

I. Introduction

This protocol provides instructions for performing single-nucleus RNA-seq with Illumina[®] Single Cell 3' RNA Prep Kits after performing the Trekker[®] workflow and nuclei isolation using the [Trekker U Single-Cell Spatial Mapping Kit User Manual](#). The final library products include the single-nucleus whole-transcriptome library, which contains the gene expression information, and the Trekker library, which contains the spatial location of each nucleus.

Compatible with Illumina Single Cell 3' RNA Prep.

II. Required Materials

This protocol applies to the following Takara Bio products:

- Trekker U 10x10 Bundle (Cat. No. SK017)

Additional Materials Required (Not Provided):

- Illumina Single Cell 3' RNA Prep
 - T2 (Illumina, Cat. No. 20135689) or
 - T10 (Illumina, Cat. No. 20135691) or
 - T20 (Illumina, Cat. No. 20135692) or
 - T100 (Illumina, Cat. No. 20135693)
- Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit (Illumina, Cat. No. 20132794)
- Illumina Single Cell Unique Dual Indexes (Illumina, Cat. No. 96 indexes, 96 samples: 20132788)
- Other kits, reagents, and equipment listed by the vendor (Illumina)
- DNA oligonucleotide primers should be ordered with desalted purification. If desired, primers can be ordered resuspended to a stock concentration of 100 μ M in 1X TE Buffer, pH 8.0, or lyophilized. If primers are ordered lyophilized, resuspend primers to a stock concentration of 100 μ M in 1X TE Buffer, pH 8.0, using best practices.

Table 1. Primer and index primer sequences.

Name	Sequence	Concentration
WTA-F-V2	CTCTTTCCCTACACGACGCTC	20 μ M
Additive Primer H	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	10 μ M
P7 Index H* for use with Additive Primer H	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	10 μ M
Universal P5 Index* for use with Additive Primer H	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXX XACTCTTTCCCTACACGACGC	10 μ M

*Refer to the table in Section 4.6 of the appropriate PIPseq V Enrichment of Synthetic Nucleotide Tags User Guide (see Table 2 below) for example index sequences for P7 Index H, and universal P5 Index for H.

III. Protocol

A. Single-nucleus RNA-seq

1. Perform single-nuclei capture and gene expression library generation following the appropriate Illumina Single Cell 3' RNA Prep User Guide (Table 2). Perform Trekker library generation following the appropriate PIPseq V Enrichment of Synthetic Nucleotide Tags Kit User Guide (Table 2), with modifications described in Step A.2.

Table 2. Illumina User Guides.

Kit	Illumina User Guides
T2	<ul style="list-style-type: none"> • Illumina Single Cell 3' RNA Prep, T2 User Guide • PIPseq V T2 Enrichment of Synthetic Nucleotide Tags Kit User Guide
T10	<ul style="list-style-type: none"> • Illumina Single Cell 3' RNA Prep, T10 User Guide • PIPseq V T10 Enrichment of Synthetic Nucleotide Tags Kit User Guide
T20	<ul style="list-style-type: none"> • Illumina Single Cell 3' RNA Prep, T20 User Guide • PIPseq V T20 Enrichment of Synthetic Nucleotide Tags Kit User Guide
T100	<ul style="list-style-type: none"> • Illumina Single Cell 3' RNA Prep, T100 User Guide • PIPseq V T100 Enrichment of Synthetic Nucleotide Tags Kit User Guide

2. Modifications for the PIPseq V Enrichment of Synthetic Nucleotide Tags Kit User Guide:
 - a. Add Additive Primer H when indicated in the User Guide. No Additive Primer A is needed.
 - b. In Step 1 of 3.3.1. SNT Sample Index PCR, use 1 ng as input instead of the recommended 10–15 ng.
 - c. In Step 5 of 3.3.1. SNT Sample Index PCR, perform a total of 7 cycles of indexing PCR. See Figure 1 for an example library trace on the TapeStation system.

