# Protocol for Single Cell Gene Expression Profiling by Multiplex RT-qPCR

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Single cell gene expression analysis is a powerful technique that provides a unique and insightful perspective on biological pathways and processes. Here we describe a validated protocol that enables fast and accurate analysis of up to 100 genes in isolated single cells using multiplex reverse transcription quantitative PCR (RT-qPCR).

## Materials

### Preparation of Cells

- 50 ml Centrifuge Tube, VWR, cat. #21008-951
- Phosphate Buffered Saline (PBS)
- Bovine Serum Albumin (BSA)
- 5 ml Tube with Cell Strainer Cap, VWR, cat. #21008-948

### Single Cell Sorting and Processing

- SingleShot™ Cell Lysis Kit, Bio-Rad cat. #1725080 (sufficient for approximately 1,000 single cells)
- Nuclease-Free Water, Integrated DNA Technologies, cat. #11-05-01-04
- IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0), Integrated DNA Technologies, cat. #11-05-01-05
- Low-Profile 0.2 ml 8-Tube Strips without Caps, Bio-Rad cat. #TLS0801
- Optical Flat 8-Cap Strips, Bio-Rad cat. #TSC0803

### cDNA Synthesis

- iScript™ Advanced cDNA Synthesis Kit for RT-qPCR, Bio-Rad cat. #1725038 (sufficient for approximately 300 single cells)

### Preamplification

- SsoAdvanced™ PreAmp Supermix, Bio-Rad cat. #1725160 (sufficient for approximately 150 single cells)
- PrimePCR™ PreAmp Assays, Bio-Rad, **bio-rad.com/primepcr** (each assay is sufficient for approximately 1,200 single cells)
- iQ™ 96-Well PCR Plates, Bio-Rad cat. #2239441
- Microseal® 'F' Foil, Bio-Rad cat. #MSF1001

### Multiplex qPCR

- PrimePCR Probe Assays for Gene Expression, Bio-Rad, **bio-rad.com/primepcr** (each assay is sufficient for approximately 1,536 qPCR reactions)
- iQ Multiplex Powermix, Bio-Rad cat. #1725849 (sufficient for approximately 768 qPCR reactions)
- Hard-Shell® Thin-Wall 384-Well Skirted PCR Plates, Bio-Rad cat. #HSP3805
- Microseal® 'B' Adhesive Seals, Bio-Rad cat. #MSB1001

## Equipment

- S3e™ Cell Sorter (488/561 nm), Bio-Rad cat. #1451006
- S3e PCR 8-Well Strip Adaptor, Bio-Rad cat. #12002449
- TC20™ Automated Cell Counter, Bio-Rad cat. #1450102
- T100™ Thermal Cycler, Bio-Rad cat. #1861096
- CFX384 Touch™ Real-Time PCR Detection System, Bio-Rad cat. #1855485

## Procedure

**Note:** Information provided is for the analysis of 96 single cells. Scale reagents accordingly if analyzing a different number of cells.

### 1 Preparation of Cells

**1.1.** Harvest cells into a 50 ml centrifuge tube and count cells using a TC20 Automated Cell Counter.

**1.2.** Centrifuge cells at 180 x g for 5 min and wash with PBS.

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**1.3.** Suspend cells in PBS with 0.1% BSA to a final concentration of 5–10 million cells/ml.

**1.4.** Filter cells into a 5 ml tube using a cell strainer to obtain a single cell suspension.

## 2 Single Cell Sorting and Processing

**2.1.** On ice, prepare the cell lysis buffer (Table 1).

**Table 1. Cell lysis buffer.**

| Component | Per Cell, µl | 96 Cells, µl* |
|---|---|---|
| IDTE | 3.2 | 352 |
| SingleShot Cell Lysis Buffer** | 0.64 | 70.4 |
| Proteinase K** | 0.08 | 8.8 |
| DNase** | 0.08 | 8.8 |
| **Total volume** | **4** | **440** |

* These volumes include overage to account for pipetting error.
** These components are included in the SingleShot Cell Lysis Kit.

**2.2.** Add 4 µl of cell lysis buffer into 12 x 8-tube strips.

**2.3.** Sort cells into tube strips containing cell lysis buffer. **Note:** It is recommended to use one 8-tube strip as a no cell control (NCC). Do not sort cells into those wells.

**2.4.** Place cap strips on tubes and vortex cell lysates for 10 sec then centrifuge briefly.

**2.5.** Transfer the cell lysates to a T100 Thermal Cycler and incubate at 25°C for 10 min and 75°C for 5 min followed by a 4°C hold. This step digests genomic DNA.

**2.6.** The cell lysates can be stored at –80°C for up to 1 week.

## 3 cDNA Synthesis

**3.1.** On ice, prepare the cDNA synthesis reaction mix (Table 2).

**Table 2. cDNA synthesis reaction mix.**

| Component | Per Cell, µl | 96 Cells, µl* |
|---|---|---|
| iScript Advanced Reaction Mix** | 1.1 | 121 |
| iScript Advanced Reverse Transcriptase** | 0.275 | 30.25 |
| Nuclease-free water** | 0.125 | 13.75 |
| **Total volume** | **1.5** | **165** |

* These volumes include overage to account for pipetting error.
** These components are included in the iScript Advanced cDNA Synthesis Kit for RT-qPCR.

**3.2.** Remove the tube strips containing cell lysates from the T100 Thermal Cycler. If cell lysates were stored at –80°C, remove lysates from the freezer and thaw on ice.

