ddSEQ™ Single-Cell 3' RNA-Seq Kit

User Guide

Version 1.0

ddSEQ Single-Cell 3' RNA-Seq Kit

User Guide

Version 1.0

Catalog No.	17009671
	12009359
	12020410
	12020632
	12020413

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Revision History

Document	Date	Description of Change
ddSEQ Single-Cell 3' RNA-Seq Kit User Guide Version 1.0 DIR No. 10000167449, Ver B	June 2024	Add bullet points to Index and Library Prep best practices, and section on preparing for sequencing
ddSEQ Single-Cell 3' RNA-Seq Kit User Guide Version 1.0 DIR No. 10000167449, Ver A	May 2024	New user guide

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ddSEQ Single-Cell 3' RNA-Seq Kit Workflow

Fig. 1 displays the hands-on time and total time for each process step in the Bio-Rad™ ddSEQ™ Single-Cell 3' RNA-Seq Kit workflow.

Workflow Time

Total Time	Step	Step Time	Hands-On Time	Pausing and Storage
Variable	Cell Preparation			
	Prepare single-cell suspension	Dependent on cell type		
2.5 hr	Step 1 — Droplet Generation and cDNA Synthesis (2.5 hr)			
	Preparing the mixes for droplet generation	25 min	25 min	
	Loading the ddSEQ M Cartridge	3 min	3 min	
	Single-cell encapsulation	5 min	1 min	
	Droplet transfer	3 min	3 min	
	In-droplet barcoding and cDNA synthesis	2 hr	5 min	Droplets: 4°C ≤24 hr or –20°C ≤7 days
4.5 hr	Step 2 — Breaking Emulsions and Purifying DO and cDNA Fractions (2 hr)			
	Breaking emulsions	5 min	4 min	
	First cleanup: cDNA purification and DO dimer size selection	1 hr	1 hr	
	Second cleanup: cDNA and DO dimer purifications	55 min	55 min	–20°C ≤7 days
7 hr	Step 3 — Generate cDNA and DO Libraries (2.5 hr)			
	cDNA tagmentation	15 min	10 min	
	cDNA and DO dimer indexing and amplification	1 hr 25 min	20 min	4°C ≤72 hr or 10°C ≤72 hr
	First library cleanup: cDNA and DO library purifications	30 min	30 min	
	Second library cleanup: cDNA and DO library purifications	30 min	30 min	–20°C long-term
7 hr 15 min to 8 hr	Check quality of libraries	15 min to 1 hr	5 min	–20°C long-term

Fig. 1: ddSEQ Single-Cell 3' RNA-Seq Kit workflow

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Chapter 1 Introduction

The ddSEQ Single-Cell 3' RNA-Seq Kit, when paired with the ddSEQ Single-Cell Isolator, provides end-users with a technology to encapsulate thousands of single cells in discrete droplet partitions. These droplet partitions include barcoded gel beads, and reagents for cell lysis, capture, and conversion of cellular mRNA (messenger RNA) into double-stranded cDNA (complementary DNA). Subsequent enzymatic reactions process the cDNA and convert it into libraries for sequencing on Illumina platforms. When paired with Omnition Analysis Software for data analysis, you can clarify biological information down to single-cell resolution (Fig. 2).

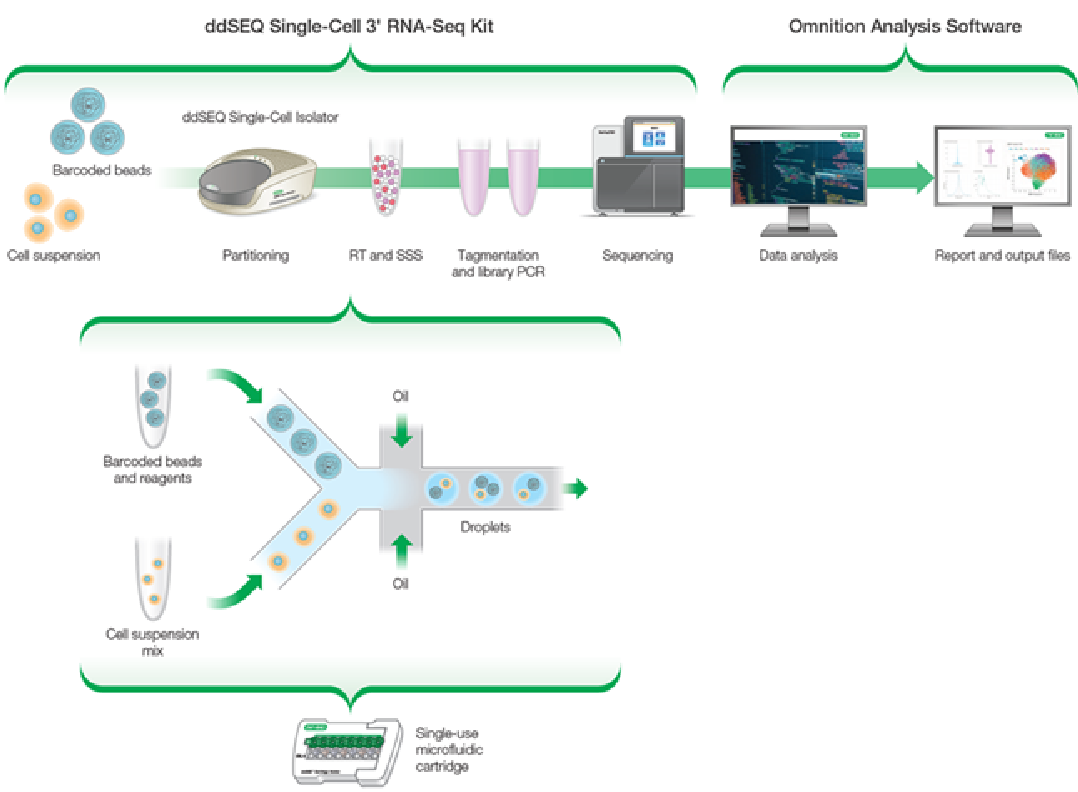

Fig. 2: ddSEQ single-cell 3' RNA-seq technology

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Chapter 2 Required Consumables and Equipment

Fig. 3 illustrates the kit contents.

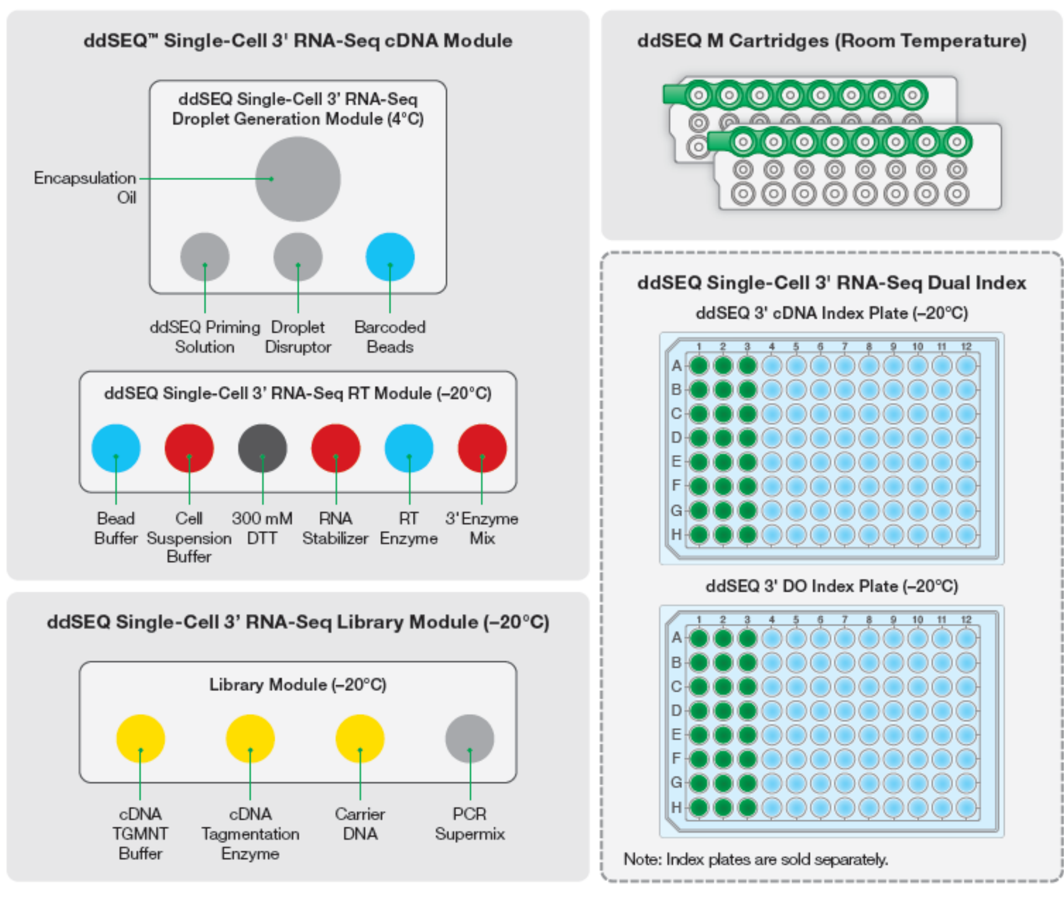

Fig. 3: ddSEQ Single-Cell 3' RNA-Seq Kit components

Table 1 defines the contents of the ddSEQ Single-Cell 3' RNA-Seq Kit.

Table 1. ddSEQ Single-Cell 3' RNA-Seq Kit (catalog no. 17009671)

Name	Catalog no.	Storage temp	Assoc doc catalog no.	Qty (ea)	Cap color
ddSEQ M Cartridges	12009359	Room temp	12008720	2	N/A
ddSEQ Single-Cell 3' RNA-Seq Droplet Generation Module (catalog no. 12020410)					
ddSEQ Priming Solution	N/A	4° C	12020075	1	Clear
Droplet Disruptor	N/A	4° C	12008761	1	Clear
Encapsulation Oil	N/A	4° C	12008762	1	Clear
Barcoded Beads	N/A	4° C	12020007	1	Blue
ddSEQ Single-Cell 3' RNA-Seq RT Module (catalog no. 12020632)					
Bead Buffer	N/A	-20° C	12019997	1	Blue
Cell Suspension Buffer	N/A	-20° C	12020074	1	Red
300 mM DTT	N/A	-20° C	10049218	1	Gray
RNA Stabilizer	N/A	-20° C	12020006	1	Red
RT Enzyme	N/A	-20° C	12020053	1	Blue
3' Enzyme Mix	N/A	-20° C	12020052	1	Red
ddSEQ Single-Cell 3' RNA-Seq Library Module (catalog no. 12020413)					
cDNA Tagmentation buffer (labeled as TGMNT on tube)	N/A	-20° C	12019996	1	Yellow

Table 1. ddSEQ Single-Cell 3' RNA-Seq Kit (catalog no. 17009671), continued

Name	Catalog no.	Storage temp	Assoc doc catalog no.	Qty (ea)	Cap color
cDNA Tagmentation Enzyme	N/A	-20° C	12019983	1	Yellow
Carrier DNA	N/A	–20° C	12020782	1	Yellow
PCR Supermix	N/A	–20° C	12020005	1	Clear

Table 2 defines the contents of the ddSEQ Single-Cell 3' RNA-Seq Dual Index Kit, **which is sold separately**.

Table 2. ddSEQ Single-Cell 3' RNA-Seq Dual Index Kit (Catalog no. 12020461)

Name	Catalog no.	Storage temp	Assoc doc catalog no.	Qty (ea)	Cap color
ddSEQ 3' cDNA Index Plate	N/A	-20° C	12020634	1	N/A
ddSEQ 3' DO Index Plate	N/A	-20° C	12020633	1	N/A

Table 3 defines the equipment that has been validated by Bio-Rad and is recommended for use.

Important: Substitutions might negatively affect system performance, although purchase from any vendor is acceptable for some equipment. For sample analyses, you can use either the Agilent 2100 Bioanalyzer or the Agilent 4150 Tapestation System, depending on preference or availability.

Table 3. Equipment (not provided)

Apparatus/equipment	Catalog no.	Supplier
ddSEQ Single-Cell Isolator for single-cell sequencing	12004336	Bio-Rad
ddSEQ Cartridge Holder	12004739	Bio-Rad
Cell counter (automated or manual hemocytometer)	N/A	Any vendor
Centrifuge (rotor for 1.5 ml tubes)	N/A	Any vendor

Table 3. Equipment (not provided), continued

Apparatus/equipment	Catalog no.	Supplier
Centrifuge (rotor for 15 ml tubes)	N/A	Any vendor
Vortex	N/A	Any vendor
96-well cooling block	N/A	Any vendor
Thermal cycler* (choose one)		Bio-Rad
■ T100 Thermal Cycler	1861096	
■ C1000 Thermal Cycler with 96-Deep Well Reaction Module)	1861097	
■ PTC Tempo Deep Well Thermal Cycler	12015392	
**Use one of the recommended thermal cyclers or an equivalent deep-well thermal cycler with up to 100 µl capacity. The default ramp rate is 2.0° C/sec for the recommended thermal cyclers.*		
Rainin Pipet-Lite LTS Pipette L-200XLS+	17014391	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-100XLS+	17014384	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-20XLS+	17014392	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-2XLS+	17014393	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-200XLS+	17013805	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-50XLS+	17013804	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-20XLS	17013803	Mettler Toledo
NEBNext Magnetic Separation Rack	S1515S	NEB
Magnetic Stand 96	AM10027	Thermo Fisher Scientific
DynaMag-96 Side Skirted Magnet	12027	Thermo Fisher Scientific
Choose one:		Agilent
■ 2100 Bioanalyzer	G2939AAR	
■ 4150 Tapestation System	G2992AA	

Table 4 defines the validated required reagents.

Table 4. Required reagents (not provided)

Reagent	Catalog no.	Supplier
Nuclease-free water	N/A	Any vendor
Trypsin or any cell dissociation reagent (dependent on cell type of interest)	N/A	Any vendor
Trypan blue or live/dead fluorescent dyes	N/A	Any vendor
Bovine Serum Albumin (BSA), molecular-biology grade/nuclease-free	B6917 126615	Millipore Sigma (or any vendor with appropriate cell culture/ molecular biology grade)
1X phosphate-buffered saline (PBS); no calcium, no magnesium, pH 7.4	N/A	Any vendor
10 mM Tris-HCl pH 8.0 Resuspension Buffer, DNAse-Free	T1173	Teknova
Ethanol, pure (200 proof, anhydrous)	N/A	Any vendor
Choose either A, or B and C: A) High-Sensitivity DNA Reagents **or** B) High-Sensitivity D5000 Reagents **and** C) High-Sensitivity D1000 Reagents	 5067-4627 5067-5593 5067-5585	Agilent
Glycerin (50% v/v glycerol solution in water)	N/A	Any vendor

Table 5 defines the validated required consumables.

