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CellCelector™ Application Note

Single Cell PCR Preparation

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From Automated Screening to the Molecular Analysis of Single Cells

The AmpliGrid system is a highly sensitive tool for the analysis of single cells. In combination with the CellCelector a standardized and automated workflow for the selection and isolation of single cells from a mixed cell population together with (RT-) PCR mix loading is guaranteed. In a first step, rare target cells are separated from a background population. Single cells are selected, isolated in vitro without any pre-treatment and transferred onto the AmpliGrid slide together with the reaction mix and sealing solution. After selection of 48 cells the amplification reaction on the AmpliGrid starts. This workflow ensures less hands-on time, high standardization and a reliable analysis of single cells.

Summary

Specific cell subpopulations from a mixture of differently stained cell types were selected in vitro automatically. Subsequently using an automated picking process selected target cells were smoothly isolated directly from their cultivation environment, transferred to and dispensed together with PCR-mix and sealing solution on the anchor positions of the AmpliGrid slide in a unique one step process (Fig. 1A). After completing the loading process the RT-PCR was started on the integrated thermal cycler. Subsequently the RT-PCR resulted cDNA was analyzed (Fig 1B).

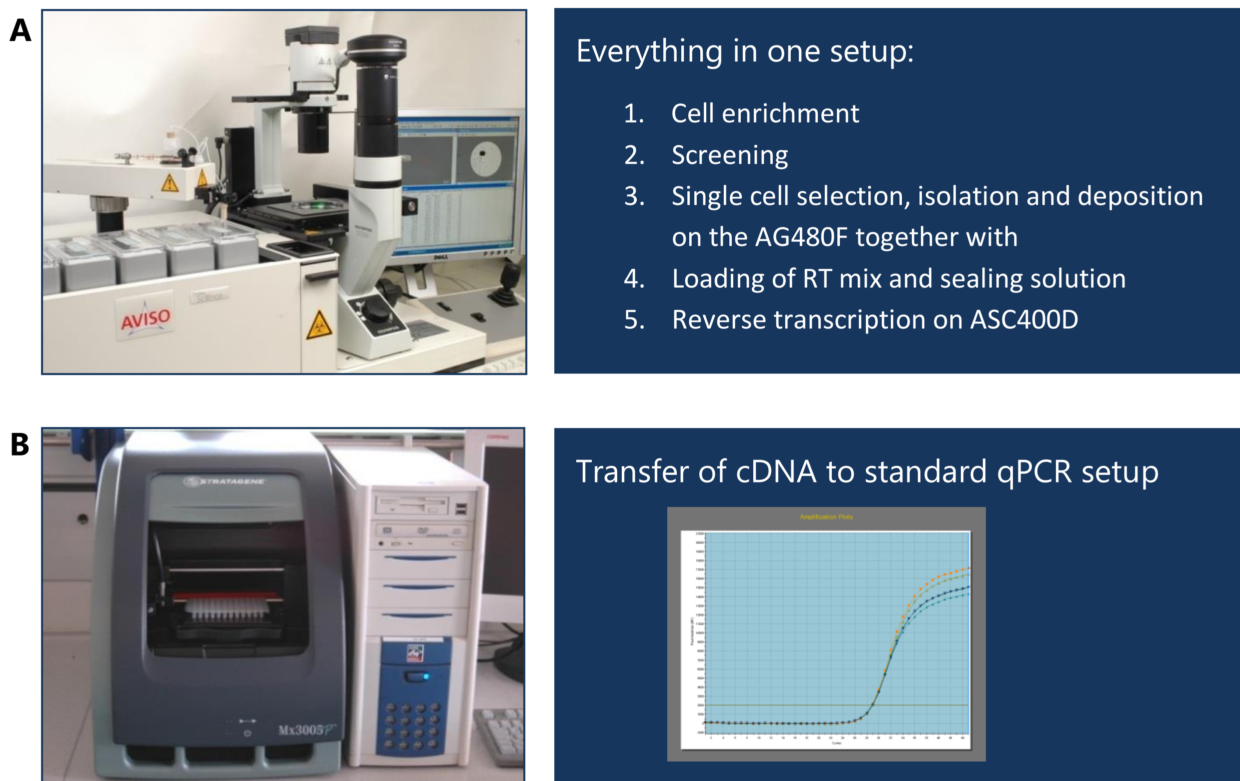

Figure 1: Workflow for automated single cell screening, single cell deposition and reverse transcription (**A**) and qPCR analysis (**B**)

Single cell detection and selection

For the experiments a cell line of human pancreatic adenoma tumour cells (ASPC-1) and Lymphocytes (Simfo GmbH Bayreuth) were used. ASPC-1 cells, derived from cell culture were harvested and stained with an Alexa-488 antibody. Lymphocytes were stained with a PE-labelled antibody. In a Petri dish 4 ml of 1xPBS were deposited and lymphocytes and ASPC-1 cells were mixed together in a 1:1 ratio. The final cell concentration was 80cells/mm^2.

