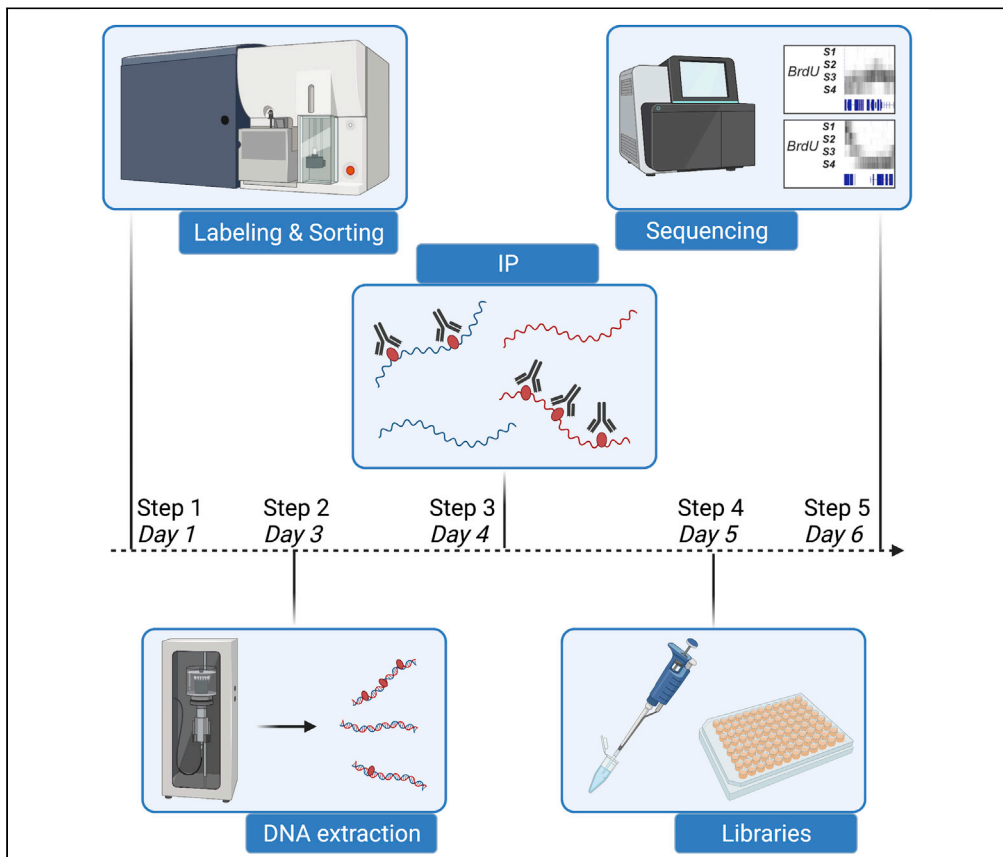


## Protocol

# Protocol to isolate cells in four stages of S phase for high-resolution replication-timing sequencing



Traditional replication timing (RT) experiments divide S phase into two phases: early and late. However, there is an increasing awareness that variation in RT can occur during the course of S phase and impact our understanding of RT patterns and regulation. Here, we describe a RT protocol in RPE-1 cells for collecting four phases within S and the library preparation that takes advantage of a commercial kit for methyl-DNA. This step allows BrdU-labeled DNA sequencing and assessment of RT genome wide.

Johnathan R. Whetstine,  
Capucine Van Rechem

johnathan.whetstine@fcc.edu (J.R.W.)  
cvrechem@stanford.edu (C.V.R.)

