EpiCypher® CUTANA™ Fiber-seq Protocol

Table of Contents

1. Overview

Fiber-seq is a multiomic long-read assay for the simultaneous analysis of chromatin accessibility, DNA methylation (DNAme), and genetic variants [1]. In the Fiber-seq workflow, isolated nuclei are incubated with Hia5 *N6*-methyladenine methyltransferase (6mA MTase) and cofactor S-adenosylmethionine (SAM) to selectively methylate adenines within regions of accessible chromatin (**Figure 1**). After a brief 10 minute labeling step, the reaction is quenched and genomic DNA (gDNA) is purified and prepared for sequencing with direct or native DNA sequencing protocols using either Pacific Biosciences® (PacBio®) HiFi or Oxford Nanopore Technologies® (ONT) Nanopore sequencing platforms.

The CUTANA™ Fiber-seq assay can be used for projects that require single molecule or haplotype phased chromatin accessibility mapping and endogenous CpG DNAme profiling. Because the accessibility signal is covalently encoded directly on individual DNA strands, both data types are intrinsically linked on sequenced DNA molecules.

This protocol is validated for labeling 1,000,000 nuclei from human cells using CUTANA™ Hia5 enzyme, a highly active 6mA MTase available exclusively from EpiCypher. CUTANA Hia5 enables rapid, 10 minute labeling of accessible chromatin delivering near-base-pair resolution maps of chromatin accessibility and nucleosome positioning.

As with any assay, optimal results depend on thoughtful experimental design and sample-specific considerations:

- This Do-It-Yourself (DIY) Fiber-seq Protocol has been optimized using human-derived samples. Users working with non-human models, particularly those with significantly larger or smaller genome sizes, may require further optimization. See the Appendix for guidance on adapting the protocol to diverse organisms.
- This protocol has been validated for mapping chromatin accessibility and nucleosome footprints. While Fiber-seq can reveal footprints of other DNA-binding proteins, optimal sequencing depth and analytical best practices for this application are still being refined. For examples on how other researchers have inferred protein binding for other chromatin associated proteins using Fiber-seq, refer to the **FAQ** Section.

