Seq-Scope Protocol version M1.0.0. (released Jul-19-2021) doi: 10.7302/1674

Seq-Scope protocol in MiSeq system for profiling hepatic and colonic spatial transcriptome.

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Method described in:

Cho CS, Xi J, Si Y, Park SR, Hsu JE, Kim M, Jun G, Kang HM, Lee JH. Microscopic examination of spatial transcriptome using Seq-Scope. Cell. 2021 Jun 24;184(13):3559-3572

Version history:

M1.0.0: The current protocol is the initial release of "Seq-Scope protocol in MiSeq for profiling hepatic and colonic spatial transcriptome".

General Notes:

- The current protocol only contains experimental procedures for Seq-Scope. Please refer to the Cho et al. 2021 paper for computational and statistical analyses. The source codes for computational analyses can be found at Github (https://github.com/leeju-umich/Cho_Xi_Seqscope). Currently, all scripts in this Github repository are written based on the SeqScope sequencing structure as indicated in our SeqScope paper. The users will need to modify the scripts to make it compatible to their experimental design. A more flexible and user-friendly software tool is under active development, and once ready, will be announced through the same Github link.
- The current protocol was optimized for MiSeq platform and for analysis of normal mouse hepatic and colonic tissues. Analysis utilizing a different sequencing platform and different tissues may require further optimization.
- We will continue working on improving the protocol. Therefore, we plan to regularly update the protocol with our most recent experiences under different conditions. The current version has already been modified to include many notes, including tips and suggestions, that were developed after the publication of our original Seq-Scope paper cited above. The protocol has yet to be fully optimized, but it will serve as a good starting point for setting up the Seq-Scope system. We would be also very happy to get feedback from the community to improve the protocol. Please email us (leeju@umich.edu) if you have any questions or suggestions.
- Key Resources Table and General References are provided at the end of this protocol as appendix.

PART I. 1st-Seq: HDMI cluster generation & sequencing

I-1. Preparation of Sequencing Libraries and Primers for 1st-Seq

(1) HDMI-oligonucleotide Library Sequences

HDMI-DraI: CAAGCAGAAGACGGCATACGAGA TTCTTTCCCTACACGACGCTCTTCCGATCT NNVNNVNNVNNVNNVNNNNN TCTTGTGACTACAGCACCCTCGACTCTCGC TTTTTTTTTTTTTTTTTTTTTTTTTT TTTAAA GACTTTCACCAGTCCATGAT GTGTAGATCTCGGTGGTCGCCGTATCATT

HDMI32-DraI: CAAGCAGAAGACGGCATACGAGAT TCTTTCCCTACACGACGCTCTTCCGATCT NNVNBVNNVNNVNNVNNVNNVNNVNNVNNNNN TCTTGTGACTACAGCACCCTCGACTCTCGC TTTTTTTTTTTTTTTTTTTTTTTTTT TTTAAA GACTTTCACCAGTCCATGAT GTGTAGATCTCGGTGGTCGCCGTATCATT

Notes:

- In the Cho et al. 2021 paper, we primarily used the HDMI-DraI oligonucleotide, which has 20-mer HDMI. However, we found that HDMI32-DraI could be more useful because of several reasons. (i) Compared to HDMI-DraI, HDMI32-DraI has a longer HDMI sequence; therefore, the barcode duplication rate will be even lower. (ii) Longer random sequences may also help cluster identification in some 2nd-Seq sequencing platforms. (iii) HDMI32-DraI was designed to completely avoid the DraI digestion site, while HDMI-DraI still has a very low chance (~0.13%) of getting cut by DraI.
- Making these oligonucleotides can be challenging because they are very long (185bp in HDMI-DraI and 197bp in HDMI32-DraI). Therefore, we used the Ultramer service from IDT or the Extremer service from Eurofins. Both worked well in our experiences.

  https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos/ultramer-dna-oligos
  https://eurofinsgenomics.com/en/products/dnarna-synthesis/extremers/
- Upon receipt, the oligonucleotide libraries were reconstituted in the Ultrapure water. The libraries were diluted to 10 nM before being sent to the sequencing facility. No further processing was necessary as we directly used them as ssDNA libraries.

