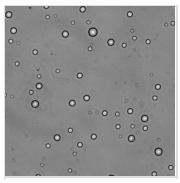

Mar 07, 2022

Single coacervate sequencing

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Wollny_Lab

Damian Wollny

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Here, we present a protocol which enables the comprehensive characterization of the RNA content of single phase-separated coacervates. We adapted single-cell RNA sequencing technology in combination with fluorescence activated cell sorting (FACS) to answer the question of how one condensate differs from the other in terms of RNA composition and how it relates to condensate features such as droplet size. This approach represents a powerful addition to labor intensive and low throughput microscopy approaches which have been the state of the art approach for coacervate RNA characterization. This protocol includes droplet production, as well as a Smart-seq2 protocol adaption for lysis, reverse transcription and cDNA amplification and sequencing library preparation. This protocol ends with the library preparation. Afterwards it got sequenced on an Illumina NextSeq500 (paired end for 300 cycles).
The Smart-seq2 protocol was originally published in Picelli, S., Faridani, O., Björklund, Å. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* **9,** 171–181 (2014). https://doi.org/10.1038/nprot.2014.006

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protocol

https://www.biorxiv.org/content/10.1101/2021.03.08.434405v1.abstract

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Single-cell sequencing, Liquid-liquid phase-separation, Coacervates

protocol ,

May 12, 2021

Mar 07, 2022

49885

Follow the general guidelines for working with RNA

- wear clean gloves all the time
- use sterile equipment and sterile disposable plasticware
- use a designated area for RNA work only
- use RNase inactivating reagents to clean equipment and surfaces
- Use RNase/DNase free filter tips instead of normal tips
- work preferably quick and on ice
- avoid handling over open bottles/tubes/etc.
- avoid RNase contamination through air
- use RNase free water

- RNA storage temperature is -80°C, avoid defrosting cycles

A	B
Primer name	**Primer sequence**
Oligo-dT	5'–AAGCAGTGGTATCAACGCAGAGTACT30VN-3'
TSO	5'-biotin-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'
ISPCR primer	5'-AAGCAGTGGTATCAACGCAGAGT-3'

All our primers were ordered from **IDT.**

1M MgCl2 **Ambion Catalog #AM9530G**

Betaine solution (5M PCR Reagent) **Sigma – Aldrich Catalog #B0300**

DNase I recombinant, RNase-free **Sigma Aldrich Catalog #000000004716728001**

dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher Scientific Catalog #R0192**

twin.tec 96-well DNA LoBind Plates **Eppendorf Catalog #0030129504**

Twin.Tec PCR Plate 96 semi-skirted, colourless wells, 25 pcs **Eppendorf Catalog #0030128575**

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Eppendorf twin.tec® PCR plate 96 LoBind **Eppendorf Catalog #0030129512**

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602**

Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Catalog #AM9938**

Recombinant RNAse Inhibitor **Takarabio Catalog #2313A**

Agencourt RNAClean XP Beads **Beckman Coulter Catalog #A63987**

RNase Zap **Sigma Aldrich Catalog #R2020-250ML**

Superscript II **Invitrogen - Thermo Fisher Catalog #18064-014** (Incl. 0.1M DTT and 5x FS Buffer)

Trizma hydrochloride solution **Sigma Aldrich Catalog #T2694** (pH 8, 1M)

Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #BP2818500**

Poly(Diallyl Dimethyl Ammonium Chloride) [Mw ~ 8500] 28 wt. % H2O **Polysciences Inc Catalog #24828-100**

CM- Dextran Natriumsalz **Merck Millipore Sigma Catalog #86524-10G-F**

Guanidine hydrochloride for molecular biology >=99% **Sigma Aldrich Catalog #G3272-500g**

Nextera XT DNA Library Preparation Kit **illumina Catalog #FC-131-1096**

Nextera XT Index Kit v2 (set A B C D) **illumina Catalog #FC-131-2001; FC-131-2002; FC-131**

Qubit™ 1X dsDNA HS Assay Kit **Thermo Fisher Catalog #Q33231**

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

Qubit® Assay Tubes **Life Technologies Catalog #Q32856**

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Invitrogen™ Qubit™ 3 Fluorometer
highly sensitive fluorescence-based Qubit quantitation assays

Invitrogen **15387293**

Invitrogen™ Q33216

2100 Bioanalyzer Instrument
Sizing, quantification, and sample quality control of DNA, RNA, and proteins on a single platform

Agilent Technologies **G2939BA**

Vortexer

VWR **97043-562**

Centrifuge
Benchtop Centrifuge

Eppendorf **5405000441**

Any benchtop centrifuge will suffice

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

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Ethanol
- H225 Highly flammable liquid and vapour.
- H319 Causes serious eye irritation.