1. *Detection of Alexa-488-positive cells*

Using the CellCelector software the culture plate was screened for Alexa-488-positive target cells utilizing the integrated fluorescence unit. Based on a reference sample area (Fig. 2A) a range of grey values was defined (Fig. 2C) used to detect the target cells by scanning the source culture plates. All areas fitting in this range of grey values are displayed as green colored event (Fig. 2B). The screening result (visualized as overview map; Fig. 2D) can be improved further by the setup of additional scan parameters. For each of them (e.g. diameter, area, shape factor etc.) specific filter ranges can be defined as well to exclude non cellular particles with the same grey value from the scan result. Scan parameters used for the detection of Alexa-488-positive cells are shown in table 1. If necessary the scan result can be sorted and filtered further by one or more scanning parameters to compile the final list of cells to pick (Fig. 3).

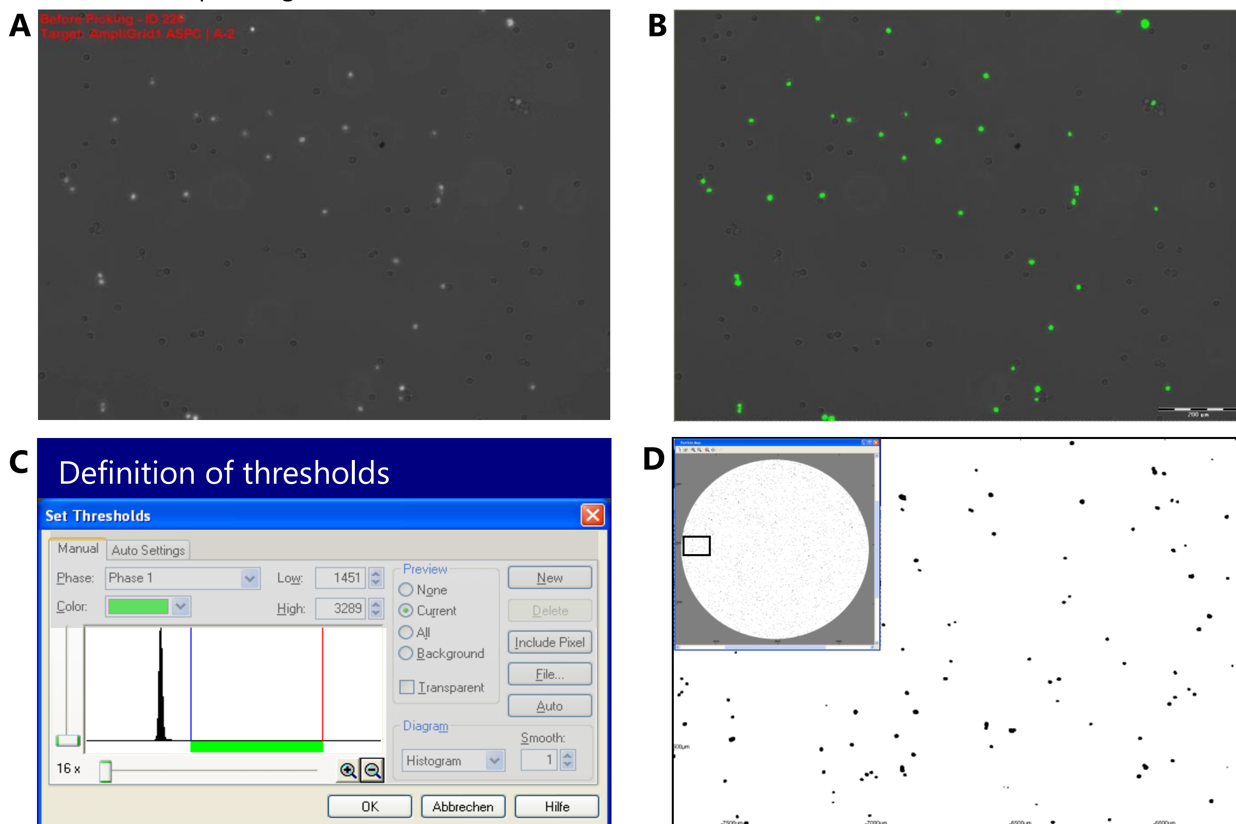

Figure 2: Detection of Alexa-488-positive target cells. Reference image before (**A**) and after (**B**) setting grey value thresholds for the scanning process. Dialog window for threshold definition (**C**): x-axis: grey value, y-axis: number of pixel with the same grey value at reference image. Setting the grey value thresholds will be done by moving the green bar (range of grey values used for target cell detection), the red line (upper limit) and/or the blue line (lower limit). **D** After scanning the culture plate the scan results will be displayed as an interactive overview map (complete scan area [small image] and enlarged section of the marked rectangular area).

Table 1: *Parameters for detection of Alexa-488-positive Cells*

Fluorescence channel	FITC (100 % excitation light intensity)
Magnification	10x
Exposure time	50 ms
Center Gravity X	No limits
Center Gravity Y	No limits
Maximum Diameter	5 µm – 20 µm
Area	No limits
Shape factor	No limits

10-08-12 Adv7 - Particle List

