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Bringing Epigenetics to Life

CUTANA[™]

Fiber-seq Kit Version 1

User Manual Version 1.0



CUTANA™

Fiber-seq Kit

Kit Version 1

Catalog No. 14-2001-8rxn: 8 Reactions

Catalog No. 14-2001-24rxn: 24 Reactions

**Upon receipt, store indicated components
at 4°C, -20°C, -80°C, and room temperature (RT)**

Stable for 6 months upon date of receipt.
See p. 7 for storage instructions.

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See EpiCypher's Tech Support Center at support.epicypher.com for additional FAQs and troubleshooting guidance.

Introduction

CUTANA™ Fiber-seq is a multiomic long-read assay for the simultaneous analysis of chromatin accessibility, DNA methylation (DNAm), protein footprinting, and genetic variants¹ (Figure 1). The Fiber-seq workflow leverages CUTANA™ Hia5, a nonspecific N⁶-methyladenine (6mA) methyltransferase to selectively methylate adenines within regions of accessible chromatin. This 6mA base, alongside 5-methylcytosine methylation (5mC), is directly readable by long-read sequencing (LRS) platforms, enabling simultaneous detection of chromatin accessibility and endogenous methylation on single DNA molecules. Features of CUTANA™ Fiber-seq include:

- **Multiomic insights from one assay** (Figure 2). Fiber-seq simultaneously profiles chromatin accessibility, DNA methylation, and genetic variants from the same sample.
- **Single-molecule protein footprinting without requiring antibodies.** Accessibility labeling is highly sensitive and captures nucleosome and protein footprints at near-basepair resolution without antibodies.
- **Unlocking hidden biology in hard-to-map regions.** Fiber-seq enables chromatin profiling of regulatory elements in repetitive or complex genomic regions that are inaccessible with short-read methods.
- **Seamless integration with existing long-read sequencing workflows.** Hia5-treated DNA is fully compatible with direct or native DNA sequencing protocols using either Pacific Biosciences® (PacBio®) HiFi or Oxford Nanopore Technologies® (ONT®) Nanopore sequencing platforms, enabling users to add epigenomic insight without retooling their workflows.
- **Reference building and decoding gene regulation all in one assay.** Fiber-seq allows you to capture chromatin features at the same time you're constructing your genome assembly, enabling high-resolution, reference-free insights into gene regulation in emerging model systems or for precision medicine applications.

Visit epicypher.com/products/fiber-seq for more resources on Fiber-seq and other LRS assays under development.

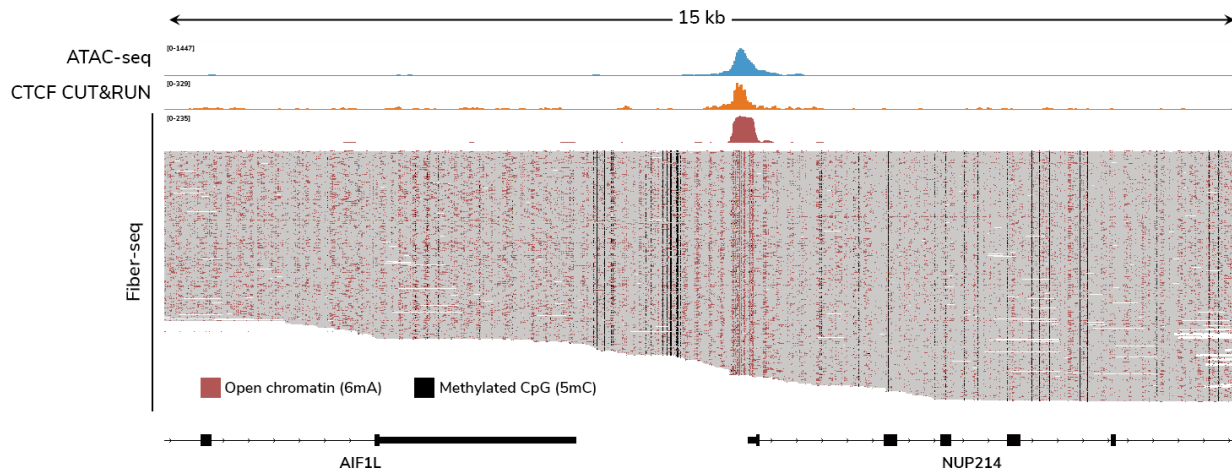


FIGURE 1

CUTANA™ Fiber-seq simultaneously detects open chromatin (marked by N⁶-methyladenine; 6mA) and DNA methylation (5mC) in human leukemia (K562) cells. Representative region compares these results with ATAC-seq and CTCF CUT&RUN data. CTCF is a DNA-binding protein that regulates chromatin structure and is often enriched at open chromatin regions. Enriched 6mA and low 5mC labeling revealed by Fiber-seq are seen compared with ATAC-seq and CTCF peaks. Each horizontal line in the Fiber-seq panel represents a single long read, providing single-DNA molecule data from 60 individual chromatin fibers.