### Highlights

Collecting four phases of S phase allows to profile replication timing in mid S phase

The use of a commercial kit for library preparation simplifies the protocol

This protocol can be adapted to any cycling cells

Whetstine & Van Rechem,  
STAR Protocols 3, 101209  
March 18, 2022 © 2022 The Author(s).  
<https://doi.org/10.1016/j.xpro.2022.101209>



## Protocol

## Protocol to isolate cells in four stages of S phase for high-resolution replication-timing sequencing

Johnathan R. Whetstine<sup>1,2,\*</sup> and Capucine Van Rechem<sup>3,4,5,\*</sup><sup>1</sup>Cancer Signaling and Epigenetics Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA<sup>2</sup>Cancer Epigenetics Institute, Fox Chase Cancer Center, Philadelphia, PA 19111, USA<sup>3</sup>Stanford Medicine Department of Pathology, Stanford, CA 94305, USA<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [johnathan.whetstine@fccc.edu](mailto:johnathan.whetstine@fccc.edu) (J.R.W.), [cvrechem@stanford.edu](mailto:cvrechem@stanford.edu) (C.V.R.)  
<https://doi.org/10.1016/j.xpro.2022.101209>

## SUMMARY

Traditional replication timing (RT) experiments divide S phase into two phases: early and late. However, there is an increasing awareness that variation in RT can occur during the course of S phase and impact our understanding of RT patterns and regulation. Here, we describe a RT protocol in RPE-1 cells for collecting four phases within S and the library preparation that takes advantage of a commercial kit for methyl-DNA. This step allows BrdU-labeled DNA sequencing and assessment of RT genome wide.

For complete details on the use and execution of this protocol, please refer to Van Rechem et al. (2021).

## BEFORE YOU BEGIN

Experimental conditions described in this protocol apply to hTERT RPE-1 cells. However, this protocol should be applicable to any cycling cell type.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-BrdU (1/250 dilution)	BD Pharmingen	Cat# 555627
<b>Chemicals, peptides, and recombinant proteins</b>		
Dulbecco's Modified Eagle's Medium - High Glucose	Sigma-Aldrich	Cat# D5648
FBS	GIBCO	Cat# 26140-079
Penicillin-Streptomycin	Life Technologies	Cat# 15140122
L-Glutamine	Life Technologies	Cat# 25030-081
Propidium Iodide	Sigma-Aldrich	Cat# P4864
Trypsin-0.25% EDTA	Life Technologies	Cat# 2520056
Ribonuclease A	Sigma-Aldrich	Cat# R4875
Proteinase K	Sigma-Aldrich	Cat# P6556
5-Bromo-2-deoxyuridine	Sigma-Aldrich	Cat# B5002
AMPure XP	Beckman Coulter	Cat# A63881
Dynabeads Protein G	Thermo Fisher Scientific	Cat# 10003D
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma-Aldrich	Cat# P2069-400ML
Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA		
Glycogen	Roche (now Sigma)	Cat# 10901393001

(Continued on next page)



<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Critical commercial assays</b>		
Accel-NGS Methyl-Seq DNA Library Kit	Swift Biosciences	Cat# 30024
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat # Q32851
High Sensitivity D1000 ScreenTape	Agilent	Cat # 5067-5584
High Sensitivity D1000 Reagents	Agilent	Cat # 5067-5585
<b>Deposited data</b>		
Raw data	( <a href="#">Van Rechem et al., 2021</a> )	GEO: GSE175752
<b>Experimental models: Cell lines</b>		
hTERT RPE-1	Nicholas Dyson's lab	N/A
RPE-GFP-CTRL	( <a href="#">Van Rechem et al., 2021</a> )	N/A
RPE-GFP-KDM4A	( <a href="#">Van Rechem et al., 2021</a> )	N/A
<b>Oligonucleotides</b>		
qPCR primers (optional step): see step 35	N/A	N/A
<b>Other</b>		
FACS sorter	N/A	N/A
Magnetic stand for 1.5 mL tubes	N/A	N/A
Magnetic stand for 96 wells plate	N/A	N/A
Sonicator	QSonica	Q700
Qubit	Thermo Fisher Scientific	N/A
TapeStation or Bioanalyser	N/A	N/A
Thermocycler	N/A	N/A

## STEP-BY-STEP METHOD DETAILS

### Cell sorting

⌚ Timing: 3–4 days

During this step cells are labeled with BrdU and sorted based on DNA content.