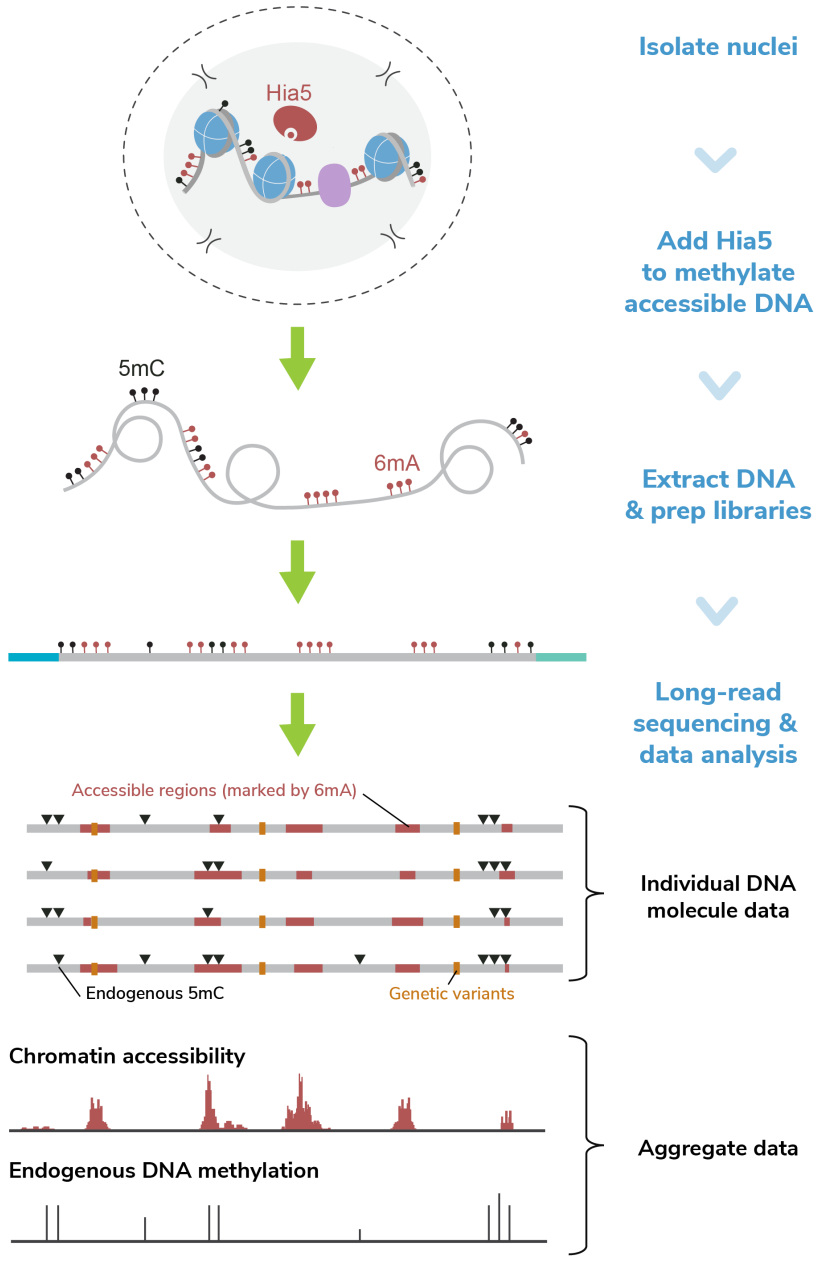

Figure 1. Overview of the CUTANA™ Fiber-seq protocol.

2. Experimental Design and Key Protocol Notes

This section is considered essential reading for CUTANA™ Fiber-seq assays.

Inputs:

- Freshly isolated, native (*i.e.*, unfixed) nuclei are the preferred input for Fiber-seq.
- The recommended input is 1,000,000 nuclei per reaction. Harvest 2,000,000 cells per reaction (plus 10% excess when possible) to account for sample loss during nuclei prep.
- To confirm high quality sample prep, see the Trypan Blue staining protocol in **Appendix I**. These quality control checks can improve experimental success. Samples are assessed at two points: at initial cell harvest and after nuclei isolation.

Control Reactions:

- Unlabelled 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.

How to know if experiment worked before sequencing:

- While there is currently no supported method to directly verify successful 6mA labeling prior to sequencing, several key indicators can help you assess whether your Fiber-seq experiment is on track before committing to a 30× coverage run: DNA yield following gDNA extraction, DNA quality control measurements recommended by the LRS platform to be used, and assessing the percent 6mA detected from a shallow coverage (5-10×) sequencing run.
- **Total DNA yields after genomic DNA (gDNA) extraction:** DNA recovery will vary depending on sample type and nuclei quantity. This protocol was optimized using 1,000,000 human K562 nuclei, which typically yield 3–4 µg of DNA when extracted with the New England Biolabs® (NEB®) Monarch® Spin gDNA Extraction Kit (Cat. No. T3010S / T3010L). If your yield is significantly lower, it may affect library prep and sequencing success.
- **DNA quality:** Assess genomic DNA quality using quality control methods recommended by your long-read sequencing platform (*e.g.*, Femto Pulse, Qubit, Nanodrop). High molecular weight DNA should show a dominant band or peak >60 kb, minimal smearing, and a high DNA Integrity Number (DIN) or equivalent metric.
- **Shallow sequencing to estimate 6mA labeling efficiency**: Performing a shallow sequencing run (*e.g.*, 5–10× genome coverage) to estimate percent 6mA labeling can indicate if further optimization is needed before committing to deeper sequencing. This protocol is optimized to achieve ~6% 6mA labeling, which supports confident detection of accessible chromatin and high-resolution protein footprinting. Significant deviation from this target may indicate a need for further protocol optimization before proceeding to deeper sequencing.