Table 5. Required consumables (not provided)

Consumable	Catalog no.	Supplier
ddPCR™ 96-Well Plates	12001925	Bio-Rad
Microseal™ F PCR Plate Seal (with pierceable foil)	MSF1001	Bio-Rad
0.2 ml Flat PCR Tube 8-Cap Strips	TCS0803	Bio-Rad
Choose one: ■ 0.2 ml TempAssure PCR Flex-Free 8-Tube Strips with Attached Individual Optical Caps ■ 0.2 ml Sapphire PCR 8-Tube Strips for qPCR, with Attached Individual Optical Flat Caps	1402-4700 608281	USA-Scientific Greiner Bio-one
0.2 µm PES filtration system	N/A	Any vendor
15 ml conical centrifuge tubes	N/A	Any vendor
1.5 ml DNA LoBind Micro-Centrifuge Tubes	022431021	Eppendorf
Pipette Tips TR LTS 200µl F 960A/10	17014963	Mettler Toledo
Pipette Tips TR LTS 20µl S 960A/10	17014960	Mettler Toledo
Purification beads (choose one): ■ SPRIselect Reagent Kit ■ Ampure XP SPRI Reagent	B23317 A63881	Beckman Coulter
Choose either A, or B and C: A) High-Sensitivity DNA Kit **or** B) High-Sensitivity D5000 Ladder and High-Sensitivity D5000 Screen Tape **and** C) High-Sensitivity D1000 Ladder and High-Sensitivity D1000 Screen Tape	5067-4626 5067-5594 and 5067-5592 5067-5587 and 5067-5582	Agilent

Chapter 3 Technology and Best Practices

Workflow Technology

The protocol in this document contains information and instructions for preparing 3’ tagged RNA-seq libraries from single cells for whole transcriptome analysis on Illumina sequencing platforms. The ddSEQ Single-Cell 3' RNA-Seq Kit is optimized for a cell input range of 1,000 to 10,000 cells per sample loaded. Each cartridge (two are provided in each kit) contains eight wells, dedicating two wells per sample. You can load a single cartridge with up to four unique samples, resulting in a typical cell output of up to 5,000 single cells per sample (Table 6).

Table 6. Viable cells loaded vs cells recovered per sample

Viable cells loaded per sample	Cells recovered per sample
1,000	500
2,000	1,000
3,000	1,500
4,000	2,000
5,000	2,500
6,000	3,000
7,000	3,500
8,000	4,000
9,000	4,500
10,000	5,000

Important: To partition single cells and barcode individual transcriptomes, you must use a ddSEQ Single-Cell Isolator and reagents provided in the ddSEQ Single-Cell 3' RNA-Seq Kit. For information, see Chapter 2, Required Consumables and Equipment.

To ensure single-cell encapsulation, the ddSEQ Single-Cell 3' RNA-Seq Kit partitions single cells into droplets at a limiting dilution. Barcoded beads are overloaded into the system to ensure that each droplet has at least one bead to capture the mRNA of an encapsulated cell. Through a deconvolution oligo (DO) strategy, cellular information is combined to single-cell resolution bioinformatically.

Each barcoded bead is covered in two types of oligos: poly(T) capture oligos and deconvolution oligos. Both types of oligos contain a bead barcode and a unique molecular identifier (UMI). Every oligo on the bead has the same bead barcode to identify the bead, and a UMI to differentiate oligos. These oligo configurations and regions are depicted with distinctly colored UMI and bead barcode regions (Fig. 4).

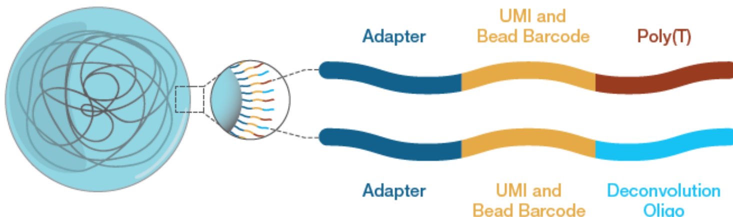

Fig. 4: Representative diagram of the barcoded gel beads

When a single cell in a droplet is paired with one or more beads, the cellular mRNA is captured by poly(T) oligos from the beads in the droplet, (Fig. 5). DOs in the droplet hybridize to form dimers with other DOs in the partition. Omnition Analysis Software assigns captured transcripts to a bead based on their bead barcode and connects beads within the same droplet based on their DO sequencing library.

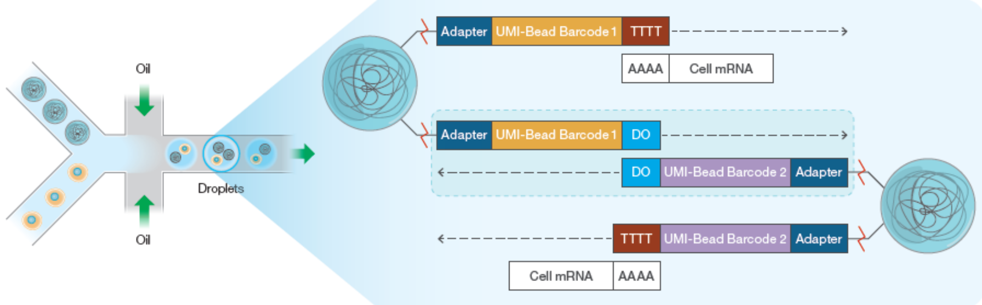

Fig. 5: Deconvolution of information from multiple beads for partition assignment

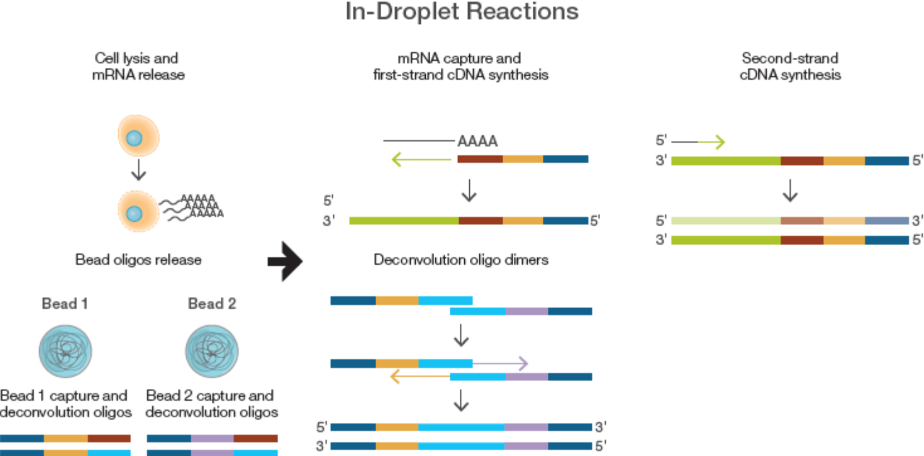

Fig. 6: In-droplet enzymatic reactions during the workflow

- Lysis occurs after cells are partitioned alongside barcoded beads, releasing mRNA into the droplet environment. Both the poly(T) capture and DOs are released from the beads.
- The cellular mRNA hybridizes to poly(T) oligos and first strand cDNA synthesis occurs, while bead connections are formed via DO hybridization and extension, resulting in DO dimers.

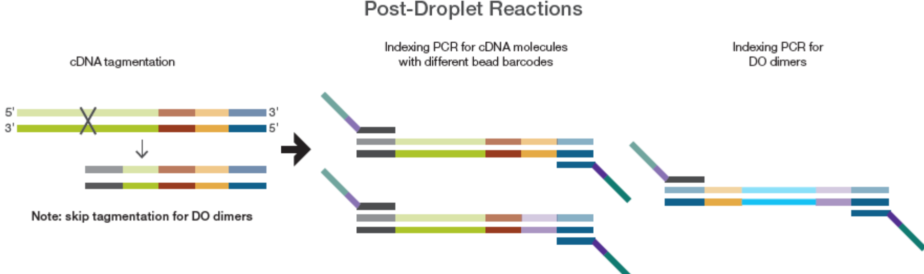

Fig. 7: Post-droplet enzymatic reactions during the workflow

- After droplet disruption and product cleanup, both cDNA and DO dimer fractions are converted to final libraries. cDNA is tagmented to incorporate adapters.

  Note: The protocol skips the tagmentation step for DO dimers.

- After cDNA tagmentation, an indexing PCR step adds final full-length adapters for sequencing.
- A parallel indexing PCR step adds final full-length adapters to the DO dimers for sequencing.

The final cDNA sequencing library structure is completed via tagmentation and index PCR (Fig. 8), while the DO sequencing library structure is achieved via index PCR only (Fig. 9). Both the cDNA and DO sequencing libraries are dual-indexed with 8-base unique dual indexes (UDI).

Note: The cDNA and DO index plates are sold separately.

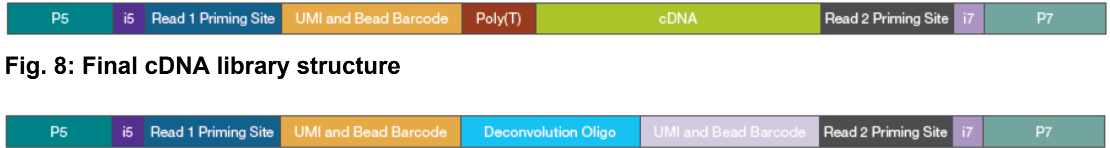

Fig. 8: Final cDNA library structure

Fig. 9: Final DO library structure

Best Practices

To ensure optimal workflow performance, Bio-Rad recommends that you comply with the best practices explained in the following subsections.

Key Workflow Considerations

- Before you begin
  - Read through the entire protocol to become familiar with the workflow.
  - Ensure that all required equipment is properly installed and functioning.
  - Ensure that all required materials and reagents are available.
- Pause the protocol only at designated safe stopping points.
- Use separate areas for appropriate steps (such as cell culture rooms, and pre-PCR and post-PCR areas).
- To avoid cross-contamination, change pipette tips between each sample and reaction.
- Use the recommended Rainin pipettes and filtered tips **only**.

  Important: Using other tips can negatively impact performance.
- Do **not** use latex gloves when making or handling droplets.

  Important: Latex gloves are static-insulative and retain the charge on their surface, which affects droplet integrity.

Dispensing the Encapsulation Oil

- Before dispensing, ensure the encapsulation oil has equilibrated to room temperature.

  Note: The oil should be equilibrated to room temperature for at least 30 min before loading the cartridge.

- Due to its viscosity, aspirate and dispense the encapsulation oil *slowly* (over 5–10 sec).

ddSEQ Single-Cell Isolator and Cartridges

- Use only ddSEQ M Cartridges for single-cell encapsulation.

  Important: ddSEQ test cartridges (amber-colored plastic) are included with the ddSEQ Single-Cell Isolator **solely** for equipment testing. **They will not produce droplets and are not suitable for this assay**.

Cartridge Assembly

1. Grip the ddSEQ M Cartridge by the tab and remove it from the package.

   Important: Do not touch the wells or gaskets.

   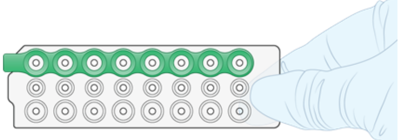

   Fig. 10: Bio-Rad ddSEQ M Cartridge

2. Insert the cartridge into the cartridge holder, as follows:

   a. Lift the cartridge holder lever.

   b. Line up the green gasket on the cartridge with the green stripe on the cartridge holder.

   c. Insert the tab under the rails, and then slide the cartridge into the holder.

   d. Before closing the lever, ensure the cartridge is fully inserted and lying flat against the bottom of the holder.

e. Close the lever. If the lever does not close completely, remove and reinsert the cartridge.

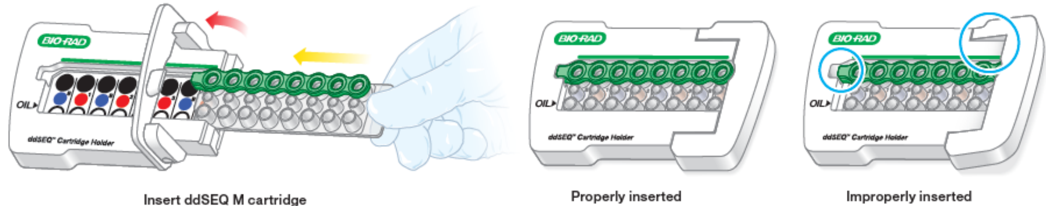

Fig. 11: Inserting ddSEQ M Cartridge into the holder

Fig. 12 shows a correctly assembled cartridge in the cartridge holder:

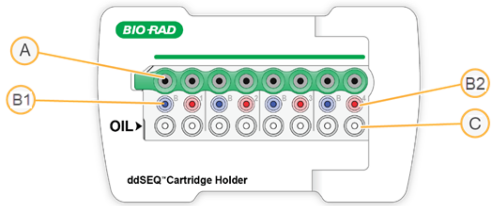

Fig. 12: Correctly assembled cartridge and cartridge holder

A) Encapsulated sample output wells
B1) Barcode suspension mix input wells (blue)
B2) Cell suspension mix input wells (red)
C) Encapsulation oil input wells

Fig. 13 shows incorrectly assembled cartridges in the cartridge holders:

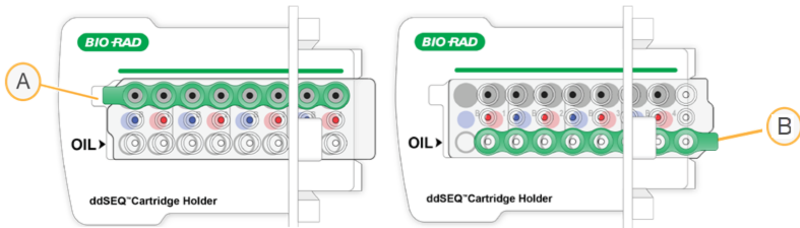

Fig. 13: Incorrectly assembled cartridge and cartridge holder

A) Cartridge misaligned
B) Cartridge upside down

Cartridge Loading

- Ensure that the ddSEQ M Cartridge is correctly assembled in the cartridge holder.
- To ensure all wells receive equal volumes of reagents, use multi-channel pipettes at all loading steps (priming, loading barcode and cell suspension mixes, and adding encapsulation oil).
- Ensure all sample wells in a cartridge are loaded with reaction mixtures.

  Important: If fewer than four samples are available, prepare and load the unused wells with 50% (v/v) glycerol solution in water in place of barcode and cell suspension mixes.

