

Quantitative RT-PCR on AmpliGrid AG480F

This process instruction describes the quantitative analysis of the housekeeping genes *Calm*, *b2m* and *GAPDH*. The reverse transcription is done on the AmpliGrid system using the Advantix Single Cell OneStep RT-PCR Kit – for a better visualization of the sample the master mixes are stained with either AdvaGold or AdvaBlue. Afterwards the quantitative amplification is done on a conventional real-time instrument (Stratagene) using SYBR green.

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, Advantix 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.

Material

- Single Cell OneStep RT-PCR Kit (OAX04515)
- Nuclease free water
- 2x Brilliant II SYBR Green Mix (Stratagene)
- AmpliGrid slides incl. Sealing Solution (e.g. OAX04503)
- Primer mix for reverse transcription: *Calm*, *b2m*, *GAPDH* antisense primer (10 μ M each)
- Primer for qPCR: *Calm*, *b2m*, *GAPDH* sense+antisense (6 μ M each)
- 0.1% AdvaGold (OAX04227)
- 0,1% AdvaBlue (OAX04243)
- Single cells deposited on the AmpliGrid and/or human total RNA (Promega); 1 ng/ μ L (store at -80°C)
- AmpliSpeed slide cyler (e.g. OAX04101)
- Electronic multistep pipette
- 96 well microtiter plate and lid (for qPCR applications)
- Real-time cyler (e.g. Stratagene Mx3005P®)

Cell isolation and sorting

Isolate lymphocytes from human peripheral blood by PANCOLL (Pan™ Biotech GmbH) density centrifugation and transfer into phosphate buffered saline (PBS). 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.

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® cyler with an established protocol (fig. 2)

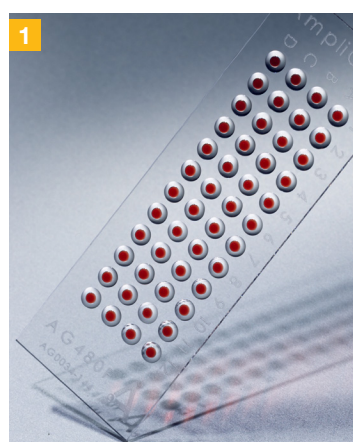
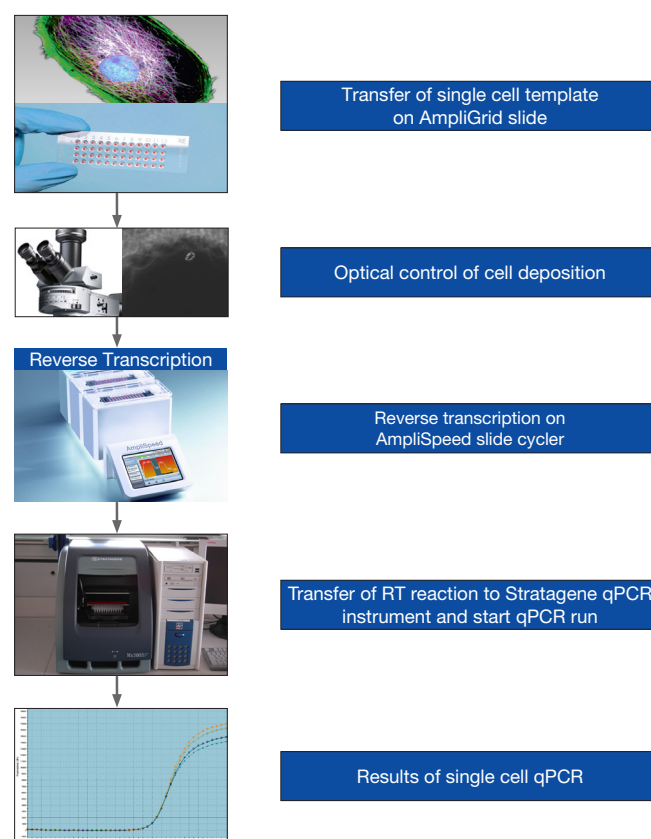


Figure 1: AmpliGrid AG480F slide

2 Convenient workflow for quantitative RT-PCR of single cells



Protocol

- Prepare the reverse transcription (RT) master mix in a fresh PCR-clean tube according to table A (keep everything at 4°C during pipetting)
- Make sure to prepare enough master mix for all the reactions, taking pipetting errors into account (dead volume of electronic multistep pipette about 10 µL)
- Mix gently and spin down shortly

A Table A: Composition of RT master mix

Component	Volume (1 reaction)	Volume (48 reaction)
2x Single Cell RT Reaction Buffer	0.50 µL	30.0 µL
RNase inhibitor (10 U/µL)	0.02 µL	1.20 µL
5x Single Cell RT Enhancer	0.15 µL	9.00 µL
Optional: RT primer mix (10 µM each)	0.06 µl	3.60 µL
Single Cell RT Enzyme Mix	0.04 µL	2.40 µL
Optional: total RNA control (10 ng/µL)	0.1 µL	6.00 µL
AdvaBlue/AdvaGold	0.1 µL	6.00 µL
Nuclease-free water	ad 1 µL	ad 60 µL
Total volume	1.0 µL	60.0 µL

- Distribute 1 µL of master mix on each reaction site
- Immediately cover with 5 µL sealing solution
- Transfer AmpliGrid slide onto the preheated AmpliSpeed slide cycler (fig 3.)



Figure 3: AmpliSpeed ASC200D slide cycler

- Incubate AmpliGrid slide for 10 min at 42°C and 30 min at 58°C
- Meanwhile prepare qPCR master mix according to table B

B Table B: Composition of PCR master mix

Component	Volume (1 reaction)	Volume (48 reaction)
2x Brilliant II SYBRGreen Mix	0.5 µL	250 µL
Nuclease-free waterr	0.3 µL	150 µL
Total volume	0.8 µL	400 µL

- Pipette 8 µL qPCR master mix into each well of a microtiter plate (three reactions per sample)
- Add 1 µL of the oppropriate primer mix (6µl sense and antisense) into the MTP wells
- After the reverse transcription has finished add 3 µL of nuclease free water to each reaction site of the AmpliGrid by pipetting onto the sealing solution and mix well by pipetting up and down (since the slide can not be used again the tip entry can touch the slide what makes the mixing easy)
- Transfer 3 times 1 µL from each reaction sites of the AmpliGrid into the microtiter plate wells; close the plate with the appropriate lid
- Run the program described in table C on a real-time instrument and analyse the data afterwards using the software

C Table C: qPCR program

Temperature	Duration	Cycle
95°C	10 min	
95°C	30 sec	
60°C	60 sec	45 cycles
72°C	60 sec	
72°C	10 min	
melting curve	55°C > 95°C	2K/min

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