**Note:** It is necessary to use BrdU rather than EdU because it will be immunoprecipitated at a later step.

1. Seed  $2 \times 10^6$  RPE in four 15 cm<sup>2</sup> plates for 48 h.

**Note:** Cells need to be at ~70% confluence when harvested.

2. Label the cells with 100 μM BrdU for 2 h at 37°C.

**Note:** BrdU incubation time depends on the growth rate of the cells and can need to be adjusted down to 1 h for very rapidly proliferating cells or even longer for slow dividing cells.

3. Rinse the cells with 1 × PBS and add 2.5 mL of trypsin per 15 cm<sup>2</sup> plate.

**Note:** A single cell suspension is critical for the sorting.

4. Wash the cells with 1 × PBS with 1% FBS.
5. Centrifuge for 2 min at 100 g and resuspend the cells in ice cold 2.5 mL 1 × PBS with 1% FBS.
6. Add 7.5 mL EtOH slowly and dropwise while vortexing.

⏸ **Pause point:** Cells can be stored in the dark (foil wrap) at –20°C for several months.

BD FACSDiva 8.0.1

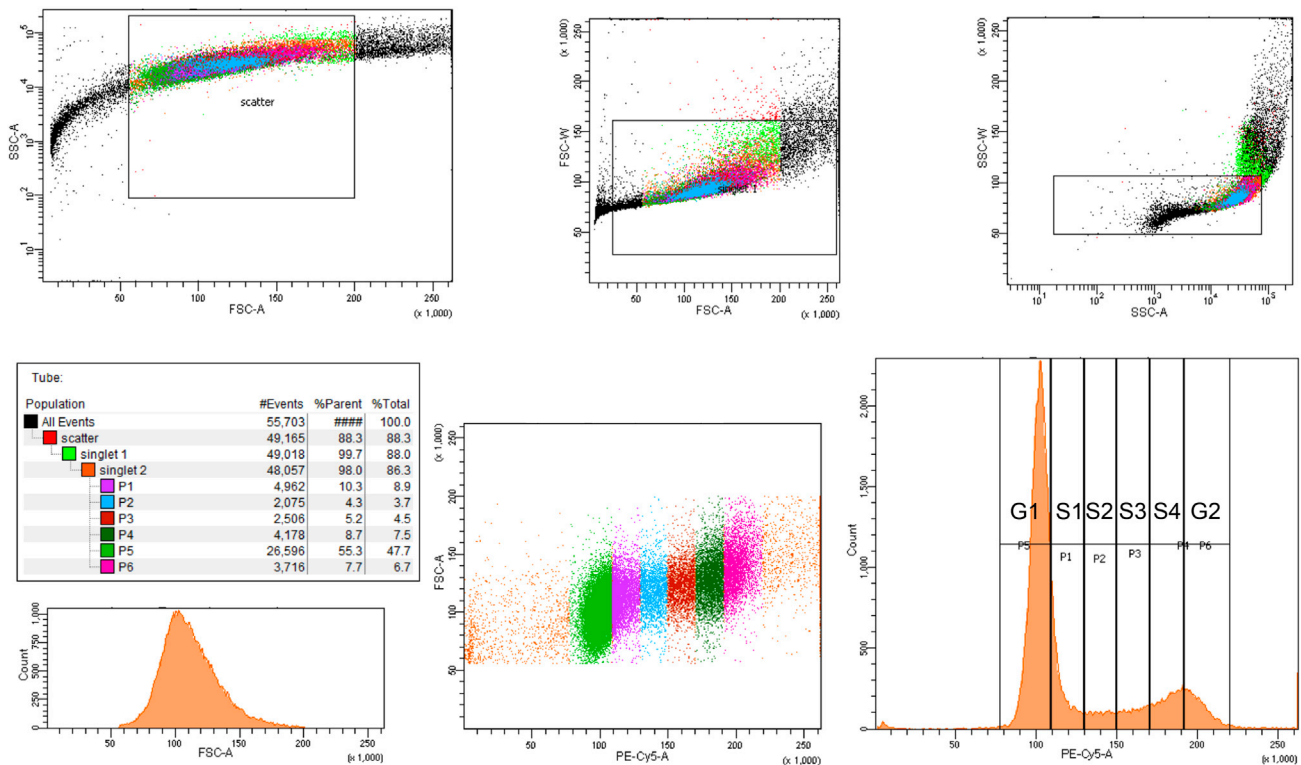


Figure 1. Gating for cell sorting

- Centrifuge the cells 5 min at 100 g at 4°C.
- Remove the supernatant and resuspend the cells in 3 mL 1 × PBS with 1% FBS.
- Add 1/100 Propidium Iodide and RNase A to 250 µg/mL. Incubate 1 h at 18°C–25°C in the dark.
- Keep the cells on ice until sorting.