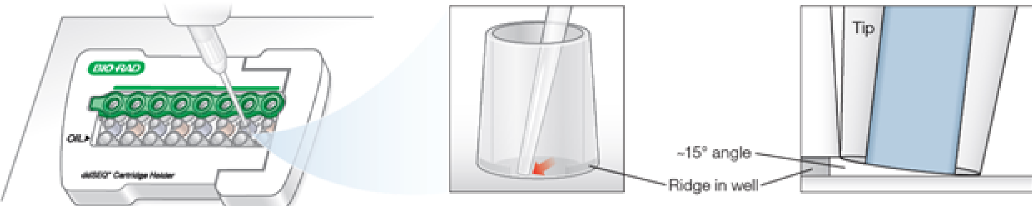

Fig. 14: Pipetting into a ddSEQ M Cartridge

- To achieve the best performance and data quality, **minimize the introduction of air bubbles into individual wells**. Air bubbles are difficult to see, but can be avoided by using the pipetting techniques that follow:
  - To dispense reagents, carefully guide the pipette tip along the side of the well at an approximate 15° angle until it slides over the lower ridge (Fig. 14).
  - While maintaining the 15° angle, anchor the pipette tip against the lower edge of the well and gradually release a small portion of the sample.

    Important: To avoid bubbles, press the pipette plunger only to the first stop. Do not pipette onto the wall or side of the wall.
  - After approximately ½ of the sample has been dispensed, gently elevate the tip while continuing to release the remaining portion of the sample.

Droplet Transfer

- Place the cartridge holder on a flat surface.
- Place the ddPCR 96-Well Plate on a cooling block that is resting on ice.
- **Without exception**, transfer the emulsion droplets using the recommended Rainin P200 filter tips. Using P200 tips from other suppliers affects the integrity of the droplets, and leads to suboptimal data quality.
- **Without exception**, incubate the emulsion droplets in the recommended Bio-Rad ddPCR 96-Well Plate, and then seal the plate with the recommended 8-strip tube caps and Bio-Rad foil seal.
- To allow for slower pipetting and finer control of droplet pickup, use a manual 8-channel L-50 Rainin pipette with filter tips to transfer droplets out of the ddSEQ M Cartridge and into a single column of a 96-Well Plate.
- Slide the multi-channel pipette tips straight down into each of the 8 top wells of the ddSEQ M Cartridge until they reach the junction where the side wall meets the bottom of the well.
- Tilt the pipette tips at an angle of approximately 30-45°.
- Avoid placing the pipette tip vertically or against any flat surface within the well; ensure the tips do not rest flat against the well bottoms.
- Carefully and slowly (~10 sec) draw the specified volume of droplets into the pipette tips (~ 5 µl of air will accrue).
- When transferring droplets, place the pipette tip along the well's side – near, but not at, the bottom of the well – and gently release the droplets over ~10 sec.

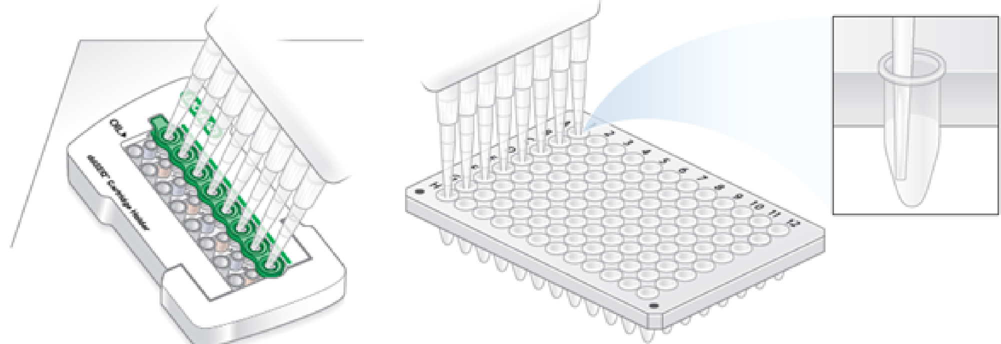

Fig. 15: Droplet transfer

Droplet and Cartridge Handling

- Do not vortex or centrifuge the droplets.
- To avoid evaporation and contamination with particulates, seal the samples immediately after transferring the droplets, as follows:
  - First, seal the droplets wells using the recommended 8-well strip-tube caps.
  - Then, cover the entire ddPCR plate (including the strip caps) with the recommended foil seal.

  Important: Using caps and seals from other suppliers affects the integrity of the droplets and leads to suboptimal data quality.
- When sealing the plate with foil, wear nitrile gloves and gently press the foil to adhere to the plate.

  Important: Do not use a roller or similar apparatus to seal the plate, since they generate static and cause droplet disruption.
- To ensure sample and droplet stability, keep the samples on the 96-well cooling block and on ice until the incubation step of In-Droplet Barcoding and cDNA Synthesis on page 44.
- Start the incubation within 30 min after droplet generation.
- The used cartridge contains residual encapsulation oil. Do not invert the cartridge when removing it from the cartridge holder.
- Discard the used cartridge according to standard laboratory procedures.

Purification Beads

- Use a single-channel pipette to transfer purification beads to sample tubes.

  Important: Using a multi-channel pipette reservoir and a multi-channel pipette can result in an inaccurate volume of purification beads reagent to complete this protocol.

Index and Library Preparation

- For index sequences and coordinates in the cDNA and DO index plates, see Sequencing Run and Loading Parameters on page 80.
- Each cDNA and DO index plate contains 24 index containing wells with indexes in the unique dual index (UDI) format.
- Index wells are for single use. If fewer than 24 indexes are used, seal the pierced index well(s) before returning to -20° C storage to prevent contamination from the used wells.
- To ensure that your libraries produce high-quality sequencing results, use the recommended number of PCR cycles.
- For NextSeq2000 two-plex pooling, pool samples with consecutive indexes from a single column of the index plate.
- For other sequencing systems, pool more than two samples with consecutive indexes down a single column of the index plate.
- Do **not** pool samples with indexes across a row of the index plate.

Chapter 4 Protocol

Preparing Single-Cell Suspension

Important Notes:

- Keep the single-cell suspension on ice until it is ready to mix with the cell enzyme cocktail.
- After the cells are prepared, there are no safe stopping points until the single-cell encapsulation and cDNA synthesis processes are completed.

Table 7. Single-cell suspension, required items

Item	Cap color	Storage	Supplier/ Catalog number	Preparation
BSA cell culture grade	N/A	Room temp or 4° C	Millipore Sigma B6917 or other	N/A
1X PBS; no calcium, no magnesium, pH 7.4	N/A	Room temp	Any vendor	N/A
0.2 µm PES filtration system	N/A	Room temp	Any vendor	N/A

Preparing 1X PBS + 0.1% BSA

Table 8. 1X PBS + 0.1% BSA

Component	Stock concentration	Final concentration	Required volume
From powder:			
1X PBS, no calcium, no magnesium, pH 7.4	1X	1X	50 ml
BSA (powder)	N/A	0.1% (w/v)	50 mg
0.2 µm PES filter	N/A	N/A	N/A
From 10% solution:			
1X PBS, no calcium, no magnesium, pH 7.4	1X	1X	49.50 ml
BSA (10%)	10%	0.1%	0.50 ml

1. Using the information in Table 8, prepare 1X PBS + 0.1% BSA.

   Important: If you are using a powdered form of BSA, or an unfiltered BSA solution, filter the final buffer through a 0.2 µm PES filter **before use**. Particulate contamination affects buffer flow in the ddSEQ M Cartridge and leads to suboptimal performance.

2. Store on ice.

Preparing the Cells

Important Notes:

- Before starting this section, ensure the sample cells are in a single-cell suspension in 1X PBS + 0.1% BSA using an optimized protocol most appropriate for the sample cells. Single-cells must be suspended in 1X PBS + 0.1% BSA solution.
- The ddSEQ Single-Cell 3' RNA-Seq Kit workflow is optimized for loading a range of 1,000 to 10,000 viable cells per sample. Each cartridge can accomodate up to four unique samples. For information on cell concentrations, see Workflow Technology on page 17.
- Cell concentration should be determined based on the **viable** cell counts.

1. After preparing the sample cells into a single cell suspension, refer to Table 9 to dilute the stock cell preparation in 1X PBS + 0.1% BSA solution to the desired number of cells input per sample.

   Important: Keep the single cell suspension on ice until use. Load onto the ddSEQ Single-Cell Isolator within 1 hr after the viable cell count is determined. Single cell stability could differ based on cell types and conditions, and might need to be processed sooner.

   Table 9 on page 29 defines the preferred number of cells loaded per sample in a volume of **5.2 µl**.

Table 9. Preferred number of cells loaded per sample in 5.2 µl total volume

<table>
<tr><th></th><th colspan="10">Estimated number of input cells</th></tr>
<tr><th>Cell suspension (cells/µl)</th><th>1,000</th><th>2,000</th><th>3,000</th><th>4,000</th><th>5,000</th><th>6,000</th><th>7,000</th><th>8,000</th><th>9,000</th><th>10,000</th></tr>
<tr><td>225</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>450</td><td>2.6 | 2.6</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>675</td><td>1.7 | 3.5</td><td>3.4 | 1.8</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>900</td><td>1.3 | 3.9</td><td>2.6 | 2.6</td><td>3.9 | 1.3</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>1,125</td><td>1.0 | 4.2</td><td>2.1 | 3.1</td><td>3.1 | 2.1</td><td>4.1 | 1.1</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>1,350</td><td>0.9 | 4.3</td><td>1.7 | 3.5</td><td>2.6 | 2.6</td><td>3.4 | 1.8</td><td>4.3 | 0.9</td><td>5.2 | 0.0</td><td></td><td></td><td></td><td></td></tr>
<tr><td>1,575</td><td>0.7 | 4.5</td><td>1.5 | 3.7</td><td>2.2 | 3.0</td><td>2.9 | 2.3</td><td>3.7 | 1.5</td><td>4.4 | 0.8</td><td>5.2 | 0.0</td><td></td><td></td><td></td></tr>
<tr><td>1,800</td><td>0.6 | 4.6</td><td>1.3 | 3.9</td><td>1.9 | 3.3</td><td>2.6 | 2.6</td><td>3.2 | 2.0</td><td>3.9 | 1.3</td><td>4.5 | 0.7</td><td>5.2 | 0.0</td><td></td><td></td></tr>
<tr><td>2,025</td><td>0.6 | 4.6</td><td>1.1 | 4.1</td><td>1.7 | 3.5</td><td>2.3 | 2.9</td><td>2.9 | 2.3</td><td>3.4 | 1.8</td><td>4.0 | 1.2</td><td>4.6 | 0.6</td><td>5.2 | 0.0</td><td></td></tr>
<tr><td>2,250</td><td>0.5 | 4.7</td><td>1.0 | 4.2</td><td>1.5 | 3.7</td><td>2.1 | 3.1</td><td>2.6 | 2.6</td><td>3.1 | 2.1</td><td>3.6 | 1.6</td><td>4.1 | 1.1</td><td>4.6 | 0.6</td><td>5.2 | 0.0</td></tr>
</table>

- The single cell suspension concentrations listed are calculated using viable cells only, not total cells.
- For each input, the **volume of cell suspension per sample (µl)** | volume of 1X PBS + 0.1% BSA per sample (µl) is listed accordingly against the number of cells input per sample:
  - **Green boxes** — Optimal cell suspension concentration to achieve the estimated number of input cells (cell suspension volume per sample is ≤ 5.2 µl and ≥ 2.0 µl)
  - **Gray boxes** — Cell suspension volume per sample (≤ 2.0 µl); adjust cell suspension concentration accordingly to minimize pipetting inaccuracies
  - **Black boxes** — Cell suspension volume (> 5.2 µl); adjust cell suspension accordingly to be ≤ 5.2 µl per sample
  - Formulas for calculating volume of cell suspension and 1X PBS + 0.1% BSA required per sample:

    Cell suspension volume (µl) = (number of cells input / viable cell count [cells/µl]) $\times$ 23/20

    1X PBS + 0.1% BSA volume (µl) = 5.2 µl – volume of cell suspension

Preparing the Mixes for Droplet Generation

Important: Work quickly to complete the cell preparation and handling tasks. **Delays can lead to sample failure. Do not stop during or between steps**.

This section explains how to

- Prepare suspension mixes containing cells, barcoded beads, and reagents for cDNA synthesis and DO dimer formation. Table 10 defines the required components for preparing the suspension mixes.
- Prepare ddSEQ Priming Solution and encapsulation oil. Table 11 on page 32 defines the required components for preparing the cartridge.

Table 10. Cell and barcode suspension mixes (required items)

Item	Cap color	Supplier/ Catalog no.	Storage	Preparation
Bead Buffer	Blue	12019997	–20° C	1. Thaw completely, on ice. 2. Using a tabletop vortexer, vortex vigorously to mix (5–10 sec). 3. Centrifuge briefly to collect volume at bottom of tube.
Barcoded Beads	Blue	12020007	4° C	1. Keep on ice. 2. Using a tabletop vortexer, pulse vortex to mix (3x, 5 sec each). **Important:** Do **not** centrifuge barcoded beads after vortexing.
Cell Suspension Buffer	Red	12020074	–20° C	1. Thaw completely, on ice. 2. Using a tabletop vortexer, vortex to mix (5–10 sec). 3. Centrifuge briefly to collect volume at bottom of tube.

Table 10. Cell and barcode suspension mixes (required items), continued

Item	Cap color	Supplier/ Catalog no.	Storage	Preparation
300 mM DTT	Gray	10049218	–20° C	1. Thaw completely, at room temperature. 2. Using a tabletop vortexer, vortex briefly to mix (5 sec). 3. Centrifuge briefly to collect volume at bottom of tube. 4. When thawed, keep on ice.
RNA Stabilizer	Red	12020006	–20° C	1. Keep on ice. 2. Gently flick the thawed tube 3–6x. 3. Centrifuge briefly to collect volume at bottom of tube.
RT Enzyme	Blue	12020053	–20° C	1. Keep on ice. 2. Gently flick the thawed tube 3–6x. 3. Centrifuge briefly to collect volume at bottom of tube.
3' Enzyme Mix	Red	12020052	–20° C	1. Keep on ice. 2. Gently flick the thawed tube 3–6x. 3. Centrifuge briefly to collect volume at bottom of tube.

Table 11. Cartridge preparation (required reagents, equipment, and consumables)

Item	Cap color	Part number	Storage	Preparation
ddSEQ Priming Solution	Clear	12020075	4° C	1. Let stand for 30 min to bring to room temperature. 2. Vortex briefly to mix (5 sec). 3. Centrifuge briefly to collect volume at bottom of tube.
Encapsulation Oil	Clear	12008762	4° C	1. Let stand for 30 min to bring to room temperature. 2. Gently flick the tube 3–5x.

Preparing the Priming Buffer and Encapsulation Oil

The following aliquot volume is sufficient for the preparation of **one** ddSEQ M Cartridge. To prepare multiple cartridges, increase the aliquot volume.

