Lab 3: Preparing a Sequencing Library

Krista Ryon, Theodore M. Nelson, Christopher E. Mason

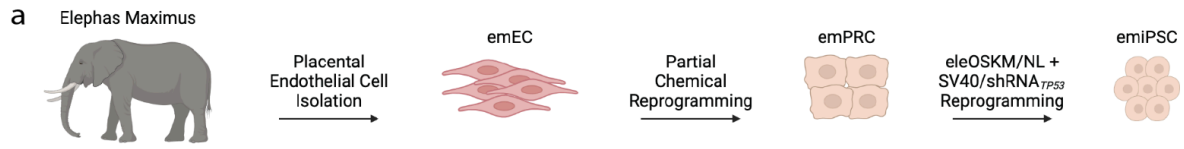
Elephant iPSC Sample Sheet

#	Sample	260/ 280	Conc. (ng/uL)	10g (uL)	Total RNA (ng)	Storage	Note	Description	Type	Received
1	WT	2.01	709	15	10635	Location: -80C Freezer I, Shelf 3, Rack WW, Column D, Row 6	Note: Qiagen RNeasy kit using Qiazol and eluted RNA in RNase/DNase-free water	4 samples total stored across 2 50mL conical tubes (within cryo box)	RNA	2024-04-24
2	Pre-iPSC	2.04	677.2	15	10158	Location: -80C Freezer I, Shelf 3, Rack WW, Column D, Row 6	Note: Qiagen RNeasy kit using Qiazol and eluted RNA in RNase/DNase-free water	4 samples total stored across 2 50mL conical tubes (within cryo box)	RNA	2024-04-24
3	C6shRNA	2.04	353.5	30	10605	Location: -80C Freezer I, Shelf 3, Rack WW, Column D, Row 6	Note: Qiagen RNeasy kit using Qiazol and eluted RNA in RNase/DNase-free water	4 samples total stored across 2 50mL conical tubes (within cryo box)	RNA	2024-04-24
4	C6iSV40	2.03	706	15	10590	Location: -80C Freezer I, Shelf 3, Rack WW, Column D, Row 6	Note: Qiagen RNeasy kit using Qiazol and eluted RNA in RNase/DNase-free water	4 samples total stored across 2 50mL conical tubes (within cryo box)	RNA	2024-04-24

Derivation of elephant induced pluripotent stem cells

Evan Appleton, Kyunghee Hong, Cristina Rodríguez-Caycedo, Yoshiaki Tanaka, Asaf Ashkenazy-Titelman, Ketaki Bhide, Cody Rasmussen-Ivey, Xochitl Ambriz-Peña, Nataly Korover, Hao Bai, Ana Quieroz, Jorgen Nelson, Grishma Rathod, Gregory Knox, Miles Morgan, Nandini Malviya, Kairui Zhang, Brody McNutt, James Kehler, Amanda Kowalczyk, Austin Bow, Bryan McLendon, Brandi Cantarel, Matt James, Christopher E. Mason, Charles Gray, Karl R. Koehler, Virginia Pearson, Ben Lamm, George Church, Eriona Hysolli

bioRxiv 2024.03.05.583606; doi: https://doi.org/10.1101/2024.03.05.583606



LABEL DEFINITIONS:

WT = emECs

Pre-iPSC = emPRC

C6shRNA = C1-loxC4-TP53shRNA (emiPSC)

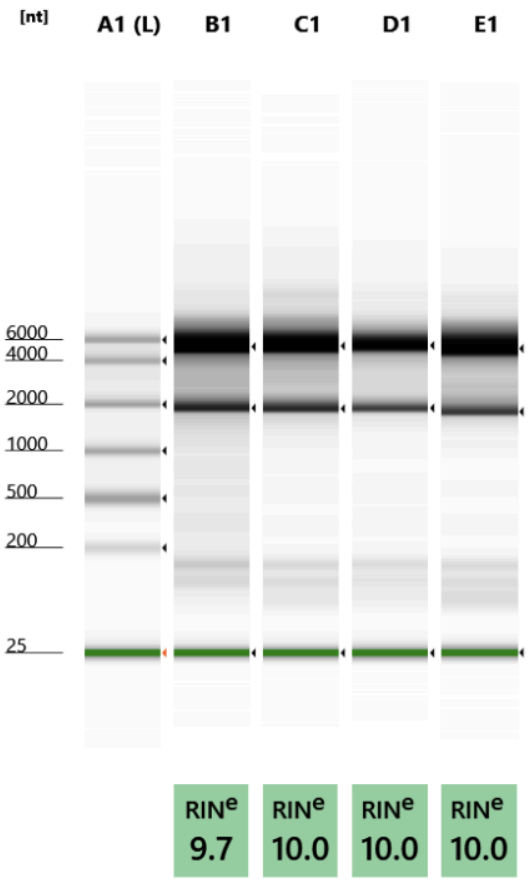
C6iSV40 = C1-loxC4/5/6-SV40 (emiPSC)

