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Library Generation using Slide-seqV2 V.2

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DISCLAIMER

ABSTRACT

This is a protocol detailing the steps necessary to generate libraries using previously manufactured Slide-seq arrays.

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MATERIALS TEXT

LIBRARY PREPARATION

- 1.5 mL Eppendorf LoBind Tubes

- UltraPure SSC, 20X (Invitrogen, 15557044)
- UltraPure Distilled Water (Invitrogen, 10977015)
- RNase-Free TE Buffer, 20X (ThermoFisher, T11493)

- NxGen RNase Inhibitor (Lucigen, F83923-1)
- Maxima H minus Reverse Transcriptase + Maxima 5X RT Buffer (Thermo Scientific, EP0752)

- Proteinase K, Molecular Biology Grade (New England BioLabs, P8107S)

- Deoxynucleotide (dNTP) solution mix (New England BioLabs, N0447L)
- Exonuclease I and 10X reaction buffer (New England BioLabs, M0293L)
- Sodium Hydroxide (NaOH), 10N aq. (Fisher Scientific, J63736)

- Klenow Fragment (New England BioLabs, M0212L)

- Terra PCR Direct Polymerase Mix (Takara, 639270)

- AmPure XP (SPRI beads) (Beckman Coulter, A63881)
- Qubit dsDNA HS Assay Kit (Thermofisher, Q32851)
- Bioanalyzer High Sensitive DNA kit (Agilent, 5067-4626)

- Nextera XT DNA Library Prep Kit (Illumina, FC-131-1096)

SOLUTIONS

6x SSC (50 mL)

15 mL 20x SSC
35 mL ultrapure water

Tissue Clearing Buffer (50 mL)

Reagents:	*Final Concentration:*
5 mL Tris (1M stock, pH 7.5)	100 mM
2 mL NaCl (5M stock)	200 mM
5 mL SDS (20% w/v stock)	2% (w/v)
500 µL EDTA (0.5M stock)	5 mM
36.5 mL ultrapure water	
(add ProK 1:50 before use)	

TE-TW (50 mL)

2.5 mL 20x TE buffer
50 µL Tween-20 (10% solution, 0.01% final concentration)
47.5 mL ultrapure water

OLIGONUCLEOTIDE SEQUENCES

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A	B
Template Switch Oligo	AAGCAGTGGTATCAACGCAGAGTGAATrG+GrG
Truseq PCR Primer	CTACACGACGCTCTTCCGATCT
SMART PCR Primer	AAGCAGTGGTATCAACGCAGAGT
Truseq-P5 Hybrid	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
dN-SMRT	AAGCAGTGGTATCAACGCAGAGTGANNNGGNNNB

DISCLAIMER:

Tissue Sectioning and RNA capture

1 Equilibrate fresh frozen tissue to -20 °C in a cryostat for ~20 minutes prior to sectioning. The tissue can be mounted onto a chuck using OCT, and the tissue should be aligned and sectioned as is standard in other protocols. Sections for Slide-seq should be 10 µm in thickness.

2 Carefully with thin forceps, place a Slide-seq array (puck) on a glass slide and set it in the cryostat to cool. Once cold, a tissue section should be moved onto the puck using a brush. Take care to position the target region of interest over the puck if the tissue section is larger than the puck.

3 Once the tissue is positioned correctly, lift the glass slide and place a finger on the slide underneath where the puck and tissue are located. As the region warms, the tissue will melt onto the puck. To avoid curling of the tissue, it's best to start from one side and slowly move your finger across the region rather than warming it from the center.

4 Using forceps, move the puck into a 1.5 mL tube containing 200 µL of hybridization buffer. Note that from this point forward, all steps must be performed using Eppendorf DNA Lo-bind tubes or equivalent in order to prevent beads from sticking to the sides of the tubes.

4.1 *Hybridization Buffer per puck:*
190 µL 6x SSC
10 µL RNase Inhibitor

Total volume 200 µL

Library Preparation

5 Incubate each puck in the hybridization buffer for **15 minutes at room temperature.** During this time, prepare the reverse transcription mix and additional 1x Maxima RT buffer. It is possible to leave the pucks in the hybridization buffer for up to 30 minutes without adversely affecting the quality of the data.

6 Using forceps, carefully remove each puck from the hybridization buffer and dip it gently into a tube of 1x Maxima RT buffer to equilibrate the puck to that buffer. After this dip, move it to a new tube containing 200 µL of reverse transcription mix. Allow pucks to incubate in the reverse transcription mix first for **30 minutes at room**

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temperature followed by an additional incubation for **90 minutes at 52 °C.** The total incubation time is 2 hours.