1. Add 25 µl of room temperature ddSEQ Priming Solution to each tube in an 8-tube strip.

   Important: Two adjacent tubes are dedicated for each sample, for a total of four samples.

   Important: If fewer than four samples are used, leave the unused tubes empty.

2. Ensure the encapsulation oil is equilibrated to room temperature.
3. Add 115 µl of room temperature encapsulation oil to each tube of another 8-tube strip.

   Important: Two adjacent tubes are dedicated for each sample, for a total of four samples.

   Important: If fewer than four samples are used, aliquot 115 µl of 50% glycerol into the unused tubes in place of encapsulation oil. **Match the empty tubes in the ddSEQ priming buffer strip to the tubes containing 50% glycerol in the encapsulation oil strip**.

4. Set aside, at room temperature, the 8-tube strips of ddSEQ Priming Solution and encapsulation oil while you prepare the cell and barcode suspension mixes (next section).

Assembling the Barcode Suspension Mix

Table 12. Barcode suspension mix

Component	Required volume, µl (1 rxn plus excess)	Required volume, µl (4 rxn plus excess)
Bead Buffer	10.40	41.60
Barcoded Beads	12.50	50.00
RT Enzyme	2.10	8.40
TOTAL	25.00	100.00

1. Add the components in Table 12 according to the order listed, in a 1.5 ml tube on ice.
2. Before combining the components, vortex the barcoded beads 3x, for 5 sec each, and then immediately add to the bead buffer.

   Important: Do **not** centrifuge the barcoded beads after vortexing.

   Important: Barcoded beads are viscous. Pipette slowly to ensure that the accurate volume is aspirated and dispensed.
3. Keep the barcode suspension mix on ice until use.

Aliquoting the Barcode Suspension Mix

Important: Keep all reagents on ice, including the bulk mixes in 1.5ml tubes and aliquoted mixes in 8-well strip tubes.

1. Vortex the barcode suspension mix 3x for 5 sec each. Vortex at medium speed to prevent formation of air bubbles.

   Important: **Do not centrifuge the barcode suspension mix after vortexing**. This creates a concentration gradient that affects the amount of barcode input.

2. Add 23 µl of the barcode suspension mix to alternate tubes (starting with tube 1) of an 8-tube strip where the positions are labeled as the Barcode tubes (Fig. 16).

   Important: To avoid bubbles, press the pipette plunger only to the first stop.

   Important: If fewer than four samples are used, aliquot 25 µl of 50% glycerol into the unused tubes in place of the barcode suspension mix. **Match the empty tubes in the ddSEQ Priming Solution strip to the tubes containing 50% glycerol in the barcode suspension mix strip**.

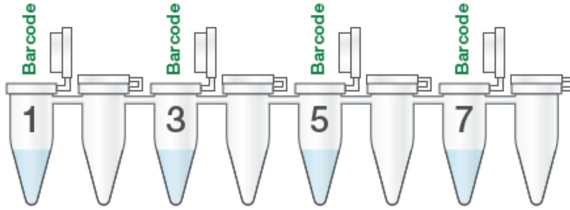

Fig. 16: Loading barcode suspension mix into an 8-tube strip

Preparing the Cell Enzyme Cocktail

Table 13. Cell enzyme cocktail

Component	Required volume (µl) (1 rxn plus excess)	Required volume (µl) (4 rxn plus excess)
Cell Suspension Buffer	12.50	50.00
300 mM DTT	1.67	6.68
3' Enzyme Mix	3.98	15.92
RNA Stabilizer	1.25	5.00
TOTAL	19.40	77.60

1. Add the components in Table 13 according to the order listed, in a 1.5 ml tube on ice.
2. Centrifuge briefly to collect the contents at the bottom of the tube.
3. Keep the cell enzyme cocktail on ice until use.

Assembling the Cell Suspension Mix

Table 14. Cell suspension mix components

Component	Required volume, µl (1 rxn plus excess)
Cell enzyme cocktail	17.8
Filtered cells	5.2
TOTAL	23.0

1. Vortex the cell enzyme cocktail 3x, for 5 sec each.
2. Add 17.8 µl of the cell enzyme cocktail to the alternate tubes (starting with tube 2) of the same 8-tube strip[1], as shown in Fig. 17 where the positions are labeled as the Cell tubes.

   Important: Do not add cells to the cell enzyme cocktail until you are ready to prime the cartridge. Cells should not sit in the cell enzyme cocktail for longer than 5 min before the cartridge is loaded.

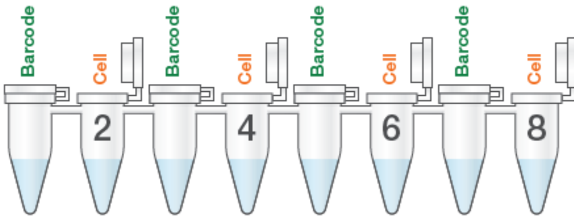

Fig. 17: Loading cell enzyme cocktail into an 8-tube strip

3. Thoroughly mix the single cell suspension (dependent on cell type).
4. To assemble the cell suspension mix, add 5.2 µl of single cell suspension to the corresponding Cell tubes in the 8-tube strip. The single cell suspension was prepared using the table in Preparing the Cells on page 28.

   Important: If fewer than four samples are used, aliquot 25 µl of 50% glycerol into the unused tubes in place of cell enzyme cocktail. **Match the empty tubes in the ddSEQ Priming Solution strip to the tubes containing 50% glycerol in the barcode and cell suspension mix strip**.

5. Immediately continue to the next section.

[1]See Assembling the Barcode Suspension Mix on page 33.

Loading the ddSEQ M Cartridges

This section explains how to prime, and then load, cartridges for cell and barcode encapsulation using the ddSEQ Single-Cell Isolator. Table 15 defines the required components for loading the cartridge.

Table 15. Cartridge loading (required reagents, equipment, and consumables)

Item	Cap color	Part number	Storage	Preparation
Encapsulation Oil	Clear	12008762	4° C	Completed in Preparing the Priming Buffer and Encapsulation Oil on page 32.
ddSEQ Priming Buffer	Clear	12020075	4° C	Completed in Preparing the Priming Buffer and Encapsulation Oil on page 32.
Barcode and cell suspension mixes in 8-tube strip	N/A	N/A	N/A	Completed in Assembling the Barcode Suspension Mix on page 33.
ddSEQ M Cartridge in cartridge holder	N/A	12008720	Room temp	See Cartridge Assembly on page 21. Keep at room temperature.

Priming the ddSEQ M Cartridge

Proper priming ensures appropriate flow of the reagent materials through the microfluidics system in the ddSEQ M Cartridge. Improper priming leads to failure in cell encapsulation and suboptimal data quality.

Before priming and loading, ensure the ddSEQ Priming Buffer and encapsulation oil are equilibrated to room temperature.

1. Insert the cartridge into the cartridge holder. For information, see Cartridge Assembly on page 21.
2. Transfer 20 µl of ddSEQ Priming Solution from the 8-tube strip to each well in the second row of the cartridge.

   To avoid missing wells during Row B cartridge priming, use a P20 multi-channel pipette (Fig. 18). When loading the cartridge, ensure that all tips contain equal volumes of priming solution.

   Important: To avoid bubbles, press the pipette plunger only to the first stop.

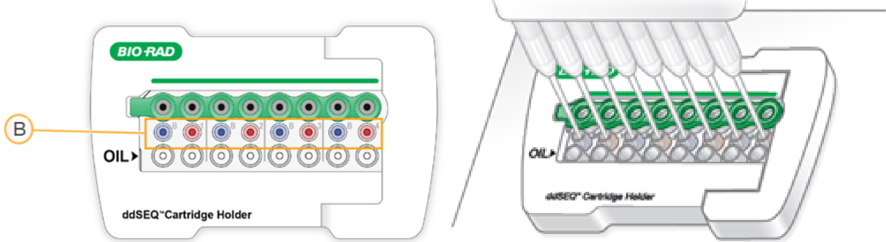

Fig. 18: Priming the ddSEQ M Cartridge

3. Allow the ddSEQ Priming Solution to remain in the wells for 1 min.
4. After incubation is finished, remove all 20 µl of ddSEQ Priming Solution in each well.

   Important: Do **not** allow the ddSEQ Priming Solution to remain in the wells longer than 3 min and ensure it is completely removed before loading. Leaving ddSEQ Priming Solution in the wells interferes with single-cell isolation.

Loading the Cartridge with Barcode and Cell Suspension Mixes

1. Using a P20 multi-channel pipette set to 20 µl, gently mix the barcode suspension and cell suspension mixes in the 8-tube strip 10–15x.

   Important: To avoid bubbles, press the pipette plunger only to the first stop.

2. Using the same pipette tips, transfer 20 µl of the barcode suspension and cell suspension mixes into the bottom of the Row B wells in the cartridge (Fig. 19).

   Important: To avoid bubbles, press the pipette plunger only to the first stop.

   Important: Load **all** Row B wells in the ddSEQ M Cartridge. If fewer than four samples are available, fill the unused wells with 50% (v/v) glycerol solution in water. You must load the wells in Row B **before** you load the oil in the bottom row of wells labeled OIL (Fig. 19).

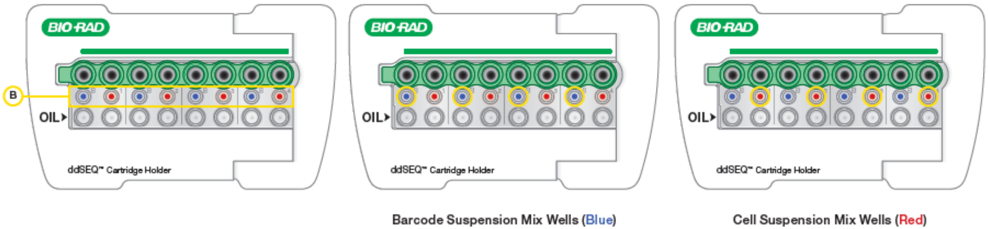

Fig. 19: Barcode suspension mix and cell suspension mix wells

3. Using a P200 multi-channel pipette, transfer 80 µl of encapsulation oil from the 8-tube strip to each well of the bottom row in the cartridge, which is labeled OIL. To avoid bubbles, press the pipette plunger only to the first stop.

   Note: One bottle of encapsulation oil is enough for two cartridges.

   Important: Load all wells in the OIL row of the ddSEQ M Cartridge. If fewer than four samples are available, add 50% (v/v) glycerol solution in water to the encapsulation oil wells. Match the glycerol-filled wells in the OIL row to the glycerol-filled wells in Row B.

   Caution: All the input wells (Row B) and oil wells (OIL) must be loaded with the corresponding reagents. Running a cartridge with empty Row B wells or empty oil wells on the ddSEQ Single-Cell Isolator damages the instrument and repairs will be required to restore it.

4. Keep the loaded cartridge in the cartridge holder for single-cell isolation on the ddSEQ Single-Cell Isolator.

Single-Cell Encapsulation

This section explains how to

- Load the cartridge into the ddSEQ Single-Cell Isolator
- Generate partitioned cells in droplets

Table 16. Dispensing to cartridge (required reagents, equipment, and consumables)

Item	Cap color	Part number	Storage	Preparation
Loaded ddSEQ M Cartridge in cartridge holder	N/A	12008720	Room temp	Prepared in previous section. Keep at room temperature.
ddSEQ Single-Cell Isolator	N/A	12004336	Room temp	Install per the instruction manual.
96-well cooling block	N/A	N/A	4° C	Set on ice to keep cold.
ddPCR 96-Well Plates	N/A	12001925	Room temp	N/A

Generating Droplets

This section explains how to use the ddSEQ Single-Cell Isolator to generate droplets.

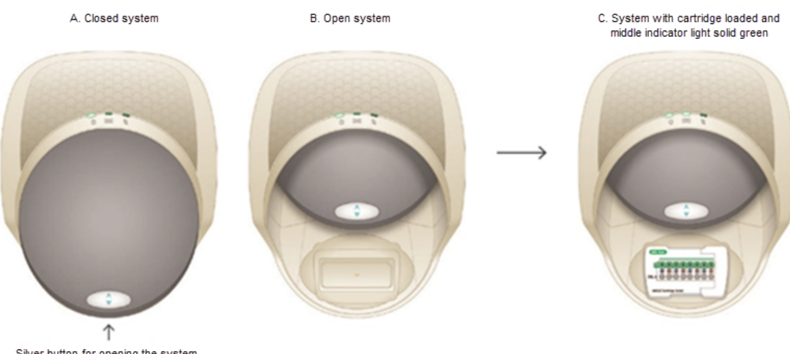

Fig. 20: ddSEQ Single-Cell Isolator

Table 17 defines information on the instrument indicator lights.

Table 17. Status indicator lights on the ddSEQ Single-Cell Isolator

Status			
Solid green	Power on	Cartridge holder in place	Run complete
Flashing green	N/A	N/A	Run in progress
Flashing amber	N/A	No seal	N/A
Off	Power off	No cartridge holder	Idle

Important: The instrument is ready when the first light is solid green and the other two lights are off.

1. Insert the cartridge as specified below and as shown in Fig. 20 on page 40.
   a. To open the instrument, press the silver button on the front of the ddSEQ Single-Cell Isolator.
   b. Place the cartridge holder into the instrument.
   c. To confirm that the cartridge holder is in the correct position, ensure that the cartridge indicator light (middle light) is solid green.

      If the cartridge indicator light is not illuminated, remove and reseat the cartridge holder on the instrument until the cartridge indicator light (middle light) is solid green.
   d. To close the instrument, press the silver button on the top of the ddSEQ Single-Cell Isolator.

      Notes:
      - After the door is closed, droplet generation and single-cell isolation begin automatically and take ~5 min.
      - While the droplet indicator flashes green, droplet generation is in progress.
      - When all three indicator lights are solid green, droplet generation is complete.

      Important: See Table 17 for information on indicator lights. **Do not open the instrument until all three indicator lights are solid green**. If the central light flashes amber, the gasket on the cartridge is not providing an adequate seal. If an error occurs, do not rerun a cartridge. For assistance, contact Bio-Rad Technical Support.

2. While the ddSEQ Single-Cell Isolator is running, set an empty ddPCR 96-Well Plate on a 96-well cooling block on ice.
3. To open the instrument after the run is complete, press the silver button.
4. Remove the cartridge holder from the ddSEQ Single-Cell Isolator. Successfully encapsulated samples are present in the top row of the cartridge (Fig. 21) and appear cloudy.

