during DNA replication. *Molecular Cell*, *72*(2), 239–249.e5. https://doi.org/10.1016/j.molcel.2018.08.010

Kolb, H. C., Finn, M. G., & Sharpless, K. B. (2001). Click chemistry: Diverse chemical function from a few good reactions. *Angewandte Chemie (International ed in English)*, *40*(11), 2004–2021. https://doi.org/10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5

Stewart-Morgan, K. R., Requena, E. C., Flury, V., Du, Q., Heckhausen, Z., Hajkova, P., & Groth, A. (2023). Quantifying propagation of DNA methylation and hydroxymethylation with iDEMS. *Nature Cell Biology*, *25*(1), 183–193.

Yu, C., Gan, H., Serra-Cardona, A., Zhang, L., Gan, S., Sharma, S., Johansson, E., Chabes, A., Xu, R.-M., & Zhang, Z. (2018). A mechanism for preventing asymmetric histone segregation onto replicating DNA strands. *Science*, *361*, 1386–1389. https://www.science.org

Zwinderman, M. R. H., Jessurun Lobo, T., van der Wouden, P. E., Spierings, D. C. J., van Vugt, M. A. T. M., Lansdorp, P. M., Guryev, V., & Dekker, F. J. (2021). Deposition bias of chromatin proteins inverts under DNA replication stress conditions. *ACS Chemical Biology*, *16*(11), 2193–2201. https://doi.org/10.1021/acschembio.1c00321

## Key References

Petryk, N., Dalby, M., Wenger, A., Stromme, C. B., Strandsby, A., Andersson, R., & Groth, A. (2018). See above.

*Study on histone deposition biased using antibody based methods in eukaryotic cells.*

Yu, C., Gan, H., Serra-Cardona, A., Zhang, L., Gan, S., Sharma, S., Johansson, E., Chabes, A., Xu, R.-M., & Zhang, Z. (2018). See above.

*Study on histone deposition biased using antibody based histone separation in yeast.*

Zwinderman, M. R. H., Jessurun Lobo, T., van der Wouden, P. E., Spierings, D. C. J., van Vugt, M. A. T. M., Lansdorp, P. M., Guryev, V., & Dekker, F. J. (2021). Deposition bias of chromatin proteins inverts under DNA replication stress conditions. *ACS Chemical Biology*, *16*(11), 2193–2201. See above.

*Original publication of the double-click seq method in which this protocol is used.*