HiFi-Slide spatial RNA-Sequencing V.2

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Human BioMolecular Atlas Program (HuBMAP) Method Development Community

Hi

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VERSION 2

MAY 10, 2023

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.3byl4by18vo5/v2

Protocol Citation: Tianyang Xu, Pei Lin, czhang, Diming Zhu, Riccardo Calandrelli, Sheng Zhong 2023. HiFi-Slide spatial RNA-Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl4by18vo5/v2Version created by Tianyang Xu

Protocol status: In development

We are still developing and optimizing this protocol

Created: Feb 06, 2023

Last Modified: May 10, 2023

PROTOCOL integer ID: 76435

Keywords: HuBMAP, TTD - UCSD/CoH, spatial transcriptome

ABSTRACT

HiFi-Slide sequencing is a super-resolution spatial transcriptomics sequencing technology. This technique captures and spatially resolves genome-wide RNA expression in a submicron resolution for fresh-frozen tissue.

MATERIALS

	A	B
	Name	Supplier, Catalog Number
	Dremel Diamond Cutter	Amazon, Allmuis
	Chambered Slides, 2 wells	Varies Suppliers
	Chambered Slides, 4 wells	Varies Suppliers
	Chamber Sealing Tape	ThermoFisher, 232702
	8 Well Proplate Module	EMS, 63484-45
	Cell Dish	Varies Suppliers
	BbsI Kit	NEB, R0539
	T4 DNA Ligase Kit	NEB, M0202
	Exonuclease I (E.coli) Kit	NEB, M0293
	Proteinase K Kit	NEB, P8107

	A	B
	Mayer's Hematoxilyn Solution	ThermoFisher, 008001
	Dako Bluing Buffer	Dako, CS70230-2
	Eosin Solution	Dako, CS70130-2
	Maxima H Minus Kit	ThermoFisher, K1652
	Klenow Frag. (exo-)	NEB, M0212
	PE-AlexaFluor conjugates	ThermoFisher, S11249
	dNTP Mix	NEB, N0447
	KAPA HIFI Hot Start Ready Mix	Roche Diagnostics, KK2602
	E-Gel 2% Agarose gel & 1kb ladder	ThermoFisher, G8142ST
	Monarch PCR Purification Kit	NEB, T1030L
	Monarch Gel Extraction Kit	NEB, T1020L
	Agilent TapeStation D5000 HS	Agilent, 5067-5592
	Thermoshaker incubator	Varies Suppliers
	Humidified Incubator	Modified from BenchMark, H2200

Hematoxylin

Slide Preparation

1 Flow Cell Generation and Collection

A recycled Illumina flow cell was used to provide spatial coordinates for RNA molecules.
For MiniSeq flow cells, a pre-run modification is necessary to replace the NaOCl solution with the Tween-20 solution in the reagent cartridge. We replaced the NaOCl solution inside the MiniSeq reagent cartridge (MiniSeq Mid Output Kit (300-cycles) FC-420-1004 or MiniSeq High Output Reagent Kit (300-cycles) FC-420-1003) by washing well #31 on the cartridge with 0.1% Tween-20 Buffer twice, then adding 1.3 mL buffer to the well. In addition, to prevent carry-over contamination between experiments, a manual wash with NaOCl solution is performed each time before using the machine.
For NextSeq 2000, no modification is required.
Collected flow cells are stored in the 4°C fridge prior to use.

2 Flow Cell Cutting

Take a photo of the flow cell surface before and after the cutting

1. Use a marker pen and carefully outline the edges for cutting.
2. Record the flow cell ID and label the flow cell on the bottom of the surfaces.
3. Use a Dremel tool with a diamond cutter and carefully remove the edges with high rpm.
4. Collect the surfaces and place them in chambered slides.

