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Split-seq with CROP-like gRNA

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We use this protocol and it's working

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Disclaimer

Based on **Rosenberg *et al.*, Science (2018)**

Abstract

Single-cell RNA sequencing is performed using Split-seq combinatorial indexing, enabling scalable profiling without droplets. Both the transcriptome and CRISPR guide identity are captured from the same cell via a CROP-like RNA polymerase II transcript.

Attachments

XLSX
Split seq primers.xl...
89KB

PDF
Molecular Chemistry....
1.6MB

Cell Fixation (Formaldehyde with BS3)

1 - set swing bucket centrifuge to 4ºC
- thaw BS3 powder (**Thermo A39266**) to room temp for at least 45min
- prepare per sample, keep on ice
  - 1X PBS + RNase Inhibitor:
    4mL PBS + 10µL Protector (**Sigma 3335399001**) + 10µL Enzymatics (**Y9240L**)
  - 0.5X PBS + RNase Inhibitor: 0.5mL 1X PBS+RI + 0.5mL H_2O

1. Prepare cells (up to **1M** cells, at least 500k) in single cell suspension, put on ice, count
2. Centrifuge at 300*g* at 4ºC for 5min, remove supernatant
3. Resuspend cells in **187.5µL** cold **1X PBS+RI**
4. Pipette cells through a 40µm strainer into a new tube, keep on ice
- for cells larger than 40µm, can use 70µm or 100µm strainer instead
- press pipette tip directly against strainer with force
5. Add **12.5µL** fresh **16% Formaldehyde** (for final 1%, **Thermo 28906**) to fix, mix by pipetting 3x, incubate on ice for 10min
- additional mixing will lead to more doublets
6. Add **4µL** **10% Triton** (for final 0.2%, **Sigma T8787**) to permeabilize, mix by pipetting 5x, incubate on ice for 3min
7. Add **40µL** **1M Tris** (**Thermo AM9856**), and **1mL** **1X PBS+RI**
8. Centrifuge at 500*g* at 4ºC for 5min, remove supernatant
9. Resuspend cells in **240µL** **1X PBS+RI**
10. Immediately before use, resuspend BS3 in water for 50mM final: 2mg BS3 + 70µL H_2O
- reconstituted BS3 quickly hydrolyzes in water, use immediately
11. Add **10µL** **50mM BS3** to resuspended cells, incubate on ice for 20min
12. Add **5µL** **10% Triton**, incubate on ice for 3min
13. Add **50µL** **1M Tris**, and **1mL** **1X PBS+RI**
14. Centrifuge at 500*g* at 4ºC for 5min, remove supernatant
15. Resuspend in **150µL** **0.5X PBS+RI**
16. Pass cells through a 40µm strainer into a new tube, keep on ice
17. Count the number of cells, keep cells on ice during counting and proceed quickly to minimize the time fixed cells are out
18. To freeze cells:
    1) add 2.5µL **DMSO**, gently flick tube 3x to mix, incubate on ice for 1min
    2) repeat twice to add a total of 7.5µL **DMSO**
    3) mix the final suspension by gently pipetting 5x with P200 pipette without creating bubbles
       - do NOT vortex cells
    4) split into aliquots (no more than 500k cells per aliquot)

5) store in Mr. Frosty (cooling at -1ºC/min) at -80ºC
- Stop: can store at -80ºC

Prepare Barcodes and Reagents

2 - to barcode 100k cells, load ~400k cells (~4X) for Round1 RT
- to sequence 100k cells, need at least 1M (10X) barcode combinations: 24×96×96 = 220k combinations after 3 rounds of barcoding, and need at least 5 sublibraries

1. Generate 3 barcoding stock plates
- oligos
  - o RT Barcode Plate (100µM, /5Phos/ACTGTGG-N8-polyT or -random_hexamer or -direct_capture)
  - o Round2 Barcode Plate (100µM, /5Phos/CATCGGCGTACGACT-N8-ATCCACGTGCTTGAG)
  - o Round3 Barcode Plate (100µM, /5Biosg/CAGACGTGTGCTCTTCCGATCT-N10-N8-GTGGCCGATGTTTCG)
  - o Round2 Linker (1mM, CCACAGTCTCAAGCACG)
  - o Round2 Blocking (1mM, CGTGCTTGAGACTGTGG)
  - o Round3 Linker (1mM, TACGCCGATGCGAAACATCG)
  - o Round3 Blocking (1mM, GTGGCCGATGTTTCGCATCGGCGTACGACT)
- prepare:
  - o 25mM NaCl to help annealing: 250µL 5M NaCl (Thermo J60434) + 49.75mL H_2O

1) Round1 RT barcode stock plate: **12.5µM** polyT + **12.5µM** random hexamer + **6µM** direct capture per well
   - o 12.5µL polyT + 12.5µL random hexamer + 6µL direct capture + (fill to 100µL) H_2O
2) Round2 ligation stock plate: **12µM** Round2 Barcode + **11µM** Round2 Linker per well
   - o overshot for 120 wells: 132µL (1.1µLx120) Round2 Linker (1mM) + 10.428mL (86.9µLx120) H_2O (with 25mM NaCl)
   - o 88µL diluted Round2 Linker + 12µL Round2 Barcode (100µM) per well
3) Round3 ligation stock plate: **14µM** Round3 Barcode + **13µM** Round3 Linker per well
   - o overshot for 120 wells: 156µL (1.3µLx120) Round3 Linker (1mM) + 10.164mL (84.7µLx120) H_2O (with 25mM NaCl)
   - o 86µL diluted Round3 Linker + 14µL Round3 Barcode (100µM) per well
4) Anneal Round2 and Round3 ligation plates of Barcode+Linker