Cellular reprogramming Primary *Elephas maximus* endothelial cells were used as the starting cell line for reprogramming. These cells are mantained in 30% FBS/1% antibiotic/antimycotic /1% Non-essential AA/ EGM-2 media (Lonza) with Laminin521 coating (5 μg/ml; Gibco A29248). Once the cells became approximately 70% confluent, a chemical cocktail medium was used for partial reprogramming to emPRCs - KO DMEM (Gibco 10829-018) + 10% KOSR (Invitrogen) + 55 μM 2-mercaptoethanol (55 mM (1000X); Gibco 21985023) + 50 ng/ml bFGF (20 μg/ml; heat stable; Life technologies PHG0369) + 0.5 mM VPA (EtOH; Selleckchem S3944) + 5 μM CHIR-99021 (DMSO; Selleckchem S1263) + 2 μM RepSox (DMSO; Selleckchem S7223) + 10 μM Tranylcypromine (2-PCPA) HCl (DMSO; Selleckchem S4246) + 20 μM Forskolin (DMSO; Selleckchem S2449) + 1 μM Ch 55 (DMSO; Tocris 2020) + 5 μM EPZ004777 (DMSO; Selleckchem S7353). The medium was changed every two days until small emPRC colonies were observed. Once colonies reached sufficient size, they were hand-picked and mechanically passaged 2-3 times. Once a 2-3 million emPRCs were avialable, they were necleofected with plasmids encoding genome-integrating (via PiggyBac), inducible, polycistronic transgene expression cassettes. These cassettes contained one of [*Loxodonta africana OCT4/SOX2/KLF4/CMYC* (C4) or *Loxodonta africana OCT4/SOX2/KLF4/CMYC/LIN28A* (C5) or *Loxodonta africana OCT4/SOX2/KLF4/CMYC/LIN28A/NANOG* (C6)] and [SV40 T-antigen or an shRNA targeting *TP53* retrogenes in *Elephas maximus*]. Cells were recovered, selected with mammalian selection markers hygromycin and puromycin, and induced for 1 month until full emiPSC morphology, growth, and molecular signature was observed.

Describe the importance of the metadata in the sample description:

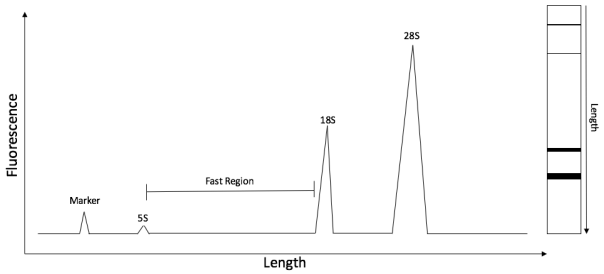
What kinds of questions can be answered by the direct-RNA sequencing we will perform to support the mission of Colossal Biosciences?

Filename: 2024-05-07 - 05.19.43.RNA



A1 = Ladder ; B1 = WT ; C1 = Pre-iPSC ; D1 = C6shRNA ; E1 = C6iSV40

RNA Integrity Numbers are calculated from 1-10 (10 being the max score) based on the prominence of rRNA peaks - 18S and 28S, as shown to the right. The prominence of these peaks indicates little rRNA degradation, proxy for total RNA degradation.



What do these peaks indicate about elephant rRNAs compared to human rRNAs?

Sample	Qubit ng/uL (RNA HS)
WT	862
Pre-iPSC	788
C6shRNA	534
C6iSV40	884

Qubit fluorometric quantification was also performed. Why are these values different from those reported in the sample table?