Sequencing:

- For human genome samples, we recommend generating 90 gigabases (Gb) of sequencing data per sample to achieve approximately 30× genome coverage.
- Native or direct long-read sequencing is required to detect 6mA and endogenous DNA methylation (5mC). Fiber-seq is not compatible with short-read sequencing workflows, as methylation marks are lost during PCR amplification.
- Both PacBio HiFi and ONT Nanopore sequencing platforms are compatible with Fiber-seq, as both can efficiently detect 6mA and 5mC when directly sequencing native DNA. For platform-specific sequencing recommendations, please refer to PacBio or ONT resources.
- For data analysis, refer to the **Appendix**.

3. Buffers, Reagents and Materials Needed

Buffer Components and Reagent Preparation

Components	Source	Cat #
KCl	Sigma-Aldrich	P3911
Molecular biology grade H_2O (RNase, Dnase free)	VWR	VWRV02-0201-0500
NaCl	Sigma-Aldrich	S5150-1L
EDTA (prepare 1 M stock at pH 8.0)	Sigma-Aldrich	E5134
EGTA (prepare 0.5 M stock at pH 8.0)	Sigma-Aldrich	E3889
Spermidine trihydrochloride	Sigma-Aldrich	S2501
Tris (pH 8.0)	JT Baker	4109-02
Trypan Blue	Thermo Fisher Scientific	T10282
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L4509
HEPES	Sigma-Aldrich	H3375
Triton X-100	Sigma-Aldrich	X100
Glycerol	Millipore Sigma	G5516
Optional Nuclei Extraction Buffer*	EpiCypher	21-1026

Reagent Prep

1M spermidine: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular biology grade H_2O. Filter sterilize using a 0.22 µM filter. *Store in single-use aliquots at -20°C for 6 months.*

Spermidine is added to compensate for the removal of chromatin stabilizing Mg^{2+}.

10% SDS (weight/volume): Dissolve 1 gram SDS powder in 10 mL molecular biology grade H_2O. *Store at room temperature for 6 months. If precipitates form, gently warm to re-dissolve.*

* Nuclei Extraction Buffer is available for purchase at epicypher.com/21-1026. The recipe is also provided on the next page.

Buffer Recipes

Stock Buffers – Can be prepared in advance

Pre-Nuclei Extraction (NE) Buffer
(**Note:** Available for purchase as part of EpiCypher 21-2026; epicypher.com/21-2026)
20 mM HEPES–KOH, pH 7.9
10 mM KCl
0.1% Triton X-100
20% Glycerol
Stable at 4ºC for up to 6 months.

Pre-1× Reaction Buffer
15 mM Tris (pH 8.0)
15 mM NaCl
60 mM KCl
1 mM EDTA (pH 8.0)
0.5 mM EGTA (pH 8.0)
Filter sterilize, 0.22 µM filter. Store at 4ºC for up to 6 months.

Buffers prepared FRESH for each Fiber-seq experiment

Nuclei Extraction (NE) Buffer (200 µL/reaction)
(**Note:** Pre-NE Buffer + spermidine available for purchase; epicypher.com/21-2026)
Pre-NE Buffer
0.5 mM Spermidine
After spermidine is added, store at 4ºC for up to 1 week.
Note: Omition of protease inhibitor is purposeful, protease inhibitors are not used in this Fiber-seq protocol.

1× Reaction Buffer (175 µL/reaction)
Pre-1X Reaction Buffer
0.5 mM Spermidine
After spermidine are added, store at 4ºC for up to 1 week.