- FACS Sort the cells with a BD FACS Fusion Tex Red A laser. A typical sort is represented in [Figure 1](#). To place the gates: place P5 (G1) and P6 (G2) first, then divide the remaining four phases across S phase in equal fractions. The P5 fraction goes from the base of the large peak (1N) to 50% of the slope on the right. The P6 fraction goes from the top of the second peak (2N) to the base on the right. Collect P1 (S1) to P4 (S4) in 500 µL of PBS (P5 and P6 are optional), at least 250,000 cells per fraction. [Troubleshooting 1](#).

**Note:** Any sorter with a laser line of 488 to excite Propidium Iodide can be used.

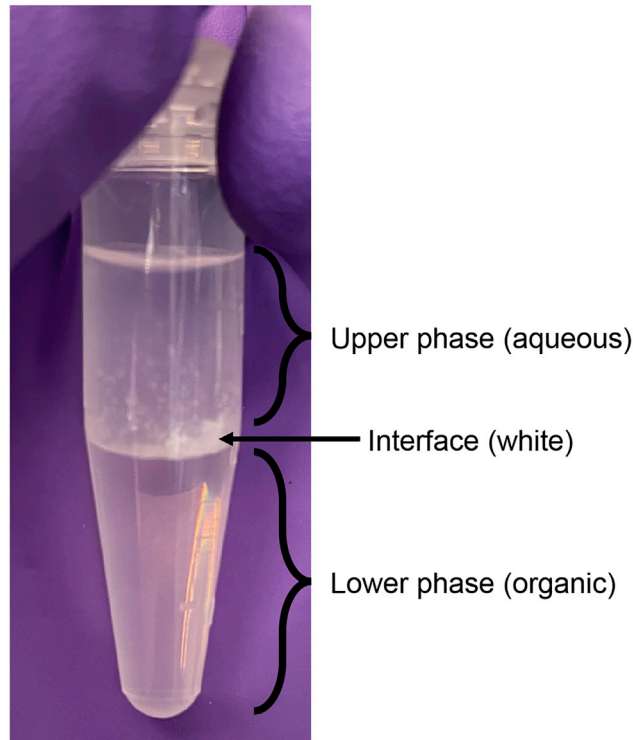
**Note:** There is no need to correct for a potential overestimation of numbers of events from the sorter compared to the actual number of collected cells.

### DNA extraction

⌚ Timing: 2 days

During this step DNA is purified and sonicated.

- Add SDS to 0.8% and proteinase K to 20 µg/mL to each fraction, incubate 2 h at 55°C .



**Figure 2.** Picture of a typical phenol-chloroform extraction

13. Add one volume of phenol-chloroform, vortex, centrifuge for 1 min at maximum speed. Collect as much as the upper phase possible and transfer in 15 mL conical tubes.

**Note:** Be careful not to transfer the interface (see [Figure 2](#)).

14. Add 1/9 volume of 3 M sodium acetate pH 5.2, 2.5 volumes of ice cold 100% EtOH and 1  $\mu$ L (20  $\mu$ g) of glycogen. Vortex and incubate 12–20 h at  $-80^{\circ}\text{C}$ .

