Quantitative RT-PCR from one single cell using the AmpliGrid technology

Pre-amplifying single cells with the AmpliGrid system followed by standard qPCR combines existing workflows with superior single cell sensitivity. This application note shows how AmpliGrid reverse transcription followed by an established Stratagene qPCR protocol allows to do gene expression analysis on single cells.

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Introduction

The analysis of single cells using quantitative RT-PCR systems often leads to unsatisfactory results due to low gene copy number contained in a single cell sample. In order to harness the sensitivity of the AmpliGrid platform, Advalytix developed a qPCR reverse transcription protocol which is compatible with any qPCR system and can be the basis for a successful quantitative single cell experiment.

Cell isolation and sorting

Isolate lymphocytes from human peripheral blood by PANCOLL (Pan Biotech) density centrifugation and transfer into phosphate buffered saline (PBS). Deposit single cells onto each of the 48 AmpliGrid reaction sites (fig. 1) by BD FACSAria™. To ensure that only vital cells are sorted on the AmpliGrid slide, use propidium iodide staining to dump dead cells.

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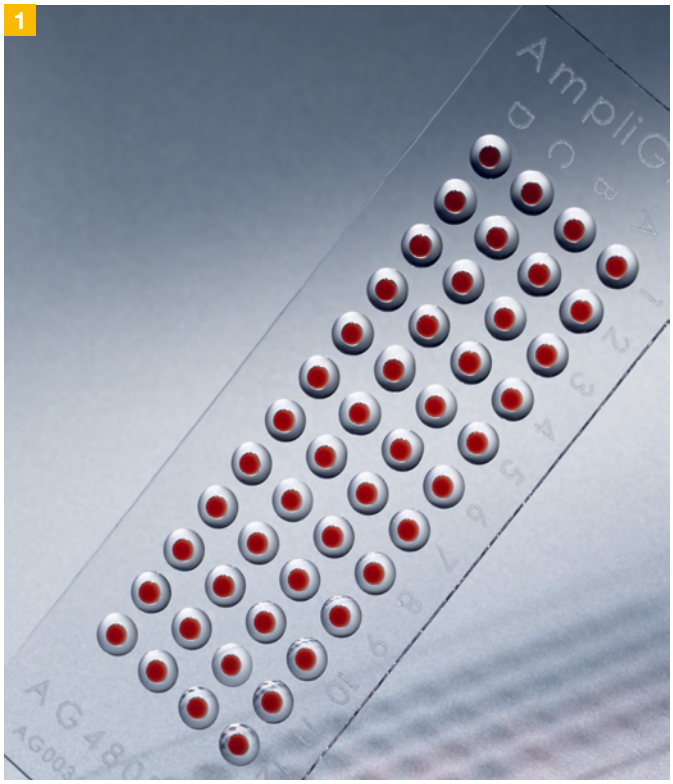
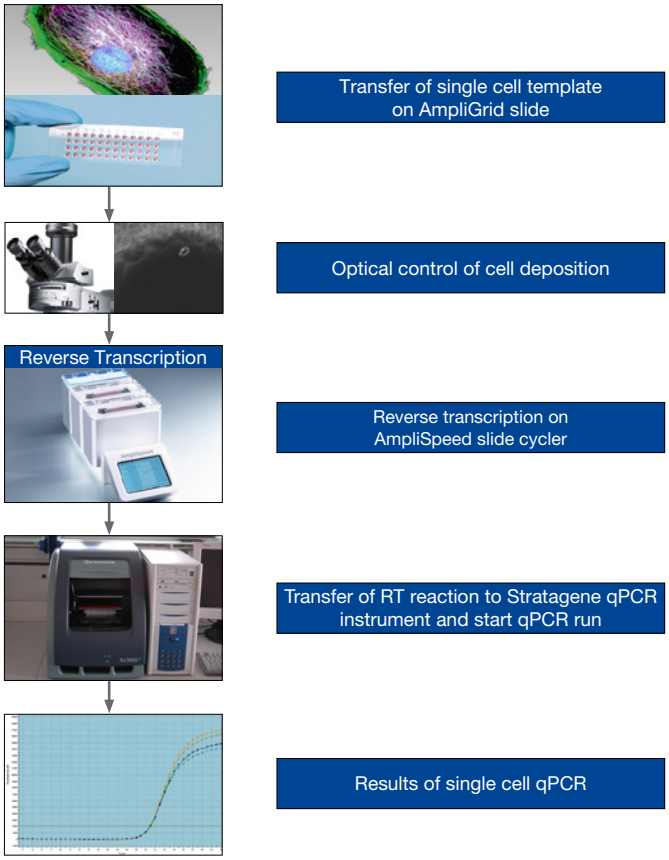