	WellPlate	Well	ID Particle	PickStatus	Pick / Scan	Target WellPlate	Target Well	Center Gravity X	Center Gravity Y	Diameter Max	Area	Shape Factor
175	10-08-12 Adv2	A-1	2	Pick OK	No	AmpliGrid9 ASPC	A-1	59090,44	46103,68	11,54	66,30	0,35
176	10-08-12 Adv2	A-1	6	Pick OK	No	AmpliGrid9 ASPC	A-2	58994,43	45295,95	13,18	84,64	0,30
177	10-08-12 Adv2	A-1	11	Pick OK	No	AmpliGrid9 ASPC	A-3	57712,97	46767,14	12,89	82,02	0,28
178	10-08-12 Adv2	A-1	13	Pick OK	No	AmpliGrid9 ASPC	A-4	58134,85	46938,22	12,68	85,17	0,27
179	10-08-12 Adv2	A-1	14	Pick OK	No	AmpliGrid9 ASPC	A-5	58433,65	45722,63	12,82	83,07	0,26
180	10-08-12 Adv2	A-1	18	Pick OK	No	AmpliGrid9 ASPC	A-6	58149,64	45725,45	13,72	87,00	0,25
181	10-08-12 Adv2	A-1	20	Pick OK	No	AmpliGrid9 ASPC	A-7	58274,96	46740,12	12,56	75,21	0,25
182	10-08-12 Adv2	A-1	28	Pick OK	No	AmpliGrid9 ASPC	A-9	56480,51	46898,87	12,98	74,16	0,24
183	10-08-12 Adv2	A-1	30	Pick OK	No	AmpliGrid9 ASPC	A-10	58017,19	47100,64	11,84	71,01	0,24
184	10-08-12 Adv2	A-1	31	Pick OK	No	AmpliGrid9 ASPC	A-11	56998,68	46045,83	16,45	131,29	0,24
185	10-08-12 Adv2	A-1	34	Pick OK	No	AmpliGrid9 ASPC	A-12	56787,01	44930,84	12,33	69,18	0,23
186	10-08-12 Adv2	A-1	35	Pick OK	No	AmpliGrid9 ASPC	B-1	56802,00	45277,98	16,92	127,62	0,22
187	10-08-12 Adv2	A-1	37	Pick OK	No	AmpliGrid9 ASPC	B-2	56267,27	45557,20	12,33	64,46	0,22
188	10-08-12 Adv2	A-1	43	Pick OK	No	AmpliGrid9 ASPC	B-3	58455,74	45890,89	12,98	84,90	0,21
189	10-08-12 Adv2	A-1	46	Pick OK	No	AmpliGrid9 ASPC	B-4	56583,56	47016,52	15,91	117,66	0,21
190	10-08-12 Adv2	A-1	53	Pick OK	No	AmpliGrid9 ASPC	B-5	58779,07	45868,29	13,23	80,19	0,20
191	10-08-12 Adv2	A-1	56	Pick OK	No	AmpliGrid9 ASPC	B-6	57699,75	47112,71	13,43	60,53	0,20
192	10-08-12 Adv2	A-1	59	Pick OK	No	AmpliGrid9 ASPC	B-7	59264,85	45705,79	15,91	97,22	0,20
193	10-08-12 Adv2	A-1	60	Pick OK	No	AmpliGrid9 ASPC	B-8	58051,66	46631,45	14,34	82,28	0,19
194	10-08-12 Adv2	A-1	63	Pick OK	No	AmpliGrid9 ASPC	B-9	57572,24	44820,70	12,88	74,95	0,19
195	10-08-12 Adv2	A-1	67	Pick OK	No	AmpliGrid9 ASPC	B-10	57570,51	43905,44	14,71	94,86	0,19
196	10-08-12 Adv2	A-1	74	Pick OK	No	AmpliGrid9 ASPC	B-12	56115,45	45796,78	16,11	97,74	0,19

Figure 3: Result list after successful picking of Alexa-488-positive cells

2. *Excluding PE-positive and Alexa-488-positive cells*

Beyond screening of culture plates with single illumination channels the CellCelector software allows for screening utilizing multiple illumination channels. Wavelength and intensity of each channel can be defined before a multiple screening process and used for selecting specific cell subpopulations. By using this multiple screening process it is also possible to exclude neighboring target cells from the result list that are situated to close to each other in the source culture plate. This guarantees single cell picking from a culture plate with a non-homogenous distributed mixed population of cells.

Using the result list of Alexa-488-positive cells that was generated using the process described in chapter 1. additional scans of this list have been performed in 20x magnification. In a circular search region of 75µm around each Alexa-488-positive cell on the result list two additional detection processes have been done (Tab. 2) – one on the FITC channel again and one on the TRITC channel of the automated fluorescence unit of the CellCelector. In case of either more than one FITC signal (Fig. 4 B) or a TRITC signal (Fig. 4 C) within that search region the corresponding Alexa-488-positive cell was disabled from the result list. Alexa-488-positive cells without any additional FITC or TRITC signal within the search region (Fig. 4 A) were kept in the result list, picked and deposited on AmpliGrids for subsequentially performed single cell PCR-Analysis.

Table 2: *Settings for detection of additional FITC- or TRITC-positive cells within the search region around the originally detected Alexa-488-positive cells*

Magnification	20x
Search region	75 µm
FITC-channel:	
Illumination intensity	100 %
Exposure time	50 ms
High sensitivity mode	gain 8
TRITC-channel:	
Illumination intensity	100 %
Exposure time	50 ms
High sensitivity mode	gain 8

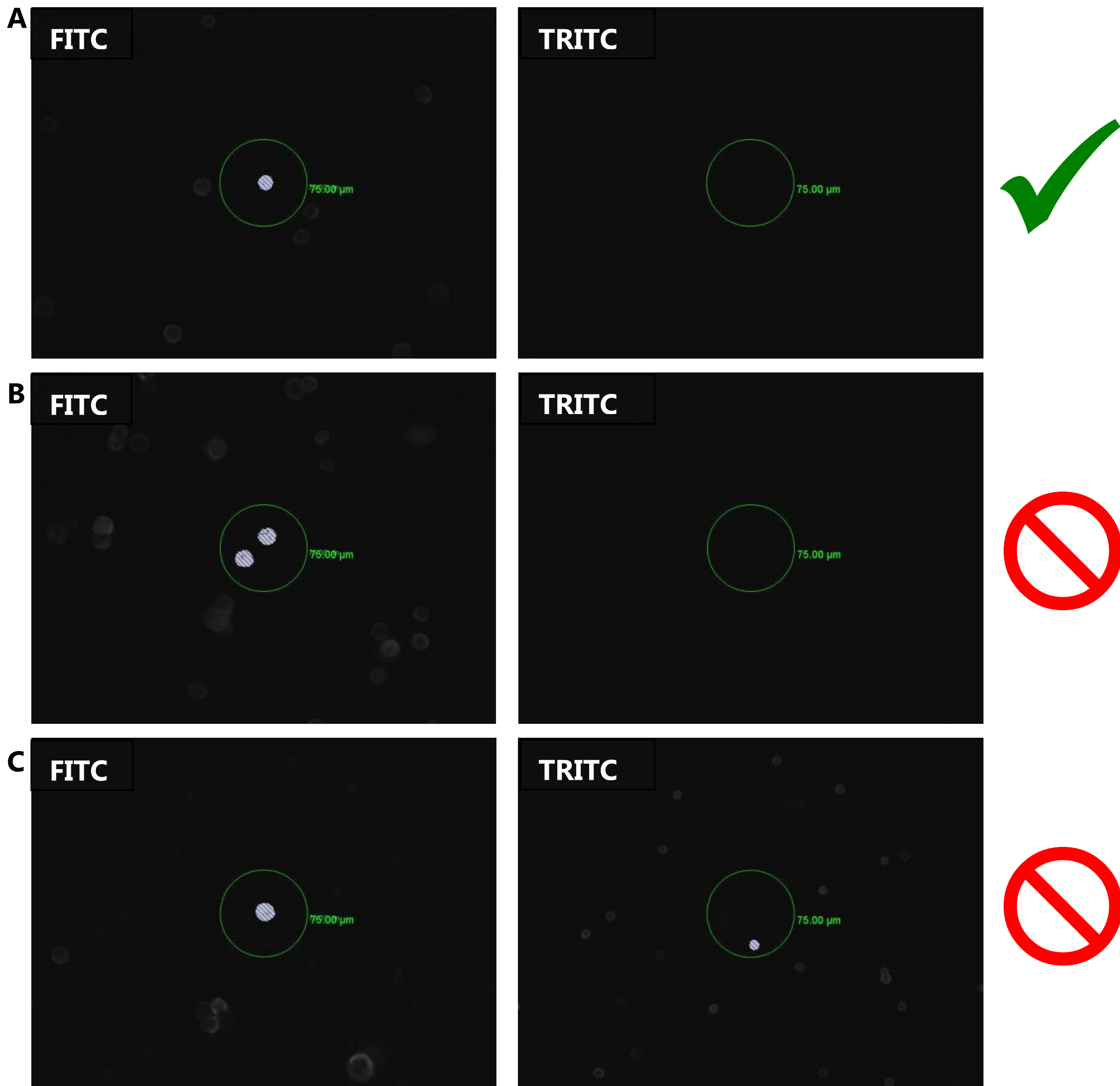

Figure 4: Detection and selection of cell subpopulation out of a cell mixture using the multi fluorescence unit of the CellCelector software. The target cell population was screened in the FITC channel. "Contaminating" cells were screened within the FITC and TRITC channel. Cells to pick have been specified by having no neighbor cell within the search region (green circled area – diameter 75 µm). **A** Only one Alexa-488 positive cell is located within the search region - cell is pickable. **B** two Alexa-488-positive single cells are located within the search region – single cell picking is not possible – cell was disabled from the original picking list. **C** Only one Alexa-488 positive single cells is located within the picking area but also one PE-positive cell – two different cells are located within the search region - single cell picking is not possible – cell was disabled from the original picking list.

Automated single cell picking, deposition and reverse transcription

After screening the culture plate and selection of pickable single Alexa-488-positive cells RT mix loading, single cell picking and covering both with a layer of sealing solution on AmpliGrids was carried out automatically by the CellCelector. Based on that automatically performed preparation three products (A, B and C) will be generated during RT-qPCR from the single cell RNA. The workflow of the automatically performed preparation of AmpliGrids for single cell PCR-Analysis includes the sequential uptake of liquids with different viscosity from different sources, which enables a one-step preparation of the sample to analyze directly on AmpliGrids. Whilst in a first step reverse transcription master mix (0,8 µl) followed by cell solution (0.2 µL) including the first single cell were aspirated by the cell picking tool directly (glass capillary) the sealing solution (wich is used as working solution inside the picking tools tubing system) was supplied via a valve from an integrated separately located reservoir (Fig. 5 A).

During the deposition process at the first reaction site on the AmpliGrid the liquid with the picked cell will be mixed with the PCR-Mix (both are hydrophilic aqueous solutions) and covered with 5µl sealing solution (hydrophobic mineral oil) to avoid evaporation of the aqueous phase (Fig. 5 B). Afterwards this process is repeated using the next selected single cell until the slide is loaded completely with cells. Subsequently the reverse transcription was started using the Thermocycler program as shown in table 4. The volume of all liquid components is freely configurable for the respective user specific application.

