

Superior sensitivity in RT-PCR of single cells using the AmpliGrid system

Single cell PCR in microplates is prone to template loss to the plastic material of the wells and lacks sensitivity due to the high reaction volume. We perform side-by-side RT-PCR analysis of flow cytometry sorted single cells in a standard microplate and on the AmpliGrid slide. RT-PCR on the AmpliGrid slide more than doubles the RT-PCR success rate due to the superior sensitivity and better efficiency of the AmpliGrid system.

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We spot single human lymphocytes on either a microtiter plate or the AmpliGrid slide by using flow cytometry. The AmpliGrid slide is chemically modified glass on the basis of a microscope slide. AmpliGrid allows for simple and convenient cell deposition control using a standard fluorescent microscope which is a clear advantage versus a microplate. In addition to that, the chemical modification of the AmpliGrid avoids template loss like it is known occurring in plastic material. We perform a one step RT-PCR on a high and a low expressed gene using the Advalytix Single Cell One-Step RT-PCR System for multiplex analysis of gene expression.

Cell Isolation

Isolate lymphocytes from human peripheral blood by PANCOLL (Pan Biotech) density centrifugation and transfer in phosphate buffered saline (PBS, 1x). Add Hoechst Bisbenzimid H 33342 (Sigma, Cat. # 14533) with a concentration of 1mg/ml (100*staining solution) 1:100 to the cell suspension and incubate for 5-10 min at room temperature prior to sorting for a nuclear staining.

Cell Sorting

Sort single cells using a MoFlo™ High Performance cell sorter (Beckman Coulter) directly onto each of the 48 AmpliGrid reaction sites (fig. 1) or in standard microplates. Sort vital cells according to their side and forward scatter signals¹.

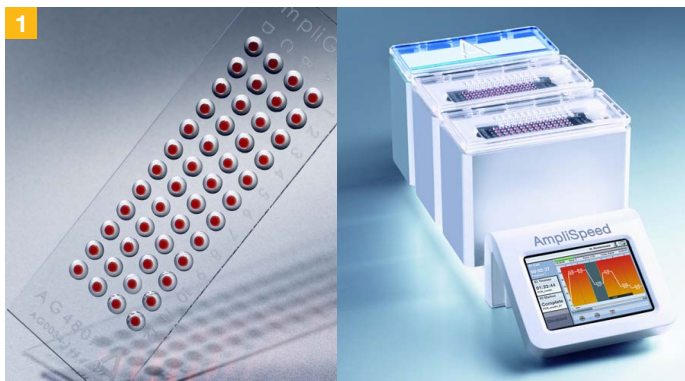


Fig. 1: AmpliGrid AG480F slide and AmpliSpeed ASC200D slide cycler

Cell deposition control

Use the nuclear staining with Hoechst dye to prove single cell deposition on the AmpliGrid reaction sites by using a standard fluorescent microscope.

The deposition of cells sorted into a microplate cannot be checked because of the opaque plastic material. It is not possible to distinguish between failure of reaction, lack in sensitivity or failure in cell deposition when full dropouts occur in the analysis. However partial dropouts clearly show lack of sensitivity

RT-PCR

The one-step RT-PCR on the AmpliGrid system works in a very low, cost saving volume of only 1µL. For the set up in a microtiter plate a standard volume of 25µL is taken.

Pipetting the AmpliGrid slide is easier, more convenient and less error prone compared to pipetting a microtiter plate as errors can be detected during the pipetting step by eye (see figure 2).

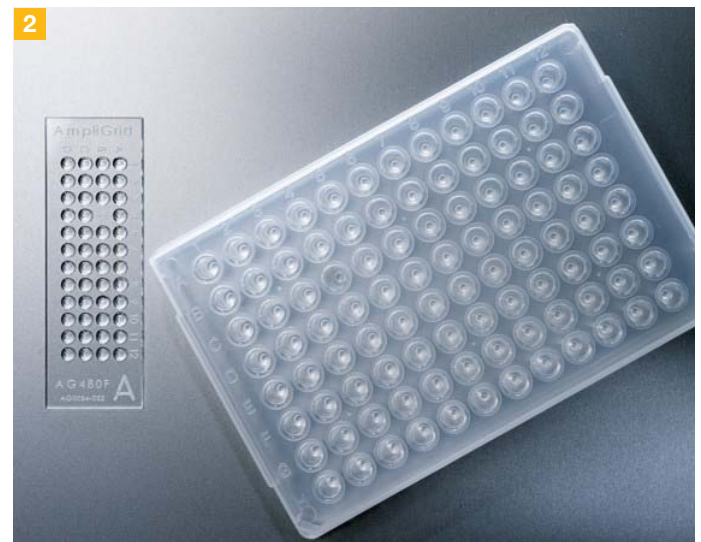


Fig. 2: Pipetting the AmpliGrid slide less error prone compared to a microtiter plate

Prepare two master mixes on ice according to table A (Advalytix Single Cell RT-PCR Kit), one for the AmpliGrid slide and one for the microplate.