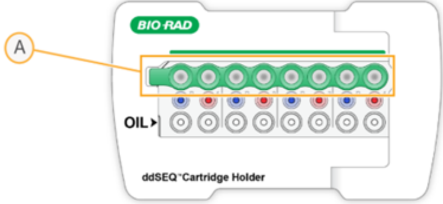

Fig. 21: Output wells containing encapsulated samples

A) Output wells

5. Check for wells that look clear or empty, which indicates droplet generation failure.

   If droplet generation failed, prepare additional cell and barcode suspension mixes and prime a second ddSEQ M Cartridge for use.

   Important: After the door opens, the instrument continues to make noise for ~5 sec until the reset is completed. Wait for the instrument to reset before running another cartridge.

   Important: **Do not rerun used cartridges and samples**.

6. Proceed immediately to the next section.

Transferring Droplets

Table 18 defines the reagents, equipment and consumables that are required during droplet transfer.

Table 18. Droplet transfer (required items)

Item	Cap color	Part number	Storage	Preparation
ddSEQ M Cartridge in cartridge holder, containing encapsulated samples	N/A	12008720	Room temp	Prepared in previous section. Keep at room temperature.
ddPCR 96-Well Plates	N/A	12001925	Room temp	To keep cold, place on 96-well cooling block.
PCR Plate Microseal F Foil Seals	N/A	MSF1001	Room temp	N/A
8-Cap Strip for 0.2ml PCR Tubes	N/A	TCS0803	Room temp	N/A
96-well cooling block	N/A	N/A	4° C	To keep cold, place on ice.

1. Place the cartridge holder on a flat surface.
2. Use a P50 multi-channel pipette, set to 43 µl, to slowy aspirate all encapsulated samples from the ddSEQ M Cartridge output wells.

   Several emulsion layers can be observed in the pipette tips, as shown in Fig. 22. The total emulsion volume transferred to each well in the ddPCR 96-Well Plate is 35– 40 µl, with ~5 µl of air.

   Important: Fast or harsh pipetting breaks encapsulated samples. Pipet slowly (5–10 sec) to avoid yield loss.
3. To transfer the encapsulated samples (Fig. 22), complete the following substeps:
   a. Slowly (5–10 sec) dispense the encapsulated samples into the empty ddPCR 96-Well Plate, on a 96-well cooling block on ice.

      Important: To avoid bubbles, press the pipette plunger only to the first stop.

   b. Hold the pipette plunger at the **first** stop and wait 5 sec for the remaining sample to collect at the tip of the pipette.

c. Press the pipette plunger to the **second** stop to slowly dispense the remaining sample into the same column of the plate.

Important: Do not discard the tips until all the encapsulated sample has been transferred to the plate. Discarding tips with sample results in yield loss.

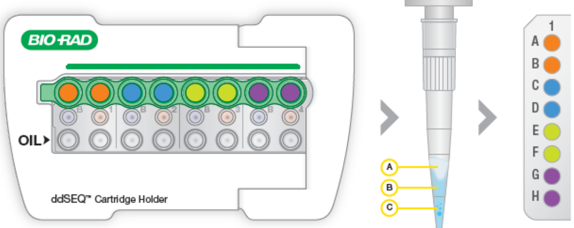

Fig. 22: Transferring encapsulated samples from cartridge to ddPCR plate

The emulsion layers from output wells result in

A) Aqueous layer containing encapsulated samples
B) Oil layer
C) Oil and air bubbles

4. To seal the plate, complete the following substeps:

   a. Cover the ddPCR 96-Well Plate samples using 8-tube strip caps.

   b. Use the PCR plate Microseal F foil pierceable seals to cover the entire ddPCR plate, including the capped wells.

   Important: You must use the recommended strip caps **and** the foil to seal the plate. Do **not** use rollers or scrapers to adhere the foil.

   Important: Do **not** wear latex gloves when handling droplets.

5. Keep the samples on the 96-well cooling block on ice until you are ready to start the in-droplet barcoding and cDNA synthesis sections.

In-Droplet Barcoding and cDNA Synthesis

This section explains the reaction steps during which partitioned cells will lyse, have their mRNA captured by barcoded bead oligos, and be converted into double-stranded cDNA. The steps also include DO dimer generation within droplets.

Table 19. In-droplet barcoding and cDNA synthesis (required reagents, equipment, and consumables)

Item	Catalog No.	Supplier
ddPCR 96-well sealed and cooled plate containing encapsulated samples (completed in previous sections)	N/A	N/A
Thermal cycler* **Use one of the recommended thermal cyclers or an equivalent deep-well thermal cycler with up to 100 µl capacity.* For information, see Required Consumables and Equipment on page 11.		

Table 20. In-droplet barcoding and cDNA synthesis program

Lid temperature	Reaction volume	Ramp rate
105° C	50 µl	2.0° C/sec, unless specified
Step	**Temperature**	**Time (hr:min:sec)**
1	4° C	00:25:00
2	50° C	00:45:00
3	65° C	00:30:00
4	80° C	00:05:00
5	Ramp rate 0.1° C/sec	
6	4° C	Hold

1. Place the sealed ddPCR 96-Well Plate with encapsulated samples, in the thermal cycler and proceed with the incubation protocol specified in Table 20.

Safe Stopping Point

- When this section is complete, you can store the sample at 4° C for ≤ 24 hours, or at -20° C for 7 days.
- Operating the instrument at 4° C should be limited to 18 hours at these conditions.
- Holds at 4° C can be performed for up to 72 hrs if humidity is 20-60% (noncondensing).

Breaking Emulsions and Purifying cDNA and DO Dimer Fractions

This section explains the steps required to

- Break the individual droplets containing barcoded sample cDNA and DO dimers
- Purify the products for library preparation

After mixing purification beads to the aqueous phase of disrupted droplets, the cDNA binds to the magnetic beads and the DO dimers remain in the supernatant. The cDNA and DO dimers are then purified independently from the bead pellet and the supernatant, respectively. At the end of the purification step, each sample will have two separate products (cDNA and DO dimers) for Illumina sequencing library preparation.

Important Notes:

- The purification protocol provides a selection step that separates and enriches the cDNA and DO dimer fractions. Both fractions are required for the ddSEQ Single-Cell 3' RNA-Seq assay.
- Pay careful attention during the cleanup step, do NOT discard the purification bead supernatant that contains the DO dimer fraction.
- The purified cDNA from the two output wells of each sample are combined after the first cDNA elution (Fig. 23).
- The purified DO dimer from the two output wells of each sample are combined after the first DO dimer elution (Fig. 23).

- Keep the purified cDNA and DO dimer separate for each sample. Do **not** combine.

Important: Experienced users can complete step 3c concurrently with steps 4b-4c. **Do not** attempt concurrent purifications if you are not experienced with the protocol.

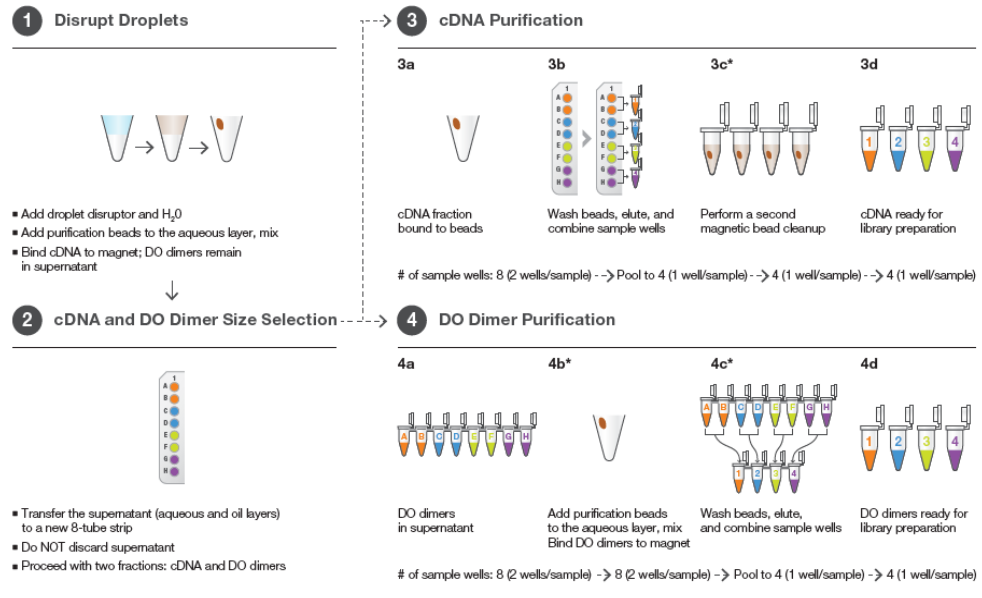

Fig. 23: Droplet disruption, cDNA and DO dimer fraction purification workflow

Table 21. Breaking emulsions and purifying cDNA and DO dimer fractions (reagents and consumables)

Item	Cap color	Part number	Storage	Preparation
ddPCR 96-Well Plate containing samples	N/A	12001925	Room temp	Created in previous section. If frozen, thaw completely to room temperature
Droplet Disruptor	Clear	12008761	4° C	1. Let stand for 30 min to bring to room temperature. 2. Immediately before use, vortex 3–5x, for 1 sec each, to mix. 3. Centrifuge briefly to collect volume at bottom of tube.
Nuclease-free water	N/A	N/A	N/A	N/A
Purification beads (choose one):				
■ Beckman Coulter SPRIselect Reagent Kit	N/A	B23317	Room temp	1. Vortex until well dispersed.
■ Beckman Coulter AMPure XP SPRI Reagent	N/A	A63881	4° C	1. Let stand for 30 min to bring to room temperature. 2. Vortex until well dispersed.
Ethanol, pure (200-proof, anhydrous)	N/A	N/A	Room temp	Prepare a fresh batch of 80% ethanol in water.

Table 21. Breaking emulsions and purifying cDNA and DO dimer fractions (reagents and-consumables), continued

Item	Cap color	Part number	Storage	Preparation
10mM Tris-HCl, pH 8.0 (Resuspension Buffer), DNAse-free (Teknova)	N/A	T1173	Room temp	N/A
NEBNext Magnetic Separation Rack	N/A	S1515S	Room temp	N/A
Thermo Fisher Scientific Magnetic Stand-96 (magnetic peg stand)	N/A	AM10027	Room temp	N/A
Thermo Fisher Scientific DynaMag 96 Side Skirted Magnet	N/A	12027	Room temp	N/A

Important: This protocol requires a **magnetic peg stand** (Thermo Fisher Magnetic Stand 96), a **skirted magnet** (Thermo Fisher DynaMag 96 Side-Skirted), and a **magnetic separation rack** (NEBNext). For information, see Required Consumables and Equipment on page 11.

Important: All magnets are required in this protocol and are not interchangeable. Pay careful attention to which magnet is used for each step.

Breaking Emulsions

1. Remove the samples from the thermal cycler.

   Important: Do not vortex or centrifuge the samples.

2. Examine the samples and confirm they have equal volumes and two distinct layers, an oil layer on the bottom and an aqueous emulsion layer on top, as shown in Fig. 24.

   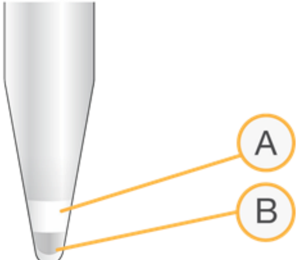

   Fig. 24: Sample emulsion layers

   A) Aqueous emulsion layer
   B) Oil layer

3. To avoid cross-contamination, gently and carefully remove the foil seal, followed by the 8-tube strip caps.

4. Add 20 µl of droplet disruptor to each sample. **Dispense slowly against the side of the well above each sample**.

5. Incubate at room temperature for 30 sec.

   Important: Do not mix or pipette the droplet disruptor into the sample. **To prevent cross-contamination**, use fresh pipette tips for each sample well .

6. Add 78 µl of nuclease-free water per well. **Dispense against the side of the well above each sample**.

   Important: Do not mix or pipette water into the sample.

cDNA and DO Dimer Fraction Purification

- The steps in this section use purification beads to clean up the cDNA and DO dimer products.

  Important: Do not discard the supernatant fraction from the first size selection step. It must be processed into a final DO library. Pay close attention at each step for proper sample handling instructions.

- At the start of the cleanup steps, sample wells contain separate oil and aqueous layers. When mixing, mix only in the specified aqueous layer.

- Use a single-channel pipette to transfer purification beads to sample wells.

  Note: Using a multi-channel pipette reservoir and a multi-channel pipette can result in an inaccurate volume of purification beads (reagent) required to complete this protocol.

First cDNA Purification and DO Dimer Size Selection

1. Prepare fresh 80% ethanol from absolute ethanol.
2. Vortex the purification beads until well dispersed.
3. Without mixing, slowly dispense 56 µl purification beads to the samples above the aqueous layer.

   Important: Do not dispense into the oil layer at the bottom of the well.

4. Gently pipette mix the purification beads in the aqueous layer until the layer is evenly distributed (10–15x).

   Tilt the pipette tip at an angle of approximately 30-45° and pipette the magnetic beads and the aqueous layer gently against the side of the wells to mix.

   Important: Pipette mix **gently** to prevent mixing the aqueous layer with the oil layer. **Forceful pipette mixing leads to sample loss**.

After sufficient mixing, the samples display two distinct layers:

- An oil layer at the bottom of the well
- A homogeneous brown aqueous layer at the top (Fig. 25C).

If parts of the aqueous layer still appear clear or a lighter brown (Fig. 25B), continue to mix until the entire aqueous layer is homogeneously brown.

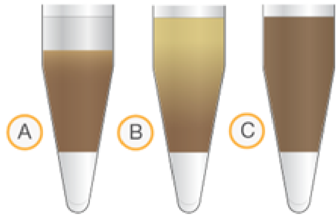

Fig. 25: Mixing states

A) Initial state with a clear aqueous layer at the top
B) A lighter brown aqueous layer at the top indicates the layer is not properly mixed
C) An entirely homogenous brown aqueous layer at the top indicates the layer is properly mixed

5. Lift the samples to closely examine the quality of mix for the aqueous layer.

First cDNA Bind

1. Incubate the sample-purification bead mixture in the ddPCR plate at room temperature for 10 min.
2. Place the samples on the **magnetic peg stand**.
3. Incubate 10 min at room temperature to allow the purification beads to bind to the magnet.

   Note: Due to bead retention in the aqueous and oil layers, the liquid might not be completely clear of beads after 10 min, but most beads should be bound to the magnet.

4. Transfer the entire volume of supernatant, including aqueous and oil layers, to a new 8-tube strip.

   Important: High profile PCR strip tubes are required to carry a total volume of ~210 µl during the purification steps.

   Important: **Do not discard the supernatant**. It contains the DO dimer fraction that must be further purified in the DO dimer purification sections. Set aside the supernatant at room temperature while you complete the cDNA purification sections.