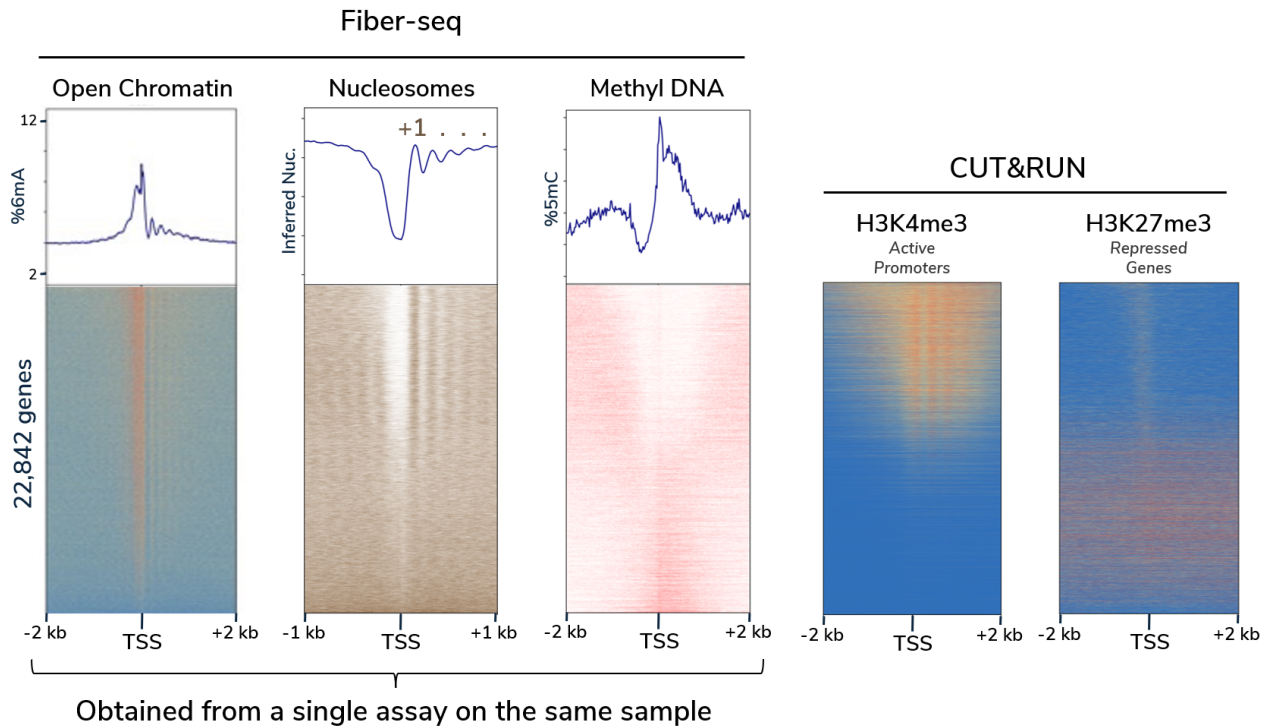


FIGURE 2

Fiber-seq reveals multiomic insights concordant with genomic features obtained from short-read sequencing approaches (CUT&RUN). Heatmaps show aligned single-molecule data for 6mA signal, endogenous 5mC signal, and inferred nucleosome positioning centered at transcription start sites (TSSs). TSSs enriched for 6mA display well-defined nucleosome organization and a depletion of 5mC, consistent with active promoter regions. These patterns align with CUT&RUN data for histone modifications, including H3K4me3 (active TSS) and H3K27me3 (repressed genes) performed in the same cell type (K562). The +1 annotation in the inferred nucleosome positioning refers to the first nucleosome downstream of an active TSS.

Outline of Workflow

Here, we review the main steps of the CUTANA™ Fiber-seq workflow (Figure 3).

- Intact nuclei are extracted and permeabilized.
- Hia5 and cofactor S-adenosylmethionine (SAM) are added to methylate accessible adenines on genomic DNA, creating 6mA at accessible regions.
- Genomic DNA is extracted and prepared for sequencing on LRS platforms.
- DNA is sequenced and analyzed to acquire information on both endogenous DNA methylation (5mC) and accessible chromatin regions marked by 6mA.

