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Quantifying Gene Expression Directly from FACS Using Hydrolysis (TaqMan) Probes on Flash-frozen Cells

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[Abstract] The TaqMan Gene Expression Cells-to-Ct™ Kit enables reverse transcription directly from cell lysates without the need for isolating RNA. The recommended input range for the lysis reaction is between 10-10^5 cells, though the upper limit may vary somewhat according to cell type. This protocol is partially adapted from the manufacturer's protocol (TaqMan Gene Expression Cells-to-Ct™ Kit User Guide) for gene expression for use with frozen cell pellets to allow for sample transfer among laboratories and involves experimentally confirmed dilution prior to lysis. Within this constraint, the recommended range for sample cell number for frozen cells is between 40-2 x 10^5 cells. This protocol is also designed for use with a 'homebrew' qPCR master mix as the volume of TaqMan Genotyping Master Mix included in the kit may be insufficient for analysis of large numbers of samples and/or assays (Smythe and Copren, 2008). Cell count is provided by fluorescence-activated cell sorting (FACS) and is a unique aspect of this protocol.

Keywords: Quantitative PCR (qPCR), Reverse transcription, Cell lysis, Flow cytometry, Cq values, Cell pellets, Cell sorting, FACS, Gene expression, TaqMan assays, Hydrolysis probes

[Background] This protocol is a modification and experimental validation of the TaqMan Gene Expression Cells-to-Ct™ Kit User Guide, Publication Number 4385117 Revision E (2012) (Thermo Fisher Scientific/Life Technologies) for flash-frozen, rather than fresh, cells to allow for transportation among core facility laboratories (Procedure B). Using fresh cells requires all steps of the process to be performed sequentially with no stopping point at one facility. Using flash-frozen cells allows the separation of the cell sorting and downstream steps at different locations while ensuring the viability of the cells. The cell sorting is unique to this protocol. It is important to maintain the viability of the cells to ensure the accurate measurement of cellular genomes and transcriptomes (Gawad *et al.*, 2016). Flash-freezing also provides a stopping point for sorting the cells and the downstream analysis. This protocol is also designed and validated for use with a 'homebrew' qPCR master mix allowing the analysis of gene expression from a larger number of samples and/or assays than originally allocated by the manufacture's kit.

We attempt to consistently use the nomenclature recommended by the MIQE guidelines for qPCR (Bustin *et al.*, 2009). Thermo Fisher Scientific uses Cts (cycles at threshold), and their kit is named as

such, but the recommended standardization is Cqs (quantification cycles). ‘TaqMan’ probes/assays are standardized as hydrolysis probes.

Materials and Reagents

1. Pipette tips
   a. 10 ml sterile pipette tip
   b. 25 ml sterile pipette tip
2. 1.5 or 1.7, 5, 10, 50 ml tubes (USA Scientific, VWR)
3. 0.2 ml strip tubes (USA Scientific)
4. 384 well optical plate (MicroAmp™ Optical 384, Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4343370)
5. Liquid nitrogen
6. TaqMan Gene Expression Cells-to-Ct™ Kit (Thermo Fisher Scientific, Invitrogen™, catalog number: 4399002):
   a. Lysis Solution (P/N 4383583)
   b. DNase I (P/N 4386321)
   c. Stop Solution (P/N 4386318)
   d. 20x Reverse Transcriptase (P/N 4386319)
   e. 2x Reverse Transcription Buffer (P/N 4383586)
7. Nuclease-free water (Sigma-Aldrich)
8. GAC 5x QPCR Buffer without Taq polymerase (see Recipes)
   a. GeneAmp™ 10x PCR Buffer II (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4379878)
   b. Glycerol (Sigma-Aldrich)
   c. Gelatin (Sigma-Aldrich)
   d. Deionized water (Sigma-Aldrich)
   e. ROX Passive Reference Dye (IDTDNA)
   f. Taqman Gene Expression Assay: Hs03003631_g1 (Eukaryotic 18S RNA) (Thermo Fisher Scientific)
   g. Universal cDNA (various vendors or homemade)

Equipment

1. Pipettes
   a. Small volume pipettor
   b. Large volume pipettor
2. Cell sorter: FACSAria II (BD, model: FACSAria II)
3. Centrifuge

4. Thermal cycler (Bio-Rad Laboratories, model: C1000 Touch™)
5. 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems™, model: 7900HT)
6. -80 °C freezer

Procedure

Please consult Figure 1 for the general workflow and order of the procedural steps of the protocol.

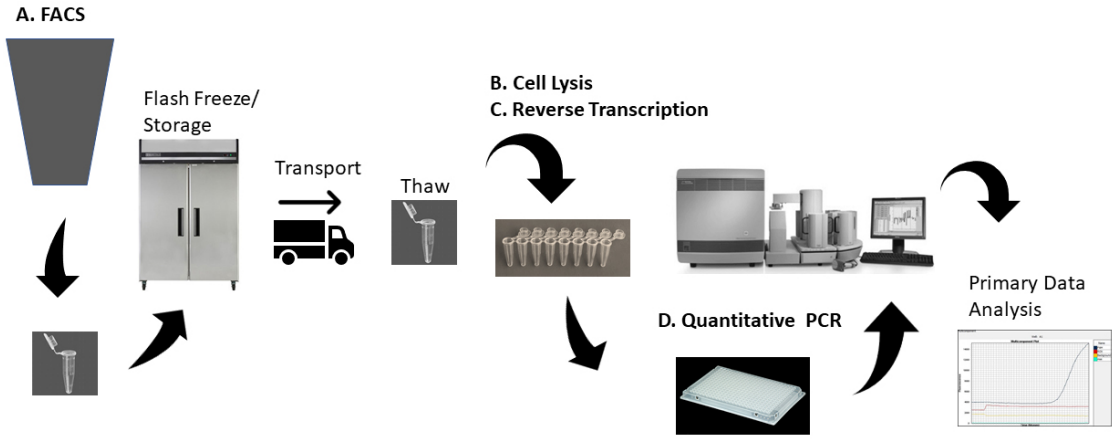

Figure 1. Protocol workflow

A. Flow cytometry: Fluorescence activated cell sorting (FACS)

1. Prepare and sort cells on the FACSAria II using a standard protocol (Kachel *et al.*, 1990; Shapiro, 2003). Isolate cell population of interest and determine the number of cells contained within this population (Ehrich and Sharova, 2001). Use the following guidelines as the sort settings, depending on cell type. The general minimum volumes per droplet with 4-way Purity Mode (or Purity 1 Drop envelope) should be approximate to:

   70 µm nozzle [90 kHz, 70 PSI]. Used for immune or smaller cells, *e.g.*, up to 15 µm.
   1 drop = 1.078 nl, therefore 1 x 10^5 = 107.8 µl

   85 µm nozzle [55 kHz, 45 PSI]. Used for similar size cells but gentler sort.
   1 drop = 1.956 nl, therefore 1 x 10^5 = 195.6 µl

   100 µm nozzle [30 kHz, 20 PSI]. Used for cell culture cells, *e.g.*, cell size up to 30 µm.
   1 drop = 3.01 nl, therefore 1 x 10^5 = 301 µl

   130 µm nozzle [14 kHz, 10 PSI]. Used for big cells, *e.g.*, cell size up to 50 or 60 µm
   1 drop = 6.141 nl, therefore 1 x 10^5 = 614.1 µl

2. Following the collection of the cells in the minimum volume possible, manually pipette off as much supernatant volume as possible without disturbing the cell pellet. The goal is to minimize the remaining volume for Step B4 to concentrate the cells. Store samples in 1.5 or 1.7 ml tubes. Flash freeze in liquid nitrogen and then store at -80 °C prior and during transportation to Procedure B.
   Note: This protocol was tested and created to ensure that cell sorting could be performed at one laboratory (in our case, the PFCC) with the cell lysis and downstream analysis performed at a second laboratory (in our case, the GAC) post short term storage. We stored cell pellets successfully at -80 °C for two weeks before proceeding to Procedure B. Flash freezing is very important to maintain the viability of the cells to achieve accurate downstream transcriptomic analysis (Gawad et al., 2016).