6.1 *Reverse Transcription Mix per puck:*
115 µL Ultrapure water
40 µL Maxima 5x RT Buffer
20 µL 10 mM dNTPs
5 µL RNase Inhibitor
10 µL 50 uM Template Switch Oligo
10 µL Maxima H- RTase Enzyne

Total volume 200 µL

7 Add proteinase K to the tissue clearing buffer stock solution at a ratio of 1:50 to create a working solution for tissue digestion. Add 200 µL of this working solution to each tube and pipet gently to mix within the tube without disrupting the puck. Incubate for **30 minutes at 37 °C.** The total volume in each tube should be 400 µL at this point.

7.1 *Tissue Clearing Buffer Stock Recipe:*
100 mM Tris-HCl pH 7.5
200 mM NaCl
2% SDS (w/v)
5 mM EDTA

Working solution for Step 7 per puck:
196 µL Tissue clearing buffer stock
4 µL Proteinase K enzyme

The tissue clearing buffer (without proteinase K) can be made ahead of time and stored for up to several months at room temperature.

8 Add 200 µL TE-TW to each tube and pipet several times to mechanical shear any tissue remaining in the tube or on the puck. At this time, the individual beads should also release from the coated glass piece which held the puck. Taking care not to introduce any bubbles, pipet several times until all beads have visibly been removed. Using forceps, quickly remove the glass shard in the tube before the beads start to pellet. **At this point, ensure all beads are still suspended in the mixture and transfer all volume to a new Lo-bind tube.** The total volume per tube should be 600 µL at this step.

9 Centrifuge each sample for 2 minutes at 3000 RCF. A white pellet should be clearly visible. Remove the supernatant and resuspend the beads carefully in 200 µL TE-TW. **It is best practice not to allow any beads to entire the pipet tip from this point forward so as to minimize any bead loss that might result from sticking to the sides of the tip.** Perform this TE-TW wash a total of 2 times at this step.

It is possible to pause here for up to 3 days. Store beads in 200 µL TE-TW at 4 °C after washes are completed.

10 Pellet beads and resuspend in 10 mM Tris-HCl, pH 7.5. Pellet once again and resuspend in 200 µL Exonuclease I mix. Incubate at **37 °C for 50 minutes.**

10.1 *Exonuclease I mix per puck:*
170 µL ultrapure water
20 µL 10x exonuclease I buffer
10 µL exonuclease I enzyme

Total volume 200 µL

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11 Add 200 µL TE-TW to each sample and pellet as before. Wash each sample a total of 2 times with TE-TW. Pellet the beads once more and resuspend in 200 µL 0.1 N NaOH and incubate for **5 minutes at room temperature.** After the incubation, add 200 µL TE-TW to quench the NaOH.

Note: 0.1 N NaOH should be prepared immediately prior to each use.

12 Pelleting as before, wash the beads once with TE-TW and then once again with 1x TE buffer (no tween added). Finally, pellet the beads once more and resuspend in 200 µL second strand synthesis mix. Incubate for **1 hour at 37 °C.**

12.1 *Second strand synthesis mix per puck:*
133 µL ultrapure water
40 µL Maxima 5x RT Buffer
20 µL 10 mM dNTPs
2 µL 1 mM dN-SMRT oligo
5 µL Klenow Enzyme

Total volume 200 µL

13 Add 200 µL TE-TW to each sample and then **transfer all beads to new Lo-bind tubes.** As before, pellet the beads and resuspend in TE-TW a total of 3 times. Pellet the beads once more and then resuspend in 200 µL ultrapure water. Finally, pellet once more and resuspend in 200 µL PCR mix.

Note: beads will not pellet as readily in water compared to the washes. It is critical to ensure that all beads have pelleted before removing the supernatant at this step. It helps to rotate the tubes in the centrifuge and perform an additional centrifugation step.

13.1 *PCR mix per puck:*
88 µL ultrapure water
100 µL Terra PCR Direct Buffer
4 µL 100 µM Truseq PCR primer
4 µL 100 µM SMART PCR primer
4 µL Terra Polymerase

Total volume 200 µL

14 After the beads are resuspended in the PCR mix, we find it works best to divide the total volume of each sample into 4 PCR tubes each containing 50 µL (25%) of the total. Check that the beads have not pelleted inside the PCR tubes and then run the following PCR protocol:

Slide-seq WTA
Start:
98 °C, 2 minutes
4 cycles of:
98 °C, 20 seconds
65 °C, 45 seconds
72 °C, 3 minutes
9 cycles of:
98 °C, 20 seconds
67 °C, 20 seconds
72 °C, 3 minutes
Then:
72 °C, 5 minutes
4 °C, Hold

It is possible to pause here. Store the samples at 4 °C.