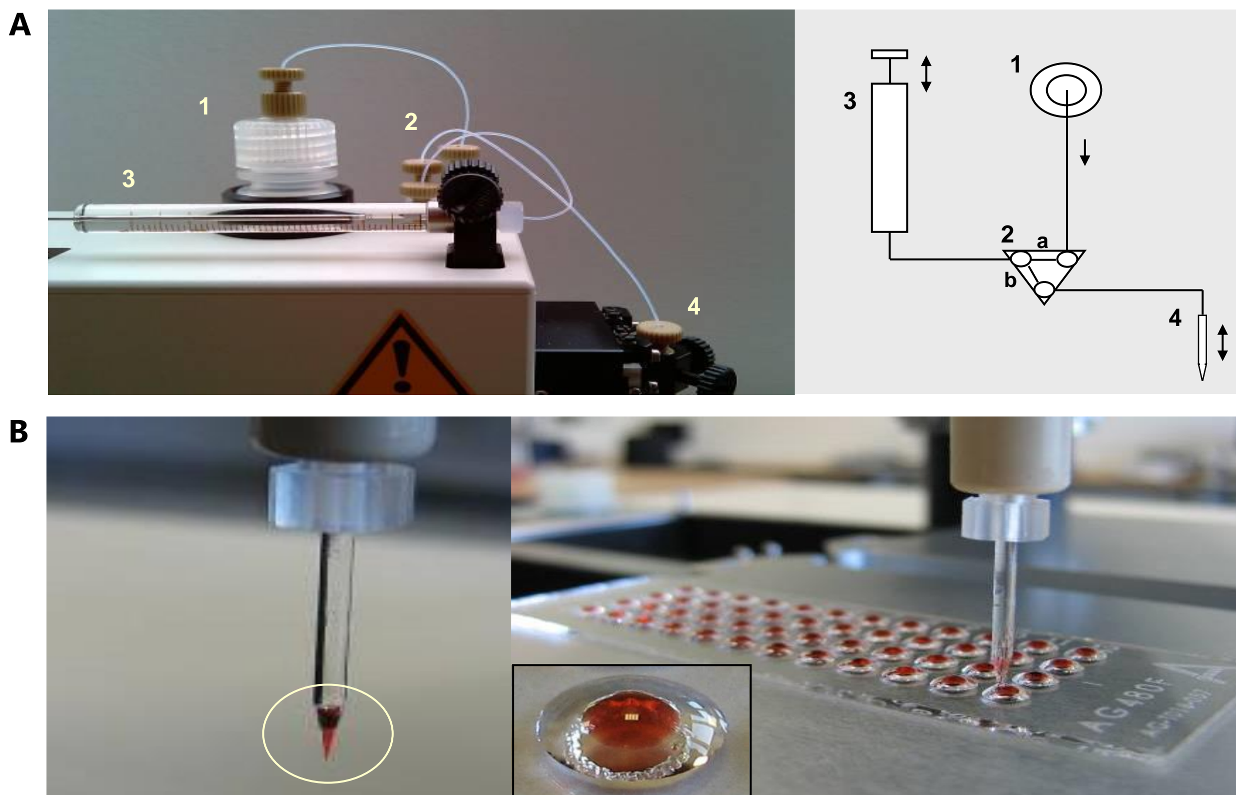

Figure 5: One-step preparation of single cells on AmpliGrids for PCR-analysis: **A** real and schematic view of the picking tool consisting of a reservoir tank for sealing solution (1), a valve (2), a syringe (3) and a holder to the glass capillary (4) for cell picking; **B** One-step preparation of the sample on an AmpliGrid slide. The PCR-Mix and the cell containing solution just before deposition are still visible as separated solutions – visualized as green (top) and red (down) solution, respectively (left). During deposition both aqueous solution will be mixed (red and green turns to brown) and covered with a layer of sealing solution (middle). Complete prepared AmpliGrid with one-step prepared samples (right).

The CellCelector software documents each cell picking process by taking an image from the picking area before and after picking the cell. That documentation works independent from the cell detection process. For example target cells can be detected using fluorescence illumination but, to prevent excessive exposure of the cells to fluorescence light, the image documentation of the picking process can be done in bright field. Examples of successful picked cells in various illumination channels are shown in figure 6.

