Quantification of 100 RNA Targets from Limited Samples and Single Cells

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Introduction

Gene expression profiling is often limited by small sample size and low target gene expression. To address these issues we developed SsoAdvanced™ PreAmp Supermix (172-5160, Bio-Rad Laboratories), a PCR-based reagent that increases the cDNA levels of up to 100 target genes at least 1000-fold. To validate our reagent, we analyzed a panel of genes that are differentially regulated during stem cell differentiation. We find that relative gene expression results generated using this product are statistically equivalent to results obtained using standard qPCR; however, 10,000-fold less starting material can be used. Importantly, SsoAdvanced PreAmp Supermix maintains patterns of gene expression changes across samples; thus the same biological insights would be derived from a pre-amplification (PreAmp) experiment and a standard gene expression profiling experiment. In addition, we demonstrate the application of this product for analyzing gene expression in single cells.

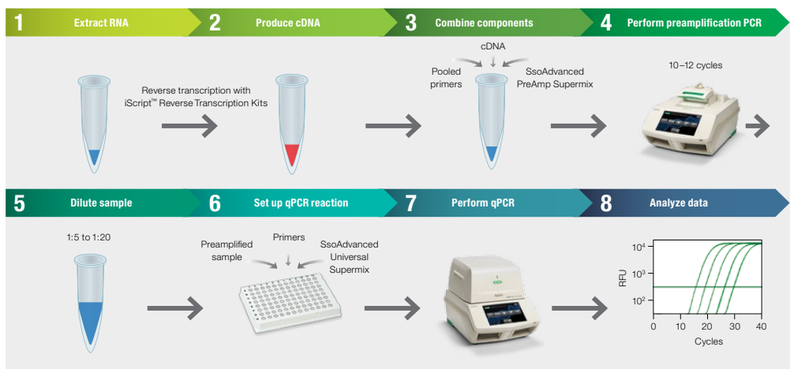
Background

Genes Analyzed

We analyzed a panel of 100 genes that are involved in stem cell differentiation. PrimePCR™ PreAmp and qPCR assays were used in the PreAmp reaction and for qPCR analysis, respectively.

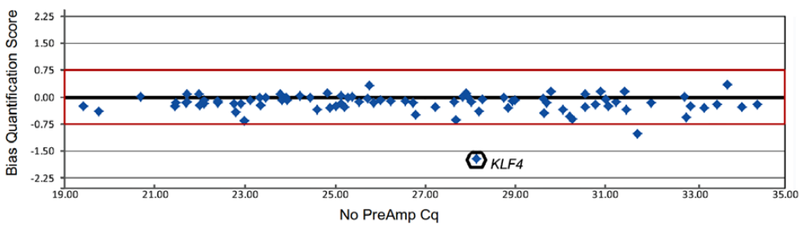
gDNA	*POU5F1*	*ZFP42*	*CD44*	*DPPA5*	*GATA2*	*KLF4*	*NKX2-5*	*SMAD1*	*TAL1*
HPRT1	*POU3F2*	*DNMT3B*	*CDC42*	*EN2*	*GATA6*	*LIN28A*	*NROB1*	*SMAD2*	*TAT*
B2M	*NEUROD1*	*NR5A2*	*CDK1*	*ENG*	*GDF3*	*MEIS1*	*NT5E*	*SMAD3*	*TCF3*
GA PDH	*LEFTY2*	*NR6A1*	*CHD1*	*ESRRB*	*GFAP*	*MESP1*	*OLIG2*	*SOX15*	*TCL1A*
TFRC	*UTF1*	*SOX2*	*CHD7*	*ETV2*	*GSC*	*MIXL1*	*OTX2*	*SOX17*	*TEK*
TBP	*TDGF1*	*ACTA2*	*CNOT3*	*FGF2*	*HAND1*	*MYBL2*	*PAF1*	*SOX3*	*TERT*
NANOG	*NEUROG2*	*AICDA*	*DES*	*FGF5*	*HNF4A*	*MYC*	*RIF1*	*SOX7*	*THAP11*
PAX6	*ASCL1*	*ALB*	*DPPA2*	*FLI*	*HSPA9*	*MYOD1*	*ALA S1*	*STAT3*	*THY1*
TBX3	*ASCL2*	*ALP L*	*DPPA3*	*FOXG1*	*ISL1*	*NCAM1*	*SALL4*	*TBP*	*TRIM28*
PRDM14	*KLF2*	*CCNA2*	*DPPA4*	*GATA1*	*KAT5*	*NE S*	*SCN1A*	*TAGLN*	*ZFX*