Figure 1: AmpliGrid AG480F slide

Quantitative RT-PCR on single cells

This application note details an easy to implement workflow for reliable, quantitative amplification of RNA from single human lymphocytes. The protocol is split into two steps. The AmpliGrid system performs highly sensitive single cell reverse transcription; the final amplification and analysis is done on a Stratagene Mx3005P® cycler with an established protocol (fig. 2)

2 *Convenient workflow for quantitative RT-PCR of single cells*



Pipette reverse transcription master mix according to table A. Pipette 1µL on the AmpliGrid with deposited single cells using only the antisense primer in a multiplex approach for the amplification of two housekeeping genes (Calm and B2M) and transfer on the preheated AmpliSpeed slide cycler (fig. 3) for 30 min at 58°C.

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Figure 3: AmpliSpeed ASC200D slide cycler

A *Table A: reverse transcription master mix*

Component	Master mix 1 sample
5x Qiagen RT-Buffer	0.2 µL
dNTP-mix (10 mM each)	0.04 µL
Enzyme mix	0.04 µL
RNase inhibitor (40 U/µL)	0.02 µl
Q-Solution, 5x	0.16 µL
RT-primer mix (6 µM)	0.1 µL
Aqua bidest	0.44 µL
Total volume	**1.0 µL**

After reverse transcription, add 4 µL of PCR-clean water to each reaction site by pipetting through the sealing solution and mix well by pipetting up and down.
Note that although the nominal volume of the AmpliGrid slide is 1 µL, adding 4 µL does cause the layer of cover oil to thin out, but does not cause the drop to run beyond its designated site. Next, pierce through the thinned out oil layer again to distribute the 4 µL of the dilution among 4 independent 96-well MTP wells (each well 1 µL). Prepare two master mixes, one for Calm and one for B2M according to table B and C below.

B *Table B: qPCR master mix Calm*

master mix	qPCR amplification
Brilliant® II SYBR® Green QPCR Master Mix (2x)	250 µL
Primer mix sense + antisense (50 µM) Calm	6 µL
PCR-clean water	244 µL
Total	**500 µL**

C *Table C: qPCR master mix B2M*

master mix	qPCR amplification
Brilliant® II SYBR® Green QPCR Master Mix (2x)	250 µL
Primer mix sense + antisense (50 µM) B2M	6 µL
PCR-clean water	244 µL
Total	**500 µL**

For the pre-amplified aliquots corresponding to one cell, transfer 2 times 10 µL of the qPCR master mix Calm and 2 times 10 µL of the qPCR master mix B2M into the appropriate MTP wells. Close the MTP with optical capable lids and run the instrument as shown in table D.

D *Table D: Stratagene Mx3005P® programme*

Temperature	Duration	
95°C	10 min	
94°C	30 sec	
60°C	60 sec	45 cycles
72°C	60 sec	
72°C	10 min	
Dissociation curve:		
95°C	1 min	
55°C	30 sec	
Heating up to 95°C	(low heating rate)	

Results

The combination of the AmpliGrid system with the Stratagene qPCR platform opens the possibility to analyse gene expression in single cells using a convenient workflow. Each bar shown in figure 4 represents the average of two qPCR measurements, with the error bar indicating the two individual data points. From the biology of expression of Calm and B2M in single cells obtained from homegenous populations, we expect distinctly different level of expressions corresponding to a log normal distribution (Kubista et. al., 2006). The graph confirms this biological expectation: three of the two cells express at the lower levels and two at the higher levels within a very narrow range. This indicates a high reproducibility of the platform, even for single cell samples.

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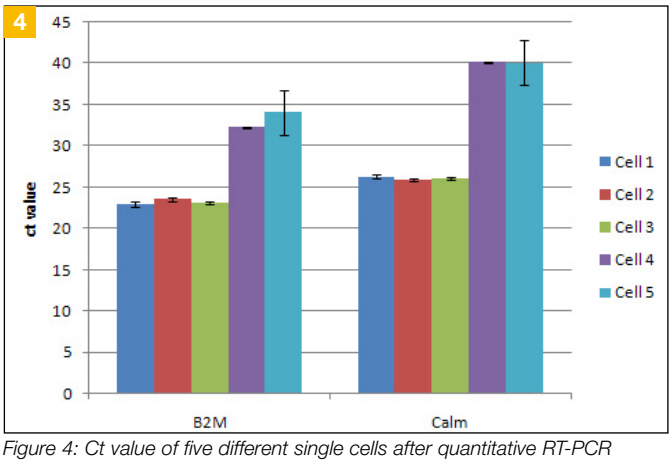

Figure 4: Ct value of five different single cells after quantitative RT-PCR

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