First cDNA Wash

1. Wash the magnet-bound purification beads, as follows:
    a. Add 200 µl of freshly prepared 80% ethanol to each well.

       Important: To avoid disturbing the pelleted purification beads, pipette against the wall opposite the beads.

    b. Incubate on the magnet for 30 sec to 1 min.
    c. Keeping the plate on the magnet, pipette to remove and discard supernatant.
    d. Place the samples on the **skirted magnet** to move the pellet closer to the bottom of the well. This improves the handling.
2. Repeat steps 1a–1c for a total of 2 washes.
3. Pipette to remove any residual ethanol at the bottom of the wells without disrupting the magnetic bead pellet.
4. Air-dry the samples on the magnet until there is no remaining liquid in each well.

   Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.

First cDNA Elute

1. Remove the plate from the magnet. Add 53 µl of resuspension buffer (10mM Tris-HCl, pH 8.0) to each sample well. Pipette to mix, ensuring all beads are resuspended.

   Important: Confirm that the solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.

2. Incubate the solution at room temperature for 5 min.
3. Place the samples on the **skirted magnet**, which pulls the bead pellet closer to the bottom of the well. This facilitates the subsequent handling.
4. Incubate at room temperature for 5 min (until the beads are completely bound to the magnet).

Combine Wells From cDNA Samples and Transfer

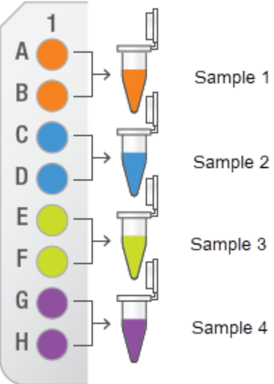

Fig. 26: Combining barcoded cDNA samples after magnetic cleanup

Important: Keep the sample on the **skirted magnet** during this procedure.

1. To combine the two wells for each sample, transfer 50 µl of supernatant from each sample well to an 8-tube strip, as shown in Fig. 26:

   - Sample 1, wells A and B, to the first tube in an 8-tube strip
   - Sample 2, wells C and D, to the second tube in an 8-tube strip
   - Sample 3, wells E and F, to the third tube in an 8-tube strip
   - Sample 4, wells G and H, to the fourth tube in an 8-tube strip

   After transferring the supernatant, the total volume in each tube is 100 µl.

   Important: Each pair of sample wells in succession represents one sample. **Proper pooling is critical for library prep indexing and sample processing**.

2. Proceed immediately to the next section.

Second cDNA Purification

1. Add 60 µl of purification beads to the tubes or wells containing 100 µl of cDNA from the previous section.
2. Pipette to mix the purification beads and cDNA until homogeneous (10–15x).

Second cDNA Bind

1. Incubate the 8-tube strip at room temperature for 5 min.
2. Place the 8-tube strip on the **magnetic separation rack**.
3. Incubate at room temperature for 5 min.
4. Keeping the 8-tube strip in the **magnetic separation rack**, pipette to remove and discard supernatant.

Second cDNA Wash

1. Wash the magnet-bound purification beads, as follows:
   a. Add 200 µl of freshly prepared 80% ethanol to each well, pipetting the ethanol against the wall opposite the beads.

      Important: Do not disturb the pelleted purification beads.

   b. Incubate on the **magnetic separation rack** for 1 min.
   c. Keeping the 8-tube strip in the **magnetic separation rack**, pipette to remove and discard supernatant.
2. Repeat steps 1a-1c for a total of 2 washes.
3. Remove all residual ethanol, as follows:
   a. Centrifuge briefly to collect purification beads at the bottom of the tubes.
   b. Place the 8-tube strip on the **magnetic separation rack**.
   c. Pipette to remove remaining ethanol.
4. Air-dry on the **magnetic separation rack** until there is no remaining liquid in each well.

   Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.

Second cDNA Elute

1. Remove the 8-tube strip from the **magnetic separation rack**.
2. Add 23 µl resuspension buffer (10 mM Tris-HCl, pH 8.0) to each sample well.
3. Pipette to mix, making sure all beads are resuspended.

   Important: Confirm the solution is homogenous. Yield loss can occur if the beads are not thoroughly resuspended.
4. Incubate at room temperature for 2 min.
5. Place the samples on the **magnetic separation rack** and incubate at room temperature for 1–5 min (until beads are no longer visible in the solution).
6. Transfer 22 µl of purified cDNA to a new 8-tube strip.

DO Dimer Purification

1. Prepare fresh 80% ethanol from absolute ethanol.
2. Vortex the purification beads until they are well dispersed.
3. Proceed with DO dimer purification at room temperature.

   Note: These are the supernatant samples that were set aside during cDNA purification. For information, see cDNA and DO Dimer Fraction Purification on page 51.
4. Without mixing, slowly dispense 56 µl purification beads to the DO dimer sample above the aqueous layer.

   Important: Do not dispense into the oil layer at the bottom of the tube.
5. Pipette mix purification beads in the aqueous layer only until the layer is evenly distributed (10–15x).

   To mix, tilt the pipette tip at an angle of ~30–45° and gently pipette the magnetic beads and the aqueous layer against the side of the tubes.

   Important: Pipette mix **gently** to prevent mixing the aqueous layer with the oil layer. **Forceful pipette mixing leads to sample loss**.
6. After mixing, the samples have two distinct layers: an oil layer on the bottom of the tube and a homogeneous brown aqueous layer on the top. If parts of the aqueous layer still appear clear or a lighter brown, continue to mix until the entire aqueous layer is homogenous brown.

DO Dimer Bind

1. Incubate the DO dimer samples in the 8-tube strip at room temperature for 5 min.
2. Place the 8-tube strip in the **magnetic separation rack**.
3. Incubate at room temperature for 5 min.
4. Keeping the strip tube in the **magnetic separation rack**, pipette to remove and discard supernatant (including aqueous and oil layers).

DO Dimer Wash

1. Wash the magnet-bound purification beads, as follows:
   a. Add 200 µl of freshly prepared 80% ethanol to each well, pipetting the ethanol against the wall opposite the beads.

      Important: Do not disturb the pelleted purification beads.

   b. Incubate on the **magnetic separation rack** for 30 sec to 1 min.
   c. Keeping the 8-tube strip in the **magnetic separation rack**, pipette to remove and discard supernatant..
2. Repeat steps 1a-1c for a total of 2 washes.
3. Remove all residual ethanol, as follows:
   a. Centrifuge briefly to collect purification beads at the bottom of the tubes.
   b. Place the 8-tube strip on the **magnetic separation rack**.
   c. Pipette to remove remaining ethanol.
4. Air-dry on the **magnetic separation rack** until there is no remaining liquid in each tube.

   Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.

DO Dimer Elute

1. Remove the 8-tube strip from the **magnetic separation rack**.
2. Add 20 µl resuspension buffer (10mM Tris-HCl, pH 8.0) to each sample tube to elute the DO dimers.
3. Pipette to mix, making sure all beads are resuspended.

   Important: Confirm that solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.
4. Incubate the 8-tube strip at room temperature for 5 min.
5. Place the sample plate on the **magnetic separation rack** and incubate at room temperature for 1–5 min (until beads are no longer visible in the solution).

Combine Wells From DO Dimer Samples and Transfer

1. To combine the two tubes for each sample, transfer 19 µl of supernatant from each sample tube to a new 8-tube strip (Fig. 27).

   Important: Keep the samples on the **magnetic separation rack** during this step.

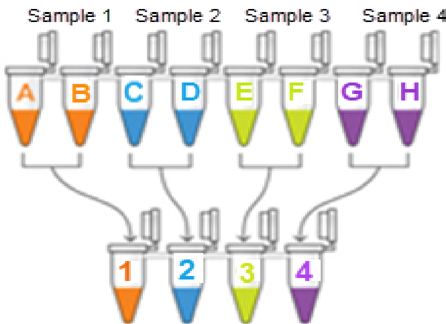

Fig. 27: Combining DO dimer samples after magnetic bead cleanup

- Sample 1, Tubes A and B, to Tube 1 of the new 8-tube strip
- Sample 2, Tubes C and D, to Tube 2 of the new 8-tube strip
- Sample 3, Tubes E and F, to Tube 3 of the new 8-tube strip
- Sample 4, Tubes G and H, to Tube 4 of the new 8-tube strip

After transferring, the total volume of supernatant in each tube is 38 µl.

Important: Each pair of sample tubes in succession represents the DO dimers from one sample. **Proper pooling is critical** for matching to each sample's partner cDNA, and for library prep indexing and sample processing.

Safe Stopping Point

After this section is complete, you can store the purified cDNA and DO dimers at -20° C for up to 7 days.

Preparing cDNA and DO Libraries

The cDNA fraction is converted into a sequencing library via tagmentation and index PCR. The DO dimers are processed into a sequencing library using indexing PCR only (Fig. 28).

Important: Follow the instructions carefully to ensure you are performing the correct library preparation procedure on the cDNA and DO dimer fractions.

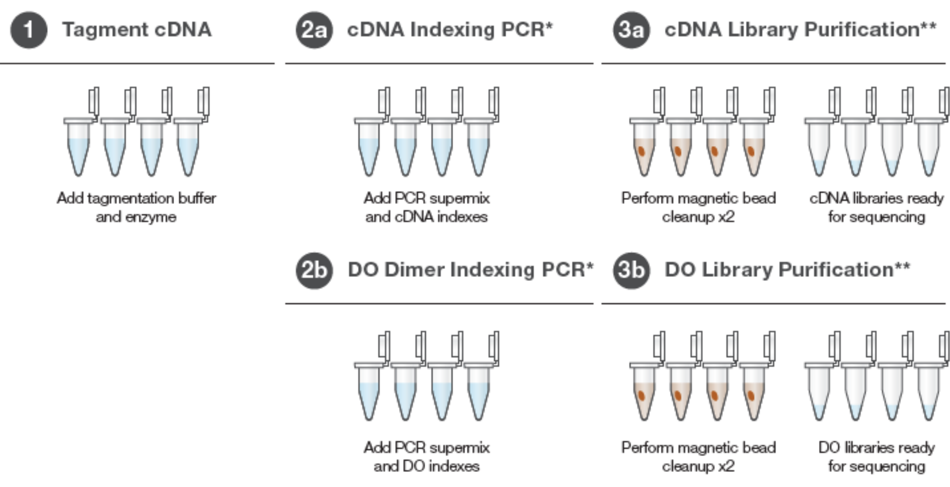

Fig. 28: cDNA and DO library preparation

cDNA Tagmentation

Important: *Perform tagmentation on cDNA samples only*. Before you begin tagmentation, you must fully thaw all necessary index PCR reagents for the next step (cDNA Indexing and Amplification on page 63).

Table 22. cDNA tagmentation (required reagents, equipment, and consumables)

Item	Cap color	Part number	Storage	Preparation
cDNA, purified (22 µl)	N/A	N/A	On ice	See previous sections.
cDNA Tagmentation Buffer (labeled as TGMNT on tube)	Yellow	12019996	-20° C	1. Thaw completely, on ice. 2. Using a tabletop vortexer, vortex to mix (5–10 sec). 3. Centrifuge briefly to collect volume at bottom of tube.
cDNA Tagmentation Enzyme	Yellow	12019983	-20° C	1. Set on ice. 2. Gently flick the thawed tube 3–6x to mix. 3. Centrifuge briefly to collect volume at bottom of tube.
Carrier DNA	Yellow	12020782	-20° C	1. Thaw completely, on ice. 2. Using a tabletop vortexer, vortex to mix (5–10 sec). 3. Centrifuge briefly to collect volume at bottom of tube.
Thermal cycler*				

**Use one of the recommended thermal cyclers or an equivalent deep-well thermal cycler with up to 100 µl capacity.For information and catalog numbers, see* *Required Consumables and Equipment on page 11*.

1. Save the following tagmentation program (TGM) on the thermal cycler.

Table 23. cDNA tagmentation protocol

Lid temperature	Reaction volume	Ramp rate
105° C	50 µl	2.0° C/sec
Step	**Temperature**	**Time (hr:min:sec)**
1	55° C	Hold
2	55° C	00:05:00
3	4° C	Hold

2. Pre-warm the thermal cycler to 55° C and hold at that temperature while preparing the tagmentation mix.
3. Before adding the cDNA tagmentation mix, mix carrier DNA (if needed) directly into each cDNA sample. For information on recommended carrier volumes, see Table 24.

   Important: Failure to mix Carrier DNA into the recommended samples might lead to low library yield and reduced gene detection sensitivity.

   Important: Keep samples on ice at all times.

Table 24. Recommended carrier DNA volumes

Carrier DNA volume		Cell input
1 µl	if	Low-expresser cells* or ≤ 2,500 high-expresser cells**
Not required	if	> 2,500 high-expresser cells**

** Based on human PBMC; actual performance may vary based on cell type and cell conditions.*

*** Based on HEK 293 and NIH 3T3 cell lines; actual performance may vary based on cell type and cell conditions.*

4. Add the components in Table 25, according to the order listed, in a 1.5 ml tube on ice.

Table 25. cDNA tagmentation components

Component	Required Volume, µl (1 rxn plus excess)	Required Volume, µl (4 rxn plus excess)
cDNA Tagmentation Buffer (labeled as TGMNT on tube)	8.80	35.20
cDNA Tagmentation Enzyme	11.00	44.00
TOTAL	19.80	79.20

5. Pipet the cDNA tagmentation mix 3–6x while on ice, and then centrifuge briefly to collect the contents at the bottom of the tube.
6. Add 18 µl of the cDNA tagmentation mix to each cDNA sample (whether or not carrier DNA is present).

   Important: Use a new pipette tip for each cDNA sample.
7. Pipette to mix the samples 3–6x and then seal or cap the samples.
8. Place on the pre-programmed thermal cycler and run the TGM program.
9. Advance the TGM program past the 55° C hold to tagment for 5 min.
10. Remove the samples from the thermal cycler as soon as the temperature reaches 4° C.
11. Place the samples on ice.

    Important: Proceed immediately to the next section.

cDNA Indexing and Amplification

Table 26. cDNA indexing and amplification (required reagents, equipment, and consumables)

Item	Cap color	Part number	Storage	Preparation
Tagmented cDNA	N/A	N/A	On ice	See previous sections
PCR Supermix	White	12020005	-20° C	1. Thaw completely, on ice. 2. Gently flick the thawed tube 3–6x. 3. Centrifuge briefly to collect volume at bottom of tube.
cDNA Index Plate	N/A	12020634	-20° C	1. Thaw completely, at room temperature. 2. Centrifuge briefly to collect volume at bottom of plate.
Thermal cycler*				

**Use one of the recommended thermal cyclers or an equivalent deep-well thermal cycler with up to 100 µl capacity.For information and catalog numbers, see* *Required Consumables and Equipment on page 11*.

