Nuclei Isolation Protocol for ddSEQ™ Single-Cell 3' RNA-Seq Kit

Protocol

Background

Single-nucleus RNA sequencing (snRNA-Seq) is a technique for profiling gene expression from nuclei and is recommended for analyzing large cells that cannot be encapsulated using droplet-based single-cell RNA sequencing (scRNA-Seq) methods. It is also recommended for archival sample types with low-quality cytoplasmic RNA and tissues that are difficult to dissociate, as it minimizes dissociation-induced gene expression.

This protocol enables tissue dissociation and single-nuclei isolation for encapsulation using the ddSEQ Single-Cell Isolator and is compatible with the ddSEQ Single-Cell 3' RNA-Seq Kit (Figure 1). Key steps include bead-based dissociation, filtration, fluorescent-activated cell sorting (FACS) of nucleated cells, and assessment of nuclei quality.

Validated in collaboration with the laboratory of Alex Shalek at the Massachusetts Institute of Technology, this procedure was tested on engineered liver grafts, following the approach described by Nadelmann et al. (2021) and Slyper et al. (2020). These grafts — composed of hepatocytes, endothelial cells, and fibroblasts — are challenging to dissociate into single cells. Thus, an alternative strategy of nuclei isolation was utilized, as described here.

Effective nuclei isolation is marked by the presence of single nuclei with intact nuclear membranes, free from debris or aggregates, resulting in high-quality sequencing data. Users attempting this protocol for the first time or applying it to a new sample type are advised to perform a pilot experiment, as optimization may be necessary.

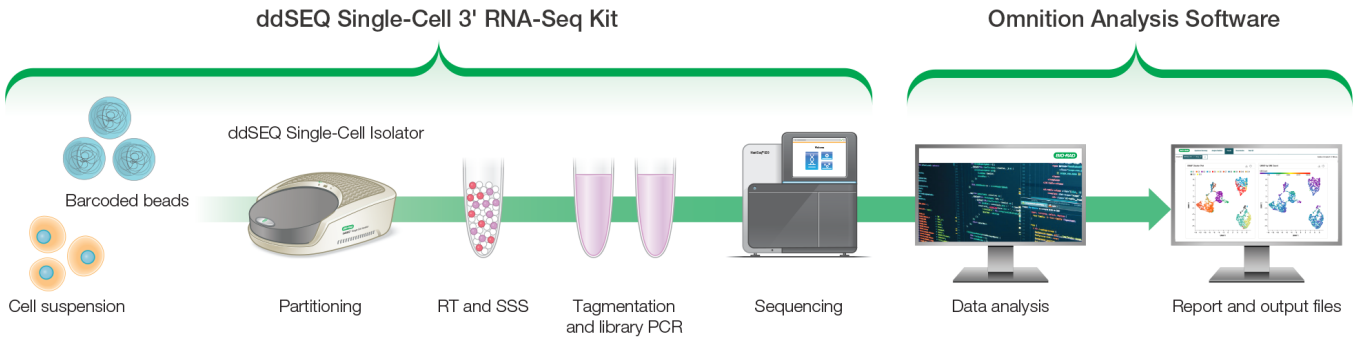

Fig. 1. Overview of the nuclei isolation workflow for the ddSEQ Single-Cell 3' RNA-Seq Kit. The workflow begins with sample isolation, followed by droplet generation to encapsulate single nuclei on the ddSEQ Single-Cell Isolator, library preparation using the ddSEQ Single-Cell 3' RNA-Seq Kit, sequencing on a standard benchtop sequencer for short-read sequencing, and secondary bioinformatics analysis with Omnition Analysis Software. Illustration created with BioRender.com.

BIO-RAD

Equipment, Consumables, and Reagents

Equipment and Consumables	Vendor	Catalog Number
TissueLyser II	QIAGEN	85300
Stainless Steel Beads, 5 mm (for TissueLyser II)	QIAGEN	69989
5 mL Tube Adapter Set (adapter plates and racks for TissueLyser II)	QIAGEN	11980
Fisherbrand Low-Retention Microcentrifuge Tubes (2 mL microcentrifuge tubes for TissueLyser II)	Thermo Fisher Scientific Inc.	02-681-332
Cell counter	Any	Any
FACS instrument	Any	Any
40 µm cell strainer	Any	Any
Refrigerated centrifuge (with swinging-bucket rotor)	Any	Any

FACS, fluorescence-activated cell sorting.