3 Terminal Priming

20m

Label the slide surfaces clearly before pooling

1. Prepare 500nM P7 primer solution in Low TE according to the slide size.
2. Immerse slides into the solution.
3. Incubate the tube at 95C for 5 minutes. Cool the tube at room temperature for 10 minutes.
4. Collect the surfaces and place them in chambered slides; use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

4 BbSI Digestion

12h

1. Prepare BbSI Mix by scaling up the following recipe:

Component	50uL RXN
BbSI (NEB, R0539)	1uL
10X NEBuffer r2.1 (10X)	5uL
Water	44uL

1. Place the slides into the tube with chilled BbSI Mix.
2. Incubate at 37C overnight, with gentle vortex if needed.
3. On the collection, place the slide on a chamfered slide and wash with pipetting water on the surfaces three times.
4. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

5 T4 Ligation

12h

1. Prepare dT solution with 100nM T4 Strand A and 100nM T4 Strand T in Low TE solution. Incubated at 95 C for 5 minutes and chilled at room temperature.
2. Prepare T4 Mix by scaling up the following recipe:

Component	20uL RXN
T4 DNA Ligase Buffer (10X)	2uL
T4 DNA Ligase (NEB, M0202)	1uL
dT Solution	1uL (5nM)
Water	16uL

1. Incubate the tube at 16C overnight. Add gentle vortex if needed.
2. Collect the surfaces and place them in chambered slides.
3. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for

temporary storage.

6 **Exonuclease I Treatment**

Incubation time shall be extended for pooled slides.

1. Prepare Exon Mix by scaling up the following recipe:

	Component	20uL RXN
	Exonuclease I (E. coli) (NEB, M0293)	2uL
	Exonuclease I Reaction Buffer	2uL
	Water	16uL

1. Place the slides inside the tube and incubate for 3 hours at 37C; add a gentle vortex if needed.
2. Collect the surfaces and place them in chambered slides.
3. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

7 **Elution**

1. Prepare 0.1 N NaOH solution with water.
2. In the cell dish or chambered glass, pipette enough NaOH solution to immerse the slide surface (NextSeq full: 150uL, half: 100uL).
3. Incubate at room temperature for 10 minutes
4. Use a pipette and remove all solutions.
5. Repeat NaOH washing three times.
6. Wash the surfaces with 200mM tris-HCl solution three times.
7. Wash the surfaces with water three times.
8. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

Tissue Application

8 **Tissue Sections**

Transport tissue applied slides with dry ice

Image GFP or other proteins fluoresce channel before submerging in MeOH

1. Prepare 1.5mL Methanol in tubes and store at -20°C.
2. Transfer the sample out of the storage and immerse it in an OCT medium with a mold.
3. While waiting for the OCT medium to fully solidify, turn on the cryostat machine and set the blade temperature to -35°C degree and chamber temperature to -20°C degree.
4. Once the sample block is fully solidified, use more OCT medium as a glue to anchor the sample block onto the specimen stage.
5. Transfer the specimen stage into the chamber and wait until the sample block is fully attached.
6. When the cryostat machine reaches its optimal temperature, place the specimen stage with the sample block onto the section stage. Start the sectioning process by trimming the block in a thicker blade setting until the sample is exposed. Make sure to clean the section stage when enough slices have been

accumulated.

7. Once the sample has been exposed to an ideal extent, adjust the blade set to the desired thickness. Carefully execute the blade to avoid slice breaking.
8. If the slice is not ideal, use a small brush to sweep off the slice and make another slice. During this process, keep an eye on the chamber and blade temperature since either too high or low can lead to slice melting/breaking.
9. When an ideal slice is sectioned, quickly flip open the glass cover slide and have the upside of the HIFI slide facing the slice.
10. The sample slice will stick onto the HIFI slide once a contact point has been established.
11. Capture any fluorescence image before the next step.
12. Retrieve the pre-chilled methanol from the storage, and immerse the slide into the tubes for 20 minutes.
13. Once the time is up, retrieve the slide out of the tube and wash the slide with water.
14. Allow the slide to air dry and proceed to the H & E staining procedure.

9 H&E

20m

1. The staining procedure is accomplished by pipetting just enough volume of the staining reagent to cover the sample and washing it with water each time after the incubation. Depending on the sample size, 50 to 100 uL reagents are used each time.
2. First, incubate the slide with Hematoxylin for 10 minutes, and dip the slide into a water reservoir to wash the slide. Be careful not to wash off any sample.
3. Then incubate the slide with a Bluing buffer for 5 minutes, and dip the slide into a new water reservoir.
4. Lastly, incubate the slide with Eosin for 2 minutes, and dip the slide into a new water reservoir to wash off any remaining staining reagent.
5. Allow the slide to air dry, and image the slide under a microscope. Capture and archive structural details for later reference.

10 Tissue Digestion

20m

Pre-warm the incubation instrument for higher efficiency

1. Prepare the proteinase K reaction mix by the following recipe:

Component	10uL RXN
Pepsin, reconstituted in 0.1M HCl	5uL
Water	5uL

1. Transfer the slides into their own individual chamber, and pipette 70uL Pepsin solution to cover the full slide and 50uL for the half slide.
2. Seal the chamber and place the chamber into the pre-warmed humidified incubation chamber for 10 minutes.
3. Remove the reaction mix with a pipette at the corner of the chamber. Wash the slide surface with 0.1X SSC one time and water two times. Remove extra liquid by gently pipetting.

11 Reverse-Transcription

12h

Add Visual TSO oligo for template switching RT and visualization of cDNA

1. Prepare Maxima H Minus Mix by scaling up the following recipe:

Component	20uL RXN

Maxima H Minus Enzyme Mix (TF, K1652)	1uL
Maxima H Minus Buffer (RT Buffer)	4uL
10 mM dNTP	1uL
Water	14uL
(Optional) Visual TSO 10uM Oligo	1uL, diluted in water

1. Add solution to the top of slide surfaces (150uL for full slide, 100uL for half slide). Incubated in a thermoshaker without vortexing or humidified chamber with sealed chambered slides at 42C overnight.
2. On the second day, remove the reaction mix by pipetting.
3. Wash the slide surface with water three times, and remove the extra liquid by pipetting.

12 Exonuclease I Treatment* 3h

1. Prepare Exon Mix by scaling up the following recipe:

Component	20uL RXN
Exonuclease I (E. coli) (NEB, M0293)	2uL
Exonuclease I Reaction Buffer	2uL
Water	16uL

1. Place the slides in separate chambered slides, and add Exon Mix to the top of the surfaces.
2. Incubation at 37C for 3 hours.
3. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

13 Elution I* 15m

1. Prepare 0.1 N NaOH solution with water.
2. In the cell dish or chambered glass, pipette enough NaOH solution to immerse the slide surface (NextSeq full: 150uL, half: 100uL).
3. Incubate at room temperature for 10 minutes
4. Use a pipette and remove all solutions.
5. Repeat NaOH washing three times.
6. Wash the surfaces with 200mM tris-HCl solution three times.
7. Wash the surfaces with water three times.
8. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

14 (cDNA Visualization) Optional 3h

1. Add 100uL 1uM Visual TSO -low TE solution to the slide surface (50uL for half slide).
2. Incubate at room temperature for 30 minutes.
3. Remove the solution by gentle pipetting, followed by washing with water three times, and centrifuge to dry.
4. Prepare Visual solution by diluting Alexa-streptavidin conjugate to 5ug/mL with 1X PBS.
5. Add 300 visual solutions to each slide individually.
6. Incubate for 2 hours in the dark at room temperature.
7. Remove the liquid by pipetting. Wash the surfaces with 1X PBS three times.

8. Place slides into a new transparent cell dish and remove extra liquid with pipetting.
9. Remove extra retentions on edges by KimWipes.
10. Image the slide with brightfield and fluorescence alternatively and stack the images.
11. After imaging, repeat the Elution I step to recover the slide for the next step.

15 Second-Strand Synthesis

3h

When scaling up the 2nd-strand mix, the concentration of random primer may be variated for optimizing outcomes.

1. Prepare Second-strand mix by scaling up the following recipe:

	A	B
	Component	20uL RXN
	Kelnow Frag, Exo- (NEB, M0212)	2uL
	NEBuffer 2	2uL
	10 mM dNTP	2uL
	Random Primer 0.3 (100uM)	1uL
	Water	13uL

1. Place the slides in separate chambered slides, and add Second Strand Mix to the top of the surfaces. For the full slide, use 150uL to 200uL. For half slide, use 100uL.
2. Incubation at 37C for 2 hours. Increasing incubation time if needed.
3. Use a pipette and remove all solutions. Wash the surfaces with water three times.
4. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.