**3.3.** Centrifuge tube strips briefly to collect cell lysates at the bottom of the tubes.

**3.4.** Carefully remove the caps and place the tube strips on ice.

**3.5.** Add 1.5 µl of cDNA synthesis buffer to each tube taking care to prevent cross-contamination.

**3.6.** Seal strip tubes with cap strip and vortex to mix reagents. Centrifuge briefly to collect all reaction components at the bottom of the tubes.

**3.7.** Transfer the strip tubes to a T100 Thermal Cycler and incubate at 46°C for 20 min and 95°C for 1 min followed by a 4°C hold.

## 4 Preamplification

**4.1.** On ice, prepare the preamplification assay pool (Table 3).

**Table 3. Preamplification assay pool.**

| Component | Per Pool, µl* |
|---|---|
| PrimePCR PreAmp Probe Assays, up to 100 per pool | 5 µl each |
| IDTE | Varies; to 500 µl total volume |
| **Total volume** | **500** |

* Each preamplification assay pool allows for the analysis of up to 288 cells. The volume of the preamplification assay pool can be scaled as desired.

**4.2.** On ice, prepare the preamplification master mix (Table 4) using the preamplification assay pool made in step 4.1.

**Table 4. Preamplification master mix.**

| Component | Per Cell, µl | 96 Cells, µl* |
|---|---|---|
| SsoAdvanced PreAmp Supermix | 7.5 | 825 |
| Preamplification assay pool | 1.5 | 165 |
| Nuclease-free water | 0.5 | 55 |
| **Total volume** | **9.5** | **1,045** |

* These volumes include overage to account for pipetting error.

**4.3.** Remove the tube strips from the T100 Thermal Cycler from step 3.7.

**4.4.** Centrifuge tubes briefly to collect the cDNA samples at the bottom of the tubes.

**4.5.** Carefully remove the caps and place the strip tubes on ice.

**4.6.** Add 9.5 µl of preamplification master mix to each tube taking care to prevent cross-contamination.

4.7. Seal the strip tubes and vortex to mix reagents. Centrifuge briefly to collect all reaction components at the bottom of the tubes.

4.8. Transfer the strip tubes to a T100 Thermal Cycler and incubate at 95°C for 3 min followed by 14 cycles of 95°C for 15 sec and 58°C for 4 min then a 4°C hold.

4.9. Remove the strip tubes containing the preamplified cDNA from the T100 Thermal Cycler.

4.10. Centrifuge tubes briefly to collect the samples at the bottom of the tubes.

4.11. Carefully remove the caps and place the strip tubes on ice.

4.12. Transfer the preamplified cDNA to an iQ 96-Well PCR Plate and add 185 µl of IDTE to each preamplified cDNA sample. This sample dilution step is necessary for successful qPCR. Seal with Microseal 'F' Foil. **Note:** This dilution will provide enough preamplified cDNA to perform up to 75 qPCR reactions. If needed, the preamplified cDNA sample may be diluted up to 600 µl.

4.13. Vortex samples to mix and centrifuge briefly to collect all reagents at the bottom of the wells.

4.14. Preamplified cDNA samples can be stored at 4°C for up to 2 weeks or at –20°C for 3 months.

## 5 Multiplex qPCR

5.1. On ice, prepare the multiplex assay pool (Table 5).

**Table 5. Multiplex assay pool.**

| Component | Per Reaction, µl | 96 Reactions, µl* |
|---|---|---|
| PrimePCR Assay–FAM | 0.25 | 27.5 |
| PrimePCR Assay–HEX | 0.25 | 27.5 |
| PrimePCR Assay–Tex 615 | 0.25 | 27.5 |
| PrimePCR Assay–Cy5 | 0.25 | 27.5 |
| **Total volume** | **1** | **110** |

* These volumes include overage to account for pipetting error.

5.2. On ice, prepare the multiplex qPCR master mixes (Table 6).

**Table 6. Multiplex qPCR master mix.**

| Component | Per Reaction, µl | 96 Reactions, µl* |
|---|---|---|
| iQ Multiplex Powermix | 5 | 550 |
| Multiplex assay pool | 1 | 110 |
| Nuclease-free water | 1.5 | 165 |
| **Total volume** | **7.5** | **825** |

* These volumes include overage to account for pipetting error.

5.3. Thaw the preamplified cDNA samples from step 4.14, if necessary.

5.4. Centrifuge the preamplified cDNA samples briefly to collect samples at the bottom of the plate.

5.5. Carefully remove the foil seal from the plate and place the plate on ice.

5.6. Add 7.5 µl of each multiplex qPCR master mix to appropriate wells of a Hard-Shell® Thin-Wall 384-Well Skirted PCR Plate.

5.7. Add 2.5 µl of preamplified cDNA to the appropriate wells of the PCR plate.

5.8. Seal the plate with a Microseal 'B' Adhesive Seal and vortex to mix reagents. Centrifuge briefly to collect all reaction components at the bottom of the plate.

5.9. Analyze the plate using a CFX384 Touch Real-Time PCR Detection System and applying this protocol: 95°C for 2 min followed by 40 cycles of 95°C for 10 sec and 60°C for 45 sec.

# Tips

- The cell sorting protocol may need to be optimized to successfully deliver a single cell to most wells. Multiplex qPCR using a set of reference genes can be used to confirm the presence of a cell during this optimization step
- Single copy RNA transcripts should have a quantification cycle (Cq) value of approximately 30 using the suggested level of preamplification and sample dilution. Cq values greater than 31 can generally be considered as background and can be confirmed using the no cell control samples
- To minimize cross-contamination, it may be advisable to use new cap strips when handling multiple strips of tubes

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