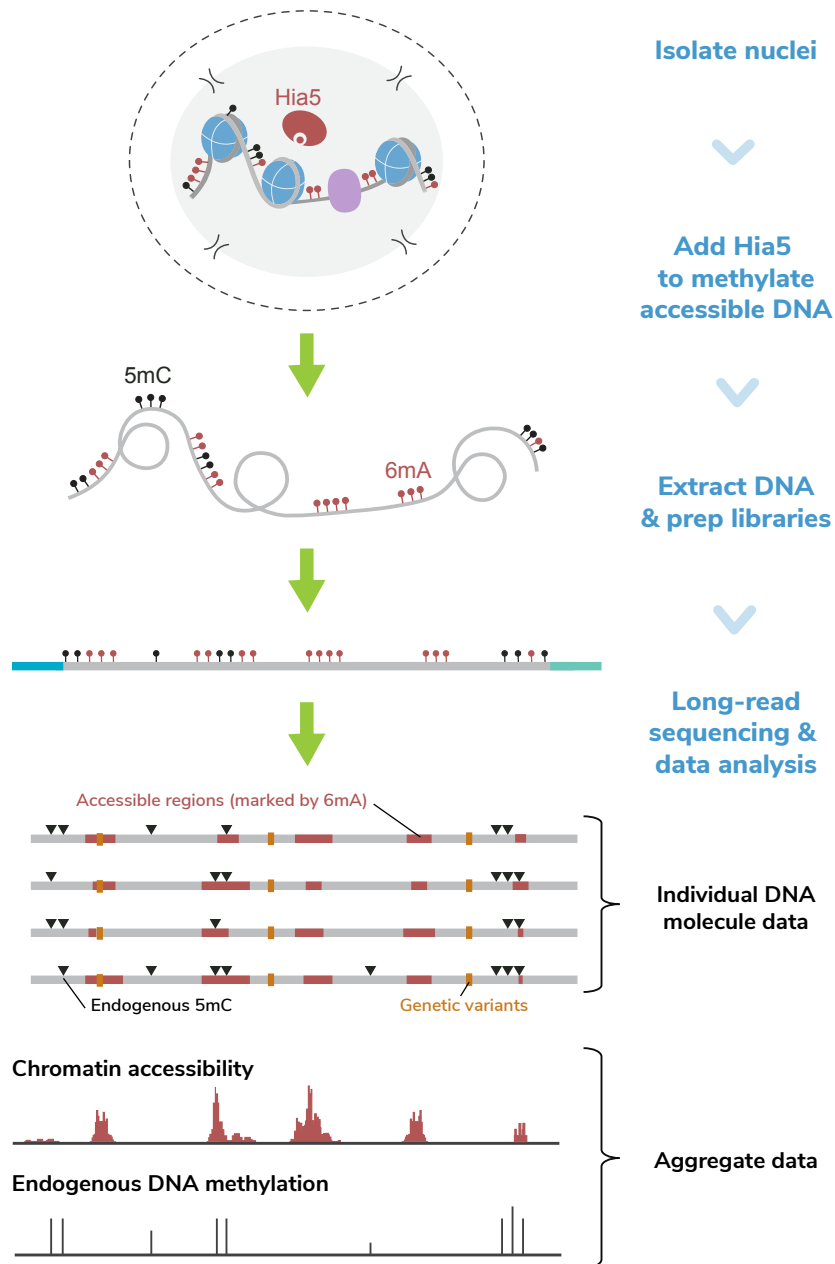


FIGURE 3

Overview of the CUTANA™ Fiber-seq workflow.

Included in the Kit

Kit components are stable for 6 months upon date of receipt. Store as outlined below.

Store at room temperature (RT) upon receipt:

Item	In 14-2001-8rxn	In 14-2001-24rxn	Notes before use
gDNA Spin Columns	10-0016-09	10-0016-10	Use for genomic DNA purification after Fiber-seq reactions.
gDNA Collection Tubes	10-0017-09	10-0017-10	
gDNA Cell Lysis Buffer	21-1029-09	21-1029-10	
gDNA Binding Buffer	21-1030-09	21-1030-10	
gDNA Wash Buffer	21-1031-09	21-1031-10	BEFORE FIRST USE: Add ethanol to bottle. For Kit 14-2001-8rxn, add 7 mL ethanol. For Kit 14-2001-24rxn, add 21 mL ethanol.
gDNA Elution Buffer	21-1032-09	21-1032-10	Use to elute genomic DNA.

Store at 4°C upon receipt:

Item	In 14-2001-8rxn	In 14-2001-24rxn	Notes before use
Pre-1X Reaction Buffer	21-1033-09	21-1033-10	Use to prepare 1X Reaction Buffer.
Pre-Nuclei Extraction Buffer	21-1021-09	21-1021-10	Use to prepare Nuclei Extraction Buffer.

Store at -20°C upon receipt:

Item	In 14-2001-8rxn	In 14-2001-24rxn	Notes before use
1 M Spermidine	21-1005-09	21-1005-10	Use to prepare Nuclei Extraction Buffer and 1X Reaction Buffer.
SAM	21-1034-09	21-1034-10	SMALL VOLUME: Quick spin before use. 32 mM stock. Use in Hia5 reactions. NOTE: SAM is a highly labile reagent and prone to degradation with repeated freeze-thaw cycles. Make single use aliquots.
Proteinase K	15-1033-09	15-1033-10	SMALL VOLUME: Quick spin before use. Use to remove protein during genomic DNA extraction.
RNase A	15-1034-09	15-1034-10	Use to remove RNA during genomic DNA extraction.

Store at -80°C upon receipt:

Item	In 14-2001-8rxn	In 14-2001-24rxn	Notes before use
Hia5 for Fiber-seq	15-1032-09	15-1032-10	SMALL VOLUME: Quick spin before use. Use for 6mA labeling in regions of accessible chromatin in Fiber-seq.