Polydiallyldimethylammonium chloride
- H410 Very toxic to aquatic organisms with long lasting effects
- H412 Harmful to aquatic organisms, with long lasting effects.

Guanidinium Hydrochlorid
- H302 Harmful if swallowed.
- H332 Harmful if inhaled.
- H315 Causes skin irritation.
- H319 Causes serious eye irritation.

Kits
Check manufacturer's safety information for the Nextera XT Library Prep Kit used in this protocol.

Check manufacturer's safety information for the Qubit RNA HS / Qubit DNA HS Kits used in this protocol.

Check manufacturer's safety information for the RNase Away used in this protocol.

:

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responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

- Take the Agencourt RNAClean XP beads out of the fridge and let them adjust to room temperature (=RT), aliquot 110µl in each tube of a 8-PCR-stripe, vortex in between to keep the beads in solution
- Defrost the reagents for the Master Mix I and Master Mix II (except enzymes and TSO)
- Unpack RNase free filter tips (10x of 10µl filter tips; 10x 200µl filter tips; 5x 20µl filter tips)
- Prepare fresh 80% EtOH p.a. (= 50ml per plate)
- Get a box full of ice

This protocol is designed for a 96-well plate (LoBind).

Buffer preparation

1

Prepare all buffers under the conditions of usage for RNA. So work RNase free.

2 Prepare [M]**6 Molarity (M)** Guanidinium hydrochloride

3 Prepare the droplet buffer consisting of [M]**10 Milimolar (mM)** Tris and [M]**4 Milimolar (mM)** $MgCl_2$ pH**8**

Droplet production

4

Calculate the droplet production according to your experiment.

The example is made for CM-Dextran:PDDA coacervates (molar ratio: 6:1)

Droplet handling only in LoBind tubes!

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A	B	C	D	E
1000µl Droplets	**Stock concentration**		**Final concentration**	
Reagents	**g/mol**	**M**	**mM**	**µl use**
CM-Dextran Sodium Salt	162.14	1	60	60
PDDA	174	1	10	5.8
		ng/µl	**ng/µl**	**µl use**
RNA		1243	50	40.2
Droplet Buffer	10mM Tris, 4mM MgCl2, pH 8			893.7

We measure the concentration of RNA with the Qubit RNA HS Kit

4.1 Calculate how much RNA you need to add to the entire solution for a final concentration of **[M]50 ng/µl**

This example is made for **1000 µL** of droplets with a RNA stock concentration of **[M]1243 ng/µl**

4.2 Mix the droplet buffer with the CM-Dextran Sodium Salt

4.3 Add the RNA and mix briefly

4.4 Finally add the PDDA [=Poly-{diallyl-dimethylammoniumchlorid}-solution] and mix by vortexing

The solution should be turbid now

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4.5 Add 4µl of **6 Molarity (M)** Guanidinium hydrochloride into each well of the full skirted 96-well LoBind plate

4.6

Perform a droplet sorting via FACS into the 96-well plate. Store the sorted droplets **immediately** at **-80 °C** or directly continue with the protocol.

The droplets got sorted by the FACS Facility.

Make sure to get a **positive control** with 1000 droplets and also a **negative control** without any droplet.

A **full skirted plate** usually gets recommended by the FACS facilty.

Smart-seq2 preparation

5 Prepare Master Mix I

A	B	C
Reagents	**Stock concentration**	**1x [µl]**
oligo-dT primer	10 µM	1
dNTPs	10 mM each	1
nuclease free H2O		2
MasterMix Total [µl]		4
Assay Total [µl]		4

add all the reagents, mix by flicking the tube and spin down

6 Prepare Master Mix II

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A	B	C
Reagents	**Stock concentration**	**1x [µl]**
nuclease free H2O		0.09
MgCl2	1 M	0.06
Betaine	5 M	2
DTT	100 mM	0.5
5xI FS Buffer	5x	2
RNAse inhibitor	40 U/µl	0.25
Superscript II RT	200 U/µl	0.5
TSO	100 µM	0.1
MM total [µl]		5.5
Assay total [µl]		9.5

add all the reagents, mix by flicking the tube and spin down

RNAse Inhibitor, SuperScript II and TSO are sensitive reagents, add them only shortly before using the master mix

Avoid unnecessary defrosting

Droplet clean up

7

Mix the amount of droplets with RNA XP beads in a 1:2.2 ratio

Vortex and spin down briefly

7.1

Preheat the thermocycler to **72 °C**

Depending on the experiment working **On ice** is required.

7.2 Incubate for **00:05:00** at **Room temperature** 5m

7.3 Pellet the beads on a magnet until solution is clear (~ **00:05:00**) 5m

Discard supernatant

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If you are losing beads during this step, leave some supernatant with beads inside the tube, rather than losing some beads

7.4 Add **180 µL** of 80% EtOH to the bead pellet

Discard EtOH

7.5 Add **160 µL** of 80% EtOH to the bead pellet

Discard EtOH

The volume might change, depending on the starting volume

7.6 Remove the EtOH completely

7.7 Resuspend the pellet in **4 µL** of Master Mix I by pipetting up and down

(The plate is not on the magnet for elution)

Do not let the pellet dry. This decreases the output drastically

Smart-seq2

8 ⚠ 3m

Incubate at **72 °C** for **00:03:00**

Finish Master Mix II during this incubation time [**go to step #6**]

9 Spin down the reaction tube

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Add **5.5 µL** of Master Mix II, mix by flicking the tube, spin down and start the following incubation

A	B	C
Cycle [Amount]	**Temp [°C]**	**Time [min]**
1	42	90
10	50	2
	42	2
1	70	15
	4	Hold

Take reagents for Master Mix III out of the freezer **00:30:00** before the incubation time ends.

10 Prepare Master Mix III

A	B	C
Reagents	**Stock concentration**	**1x [µl]**
KAPA HiFi HS ReadyMix	2 x	12.5
IS PCR primer	10 µM	0.25
nuclease free H2O		2.25
MM total [µl]		15
Assay total [µl]		24.5

Mix by flicking the tube and spin down

10.1 Add **15 µL** of Master Mix III to the reaction

Mix by flicking the tube and spin down

10.2

Start the following incubation

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A	B	C
Cycle [Amount]	**Temp [°C]**	**Time**
1	98	3 min
12 - 23	98	20 sec
	67	15 sec
	72	6 min
1	72	5 min
	4	Hold

The amount of cycles can be varied. For single coacervate sequencing we have used 23 cycles.

11 Add SPRIselect beads in a 1:0.7 ratio, vortex and spin down briefly

Bring the SPRIselect beads to **Room temperature** and vortex properly before usage

11.1 Incubate for **00:05:00** at **Room temperature** 5m

11.2 Pellet the beads on a magnet until solution is clear (~ **00:05:00**) 5m
Discard supernatant

If you are losing beads during this step, leave some supernatant with beads inside the tube, rather than losing some beads.

11.3 Add **180 µL** of 80% EtOH (= Ethanol, molecular biology grade) to the bead pellet

Discard EtOH

11.4 Add **160 µL** of 80% EtOH to the bead pellet

Discard EtOH

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11.5 Remove the EtOH completely

11.6 Resuspend the pellet in **17.5 µL** nuclease free H_2O by pipetting up and down
(The plate is not on the magnet for elution)

11.7 Transfer the eluate into a fresh 96-well plate

11.8

Store at **-20 °C** until further usage

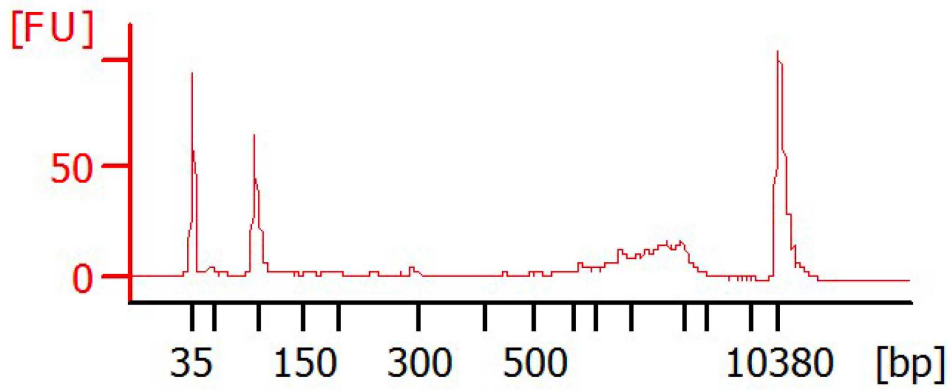
First Quality control

12 Quality Control

First measure **1 µL** of cDNA with the Qubit HS DNA Kit according to the manufacturer's protocol

12.1 Use **1 µL** - **2 µL** of the cDNA and load it on Tapestation/Bioanalyzer

Representative Bioanalyzer trace of amplified cDNA prepared from a single coacervate



Sample: cDNA of a single droplet ; Kit: Bioanalyzer HS 2100 Expert

Tagmentation

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13 Get a box full of ice, bring Tagment DNA buffer and NT buffer (Illumina, Nextera XT Library Prep Kit) to room temperature

Decide already about the index combinations that you want to use

14 Predilute cDNA to a final concentration of [M]**0.1 ng/µl** to [M]**0.3 ng/µl** in nuclease free H_2O

For single-droplet experiments or negative controls we use **1 µL** by standard, in case of positive controls the input quantity has to be adapted if necessary.

15 Prepare tagmentation pre-mix as described in the following table

A	B
Reagent	**1x (µL)**
Tagment DNA buffer	2.5
Amplicon Tagmentation mix (Tn5)	1.25
MM total (µL)	**3.75**

Mix by vortexing and spin down briefly

16 Mix tagmentation pre-mix with pre-diluted cDNA as described following

A	B
Reagent	**1x (µL)**
tagmentation pre-mix	3.75
pre-diluted cDNA	1.25
Assay total [µl]	**5**

17 Incubate in a thermocycler as described following

A	B	C
Cycle [Amount]	**Temp [°C]**	**Time [min]**
1	55	10
1	10	Hold

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18 Spin down briefly

Add **1.25 µL** NT buffer to each reaction,

Mix by vortexing and spin down briefly

Tagmentation_Indexing

19 

Latest possibility to decide for indexes!

Prepare the indexing reaction as described in the following table and add to the reaction

A	B
Reagents	**1x (µL)**
Index primer 1	1.25
Index primer 2	1.25
Nextera PCR Master mix	3.75
MM total [µl]	6.25
Assay total [µl]	12.5

Mix by flicking the tube and spin down

20 

Start incubation in a thermocycler as described following

A	B	C
Cycle [Amount]	**Temp [°C]**	**Time**
1	72	3 min
1	95	30 sec
12	95	10 sec
	55	30 sec
	72	60 sec
1	72	5 min
1	10	hold

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First bead clean up 10m

21 Pool all the samples

Vortex the SPRIselect beads carefully before use

Add SPRIselect beads in a 1:1 ratio, mix by vortexing

22 Incubate on a rotator for **00:05:00** at **Room temperature** 5m

23 Pellet the beads on a magnet until the solution is clear (~ **00:05:00**) 5m

Discard the supernatant

24 Wash pellet with fresh 80% EtOH p.a.

Discard the supernatant

25 Repeat for a total of two washes

26 Remove the remaining EtOH 1m 30s

Wait **00:00:30** to **00:01:00**

27 Elute in 1/5 of the sample volume in nuclease free H_2O 5m

Incubate for **00:05:00** at **Room temperature**
(The incubation tube is not on magnet for elution)

28 Incubate on a magnet until the solution is clear (~ **00:05:00**) 5m

Prepare a fresh tube with SPRIselect beads in a 1:0.8 ratio

Second bead clean up 21m 30s

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29 Transfer the supernatant into the prepared SPRIselect beads (1:0.8), mix by vortexing

30 Incubate on a rotator for **00:05:00** at **Room temperature** 5m

31 Pellet the beads on a magnet until the solution is clear (~ **00:05:00**) 5m

Discard the supernatant

32 Wash pellet with fresh 80% EtOH

Discard the supernatant

33 Repeat for a total of two washes

34 Remove the reminaing EtOH 1m 30s

Wait **00:00:30** to **00:01:00**

35 Elute in 1/5 of the sample volume in nuclease free H_2O 5m

Incubate for **00:05:00** at **Room temperature**

36 Incuabte on a magnet until the solution is clear (~ **00:05:00**) 5m

Transfer the clear supernatant to a fresh tube

37 ⏸

Store at **-20 °C** until further usage

Second Quality control

38 Quality control

First measure **1 µL** of cDNA with the Qubit HS ds Kit according to the manufacturer's protocol

39 Use **1 µL** - **2 µL** of the cDNA and load it on Tapestation/Bioanalyer

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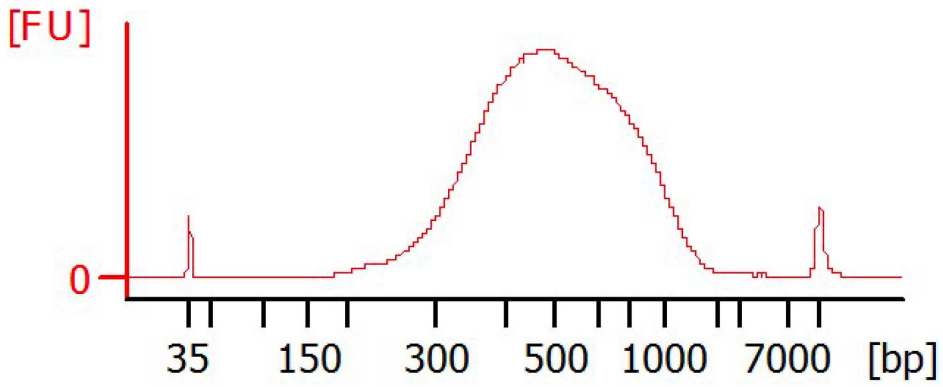

Sample: Pool of an entire plate (1 positive control; 1 blank, 94 single droplets); Kit: Bioanalyzer HS 2100 Expert

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