(2) Custom Read1 primer sequence

Read1-DraI: ATCATGGACTGGTGAAAGTC TTTAAA AAAAAAAAAAAAAAAAAAAAAAAAA GCGAGAGTCGAGGGTGCTGTAGTCACAAGA

Notes:

- Read1-DraI is long; however, it is not as long as the HDMI library oligonucleotides. Initially, we generated this using the Ultramer and Extremer options. However, we are now using a standard oligonucleotide synthesis options with the PAGE purification.
- Upon receipt, Read1-DraI primer was reconstituted in the Ultrapure water. The libraries were diluted to 100 μM stock before being sent to the sequencing facility.

I-2. HDMI Cluster Generation & Sequencing – 1st-Seq Running condition

Any standard sequencing facilities operating MiSeq should be able to run the 1st-Seq without major problems. Below are the instructions, so that they are able to produce 1st-Seq data and flow cells.

1) Library: ssDNA library of HDMI-DraI (Cho et al. 2021) or HDMI32-DraI (our preferred condition)

2) Custom Read 1 Primer: Read1-DraI

3) MiSeq v3 regular platform

4) Manual Mode Condition: 25bp single end reading without indexing (for HDMI-DraI) or 37bp single end reading without indexing (for HDMI32-DraI)

5) The run must be completed right after the first read (no denaturation or re-synthesis steps). Retrieve the flow cell right after the completion of the 37bp single-end reading. Routine after-run washing with water is fine.

6) Library loading concentration: This needs to be empirically determined. See the notes below. For liver and colon experiments in Cho et al. 2021, we used 100 pM (HDMI-DraI).

7) Output – Obtain the standard FASTQ file that contains tile and XY coordinate information along with the sequencing information, as well as all raw and temporary files saved in MiSeqTemp, MiSeqOutput and MiSeqAnalysis folders.

9) Flow cell -- After running, recover the flow cell and store it in the original buffer or in water.

Notes:

- HDMI-DraI and HDMI32-DraI produces extreme nucleotide diversity because Read1 will consist of the random sequence (HDMI) plus 5 conserved bases. Therefore, PhiX spike-in is not necessary. Do NOT add PhiX because it will decrease HDMI cluster concentration and may interfere with downstream processes.
- Library loading concentration needs to be empirically determined. ssDNA libraries (HDMI-DraI and HDMI32-DraI) seem to behave quite differently from dsDNA. In addition, there are batch-to-batch variations in the capabilities of the oligonucleotide library in generating clusters in MiSeq. We suggest that the titration of library concentration is performed with 60pM, 80pM, 100pM and 120pM. The concentration showing more than 1 million passing filter (PF) clusters per mm^2 would be ideal. We were able to obtain up to 1.5 million PF clusters per mm^2 in our titration experiments. Once the concentration is titrated, the same concentration can be used for the whole library in the same batch; the variation between sequencing runs is relatively minimal. Since MiSeq v3 regular flow cell is costly, the titration experiments can be done in MiSeq v2 nano flow cell, whose price is much lower than MiSeq v3. According to our experiences, cluster forming capabilities of a single library were similar between MiSeq v2 and MiSeq v3.
- FASTQ file (the 1st-Seq run only produces Read1 file) is sufficient for the downstream analysis. However, we routinely obtain the whole output folders so that we can analyze the sequencing quality statistics in the Illumina Sequence Analaysis Viewer (SAV). In an ideal running, PF clusters will be over 1 million, and the rate of PF/total clusters will be over 80%. %Q30 will be over 95%. Q30 may be low in the last 5-base of Read1 sequence because of the low sequence diversity.
- From our experience, the 1st-Seq completed flow cell was stable at 4℃ for around a month.

PART II. Flow Cell Processing to Generate HDMI-Array

II-1. Chemical Flow Cell Processing

Using the flow cells recovered from 1st-Seq, run this section to expose the oligo-dT domain for RNA capture.

1. Wash the flow cell with 20 µl of Ultrapure water three times. Between each solution change, empty the flow cell by gently pipetting out the previous solution.
2. Load the flow cell with DraI cocktail to cut out the P5 sequence and expose oligo-dT

DraI cocktail (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
10x CutSmart buffer	1x	2
Dra I (20 U/µl)	2 U/µl	2
Ultrapure water		16
Total		**20**

3. Incubate at 37℃ overnight (16-20 hours)
4. Load the flow cell with Exonuclease I cocktail to eliminate non-specific ssDNA. HDMI-DraI will still make a duplex with Read1-DraI, so it will be protected.

