

# Quantitative multiplex TaqMan® qPCR from single cells using the AmpliGrid platform

The AmpliGrid in combination with the Ambion TaqMan® Gene Expression Cells-to-CT™ Kit offers superior single cell sensitivity in a simple and seamless workflow. In this application note we demonstrate the reproducibility of single cell housekeeping gene expression using a convenient multiplex TaqMan® assay.

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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. To overcome this lack of sensitivity Advalytix has been developing protocols that combine the sensitivity of the AmpliGrid platform with qPCR analysis. This application report shows how to achieve single cell quantitative analysis using TaqMan® chemistry; another qPCR application report shows the usage of SYBR® Green chemistry. Scaling down to one single cell the reproducibility of the system is shown with the expression pattern of the two housekeeping genes B2M and Calm in single human male and female lymphocytes.

## Cell isolation and sorting

Isolate lymphocytes from human peripheral blood by PANCOLL (PAN™ Biotech GmbH) density centrifugation and transfer into phosphate buffered saline (1 x PBS pH 7.4). Deposit single cells onto each of the 48 AmpliGrid reaction sites (fig. 1) by Beckman Coulter MoFlo™. To ensure that only vital cells are sorted on the AmpliGrid slide, use propidium iodide staining to dump dead cells.

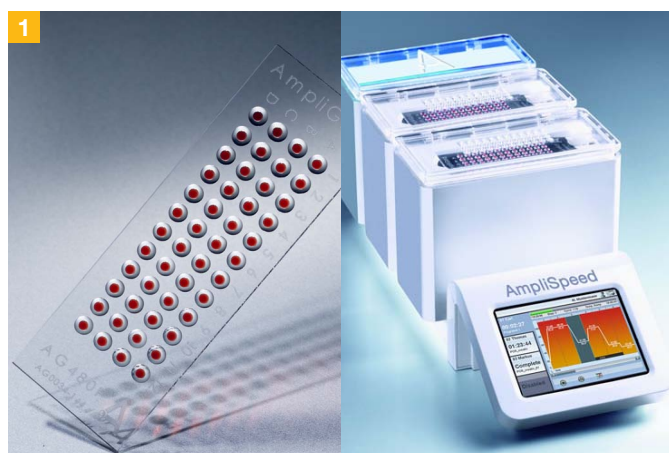


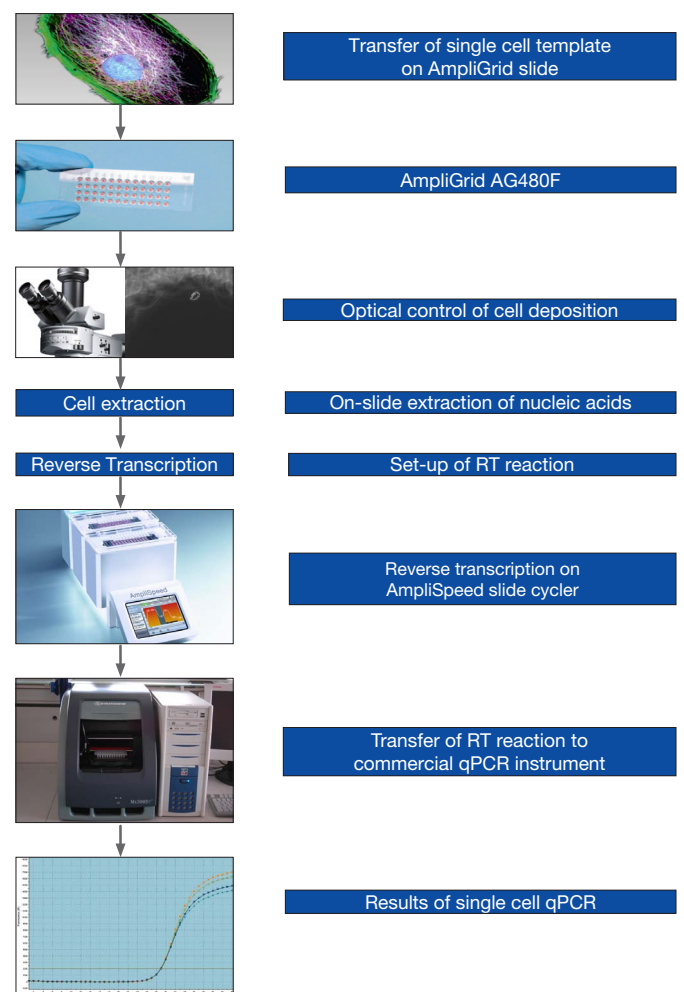
Figure 1: AmpliGrid AG480F slide and AmpliSpeed ASC200D slide cycler

## 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 Figure 2: Convenient workflow for quantitative RT-PCR of single cells



For improvement of amplification results through enhanced accessibility of the template material, prepare the cell extraction working solution as described in Table A.