B. Cell lysis
   1. Dilute DNase 1:100 in lysis solution at room temperature (~20 °C).
   2. Remove cell pellet samples (in 1.5 or 1.7 ml tubes) from the -80 °C freezer and transfer to dry ice.
   3. Add 50 µl DNase + lysis solution to new, 0.2 ml strip tubes for incubation. Set aside at room temperature.
   4. Thaw and resuspend cell pellets. One sample at a time, add 10 µl DNase + lysis solution to each FACs sorted cell pellet (in 1.5 or 1.7 ml tubes). Pipette up and down thoroughly, avoiding bubbles.
   5. Calculate and record initial volume of cell pellet: Reverse pipette to measure the total volume of thawed sample, subtracting 10 µl to derive initial sample volume (for use in calculating the total number of cell input).
   6. Add a 5 µl aliquot of the resuspended cell pellets to the 0.2 ml strip tubes. Thoroughly mix by pipetting, avoiding bubbles.
   7. Incubate lysates 5-8 min at room temperature.
      Note: Frozen cell pellets from FACs are often delivered to the GAC at volumes higher than 5 µl because of variability in manually removing supernatant via pipette in Step A2, which is why we added Step B4. The kit is optimized for no more than a 5 µl input volume (regardless of total cell count) into 50 µl DNase + lysis solution, which we verified in our experimental data. Excess liquid volume in the initial cell pellet sample will result in a dilution of the DNase + lysis solution, altering buffering conditions and diminishing the activity of the DNase enzyme.
   8. Incubate lysates 5-8 min at room temperature.
   9. Add 5 µl stop solution (do not vortex stop solution). Thoroughly mix by pipetting, avoiding bubbles.
   10. Incubate for 2 min at room temperature (after 20 min, transfer to ice).
      Note: Lysates can be stored on ice ≤ 2 h, at -20 °C or -80 °C for ≤ 5 months (TaqMan Gene Expression Cells-to-Ct™ Kit User Guide).

11. For the following reverse transcription (RT) step, calculate the total volume of cDNA that will be required for each lysate sample given the final number of gene expression assays using hydrolysis probes (Bustin *et al.*, 2009) to be run based on a 2 µl input of cDNA into a final volume of 20 µl qPCR reaction. Each 50 µl RT reaction creates 50 µl cDNA, enough for 25 reactions of qPCR (50 µl/2 µl = 25 rxns). 25 reactions of qPCR are enough for 8 gene expression assays to be run in triplicate (8 x 3 = 24 rxns) on each sample. The amount of lysate that goes into each 50 µl RT reaction can range from 10 µl-22.5 µl (TaqMan Gene Expression Cells-to-Ct™ Kit User Guide). If running more than 7 assays, the amount of lysate should be spread over multiple RT reactions. In Procedure C, the reverse transcription step is based on an 18 µl input of lysate and 3 RT reactions are performed on each sample. At this volume, the maximum number of assays that we are able to run in triplicate per sample during qPCR is 22 assays.

C. Reverse transcription

1. Assemble reverse transcription (RT) master mix (2x RT buffer, 20x RT enzyme, nuclease-free water. Include 20% overhang in the master mix as shown below).
2. For each sample, combine 32 µl RT master mix and 18 µl lysate in the PCR tube. If lysate samples require multiple RT reactions as described above, combine total RT master mix first, followed by lysate in a 0.2 ml PCR tube, then partition (For example, if making 2 RT reactions per lysate sample add 32 x 2 = 64 µl RT master mix to a 0.2 ml tube, then add 36 µl lysate). Vortex, spin, and aliquot 50 µl RT reaction mix into separate PCR tubes. Similarly, prepare a No-RT negative control sample with nuclease-free water substituted for 20x RT enzyme.

RT master mix component	**Per reaction (µl)**	
	RT test samples	**No-RT control**
2x RT buffer	25	25
20x RT enzyme mix	2.5	-
Nuclease-free water	4.5	7
Final volume	32	32

Example calculation for the master mix for 8 reverse transcription reactions with 20% overhang.