Exonuclease I cocktail (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
10x Exo I Buffer	1x	2
Exo I (20 U/µl)	1 U/µl	1
Ultrapure water		17
Total		**20**

5. Incubate 45 min at 37℃.
6. Wash the flow cell in the following order
    a. Wash the flow cell with Ultrapure water three times.
    b. Wash the flow cell with 20 µl of 0.1N NaOH three times (each with 5 min incubation at room temperature).
    c. Wash the flow cell with 20 µl of 0.1M Tris (pH7.5) three times.
    d. Wash the flow cell with 20 µl of Ultrapure water three times.
7. The flow cell is now ready for physical disassembly. The flow cell can be stored at 4℃ for several days

Notes:

- Video demonstration of the liquid handling in this step can be found at the following link. https://www.youtube.com/watch?v=fhND2zxziBI
- It is very important to eliminate all the bubbles before 37℃ incubation. Bubbles may interfere with digestion processes, leading to loss of information in the corresponding area.
- The inlet/outlet holes in the flow cell can be sealed using Scotch Heavy Duty Packaging Tape for 37℃ incubation to prevent evaporation. For sealing the holes, remove the plastic adapter encasing the glass flow cell. A humidity chamber can be also used.
- After MiSeq sequencing, the P5 primer lawn on the flow cell is 3'-phosphorylated, which will render them resistant to the Exonuclease I digestion. Adding 1 µl of calf intestinal phosphatase to the Exonuclease I cocktail may help eliminate the P5 primer lawn.
- 0.1N NaOH wash denatures the dsDNA structure and removes the complementary strand. 0.1M Tris (pH7.5) wash neutralizes the solution, and water wash removes the salt. For our previous experiments, these washing steps were conducted three times to completely eliminate the complementary strands.

II-2. Physical Flow Cell Disassembly

1. Prepare 1.5% agarose suspension (15 mg in 1 mL) in Ultrapure water.
2. Incubate it in 95°C (5 min) to dissolve the Agarose.
3. Load the 1.5% melted agarose solution into the flow cell before it is chilled.
4. Remove the plastic adapter encasing the flow cell, and seal the inlet/outlet holes with Scotch Tape.
5. Keep the flow cell at room temperature for 15 min.
6. Using the Tungsten Carbide Tip Scriber, remove the top glass layer to expose the lower layer for tissue attachment. The top-exposed flow cell will be referred to as "HDMI-slide". All these procedures should be conducted under a stereomicroscope.
    a. Score all the boundary lines of the channel (corresponding to the imaging area) using the Tungsten Carbide Tip Scriber.
    b. Score additional lines inside of the boundaries to help break the glass into small pieces.
    c. Using the scriber, apply the pressure around the scored lines to break the glass out. Large glass particles can be removed by fine forceps
    d. Then, remove the glass particles and agarose debris by washing with water. If some glass particles are not removed by water pressure, use fine forceps to carefully remove them without damaging the bottom.
    e. Aspirate and dry-off the extra water in the flow cell channel using a vacuum.
    f. The top-exposed flow cell (HDMI-slide) is now ready for tissue attachment.

Notes:

- Although not absolutely necessary, the flow cell can be placed on a slide warmer (temperature set around 60°C) when melted agarose solution is loaded. This can help prevent premature solidification of agarose in the flow cell.
- Video demonstration of the flow cell disassembly can be found at the following links:

  https://www.youtube.com/watch?v=b2aCu_1Tl-8 (Step 6a-6c)
  https://www.youtube.com/watch?v=GGDb6tdCiJw (Step 6d)
  https://www.youtube.com/watch?v=RNmq_0tuDA0 (Step 6e)
  https://www.youtube.com/watch?v=LyxcCubuSmA (Step 6f)
- The disassembly process can be practiced with used MiSeq flow cells, which can be obtained as a byproduct of conventional sequencing (typically obtainable with no cost). After the practice flow cell disassembly, the quality of cluster arrays can be inspected by staining with DNA dye, such as SYBR Gold. An exemplary SYBR Gold staining image of the disassembled flow cell with minimal array damage was provided as a reference (See Figure S2K in Cho et al. 2021). It is critical to avoid scratches that damage the HDMI cluster array.