	Temp	Tim

		e
	95ºC	2 min
	cool down to 25ºC at -0.1ºC/s	
	25ºC	10 min
	4ºC	Hold

- each Split-seq experiment only requires 8µL/well of RT Barcode and 10µL/well of Round2 and Round3 Barcode+Linker, store stock plates in -20ºC

2. Prepare **Reverse Transcription Mix**:

	A	B	C	D	E	F	G
			Storage	Volume per well	24 +4 wells	48 +7 wells	96 + 9 wells
	Maxima H Minus RT (200U/µL)	Thermo EP0753	-20 ºC	2µL	56 µL	110 µL	210 µL
	5X RT Buffer	-20ºC	8µL	224 µL	440 µL	840 µL	
	dNTP (10mM)	NEB N0447L	-20 ºC	2µL	56 µL	110 µL	210 µL
	Protector RNase Inhibitor (40U/µL)	Sigma 3335399001	-20 ºC	0.5µL	14µL	27.5µL	52.5µL
	Enzymatics RNase Inhibitor (40U/µL)	Enzymatic Y9240L	-20 ºC	0.5µL	14µL	27.5µL	52.5µL
	H2O		room	5µL	140µL	275µL	525µL
			Total	**18µL**	**504µL**	**990µL**	**1890 µL**

3. Prepare 3 barcoding plates for experiment
- can prepare in advance, then store at -20ºC

1) **Round1 plate**:
   - 18µL **Reverse Transcription Mix** + 8µL primer mix from Round1 RT barcode stock plate
   - final volume should be 26µL per well, will add 14µL cells for total 40µL RT reaction

2) **Round2 and Round3 plates**:
   - 10µL Barcode+Linker from Round2 and Round3 ligation stock plates

4. Prepare **Spin Additive**: 10% Triton X-100
- 1mL Triton X-100 (**Sigma T8787**, room temp) + 9mL H_2O, store at 4ºC

5. Prepare **Dilution Buffer**: 0.5X PBS + RNase Inhibitor
- can be prepared in advance, then store at -20ºC, keep on ice after thaw

	A	B	C	D
			Storage	Volume
	10X PBS	Thermo AM9625	room	100µL
	Protector RNase Inhibitor (40U/µL)	Sigma 3335399001	-20º C	7.5µL
	Enzymatics RNase Inhibitor (40U/µL)	Enzymatic Y9240L	-20º C	7.5µL
	H2O		room	1.9mL
			Total	**2mL**

6. Prepare **Resuspension Buffer**: NEBuffer r3.1 + RNase Inhibitor
- can be prepared in advance, then store at -20ºC, keep on ice after thaw

	A	B	C	D
			Storage	Volume
	10X NEBuffer r3.1	NEB B6003S	-20º C	200µL
	Protector RNase Inhibitor (40U/µL)	Sigma 3335399001	-20º C	20µL
	Enzymatics RNase Inhibitor (40U/µL)	Enzymatic Y9240L	-20º C	20µL

	A	B	C	D
	H2O		room	1.8mL
			Total	**2.04 mL**

7. Prepare **Ligation Mix**: T4 Ligase + BSA + RNase Inhibitor
- for 96 wells, 2.04mL total
- if want to prepare in advance, do NOT add T4 DNA Ligase, store at -20ºC

	A	B	C	D
			Storage	Volume
	T4 DNA Ligase (2,000U/µL)	NEB M0202M	-20º C	20µL
	10X T4 Ligase Buffer	NEB B0202S	-20º C	500µL
	BSA (Recombinant Albumin, 20mg/mL)	NEB B9200S	-20º C	50µL
	Protector RNase Inhibitor (40U/µL)	Sigma 3335399001	-20º C	40µL
	Enzymatics RNase Inhibitor (40U/µL)	Enzymatic Y9240L	-20º C	40µL
	H2O		room	1.39mL
			Total	**2.04 mL**

8. Prepare **Round2 Stop Mix**: 26.4µM Round2 Blocking
- can be prepared in advance, then store at -20ºC, keep on ice after thaw
- 37µL Round2 Blocking (1mM) + 350µL 10X Ligase Buffer + 1013µL H_2O → total 1.4mL

9. Prepare **Round3 Stop Mix**: 11.5µM Round3 Blocking, 125mM EDTA
- can be prepared in advance, then store at -20ºC, keep on ice after thaw
- 37µL Round3 Blocking (1mM) + 800µL 0.5M EDTA + 2363µL H_2O → total 3.2mL

10. Prepare **Pre-Lyse Wash Buffer**: 0.1% Triton X-100/PBS + RNase Inhibitor
- can be prepared in advance, then store at -20ºC, keep on ice after thaw

	A	B	C	D
			Storage	Volume
	10X PBS	Thermo AM9625	room	400µL
	10% Triton X-100	Sigma T8787	room	40µL
	Protector RNase Inhibitor (40U/µL)	Sigma 3335399001	-20° C	10µL
	Enzymatics RNase Inhibitor (40U/µL)	Enzymatic Y9240L	-20° C	10µL
	H2O		room	3.6mL
			Total	**4.06 mL**

Barcoding Single Cells

3 - use swing bucket centrifuge for all spins, fixed-angle centrifuge will lead to substantial cell loss
- use polypropylene tubes, polystyrene tubes will lead to substantial cell loss
- maximize cell retention during pooling by pipetting up and down several times (in the middle and on the front & back sides) in each well before pooling
- avoid excess bubble formation (not affect quality) by pipetting up and down with pipette set to 10µL less volume, pooling, then collecting any remaining liquid

