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BioMark Single Cell Protocol (Two-Step RTSTA)

In 1 collection

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1 Works for me dx.doi.org/10.17504/protocols.io.wdsfa6e

SPARC

ABSTRACT

This protocol utilizes Fluidigm's Biomark system which performs high-throughput real-time PCR that can assay 48 or 96 genes for 48 or 96 samples respectfully. This protocol is used for gene expression targeting samples at the single-cell scale and can be used with the 48.48 Dynamic Array integrated fluidic circuit (IFC) or the 96.96 Dynamic Array IFC. Note that this protocol assumes that single-cell samples are captured using the Arcturus Laser Capture Microdissection system.

MATERIALS

NAME	CATALOG #	VENDOR
T4 Gene 32 Protein - 100 ug	M0300S	New England Biolabs
Exonuclease I (E.coli) - 3,000 units	M0293S	New England Biolabs
Thermocycler		
TaqMan™ PreAmp Master Mix	4391128	Applied Biosystems, Foster City, California, USA
TaqMan Universal PCR Master Mix	4304437	Life Technologies
TE Buffer	AM9861	Invitrogen - Thermo Fisher
CapSure HS Caps	LCM0214	Applied Biosystems
CellsDirect™ One-Step qRT-PCR Kit	11753100	Invitrogen - Thermo Fisher
SuperScript™ VILO™ cDNA Synthesis Kit	11754050	Invitrogen - Thermo Fisher
2X SsoFast EvaGreen Supermix with Low ROX	1725211	BIO-RAD
20x DNA Binding Dye Sample Loading Reagent	100-7609	Fluidigm
2X Assay Loading Reagent	100-7611	Fluidigm
Control Line Fluid Kit—96.96	89000021	Fluidigm
Control Line Fluid Kit—48.48	89000020	Fluidigm
48.48 Dynamic Array™ IFC for Gene Expression	BMK-M-48.48	Fluidigm
96.96 Dynamic Array™ IFC for Gene Expression	BMK-M-96.96	Fluidigm
IFC Controller HX	View	Fluidigm
BioMark HD System	View	Fluidigm

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LCM Extraction

1 Prepare lysis buffer as follows:

- **0.5 µl** Lysis enhancer (CellDirect kit)
- **5 µl** Resuspension buffer (CellDirect kit)
- **5.5 µl** Total lysis buffer per sample

2 Capture single cells on CapSure HS caps.

Add **5.5 µl** lysis buffer per cap

Incubate at **75 °C on heat block** for **00:15:00** cap-side down

Incubate on ice for **00:05:00** cap-side down

Incubate on ice for **00:05:00** right-side up

Spin down and transfer lysate to PCR tube or plate or freeze at **-80 °C**

Sample size will vary, i.e., single cell, 10-cell pool, 100-cell pool, etc.

RNA Dilution Series (Optional)

3 Prepare total RNA dilutions of 1 ng/ul, 300 pg/ul, 100 pg/ul, 30 pg/ul, 10 pg/ul, 3 pg/ul, 1 pg/ul, 300 fg/ul and H2O.

Add **1 µl** of RNA to **5.5 µl** lysis buffer.

Treat RNA dilution samples the same as the LCM samples.

We assume the average RNA yield from single cell LCM is approximately 10 pg.

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Reverse Transcription

4 Transfer all samples to a PCR plate.

Add **1.2 µl** 5X VILO Reaction Mix to each cell lysate (including dilution series).

VILO Reaction Mix is part of the VILO cDNA synthesis kit and always runs short. Make sure sufficient reagent is available before RT.

5 Incubate plate at **65 °C** for **00:01:30** , snap cool on ice and spin down.

6 Mix the following reagents and add **1 µl** to each sample (for 96.96 Biomark chip):

- 10X Superscript III Mix (VILO kit) **0.15 µl** x 110 = **16.5 µl**
- T4 Gene 32 Protein **0.12 µl** x 110 = **13.2 µl**
- H2O **0.73 µl** x 110 = **80.3 µl**

Final sample volume including lysate = **7.5 µl**

7 Incubate PCR plate using the following thermocycles:

- **25 °C** for **00:05:00**
- **50 °C** for **00:30:00**
- **55 °C** for **00:25:00**
- **60 °C** for **00:05:00**
- **70 °C** for **00:10:00**
- Hold at **4 °C**

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Preparing Pre-Amp Primer Pool

8 For 96.96 chip, each primer pair is diluted to 50 uM

- Primer pairs (50 uM) **2 µl** x 96 = **192 µl**
- H2O **8 µl**
- Total = **200 µl** of primer pool (500 nM concentration)

PreAmp

9 Mix together and add **10.5 µl** to each RT product (for 96.96 chip):

- Taqman PreAmp Master Mix **9 µl** x 110 = **990 µl**
- 500 nM primer pool **1.5 µl** x 110 = **165 µl**

Final sample volume = **18 µl**

10 Incubate PCR plate using the following thermocycles:

- **95 °C** for **00:10:00**

22 cycles of:

- **96 °C** for **00:00:05**
- **60 °C** for **00:04:00**

We consider 22 cycles of pre-amp optimal for LCM captured neurons. Number of cycles can be adjusted acccording to sample type.