PART III. Tissue Attachment and Imaging

III-1. Tissue attachment

1. Place the OCT-mounted fresh frozen tissue in a cryostat (Leica CM3050S, -20℃) for 20 minutes
2. Slice the tissue at a 5° cutting angle at 10 μm thickness
3. Place HDMI-slide on the cutting stage and maneuver tissue onto the HDMI-slide.
4. Immediately thereafter, melt the tissue onto the HDMI-slide by moving the tissue-attached HDMI-slide off the stage and placing a finger on the bottom side of the HDMI-slide.
5. Repeat the tissue attachment until the HDMI-slide surface is fully covered by tissue sections.

Notes:

- MiSeq imaging areas are arranged in a long rectangle. To generate a long rectangular section, it is helpful to make a notch on the block using a blade. Video demonstration of the tissue section procedure can be found at the following links.

  https://www.youtube.com/watch?v=7Fs4taviqYI (3-1. Tissue Sectioning Preparation)

  https://www.youtube.com/watch?v=rgJSWWQK-Oc (3-2. Tissue Sectioning)

  https://www.youtube.com/watch?v=N4BX7s8FM3c (3-3. Tissue Attachment)
- It might be necessary to adjust the thickness of the tissue slice for different tissues. For mouse liver and colon, 10 μm thickness worked well.

III-2. Fixation

1. Cover the tissue sections on the HDMI-slide with 4% formaldehyde (100 μl, diluted from EM-grade 16% paraformaldehyde), and incubate for 10 min.

4% formaldehyde (make fresh)		
Reagent	**Final concentration**	**Volume (μl)**
16% paraformaldehyde	4%	5
1x PBS		15
Total		**20**

2. Wash the HDMI-slide with PBS, 3 times

Notes:

- Video demonstration of liquid handling on the HDMI-slide (disassembled HDMI-array) can be found at the following link.

  https://www.youtube.com/watch?v=8I9ayK6Q-UU

III-3. H&E staining

1. Add 40 µl of isopropanol to the tissues on the HDMI-slide, and incubate for 1 min, then remove the solution and dry the tissues.
2. Add 40 µl hematoxylin, and incubate for 5 min
3. Wash the HDMI-slide with Ultrapure water until the hematoxylin dye is completely removed.
4. Add 40 µl bluing buffer, and incubate for 2 min.
5. Wash the HDMI-slide with Ultrapure water 3 times.
6. Add 40 µl buffered eosin onto tissue section.

Buffered eosin (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
Eosin	1/10	4
0.45M Tris-Acetate buffer pH 6.0		36
Total		**40**

7. Wash the slide with Ultrapure water 3 times.
8. Let the HDMI-slide air-dry at room temperature for 10 min. Dry the tissues completely with no residual water.
9. Add 60 µl of 85% glycerol onto the tissues and slowly place a coverslip on top of the HDMI-slide,
10. Take images of the tissue samples under an optical microscope.
11. Remove the coverslip by dipping the HDMI-slide in Ultrapure water a few times.
12. Then add 80% ethanol and wash it out, then let it air-dry

Notes:

- Before optical imaging, fiducial marks could be introduced to the MiSeq imaging area so that it can facilitate the alignment of optical images and transcriptome images. Fiducial marks can be made by intentionally scratching the imaging area surface with the Tungsten Carbide Tip Scriber in a unique and specific pattern. The glass scratches are visible in optical images, and detectable in transcriptome images as the HDMI loss pattern.
- It is desired that the entire MiSeq imaging area is imaged under a microscope as a stitched image. This will facilitate the tissue boundary detection and image alignment.

PART IV. RNA Capture and Reverse Transcription

IV-1. Pre-permeabilization (collagen I treatment)

1. Pre-warm 249 µl of HBSS at 37°C. Add 1 µl of collagenase I (50 U/µl) to the tube just before use, and mix well by pipetting.

Collagenase I-HBSS solution (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
collagenase I (50 U/µl)	0.5 U/µl	1
1X HBSS		249
Total		**250**

2. Pipette 20 µl of collagenase I-HBSS solution onto the HDMI-slide, then leave the HDMI-slide in humidified chamber (petri dish with water drops) and incubate it at 37℃ for 20 min.