1. Thaw **Round1, Round2, Round3 plates** in a thermocycler
   - Lid Temperature: 70°C
   - Reaction Volume: 26µL for Round1, 10µL for Round2 & Round3

	Temp	Time
	25°C	10min
	4°C	Hold

2. Sample Counting and Loading Setup
   1) thaw fixed cell samples at 37°C until all ice crystals dissolve, then put on ice
      - important to fully thaw samples before placing on ice

2) count the number of cells in each sample
3) dilute samples with **Dilution Buffer** (0.5X PBS + RNase Inhibitor), put on ice

3. Round1 Reverse Transcription Barcoding
1) centrifuge **Round1 plate** at 100*g* for 1min, put on ice
2) add **14µL** diluted cells to each well of **Round1 plate** according to the Loading Table; immediately after adding cells, mix gently by pipetting up and down exactly 3x
o when pipetting same sample into many wells, periodically mix sample by gentle pipetting to avoid cell settling; do NOT vortex cells
o use different tips when pipetting cells into each well
3) reverse transcription (~**40min**)
o Lid Temperature: 70ºC
o Reaction Volume: 40µL (14µL cells + 18µL RT Mix + 8µL Primer Mix)

	A	B	C
	Cycles	Temp	Time
	1	50ºC	10min
	3	8ºC	12s
		15ºC	45s
		20ºC	45s
		30ºC	30s
		42ºC	2min
		53ºC	3min
	1	53ºC	5min
	1	55ºC	5min
	1	4ºC	Hold

4) pool all wells from **Round1 Plate** into a 2mL tube on ice
o set pipette to 30µL, mix up and down at least 3x on each side to retain settled cells
o both Round1 Plate and 2mL tube with pooled cells should be kept on ice during pooling
5) discard **Round1 Plate**
6) add **9.6µL** (for 24-well) or **19.2µL** (for 48-well) **Spin Additive** (10% Triton X-100) for final concentration of 0.1% to the pooled cells, gently invert once to mix
7) centrifuge the pooled cells at 200*g* at 4ºC for 10min in a swinging bucket
o proceed to the next step immediately, avoid dislodging the cell pellet
8) remove supernatant with P1000 and P200 pipettes, leave ~40µL of liquid

- do not disturb the pellet

9) gently resuspend with 1mL **Resuspension Buffer** (NEBuffer r3.1 + RNase Inhibitor), once cells are fully resuspended, add an additional 1mL for 2mL total, keep on ice

4. Round2 Ligation Barcoding

1) make sure 20µL T4 DNA Ligase (2,000U/µL) have been added to the **Ligation Mix**
   - do not vortex
2) add 2mL of cells in **Resuspension Buffer** into 2.04mL **Ligation Mix**, mix 10x with P1000, keep on ice
3) centrifuge **Round2 Plate** at 100*g* for 1min, keep at room temp
4) add entirety of cells to a basin, add **40µL** cell mix to each well of **Round2 plate**; as adding cells, pipetting up and down exactly 2x to ensure proper mixing
   - avoid cells settling in basin by gently pipetting up and down 2x before transferring cells
   - if volume is insufficient to fill every well, a few wells can be left empty
   - use different tips when pipetting cells into each well
5) incubate at 37ºC for **30min** while shaking (15s at 900RPM, 1min45s pause)
   - Lid Temperature: 50ºC
   - Reaction Volume: 50µL (40µL cell + 10µL R2 primer)
6) vortex **Round2 Stop Mix** briefly, add all to a basin
7) transfer **Round2 Plate** from thermocycler, keep at room temp
8) add **10µL Round2 Stop Mix** to each well of **Round2 Plate**, pipette up and down exactly 3x to ensure proper mixing
   - use different tips when pipetting Stop Mix into each well
9) incubate at 37ºC for **30min** while shaking (15s at 900RPM, 1min45s pause)
   - Lid Temperature: 50ºC
   - Reaction Volume: 60µL (40µL cell + 10µL R2 primer + 10µL R2 Stop)
10) transfer **Round2 Plate** from thermocycler, keep at room temp
11) pool all wells from **Round2 Plate** into a new basin
   - set pipette to 50µL, mix up and down at least 3x on each side to retain settled cells
12) discard **Round2 Plate**
13) pass all cells through a 40µm strainer into a new basin
   - press the tip of pipette against the filter to ensure all liquid passes

5. Round3 Ligation Barcoding

- keep on ice: T4 DNA Ligase (2,000U/µL, **NEB M0202M**, store at -20ºC)

1) add **20µL** T4 DNA Ligase (2,000U/µL) to the basin with strained cells, mix by gently pipetting up and down ~20x
2) centrifuge **Round3 Plate** at 100*g* for 1min, keep at room temp
3) add **50µL** cell mix to each well of **Round3 plate**; as adding cells, pipetting up and down exactly 2x to ensure proper mixing

o avoid cells settling in basin by gently pipetting up and down 2x before transferring cells
o if volume is insufficient to fill every well, a few wells can be left empty
o use different tips when pipetting cells into each well

4) incubate at 37ºC for **30min** while shaking (15s at 900RPM, 1min45s pause)
o Lid Temperature: 50ºC
o Reaction Volume: 60µL (50µL cell + 10µL R3 primer)

5) vortex **Round3 Stop Mix** briefly, add all to a basin

6) transfer **Round3 Plate** from thermocycler, keep at room temp

7) add **20µL Round3 Stop Mix** to each well of **Round3 Plate**, pipette up and down exactly 3x to ensure proper mixing
o use different tips when pipetting Stop Mix into each well
o no incubation required, proceed directly to pooling

8) pool all wells from **Round3 Plate** into a new basin
o set pipette to 70µL, mix up and down at least 3x on each side to retain settled cells

