Version 2

May 04, 2020

SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL V.2

In 1 collection

Sirisha Achanta[1]

[1]Thomas Jefferson University

1 Works for me dx.doi.org/10.17504/protocols.io.bftdjni6

SPARC
Tech. support email: info@neuinfo.org

ABSTRACT

Single cell high-throughput qRT-PCR protocol combines high sensitivity technique of single cell qPCR with high-throughput qPCR technology that can generate data from 96 samples and 96 genes in a single experiment. It can be adapted for various sample types- cell culture, tissue samples and extracted RNA (10pg) and measured on traditional qPCR and high-throughput qPCR platforms. The workflow is comprised of four steps – cell lysis, reverse transcription, pre-amplification and qPCR. Key features of this protocol are; processing low input samples directly to reverse transcription without RNA extraction which minimizes sample loss, pre-amplification enables amplification of cDNA from single cells to detectable levels for qPCR and measuring up to 400 genes from a single cell sample/10pg of RNA (starting material). Robust reproducible and versatile this protocol can be adapted to several upstream and downstream techniques.

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		Fluidigm
BioMark HD System		Fluidigm

Reagent Preparation 2h

1 **Lysis Buffer:** Combine Lysis enhancer and Resuspension buffer (CellsDirect Kit) as detailed in Table 1 (below)

	Volume for one sample (µl)	Volumes for 96 samples with overage (for 110 samples) (µl)
Lysis enhancer (CellDirect kit)	0.5	55

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Resuspension buffer (CellDirect kit)	5	550
Total	5.5	605

Table 1: Lysis Buffer Preparation

Lysis Buffer can be stored at -20^{o}C for upto a year

2 Primer Dilutions

Forward and reverse primers need to be ordered at a stock concentration of 400µM to obtain a forward reverse mix concentration of 200µM.

Prepare primer dilutions:

- [M]**100 Micromolar (µM)** , [M]**200 Micromolar (µM)** (forward and reverse primer mix) – Used in the pre-amplification step (Step 9). Primer pool preparation see STEP 3
- [M]**20 Micromolar (µM)** (forward and reverse primer mix) – Used for BioMark Chip loading (STEP 14)
- [M]**2 Micromolar (µM)** used for traditional qPCR (STEP 17)

3 Primer Pool Preparation - [M]500 Nanomolar (nM) . For primer pool with fewer than 200 primers, see Table 2. For primer pool greater than 200 primers and less than 400 see Table 3 (below)

Primer pool upto 200 primers	Volume
Primer pairs (100 µM)	1 ul from each primer pair- X µl
DNA Suspension Buffer	200 – X µl
Total	200 µl

Table 2: 500 nM primer pool for 1-200 primers- Each primer is at a 500nM concentration.

Primer pool 201-400 primers	Volume
Primer pairs (200 µM)	1 ul from each primer pair- X µl
DNA Suspension Buffer	400 – X µl
Total	400 µl

Table 3: 500 nM primer pool for 201-400 primers- Each primer is at a 500nM concentration.

Primer pool can be generated and used for multiple experiments. Store at -20^{o}C for a year. With 200 µM stock primers up to 400 primer pairs can be included. For generating primer pools with greater than 400 primer pairs, primers will have to be ordered at a higher concentration.

For generating primer pools with greater than 400 primer pairs, primers will have to be ordered at a higher concentration. We have tested up to 400 primers in the primer pool.

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Positive and Negative controls

4 RNA Dilution series- This is used as a positive control for the experiment and must contain known amounts of RNA obtained from whole tissue and include all the conditions tested in the experiment. This is to ensure that the positive control has expression of all the genes measured. Dilution series is also used as a metric to test primer efficiency. For example in a 2X dilution series each point contains twice the starting material as the previous point, therefore if the primer scales accurately there should be 1 Ct difference between 2 dilution points.

Prepare 2X or 3X RNA dilution series for 6-8 dilution points. The mean sample input should correspond to the middle points in the dilution series.
For example, dilution series for an experiment with single cell samples (we consider a cell to contain approximately 10pg of RNA):
2X dilution series – 2pg/ µl, 4pg/ µl, 8pg/ µl, 16pg/ µl, 32pg/ µl and 64pg/ µl.

Sample Plate Setup

5 **Cell Lysis**

This step is for processing samples collected using LCM. For cell culture samples lyse cells in tubes instead of LCM cap.