Materials Required but Not Supplied

REAGENTS:

- 1X PBS (free of calcium and magnesium), any vendor
- SDS, any vendor
- Molecular biology grade water, any vendor
- 0.4% Trypan Blue, any vendor

RECOMMENDED LIBRARY PREP REAGENTS:

For PacBio HiFi platform:

- PacBio SMRTbell Library Prep Kit (e.g., SMRTbell prep kit 3.0)

For ONT Nanopore platform:

- ONT Ligation Sequencing Kit (e.g., Ligation Sequencing Kit V14)

EQUIPMENT:

- 1.5, 15, and 50 mL tubes
- Low-retention filter pipette tips
- Automated cell counter or brightfield / phase contrast microscope
- Cell counting slides
- Vortex (e.g., Vortex-Genie[®] 2, Scientific Industries SI-0236)
- Heatblock or thermomixer
- Benchtop centrifuge/mini-centrifuge (e.g., from Fisher Scientific)
- Thermocycler with heated lid (e.g., from BioRad, Applied Biosystems, Eppendorf)
- Qubit[™] 4 Fluorometer (or previous version) and 1X dsDNA HS Kit (Invitrogen Q33230)
- Capillary electrophoresis machine and required reagents for DNA fragment size analysis, e.g., Agilent TapeStation[®] with Genomic DNA ScreenTape (5067-5365) and Genomic DNA Reagents (5067-5366).

SAMPLE INPUTS FOR FIBER-SEQ

- Freshly isolated, unfixed (i.e., native) cells are the preferred input for Fiber-seq.
- 2,000,000 cells per reaction is recommended to achieve the recommended 1,000,000 nuclei required per reaction. Harvest at least 10% excess cells if possible to account for sample loss and allow for quality control checks.
- The Fiber-seq protocol is optimized for native human nuclei. If using cross-linked samples, frozen nuclei/cells, or other organisms, visit support.epicypher.com for a list of other compatible sample types.

EXPERIMENTAL CONTROLS & SUCCESS METRICS

- Use fibertools and fiberseq-qc (github.com/fiberseq) for Fiber-seq data analysis.
- Optimized Fiber-seq conditions should reveal high resolution nucleosome footprinting patterns from labeling linker DNA (Figure 4).
- For human samples, the average nucleosome footprint should be 147 base pairs. The 6% 6mA labeling is recommended as it is ideal to generate nucleosome footprints of this length with 1,000,000 human nuclei when performing Fiber-seq as described in this protocol.

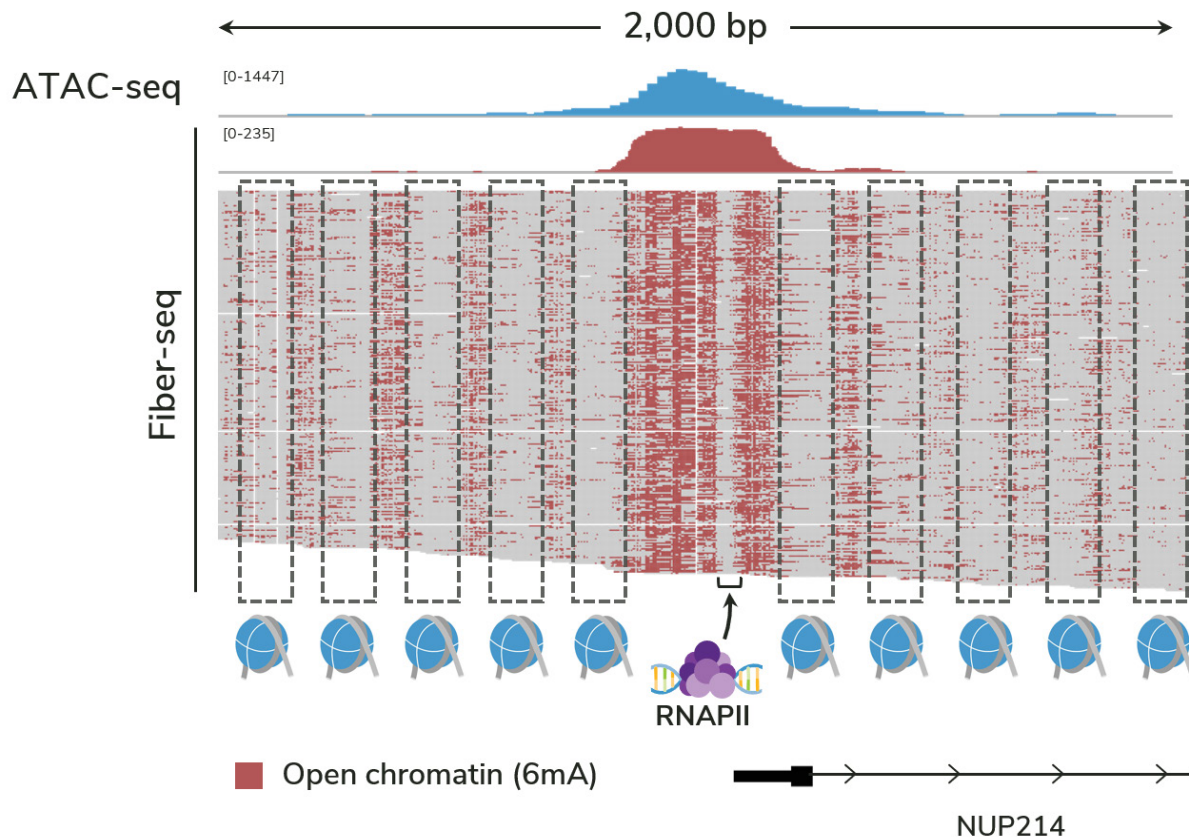


FIGURE 4

Example Fiber-seq data analysis using fibertools. Phased nucleosome footprints around 147 bp are revealed by Fiber-seq flanking a gene promoter. Sub-nucleosomal footprint at the nucleosome-free region represents engagement by chromatin interacting proteins, such as RNA Polymerase II (RNAPII).