RT master mix component	**Per rxn (µl)**	**8 samples**	**Overhang 20%**	**Total working volume**
	RT test samples	**(A) x 8**	**(B) A x 0.2**	**A + B**
2x RT buffer	25	200	40	240
20x RT enzyme mix	2.5	20	4	24
Nuclease-free water	4.5	36	7.2	43.2
Final volume	32	256	51.2	307.2

3. Run samples on a thermal cycler with the following conditions: 37 °C (60 min), 95 °C (5 min), 4 °C (indefinite).

D. qPCR

Following the RT reactions, perform a standard qPCR protocol using a fixed volume of 2 µl cDNA input per reaction into a 20 µl final qPCR reaction volume. Each assay is run in triplicate qPCR reactions under best practices. Nomenclature for quantitative Real-Time PCR (qPCR) is standardized to meet MIQE guidelines (Bustin *et al.*, 2009). Thermo Fisher Scientific uses Cts, but the recommended standardization is Cqs. 'TaqMan' probes/assays are standardized as hydrolysis probes.

Data analysis

Figure 2 outlines the molecular steps of the protocol. We varied the volume input of the cell pellet into the lysis which includes DNase to remove gDNA and measured that change downstream via qPCR. The cell lysis + DNase reaction (lysate) was added to a reverse transcription reaction, converting mRNA to cDNA, which was then measured by qPCR using assays that can detect gDNA and mRNA. gDNA should be removed by the DNAse in the lysis reaction, leaving mRNA for measurement only. We used negative controls for the reverse transcription (No-RT) and the qPCR (NTC) to monitor for remaining gDNA in the final reaction. Unknown test samples could be positive or negative depending on the presence of the transcript.

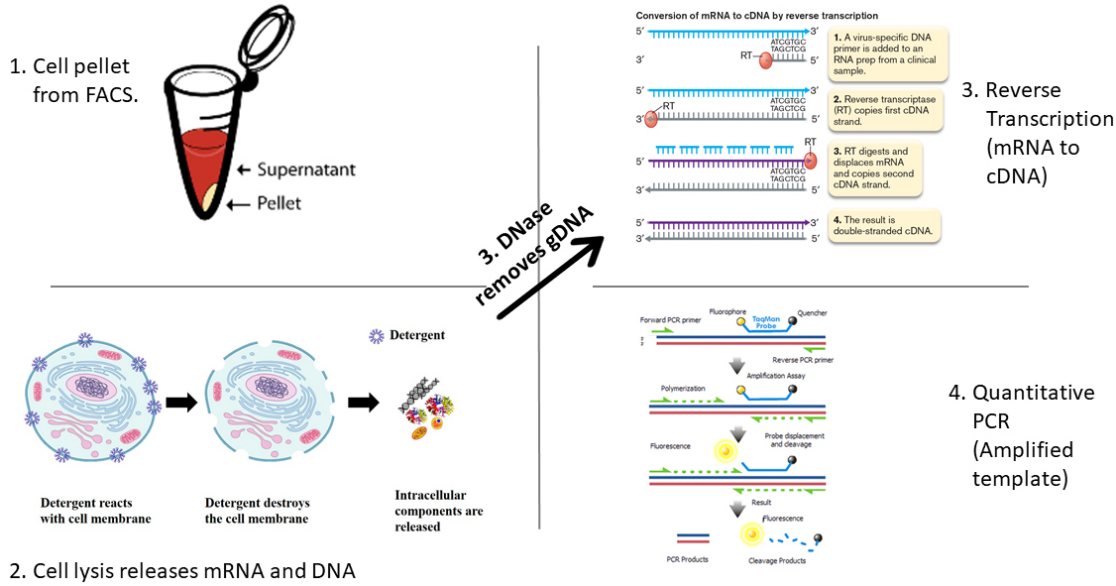

Figure 2. Molecular steps of the protocol. The DNase treatment is included in the same reaction as the lysis.