9) discard **Round3 Plate**

10) pass all cells through a 40µm strainer into a new 15mL tube
o press the tip of pipette against the filter to ensure all liquid passes

6. Lysis and Sublibrary Generation

- keep on ice: Proteinase K (20mg/mL, **Thermo 25530049**, store at -20ºC)

1) prepare **2X Lysis Buffer**: 20mM Tris, 400mM NaCl, 100mM EDTA, 4.4% SDS
o can be prepared in advance, store at 4ºC, keep at 37ºC until use to dissolve precipitate

	A	B	C	D
			Storage	Volume
	1M Tris, pH8	Thermo AM9856	room	20µL
	5M NaCl	Thermo J60434	room	80µL
	0.5M EDTA, pH8	Thermo 15575020	room	200µL
	10% SDS	Sigma 71736	room	440µL
	H2O		room	260µL
			Total	**1mL**

2) add **60µL Spin Additive** (10% Triton X-100) for final concentration of 0.1% to the pooled cells, gently invert once to mix

3) centrifuge the pooled cells at 200*g* at 4ºC for 10min in a swinging bucket

4) remove supernatant with P1000 and P200 pipettes, leave ~40µL of liquid

5) gently resuspend with 1mL **Pre-Lyse Wash Buffer** (0.1% Triton X-100/PBS + RNase Inhibitor), pipette slowly to prevent mechanical damage to cells; once cells are fully resuspended, add an additional 3mL for 4mL total

6) centrifuge the pooled cells at 200*g* at 4ºC for 10min in a swinging bucket

7) remove supernatant with P1000 and P200 pipettes, leave ~40µL of liquid

8) gently resuspend pellet with 100µL **Dilution Buffer** to bring the volume to ~100µL, transfer to a 1.5mL tube, keep on ice

9) set pipette to 80µL, gently pipette up and down 5x, immediately use 6µL to count
   - 6µL cells + 6µL 2.4nM YOYO-1 (**Thermo Y3601**, 2.4µL 1mM stock + 997.6µL PBS, 4ºC)
   - some level of debris is normal

10) aliquot sublibraries, record sublibrary sizes and labels, add **Dilution Buffer** to **25µL**, keep on ice
   - each sublibrary will have separate sequencing index
   - useful to have at least one sublibrary with few cells (200-500) with deep sequence (>50,000 reads per cell), this sublibrary provides good estimate of transcript detection per cell that would be expected if other sublibraries were also sequenced deeply
   - do not overload a sublibrary, 12,500 cells/sublibrary is the maximum

11) per sublibrary, add **25µL 2X Lysis Buffer** and **5µL Proteinase K**, keep at room temp

12) vortex for 10s to initiate lysis, briefly centrifuge

13) incubate at 65ºC for **60min** while shaking (15s at 900RPM, 1min45s pause)
   - Lid Temperature: 80ºC
   - Reaction Volume: 55µL (25µL cell + 25µL Lysis Buffer + 5µL Proteinase K)

- Stop: can store sublibrary lysates at -80ºC for up to 6 months

Amplification of Barcoded cDNA

4 1. Prepare reagents

1) prepare **2X Binding&Washing Buffer**: 10mM Tris, 2M NaCl, 1mM EDTA
   - keep at room temp

	A	B	C	D
			Storage	Volume
	1M Tris, pH8	Thermo AM9856	room	500 µL
	5M NaCl	Thermo J60434	room	20mL

	A	B	C	D
	0.5M EDTA, pH8	Thermo 15575020	room	100µL
	H2O		room	29.4 mL
			Total	**50mL**

2) prepare **Bead Wash Buffer**: 0.05% Tween in 1X B&W
   - o keep at room temp

	A	B	C	D
			Storage	Volume
	2X Binding&Washing Buffer		room	10mL
	10% Tween20	Sigma P9416	room	100µL
	H2O		room	9.9mL
			Total	**20mL**

3) prepare **Bind Buffer A**: 2X B&W + RNase Inhibitor
   - o store at -20ºC, keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8
	2X B&W Buffer	60	120	180	240	300	360	420	480
	Protector RNase Inhibitor	1	2	3	4	5	6	7	8

4) prepare **Bind Buffer B**: 0.05% Tween in 1X B&W + RNase Inhibitor
   - o store at -20ºC, keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8

	A	B	C	D	E	F	G	H	I
	Bead Wash Buffer	300	600	900	1200	1500	1800	2100	2400
	Protector RNase Inhibitor	0.5	1	1.5	2	2.5	3	3.5	4

5) prepare **Bead Storage Buffer**: 0.1% Tween in 10mM Tris + RNase Inhibitor
   - store at -20ºC, keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8
	10mM Tris, pH8	200	400	600	800	1000	1200	1400	1600
	10% Tween20	2	4	6	8	10	12	14	16
	Protector RNase Inhibitor	0.5	1	1.5	2	2.5	3	3.5	4

2. Prepare Streptavidin beads

- obtain:
  - Streptavidin C1 beads (**Thermo 65001**, 4ºC)

1) vortex **Streptavidin beads**, take (**44µL** x # sublibrary) beads to a 1.5mL tube
2) put on magnet until liquid becomes clear (~2min), discard supernatant
3) remove from magnet, resuspend with (**100µL** x # sublibrary) **Bead Wash Buffer**, ensure all beads are fully resuspended, not stuck to the side
4) put on magnet until liquid becomes clear (~2min), discard supernatant
5) repeat wash twice more for a total of three washes
6) resuspend with (**55µL** x # sublibrary) **Bind Buffer A**, keep at room temp

3. Apply Streptavidin beads to sublibrary lysates

- obtain:
  - **Lysis Neutralizer**: 100mM PMSF (**Thermo 36978**, -20ºC): add isopropanol to dissolve, make sure at saturate level by still having white undissolved in the bottom

1) remove sublibrary lysates from -80ºC, incubate at 37ºC for 5min, ensure no precipitate before proceeding, quick centrifuge sublibrary lysates
2) per sublibrary, add **2.5µL Lysis Neutralizer** (100mM PMSF), mix 5x (set to 40µL); quick centrifuge, incubate at room temp for 10min
3) per sublibrary, add **50µL Streptavidin beads in Bind Buffer A**, mix 5x (set to 90µL)

4) incubate at 25ºC for **60min** while shaking (30s at 1400RPM, 30s pause)
5) quick centrifuge, put on magnet High until liquid becomes clear, discard supernatant
    - cDNA is unamplified, discarding any beads will result in reduction of transcripts detected
6) resuspend beads with **125µL Bind Buffer B**, keep at room temp for 1min
7) put on magnet High until liquid becomes clear, discard supernatant
8) repeat wash with **125µL Bind Buffer B**
9) resuspend beads with **125µL Bead Storage Buffer**, keep at room temp for 1min

4. Template Switch

- oligo:
    - Split_TSO (100µM, /5dSp/AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG, order as RNA with HPLC purification)
- obtain:
    - Maxima H Minus RT (200U/µL) and 5X RT Buffer (**Thermo EP0753**, -20ºC)
    - dNTP (10mM, **NEB N0447L**, -20ºC)
    - PEG 8000 (25% at -20ºC): from 5g powder (**Sigma P5413**) in H_2O (~15mL) for total 20mL

1) prepare **Template Switching Mix**:
    - keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8
	Split_TSO (100µM)	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6
	Maxima H Minus RT	5.5	11	16.5	22	27.5	33	38.5	44
	5X RT Buffer	22	44	66	88	110	132	154	176
	dNTP (10mM)	11	22	33	44	55	66	77	88
	PEG 8000 (25%)	33	66	99	132	165	198	231	264
	Protector RNase Inhibitor	5.5	11	16.5	22	27.5	33	38.5	44
	H2O	30.8	61.6	92.4	123.2	154	184.8	215.6	246.4
	Total	**110**	**220**	**330**	**440**	**550**	**660**	**770**	**880**

2) put on magnet High until liquid becomes clear, discard supernatant
   - cDNA is unamplified, discarding any beads will result in reduction of transcripts detected
3) without resuspending beads, add **125µL H_2O**, wait 1min, discard supernatant
4) resuspend with **100µL Template Switch Mix**, quick centrifuge
   - Template Switch Mix is viscous, ensure beads are fully resuspended before proceeding
5) incubate at 25ºC for **30min** while shaking (30s at 1400RPM, 30s pause)
6) mix by pipetting 5x to resuspend settled beads, incubate at 42ºC for **90min** while shaking
7) optional: to help TSO RT extension (**30min**): 55ºC for 15min, 42ºC for 15min

5. cDNA Amplification

- oligo:
  - Split_Partial-TSO (100µM, AAGCAGTGGTATCAACGCAGAGT)
  - Split_TruSeq-Read2 (100µM, CAGACGTGTGCTCTTCCGATCT)
  - Split_hU6_outer (100µM, GGGCCTATTTCCCATGATTCCTTC)
- obtain:
  - KAPA HiFi 2X Master Mix (**Roche KK2602**, -20ºC)

1) prepare **Amplification Reaction Solution**:
   - keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8
	KAPA 2X MM	55	110	165	220	275	330	385	440
	Split_Partial-TSO (100µM)	0.5	1	1.5	2	2.5	3	3.5	4
	Split_Read2 (100µM)	0.5	1	1.5	2	2.5	3	3.5	4
	Split_hU6_outer (100µM)	0.5	1	1.5	2	2.5	3	3.5	4
	H2O	53.5	107	160.5	214	267.5	321	374.5	428
	Total	**110**	**220**	**330**	**440**	**550**	**660**	**770**	**880**

2) mix by pipetting to resuspend settled beads, put on magnet High until liquid becomes clear, discard supernatant
3) without resuspending beads, add **125µL H_2O**, wait 1min, discard supernatant
4) resuspend with **100µL Amplification Reaction Solution**, keep on ice
5) incubate (~**60min**)

- adjust the number of 2nd cycles based on number of cells in each sublibrary
- Lid Temperature: 105ºC
- Reaction Volume: 100µL

	A	B	C
	Cycles	Temp	Time
	1	95º C	3 min
	5	98º C	20 s
		65º C	45 s
		72º C	3 min
	12 for 200-1,000 cells 10 for 1,000-2,000 cells 8 for 2,000-6,000 cells 6 for 6,000-12,500 cells 1-2 additional for cells with low RNA	98º C	20 s
		67º C	20 s
		72º C	3 min
	1	72º C	5 min
	1	4ºC	Hold

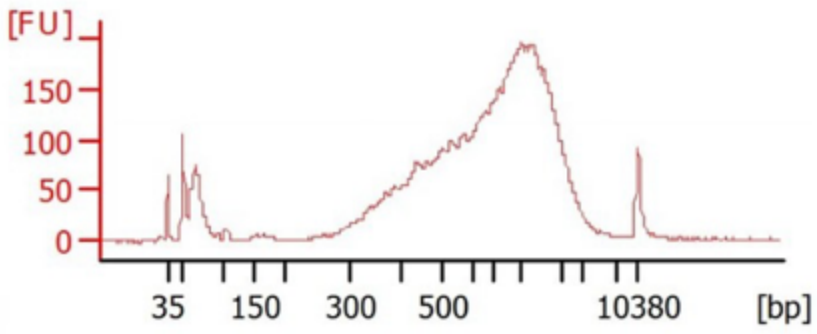
- Stop: can store sublibrary at 4ºC overnight