A Table A: Composition of cell extraction reaction mix

Component	Volume (1 slide)	Volume (5 slides)
Lysis Enzyme	1 µL	5 µL
10x Lysis Buffer	6 µL	30 µL
Nuclease free water	53 µL	265 µL
<b>Total volume</b>	<b>60 µL</b>	<b>300 µL</b>

Mix gently by vortexing and spin down shortly. After preparing the working solution of the cell extraction kit keep it on ice and use within 6 hours. Dispense 0.75  $\mu\text{L}$  of the cell extraction reaction mix on AmpliGrid reaction sites and cover immediately with 5  $\mu\text{L}$  of sealing solution. Place the AmpliGrid slide on the AmpliSpeed slide cycler (fig. 1) and run the programme shown in Table B.

**B** Table B: Recommended incubation conditions

Temperature	Time
75° C	5 min
95° C	2 min
Ambient	$\infty$

Prepare the reverse transcription master mixes for the samples as well as for the negative control (without reverse transcriptase) in an approximate two-fold concentration (Table C).

**C** Table C: Reverse transcription master mix

Component	Volume per reaction
2x RT buffer	0.675 $\mu\text{L}$
20x reverse transcriptase (substitute with water in negative control)	0.075 $\mu\text{L}$
<b>Total volume</b>	<b>0.75 <math>\mu\text{L}</math></b>

Pipette 0.75  $\mu\text{L}$  reverse transcription master mix on the AmpliGrid with deposited single cells in cell extraction working solution by pipetting through the sealing solution. Transfer the AmpliGrid on the preheated AmpliSpeed slide cycler for 30 min at 37°C.

After reverse transcription, add 4  $\mu\text{L}$  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  $\mu\text{L}$ , adding 4  $\mu\text{L}$  does cause the layer of cover sealing solution 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  $\mu\text{L}$  of the dilution among 4 independent 96-well MTP wells (each well 1  $\mu\text{L}$ ). Prepare two master mixes, one for Calm and one for B2M according to table D below.

**D** Table D: real-time amplification master mix

Component	Volume per reaction
2x TaqMan GeneExpression master mix	10 $\mu\text{L}$
Expression assay mix (B2M or Calm)	1 $\mu\text{L}$
Nuclease free water	8 $\mu\text{L}$
<b>Total volume</b>	<b>19 <math>\mu\text{L}</math></b>

Pipette 19  $\mu\text{L}$  master mix into each well of the microtiter plate. Close plate with optical lids and mix by vortexing the plate. Centrifuge shortly to collect the liquid at the bottom of the wells. Perform real-time PCR on a Stratagene® Mx3005P® cycler as described in table E.

**E** Table E: real-time amplification programme for Stratagene® Mx3005P® cycler

Temperature	Time
50° C	2 min.
95° C	10 min
95° C	25 sec      45 cycles
60° C	1 min

The analysis shown in figure 3 was done using the MxPro – Mx3005P v4.00 Build 367, Schema 80 software (Stratagene).

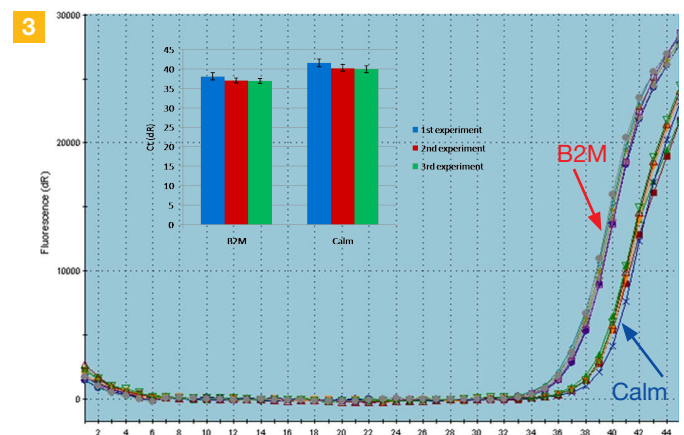


Figure 3: Mean  $C_t$  (dR) for B2M (left side) and Calm (right side) and standard deviation for three independent experiments, each run with 6 single cells run independently from each other. The results show reproducible  $C_t$  values for the single cells displaying equal expression levels of the analysed housekeeping genes.

## Results

The combination of the AmpliGrid system with the Stratagene® real-time PCR platform opens the possibility to analyse gene expression in single cells using a convenient workflow. In three independent experiments (6 single cells each) the analysis of two housekeeping genes is shown. As expected the expression level of the housekeeping genes is equal in single individual cells analysed for the two housekeeping genes B2M and Calm. The low standard deviations in figure 3 prove the high reproducibility of the AmpliGrid system for single cell handling and amplification.

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