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MANUSCRIPT CITATION:

Protocol status: Working
We use this protocol and it's working

Fixed RNA - GEM Recovery - Sequencing

Forked from Fixed RNA - FFPE Resection Tissue (gentleMACS dissociation)

Ksenija Sabic[1]

[1]Icahn School of Medicine at Mount Sinai

Ksenija Sabic

ABSTRACT

This protocol is a continuation of CG000527 (Step 3 and onward).

IMAGE ATTRIBUTION

10x Genomics

GUIDELINES

Please review and consult the full 10x Genomics protocols prior to starting and at any point during the procedure if needed.

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PROTOCOL integer ID: 92741

MATERIALS

From 10x Genomics:

- Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC **10x Genomics Catalog #1000475**

OR

- Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC **10x Genomics Catalog #1000476**
- Dual Index Kit TS Set A, 96 rxns **10x Genomics Catalog #1000251**

From Agilent:

- Bioanalyzer High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

Miscellaneous:

- SPRIselect **Beckman Coulter Catalog #B23318**
- 10% Tween 20 solution **Teknova Catalog #T0710** or similar
- Ethyl alcohol, 200 proof, anhydrous, ≥99.5% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #459836**
  or similar
- Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #AM9937**
  or similar
- Buffer EB **Qiagen Catalog #19086** or similar

BEFORE START INSTRUCTIONS

Prepare daily fresh 80% Ethanol for wash steps.

GEM Recovery and Pre-Amplification

3d 0h 9m

1 Reagent Preparation (~15 mins):

1.1 Thaw **Reducing Agent B** at Room temperature, vortex, verify no precipitate, centrifuge briefly.

1.2 Thaw **Pre-Amp Primers B** at Room temperature, vortex, centrifuge briefly.

1.3 Keep **Amp Mix** On ice. Vortex and centrifuge briefly.

1.4 Prepare fresh 80% Ethanol (2.5 mL for 4 GEM reactions).

2 Add 125 µL Recovery Agent to each sample at room temperature.

DO NOT pipette mix or vortex the biphasic mixture.

3 Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x.

DO NOT invert without firmly securing the caps.

4 Wait 2 min.

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (translucent/opaque). A smaller aqueous phase volume indicates a clog during GEM generation.

5 Centrifuge briefly.

6 Slowly remove and discard 125 µL Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. *DO NOT aspirate any aqueous sample.*

7 Proceed directly to Pre-Amplification PCR. No cleanup step is required.

8 Prepare Pre-Amplification Mix on ice. Vortex and centrifuge briefly.

	A	B	C	D
	Pre-Amp Mix (add reagents in order listed)	PN	1X (µl)	4X + 10% (µl)
	Amp Mix	2000103	25.0	110.0
	Pre-Amp Primers B	2000529	10.0	44.0
	Total		35.0	154.00

9 Add 35 µL Pre-Amplification Mix to aqueous sample from step 6.

10 Cap firmly and invert 8x to mix. Centrifuge briefly.

11 Incubate in a thermal cycler with the following protocol:

	A	B	C
	Step	Temperature	Time (hh:mm:ss)
	1	98C	00:03:00
	2	98C	00:00:15
	3	63C	00:00:20
	4	72C	00:01:00
	5	Go to Step 2, 7x (total 8 cycles)	

	A	B	C
	6	72C	00:01:00
	7	4C	Hold

Saved as 'pre-amp' under Fixed RNA folder.
Lid Temperature: 105C
Reaction Volume: 100µl
Run Time: ~30-45 min

Store at 4°C for up to 72 h or −20°C for ≤1 week, or proceed to the next step.

12 Prepare Elution Solution. Vortex and centrifuge briefly.

	A	B	C
	Elution Solution (add reagents in order listed)	PN	1000µl
	Buffer EB		980
	10% Tween 20		10
	Reducing Agent B	2000087	10
	Total		1000

13 Centrifuge the sample (PCR product) for 00:00:30 sec in a microcentrifuge and transfer 70 µL 30s of the upper layer to a new tube.

Presence of a cloudy precipitate at the interface between phases is normal. Avoid transferring the precipitate when transferring 70 µl at this step.