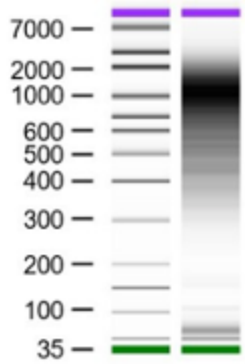
6. Post-Amplifcation SPRI Clean Up (**0.8X**)

- prepare fresh 85% EtOH

1) put on magnet High until liquid becomes clear
   - do NOT discard supernatant
2) transfer **90µL** clear supernatant to a new tube, discard original tubes with beads
3) vortex SPRI, add **72µL** SPRI (**0.8X**), brief vortex, incubate at room temp for 5min
4) put on magnet High until liquid becomes clear, discard supernatant
5) without resuspending beads, add **180µL** **85% EtOH**, wait 1min, discard supernatant
6) repeat wash with **85% EtOH**

7) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 2min
   - o do NOT over-dry beads as this will loss yield, cracking of beads is over-drying
8) resuspend beads in **25µL** **H_2O**, incubate at 37ºC for 10min to maximize elution
9) put on magnet Low until liquid becomes clear
10) transfer **25µL** eluted cDNA to a new tube, discard original tubes with beads
11) measure the concentration of cDNA using Qubit dsDNA HS, record for Index PCR
12) run 1µL cDNA (1:10 dilution) on Bioanalyzer





- Stop: can store at 4ºC for up to 2 days or -20ºC for up to 3 months

Preparing Gene Expression Libraries for Sequencing

5 1. **Gene Expression** Fragmentation, End Repair, A-Tailing
- obtain:
  - o **Fragmentation Enzyme**: 5X WGS Fragmentation Mix (**Enzymatics Y9410L**)
  - o **Fragmentation Buffer**: 10X Fragmentation Buffer (**Enzymatics B0330L**)

1) brief vortex cDNA, quick centrifuge, take **10µL** **cDNA** (at least **125ng** in total), add **25µL** **H_2O** to bring total volume to 35µL, keep on ice
   - o concentration based on Qubit, not Bioanalyzer

- remaining cDNA can be stored at -20ºC

2) prepare thermal cycler (**40min**)
   - Lid Temperature: 70ºC
   - Reaction Volume: 50µL

	Step	Temp	Time
	pre-cool	4ºC	Hold
	Fragmentation	32º C	10min
	End Repair & A-tailing	65ºC	30min
		4ºC	Hold

   - pre-cool block prior to preparing Fragmentation Mix

3) prepare **Fragmentation Mix**:
   - confirm reagents fully thawed and mixed well before using
   - mix well by pipetting 10x, keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							
		1	2	3	4	5	6	7	8
	Fragmentation Buffer	5.5	11	16.5	22	27.5	33	38.5	44
	Fragmentation Enzyme	11	22	33	44	55	66	77	88
	Total	**16.5**	**33**	**49.5**	**66**	**82.5**	**99**	**115.5**	**132**

4) add **15µL Fragmentation Mix**, mix 10x (set to 40µL) on ice, quick centrifuge
5) transfer to pre-cooled thermal cycler and press skip to initiate protocol

2. Post-Fragmentation Double-Sided SPRI Selection (**0.6X-0.8X**)

1) vortex SPRI, add **30µL** SPRI (**0.6X**), brief vortex, incubate at room temp for 5min
2) put on magnet High until liquid becomes clear
   - do NOT discard supernatant
3) transfer **75µL** clear supernatant to a new tube, discard original tubes with beads
4) add **10µL** SPRI (**0.8X**), brief vortex, incubate at room temp for 5min
5) put on magnet High until liquid becomes clear, discard supernatant
   - this may take longer due to low volume of beads
6) without resuspending beads, add **180µL 85% EtOH**, wait 1min, discard supernatant

7) repeat wash with **85% EtOH**

8) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 30s

- only 30s due to small amount of beads, do NOT over-dry beads as this will loss yield

9) resuspend beads in **50µL H_2O**, incubate at room temp for 5min to elute

10) put on magnet High until liquid becomes clear

11) transfer **50µL** fragmented DNA to a new tube, discard original tubes with beads

- Stop: can store at 4ºC overnight or -20ºC for up to 2 weeks

3. **Gene Expression** Adapter Ligation

- oligo:
  - Split_Adaptor_Top (100µM, ACACTCTTTCCCTACACGACGCTCTTCCGATC*T, *phosphorothioated)
  - Split_Adaptor_Bottom (100µM, GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT)
- obtain:
  - **Adaptor Ligase**: WGS Ligase (**Enzymatics L6030-W-L**)
  - **Adaptor Ligation Buffer**: 5X Rapid Ligation Buffer (**Enzymatics B9020L**)

1) anneal **Adaptor Duplex**:

- per sublibrary: 2.5µL Split_Adaptor_Top + 2.5µL Split_Adaptor_Bottom (100µM) +
0.125µL 1M NaCl (final 25mM NaCl to help annealing)

	Temp	Time
	95ºC	2 min
	cool down to 25ºC at -0.1ºC/s	
	25ºC	10 min
	4ºC	Hold

2) prepare **Adaptor Ligation Mix**:

- confirm reagents fully thawed and mixed well before using
- mix well by pipetting, keep on ice