IV-2. Permeabilization and RNA Capture (pepsin treatment)

1. Mix 1 µl of pepsin (0.1 g/1 ml) with 99 µl of 0.1M HCl and prewarm at 37℃.

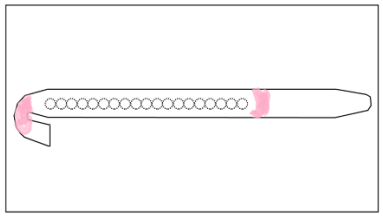
Pepsin solution (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
pepsin (0.1 g/ml)	1 µg/µl	1
0.1M HCl		99
Total		**100**

2. Add 20 µl of pepsin solution onto the HDMI-slide and incubate at 37℃ for 10 min

Notes:

- The pre-permeabilization and permeabilization protocols described above worked well for profiling liver and colon spatial transcriptome. However, different tissues may require different methods for tissue permeabilization and RNA capture. The tissue optimization conditions obtained from the other spatial transcriptomics platforms (e.g. the method described in Salmen et al. 2018 or the method offered by 10X VISIUM Tissue Optimization) might work well for the Seq-Scope experiments, because the biochemical principles of tissue permeabilization, RNA release, and reverse transcription are similar between the methods.

- To save the solution and make sure that the tissue coverage and solution exchange are complete, it could be helpful to use a DAKO pen to limit the area covered by the enzymatic solutions. For instance, in the HDMI-slide diagram below (circles represent the MiSeq imaging areas), DAKO could be applied to the pink-colored area, so that enzymatic solutions will only cover the MiSeq imaging areas, which are overlaid by the tissues.



IV-3. Reverse Transcription

1. Wash the slide with 40 µl RT Buffer once.

RT Buffer (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
Maxima 5x RT Buffer	1x	8
RNase Inhibitor (40 U/ µl)	1 U/µl	1
Ultrapure water		31
Total		**40**

2. Subsequently, perform the first strand synthesis by incubating the tissue-attached HDMI-slide in RT solution for overnight at 42℃ in a humidified chamber.

RT reaction solution		
Reagent	**Final concentration**	**Volume (µl)**
Maxima 5x RT Buffer	1x	8
RNase Inhibitor (40 U/µl)	1 U/µl	1
20% Ficoll PM-400	4%	8
10 mM dNTPs	1mM	4
Maxima H- RTase(200 U/µl)	10 U/µl	2
Ultrapure water		17
Total		**40**

3. Remove the RT solution
4. The HDMI-slide will be overlaid with Exonuclease I cocktail (see above for composition, scale up to 40 µl) to eliminate DNA that does not hybridize with mRNA. Incubate 45 min at 37℃ in a humidified chamber.

Notes:

- A humidified chamber can be made through many different methods. Video demonstration of humidified chamber setup can be found at the following link. In this demonstration, the humidified chamber was constructed using multiple layers of Frame-Seal incubation chambers (Bio-Rad, #SLF3001). Other configurations that maintain humidity should also work. https://www.youtube.com/watch?v=LwpquB7bHqw

IV-4. Tissue Removal

1. Remove the Exonuclease I cocktail solution.
2. Add 40 µl of 1x tissue digestion buffer to the tissue-HDMI-slide and incubate the mixture for 40 minutes at 37℃ in a humidified chamber.

Tissue digestion buffer (make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
1M Tris-Cl pH 8.0	100 mM	4
2M NaCl	200 mM	4
20% SDS	2%	4
0.1M EDTA	5 mM	2
Proteinase K (800 mU/µl)	16 mU/µl	0.8
Nuclease -free water		25.2
Total		**40**

3. Wash the flow cell in the following order
    a. Wash the flow cell with Ultrapure water three times.
    b. Wash the flow cell with 20 µl of 0.1N NaOH three times (each with 5 min incubation at room temperature).
    c. Wash the flow cell with 20 µl of 0.1M Tris (pH7.5) three times.
    d. Wash the flow cell with 20 µl of Ultrapure water three times.
4. The reverse transcription-completed HDMI-slide (RT-slide) is now ready for library preparation.

Notes:

- When a fibrous tissue is examined, pre-treating samples with β-mercaptoethanol may promote tissue removal. See Salmen et al. 2018 for the β-mercaptoethanol treatment procedure.

PART V. 2nd-Seq Library Preparation

V-1. Secondary Strand Synthesis

1. Prepare 40 µl of Primer Extension Mix (PEM).

Primer Extension Mix (PEM, make fresh)		
Reagent	**Final concentration**	**Volume (µl)**
NEBuffer-2		3
100 µM Randomer	10 µM	3
10 mM dNTPs	1 mM	3
Klenow exo (-) Fragment (5 U/µl)	0.5 U/µl	3
Ultrapure water		18
Total		**30**

2. Cover the RT-slide surface with PEM and put in a humidity chamber.
3. Incubate PEM solution/RT-slide at 37℃ for 2 hr.
4. Wash the RT-slide surface with Ultrapure water 3 times.
5. Load the RT-slide with 30 µl of 0.1N NaOH
6. After 5 min, recover 30 µl of random primer extension (RPE) product.
7. Repeat (5-6) to get another 30 µl of RPE product (total 60 µl).
8. Mix 60 µl of RPE product with 30 µl of neutralization buffer (3M potassium acetate, pH5.5 – adjusted using acetic acid). Proceed directly to next step.

Notes:

- The Randomer sequence we used was 5'-TCAGACGTGTGCTCTTCCGATCTNNNNNNNNN-3'. Considering that our datasets described in Cho et al. 2021 contained many poly-A transcripts, the randomer sequence could be modified to avoid the annealing to the poly-A region (oligo-dT in the first cDNA strand). For instance, 5'-TCAGACGTGTGCTCTTCCGATCTNNNNNNNNB-3' could be used to avoid annealing to oligo-dT. Also, 5'-TCAGACGTGTGCTCTTCCGATCTNNNGGNNNB-3', which is similar to the randomers used in Seq-Well S^3 (Hughes et al. 2020) or Slide-SeqV2 (Stickels et al. 2021) could also be used instead to promote annealing to the complex cDNA sequences.
- The RT slide can theoretically be recycled for another round of secondary strand synthesis, potentially diversifying the library content. However, we do not recommend doing this because the yield is generally lower, and the library will generate a completely new set of UMI from the same molecules, making further data analysis complicated.

V-2. Purifying RPE product

1. In a new 15 mL conical tube, prepare 10 mL of fresh 80% (v/v) ethyl alcohol by pipetting 8.0 mL of absolute ethyl alcohol to 2.0 mL of Ultrapure water. Vortex the tube for 10 seconds.
2. Bring AMPure XP magnetic beads to room temperature (15℃ to 25℃). Vortex the AMPure XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
3. Bring the RPE product up to exactly 100 µl with Ultrapure water and transfer to a 1.5-mL Lobind tube.
4. Pipet 180 µL of AMPure XP magnetic beads into the tube containing the 100 µL of RPE product. Pipet-mix at least 10 times.
5. Incubate the suspension at room temperature for 10 minutes.
6. Place the suspension on the 1.5-mL tube magnet for 5 minutes. Remove the supernatant.
7. Keeping the tube on the magnet, gently add 1 mL of fresh 80% ethyl alcohol to the tube.
8. Incubate the sample on the magnet for 30 seconds. Remove the supernatant.
9. Repeat the 80% ethyl alcohol wash for a total of two washes.
10. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.
12. Remove the tube from the magnet and pipet 40 µL of nuclease-free water into the tube. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
13. Incubate the sample at room temperature for 2 minutes. If the solution is splashed to the wall, briefly centrifuge the tube to collect the contents at the bottom.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product.

Notes:

- 80% ethyl alcohol should be made fresh and used within 24 hours.

V-3. RPE PCR

1. Run RPE PCR in two PCR tubes, each with 50 µL volume.

RPE PCR mix (make fresh, for two PCR tubes)		
Reagent	**Final concentration**	**Volume (µl)**
2x Kapa HiFi Hotstart Readymix	1x	50
100 µM RPEPCR*F primer	1 µM	1
100 µM RPEPCR*R primer	1 µM	1
Purified RPE product		40
Ultrapure water		8
Total		**100**

RPE PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	13-15 cycles
Annealing	60 °C	1 min	
Extension	72 °C	1 min	
Final extension	72 °C	2 min	1
Hold	4 °C	Open-ended	

