



QX100™ Droplet Digital™ PCR System XXX

Version 2.0

Released Date: 9/14/2012

Pilot Protocol for Single-cell GeX analysis via ddPCR

Background and Disclaimer: This is a preliminary protocol that has been found to be compatible with iScript Advanced RT (or SuperScript III) and ddPCR (QX100). Single cells are sorted into 10ul of lysis buffer and cDNA is made in a final volume of 15.5ul (or 16.5ul with IVT spike). The protocol enables up to ~50% of a cell's cDNA to be assayed in a single ddPCR well, *without any pre-amplification*. The ddPCR counts are proportional to the volume of cDNA ($\leq 8\text{ul}$) added to the 25ul ddPCR reaction, suggesting that the lysis buffer has no inhibitory effect on ddPCR. Singleplex or duplex assays show ~85-100% of their activity (counts) as compared to a mock lysis with control RNA, indicating little or no inhibition of the RT reaction by the lysis buffer.

Preliminary studies on flow-sorted Jurkat cells using the BD FACSAria II show that the average transcript levels from single cells ($n=24$) is proportional to that from groups of 10 or 100 cells obtained by the same flow-sorting procedure. The average GAPDH counts per cell is comparable to levels seen in purified RNA from various tissues suggesting that the cell is effectively lysed and RNAs are made accessible to the reverse transcriptases mentioned above.

Overview:

- Cells are resuspended in 1xPBS (phosphate-buffered saline) containing 2% FBS (fetal bovine serum) before sorting
- Cell sorting receiving buffers are:
 - 9ul Ambion's Single Cell-to-CT lysis buffer and 1ul Single cell DNaseI added right before cell sorting, OR
 - 9.9ul Ambion's TaqMan Gene Expression Cells-to-CT lysis buffer and 0.1ul DNaseI from the same kit
- Lysis is stopped with 1ul stop solution from the respective kit
- 4ul of RT reaction mix is added to above cell lysate to convert RNA to DNA (total reaction volume $\sim 16.5\text{ul}$)
- ddPCR is performed with the above cDNA ($\leq 8\text{ul}$) to measure transcript copies

Protocol:

Cell suspension:

- Jurkat cell are grown to 10^5 - 10^6 cell/ml and counted with the TC10 Automated cell counter (Bio-Rad).



- Spin down cells at 1000rcf for 5min at room temperature. Aspirate medium.
- Resuspend cells in 1x PBS and 2% FBS.
- Sort cells into PCR wells containing cell lysis buffers using the BD FACSAria II.

Cell lysis buffer mix :

Prepare the following cell lysis buffer mix just before cell sorting (within 10 min):

- a. 9ul Single cell lysis solution and 1ul Single Cell DNaseI (Ambion Single Cell Lysis kit, Cat# 4458235).
- b. 9.9ul lysis solution and 0.1ul DNaseI (Ambion TaqMan Gene Expression Cells-to-CT kit, Cat# AM1728).

Cell lysis

Sort cells into a 96-well PCR plate containing 10ul cell lysis buffer in each well as a receiving buffer and incubate at room temperature for 5-10min. Add 1ul stop solution (and optionally, 1ul IVT RNA spike-in for AM1728 only). Pipet up and down 5 times to make sure the stop solution/spike-in mix is fully delivered into the cell lysate. Place the PCR plate onto dry ice. Store the plate at -20C until ready to perform RT reaction.

Reverse transcription mix and reaction (iScript Advanced)

To the above ~12ul cell lysate (11.5ul w/o IVT spike or 12.5ul with spike), add 4ul RT reaction mix (3.2ul 5x iScript Advance Reaction Mix and 0.8ul iScript Advanced Reverse Transcriptase, Bio-Rad, Cat# 170-8843). Mix well by pipetting 10ul up and down 10 times, then centrifuge the plate before putting it into the thermocycler.

Thermocycle at: 42°C x 30min, 85°C x 5min, 2°C hold

ddPCR

Prepare each ddPCR reaction mix as follows:

- 12.5ul 2X ddPCR™ Super Mix for Probes
- 1.25ul 20x assay 1 (primers + probe-FAM)
- 1.25ul 20x assay 2 (primers + probe-VIC or probe-HEX)
- Up to 5ul cDNA (from the RT reaction above)
- Water to 25ul final volume.

Generate droplets and thermocycle in Bio-Rad C1000 Touch thermocycler as follows:

95C x 10min; 40cycles of [94C x 30s and 60C x 1min, (ramp rate set to 2C/second)];
98C x 10min.

Place ddPCR plate in the QX100 Droplet Reader for droplet counting.