Reagents	Vendor	Catalog Number
Sucrose (1.5 M)	Sigma-Aldrich	S0389
Invitrogen KCl (2 M), RNase-Free	Fisher Scientific	AM9640G
$MgCl_2$ (1 M)	Fisher Scientific	AM9530G
Tris (1 M), pH 8.0, RNase-Free	Fisher Scientific	AM9855G
Invitrogen DTT, 1M	Fisher Scientific	P2325
cOmpete, EDTA-Free Protease Inhibitor Cocktail (50x)	Roche	11873580001
Ambion RNase Inhibitor, cloned, 40 U/µL	Fisher Scientific	AM2684
Protector RNase Inhibitor (40 U/µL)	Sigma-Aldrich	03335402001
20 U/µL Superase Inhibitor	Invitrogen	AM2696
Octylphenol ethoxylate (10%, v/v)	Sigma-Aldrich	T8787-100ML
PBS, pH 7.4 (1x)	Invitrogen	10010049
Thermo Scientific Blocker BSA (10%)	Fisher Scientific	PI37525
Nuclease-free water (not DEPC-treated)	Any	Any
NucBlue Live Ready Probes Reagent	Fisher Scientific	R37605

BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline.

Tissue Dissociation and Nuclei Isolation Workflow

Prepare the following reagents:

Nuclei Isolation Buffer 1

Component	[Initial]	[Final]	Volume (per sample), µL
Sucrose	1,500 mM	250 mM	625.0
KCl	2,000 mM	25 mM	46.8
$MgCl_2$	1,000 mM	5 mM	18.8
Tris, pH 8	1,000 mM	10 mM	37.5
Nuclease-free water	N/A	N/A	3,021.9
Total			3,750.0

NA, not applicable.

Nuclei Isolation Buffer 2

Component	[Initial]	[Final]	Volume (per sample), µL
Nuclei Isolation Buffer 1	N/A	N/A	1,223.75
DTT	1 mM	0.001 mM	1.25
Protease Inhibitor	50x	25x	25.00
Total			1,250.00

DTT, dithiothreitol; NA not applicable.

Homogenization Buffer

Component	[Initial]	[Final]	Volume (per sample), µL
Nuclei Isolation Buffer 2	N/A	N/A	1,212.5
RNase Inhibitor*	40 U/µL	0.4 U/µL	12.5
Superase Inhibitor	20 U/µL	0.2 U/µL	12.5
Octylphenol ethoxylate	10%	0.10%	12.5
Total			1,250.0

Storage Buffer

Component	[Initial], mM	[Final], mM	Volume (per sample), µL
BSA	10%	4%	400
Protector RNase Inhibitor*	40 U/µL	0.2 U/µL	5
1x PBS	N/A	N/A	595
Total			1,000

BSA, bovine serum albumin; N/A, not applicable; PBS, phosphate buffered saline.

* The listed RNase inhibitors and their specified concentrations are critical for successful nuclei isolation using this protocol. Do not reduce the recommended amount, as its cost is minimal compared to the overall expense of repeating the library preparation and sequencing procedure.

Perform the following steps to dissociate the samples and obtain nuclei for library preparation:

1. Precool the TissueLyser II adapter plates and racks at 4°C.
2. In a 2 mL microcentrifuge tube, add a tissue sample (~25 mm^3) and two 5 mm stainless steel beads for use with the TissueLyser II.
3. Add 500 µL Homogenization Buffer to the tube.
4. Remove the adapter plates and racks from 4°C and set up the TissueLyser II.
5. Load the sample and run the TissueLyser II for 1 min at 25 Hz. If the tissue is not homogenously dissociated, run for an additional 1 min.
6. Remove the sample and place on ice for 5 min.
7. After 5 min, filter the sample through a 40 µm cell strainer into a 50 mL conical tube.
8. Add 5 µL of trypan blue to help visualize the nuclei pellet. Centrifuge at 1,500 x g for 5 min at 4°C using a swinging bucket centrifuge.
9. Carefully remove and discard the supernatant without disturbing the pellet.
10. Resuspend the pellet in 500 µL Storage Buffer.
11. Take a 100 µL aliquot from the suspension and set it aside to use as an unstained negative control.
12. Add two drops of NucBlue Reagent to the remainder of the sample. Mix well by vortexing for 5 sec, then place on ice for 15 min.