V-4. Purification of the RPE PCR amplification product

1. Combine the two RPE PCR reactions into a new 1.5-mL tube.
2. Briefly centrifuge the tubes with the RPE PCR product. Bring the volume up to exactly 120 μL with Ultrapure water.
3. Bring AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex the AMPure XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
4. Pipet 120 μL of AMPure XP magnetic beads into the tube containing 120 μL of RPE PCR product. Pipet-mix at least 10 times, then briefly centrifuge the samples.
5. Incubate the suspension at room temperature for 5 minutes.
6. Place the suspension on the strip tube magnet for 3 minutes. Discard the supernatant.
7. Keeping the tubes on the magnet, gently pipet 200 μL of fresh 80% ethyl alcohol to the tube.
8. Incubate the samples for 30 seconds on the magnet. Remove the supernatant.
9. Repeat the 80% ethyl alcohol wash for a total of two washes.
10. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.
12. Remove the tube from the magnet and pipet 40 μL of nuclease-free water into the tube. Pipet-mix the suspension at least 10 times until beads are fully suspended.
13. Incubate the samples at room temperature for 2 minutes. Briefly centrifuge the tubes to collect the contents at the bottom.
14. Place the tubes on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 μL) into new 1.5-mL LoBind tubes. The RPE PCR product is ready for Index PCR.
16. Examine the eluate using Qubit. Estimate the molar concentration of the RPE PCR product (see Notes).

Notes:

- The RPE PCR libraries can be stored at –20°C for up to 6 months or 4°C for up to 6 weeks.
- For successful experiments, library concentrations were between 10-30 ng/μL. But it will be possible to use libraries with lower concentrations as long as they are amplifiable in the indexing PCR.
- (optional) After Qubit quantification, RPE PCR libraries can be examined through agarose gel running or bioanalyzer. Typically, the gel running pattern shows a broad smear between ~200 bp to 2,000 bp.
- The molar concentration of the library can be calculated using the following formula.
  (concentration ng/μL) / (660 g/mol * average library size bp) * 10^6 = (concentration nM)
  For most RPE PCR, we roughly estimate the average library size to be 500 bp. So, the estimation formula can be simplified as below.
  (concentration ng/μL) * 3 = (concentration nM)
  This molar concentration will be used in the following indexing PCR step to calculate the amount of template necessary to add in the PCR reaction.

V-5. Indexing PCR

1. Dilute the purified RPE PCR product to 2 nM, if higher than 2 nM.
2. Run RPE PCR in eight PCR tubes, each with 40 µL volume.

Indexing PCR mix (make fresh, for each tube)		
Reagent	**Final concentration**	**Volume (µl)**
2x Kapa HiFi Hotstart Readymix	1x	20
10 µM WTA1*F primer	1 µM	4
10 µM WTA1*R primer	1 µM	4
2 nM Purified RPE PCR product	0.5 nM (or lower if the product is limited)	10
Ultrapure water		2
Total		**40**

RPE PCR cycling conditions			
Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	8-9 cycles
Annealing	60 °C	30 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	2 min	1
Hold	4 °C	Open-ended	

V-6. Final Size Selection

1. Mix 40 µl of indexing PCR product with 8 µl of 6x orange gel loading dye
2. Load the mixture on 2% agarose gel. Run until the orange dye is at the bottom of the gel.
3. Incubate the gel in 1x SYBR Gold staining solution.
4. Cut the gel between 400-850bp in size, on a blue light illuminator.
5. Gel elution using the Zymogen Gel DNA Recovery kit according to the manufacturer's recommendation
6. Final elution with 40 µL of Ultrapure water
7. Add ~60 µL of Ultrapure water to the Indexing PCR product to make a final volume of 100 µL.
8. Transfer 100 µL of Indexing PCR product into a new 1.5-mL tube.
9. Bring AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex the AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
10. Add 75 µL of AMPure XP magnetic beads to the 1.5-mL tube from step 2.
11. Pipet-mix at least 10 times, then briefly centrifuge the samples.
12. Incubate the suspensions at room temperature for 5 minutes.
13. Place the suspension on the strip tube magnet for 3 minutes. Discard the supernatant.
14. Keeping the tubes on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the tube.
15. Incubate the samples for 30 seconds on the magnet. Remove the supernatant.
16. Repeat the 80% ethyl alcohol wash for a total of two washes.
17. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
18. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.
19. Remove the tube from the magnet and pipet 40 µL of Ultrapure water into the tube. Pipet-mix the suspension at least 10 times until beads are fully suspended.

20. Incubate the samples at room temperature for 2 minutes. Briefly centrifuge the tubes to collect the contents at the bottom. Place the tubes on the magnet until the solution is clear, usually ~30 seconds.
21. Pipet the eluate (~40 μL) into new 1.5-mL tubes.
22. Repeat the steps 7-21
23. Examine the eluate using Qubit.