16 Elution and Purification

1. Prepare 0.1 N NaOH solution with water.
2. In the cell dish or chambered glass, pipette enough NaOH solution to immerse the slide surface (NextSeq full: 50uL, half: 30uL).
3. Incubate at room temperature for 10 minutes
4. Use a pipette and move all liquids to a 1.5 mL tube. Label the tubes.
5. Repeat NaOH washing twice for half and twice for single slides. Pool the elutions to the same 1.5mL for each slide. The total volume shall be less than 150uL.
6. Wash the surfaces with 200mM tris-HCl solution three times.
7. Wash the surfaces with water three times.
8. Use a pipette to collect the extra liquid, then centrifuge to dry. Place the slides in a cell dish for temporary storage.
9. Add 10uL 3M sodium acetate to the tube, mix by vortex, and spin it down.
10. Purification with Monarch PCR & DNA purification kit, use 7:1 binding buffers and 30uL elution volume.
11. **Repeat the Second Strand Step to increase the combined diversity of random priming libraries. Combine the libraries after PCR purification. (Three to four times.)**

17 PCR *

1. Prepare Primer Mix by diluting P7 0.2 Primer and P5 0.3 Primer to 3uM.
2. With 30uL elution strands, prepared PCR with thefollowing recipe:

Component	50uL *2 RXN
Primer Mix	20uL
Elution DNA	30uL
Kapa HIFI Hotstart ReadyMix (Roche Diagnostics, KK2602)	50uL

1. Dispense 100uL reaction mix to 2 PCR tubes with 50uL each, then perform the initial PCR with

Initial	95C	180 sec	
Denature	98C	20 sec	5 Cycles
Anealing	65C	20 sec	
Extension	72C	180 sec	
Denature	98C	20 sec	20-22 Cycles
Anealing	65C	20 sec	
Extension	72C	180 sec	
Final Extension	72C	180 sec	
Hold	4C	Infinite	

1. Gathered PCR products and purified with Monarch PCR & DNA purification kit, using 4:1 binding buffers and 80uL elution volume.

18 Size Selection

Setup replicates adjacent to each other can increase product

1. Load eluted product to E-Gel EX 2% Agarose gel cartridge (or prepared 2% gels). Load 1kb+ ladder on edge.
2. Run the electrophoresis until clear separations.
3. Cut and isolate the bands within 400 to 2000 bp; the range is changed based on the recycled flow cell, with a minimum of 300 bp and a maximum of 3kb.
4. Use a Monarch Gel Extraction kit with 30uL elution volume to extract the cut gels.

19 PCR II(Optional)*

Conduct a second PCR only if the size selection concentration is not enough

1. Prepare Primer Mix by diluting P7 0.2 Primer and P5 0.3 Primer to 2uM.
2. With 30uL elution strands, prepared PCR with following recipe:

Component	50uL *2 RXN
Primer Mix	20uL
Elution DNA	30uL
Kapa HIFI Hotstart ReadyMix	50uL

1. Dispense 100uL reaction mix to 2 PCR tubes with 50uL each, then perform the initial PCR with

Initial	95C	180 sec	

	Denature	98C	20 sec	5 Cycles to 8 Cycles
	Anealing	70C	20 sec	
	Extension	72C	180 sec	
	Final Extension	72C	180 sec	
	Hold	4C	hold	

1. Gathered PCR products and purified with Monarch PCR & DNA purification kit, using 4:1 binding buffers and 80uL elution volume.

20 **Library QC and Sequencing**

1. Dilute eluted products with water 5 times and 10 times, and quantify with 1X Qubit dsDNA working solution.
2. Set up a TapeStation DNA 5K kit (Agilent, 5067) and estimate the average length.
3. Calculate the molecular amount of the diluted library and stock library.
4. Check the library size distribution complies with the size selection.
5. Prepare samples for sequencing.

Data Analysis

21 **Data processing and analysis**

HiFi Slide sequencing basecell files were converted to raw reads by illumina software bcl2fastq (version 2.20.0.422).
HiFi Slide R1 reads were indexed by BWA (version 0.7.17-r1188).
Then de-duplicated spatial barcodes from the recycled flow cell were mapped to R1 reads using BWA-MEM. It is necessary to invoke the BWA-MEM parameter "-a".
HiFi Slide R2 reads were mapped to human genome by STAR (version 2.7.5c).
Reads uniquely aligned to human genome were extracted from the resultant sam file.
We used bedtools (version 2.30.0) to identify genes mapped by HiFi Slide R2 reads.
Summary statistics such as number of spatially resolved HiFi Slide read pairs were calculated by in-house script. Visualization of results were performed by R (version 4.1.2).