13. Run the sample on a FACS instrument following the manufacturer's instructions. Sort for Hoescht-positive cells and collect into a 1.5 mL Eppendorf tube precoated overnight with 1% BSA to minimize nuclei loss. Keep the sample on ice.
14. Use a cell counter to determine nuclei concentration and viability. Effective nuclei isolation should yield viability of <10%. Adjust the concentration to the desired throughput as described in the Bio-Rad ddSEQ Single-Cell 3' RNA-Seq Kit User Guide.
15. Use a brightfield microscope to assess nuclei quality. Efficient lysis is indicated by single nuclei with intact nuclear membranes and minimal debris and aggregates.
16. Proceed with library preparation following the Bio-Rad ddSEQ Single-Cell 3' RNA-Seq Kit User Guide.

Example Data

snRNA-Seq libraries from engineered liver grafts were prepared by following the nuclei isolation protocol using the ddSEQ-Single-Cell 3' RNA-Seq Kit. This resulted in high-quality libraries (Figure 2), yielding ~2,000 nuclei and demonstrating a high number of genes detected at the minimum recommended sequencing depth. Sequencing data analysis using Omnition Analysis Software requires modification of the intron setting to include intronic reads. Tertiary analysis of the sequencing data identified the major cell type composition as 40% endothelial cells, 36% fibroblasts, and 25% hepatocytes (Figure 3), consistent with expectations for this sample type.

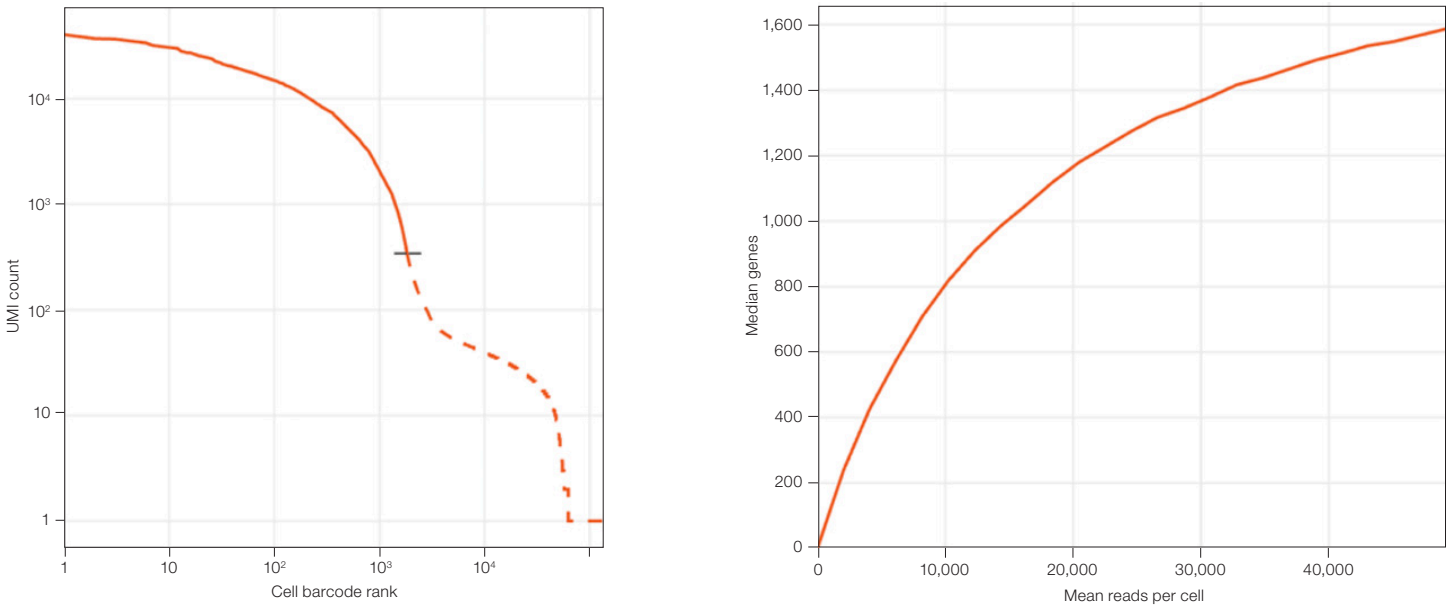

Fig. 2. Omnition Analysis Software report plots. The barcode rank plot (left) shows a clear distinction between signal and background. The median genes per cell plot (right) demonstrates high sensitivity at the minimum recommended sequencing depth. UMI, unique molecular identifier.