	A	B	C	D	E	F	G	H	I
		Volume (µL) for number of sublibraries							

	A	B	C	D	E	F	G	H	I
		1	2	3	4	5	6	7	8
	Adaptor Ligation Buffer	22	44	66	88	110	132	154	176
	Adaptor Ligase	11	22	33	44	55	66	77	88
	Adaptor Duplex	5.5	11	16.5	22	27.5	33	38.5	44
	H2O	16.5	33	49.5	66	82.5	99	115.5	132
	Total	**55**	**110**	**165**	**220**	**275**	**330**	**385**	**440**

3) add **50µL** **Adaptor Ligation Mix** to 50µL fragmented DNA, mix 10x (set to 80µL), quick centrifuge

4) incubate (**15min**)

- Lid Temperature: 30ºC
- Reaction Volume: 100µL

	Step	Temp	Time
	1	20ºC	15min
	2	4ºC	Hold

- proceed directly to next step, do NOT leave in thermal cycler for longer than indicated

4. Post-Ligation SPRI Clean Up (**0.8X**)

1) vortex SPRI, add **80µL** SPRI (**0.8X**), brief vortex, incubate at room temp for 5min
2) put on magnet High until liquid becomes clear, discard supernatant
3) without resuspending beads, add **180µL** **85% EtOH**, wait 1min, discard supernatant
4) repeat wash with **85% EtOH**
5) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 3min
   - do NOT over-dry beads as this will loss yield
6) resuspend beads in **23µL** **H_2O**, incubate at room temp for 5min to elute
7) put on magnet Low until liquid becomes clear
8) transfer **21µL** eluted DNA to a new tube, discard original tubes with beads

5. **Gene Expression** Sublibrary Index PCR

- oligo:
  - Split_P5 (10µM, AATGATACGGCGACCACCGAGATCTACAC-N6-ACACTCTTTCCCTACACGACGC)

- Split_P7 (10µM, CAAGCAGAAGACGGCATACGAGAT-N6-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT)

- obtain:
  - KAPA HiFi 2X Master Mix (**Roche KK2602**, -20ºC)

1) set up Index PCR per sublibrary on ice, record P5 & P7 index primer pairs used
   - confirm reagents fully thawed and mixed well before using
   - ensure no two sublibraries contain the same index primer pair
   - mix well by pipetting, keep on ice

	A	B
		Volume (µL) per sublibrary
	KAPA 2X MM	25
	Split_P5 (10µM)	2.5
	Split_P7 (10µM)	2.5
	cDNA	20
	Total	50

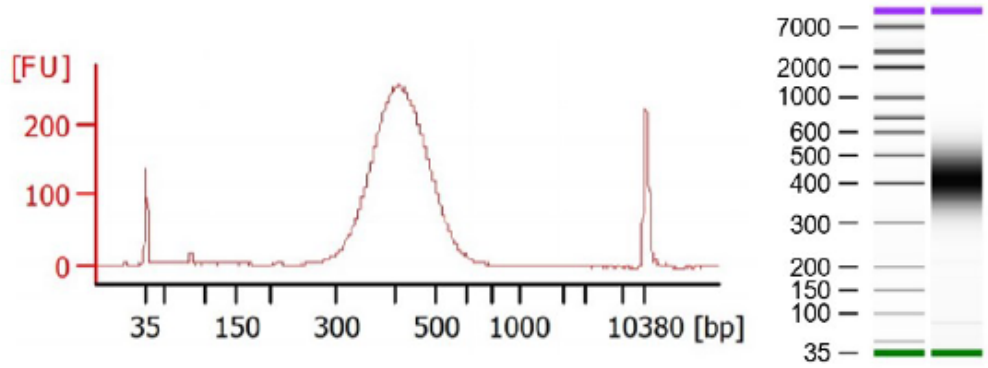
2) incubate (~**30min**)
   - Lid Temperature: 105ºC
   - Reaction Volume: 50µL
   - adjust cycles depending on the amount of cDNA during fragment based on Qubit

	A	B	C
	Cycles	Temp	Time
	1	95º C	3 min
	13 for 10-24ng 12 for 25-49ng 11 for 50-99ng 10 for 100-299ng 8 for 300-999ng 7 for 1000+ng	98º C	20 s
		67º C	20 s
		72º C	1min
	1	72º C	5 min

	A	B	C
	1	4ºC	Hold

- Stop: can store at 4ºC overnight

6. Post-Amplification Double-Sided Size Selection (**0.6X-0.8X**)
   1) vortex SPRI, add **30µL** SPRI (**0.6X**), brief vortex, incubate at room temp for 5min
   2) put on magnet High until liquid becomes clear
      - o do NOT discard supernatant
   3) transfer **75µL** clear supernatant to a new tube, discard original tubes with beads
   4) add **10µL** SPRI (**0.8X**), brief vortex, incubate at room temp for 5min
   5) put on magnet High until liquid becomes clear, discard supernatant
      - o this may take longer due to low volume of beads
   6) without resuspending beads, add **180µL** **85% EtOH**, wait 1min, discard supernatant
   7) repeat wash with **85% EtOH**
   8) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 30s
      - o only 30s due to small amount of beads, do NOT over-dry beads as this will loss yield
   9) resuspend beads in **20µL** H_2O, incubate at room temp for 5min to elute
   10) put on magnet Low until liquid becomes clear
   11) transfer **20µL** eluted DNA to a new tube, discard original tubes with beads
   12) measure the concentration of GEX sequencing library using Qubit dsDNA HS
   13) run 1µL cDNA (1:10 dilution) on Bioanalyzer



      - o there should be a peak between 400-500bp
- Stop: can store at -20ºC for up to 3 months