1. Save the cDNA library amplification (CLA) program (Table 27) on the thermal cycler.

Table 27. CLA protocol

Lid temperature	Reaction volume	Ramp rate
105° C	100 µl	2.0° C/sec
Step	**Temperature**	**Time (hr:min:sec)**
1	95° C	00:03:00
2	98° C	00:00:20
3	60° C	00:00:45
4	72° C	00:01:00
5	Go to Step 2 for a total of **X** cycles (Table 28 on page 65)	
6	72° C	00:05:00
7	10° C	Hold

Important: To amplify cDNA libraries based on cell input and expression level, **follow the PCR cycle recommendations in Table 28 on page 65**. Use the recommended number of PCR cycles as the starting point to determine the optimal number of cycles. Upon evaluation of the performance of similar sample types, the number of PCR cycles can be adjusted for optimal performance.

Table 28. Recommended number of cDNA index PCR cycles

Recommended cDNA index PCR cycle number		Cell input
14	if	≤ 5,000 low-expresser cells * or ≤ 4,000 high-expresser cells **
13	if	> 5,000 low-expresser cells * or >4,000 high-expresser cells **

** based on human PBMC; actual performance may vary based on cell type and cell conditions.*

*** based on HEK 293 and NIH 3T3 cell lines; actual performance may vary based on cell type and cell conditions.*

2. Add 50 µl of PCR supermix to each of the cDNA-tagmented samples.

   Important: To prevent cross-contamination, use a fresh tip for each sample.

3. Open the lid of the index plate **marked for cDNA indexing only** and gently wipe the surface (foil) of the cDNA index plate with 80% ethanol.

   Important: The cDNA and the DO Index plates are NOT interchangeable. **Ensure you are using the cDNA index plate for the cDNA libraries you are preparing**.

4. Using a clean pipette tip, gently pierce the foil over the well containing the index adapters to be used with your cDNA sample.

   Important: To prevent cross-contamination, do the following:

   - Use a new pipette tip for each index well
   - Indexes are for single use. If fewer than 24 indexes are used, seal the pierced index wells before returning to -20° C storage to prevent contamination from the pierced wells.

   Important: Because the foil may require slight pressure to pierce, ensure the plate is on a flat surface to prevent spills and cross-contamination.

5. Using a pipette set to 10 µl, **with a clean tip**, pipette mix the cDNA indexes in the pre-pierced index well 5x, and then add 10 µl of a unique cDNA index to each cDNA tagmented sample.

   Important: Use a different cDNA index for each sample, and record the index used. The information is required to match the index to the paired DO dimer sample during DO library preparation, and to set up the sequencing run.

   Fig. 29 illustrates the cDNA index plate map. For index sequences, see Library Preparation for Sequencing on page 79.

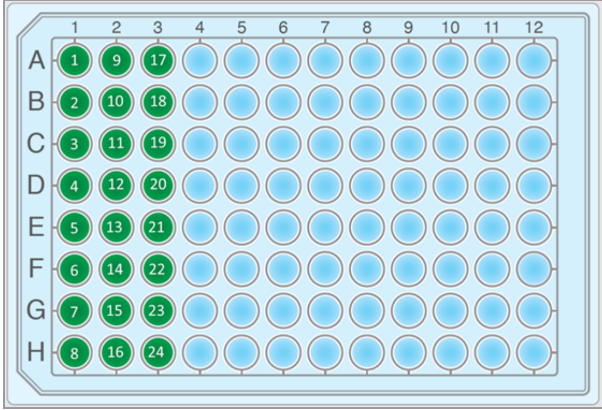

Fig. 29: cDNA index map

6. Using a P200 multi-channel pipette set to 80 µl, mix the cDNA PCR reaction 10x.
7. Seal or cap the cDNA PCR reactions and centrifuge.
8. Place the cDNA PCR reactions on the pre-programmed thermal cycler and run the CLA program.
9. If a second thermocycler is available, proceed to DO indexing and amplification once the CLA program is initiated.

Safe Stopping Point

- **After this section is complete, you can store the sample on the thermal cycler at 10° C or at 4° C for up to 72 hr.**
- **Operating the instrument at 4° C should be limited to 18 hours at these conditions.**
- **Holds at 4° C can be performed for up to 72 hrs if humidity is 20-60% (noncondensing).**

DO Dimer Indexing and Amplification

Table 29. DO dimer indexing and amplification (required items)

Item	Cap color	Part number	Storage	Preparation
Purified DO dimer samples	N/A	N/A	On ice	See previous section.
PCR Supermix	White	12020005	-20° C	1. Thaw on ice. 2. Gently flick the thawed tube 3–6x. 3. Centrifuge briefly to collect volume at bottom of tube.
DO Index Plate	N/A	12020634	-20° C	1. Thaw completely, at room temperature. 2. Centrifuge briefly to collect volume at bottom of plate.
Nuclease-free water	N/A	N/A	N/A	N/A

Thermal cycler*

**Use one of the recommended thermal cyclers or an equivalent deep-well thermal cycler with up to 100 µl capacity. For information and catalog numbers, see* *Required Consumables and Equipment on page 11*.

1. Save the following DO library amplification (DLA) program on the thermal cycler:

Table 30. DLA protocol

Lid temperature	Reaction volume	Ramp rate
105° C	100 µl	2.0° C/sec
Step	**Temperature**	**Time (hr:min:sec)**
1	95° C	00:03:00
2	98° C	00:00:20
3	50° C	00:00:45
4	72° C	00:00:45
5	Go to Step 2 for a total of 3 cycles	
6	98° C	00:00:20
7	65° C	00:00:20
8	72° C	00:00:25
9	Go to Step 6 for a total of 13 cycles	
10	72° C	00:05:00
11	10° C	Hold

2. Add 50 µl of PCR supermix to each of the purified DO dimer samples.

   Important: To prevent cross-contamination, use a fresh tip for each sample.

3. Add 2 µl of nuclease-free H_2O to the DO dimer samples.

   Important: To prevent cross-contamination, use a fresh tip for each sample.

4. Open the lid of the index plate, **marked for DO indexing**, and gently wipe the surface of the DO index plate (foil) with 80% ethanol.

   Important: The cDNA and the DO Index plates are NOT interchangeable. Ensure you are using the correct index plate for the type of libraries you are preparing.

   Fig. 30 on page 69 illustrates the cDNA and DO index plate maps. For index sequences, see Library Preparation for Sequencing on page 79.

5. Using a clean pipette tip, gently pierce the foil over the well containing the indexes to be used with your DO dimer sample.

   Important: To prevent cross-contamination, do the following:

   - Use a new pipette tip for each index well
   - Indexes are for single use. If fewer than 24 indexes are used, seal the pierced index wells before returning to -20°C storage to prevent contamination from the pierced wells

   Important: Because the foil may require slight pressure to pierce, ensure the plate is on a flat surface to prevent spills and cross-contamination.

   Important: In Step 6, **you must use the same index for each cDNA and DO sample pair**. Matching is crucial for deconvoluting sample information bioinformatically. Each sample must receive a unique index from the plate, matching back in coordinate position to the cDNA plate used for cDNA libraries.

6. Complete the following substeps:

   a. Using a pipette set to 10 µl, **with a clean tip**, pipette mix the DO indexes in the pre-pierced index well 5x.

   b. Transfer 10 µl of a unique DO Index to each DO dimer sample.

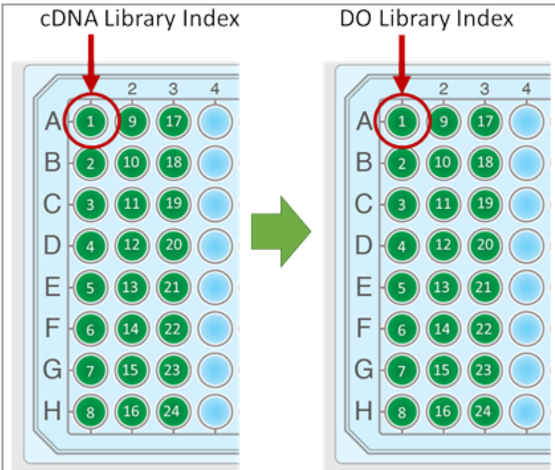

Fig. 30: Index coordinates

7. Use a P200 multi-channel pipette, set at 80 µl, to mix the DO dimer PCR reaction 10x.
8. Seal or cap the samples DO dimer PCR reactions and centrifuge.
9. Place the DO dimer PCR reactions on the pre-programmed thermal cycler and run the DLA program.

Safe Stopping Point

- **After this section is complete, you can store the sample on the thermal cycler at 10° C or at 4° C for up to 72 hr.**
- **Operating the instrument at 4° C should be limited to 18 hours at these conditions.**
- **Holds at 4° C can be performed for up to 72 hrs if humidity is 20-60% (noncondensing).**

cDNA and DO Library Purification

Follow the instructions in this section carefully to ensure the correct amount of purification beads are used to purify the cDNA and DO libraries. Inexperienced users may purify the cDNA and DO libraries sequentially.

Table 31. cDNA and DO library purification, required equipment and consumables

Item	Cap color	Part number	Storage	Preparation
cDNA libraries	N/A	N/A	4° C or 10° C	Preparation completed in cDNA Indexing and Amplification on page 63.
DO libraries	N/A	N/A	4° C or 10° C	Preparation completed in DO Dimer Indexing and Amplification on page 67.
Purification beads (choose one):				
■ Beckman Coulter SPRIselect Reagent Kit	N/A	B23317	Room temp	1. Vortex until well dispersed.
■ Beckman Coulter AMPure XP SPRI Reagent	N/A	A63881	4° C	1. Let stand for 30 min to bring to room temperature. 2. Vortex until well dispersed.
10mM Tris-HCl, pH 8.0 (Resuspension Buffer), DNAse-free (Teknova)	N/A	T1173	Room temp	N/A
Ethanol, pure (200 proof, anhydrous)	N/A	N/A	Room temp	Prepare a fresh batch of 80% ethanol in water.
NEBNext Magnetic Separation Rack	N/A	S1515S	Room temp	N/A

First Library Purification

1. Prepare fresh 80% ethanol.
2. Vortex the purification beads until well dispersed.
3. Add purification beads to the tubes containing 100 µl of libraries prepared from the previous index PCR sections, as follows:
   - **cDNA library:** 60 µl of purification beads
   - **DO library:** 80 µl of purification beads
4. Pipette mix the purification beads and samples until homogenous (10–15x).

First Library Bind

1. Incubate the samples at room temperature for 5 min.
2. Place the strip tubes in the **magnetic separation rack**.
3. Incubate at room temperature for 5 min.
4. Keeping the strip tubes in the **magnetic separation rack**, pipette to remove and discard the supernatant.

First Library Wash

1. Wash the magnet-bound purification beads, as follows:
    a. Add 200 µl of freshly prepared 80% ethanol to each well, pipetting the ethanol against the wall opposite the beads.

       Important: Do not disturb the pelleted purification beads.

    b. Incubate on the **magnetic separation rack** for 1 min.
    c. Keeping the 8-tube strip in the **magnetic separation rack**, pipette to remove and discard the supernatant.
2. Repeat steps 1a-1c, for a total of 2 washes.
3. Remove all residual ethanol, as follows:
    a. Centrifuge briefly to collect purification beads at the bottom of the tubes.
    b. Place the 8-tube strip on the **magnetic separation rack**.
    c. Pipette to remove remaining ethanol.
4. Air-dry on the **magnetic separation rack** until there is no remaining liquid in each tube.

   Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.

First Library Elute

1. Remove the sample tubes from the magnet.
2. Add 51 µl resuspension buffer (10mM Tris-HCl, pH 8.0) to each sample.
3. Pipette to mix, making sure all beads are resuspended.

   Important: Confirm that solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.

4. Incubate at room temperature for 2 min.
5. Place the strip tubes on the **magnetic separation rack** and incubate for 1–5 min (until the beads have attached to the magnet and are no longer visible in the solution).
6. Keeping the strip tube in the **magnetic separation rack**, transfer 50 µl of library from each sample tube to a new 8-tube strip.

Second Library Purification

1. Vortex the purification beads until well dispersed.
2. Add purification beads to the tubes containing 50 µl of first purification eluates from the previous section:
   - **cDNA library first purification eluate**: 30 µl of purification beads
   - **DO library first purification eluate**: 40 µl of purification beads
3. Pipette mix the purification beads and library until homogenous (10–15x).

Second Library Bind

1. Incubate the library samples at room temperature for 5 min.
2. Place the strip tubes in the **magnetic separation rack**.
3. Incubate at room temperature for 5 min.
4. Keeping the strip tubes in the **magnetic separation rack**, pipette to remove and discard the supernatant.

Second Library Wash

1. Wash the magnet-bound purification beads, as follows:
   a. Add 200 µl of freshly prepared 80% ethanol to each tube, pipetting the ethanol against the wall opposite the beads.

      Important: Do not disturb the pelleted purification beads.

   b. Incubate on the **magnetic separation rack** for 30 sec to 1 min.
   c. Remove and discard all the supernatant from each tube.
2. Repeat steps 1a-1c, for a total of 2 washes.

3. Remove all residual ethanol, as follows:
   a. Centrifuge briefly to collect purification beads at the bottom of the tubes.
   b. Place the 8-tube strip on the **magnetic separation rack**.
   c. Pipette to remove remaining ethanol.
4. Air-dry the samples on the **magnetic separation rack** until there is no remaining liquid in each tube.

   Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.

Second Library Elute

1. Remove the 8-tube strip from the **magnetic separation rack**.
2. Add 21 µl resuspension buffer (10mM Tris-HCl, pH 8.0) to each sample tube.
3. Pipette to mix, making sure all beads are resuspended.

   Important: Confirm that solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.
4. Incubate at room temperature for 2 min.
5. Place the strip tube on the **magnetic separation rack** and incubate for 1–5 min (until the beads have attached to the magnet and are no longer visible in the solution).
6. Keeping the strip tube in the **magnetic separation rack**, transfer 20 µl of library from each sample tube to a new 8-tube strip.

Safe Stopping Point

After this section is complete, you can store the sample long-term at -20° C.

Library Processing

Table 32. cDNA and DO library analysis (required items)

Item	Part number	Supplier
cDNA and DO library samples	N/A	See previous section.
*2100 Bioanalyzer Instrument ■ High Sensitivity DNA Kit ■ High Sensitivity DNA Reagents	G2939AAR 5067-4626 5067-4627	Agilent
*4150 TapeStation System ■ High Sensitivity D5000 Ladder ■ High Sensitivity D5000 Reagents ■ High Sensitivity D5000 Screen Tape	G2992AA 5067-5594 5067-5593 5067-5592	Agilent

**Use either the 2100 Bioanalyzer Instrument or the 4150 TapeStation System and their respective consumables.*

Check Quality of Libraries

This section contains the procedure for processing libraries for Illumina NGS. Before proceeding with sequencing, complete the following procedure for quality control analysis on your cDNA and DO libraries:

1. Load the libraries onto the 2100 Bioanalyzer or the 4150 Tapestation System. You can often run

   - cDNA libraries from low-expresser cells, undiluted
   - cDNA libraries from high-expresser cells at 1:4 to 1:10 dilution
   - DO libraries at a 1:4 dilution

   Important: Follow the recommended loading ranges for each instrument, and perform appropriate dilutions before loading. If you dilute libraries following our recommended guidelines and observe that Bioanalyzer and/or Tapestation marker peaks are not in the appropriate signal range for accurate quantification (following Agilent guidelines), then dilute the libraries further.