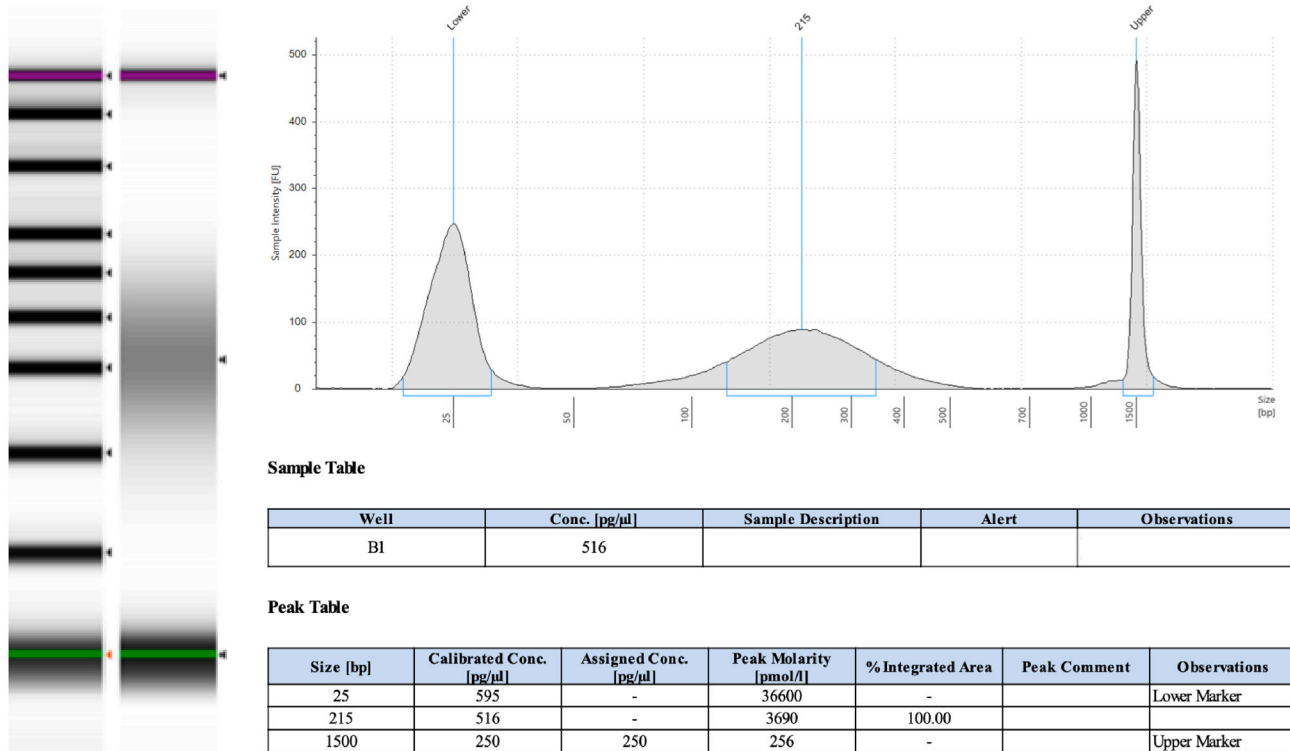
**Note:** volume refers to the volume of upper phase collected in the previous step.

▣ **Pause point:** DNA can be precipitated at  $-80^{\circ}\text{C}$  for a few days if needed.

15. Transfer 2 mL of each sample to 2 mL low-bind DNA tubes, centrifuge 17 min at maximum speed at  $4^{\circ}\text{C}$ . Remove the supernatant without disturbing the pellet.
16. Repeat step 15 until all the material has been precipitated in the same tube.
17. Wash the pellet with 500  $\mu$ L of 70% EtOH, centrifuge 2 min at maximum speed at  $4^{\circ}\text{C}$ .
18. Remove the supernatant and air dry the pellet.
19. Resuspend the DNA in 100  $\mu$ L of 10 mM Tris pH 8 and in 0.5 mL thin wall PCR tubes.
20. Sonicate at amplitude 70% for 20 s ON, 40 s OFF for 12 min of total time on, using a QSonica Q700 containing cold water (ideally in a cold room).