- Add **5.5 µl** lysis buffer (*Reagent Preparation*) to the capture surface on the LCM Cap
- Cover the cap with a 0.2 ml tube, ensure tight seal
- Incubate at **75 °C** on a heat block (Cap surface in contact with the heating block) for **00:15:00**
- Cool Cap and tube on ice **On ice** for **00:05:00**
- Spin down to collect lysate in the PCR tube and transfer cell lysate plate

Refer to the LCM protocol for details on how to aquire samples from tissue sections.

6 **Sample plate setup**

This step is performed on **On ice**

- Unknown samples: Transfer cell lysate to PCR plate. If using extracted RNA transfer 1 µl of RNA from sample (10-100pg) add **4.5 µl** of lysis buffer (*Reagent Preparation*).
- Positive control: Transfer **1 µl** of RNA standard (*Reagent Preparation*) to PCR plate and add **4.5 µl** of lysis buffer.
- Negative control: Add **1 µl** of molecular grade water to a single well on the PCR plate and add **4.5 µl** of lysis buffer.

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Reverse Transcription

7 Vilo activation

This step is prepared **On ice**

- Add 1.8 µl of 5X VILO Reaction Mix (*Component of Superscript VILO cDNA synthesis kit*) to each sample (unknown sample, positive control and negative control).
- Spin down for **00:02:00** at 2000 rpm and proceed to Thermocycler
- **Thermocycler : VILO activation**

  65 °C – **00:01:30**

  Hold at **4 °C**

8 **RT Mix**: To be prepared immediately prior to use in STEP 8. Mix the following reagents using the table below (Table 4)

Component	Volume for one sample (µl)	Volumes for 96 samples with overage (for 110 samples) (µl)
10X Superscript III Mix (VILO kit)	0.30	33
T4 Gene 32 Protein	0.20	22
DNA Suspension Buffer	1	110
Total	1.5	

Table 4: Reaction mix for Reverse Transcription

- Add **1.5 µl** of RT mix to each sample (*Table 4*)
- Spin down for **00:02:00** at 2000 rpm and proceed to Thermocycler
- **Thermocycler : RT**

  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**
- Spin down for **00:02:00** at 2000 rpm and place on ice

This is an optional stopping point. Samples can be stored overnight at **4 °C** or stored at **-20 °C** for a year.

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Pre-amplification

9 **PreAmp Mix** To be prepared immediately prior to use in STEP 10. Mix the following reagents using the table below (Table 5)

Component	Volume for one sample (µl)	Volumes for 96 samples with overage (for 110 samples) (µl)
TaqMan PreAmp Master Mix	10	1100
500 nM primer pool	1.6	176
Total	11.6	

Table 5: Reactiom Mix for Pre-amplification

10 **Pre-amplification**

This step is performed on **On ice**

- Add **11.6 µl** of PreAmp Mix to each sample
- Spin down for 2 minutes at 2000 rpm and proceed to Thermocycler
- **Thermocycler : PreAmp22**

95 °C for **00:10:00**

22 cycles of:

96 °C for **00:00:05**

60 °C for **00:04:00**

Hold at **4 °C**

- Spin down for **00:02:00** at 2000 rpm and place on ice

This is an optional stopping point. Samples can be stored overnight at **4 °C** .

We consider 22 cycles of pre-amp optimal for single cells collected using LCM. We do not recommended to pre-amplification of samples over 22 cycles due to observed increase in production of non-specific PCR fragments and primer-dimers.

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Exonuclease

11 **Exonuclease Mix:** To be prepared immediately prior to use in STEP 12. Mix the following reagents using the table below (Table 6)

Component	Volume for one sample (µl)	Volumes for 96 samples with overage (for 110 samples) (µl)
Exonuclease I reaction buffer 10X	0.8	88
Exonuclease I	1.6	176
DNA Suspension Buffer	5.6	616
Total	8	

Table 6: Reaction Mix for Exonuclease

Catalyzes the removal of nucleotides from linear single-stranded DNA in the 3' to 5' direction. It is used for sample cleanup post pre-amplification to remove unincorporated primers and other single stranded cDNA.