A Table A: RT-PCR master mixes for AmpliGrid or microtiter plate

Component	1 reaction AmpliGrid	1 reaction microtiter plate
2x Single Cell RT Reaction Buffer	0.50 µL	12.5 µL
RNase Inhibitor (40 U/µL)	0.02 µL	0.25 µL
5x Single Cell RT Enhancer	0.15 µL	5 µL
Single Cell RT Enzyme Mix	0.04 µL	1 µL
2-plex Primer (20µM)	0.015 µL	0.375 µL
Nuclease-free water	0.29 µL	5.875 µL
Total Volume	1 µL	25 µL

Pipet 1 µL of the master mix on each AmpliGrid reaction site and immediately cover with 5µL of sealing solution. Pipet 25 µL master mix into each well of the microtiter plate and close it using an adhesive foil. Transfer the AmpliGrid to the AmpliSpeed slide cycler (fig. 1), the microtiter plate to a standard cycler (Eppendorf Mastercycler) and start the amplification program described in table B

B Table B: One-step RT-PCR program

Temperature	Time	Cycle
42°C	10 min	
50°C	10 min	
58°C	30 min	
95°C	10 min	
94°C	30 sec	
60°C	60 sec	30
72°C	60 sec	
72°C	10 min	

After amplification apply 4 µL of 1.5x gel loading dye on the AmpliGrid reaction sites or into the microtiter plate wells. Afterwards load samples directly onto a polyacrylamide gel and start the electrophoresis. Afterwards do a silver staining to visualize the bands on the polyacrylamide gel.

Results

Count the bands on the stained polyacrylamide gel independently of the intensity – to visualize differences in sensitivity, the number of cycles in the PCR reaction is chosen in a way that the reaction has not achieved saturation. Figure 3 shows a typical example of gel images of a polyacrylamide gel electrophoresis followed by silver staining after RT-PCR of a low expressed and a high expressed gene on the AmpliGrid system or a standard microplate.

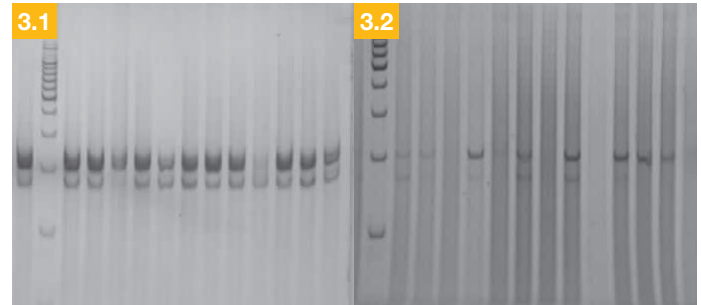


Figure 3: Examples of typical gel images of a polyacrylamide gel electrophoresis followed by silver staining after RT-PCR of a low expressed and a high expressed gene on either the AmpliGrid system (3.1) or a standard microplate (3.2)

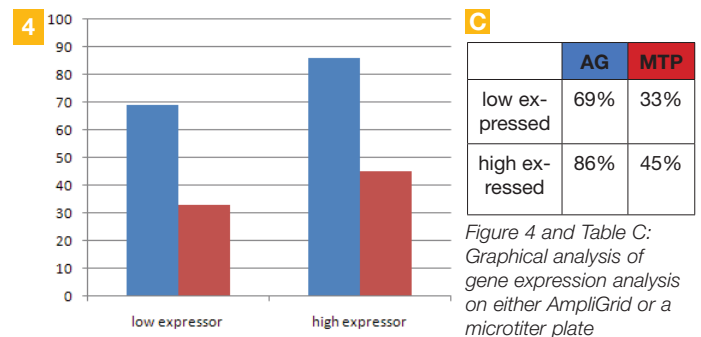


Figure 4 and Table C: Graphical analysis of gene expression analysis on either AmpliGrid or a microtiter plate

Discussion

The results clearly show the superior sensitivity of the AmpliGrid in comparison to the microplate. A higher success rate of the experiment can be achieved when using the AmpliGrid platform because cell deposition can be optically controlled to ensure RT-PCR success, even if sorting dropouts occur, improving the overall quality of the experiment. In the microplate the sensitivity is less in comparison to the AmpliGrid because of lower PCR efficiency. Drop outs in the reaction cannot be further verified because it is not possible to discriminate between sorting failure or RT-PCR failure.

Besides the quality of the reaction also cost aspects make the AmpliGrid the platform of choice. The reaction volume can be reduced by a factor of 10-25 which is especially important when using high price RT-PCR kits.

¹ Cell sorting was done by Dr. J.W. Ellwart, Helmholtz Zentrum München GmbH, Ingolstädter Landstraße 1, D-85764 Neuherberg

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