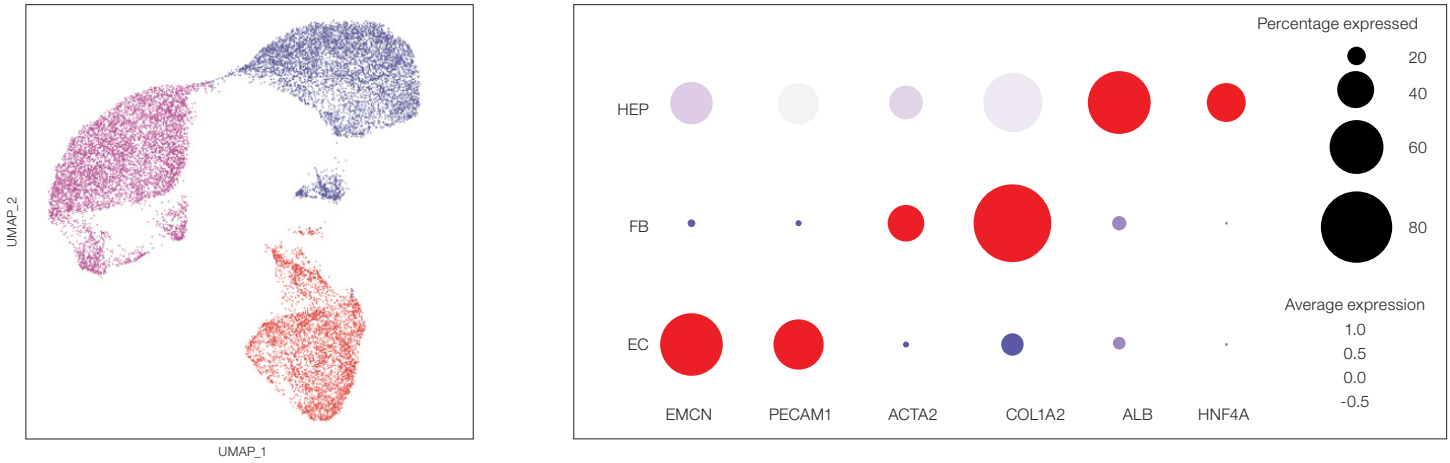

Fig. 3. Cell-type clustering and marker expression. The uniform manifold approximation and projection (UMAP) plot (left) shows cell-type clustering generated from engineered liver grafts, with cell-type distribution consistent with expected proportions. The dot plot (right) displays gene expression specificity using signature markers for each cell type: endomucin (EMCN) and platelet endothelial cell adhesion molecule 1 (PECAM1) for endothelial cells, actin alpha 2 (ACTA2) and collagen type I alpha 2 chain (COL1A2) for fibroblasts, and albumin (ALB) and hepatocyte nuclear factor 4 alpha (HNF4A) for hepatocytes. UMAP plot (left): endothelial cells (●), fibroblasts (●), hepatocytes (●). EC, endothelial cell; FB, fibroblast; HEP, hepatocyte.

Acknowledgement

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References

1. Nadelmann ER et al. (2021). Isolation of nuclei from mammalian cells and tissues for single-nucleus molecular profiling. Curr Protoc 1, e132.

2. Slyper M et al. (2020). A single-cell and single-nucleus RNA-Seq toolbox for fresh and frozen human tumors. Nat Med 26, 792–802.

Note: Slyper et al. provide a systematic approach for both scRNA-Seq and snRNA-Seq, specifically designed for nuclei isolation from frozen tissue across multiple cancer types.

Visit bio-rad.com/3scRNA-seq or see bulletin 3684 for more information.

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