SECTION I: FIBER-SEQ BUFFER PREP (~30 MIN)

IMPORTANT NOTES ON BUFFER PREP

- * Buffers are prepared **FRESH** for every Fiber-seq experiment.
- * Volumes in [Table 1](#) are per Fiber-seq reaction and include 20% excess to account for pipetting errors. You do **NOT** need to add additional volume.

1. Gather kit reagents stored at 4°C, -20°C, and -80°C needed for Day 1: **Pre-1X Reaction Buffer, Pre-Nuclei Extraction Buffer, Spermidine, SAM, Proteinase K, RNase A, and Hia5 for Fiber-seq**. Place on ice to thaw or equilibrate. Note that protease inhibitors are not used in this Fiber-seq protocol.
2. Prepare **Nuclei Extraction Buffer** by combining Pre-Nuclei Extraction Buffer and 1 M Spermidine as outlined in [Table 1](#). Place on ice.
3. To a new tube labeled **1X Reaction Buffer**, add Pre-1X Reaction Buffer and 1 M Spermidine as outlined in [Table 1](#). Place on ice.

Buffer Sample Scaling Calculations:

COMPONENT	[FINAL]	1rxn	4rxn	8rxn
Nuclei Extraction Buffer - store on ice for use				
Pre-Nuclei Extraction Buffer	-	240 µL	960 µL	1.92 mL
1 M Spermidine	0.5 mM	0.12 µL	0.48 µL	0.96 µL
1X Reaction Buffer - store on ice for use				
Pre-1X Reaction Buffer	-	210 µL	840 µL	1.68 mL
1 M Spermidine	0.5 mM	0.11 µL	0.44 µL	0.88 µL

TABLE 1

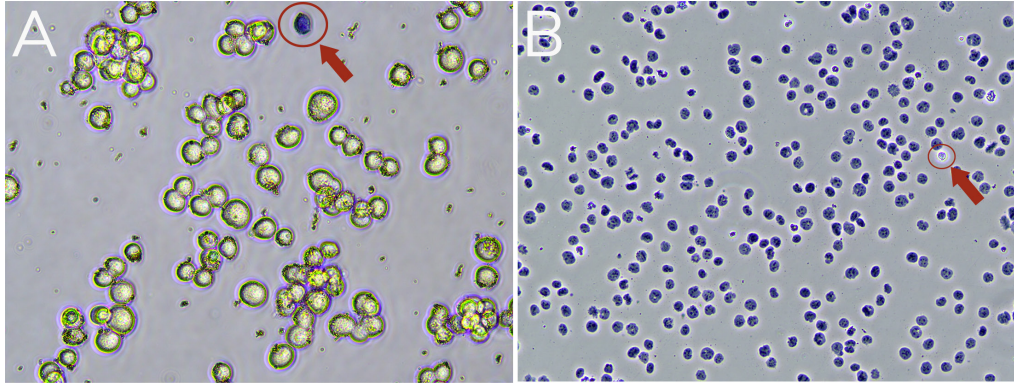
Combine reagents as instructed in the table to prepare Fiber-seq Buffers. Calculations for 4 and 8 reactions are provided. **All buffers include 20% extra volume to account for pipetting error - no additional overage is needed.**

SECTION II: NUCLEI EXTRACTION (~30 MIN)

FIBER-SEQ SAMPLE PREP NOTES

- * **High-quality sample prep is essential to Fiber-seq.** This Fiber-seq protocol is optimized for fresh, native human suspension cells. For adherent cells, cross-linked, frozen, or other sample types, further optimization may be required. Visit support.epicypher.com for current recommendations.
- * Ideal cell viability may vary by sample type, treatments, or processing conditions. The goal is to harvest cells with good integrity and minimal lysis.
- * We recommend sample quality control checks for every Fiber-seq experiment. If nuclei are of poor quality, DNA yields may be reduced and Fiber-seq data quality may be affected.

4. Collect starting cells (in tissue culture flask, tube, etc.) by spinning at 600 x g for 3 min at room temperature (RT). Remove supernatant, flick tube to loosen cell pellet, and resuspend in 1-2 mL 1X PBS. Count and confirm cell integrity as follows:
 - A. Transfer 10 μ L cells to a fresh tube.
 - B. Add 10 μ L 0.4% Trypan Blue. Pipette 3 times to mix.
 - C. Transfer 10 μ L of the cell-Trypan Blue mixture to a cell counting slide. Obtain cell counts, determine viability, and confirm expected cellular morphology using a brightfield / phase microscope or cell counter. See [Figure 5A](#) (p. 12).
5. Transfer 2,000,000 starting cells per reaction (plus 10% excess if possible) to a 15 mL tube. This ensures having enough cells to meet the required nuclei counts at ~1,000,000 per reaction.
6. Spin cells at 600 x g for 3 min at room temperature (RT). Remove supernatant.
7. Resuspend cells in 0.5 mL 1X PBS and transfer to a 1.5 mL tube.
8. Spin cells at 600 x g for 3 min at RT. Remove supernatant and gently resuspend cells in 200 μ L per reaction cold **Nuclei Extraction Buffer**.
9. Incubate for 10 min on ice.
10. Spin 600 x g for 3 min at 4°C. The pellet should change from pale yellow (cells) to a white, fluffy pellet (nuclei). Pipette to remove supernatant.
11. Resuspend nuclei in 75 μ L per reaction cold **1X Reaction Buffer**.
12. Take 10 μ L nuclei and perform Trypan Blue staining as in step 4. Obtain nuclei counts and confirm nuclei integrity. Nuclei should be >95% Trypan Blue positive, unclumped, and show minimal lysis. See [Figure 5B](#) for expected results.