14 Vortex to resuspend the SPRIselect reagent. Add 126 µL **SPRIselect reagent** (1.8X) to each sample and pipette mix 15x (pipette set to 180 µl).

15 Incubate 00:05:00 min at Room temperature . 5m

16 Place on the magnet (high position) until the solution clears.

17 Remove the supernatant. ***DO NOT discard any beads.***

18 With the tube still in the magnet, add 200 µL **80% ethanol** to the pellet. Wait 00:00:30 sec. 30s

18.1 Remove the ethanol.

18.2 Repeat steps 18 and 18.1 for a total of 2 washes.

19 Centrifuge briefly and place on the magnet (low position).

20 Remove any remaining ethanol. ***DO NOT let the sample dry to ensure maximum elution efficiency.***

21 Remove from the magnet. Add 101 µL **Elution Solution** (from step 12). Wait 00:01:00 min 1m before resuspending. Pipette mix 15x.

22 Incubate 00:02:00 min at Room temperature . 2m

23 Place the tube strip on the magnet (high position) until the solution clears.

24 Transfer 100 µL sample to a new tube strip.

25 Store at 4 °C for ≤ 72:00:00 hours or at -20 °C for ≤4 weeks, or proceed to the next ste 3d

Library Construction 7m 30s

26 **Reagent Preparation (~10 min):**

26.1 Thaw **Dual Index Plate TS Set A** at Room temperature.

26.2 Keep **Amp Mix** On ice. Vortex and centrifuge briefly.

26.3 If planning to assess quality and concentration of library immediately after completion, thaw **Agilent Bioanalyzer High Sensitivity Kit** at Room temperature, keeping prepared gel and gel-dye away from light sources. Consult the following protocol for details:

Protocol

NAME
Bioanalyzer High Sensitivity DNA Kit

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27 Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000511 Dual Index Plate TS Set A well ID) used.

28 Prepare Sample Index PCR Mix on ice:

	A	B	C	D	E
	Sample Index PCR (add reagents in the order listed)	PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
	Amp Mix	2000103	50	55	220
	Nuclease-free water		10	11	44
	Total		60	66	264

29 Transfer **ONLY** 20 µL of sample from the step 24 to a new tube strip.

30 Add 60 µL **Sample Index PCR Mix** to 20 µl sample.

31 Add 20 µL of an individual **Dual Index TS Set A** to each sample. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

32 Incubate in a thermal cycler with the following protocol:

	A	B	C
	Step	Temperature	Time (hh:mm:ss)
	1	98C	00:00:45
	2	98C	00:00:20
	3	54C	00:00:30
	4	72C	00:00:20

	A	B	C
	5	Go to step 2, see table below for total # of cycles	
	6	72C	00:01:00
	7	4C	Hold

Saved as 'Sample Index PCR' under Fixed RNA folder.
Lid Temperature: 105C
Reaction Volume: 100µl
Run Time: ~25 - 40 min

32.1

	A	B	C	D	E
	Targeted Cell Recovery	Total Cycles*			
		for Cell Lines	for PBMCs & Nuclei	for Cells from Fixed & Dissociated Tissues**	for Cells from FFPE Tissue Sections
	500 - 2,000	11	15	14 - 15	16
	2,000 - 4,000	10	14	13 - 14	15
	4,000 - 7,000	9	13	12 - 13	14
	7,000 - 12,000	8	12	11 - 12	13
	12,000 - 25,000	7	11	10 - 11	12
	25,000 - 50,000	6	10	9 - 10	11
	50,000 - 128,000	5	9	8 - 9	10

**Optimization of cycle number may be needed based on the total RNA content of the sample. The ideal target library concentration is 50 - 200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed. If optimization is needed, additional Amp Mix can be obtained using the Fixed RNA Feature Barcode Kit (PN-1000419). For dissociated tumor cells, cycle numbers for cell lines can be used as a starting point. For dissociated primary cells, cycle numbers for PBMCs can be used as a starting point.*
***For cells derived from the fixed and dissociated tissue samples, the cycle number will depend on the RNA expression level of the tissue and on overall quality of the tissue prior to fixation. Additional optimization may be required.*

33 Store at 4 °C for ≤72 h, or proceed to the next step.

34 Vortex to resuspend the SPRIselect reagent. Add 100 µL **SPRIselect Reagent** (1.0X) to each sample. Pipette mix 15x (pipette set to 180 µl).