Pre-amplification workflow



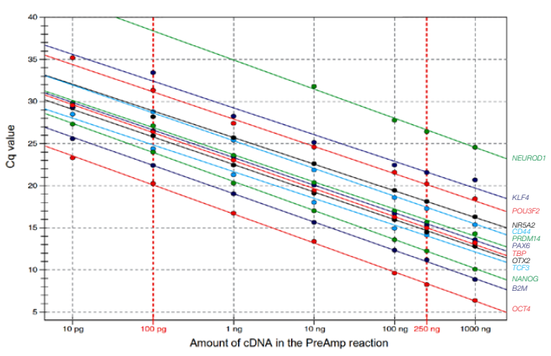
SsoAdvanced PreAmp Supermix generates minimal bias

To assess bias introduced by PreAmp, we compared target gene levels in a cDNA sample before and after PreAmp. For each target, we calculated the bias introduced by PreAmp and plotted it against the no PreAmp Cq values for the target genes. For 98% of genes with a no PreAmp Cq<35, PreAmp did not introduce bias by more than 0.75 Cq.



SsoAdvanced PreAmp Supermix has a wide dynamic range

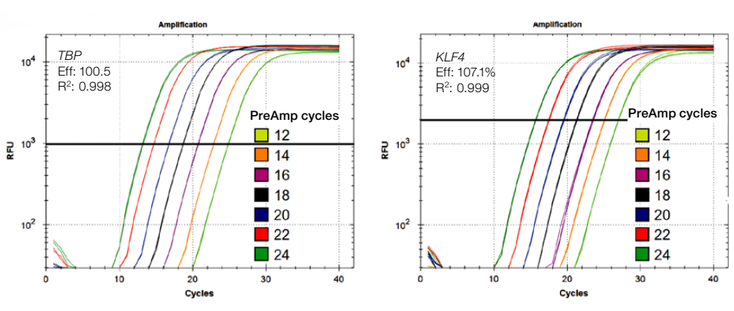
PreAmp was performed on 10 pg to 1 µg cDNA. Selected target genes with varied expression levels were analyzed by qPCR. The results (efficiency, R^2) show that a wide linear dynamic range of cDNA input can be achieved regardless of target expression level.



Gene	Eff	R^2
NEUROD1	88.6	0.986
KLF4	108.5	0.958
POU3F2	100.8	0.994
NR5A2	114.6	0.998
CD44	98.3	1.000
PRDM14	105.0	1.000
PAX6	105.0	0.999
TBP	100.6	0.999
OTX2	100.4	1.000
TCF3	110.1	0.991
NANOG	95.2	1.000
B2M	99.7	1.000
OCT4	95.2	0.999

SsoAdvanced PreAmp Supermix is efficient for up to 24 PreAmp cycles

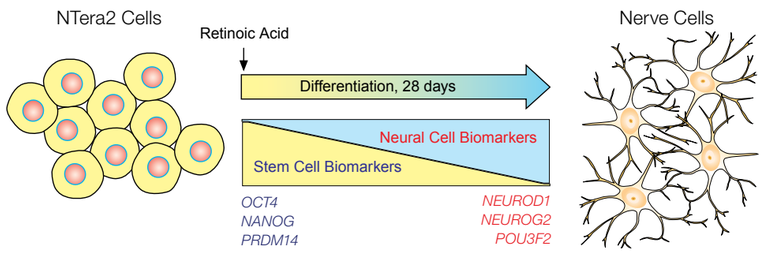
PreAmp was performed on 10 ng cDNA for 12-24 cycles. qPCR traces for *TBP* and *KLF4* are shown. Efficiency and R^2 values show that this product maintains exponential amplification for up to 24 PreAmp cycles.



Gene expression profiling during differentiation

Analysis of cell populations undergoing differentiation

NTera2 cells (NT2) are a well-established model system used to study human stem cell behavior (Andrews 1984). When treated with retinoic acid (RA), NT2 differentiate into neurons (Pleasure and Lee 1993). As NT2 differentiate, the level of stem cell specific biomarkers decrease and the level of neuron-specific biomarkers increase (Deb-Rinker et al. 2005). We used the NT2 model system to determine if PreAmp can accurately quantify gene expression changes in limited samples.

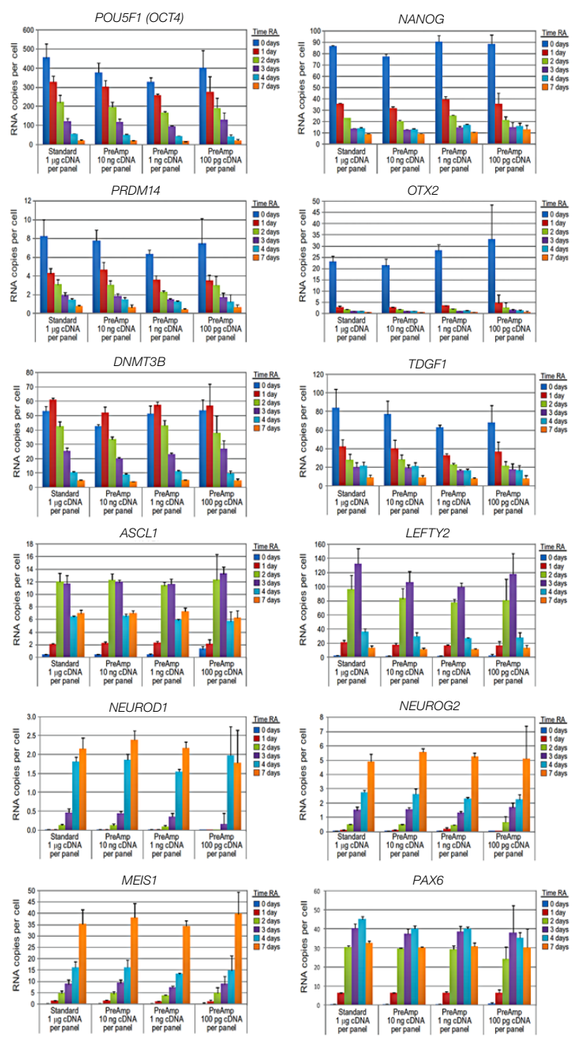


Assessment of PreAmp bias

NT2 cells were treated with RA for 0 to 7 days; RNA was isolated and converted to cDNA. Varied amounts of cDNA (100 pg, 1 ng and 10 ng) were pre-amplified with SsoAdvanced PreAmp Supermix and all 100 target genes were subsequently analyzed by qPCR. In parallel, as a no-PreAmp control, 1 µg cDNA was used to quantify the 100 gene targets directly by qPCR (10 ng/target). The experiment was performed three times independently.

The level of expression of each target gene was determined and expressed as "RNA copies per cell" after normalizing to *TBP* with the assumption that there are 10 *TBP* copies per cell. Error bars represent standard deviation of the three independent experiments. Data for target genes that show significant differentiation induced changes are shown.

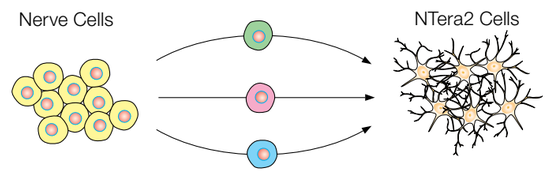
We find that, relative to standard gene expression analysis, PreAmp does not cause statistically significant bias in the gene expression profiles of all target genes analyzed, even when 10,000-fold less cDNA is used These findings demonstrate that SsoAdvanced PreAmp Supermix provides accurate and reproducible quantification of both stem cell- and neural cell-specific biomarkers and does not bias gene expression profiling results.



Single cell analysis

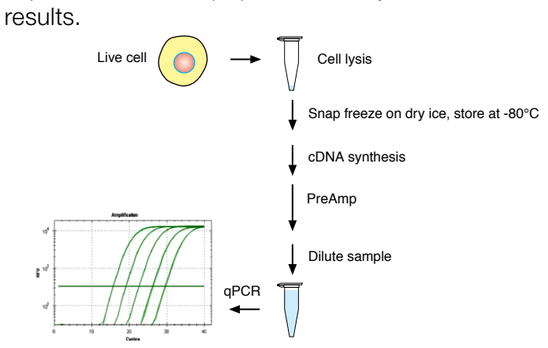
Analysis of single cells undergoing differentiation

Most studies that address how cells change are based on population analyses. It is, however, also important to understand how individual cells behave in a changing population. For example, as a NT2 population moves from a pluripotent to a neuronal state, do individual cells change in unison, or at varying rates?



Single cell analysis

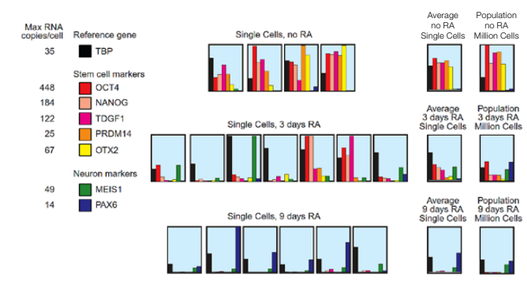
We developed a protocol that uses SsoAdvanced PreAmp Supermix to quantify gene expression in individual NT2 cells at various time points following RA treatment. We find that cells at the 0 day time point express primarily stem cell biomarkers and cells at the 9 day time point express mainly neural cell biomarkers. Interestingly, at the 3 day time point we observe that the gene expression profiles are heterogeneous – some cells are similar to the 0 day cells while others are similar to the 9 day RA treated cells. These findings imply that NT2 cells do not undergo synchronized differentiation; instead the differentiation process is heterogeneous and cell dependent. In addition, the average gene expresssion profiles from the individual cells correlate with the gene expresssion profiles from the population analysis; this validates our single cell results.



Gene expression in differentiating single cells

Sample		RNA copies per cell							
RA		*TBP*	*OCT4*	*NANOG*	*TDGF1*	*PRDM14*	*OTX2*	*MEIS 1*	*PAX6*
No cells		0	0	0	0	0	0	0	0
None	Cell 1	25	131	66	69	9	9	2	0
None	Cell 2	10	443	115	84	11	8	0	0
None	Cell 3	23	287	104	29	25	52	0	1
None	Cell 4	14	368	147	105	19	67	0	0
No cells		0	0	0	0	0	0	0	0
3d	Cell 5	9	161	19	4	0	2	18	0
3d	Cell 6	14	8	8	1	0	9	4	0
3d	Cell 7	35	67	19	7	0	6	49	1
3d	Cell 8	25	5	7	14	0	11	3	0
3d	Cell 9	25	448	184	34	7	8	6	0
3d	Cell 10	18	330	105	122	2	1	4	0
3d	Cell 11	22	43	13	7	0	2	15	2
No Cells			0	0	0	0	0	0	0
9d	Cell 12	6	0	0	1	0	0	5	2
9d	Cell 13	18	0	1	3	0	0	4	14
9d	Cell 14	10	0	0	0	0	0	4	6
9d	Cell 15	6	3	1	2	0	0	2	4
9d	Cell 16	10	0	5	8	0	0	5	9
9d	Cell 17	19	0	5	5	0	0	9	0

Graphical representation of gene expression in differentiating single cells



Summary and Conclusions

We have developed a new reagent, SsoAdvanced PreAmp Supermix, which allows for accurate quantification of up to 100 target genes from very small sample amounts including single cells. We envision that this product can benefit researchers who work with limited or rare samples, and can lead to advances in the single cell analysis field.

References

1. Andrews PW (1984). Retinoic acid induces neuronal differentiation of a cloned human embryonalcarcinoma cell line in vitro. Dev. Biol 103(2):285-293.
2. Pleasure, SJ, and Lee, VM (1993). NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell, J Neurosci Res 35, 585-602.
3. Deb-Rinker, et al. (2005). Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation, J Biol Chem 280, 6257-60.

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