Six experiments were run using the Cells-to-Ct kit, three with cultured cells (pilot, C, E) and three with dissociated cells from mice organs (A, B, D) to determine the best input of cell pellet volume to maximize cell number. Raw data can be found in Table S1 in the Appendix. The expected and measured amplification (positive or negative) in qPCR of control and positive test samples are shown in Table 1.

Table 1. Expected and measured amplification of different input volumes of the cell pellet and cell lysis buffer as measured downstream by qPCR

Cell pellet volume (µl) to lysis	Lysis + DNase buffer volume	Positive test samples (mRNA) Expected/Measured	No-RT negative control (RT reaction) Expected/Measured	Water negative control (qPCR reaction) Expected/Measured
3-5 µl	45-47 µl	+/+	-/-	-/-
8-33 µl	17-42 µl	+/+	-/+	-/-

Amplification was observed in the No-RT negative controls for two assays capable of detecting genomic DNA [Thermo Fisher Scientific: Mm02619580_g1 (Actb), Mm00479862_g1 (Aif1)] in two experiments that had the largest variation of cell pellet input volume to the lysis + DNase reaction (Table 2, Figure 3, Experiments C and D). No amplification was observed in the No Template Control (NTC) in these experiments, so this false positive signal suggested genomic DNA contamination remaining in the initial lysate, despite DNase treatment prior to the RT step. In these experiments, the total initial cell pellet sample volume was included in the lysis reaction to maximize cellular input (range of 8-33 µl) for higher sensitivity of mRNA detection. However, the false positives indicated a narrower range of inputs, so a final experiment (E) was run to confirm cell pellet input volume. Following the final experiment, the protocol was amended to specify a diluted fraction of initial sample volume input to ensure proper buffering conditions for the DNase and lysis reaction. This set the specifications for the current protocol.