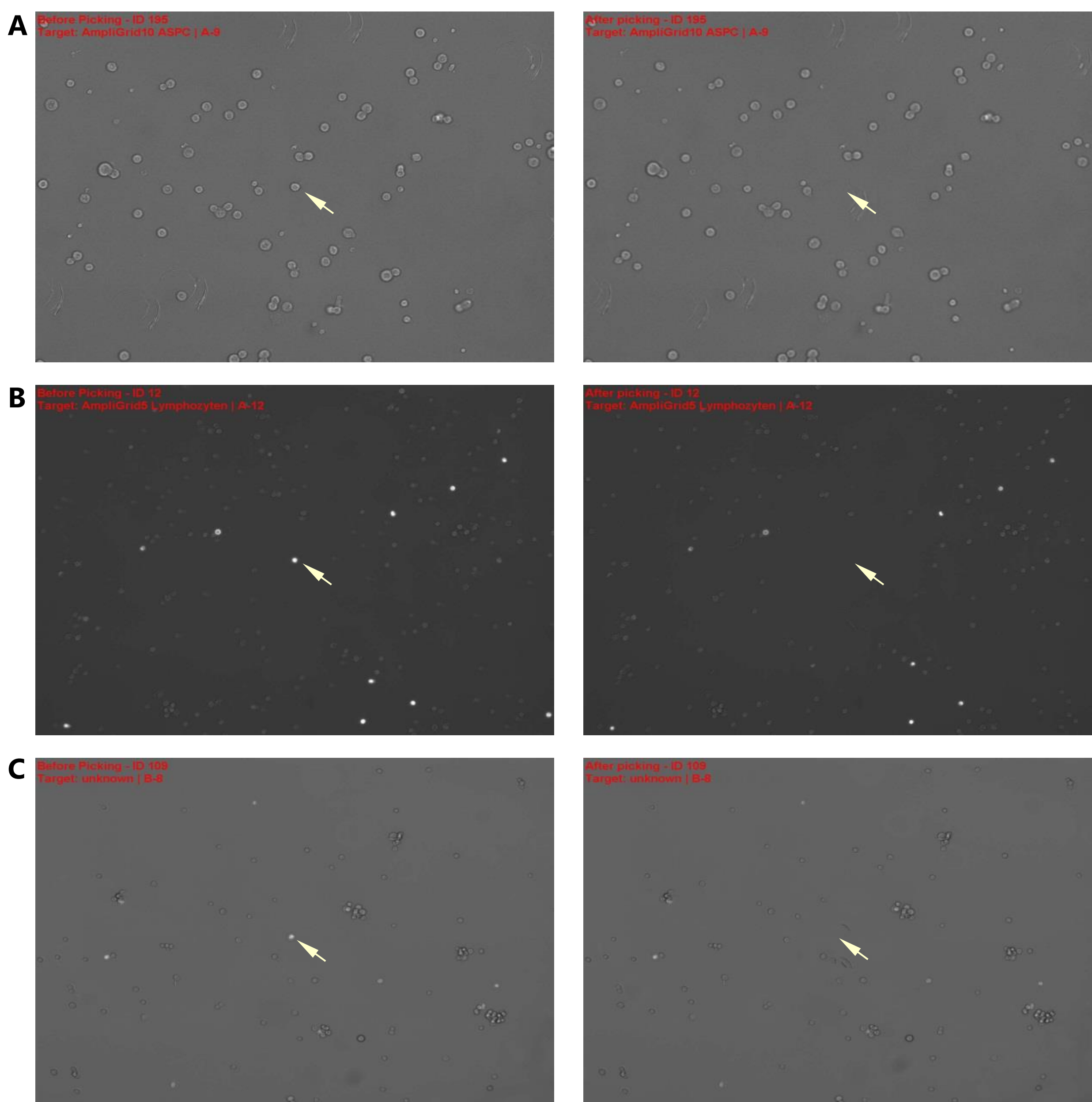

Figure 6: Pictures taken automatically from the picking area before (left) and after (right) the single cell picking: **A** in bright field; **B** in fluorescence illumination; **C** combination of A and B

The reverse transcription master mix was prepared in a MTP according to table 3. The MTP was placed onto the CellCelector.