In preparing the library, we load 1 µg of total RNA. Similar steps to the following were performed in a Day 1 preparation [Nanopore 002 direct-RNA sequencing protocol]:

1.] Prepare the RNA in nuclease-free water. Transfer 1 µg of total RNA into a 1.5 ml Eppendorf DNA LoBind tube Adjust the volume to 9 µl with nuclease-free water. Mix thoroughly by flicking the tube to avoid unwanted shearing. Spin down briefly in a microfuge.

2.] In a 0.2 ml thin-walled PCR tube, mix the reagents in the following order:

Reagent	Volume
NEBNext Quick Ligation Reaction Buffer	3.0 µl
RNA	9.0 µl
RNA CS (RCS), 110 nM	0.5 µl
Custom-ordered Reverse Transcription Adapter	1.0 µl
T4 DNA Ligase	1.5 µl
Total	**15 µl**

3.] Mix by pipetting and spin down.

4.] Incubate the reaction for 10 minutes at room temperature.

Reagent	Volume
Nuclease-free water	9.0 µl
10 mM dNTPs	2.0 µl
5x first-strand buffer	8.0 µl
0.1 M DTT	4.0 µl
Total	**23.0 µl**

5.] Mix the following reagents together to make the reverse transcription master mix:

6.] Add the master mix to the 0.2 ml PCR tube containing the RT adapter-ligated RNA from the "RT Adapter ligation" step. Mix by pipetting.

7.] Add 2 µl of SuperScript III reverse transcriptase to the reaction and mix by pipetting.

8.] Place the tube in a thermal cycler and incubate at 50°C for 50 min, then 70°C for 10 min, and bring the sample to 4°C before proceeding to the next step.

9.] Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

10.] Resuspend the stock of Agencourt RNAClean XP beads by vortexing.

11.] Add 72 µl of resuspended RNAClean XP beads to the reverse transcription reaction and mix by pipetting.

Questions

What occurs in the thermocycler at 50˚C? 70˚C?

What is the purpose of the Agencourt RNAClean XP beads?

Today's Protocol [Source: RNA -004 Direct-RNA sequencing protocol]:

1. In the same 1.5 ml Eppendorf DNA LoBind tube, combine the reagents in the following order: ☐

Reagent Volume
RT-RNA sample: 20 µl
NEBNext Quick Ligation Reaction Buffer: 8 µl
RNA Ligation Adapter (RLA): 6 µl
Nuclease-free water: 3 µl
T4 DNA Ligase: 3 µl
Total: 40 µl

2. Mix by pipetting. ☐

3. Incubate the reaction for 10 minutes at room temperature. ☐

4. Resuspend the stock of Agencourt RNAClean XP beads by vortexing. Add 16 µl of resuspended Agencourt RNAClean XP beads to the reaction and mix by pipetting. ☐

5. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature. ☐

6. Spin down the sample and pellet on a magnet. Keep the tube on the magnet for 5 minutes, and pipette off the supernatant when clear and colorless. ☐

7. Add 150 µl of the Wash Buffer (WSB) to the beads. ☐

8. Close the tube lid and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow the beads to pellet for 5 minutes and pipette off the supernatant when clear and colorless. ☐

9. Repeat the previous step. ☐

10. Spin down the tube and replace onto the magnetic rack until the beads have pelleted to pipette off any remaining Wash Buffer (WSB). ☐

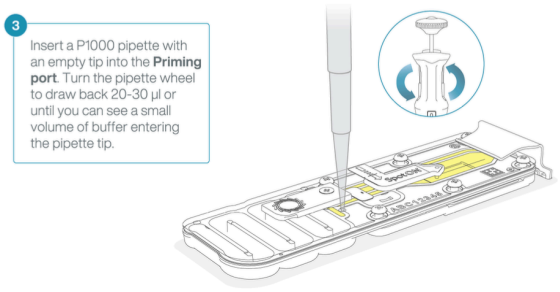
11. Remove the tube from the magnetic rack and resuspend pellet in 33 µl RNA Elution Buffer (REB) by the gently flicking the tube. Incubate for 10 minutes at room temperature. ☐

Post-elution, we perform quality control with the Qubit HS DNA assay. **Why**?

Loading the Flow Cell [according to Krista's demonstration / instructions]:

6 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip
   Note: Visually check that there is continuous buffer from the priming port across the sensor array.



Consider possible variables within the sequencing preparation process that you may be able to detect in downstream computational analysis: