High Throughput Single Cell Gene Expression Profiling by Multiplex qPCR

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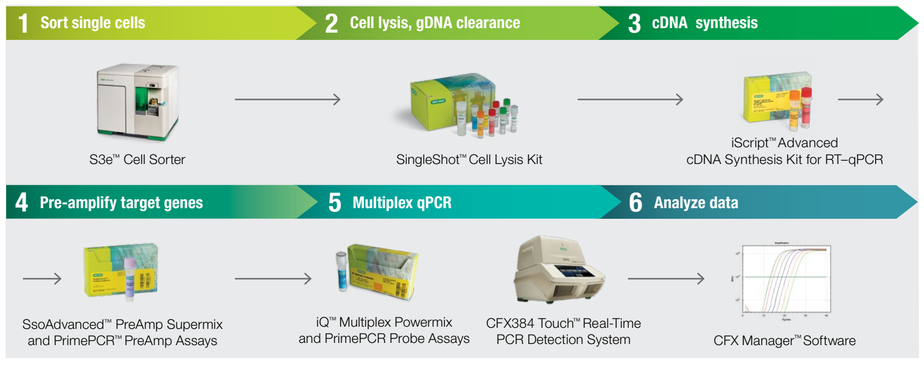
1 Abstract

Single cell gene expression analysis is a powerful technique that provides a unique and insightful perspective on biological pathways and processes. Here we present a robust workflow that enables fast and accurate analysis of up to 100 genes in isolated single cells. Our workflow is highly sensitive. By assessing RNA reference standards we find that a single RNA transcript is detected with about 80% efficiency. We used this workflow to study differentiation in cultured NTera2 cells (NT2), a human embryonic stem cell model system. We analyzed untreated NT2 cells, and NT2 cells treated with low and high doses of retinoic acid (RA) for 8 days to initiate differentiation to a neuronal lineage. The expression levels of 16 genes were quantified in 164 single cells by multiplex real-time qPCR with two technical replicates. The entire experiment, from cultured cells to results, can be completed in 2-3 days and requires four 384-well qPCR plates for gene expression quantification. We find that control cells and cells treated with a high dose of RA (10 µM) are relatively homogeneous in the expression levels of the targeted genes. However cells treated with a low dose of RA (0.25 µM) exhibit significant heterogeneity with respect to gene expression; about half of the cells are similar to the high-dose RA cells, the other cells exhibit a wide range of partial differentiation. Interestingly, we find that LEFTY2 expression is almost exclusive to the low dose RA cells and strongly correlates with partial differentiation. A time-course study analyzing cell populations reveals that LEFTY2 is only transiently expressed in differentiating NT2 cells with peak expression at 3 days of high dose RA treatment. These findings imply that, in NT2 cells, LEFTY2 is a potential biomarker of early differentiation. In summary, we present an accurate, sensitive and robust single cell analysis procedure that uses standard reagents and platforms. We envision that this workflow will enable researchers to investigate cell heterogeneity in biological pathways in a cost-effective way.

2 Single cell analysis workflow

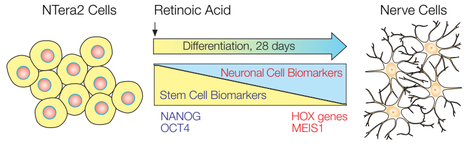
Bio-Rad's single cell analysis workflow uses reagents and instruments designed to work together.



3 Model systems utilized

Model system of stem cell differentiation

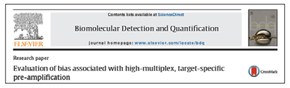
NTera2 cells (NT2) are a model system of human stem cell behavior. When treated with retinoic acid (RA), NT2 differentiate into neurons. During differentiation the expression of stem cell biomarkers decrease and the expression of neuronal biomarkers increase.



ERCC model system

We utilized ERCC RNA reference standards, an RNA measurement model system developed by the National Institute of Standards and Technology. The ERCC standards are mixtures of 92 synthetic RNAs that are spiked into RNA samples and processed and quantified along with the natural RNAs. The amount of each ERCC RNA is precisely defined; the performance of an RNA quantification workflow is determined by comparing the measured amount with the actual, defined amount of each ERCC control RNA.

Appendix 1

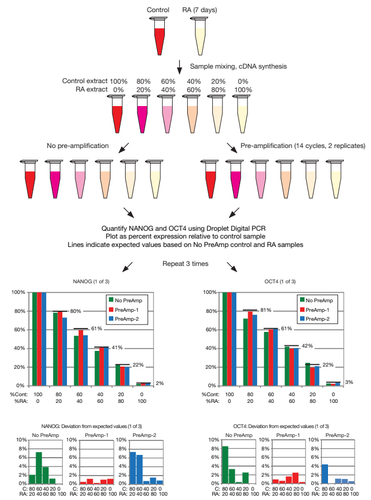


Bias associated with Bio-Rad's pre-amplification reagent, SsoAdvanced PreAmp, in a non single cell workflow.

4 Pre-amplification bias in the single cell analysis workflow

The single cell analysis workflow utilizes a pre-amplification step that may bias gene expression results. To assess pre-amplification bias we analyzed mixed samples of RNA isolated from control and RA-treated NT2 cells, both with and without pre-amplification for 96 targets. Analysis was performed using Droplet Digital PCR™ (ddPCR™) due to its outstanding precision and accuracy. NANOG and OCT4 were chosen as targets because they are highly expressed in control NT2 cells and show only trace expression after RA treatment.

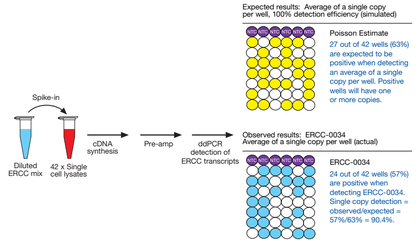
In our single cell workflow NANOG pre-amplification introduces about 2.2% bias, for OCT4 no bias is introduced. This suggests that pre-amplification introduces minimal bias in single cell gene expression results.



Average deviation from expected value (bias)			
NANOG		OCT4	
No PreAmp	PreAmp	No PreAmp	PreAmp
2.4%	4.6%	1.7%	1.7%

5 RNA detection sensitivity in the single cell analysis workflow

To assess RNA detection sensitivity we utilized ERCC RNA standards - a mixture of 92 synthetic RNAs with defined copy numbers. We precisely diluted the RNA standards and added them to 42 different single cell lysates. We then processed the samples and assessed specific ERCC transcripts that were added at 1 to 16 copies per reaction by ddPCR. Sensitivity of RNA detection was calculated using the Poisson distribution and the observed to expected target detection ratio.



Average copies per well	ERCC#	Observed detection percent	Expected detection percent	Detection sensitivity (Obs/Exp)	Average detection sensitivity
1	ERCC-00034	57.1%	63.2%	90.4%	82.9%
	ERCC-00154	33.3%		52.7%	
	ERCC-00160	69.0%		109.2%	
	ERCC-00157	50.0%		79.1%	
2	ERCC-00059	52.4%	86.5%	60.6%	75.7%
	ERCC-00099	47.6%		55.1%	
	ERCC-00170	88.1%		101.9%	
	ERCC-00163	73.8%		85.4%	
4	ERCC-00019	90.5%	98.2%	92.2%	80.0%
	ERCC-00053	92.9%		94.6%	
	ERCC-00078	88.1%		89.7%	
	ERCC-00144	42.9%		43.7%	
8	ERCC-00025	100.0%	100.0%	100.0%	98.8%
	ERCC-00071	95.2%		95.3%	
	ERCC-00162	100.0%		100.0%	
	ERCC-00062	100.0%		100.0%	
16	ERCC-00095	100.0%	100.0%	100.0%	100.0%
	ERCC-00112	100.0%		100.0%	
	ERCC-00131	100.0%		100.0%	
	ERCC-00035	100.0%		100.0%	

Our single cell workflow detects a single copy RNA transcript with an average efficiency of about 80%. We do observe significant variation when different ERCC transcripts with the same copy number are analyzed. Our detection sensitivity results should thus be viewed as "ballpark" estimates.