Assay Reagents

Item	Vendor, Cat. No.	Notes
CUTANA™ Hia5 for Fiber-seq	EpiCypher 15-1032-8RXN / 15-1032-24RXN	30X stock. 8 & 24 reaction pack sizes available.
S-adenosylmethionine (SAM)	New England Biolabs B9003S	Methyl-donor cofactor required for Hia5 methylase activity. *NOTE: SAM is a critical component of the Fiber-seq labelling reaction. Be sure to source only high-grade SAM and follow recommendations for handling and storage.*
Monarch Spin gDNA Extraction Kit	New England Biolabs T3010S / T3010L	For purification of genomic DNA following Hia5 incubation.
Library preparation and instrument loading components	Variable	Dependent on your preferred LRS platform. Choose reagents and components recommended by your chosen LRS platform intended for direct or native DNA whole genome sequencing.

Equipment

Item	Vendor, Catalog No.	Notes
1.5, 15, 50 mL tubes and centrifuge	Various	For making buffers, harvesting cells, etc.
PCR Tubes	Various / EpiCypher 10-0009	For performing the Fiber-seq reaction.
Vortex	Various, *e.g.* Vortex Genie, Scientific Industries SI-0236	For mixing after adding 10% SDS to stop the Fiber-seq labeling reaction.
Small benchtop centrifuge	Various, *e.g.* from Fisher Scientific, Benchmark Scientific	For pelleting cells/nuclei and collecting liquid from sides of tubes/caps after vortexing.
Thermocycler with a heated lid	Various: *e.g.* from Biorad, Applied Biosystems, Eppendorf.	For incubation step.

Note: Please consult the technical documentation provided by your chosen LRS platform provider for platform-specific equipment requirements.

4. EpiCypher CUTANA™ Fiber-seq Protocol

Section I: Nuclei Extraction (~30 min)

Good sample prep is critical for Fiber-seq. In this section, you will harvest cells and extract nuclei. ***Note*** *that this protocol is designed for fresh, native human suspension cells.*

1. Count cells, confirm expected morphology, and determine cell viability (as in **Appendix I**). Ideally, cells should be at least 80% viable, unclumped and with minimal cell lysis or debris. However, ideal viability may vary by sample type, treatments, or processing conditions. The goal is to harvest cells with good integrity and minimal lysis.
2. Transfer 2,000,000 cells per reaction (plus 10% excess if possible) to a 15 ml tube.
3. Spin cells 600 x g for 3 min at room temperature (RT). Aspirate supernatant and resuspend cells in 500 µL PBS to wash once.
4. Transfer cells in 500µL PBS to a 1.5 mL tube.
5. Spin cells 600 x g for 3 min at room temperature (RT). Remove supernatant and resuspend cells in 200 µL per reaction cold Nuclei Extraction Buffer.
6. Incubate for 10 min on ice.
7. Spin 600 x g for 3 min at 4ºC. Pipette to remove supernatant. The pellet should change from pale yellow (cells) to a white, fluffy pellet (nuclei).
8. Gently resuspend nuclei in 75 µL per reaction cold 1× Reaction Buffer.
9. Take a 10 µL aliquot to examine nuclei integrity and count nuclei using Trypan Blue staining (as in **Appendix I**). Nuclei should be >95% Trypan Blue positive, unclumped, and show minimal lysis.

Section II: Fiber-seq Reaction (~15 min)

Nuclei are incubated with Hia5 6mA MTase for labeling adenines in accessible chromatin.

10. Transfer 1,000,000 nuclei to a PCR tube and bring the volume up to 56.5 µL with 1× Reaction Buffer.
11. Add 1.5 µL 32 mM SAM and 2 µL Hia5 to each reaction. The final reaction volume per tube should now be 60 µL.

    Note: SAM is a highly labile reagent and prone to degradation with repeated freeze-thaw cycles. Always use fresh, high-grade SAM for Fiber-seq labeling reactions to ensure optimal performance.
12. Pipette gently to mix.
13. Incubate reaction for 10 min at 25°C. We recommend using a thermocycler for this incubation for optimal labeling efficiency.
14. After 10 min at 25°C, stop the reaction by adding 6 µL 10% SDS. Vortex to mix.
15. Add 34 µL of 1× Reaction Buffer to bring the volume to 100 µL and proceed to gDNA extraction

Section III: Genomic DNA extraction (~30 min)

Following addition of SDS to stop the MTase reaction, gDNA should be purified. We recommend using NEB Monarch Spin gDNA purification kit (Cat. Nos. T3010S, T3010L).

***Safe pause point**. Store DNA at -20˚C or continue to library preparation and sequencing*

Section IV: Library preparation and sequencing (platform dependent)

*Follow LRS provider recommendations for shearing, library preparation, and instrument loading. Each sample should be sequenced to achieve 30× coverage. If haplotype phased data is desired, samples should be sequenced to achieve 30× coverage per haplotype (*i.e.*, 60× coverage for diploid cell types).*

5. FAQs

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 II** for more information on adapting Fiber-seq to alternative samples and nuclei quantities.

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

We have not yet tested cells in Fiber-seq and recommend nuclei to avoid potential interference from cytosolic proteins or RNA.

How many micrograms (µg) of gDNA are needed for LRS of Fiber-seq libraries?

In general, 0.5-2 µg of gDNA is typically required for library preparation. However, this is dependent on the LRS platform you intend to use to sequencing your Fiber-seq libraries. Please refer to the recommendations provided by the LRS platform you are using for applications involving direct or native whole genome sequencing.

What organisms are compatible with Fiber-seq?

Fiber-seq is a LRS-compatible open chromatin mapping assay, therefore potentially any nucleated eukaryotic cell would be compatible. Fiber-seq has been published for multiple organisms and cell / tissue types [2-5]. Please refer to the **Appendix** for best-practices adapting Fiber-seq to samples other than human cells.

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

Thus far we have only used fresh material (but such studies are underway!).

Can you share your analysis pipeline code?

All the tools EpiCypher uses to analyze LRS data are publicly available. We generally do not share the ever-evolving code from our programming environment. Please refer to **Appendix III** for more information on sequencing and data analysis.

How can I assess the 6mA-labelling 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.

I have no idea how to deal with LRS datasets but I want to use Fiber-seq! Where should I start?

We recommend starting with two recent review articles to get a general overview: [6, 7]. PacBio and ONT both offer excellent resources to help you get familiar with whole genome sequencing applications using their platforms. And keep an eye out—we'll be releasing blogs, tutorials, and other helpful materials to support your Fiber-seq journey. Be sure to sign up for our emails and follow us on social media so you don't miss out!

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, 4, 5].

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).

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).

6. Appendix I: Quality Control Checks for Sample Prep

Description: This Appendix provides detailed instructions on checking the quality of starting cells and isolated nuclei. All QC checks were developed using native K562 cells and nuclei.

We recommend these quality control checks for every Fiber-seq experiment. This is a key step in the Fiber-seq protocol, and if nuclei are of poor quality, DNA yields may be reduced and Fiber-seq data quality may be affected.

Reagents, Materials & Equipment Needed

Item	Vendor
1X PBS	Any
0.4% Trypan blue	Any
Hemacytometer	Any
Cell counting slides	Any
Brightfield/phase contrast microscope or automated cell counter	Any

Protocol: Harvesting cells and nuclei for Fiber-seq with built-in quality control checks

Starting from Section I of the CUTANA Fiber-seq Protocol, Step 1.

1. Spin cells 600 x g, 3 min, at room temperature (RT). Remove supernatant, flick tube to loosen cell pellet, and resuspend in 1-2 mL 1X PBS. Transfer 10 µL to a 1.5 mL tube.
2. Add 10 µL 0.4% Trypan Blue to 10 µL cells. Pipette 10 times to mix.
3. Transfer 10 µL to a cell counting slide. Obtain cell counts and determine viability using a brightfield/phase microscope or cell counter. See **Figure 2** for expected results.
4. Transfer 2,000,000 cells per reaction (plus 10% excess if possible) to a 15 ml tube.
5. Spin cells 600 x g for 3 min at room temperature (RT). Aspirate supernatant and resuspend cells in 500µL PBS to wash once.
6. Transfer cells in 500µL PBS to a 1.5 mL tube.
7. Spin cells 600 x g for 3 min at room temperature (RT). Remove supernatant and resuspend cells in 200 µL per reaction cold Nuclei Extraction Buffer.
8. Incubate for 10 min on ice.
9. Spin 600 x g, 3 min, 4˚C. Pipette to remove supernatant. Resuspend nuclei in 75 µL per reaction cold 1× Reaction Buffer.
10. Transfer 10 µL nuclei to a new 1.5 mL tube. Evaluate integrity using Trypan Blue staining as in steps 2 and 3 above. See **Figure 2** for expected results.
11. Continue with the Fiber-seq Protocol, Section II (Fiber-seq Reaction).

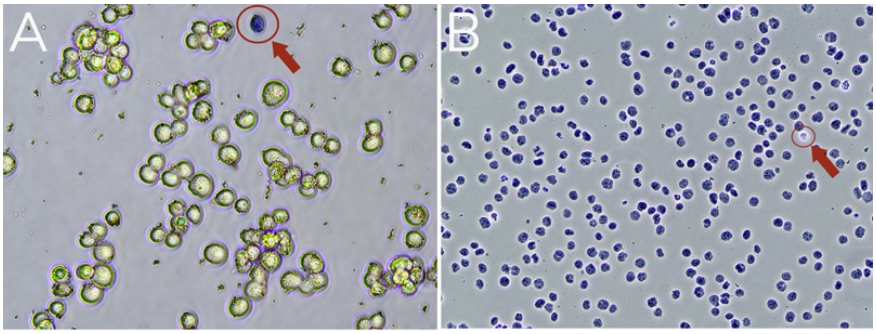

Figure 2: Representative images of cell and nuclei samples for Fiber-seq. Samples were stained with Trypan Blue and visualized under brightfield microscope. **(A) Cells** before nuclei extraction. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white and round. **(B) Nuclei** after extraction. An intact cell (Trypan negative) is circled in red, whereas isolated nuclei are Trypan stained.

Sample	Figure	Success Metric (Trypan Blue staining)	Troubleshooting Tips
Cells	**Fig. 2A**	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	**Fig. 2B**	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).

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

Description: 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)

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.

Important: 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, making it difficult or impossible to load your sample onto a long-read sequencer. Always refer to your sequencing platform's guidelines (PacBio or ONT) 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 60µL 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

8. Appendix III: Sequencing Platforms and Data Analysis

Platform Compatibility: Fiber-seq is compatible with both PacBio HiFi and ONT Nanopore LRS platforms [2, 8]. 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 tools and *fibertools* for downstream analysis. These tools provide Fiber-seq-specific quality control metrics, detection of accessible DNA regions, and nucleosome positioning. Please refer to the *fibertools* publication [9] and Fiber-seq github resource (github.com/fiberseq) for detailed guide on how to use *fibertools* and expected Fiber-seq results.

9. References

1. Stergachis et al. (2020) *Science*. PMID: 32587015.
2. Vollger et al. (2025) *Nat Genet.* PMID: 39880924.
3. Peter et al. (2024) *Cell Rep Methods.* PMID: 39631398.
4. Tullius et al. (2024) *Mol. Cell*. PMID: 39191261.
5. Dubocanin et al. (2025) *Cell Genom.* PMID: 40147439.
6. Liu & Conesa. (2025) *Nat Genet.* PMID: 39779955.
7. Fiu et al. (2025) *Nat Rev Genet.* PMID: 40155770.
8. Boltengagen et al. (2025) *Nucleic Acids Res.* PMID: 37994698.
9. Jha et al. (2024) *Genome Res.* PMID: 38849157.