35 Incubate 00:05:00 min at Room temperature. 5m

36 Place on the magnet (high position) until the solution clears.

37 Remove the supernatant. **DO NOT discard any beads.**

38 With the tube still in the magnet, add 200 µL **80% ethanol** to the pellet. Wait 00:00:30 sec. 30s

38.1 Remove the ethanol.

38.2 Repeat steps 38 and 38.1 for a total of 2 washes.

39 Centrifuge briefly and place on the magnet (low position).

40 Remove any remaining ethanol. ***DO NOT let the sample dry to ensure maximum elution efficiency.***

41 Remove from the magnet. Add 41 µL **Buffer EB**. Pipette mix 15x.

42 Incubate 00:02:00 min at Room temperature . 2m

43 Place on the magnet (low position) until the solution clears.

44 Transfer 40 µL to a new labeled DNA LoBind tube.

44.1 Make a 1:80 dilution of the library for quantification.

45 Store at 4 °C for up to 72 h, -20 °C or at -80 °C for long-term storage.

46 Run 1 µL **sample at 1:80 dilution** on an **Agilent Bioanalyzer High Sensitivity chip**. Select the region between 150-300 bp to determine average size of the library. Consult the following protocol:

Protocol

NAME

Bioanalyzer High Sensitivity DNA Kit

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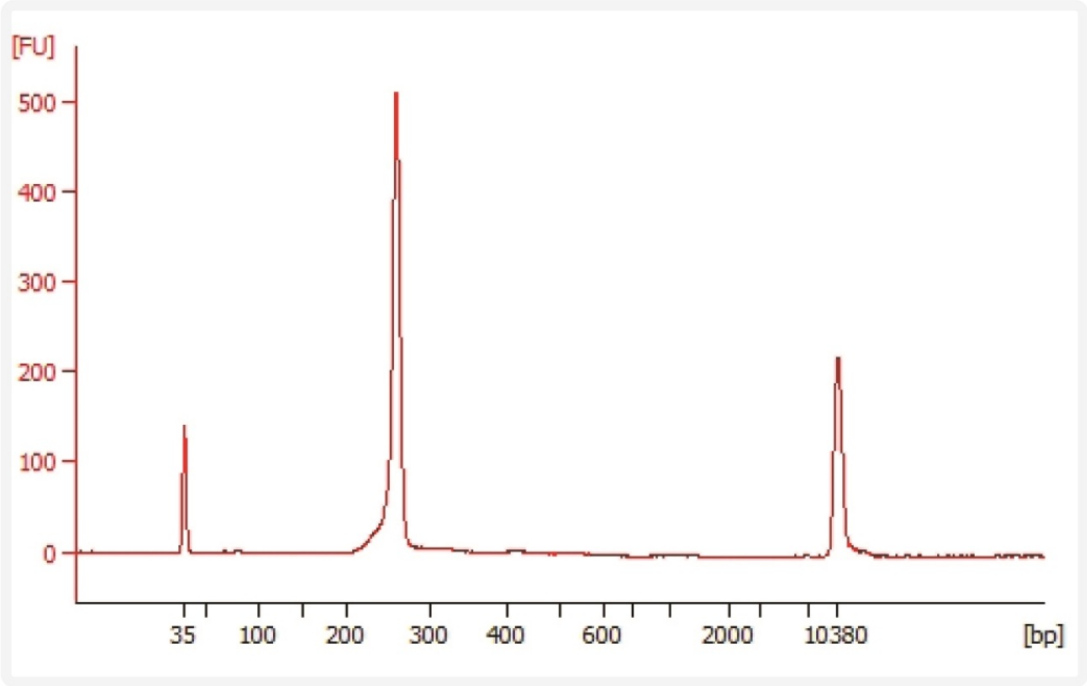
46.1 See image below for representative trace:



Sequencing

47 Consult the following protocol to send the libraries for sequencing:

Protocol

NAME

Sequencing 10x Single Cell Libraries (UMGC Workflow)

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