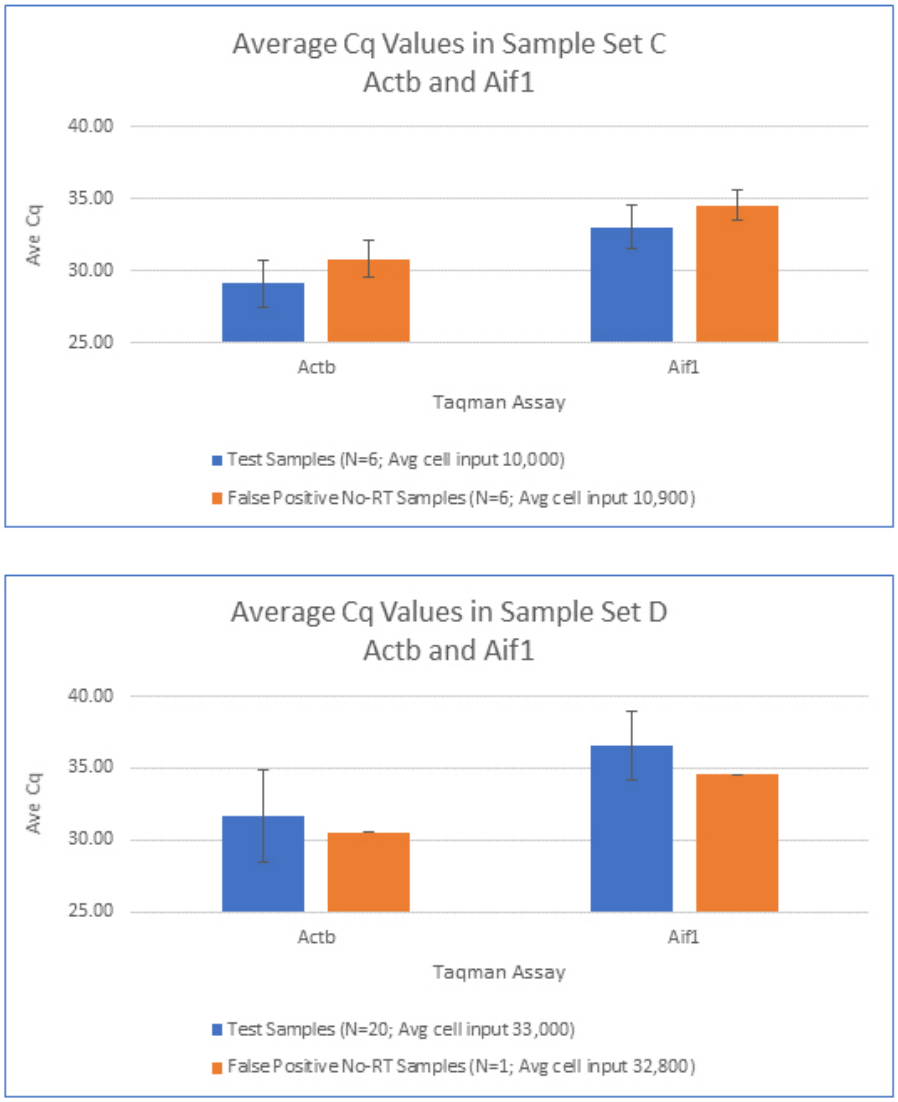

Figure 3. The average Cq values and standard deviations of test samples compared to false positive No-RT controls in experiments C and D. Cq values ≤ 37 indicate a positive result. The Cq values for the No-RT controls shown fall within the positive range exhibited by the test samples at comparable cell inputs and therefore compromise interpretation of test results for these assays. Error bars represent one standard deviation of Cq values in each sample set for each of the two assays.

Table 2. Summary of false positives in experimental data

Sample ID	Sample Type	Initial Sample Volume (µl)	Dnase + lysis volume (µl)	Cell Number	ACTB Mean Cq	AIF1 Mean Cq	ACTB Ave Cq	AIF1 Ave Cq	Template Conclusion for mRNA
No-RT (Pilot)	Negative	5	50	91,667	NA	NA	No Amp	No Amp	True Negative
No-RT (A)	Negative	5	50	91,667	NA	NA			True Negative
No-RT (B)	Negative	1	54	48,000	NA	NA			True Negative
Sample C5	Test Sample	5	50	10,000	NA	NA			True Negative
Sample C1	Test Sample	20	35	2,500	30.42	34.23	30.76	34.55	Inconclusive
Sample C2	Test Sample	20	35	5,000	31.22	34.84			Inconclusive
Sample C4	Test Sample	15	40	20,000	29.61	33.83			Inconclusive
Sample C6	Test Sample	23	32	7,143	33.21	36.49			Inconclusive
No-RT1 (C)	Negative	33	22	15,278	29.98	34.15			**False Positive**
No-RT2 (C)	Negative	33	22	15,278	30.34	33.75			**False Positive**
No-RT3 (C)	Negative	21	34	32,805	30.53	34.54			**False Positive**

Table 2 summarizes the qPCR data for No-RT negative controls along with unknown test samples from experiment C. It shows the correlation between the initial cell pellet volume and the likelihood of amplification in negative controls, indicating remaining gDNA. The higher input volumes of initial negative lysate controls (> 21 µl) show positive amplification similar to the test samples. Therefore, one cannot tell if unknown test samples with positive amplification are true positives for mRNA or might actually be negative like Sample C5. The kit is optimized for a maximum input of 10^5 fresh cells in 5 µl wash buffer, treated with 50 µl of a lysis solution into which DNase has been diluted at 1:100. In practice, frozen samples will generally contain more than 5 µl of starting volume based on the variability inherent in manually removing supernatant with a pipettor following cell sorting. Because the total lysate volume was included for some reactions, some samples were treated with as little as half the recommended volume of DNase + lysis solution. While the DNase was likely present in sufficient quantity (according to the manufacturer it is present in excess), its activity may have been reduced by dilution of the lysis buffer in the collection media present in the frozen pellet. Like many enzymes, DNase is sensitive to the composition of the reaction buffer, such as the presence of specific divalent cations or chelating agents like EDTA.

Therefore, our protocol for use of the Cells-to-Ct kit on flash-frozen cell pellets has been altered and experimentally validated to ensure a fixed 5 µl volume of sample input to a 50 µl DNase + lysis solution treatment. This is achieved with an initial input of 10 µl DNase + lysis solution used to thaw and mix the cell pellet, followed by extraction of a 5 µl aliquot to be treated with an additional 50 µl DNase + lysis solution.