***Table 3**: Reverse transcription master mix*

Component	**1 sample**	**120 samples**
2x Single Cell RT Reaction Buffer	0.50 µL	60.0 µL
RNase inhibitor (10 U/µL)	0.02 µL	2.4 µL
5x Single Cell RT Enhancer	0.15 µL	18.0 µL
RT Primer mix (20 µM)	0.02 µL	2.4 µL
Single Cell RT Enzyme Mix	0.04 µL	4.8 µL
Nuclease-free water	0.07 µL	8.4 µL

***Table 4**: Reverse transcription on ASC 400D*

Temperature	**Time**
42°C	10 min
50°C	10 min
58°C	30 min

Standard qPCR in commercial qPCR cyclers

After reverse transcription, 3 µL of PCR-clean water were added to each reaction site by pipetting through the sealing solution and mixing by pipetting up and down. One master mix for each of the 3 transcripts was prepared according to table 5. For the qPCR 9 µL of the qPCR master mixes were transferred into the MTP wells. 1 µL cDNA for each qPCR was transferred from the AmpliGrid into the appropriate MTP well by piercing through the thinned out oil layer. The MTP was closed with optical capable lids and the run was started as shown in table 6.

***Table 5**: qPCR master mix*

Component	**1 sample**	**10 samples**
Brilliant® II FAST SYBR® Green QPCR Master Mix (2x)	5.0 µL	50 µL
Primer sense (10 µM)	0.6 µL	6 µL
Primer antisense (10 µM)	0.6 µL	6 µL
PCR-clean water	2.8 µL	28 µL
Total	9 µL	90 µL

***Table 6**: Stratagene Mx3005P® program*

Temperature	**Time**	
95°C	2 min	
95°C	5 sec	45 cycles
60°C	20 sec	
Dissociation curve:		
95°	1 min	
55°C up to 95°C		

The qPCR analysis was done using the MxPro – Mx3005P v4.00 Build 367, Schema 80 software (Stratagene). Data was obtained from 22 independent single cells and for each of the three transcripts (A, B or C). Each transcript shows a specific ct range and melting temperature. For a better visualization figure 7 shows the data of 8 of the 22 single cell reactions. Reproducible ct values were obtained and the corresponding melting curves are overlapping perfectly.

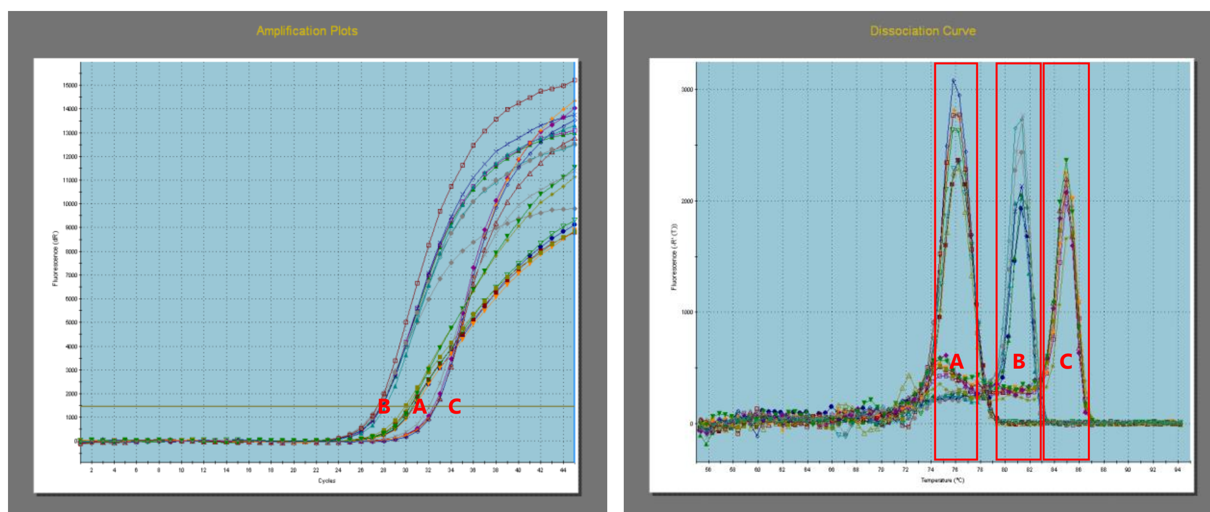

Figure 7: Ct (dR) values (left) and melting curves (right) for genes A, B and C from 8 independent single cell reactions

Results

The combination of the AmpliGrid system with the CellCelector platform opens the way for standardized cell screening and selection together with automated high-throughput single cell analysis. It was shown that single target cells could be selected from a mixed population and deposited on the AmpliGrid slide in a reproducible manner. The mix for reverse transcription and a sealing solution was loaded at the same time minimizing hands-on times and costs. In a subsequent RT-qPCR analysis the single cells showed reliable results for ct values and melting temperatures.

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Notes:

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