Preparing CROP CRISPR Libraries for Sequencing

6 1. **CRISPR** Feature PCR

- oligo:
  - Split_hU6_inner (100μM, ACACTCTTTCCCTACACGACGCTCTTCCGATCT**CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACG**)
  - Split_TruSeq-Read2 (100μM, CAGACGTGTGCTCTTCCGATCT)
- obtain:
  - KAPA HiFi 2X Master Mix (**Roche KK2602**, -20ºC)

1) obtain amplified cDNA, quick centrifuge, aliquot out **50ng cDNA** (at least **10ng**), add **H_2O** to bring total volume to 25μL

2) set up Feature PCR per sublibrary on ice
   - confirm reagents fully thawed and mixed well before using
   - expected size: 268bp (scaffold), 293bp (CS1), ~600bp (polyT)

	Volume (μL) per sublibrary
KAPA 2X MM	25
Split_hU6_inner (100μM)	0.25
Split_TruSeq-Read2 (100μM)	0.25
cDNA	24.5
Total	50

3) incubate (~**50min**)
   - Lid Temperature: 105ºC
   - Reaction Volume: 50μL

A	B	C
Cycles	Temp	Time
1	95ºC	3min
5	98ºC	20s
	65ºC	20s
	72ºC	1min
13	98ºC	20s

	A	B	C
		67ºC	20s
		72ºC	1min
	1	72ºC	5min
	1	4ºC	Hold

- Stop: can store at 4ºC overnight

2. Post-Amplification Double-Sided Size Selection (**0.5X-1X**)
   1) vortex SPRI, add **25µL** SPRI (**0.5X**), brief vortex, incubate at room temp for 5min
   2) put on magnet High until liquid becomes clear
      o do NOT discard supernatant
   3) transfer **70µL** clear supernatant to a new tube, discard original tubes with beads
   4) add **25µL** SPRI (**1X**), brief vortex, incubate at room temp for 5min
   5) put on magnet High until liquid becomes clear, discard supernatant
      o this may take longer due to low volume of beads
   6) without resuspending beads, add **180µL** **85% EtOH**, wait 1min, discard supernatant
   7) repeat wash with **85% EtOH**
   8) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 30s
      o only 30s due to small amount of beads, do NOT over-dry beads as this will loss yield
   9) resuspend beads in **20µL** **H_2O**, incubate at room temp for 5min to elute
   10) put on magnet Low until liquid becomes clear
   11) transfer **20µL** eluted DNA to a new tube, discard original tubes with beads
   12) measure the concentration of CRISPR Feature PCR DNA using Qubit dsDNA HS
- Stop: can store at 4ºC for up to 2 days or -20ºC for up to 3 months

3. **CRISPR** Sublibrary Index PCR
- oligo:
  o Split_P5 (10µM, AATGATACGGCGACCACCGAGATCTACAC-N6-ACACTCTTTCCCTACACGACGC)
  o Split_P7 (10µM, CAAGCAGAAGACGGCATACGAGAT-N6-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT)
- obtain:
  o KAPA HiFi 2X Master Mix (**Roche KK2602**, -20ºC)
   1) obtain post Feature PCR cDNA, quick centrifuge, aliquot out **10ng** **cDNA**, add **H_2O** to bring total volume to 21µL
   2) set up **CRISPR** Index PCR per sublibrary, record P5 & P7 index primer pairs used
      o confirm reagents fully thawed and mixed well before using

- ensure no two sublibraries contain the same index primer pair
- expected size: 345bp (scaffold), 370bp (CS1), ~700bp (polyT)

	A	B
		Volume (µL) per sublibrary
	KAPA 2X MM	25
	Split_P5 (10µM)	2.5
	Split_P7 (10µM)	2.5
	cDNA	20
	Total	50

3) incubate (~**30min**)
   - Lid Temperature: 105ºC
   - Reaction Volume: 50µL

	A	B	C
	Cycles	Temp	Time
	1	95ºC	3min
	9	98ºC	20s
		67ºC	20s
		72ºC	1min
	1	72ºC	5min
	1	4ºC	Hold

4. Post-Amplification Double-Sided Size Selection (**0.5X-0.9X**)
   1) vortex SPRI, add **25µL** SPRI (**0.5X**), brief vortex, incubate at room temp for 5min
   2) put on magnet High until liquid becomes clear
      - do NOT discard supernatant
   3) transfer **70µL** clear supernatant to a new tube, discard original tubes with beads
   4) add **20µL** SPRI (**0.9X**), brief vortex, incubate at room temp for 5min
   5) put on magnet High until liquid becomes clear, discard supernatant
      - this may take longer due to low volume of beads
   6) without resuspending beads, add **180µL** **85% EtOH**, wait 1min, discard supernatant

7) repeat wash with **85% EtOH**

8) centrifuge briefly, put on magnet Low, remove remaining EtOH, dry for 30s

o only 30s due to small amount of beads, do NOT over-dry beads as this will loss yield

9) resuspend beads in **20µL H_2O**, incubate at room temp for 5min to elute

10) put on magnet Low until liquid becomes clear

11) transfer **20µL** eluted DNA to a new tube, discard original tubes with beads

12) measure the concentration of CRISPR sequencing library using Qubit dsDNA HS

13) run 1µL cDNA (1:10 dilution) on Bioanalyzer

- Stop: can store at -20ºC for up to 3 months

Sequencing

7

	A	B	C	D
			Gene Expression	**CRISPR gRNA**
	Sequencing Depth/cell		30-50k read pairs	2-5k read pairs
	Library Pooling Ratio		6-10	1
	Recommended Number of Cycles	Read 1	70nt	at least 64nt
		i7 Index 1	6nt	6nt
		i5 Index 2	6nt	6nt
		Read 2	86nt	86nt