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Gene Expression Dual Index Library Construction

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Abstract

The Chromium Single Cell Gene Expression Solution upgrades short read sequencers to deliver a scalable microfluidic platform for 3’ digital gene expression by profiling 500-10,000 individual cells per sample.

Once cDNA is amplified, enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, i7 and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

This protocol details the gene expression dual index library construction and sequencing.

Attachments

DOCX

snRNAseq_ProtocolsIO..

66KB

Image Attribution

Nayra Cardenes, PhD

Materials

- Fragmentation Buffer (2000091)
- Adaptor Oligos (2000094)
- Ligation Buffer (2000092)
- Dual Index Plate TT Set A (3000431)
- Fragmentation Enzyme (2000090/2000104)
- DNA Ligase (220110/220131)
- Amp Mix (2000047/2000103)
- Cleanup Buffer (2000088)

Fragmentation Mix:

	A	B	C	D
	Reagents	1X (µl)	4X+10% (µl)	8X+10% (µl)
	Fragmentation Buffer	5	22	44
	Fragmentation Enzyme	10	44	88
	Total	15	66	132

Ligation Mix:

	A	B	C	D
	Reagents	1X (µl)	4X+10% (µl)	8X+10% (µl)
	Ligation Buffer	20	88	176
	DNA Ligase	10	44	88
	Adaptor Oligos	20	88	179
	Total	50	220	440

Safety warnings

Before start

- **Equilibrate to Room temperature (RT) –** Fragmentation Buffer (2000091), Adaptor Oligos (2000094), Ligation Buffer (2000092) and Dual Index Plate TT Set A (3000431)
- **Place on ice–** Fragmentation Enzyme (2000090/2000104), DNA Ligase (220110/220131) and Amp Mix (2000047/2000103)
- **Thaw at 65°C-** Cleanup Buffer (2000088)

Gene Expression Dual Index Library Construction

1w 2d 0h 34m 30s

1 Fragmentation, End Repair & A-tailing

1.1 Prepare a thermal cycler with the following incubation protocol:

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	65°C	50 µl	~35 min
	Step	Temperature	Time
	Pre-cool block	4°C	Hold
	Fragmentation	32°C	5 min
	End Repair & A-tailing	65°C	30 min
	Hold	72°C	Hold

Thermocycler protocol.

1.2 Vortex fragmentation buffer. Verify there is no precipitate.

1.3 Prepare fragmentation mix On ice . Pipette mix and centrifuge briefly.

Fragmentation Mix

	A	B	C	D
	Reagents	1X (µl)	4X+10% (µl)	8X+10% (µl)
	Fragmentation Buffer	5	22	44
	Fragmentation Enzyme	10	44	88
	Total	15	66	132

Calculation for the Fragmentation Mix preparation.

1.4 Transfer ONLY 10 µL purified cDNA sample from pellet cleanup to a tube strip. The remaining 30 µL (75%) cDNA sample can be stored at 4 °C for up to 72:00:00 or at -20 °C for up to 4 weeks for generating additional 3’ Gene Expression libraries.

3d

1.5 Add 25 µL buffer EB to each sample.

1.6 Add 15 µL fragmentation mix to each sample.

1.7 Pipette mix 15× (pipette set to 35 µl) On ice . Centrifuge briefly.

1.8 Transfer into the pre-cooled thermal cycler (4 °C) and press “SKIP” to initiate the protocol.

2 **Post Fragmentation,End Repair & A-tailing Double Sided Size Selection – SPRIselect:**

2.1 Vortex to resuspend SPRIselect reagent. Add 30 µL SPRIselect (0.6X) reagent to each sample. Pipette mix 15× (pipette set to 75 µl).

2.2 Incubate 00:05:00 at Room temperature . 5m

2.3 Place on the magnet. High until the solution clears. DO NOT discard supernatant.

2.4 Transfer 75 µL supernatant to a new tube strip.

2.5 Vortex to resuspend SPRIselect reagent. Add 10 µL SPRIselect reagent (0.8X) to each transferred supernatant. Pipette mix 15× (pipette set to 80 µl).

2.6 Incubate 00:05:00 at Room temperature . 5m

2.7 Place on the magnet. High until the solution clears.

2.8 Remove 80 µL supernatant. DO NOT discard any beads.

2.9 Add 125 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

2.10 Remove the ethanol.

2.11 **Repeat sub-steps 2.9 and 2.10 for a total of 2 washes.**

2.12 Centrifuge briefly. Place on the magnet. Low until the solution clears. Remove remaining ethanol.

2.13 Remove from the magnet. Add 50.5 µL buffer EB to each sample. Pipette mix 15× (pipette set to 45 µl).

2.14 Incubate 00:02:00 at Room temperature . 2m

2.15 Place on the magnet. High until the solution clears.

2.16 Transfer 50 µL sample to a new tube strip.

3 Adaptor Ligation

3.1 Prepare adaptor ligation Mix. Pipette mix and centrifuge briefly.

Adaptor Ligation Mix:

	A	B	C	D
	Reagents	1X (µl)	4X+10% (µl)	8X+10% (µl)
	Ligation Buffer	20	88	176
	DNA Ligase	10	44	88
	Adaptor Oligos	20	88	179
	Total	50	220	440

3.2 Add 50 µL adaptor ligation mix to 50 µL sample. Pipette mix 15× (pipette set to 90 µl). Centrifuge briefly.

3.3 Incubate in a thermal cycler with the following protocol:

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	30°C	100 µl	15 min
	Step	Temperature	Time
	1	20°C	15 min
	2	4°C	Hold

4 Post Ligation Cleanup – SPRIselect:

4.1 Vortex to resuspend SPRIselect reagent. Add 80 µL SPRIselect (0.8X) reagent to each sample. Pipette mix 15× (pipette set to 75 µl).

4.2 Incubate 00:05:00 at Room temperature . 5m

4.3 Place on the magnet. High until the solution clears.

4.4 Remove the supernatant.

4.5 Add 200 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

4.6 Remove the ethanol.

4.7 **Repeat sub-steps 4.5 and 4.6 for a total of 2 washes.**

4.8 Centrifuge briefly. Place on the magnet. Low until the solution clears.

4.9 Remove remaining ethanol. Air dry for 00:02:00 . 2m

4.10 Remove from the magnet. Add 30.5 µL buffer EB to each sample. Pipette mix 15x.

4.11 Incubate 00:02:00 at Room temperature . 2m

4.12 Place on the magnet. Low until the solution clears.

4.13 Transfer 30 µL sample to a new tube strip.

5 Sample Index PCR

5.1 Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10× Sample Index name (PN-3000431 Dual Index Plate TT Set A well ID) used.

5.2 Add 50 µL Amp mix to 30 µL sample.

5.3 Add 20 µL of an individual Dual Index TT Set A to each sample and record the well ID used. Pipette mix 5× (pipette set to 90 µl). Centrifuge briefly.

5.4 Incubate in a thermal cycler with the following protocol:

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	105°C	100 µl	~25-40 min
	Step	Temperature	Time
	1	98°C	45 sec

	A	B	C
	2	98°C	20 sec
	3	54°C	30 sec
	4	72°C	20 sec
	5	Go to Step 2 – * # cycles calculated below	
	6	72°C	1 min
	7	4°C	Hold

Note

The total cycles should be optimized based on 25% carry forward cDNA yield/input calculated during cDNA QC & Quantification

	A	B
	cDNA Input	Total cycles
	0.25-25 ng	14-16
	25-150 ng	Dec-14
	150-500 ng	10-Dec
	500-1,000 ng	08-Oct
	1,000-1,500 ng	06-Aug
	>1,500 ng	5

5.5 Store at 4 °C for up to 72:00:00 or proceed to the next step. 3d

6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

6.1 Vortex to resuspend SPRIselect reagent. Add 60 µL SPRIselect (0.6X) reagent to each sample. Pipette mix 15× (pipette set to 150 µl).

6.2 Incubate 00:05:00 at Room temperature . 5m

6.3 Place on the magnet. High until the solution clears. DO NOT discard supernatant.

6.4 Transfer 150 µL supernatant to a new tube strip.

6.5 Vortex to resuspend SPRIselect reagent. Add 20 µL SPRIselect reagent (0.8X)to each transferred supernatant. Pipette mix 15× (pipette set to 150 µl).

6.6 Incubate 00:05:00 at Room temperature . 5m

6.7 Place on the magnet. High until the solution clears.

6.8 Remove 165 µL supernatant. DO NOT discard any beads.

6.9 Add 200 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

6.10 Remove the ethanol.

6.11 **Repeat sub-steps 6.9 and 6.10 for a total of 2 washes.**

6.12 Centrifuge briefly. Place on the magnet. Low until the solution clears. Remove remaining ethanol.

6.13 Remove from the magnet. Add 35.5 µL buffer EB to each sample. Pipette mix 15× (pipette set to 35 µl).

6.14 Incubate 00:02:00 at Room temperature . 2m

6.15 Place on the magnet. Low until the solution clears.

6.16 Transfer 35 µL sample to a new tube strip.

6.17 Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage. 3d

7 **Post Library Construction QC**

7.1 Run 1 µL sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip.

7.2 Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Sequencing

8 3’ Gene Expression Library Sequencing Depth & Run Parameters:

	A	B
	Sequencing Depth	Minimum 20,000 read pairs per cell
	Sequencing Type	Pair-end, dual indexing
	Sequencing Read	Recommended Number of cycles
	Read 1	28 cycles
	i7 Index	10 cycles
	i5 Index	10 cycles
	Read 2	90 cycles

9 Once quantified and normalized, the 3’ Gene Expression libraries should be denatured and diluted as recommended for Illumina sequencing platforms.

Protocol references

https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000505_Chromium_Nuclei_Isolation_Kit_UG_RevA.pdf