Sample	Success Metrics	Troubleshooting Tips
Cells Figure 5A	Cells should be bright white (Trypan Blue excluded), round, unclumped, and ideally show >80% viability. Excess dead cells may reduce DNA yields and impact Fiber-seq data quality.	Optimize cell culture conditions; use fresh media, evaluate potential contamination issues.
Nuclei Figure 5B	Nuclei should be >95% Trypan Blue positive and unclumped.	Monitor cells during nuclei extraction by Trypan Blue staining to optimize incubation time. Increase spin time if you are losing nuclei during spins (keep at 600 x g).

FIGURE 5

Representative images of K562 cell and nuclei samples for Fiber-seq. Samples were stained with Trypan Blue and visualized under brightfield microscope. **(A)** Cells before nuclei extraction. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white and round. **(B)** Nuclei after extraction. An intact cell (Trypan negative) is circled in red, whereas isolated nuclei are Trypan stained.

NOTE

- * If severe sample clumping is observed, consider counting nuclei in 1X PBS instead for better accuracy: spin extracted nuclei **after step 9** at 600 x g for 3 min at RT, remove supernatant, and thoroughly resuspend nuclei in ~1 mL/reaction cold 1X PBS (supplemented with 0.5 mM Spermidine) before taking 10 μ L for Trypan Blue staining and counting. After counting, spin nuclei at 600 x g for 3 min at 4°C and resuspend with 75 μ L/reaction cold **1X Reaction Buffer**. Proceed to **step 13**.
- * **Take caution** as an extra wash step may cause additional loss of nuclei. Having sufficient nuclei (1,000,000 per reaction) is critical to the success of a Fiber-seq reaction.

SECTION III: FIBER-SEQ REACTION (~20 MIN)

NOTE

- * SAM is a critical component of the Fiber-seq labeling reaction but is a highly labile reagent and prone to degradation with repeated freeze-thaw cycles. Be sure to always use fresh aliquots made from original stock for Fiber-seq labeling reactions to ensure optimal performance.
- * The standard Fiber-seq labeling condition is optimized to achieve ~6% 6mA labeling in 1,000,000 human nuclei. See [Appendix](#) for scaling recommendations.

13. For each reaction, pipette mix and transfer 1,000,000 nuclei from step 11 to a PCR tube and bring the volume up to 56.5 μ L with **1X Reaction Buffer**. Equilibrate to RT.
14. Add 1.5 μ L 32 mM SAM to each reaction, pipette gently to mix, followed by addition of 2 μ L **Hia5 for Fiber-seq** to initiate each reaction. The final reaction volume per tube should now be 60 μ L. Pipette gently to mix again.
15. Incubate reaction for 10 min at 25°C using a thermocycler for optimal labeling efficiency.
16. After incubation, stop the reaction by adding 6 μ L 10% SDS to each reaction. Vortex to mix.
17. Add 34 μ L **1X Reaction Buffer** to bring the volume to 100 μ L, transfer to a 1.5 mL tube and proceed to gDNA extraction.

SECTION IV: GENOMIC DNA EXTRACTION (~1 HOUR)

BEFORE FIRST USE PER KIT: Add ethanol (\geq 95%, not supplied in kit) directly to the **gDNA Wash Buffer** bottle.

For Kit 14-2001-8rxn, add 7 mL ethanol.

For Kit 14-2001-24rxn, add 21 mL ethanol.

18. Add 1 μ L **Proteinase K** and 3 μ L **RNase A** to each reaction mix from step 17. Vortex to mix.
19. Add 100 μ L **gDNA Cell Lysis Buffer** to each reaction. Vortex immediately and thoroughly to mix. The solution will become viscous at this point.
20. Incubate for 30 min at 56°C in a thermomixer with full speed agitation (~1,400 rpm). If a thermomixer is not available, use a heating block and vortex occasionally.
21. Add 400 μ L **gDNA Binding Buffer** to each reaction. Pulse-vortex for 5-10 seconds to mix thoroughly.
22. For each reaction, transfer the entire mix (~600 μ L) to a **gDNA Spin Column** pre-inserted into a **gDNA Collection Tube**, without touching the upper column area. Proceed immediately to next step.
23. Close cap and centrifuge for 3 min at 1,000 x g to bind gDNA, then for 1 min at max speed ($>$ 12,000 x g) without taking tubes out of the centrifuge. Discard the flow through and the collection tube.