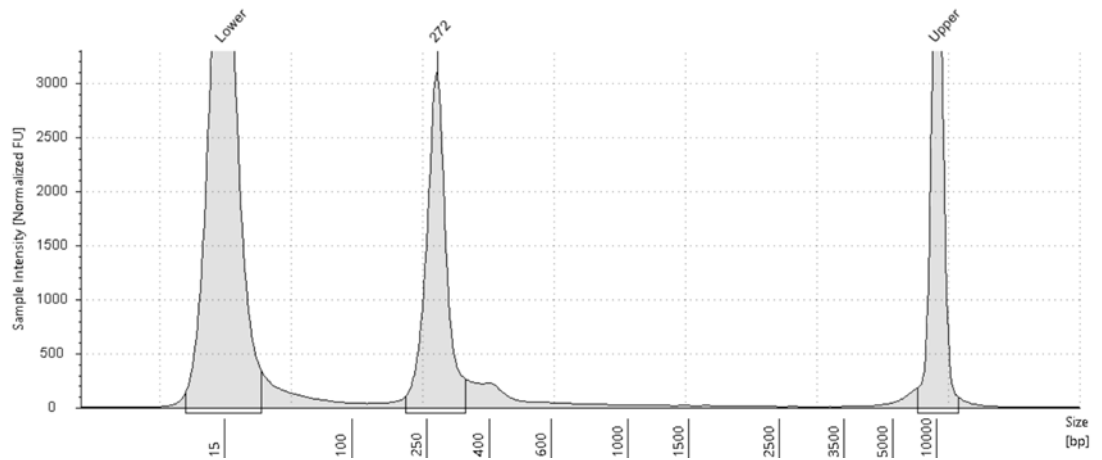


Figure 1. Example Trekker library trace on the TapeStation system (D5000).

B. Sequencing

1. Sequence the gene expression library according to the Illumina guides listed in Table 2.
2. Sequence the Trekker library at ~5,000 read pairs per nucleus captured. For example, if 10,000 nuclei were captured, allocate 5.0×10^7 reads for the Trekker library.

Table 3. Read lengths for Illumina Single Cell 3'RNA Prep.

Sequencing read	Recommended read length (bp)
Read 1	45
I7 index	10
I5 index	10
Read 2	72

- If the Trekker library is sequenced on its own, additional PhiX spike-in may be needed. If the Trekker library is sequenced with gene expression libraries, follow the sequencing recommendations in the Parse Evercode WT v3 User Guide for PhiX spike-in.

 - NextSeq 1000/2000: 10% PhiX spike-in when pooling with only Trekker libraries
 - NextSeq 500/550: 10% PhiX spike-in when pooling with only Trekker libraries
 - NovaSeq 6000 and Novaseq X:
 - 5% PhiX spike-in when pooling with non-Trekker libraries
 - 10% PhiX spike-in when pooling with only Trekker libraries

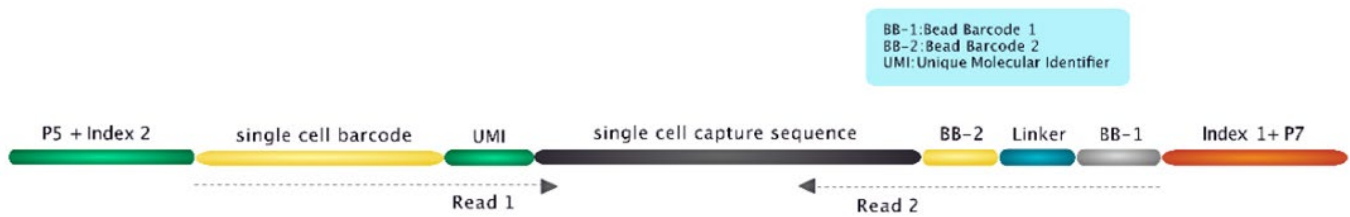


Figure 2. Trekker library structure.

IV. Bioinformatics

For details on data processing and output interpretation, refer to the [Trekker Bioinformatics Solutions](#) for local analysis, and the [Takara Bio Spatial Bioinformatics Portal](#) for cloud analysis, which can both be found at takarabio.com.

Appendix A. Troubleshooting Guide

Table 4. Troubleshooting guide.

Problem	Possible Explanation	Solution
Trekker final library missing expected peak	UV cleavage unsuccessful	Ensure UV lamp settings are correct and the UV lamp is functioning
	Incorrect primers were used in library prep	Ensure correct primers and cycle numbers were used
Trekker final library contains significant off-target peaks	Over amplification	Ensure the correct input amount and cycle number (different from 10x user guide) was used for index PCR.

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