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Purification and Quantification

15 Recombine the samples that were split into 4 parts in the previous step and perform a PCR clean-up using AMPure XP beads following the manufacturer's guidelines. In particular, we use a ratio of 0.6x AMPure beads (120 µL AMPure beads into 200 µL sample volume) for all steps. We perform the purification **twice**, eluting first in 50 µL water, proceeding again with a ratio of 0.6x (30 µL AMPure beads into 50 µL sample volume), and finally eluting into 20 µL water to obtain the final product.

16 To quantify the cDNA libraries, we use both the Qubit dsDNA high sensitivity kit and Bioanalyzer High Sensitivity DNA kit following the manufacturer protocols.

As a general guideline, little variability is expected between replicates within a single tissue sample, but larger variability may be observed between tissues. Concentrations in the range of approximately 0.3 ng/µL and above are acceptable, and expected average fragment sizes fall roughly within the range of 1300-1700 base pairs when following this protocol. There should be no significant amount of primer dimer present.

Tagmentation

17 The tagmentation is performed using the Nextera XT DNA Library prep kit largely following the manufacturer protocols. However, some modifications are necessary and are outlined in a brief protocol as follows:

18 Pre-heat a thermocycler to **55 °C.** While the block is heating, prepare 600 picogram dilutions of all samples into a total volume of 5 µL of ultrapure water. Add tagmentation buffer to each sample and then add the transposase enzyme immediately before moving to 55 °C. Incubate for **5 minutes.**

Note: steps in the tagmentation protocol are **highly time-sensitive.** Use of a multi-channel pipet is recommended to hasten pipetting steps and facilitate rapid mixing at each step.

18.1 *Tagmentation Mix per sample:*
600 pg cDNA in total volume of 5 µL ultrapure water
10 µL Tagment DNA Buffer
5 µL Amplicon Tagment Enzyme

Total volume 20 µL

19 Add 5 µL of NT buffer as quickly as possible following the tagmentation reaction. Mix quickly using a multi-channel pipet and allow to incubate at **room temperature for 5 minutes.**

20 Prepare the Nextera PCR Master mix with water, NPM, and P5-Truseq Hybrid oligo. Add 24 µL of this master mix to each sample, followed by 1 µL of i7 indexing primer from the Nextera XT kit. Gently mix by pipetting and run the PCR program below.

Note: Each sample must use a different i7 index if you intend to pool samples for multiplexed sequencing. We do not recommend dual-indexing of samples.

It is possible to pause here. Store the samples at 4 °C after the PCR.

20.1 *Nextera PCR Mix per sample:*
8 µL ultrapure water
15 µL NPM
1 µL of 10 µM P5-Truseq Hybrid oligo
1 µL of Nextera N7XX indexing primer or custom i7 index
25 µL from tagmentation reaction

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Total volume 50 µL

20.2 *Nextera XT PCR Amplification Protocol*

Start
72 °C, 3 minutes
95 °C, 30 seconds
12 cycles of:
95 °C, 10 seconds
55 °C, 30 seconds
72 °C, 30 seconds
Then:
72 °C, 5 minutes
4 °C, Hold

21 Purification and Quantification of DNA sequencing libraries is performed similarly to the cDNA purification and quantification in steps 15 and 16. However, it is only necessary to perform the AMPure clean-up **once.** Additionally, final product should be eluted in **10 µL.**

Variability between samples is common. As a general guideline, acceptable concentrations for libraries are approximately 3 ng/µL or higher and expected average fragment sizes are in the range 400-700 base pairs.

Sequencing

22 Sequencing is standard following Illumina's protocols, and submission should proceed as outlined by your institution's core facility or lab policies. We recommend preparing pools of libraries at 4 nM concentration if possible.

For best results, it's generally advised to sequence each puck to a depth of 100-200 million reads, though smaller sequencing runs can be used to assess the quality of new samples. Read structure is as follows:

Read 1: 42 bp
Index 1: 8 bp
Read 2: 41 - 60 bp
Index 2: 0 bp

For read 2, the read length is dependent on the capacity of the sequencing kit. For a Nextseq 75-cycle kit, the maximum possible is 41 bp, which is the minimum recommended length. For a 100-cycle kit, use a read length of 60 bp.

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