Fiber-seq Experimental Protocol

24. Transfer each column to a new **gDNA Collection Tube** and add 500 μ L **gDNA Wash Buffer (with supplemented ethanol)**. Close the cap and invert a few times so that the wash buffer touches the cap. Centrifuge immediately for 1 min at max speed ($>12,000 \times g$), then discard the flow through.
25. Reinsert the column into the collection tube. Repeat the wash with 500 μ L **gDNA Wash Buffer**.
26. Place each gDNA Spin Column in a DNase-free 1.5 mL tube. Add 100 μ L **gDNA Elution Buffer** preheated at 60°C. Close cap and incubate for 1 min at RT.
27. Centrifuge at max speed ($>12,000 \times g$) for 1 min to elute the gDNA.
28. Quantify DNA with the Qubit fluorometer and assess gDNA quality on the Agilent TapeStation using Genomic DNA ScreenTape and Reagents (Figure 6), or use Agilent Femto Pulse® system for more quantitative evaluations for larger DNA fragments.

Safe pause point. DNA can be stored at -20°C, or proceed to LRS library prep and sequencing based on individual platform instructions.

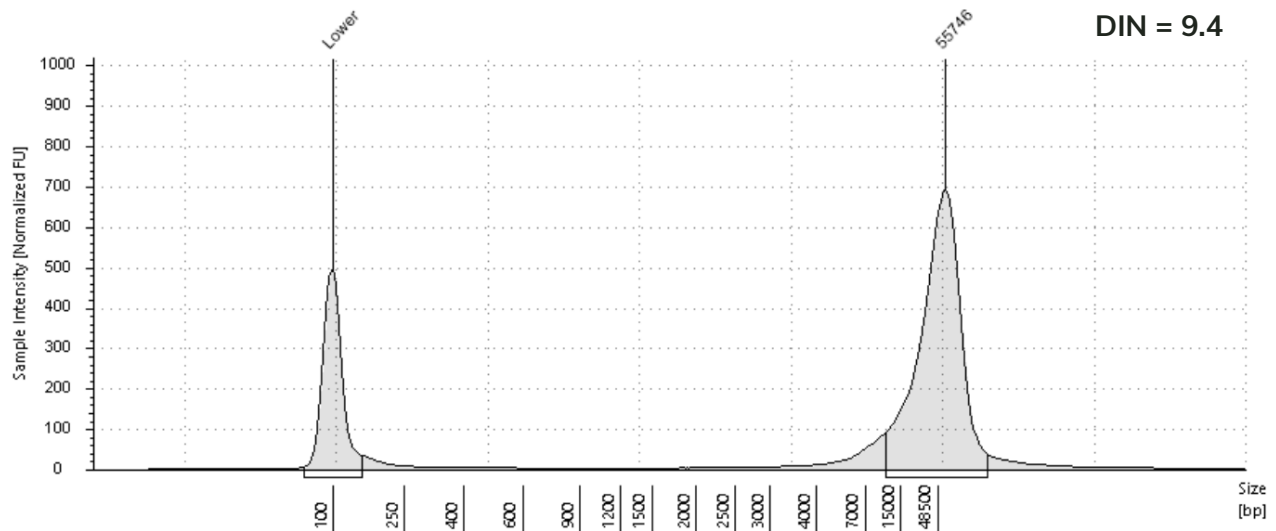


FIGURE 6

Representative Genomic DNA TapeStation track from a standard Fiber-seq reaction using 1,000,000 K562 nuclei. Extracted genomic DNA shows a dominant band around or above 60 kb with minimal smearing and a high DNA Integrity Number (DIN) or equivalent metric.

RECOMMENDATIONS FOR LIBRARY PREP AND SEQUENCING

Extracted gDNA can be used for library prep. Follow LRS provider recommendations for input requirement, shearing, library preparation, QC metrics and instrument loading.

For human genome samples, we recommend achieving 30× genome coverage per sample. If haplotype phased data is desired, samples should be sequenced to achieve 30× coverage per haplotype (i.e., 60× total coverage for diploid cell types).

Platform Compatibility

- Fiber-seq is compatible with both PacBio HiFi and ONT Nanopore LRS platforms^{2,3}. Both platforms support 6mA and 5mC base calling, allowing for simultaneous detection of Fiber-seq labeled chromatin accessibility (6mA) and endogenous DNA methylation (5mC).
- Fiber-seq records sites of chromatin accessibility directly onto DNA via 6mA labeling. Because neither 6mA nor endogenous 5mC modifications are preserved during PCR, these marks can only be detected using native (amplification-free) DNA sequencing. As a result, only direct or native DNA sequencing workflows are compatible with Fiber-seq. When preparing libraries, use kits and reagents specifically validated for **whole-genome native DNA sequencing**.

NOTE

- * Base calling accuracy varies by platform and software version. For optimal results, ensure you are using the latest base calling algorithms provided by your sequencing platform.

Data Analysis

- All tools required to analyze Fiber-seq data are publicly available. After sequencing, users should follow platform-specific guidance for base calling and read alignment using the most up-to-date tools provided by your chosen platform.
- Once reads are aligned, we recommend using Fiber-seq-specific QC tools (github.com/fiberseq/fiberseq-qc) for downstream analysis. These tools provide Fiber-seq-specific quality control metrics, detection of accessible DNA regions, and inferred nucleosome positioning. Please refer to the fibertools publication⁴ and Fiber-seq GitHub resource (github.com/fiberseq) for detailed guides on how to use fibertools and expected Fiber-seq results.