Notes

As this protocol necessarily involves a dilution of the initial sample, it raises the theoretical lower limit of initial sample cell count from 10 to 20. In practice, when manually removing supernatant via pipette following FACS sorting and pelleting, it is difficult to leave less than ~10 µl in the sample tube without disturbing the cell pellet. Therefore, the effective lower limit of the initial sample cell number

is approximately 40 cells. By contrast, because there will be a minimum sample dilution by a factor of 2 (theoretically, a sample with zero volume will be thawed in 10 µl DNase/lysis solution, followed by extraction of 5 µl for input into final lysate), the maximum initial sample cell count that can be accommodated by this protocol without additional dilution steps is twice the upper limit of the kit's detection range, or 2 x 10^5 cells. We therefore recommend samples sorted via FACS contain a range of cells between 40-2 x 10^5 before proceeding to downstream gene expression analysis.

Recipes

1. GAC 5x qPCR buffer without Taq polymerase

Reagents	**Volume**
GeneAmp™ 10x PCR Buffer II (P/N 4379878) (Thermo Fisher Scientific)	25 ml
Glycerol	10 ml
Gelatin	1,250 µl
Deionized water	13 ml
Deionized water	720 µl
500 µM ROX Passive Reference Dye (IDTDNA)	30-33 µl
Total	**50 ml**

   a. Making GAC 5x qPCR buffer without Taq polymerase

      Pipet 25 ml of 10x PCR buffer into a 50 ml tube. Pipet 10 ml glycerol into the 50 ml tube paying attention to viscosity, then add gelatin, water, and ROX .Vortex to mix thoroughly

   b. Buffer test

      Test the new buffer in laboratory standard 20 µl qPCR reaction using an 18S gene expression control assay on a universal cDNA with a minimum of 6 replicates and a 5 ng input. Record the expected Cqs of the control 18S assay for the given input. Typically, a 5 ng input of cDNA results in ~18 Cqs but is dependent on the assay used. Record the RFUs (Raw Fluorescent Units on AB7900HT) of ROX on the multicomponent graph (Figure 2) and the average Cqs of the 18S control assay

      i. The RFUs should be between 3,000-4,000 (ideally 3,400-3,600) after cycle 6. An example is shown in Figure 4

      ii. Record the RFUs in the lot notebook. If too low (below 3,000), add 1 µl ROX and test again with new qPCR reactions. Don’t try to increase fluorescence of ROX more than 2 times as it will negatively impact the overall composition of the buffer

      iii. New tubes of lyophilized ROX from the vendor (IDTDNA) will always start brighter then decrease as they age. The starting input of ROX therefore may increase over time to 33 µl as the stock ages. Aliquot 50 ml stock into 5, 10 ml aliquots. Record lot number in the record and on the tube. Store at -20 °C

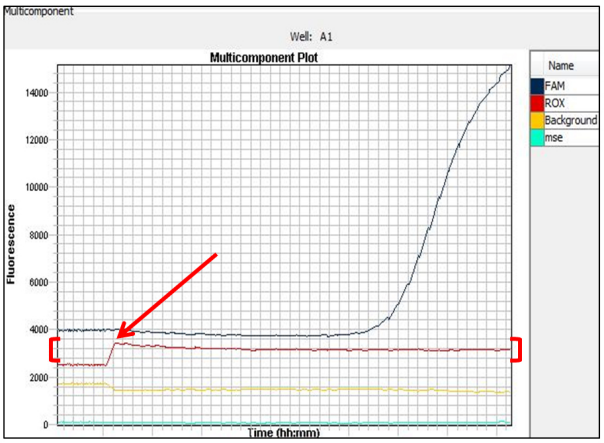

Figure 4. Multicomponent plot of the AB7900HT SDS 2.3 Software. Example of amplification curve post qPCR run for buffer test indicating the ideal fluorescence of ROX (~3,200-3,600 RFUs indicated by the red arrow and parentheses) for the GAC homebrew 5x qPCR buffer.

Acknowledgments

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