Apr 10, 2020 Version 1

BioMark Single Cell Protocol (Two-Step RTSTA) V.1

DOI
dx.doi.org/10.17504/protocols.io.wdsfa6e

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.wdsfa6e

Protocol Citation: Shaina Robbins, Alison Moss, Sean Nieves 2020. BioMark Single Cell Protocol (Two-Step RTSTA). **protocols.io https://dx.doi.org/10.17504/protocols.io.wdsfa6e**

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

Created: December 12, 2018

Last Modified: April 30, 2020

Protocol Integer ID: 18578

Keywords: Biomark Fluidigm High Throughput Real Time PCR

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

MATERIALS

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

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

Note

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.

Note

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

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).

Note

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

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

Note

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

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.

Note

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.

Prepare Assay Mix

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Note

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.

Note

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.

Priming and Loading the Dynamic Array IFC

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Note

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.

Note

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.

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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 48×48 PCR+Metl v2.pcl or GE Fast 96×96 PCR+Melt v2.pcl. For BioMark- GE 48×48 PCR+Metl v2.pcl or GE 96×96 PCR+Melt v2.pcl.
10. Confirm **Auto Exposure** is selected.
11. Click **Next**.

12. Verify the chip run information.
13. Click **Start Run**.