Notes:

- 6x orange loading dye can be purchased (NEB B7022S) or made in house (doi:10.1101/pdb.rec11112)
- To prevent contamination, make sure that gel tank and imaging station are thoroughly cleaned. Use a new blade for cutting the gel.
- The goal of this procedure is to produce 2nd-Seq library whose size is roughly between 300-1,000 bp. Therefore, alternative size selection methods (e.g. bead-based double-sided size selection) could be also used. But we found that the agarose-based size selection was the most robust and gave higher yield.
- The final libraries can be stored at -20°C for up to 6 months until sequencing.
- Sequencing companies ask to submit at least 10 uL of 4 nM (HiSeq4000) or 30 uL of 3 nM (NovaSeq) library. Bioanalyzer or Tapestation QC should be performed before the library loading.
- If contaminant bands are still observed at the low molecular weight, gel extraction and/or 0.75X AMPure XP purification could be repeated to further remove them.

Appendix

A. Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
DraI enzyme	NEB	R0129
Exo I enzyme	NEB	M2903
Agarose	Fisher	BP160
Paraformaldehyde	Electron Microscopy Sciences	15170
Isopropanol	Sigma-Aldrich	19516
Hematoxylin	Agilent	S3309
Bluing buffer	Agilent	CS702
UltraPure Distilled water	Invitrogen	10977-015
Eosin	Sigma	HT110216
Collagenase I	Thermo Fisher	17018-029
Pepsin	Sigma	P7000
Maxima 5x RT Buffer	Thermo fisher	EP0751
Maxima H- RTase	Thermo fisher	EP0751
RNase Inhibitor	Lucigen	30281
Ficoll PM-400	Sigma	F4375-10G
dNTPs	NEB	N0477L
Proteinase K	NEB	P8107S
Klenow Fragment (exonuclease-deficient)	NEB	M0212
AMPure XP purification	Beckman Coulter	A63881
Kapa HiFi Hotstart Readymix	KAPA Biosystems	KK2602
Zymoclean Gel DNA Recovery Kit	Zymo Research	D4001
dNTP Solution Set	NEB	0446S
Oligonucleotides		
HDMI-DraI : CAAGCAGAAGACGGCATACGAGATTCTTTCCCTACACGACGCTCTTCCGATCTNNVNNVNNVNNVNNVNNNNNTCTTGTGACTACAGCACCCTCGACTCTCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAGACTTTCACCAGTCCATGATGTGTAGATCTCGGTGGTCGCCGTATCATT		
Read1-DraI : ATCATGGACTGGTGAAAGTCTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCGAGAGTCGAGGGTGCTGTAGTCACAAGA		
Randomer: TCA GAC GTG TGC TCT TCC GAT CTNNNNNNNNN	Mixed bases are achieved by handmixing of 25% per nucleotide content	
RPEPCR*F: TCT TTC CCT ACA CGA CGC*T*C	Stars denote phosphorothioate bonds, which are resistant to 3'-to-5' proofreading exonuclease activities of high-fidelity DNA polymerases.	
RPEPCR*R: TCA GAC GTG TGC TCT TCC*G*A		
WTA1*F: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CT*T *C		
WTA1*R: CAA GCA GAA GAC GGC ATA CGA GAT [8-mer index sequence] GTG ACT GGA GTT CAG ACG TGT GCT CTT CC*G*A		
Other		
Tungsten Carbide Tip Scriber	IMT	IMT-8806,
Frame-Seal incubation chambers	BIORAD	SLF-3001

B. References

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- 10X Genomics Visium Spatial Gene Expression Reagent Kits User Guide
- BD Rhapsody System Whole Transcriptome Analysis Protocol
- Illumina MiSeq System Guide
- Agencourt AMPure XP Information For Use Guide, PCR Purification

C. Additional Resources

- Seq-Scope Resources Summary: https://lee.lab.medicine.umich.edu/seq-scope
- Custom Codes used for Seq-Scope Analysis: https://github.com/leeju-umich/Cho_Xi_Seqscope
- Video Demonstration of Key Procedures: https://www.youtube.com/playlist?list=PLRwwF9JZ_f5P7wXjgt90o52Jz9JyMYWf4