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11 Exonuclease Treatment

Mix the following and add **6 µl** to each sample (for 96.96 chip):

- Exonuclease I reaction buffer 10X **0.6 µl** x 110 = **66 µl**
- Exonuclease I **1.2 µl** x 110 = **132 µl**
- H2O **4.2 µl** x 110 = **462 µl**

Final sample volume = **24 µl**

12 Incubate PCR plate using the following thermocycles:

- **37 °C** for **00:30:00**
- **80 °C** for **00:15:00**

Add **51 µl** of TE buffer to each reaction.

Water can be used if long term storage of amplified cDNA is not required. Samples can be stored in **-20 °C** if they will not be taken to the next step right away.

Prepare Sample Pre-Mix and Samples

13 Prepare the sample pre-mix:

- 2X SsoFastEvaGreen Supermix with low ROX **4 µl**
- 20X DNA Binding Dye Sample Loading Reagent (green cap) **0.4 µl**

Add **4.4 µl** sample pre-mix to **3.6 µl** of sample **after PreAmp** for a total of **8 µl** per sample.

Vortex the Sample Mix solution for a minimum of **00:00:20** , and centrifuge for at least **00:00:30** .

Prepared reactions can be stored for short times at **4 °C** until the samples are ready to be loaded into the chip.

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Prepare Assay Mix

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The same preparation of primers can be used for the Fluidigm 48.48 Dynamic Array IFC and the Fluidigm 96.96 Dynamic Array IFC.

Combine the following:

- 2X Assay Loading Reagent (yellow cap) **4 µl**
- 20 uM primers **4 µl**

Vortex the Assay Mix for a minimum of **00:00:20** and centrifuge for at least **00:00:30** to spin down all the components.

Vortex thoroughly and centrifuge all samples and assay soltions before pipetting into the chip inlets. Failure to do so may result in a decrease in data quality.
The final concentration of each primer is 5 uM in the inlet, and 500 nM in the reaction chamber.

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Priming and Loading the Dynamic Array IFC

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CAUTION! Due to different accumulator volumes, use the appropriate control syringe for your chip type: **300 µl** for the 48.48 chip or **150 µl** for the 96.96 chip.

- Inject control line fluid into each accumulator on the chip (see diagram).
- Remove and discard the blue protective film from the bottom of the chip.
- Place the chip in the IFC Controller MX for the 48.48 chip or the IFC Controller HX for the 96.96 chip, then run the **Prime (113x)** script for the 48.48 chip or the **Prime (136x)** script for the 96.96 chip.
- When the prime script has finished, press **Eject** to remove the primed chip from the IFC Controller.

CAUTION! While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.

- Pipette **5 µl** of each assay and **5 µl** of each sample into their respective inlets on the chip (refer to diagram).
- IMPORTANT: You must pop/remove any air bubbles that are in the inlets. This must be done in under **00:30:00** .
- Return the the chip to the IFC Controller.
- Using the software, run the **Load Mix (113x)** script for the 48.48 chip or **Load Mix (136x)** script for the 96.96 chip to load the samples and assays into the chip.
- When the Load Mix script has finished, remove the loaded chip from the IFC Controller.
- Remove any dust particles or debris from the chip surface using scotch tape. you are now ready for the chip run.

96.96chip with well number and control line fluid diagram.pdf

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BioMark Run

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1. Double-click the Data Collection Software Icon on the desktop to launch the software.
2. Click **Start a New Run**.
3. Check the staus bar to verify the lamp and the camera are ready. Make sure both are green before proceeding.
4. Place the chip into the reader.
5. Click **Load**.
6. Verify chip barcode and chip type. Choose project settings (if applicable). Click **Next**.
7. Chip run file: Select **New**. Browse to a file location for data storage. Click **Next**.
8. Application, Reference, Probes: Select Aplication Type--**Gene Expression** for version 3.1.2 or higher software (for earlier versions, contact Fluidigm Technical Support). Select Passive References- **ROX**. Select Probe--**Single probe**. Select probe type- **EvaGreen**. Click **Next**.
9. Click **Browse** to find the thermal cycling protocol file. For BioMark HD- GE Fast 48x48 PCR+Metl v2.pcl or GE Fast 96x96 PCR+Melt v2.pcl. For BioMark- GE 48x48 PCR+Metl v2.pcl or GE 96x96 PCR+Melt v2.pcl.
10. Confirm **Auto Exposure** is selected.
11. Click **Next**.
12. Verify the chip run information.
13. Click **Start Run**.

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