12 **Exonuclease treatment:**

- Add **8 µl** of Exonuclease Mix (*reagent preparation*) to each sample
- Spin down for 2 minutes at 2000 rpm and proceed to Thermocycler
- **Thermocycler : Exonuclease**

  37 °C for **00:30:00**

  80 °C for **00:15:00**

  Hold at **4 °C**
- Spin down for **00:02:00** at 2000 rpm andplace on ice
- Add **54 µl** of TE buffer to each sample

This is an optional stopping point. Samples can be stored overnight at **4 °C** or stored at **-20 °C** for a year.

High-throughput qRT-PCR : BioMark Chip Run

13 **Chip Sample plate preparation**

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

Prepare Chip Sample plate on the day of Chip run

- Combine 2X SsoFast EvaGreen Supermix with Low ROX and 20X DNA Binding Dye Sample Loading Reagent for a 48.48 or 96.96 IFC Biomark chip using the table below (Table 7):

Component	Volume per inlet with overage (µl)	Volume for 48.48 IFC (µl) with overage	Volume for 96.96 IFC (µl) with overage
2X SsoFast EvaGreen Supermix with Low ROX	4	220	440
20X DNA Binding Dye Sample Loading Reagent	0.4	22	44
Total	4.4		

Table 7: Chip Sample Plate Reagent mix

- In a new PCR plate aliquot 4.4ul of the above mix into sample wells in the following order
- 96.96 IFC – fill wells A1 to H12 (all the wells)
- 48.48 IFC – fill wells 1 to 6 in each row (A1-A6, B1-B6….H1-H6)
- Add **3.6 µl** of Exonuclease treated sample to the plate with the above reagents
- Spin down for **00:02:00** at 2000 rpm and place **On ice**

14 Chip Assay plate

Chip assay plate can be prepared a day before chip run and stored at 4°C.

- In a new PCR plate aliquot **4 µl** 2X Assay Loading Reagent into 48 or 96 wells for a 48.48 or 96.96 IFC Biomark chip using the corresponding layout as described in step 13 for the two types of IFC BioMark Chips.
- Add **4 µl** of corresponding primer pairs at 20µM concentration to the wells
- Spin down for **00:02:00** at 2000 rpm and place **On ice**

The final concentration of each primer pair is 5 µM in the inlet and 500 nM in the reaction chamber

15 Priming and loading BioMark Chip:

- Inject control line fluid into each accumulator on the chip (see Figure 1).
- Place the chip into 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.
- Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip (see Figure 1).
- Using an 8 channel pipette load column 1 (A1, B1..H1) in the alternate wells starting with the top left well. Similarly load the next 5 columns for 96.96 chip and next 2 for 48.48 chip. For the seventh(96.96) or the fourth (48.48 chip) column load starting with second from the top on the chip.
- Return the chip to the IFC Controller.
- Using the IFC Controller 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 chambers.
- 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.

Single Cell HT-qRT-PCR Figure 1.pdf

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6

16 **Data Collection software settings:**

- Double-click the Data Collection Software icon on the desktop to launch the software.
- Click **Start a New Run**.
- Check the status bar to verify that the lamp and the camera are ready. Make sure both are green before proceeding.
- Remove and discard the blue protective film from the bottom of the chip. Place the chip into the reader. Click **Load**.
- Verify chip barcode and chip type. Click **Next**.
- Chip Run file: Select **New**. Browse to a file location for data storage. Click **Next**.
- Application, Reference, Probes:
- Select Application Type--**Gene Expression** for version 3.1.2 or higher software (for all earlier versions, contact Fluidigm Technical Support).
- Select Passive Reference: **ROX**.
- Select Probe--**Single probe**.
- Select probe type: **EvaGreen**. Click **Next**.
- Click **Browse** to find the thermal cycling protocol file.
  For BioMark HD:
- GE Fast 48x48 PCR+Melt v2.pcl
- GE Fast 96x96 PCR+Melt v2.pcl

Traditional qPCR Setup

17 Samples can be measured using a traditional qPCR instrument using the following reaction mix for a 20 µl qPCR reaction see Table 8 (below)

Component	Volume (µl)
Pre-amplified and Exonuclease treated sample	2
Primer pair (2µM)	2
TaqMan Universal PCR Master Mix	10
Molecular grade water	6
Total	20

Table 8: PCR reaction components and volumes

Run a standard 30-35 cycle PCR for gene expression.

Citation: Sirisha Achanta (05/04/2020). SINGLE CELL HIGH-THROUGHPUT QRT-PCR PROTOCOL. https://dx.doi.org/10.17504/protocols.io.bftdjni6