**Note:** We found this sonicator optimal for DNA sonication, however equivalent bath sonicators could be used and will need adapting for the sonication time and amplitude.

**Note:** The bath should never get hot, change the water if necessary.



**Figure 3.** TapeStation analyses for a typical DNA extraction performed with this protocol

21. Evaluate the size of the DNA using a TapeStation D1000 or a Bioanalyzer. The DNA should be < 300 bp fragments. [Troubleshooting 2](#) and [3](#).

**Note:** A typical TapeStation result is presented in [Figure 3](#).

**Note:** Refer to [Figure 3](#) for a typical yield of DNA given by Tape Station, please note that one can proceed with at least twice as less DNA than presented in the figure.

⏸ **Pause point:** DNA can be stored at  $-80^{\circ}\text{C}$  for several months.

### Immunoprecipitation

⌚ **Timing:** 2 days

During this step the DNA labeled with BrdU is immunoprecipitated and purified.

22. Heat denature the DNA 2 min at  $95^{\circ}\text{C}$ . Move immediately on ice and spin down the contents when cold.

**Note:** The BrdU antibody only works on single-stranded DNA (see antibody's technical data sheet).

23. Take  $75\ \mu\text{L}$  of DNA, add  $45\ \mu\text{L}$  of  $10\times$  IP buffer ( $10\times$  PBS, 0.5% Triton-X100) and  $330\ \mu\text{L}$  of RNase free  $\text{H}_2\text{O}$ .
24. Wash  $50\ \mu\text{L}$  of Protein G magnetic beads with  $1\times$  IP buffer and resuspend in  $1\times$  IP buffer.

25. Add 50  $\mu\text{L}$  of washed beads and 2  $\mu\text{L}$  of anti-BrdU antibody to the DNA prepared in step 23. Incubate 2.5 h under rotation at 18°C–25°C in the dark.
26. Place the tubes on a magnetic rack and remove the supernatant. Wash the beads with 1 mL 1 $\times$  IP buffer, vortex thoroughly, place the tubes on the magnetic stand and remove the supernatant.
27. Repeat the previous step for 4 more washes.
28. Elute the samples by resuspending the beads in 200  $\mu\text{L}$  1 $\times$  IP buffer containing 250  $\mu\text{g}/\text{mL}$  proteinase K. Incubate 2 h at 55°C in the dark.
29. Take the eluates off the beads, add 200  $\mu\text{L}$  phenol/chloroform, vortex, centrifuge 1 min at maximum speed. Collect as much as the upper phase possible.

**Note:** Be careful not to transfer the interface.

30. Add 1/9 volume of 3 M sodium acetate pH 5.2, 2.5 volumes of ice cold 100% EtOH and 1  $\mu\text{L}$  of glycogen. Vortex and incubate o/n at  $-80^\circ\text{C}$ .

**Note:** Volume refers to the volume of upper phase collected in the previous step.

**▮▮ Pause point:** DNA can be precipitated at  $-80^\circ\text{C}$  indefinitely.

31. Centrifuge 17 min at maximum speed at 4°C. Remove the supernatant without disturbing the pellet.
32. Wash the pellet with 500  $\mu\text{L}$  of 70% EtOH, centrifuge 2 min at maximum speed at 4°C.
33. Remove the supernatant and air dry the pellet.
34. Resuspend the DNA in 75  $\mu\text{L}$  of 10 mM Tris pH 8.

**Note:** If the pellet is difficult to resuspend the tubes can be placed 20 min at 55°C.

35. *Optional step: qPCR verification of IP.* Set up 10  $\mu\text{L}$  qPCR reactions using 0.5  $\mu\text{L}$  of IP and 1% input (1% input: 0.75  $\mu\text{L}$  denatured DNA (non-IPed) diluted to 75  $\mu\text{L}$ ) and actin and satellite 2 primers. [Troubleshooting 4](#) and [5](#).

**Note:** Actin replicates early so the qPCR should be prominent in early S phases while satellite 2 replicates late so should be enriched in late S phases ([Black et al., 2010](#)).

**Note:** Actin genomic primers *forward* TCCAAAGGAGACTCAGGTCAG *reverse* CGCCCTTTCTCACTGGTTC, satellite 2 primers *forward* CATCGAATGGAAATGAAAGGAGTC *reverse* ACCATTGGATGATTGCAGTCAA.

**Note:** Expected qPCR results should be 1.5%–10% input in the fractions the regions are expected to replicate.

### Libraries preparation for sequencing

⌚ Timing: 1 day

During this step the DNA will be prepped to be sequenced with Illumina Nextseq 550 or related Illumina sequencer.

We used the Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences).

Swift Biosciences- <https://swiftbiosci.com/wp-content/uploads/2020/02/PRT-019-Methyl-Seq-Protocol-Rev-3.pdf>

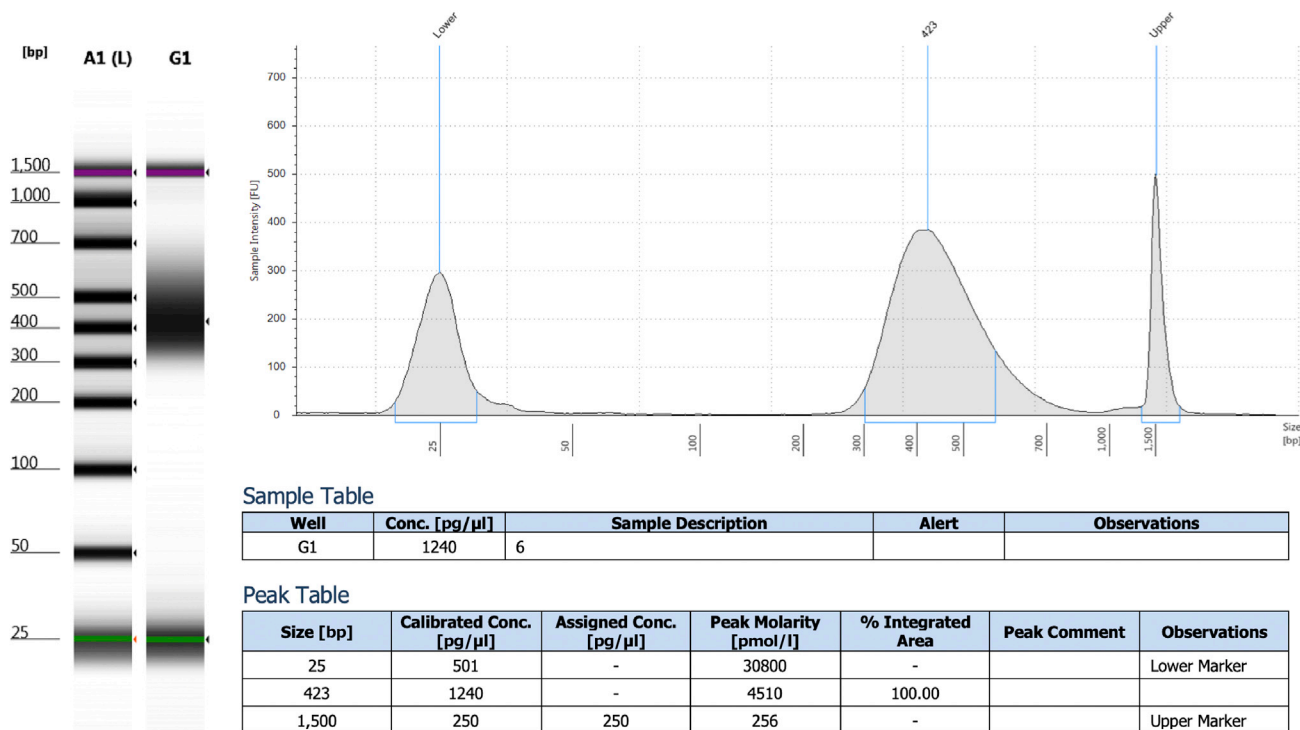


Figure 4. TapeStation analyses for a typical library performed with this protocol

**Note:** This is the only library prep kit that we optimized successfully with BrdU labeled DNA.

We are annotating the main steps below as an overview, please refer to the guide for details.