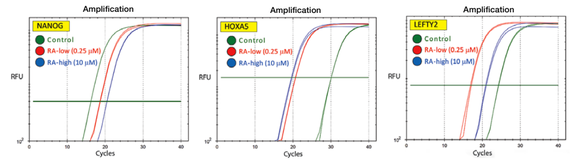
6 Studying cell differentiation using the single cell analysis workflow

Control NT2 cells or NT2 cells treated for eight days with low and high doses of RA (0.25 µM and 10 µM respectively) were analyzed using the single cell workflow. The expression of 16 genes involved in pluripotency, neuronal cell differentiation, or housekeeping were quantified using multiplex qPCR.

7 Sixteen genes analyzed in four 4-plex reactions using PrimePCR Assays

Gene	Type	Probe	Gene	Type	Probe	Gene	Type	Probe	Gene	Type	Probe
TBP	Ref	FAM	TDGF1	Pluripot	FAM	LEFTY2	Neuronal	FAM	HOXB3	Neuronal	FAM
TFRC	Ref	HEX	OTX2	Pluripot	HEX	MEIS1	Neuronal	HEX	HOXB2	Neuronal	HEX
B2M	Ref	Tex615	NANOG	Pluripot	Tex615	HOXD4	Neuronal	Tex615	HOXA5	Neuronal	Tex615
PGK1	Ref	Cy5	OCT4	Pluripot	Cy5	PAX6	Neuronal	Cy5	HOXA4	Neuronal	Cy5

8 Gene expression changes in differentiating cell populations



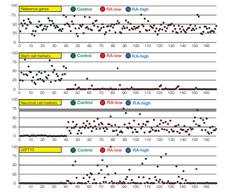
Population	NANOG	HOXA5	LEFTY2
Control	100%	0%	1%
RA-Low	18%	50%	100%
RA-High	5%	100%	6%

Methods: Populations (about 1 million cells) of control NT2 cells and NT2 cells treated with low and high doses of RA for 8 days were analyzed by multiplex qPCR.

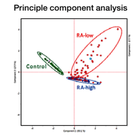
Findings: We observe different patterns of gene expression changes in cell populations during RA-induced NT2 differentiation.

Questions: What happens at the single cell level?
Is there heterogeneity within the RA-low population?

9 Gene expression in 168 differentiating single cells

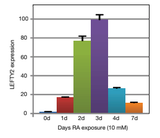


Each circle represents a single cell, there are 168 cells in three treatment groups plotted on the X-axis. Gene expression is plotted on the Y-axis and is an average of several genes within a specific group (reference, stem cell markers, and neuronal cell markers). For each gene, the cell with highest expression was set at 100 and the expression in other cells was normalized accordingly. The expression of LEFTY2 in single cells is plotted on a separate chart. We observe that high LEFTY2 expression is found in a subset of cells within the RA-low group.



Findings:
1. Control cells are different than the RA treated cells.
2. Some RA-low cells are similar to the RA-high cells.
3. Other RA-low cells constitute a unique group.

10 LEFTY2 marks early differentiation



Findings: In a cell population treated with a high dose of RA, LEFTY2 expression peaks at 3 days and declines thereafter. LEFTY2 is thus a potential marker of early differentiation. (Note: Single cell analysis occurred at day 8 of RA exposure).

11 Single cell gene quantification is accurate

Single Cell	NANOG	HOXA5	LEFTY2
Control	100%	1%	1%
RA-Low	7%	61%	100%
RA-High	2%	100%	16%

Methods: Target gene expression was averaged over all single cells in a treatment group. The results were normalized and compared with similar data from the population study (table on left).

Findings: Because population data can be modeled by averaging its component single cells, it implies that (a) the single cell data are accurate and (b) the number of single cells analyzed is sufficient to reflect the population.

12 Summary and conclusions

1. We have developed a protocol for single cell gene expression analysis using Bio-Rad products and instruments designed to work together.
2. There is minimal bias introduced by target gene pre-amplification in the single cell workflow.
3. The single cell workflow can detect a single RNA transcript with approximately 80% efficiency.
4. Because gene expression quantification is by multiplex qPCR the protocol is fast and cost efficient. 168 single cells can be analyzed for 16 targets in 2-3 days using 384-well qPCR plates.
5. By comparing population data with single cell data we conclude that (a) the single cell data is accurate and (b) analysis of hundreds of single cells is sufficient to reflect the population.
6. In NT2 cells, LEFTY2 is a potential marker of early stage stem cell differentiation.

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