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Evercode Dual Index PCR

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We use this protocol and it's working

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Abstract

This protocol describes the **dual-index** PCR procedure for Parse Biosciences Evercode WT and WT Mega v2 kits. Each subpool is barcoded with two Illumina indices on the 5' and 3' ends of the cDNA library. These indices acts as the fourth "round" of cell barcoding and must be included in the final cell ID/barcode in order to ensure unique barcodes across subpools within an experiment. The numerical ID and sequence of the Illumina barcode used for each subpool are recorded in experiment metadata and used downstream for subpool demultiplexing after the sequencing run.

Sublibrary Dual Index PCR

1 If using the alternative version of Evercode Mega WT v2 (ECW02050) that includes RX100 instead of RX200, follow the protocol modifications described in Appendix D.

Multiple thermocyclers may be needed for this section depending on the amount of cDNA added to each sublibrary during the fragmentation reaction. Refer to the step 3.5.6.

2 Ensure that each well of the **UDI Plate - WT** is properly thawed. Centrifuge the plate at **100 x g** for **1**
minute.

Critical! Double-check the label on the plate as specific plates are used in different protocols.

3 Thoroughly wipe the UDI Plate - WT seal with 70% ethanol and allow it to dry completely.

Note: Before proceeding, ensure the UDI Plate is properly orientated. The notched corner should be in the bottom left (see image on the right). Only wells from columns 1-6, and only one well/sublibrary can be used.

4 Add well-specific index primers from the **UDI Plate - WT** to your sublibraries as follows:

Using a multichannel P20 pipette set to 4 µL, pierce new, unused wells of the **UDI Plate - WT.** Mix 5x then transfer **4 µL** of the index primer solution into your sublibraries.

Note: Ensure that no two sublibraries contain index primers from the same well. To minimize cross-contamination, use a new pipette tip for each sublibrary and avoid splashing or mixing the liquid between individual wells.

For each sublibrary, record the **UDI Plate - WT**'s well position (e.g., 'A1', 'B1') and sublibrary index ID (see Section 4.1) for sequencing and demultiplexing purposes.

5 If the **UDI Plate - WT** has unused wells, store it at -20°C for future use.

6 Add **25 µL** of the **Index PCR Mix** to each sublibrary, bringing the total volume to 50 µL. Pipette up and down 10x with the pipette set to 25 µL to ensure proper mixing, followed by brief centrifugation (~2 sec).

Critical! Different tips must be used when pipetting Index PCR Mix into each sublibrary. Never place a tip that has entered a sublibrary back into the Index PCR Mix.

7 Place the sample(s) into a thermocycler and run the program below. The number of cycles (**X**) should be adjusted based on the amount of cDNA added to the fragmentation reaction.

	Run Time	Lid temperature	Sublibrary Volume
	~30 min	105C	50 uL

Sublibrary Index Amplification Overview

	Step	Time	Temperature
	1	3 min	95C
	2	20 sec	98C
	3	20 sec	67C
	4	1 min, then go to step 2, repeat X-1 times (X cycles total)	72C
	5	5 min	72C
	6	Hold	4C

Sublibrary Index Amplification

	A	B	C	D	E	F	G
	cDNA in fragmentation (ng)	10-24	25-49	50-99	100-299	300-899	1000+
	Total PCR cycles required (X)	13	12	11	10	8	7

PCR Cycles based on cDNA in Fragmentation

Note: cDNA concentration was recorded in step 2.5.18, and 10 µL from each sublibrary should have been added into the fragmentation reaction (step 3.1.2).

8 Sublibraries can be stored at this point at 4C overnight. If you wish to continue, proceed directly to Section 3.6: Post-Amplification Double-Sided Selection.
[STOPPING POINT]