36. Start with 15 μL of immunoprecipitated DNA.
37. Denaturation (page 9).
38. Adaptase (page 9).
39. Extension (page 10).

**Note:** To cleanup follow the instructions for < 10 ng gDNA.

40. Ligation (page 10).

**Note:** To cleanup follow the instructions for gDNA.

41. Indexing PCR (page 11).

**Note:** We performed 7 PCR cycles. The numbers of PCR cycles could be increased slightly if very little DNA was obtained at step 21 (~2 cycles); however too many cycles will create PCR duplicates that will need to be removed from the sequencing results.

**Note:** To cleanup follow the instructions for gDNA.

42. Validate the libraries using a TapeStation or a Bioanalyzer and proceed to sequencing.

**Note:** A typical TapeStation result is presented in Figure 4.



**Note:** Libraries should yield at a minimum 800 ng/mL (Qubit concentration).

## EXPECTED OUTCOMES

Expected outcomes were annotated in the protocol. The final libraries should yield at a minimum 800 ng/mL (Qubit concentration).

## LIMITATIONS

N/A.

## TROUBLESHOOTING

### Problem 1

Step 1. Cells are clumpy during sorting.

#### Potential solution

Make sure to resuspend the cells in a single cell suspension and dilute more the cells before sort (sort in a higher volume).

### Problem 2

Step 21. DNA fragments above 300 bp after sonication.

#### Potential solution

Sonicate more.

### Problem 3

Step 21. No or very little DNA observed on TapeStation or Bioanalyzer after DNA extraction.

#### Potential solution

Collect more cells per fraction during the sort.

### Problem 4

Step 35. qPCR test of replication timing did not work: no amplification in the expected fractions.

#### Potential solution

Increase the labeling time with BrdU.

Increase the incubation time with the antibody.

### Problem 5

Step 35. qPCR test of replication timing did not work: amplification everywhere.

#### Potential solution

Increase the washes after IP: vortex more thoroughly and add more washes.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Capucine Van Rechem ([cvrechem@stanford.edu](mailto:cvrechem@stanford.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The published article includes all datasets and codes generated during this study.

### ACKNOWLEDGMENTS

This study is supported by R01GM097360 and R35GM144131 (J.R.W.), NIH/NCI Cancer Center Support grant P30 CA006927 (J.R.W.), and the American Lung Association Lung Cancer Discovery Award. Graphical Abstract created with [BioRender.com](https://www.biorender.com).

### AUTHOR CONTRIBUTIONS

C.V.R. and J.R.W. worked together to optimize the conditions that are described in this methods article. C.V.R. conducted the methods.

### DECLARATION OF INTERESTS

In the past year, J.R.W. has consulted for Qsonica (manufacturer of Q800R system). J.R.W. has served as a consultant or advisor for Salaria Pharmaceuticals, Daiichi Sankyo, and VYNE Therapeutics. J.R.W. has also received sponsored research from Salaria Pharmaceuticals. C.V.R. declares no competing interests.

### REFERENCES

Black, J.C., Allen, A., Van Rechem, C., Forbes, E., Longworth, M., Tschop, K., Rinehart, C., Quito, J., Walsh, R., Smallwood, A., et al. (2010). Conserved antagonism between JMJD2A/KDM4A and HP1gamma

during cell cycle progression. *Mol. Cell* 40, 736–748.

Van Rechem, C., Ji, F., Chakraborty, D., Black, J.C., Sadreyev, R.I., and Whetstone, J.R. (2021).

Collective regulation of chromatin modifications predicts replication timing during cell cycle. *Cell Rep.* 37, 109799.