Appendix: Adapting Fiber-seq to other organisms or sample quantities

This appendix provides guidance on how to adjust the Fiber-seq protocol for:

- * Organisms with genome sizes that differ significantly from human (~3,200 Mb)
- * Sample inputs other than the standard 1,000,000 human nuclei (or human nuclei equivalents)
- * Check support.epicypher.com for the latest validated Fiber-seq protocol adaptations from our continuous research and innovations.

SCALING THE FIBER-SEQ REACTION

The standard Fiber-seq labeling reaction is optimized to achieve ~6% 6mA labeling in 1,000,000 human nuclei. The Hia5 reaction generally follows Michaelis-Menten kinetics, meaning it scales proportionally with the amount of substrate (i.e., DNA). If you are using more than the equivalent of 1,000,000 human nuclei (e.g., more than ~1,200,000 mouse nuclei) in a Fiber-seq reaction, the reaction volume and reagents should be increased proportionally to the additional nuclei to be used.

- Example: For 2,000,000 human nuclei, double the reaction volume and all reagents used.

NOTE

- * We do not recommend scaling the reaction down below 1,000,000 human nuclei (or equivalent DNA content). Reducing the nuclei used may result in insufficient DNA recovery for long-read sequencing, making it difficult or impossible to load your sample onto a long-read sequencer and achieve the recommended 30x coverage. Always refer to your sequencing platform's guidelines for the minimum DNA input required for whole genome sequencing.

ADJUSTING FOR GENOME SIZE

For organisms with smaller genomes, you'll need more nuclei to match the total DNA content of 1,000,000 human nuclei. This ensures consistent labeling and yields enough DNA for long-read sequencing.

- Recommendation: Keep the reaction volume and enzyme concentration the same, but adjust the number of nuclei to match total DNA input of 1,000,000 human nuclei.
- Below is a table of common research organisms and the recommended number of nuclei to use per Fiber-seq reaction.

Organism	Genome Size	Genome Size Relative to Human	Recommended Nuclei per Reaction
Human (Hs)	3,200 Mb	100%	1,000,000
Mouse (Mm)	2,700 Mb	84%	1,185,000
Drosophila (Dm)	143.7 Mb	4.5%	22,270,000
Yeast (Sc)	12.07 Mb	0.38%	265,120,000

Frequently Asked Questions

1. How many cells are needed for Fiber-seq?

This depends on several factors and should be determined for each cell type and organism. In general, sufficient cells are required to yield approximately the same quantity of DNA in 1,000,000 human nuclei and yield the platform recommended starting material for library prep. Please refer to [Appendix](#) for more information on adapting Fiber-seq to alternative samples and nuclei quantities.

2. Is it necessary to use nuclei? What about permeabilized cells?

We recommend nuclei to avoid potential interference from cytosolic proteins or RNA. Please check support.epicypher.com for continuously updated information on using other input samples for Fiber-seq.

3. Can I use nuclei that have been cryopreserved, flash frozen, or cross-linked in Fiber-seq?

Yes. We have validated that Fiber-seq works for cryopreserved and cross-linked nuclei with some slight differences in labeling compared to native. Please refer to support.epicypher.com for the most up-to-date recommendations for these sample types.

4. How can I assess the 6mA-labeling before I proceed to library preparation and sequencing?

As of yet we do not have a solution for users to directly assess if their sample chromatin was properly labeled with 6mA. Please refer to the Experimental Design and Key Protocol Notes for current recommendations for determining if the experiment worked prior to sequencing.

5. Should I include an unlabeled sample as negative control?

Unlabeled libraries are generally not necessary as a negative control unless you suspect the presence of endogenous 6mA in your model system. Fiber-seq is not recommended for organisms known to naturally contain high levels of 6mA, such as bacteria. However, if background labeling is a concern, we suggest including a Hia5-negative control in parallel to assess endogenous 6mA levels and distinguish true accessibility signals from background.

6. I want to use Fiber-seq to study my favorite DNA-binding protein. What should I change in the protocol and data analysis to do this?

Fiber-seq offers near-nucleotide resolution of chromatin accessibility, making it a promising approach for studying protein-DNA interactions. However, this application is still being actively explored, and we do not yet offer formal recommendations for detecting and analyzing footprints for specific DNA-binding proteins. The feasibility of detecting a given protein's footprint depends on several biological factors, including binding dynamics, occupancy frequency, and footprint size. That said, multiple labs have successfully used Fiber-seq for this purpose, and we recommend reviewing published examples to guide experimental design and analysis^{2,5,6}.

7. I don't currently have a preference between PacBio and ONT for my LRS platform. Which one is better for Fiber-seq?

Fiber-seq is fully compatible with both PacBio and ONT platforms. Each can detect 5mC and 6mA on native DNA and produces comparable results for Fiber-seq applications. If you're interested in comparing performance across platforms, direct comparisons can be found on GitHub (<https://fiberseq.github.io/analyses/ont.html>).

8. Do you have example data I can use to establish my own Fiber-seq data analysis pipeline?

Absolutely! We have deposited an example Fiber-seq bam file (unaligned) on SRA and can be found here ([SRX28885063](https://www.ncbi.nlm.nih.gov/sra/SRX28885063)).

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