2. Use the following 2100 Bioanalyzer and 4150 Tapestation System guidelines to determine library size ranges and concentration for sequencer loading:

   - For cDNA libraries, capture the 200–1,200 bp region.

     The average size is used for nM calculation for sequencing. *Expect libraries to have an average of 700–900 bp.*

   - For DO libraries, capture the 200–500 bp region.

     The average size is used for nM calculation for sequencing. *Expect libraries to have a predominant peak at around 300bp*.

     Fig. 31 shows the representative cDNA traces from the Agilent Tapestation system software on the left and from the Agilent Technology 2100 Bioanalyzer Software on the right.

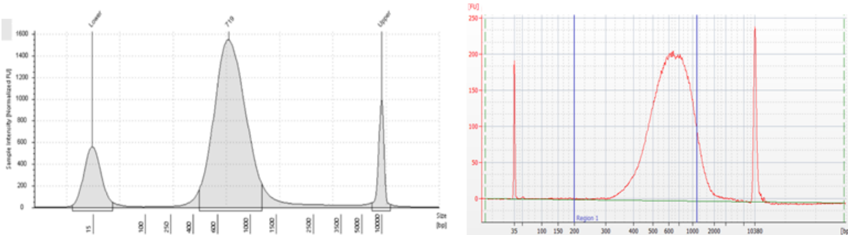

Fig. 31: Representative cDNA library traces

Fig. 32 shows representative DO library traces from A) Agilent Tapestation System Software or B through E) Agilent Technology 2100 Bioanalyzer Software. Most DO library traces will present like A and B, with a single predominant peak. Occasionally, DO library traces may present with multiple peaks, such as those observed in C through E. You can still proceed to sequence with these libraries.

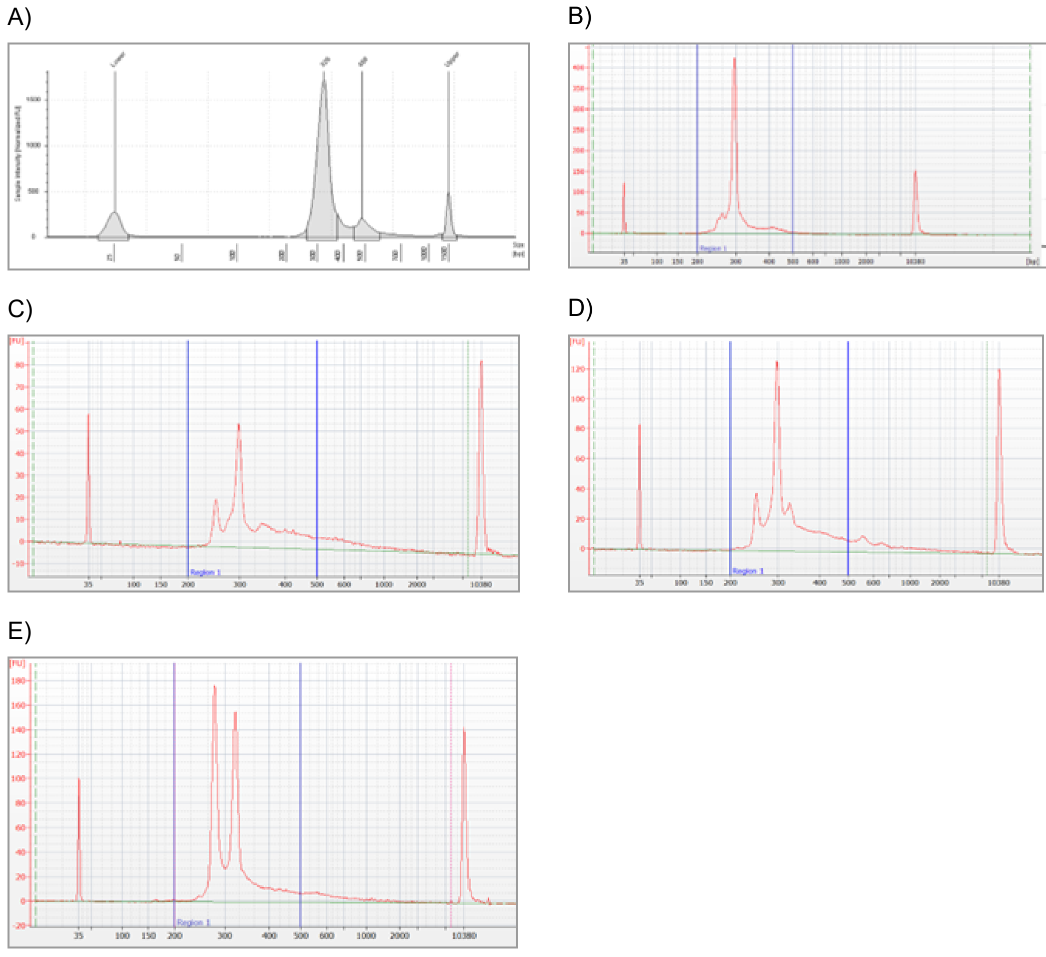

Fig. 32: Representative DO library traces

Preparing for Sequencing

Library Preparation for Sequencing

Bio-Rad recommends that you sequence libraries on Illumina platforms targeting sufficient depth for subsequent analyses. You should expect to recover 50% (minimum) of the original cell input. A minimum of 20,000 read pairs per cell might provide adequate depth for high-expressor cell types, but at least 50,000 reads per expected cell output are recommended for single-cell transcriptomic analyses.

Depending on the sequencing platform and kit chosen, properly account for the number of samples being tested to achieve proper sequencing depth. You must prepare two sequencing pools separately (the cDNA and the DO library pools), and then combine them to achieve the desired read distribution.

Note the following:

- For NextSeq2000 two-plex pooling, pool samples with consecutive indexes from a single column of the index plate.
- For other sequencing systems, pool more than two samples with consecutive indexes down a single column of the index plate.

Important: Do **not** pool samples with indexes across a row of the index plate.

Note: To ensure accurate bead deconvolution, DO libraries must be allocated 5 million reads per DO library during sequencing. The following is an example read pair allocation.

Cell input = 10,000 viable cells

Expected cells recovered = 5,000 cells

Recommended cDNA library read pairs per sample = 5,000 cells per sample * 50,000 read pairs per cell = 250M read pairs per sample

Recommended DO library read pairs per sample = 5M read pairs per sample

Total read pairs required per sample = 255M read pairs

For each Illumina platform being used, follow the platform-specific protocol for denaturing, diluting, and loading libraries.

Sequencing Run and Loading Parameters

The sequencing parameters in Table 33 are recommended when sequencing on Illumina NextSeq2000.

Table 33. Sequencing run parameters

Parameter	Recommended
Pool concentration	725 pM
Sequencing depth	Minimum: 20,000 read pairs per cell Recommended: 50,000 read pairs per cell
Sequencing type	Paired-end, dual indexing
Sequencing reads ■ Read 1 ■ i7 index ■ i5 index ■ Read 2	Recommended number of cycles 54 8 8 68

Notes:

- A PhiX spike-in control is not required for sequencing the ddSEQ single cell 3' RNA-seq libraries.
- A low concentration of PhiX spike-in control could be included for sequencing run spec monitoring purposes if needed.

Table 34. Matched cDNA and DO library indexes corresponding to well position

Sample index	Well position	i7 index in adapter (Index 1)	i5 index in adapter (Index 2)
UDI_001	A1	CCGCGGTT	AGCGCTAG
UDI_002	B1	TTATAACC	GATATCGA
UDI_003	C1	GGACTTGG	CGCAGACG
UDI_004	D1	AAGTCCAA	TATGAGTA
UDI_005	E1	ATCCACTG	AGGTGCGT
UDI_006	F1	GCTTGTCA	GAACATAC

Table 34. Matched cDNA and DO library indexes corresponding to well position, continued

Sample index	Well position	i7 index in adapter (Index 1)	i5 index in adapter (Index 2)
UDI_007	G1	CAAGCTAG	ACATAGCG
UDI_008	H1	TGGATCGA	GTGCGATA
UDI_009	A2	AGTTCAGG	CCAACAGA
UDI_010	B2	GACCTGAA	TTGGTGAG
UDI_011	C2	TCTCTACT	CGCGGTTC
UDI_012	D2	CTCTCGTC	TATAACCT
UDI_013	E2	CCAAGTCT	AAGGATGA
UDI_014	F2	TTGGACTC	GGAAGCAG
UDI_015	G2	CAGTAGGC	TGACGAAT
UDI_016	H2	TGACGAAT	CAGTAGGC
UDI_017	A3	TAATACAG	ATATTCAC
UDI_018	B3	CGGCGTGA	GCGCCTGT
UDI_019	C3	ATGTAAGT	ACTCTATG
UDI_020	D3	GCACGGAC	GTCTCGCA
UDI_021	E3	GGTACCTT	AAGACGTC
UDI_022	F3	AACGTTCC	GGAGTACT
UDI_023	G3	GCAGAATT	ACCGGCCA
UDI_024	H3	ATGAGGCC	GTTAATTG

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Chapter 5 Data Analysis

You can analyze your data using Omnition Analysis Software, which performs the following:

- Read QC
- Sample deconvolution
- Single-cell identification
- Transcriptome alignment
- 3' gene and unique molecular identifier (UMI) counting
- Cell clustering

The Omnition Analysis Software user guide, which explains how to set up and use the analysis software, is available at the following hyperlink:

Omnition Analysis Software User Guide (3' RNA Module)

Omnition supports human and mouse genomes. You can use other ENSEMBL genome assemblies, but they are **not** supported.

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Appendix A Checking cDNA and DO Dimer Samples Before Library Preparation

Important: Do **not** dedicate >1 µl of cDNA or DO dimer sample for this optional QC step. Sample loss before library preparation negatively impacts gene detection sensitivity.

This quality control check is an optional step in the protocol. Bio-Rad recommends that you skip this section and refer instead to Preparing cDNA and DO Libraries on page 59, since low or undetectable cDNA does not indicate that sample preparation has failed.

To assess the presence of cDNA and DO dimers before library preparation, you can run a Bioanalyzer or TapeStation electropherogram test if the samples used were from high-expresser cell types. For high-expresser samples with cell input ≥ 2,500 cells, cDNA is detectable and can be analyzed for size distribution and yield. However, cDNA that is isolated from low-expresser cell types cannot be detected at this step, regardless of cell input. The same is true for low input high-expresser cells. To conduct cDNA quality analysis, complete the following procedure on your cDNA and DO dimer samples. For technical assistance, contact Bio-Rad Technical Support.

Table 35. cDNA and DO dimer sample analysis (required equipment and consumables)

Item	Part number	Supplier
cDNA and DO dimer samples	N/A	See previous section.
*2100 Bioanalyzer Instrument	G2939AAR	Agilent
■ High Sensitivity DNA Kit	5067-4626	
■ High Sensitivity DNA Reagents	5067-4627	

Table 36. cDNA and DO sample analysis (required equipment and consumables)

*4150 TapeStation System	G2992AA	Agilent
■ High Sensitivity D5000 Ladder	5067-5594	
■ High Sensitivity D5000 Reagents	5067-5593	
■ High Sensitivity D5000 Screen Tape	5067-5592	
■ High Sensitivity D1000 Ladder	5067-5587	
■ High Sensitivity D1000 Reagents	5067-5585	
■ High Sensitivity D1000 Screen Tape	5067-5582	

**Use either the 2100 Bioanalyzer Instrument or the 4150 TapeStation System and their respective consumables.*

1. Run 1 µl of undiluted cDNA and DO dimer sample on separate wells of an Agilent Technology 2100 Bioanalyzer or an Agilent 4150 TapeStation. Assess as follows:
   - Assess cDNA using the High Sensitivity DNA Kit and Reagents (2100 Bioanalyzer), or the High Sensitivity D5000 kit and reagents (4150 TapeStation)
   - Assess DO dimers using the High Sensitivity DNA Kit and Reagents (2100 Bioanalyzer), or the High Sensitivity D1000 kit and reagents (4150 TapeStation)
2. Determine concentrations using the instrument's incorporated software.
   - For cDNA samples, capture the 200–8,000 bp region. Yields can range from undetectable to > 2 ng.
   - For DO dimer samples, capture the 200–500 bp region. You should expect two to three peaks in the region (~200 bp, ~250 bp, ~275 bp) at varying levels of intensity.

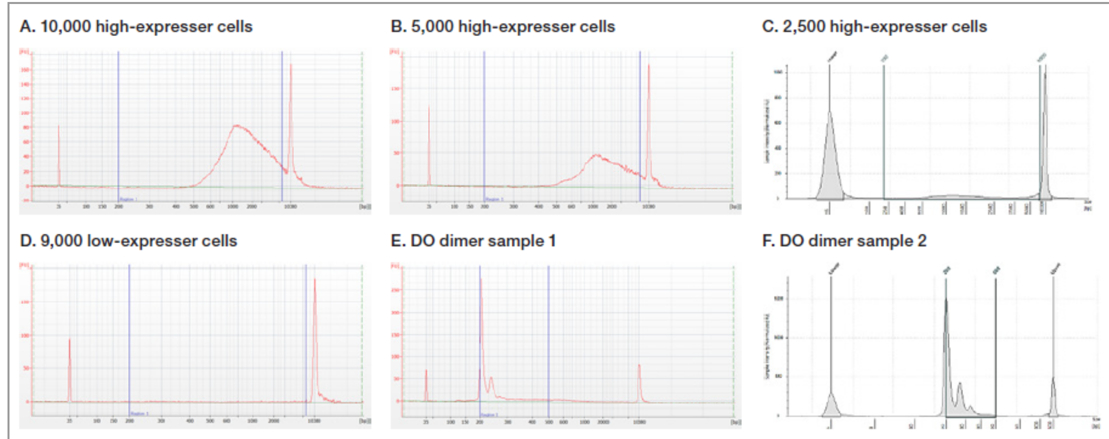

Fig. 33: Representative cDNA and DO dimer sample traces analyzed using an Agilent Technology 2100 Bioanalyzer

LEGEND

A–C: cDNA samples from high-expresser cell lines
D: Lack of quantifiable cDNA from 9,000 low-expresser cells
E–F: Representative DO dimer sample traces

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