

Identifying virus copies in single cells

The superior single cell sensitivity of the AmpliGrid system allows the detection of single genome copies in single infected cells. Using standard optical microscope systems individual cells can be monitored prior to PCR which leads to reproducible single cell PCR results. Single cell sensitivity is even possible in a high background of non-infected cells. The present application note describes the detection of spiked-in HPV (human papillomavirus) sequences in a cell culture as a model system showing the high potential of the AmpliGrid technology.

Norman Häfner and Matthias Dürst, Gynäkologische Molekularbiologie, Frauenklinik FSU Jena, Bachstr. 18, 07743 Jena

In the note presented here we show the single molecule sensitivity of the AmpliGrid and its ease of use for validation of a specific PCR application. In addition, the detection of single infected cells is shown in a high background of non-infected cells making the AmpliGrid an ideal tool for screening large cell populations.

Cells and deposition

SiHa cells are an established cell line derived from a HPV16 positive squamous cervical carcinoma. Each cell contains a single partially deleted HPV16 genome integrated at 13q21-31. In most cells the chromosome affected by integration is duplicated.

For the experimental procedure prepare SiHa cells in ThinPrep® solution (Cytoc Corp., Germany), wash two times in 0.05x phosphate buffered saline and stain with Hoechst dye (making the nucleus visible). Micromanipulation for single cell deposition is carried out with 20µm capillaries on a Patchman system (Eppendorf, Germany) that is connected to an Olympus Microscope IX81.

Non-infected, HPV negative C33 cells are placed in known amounts onto reaction sites of the AmpliGrid by means of limiting dilution series. The total volume per reaction site was 1µl. After 10 minutes at room temperature, spots are dried completely (no volume visible). The presence of individual cells can be determined by identifying individual nuclei on the AmpliGrid (fig. 1, 3).

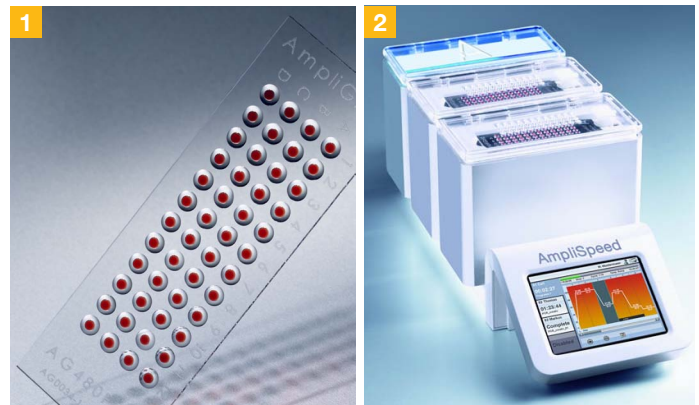


Fig. 1: AmpliGrid AG480F slide; Fig. 2: AmpliSpeed ASC200D slide cycler

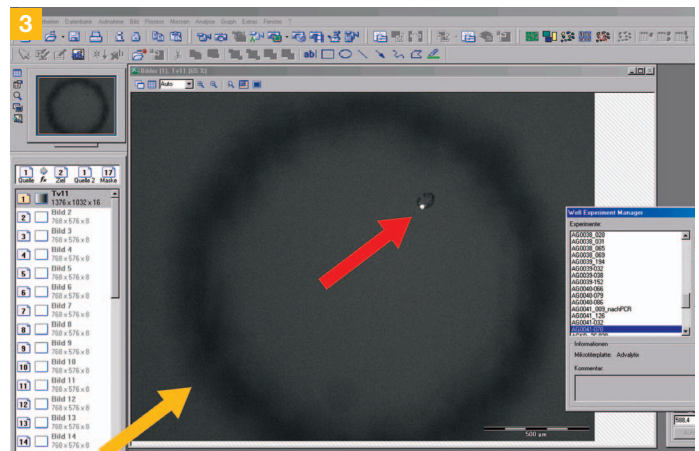


Fig 3: Single cell made visible in one of the reaction sites

PCR:

Perform PCR on single SiHa cells using two primer sets that amplify specific regions of E6 and E2 genes yielding PCR products of 124bp and 110bp (E6-F 5'-AATGTTTCAGGACCC-ACAGG-3'; E6-R 5'-CTCACGTCGCAGTAACTGTTG-3'; E2-F 5'-GCCAACGTTTAAATGTGTGTCAG-3'; E2-R 5'-CAC-ATTCTAGGCGCATGTGTTT-3'). Negative controls have been run with different numbers of C33 cells.

After deposition of the cells primer solutions (0.3µM each) are also placed on the reaction sites and dried down. The master mix for PCR contains the following:

A Table A: PCR master mix

Component	Volume / reaction site
2x Qiagen Multiplex PCR Master Mix	0.67 μ L
Q-Solution, 5x	0.08 μ L
ddH ₂ O	0.75 μ L
Total volume	1.5 μL

Cover each reaction site with 5 μ L of sealing solution. For PCR use an AmpliSpeed slide cycler using the following programme:

B Table B: PCR programme

Temperature	Duration	
95 °C	10 min	
95 °C	30 sec	
58 °C	60 sec	40 cycles
72 °C	60 sec	
72 °C	10 min	

After PCR is finished, pipette 4 μ L of 1x gel loading dye on top of the sealing solution of each reaction site of the AmpliGrid. The aqueous phase mixes within a second and the total reaction volume can easily be retrieved and loaded on an 8% PAA gel. Gel electrophoresis is carried out in 1x TBE followed by fast silver staining.

Results:

Amplification and detection of single HPV sequences is shown in Fig 4. Both fragments of expected sizes can be amplified with single cells as target. Negative controls (genomic background without integrated virus) do not generate any specific PCR product.

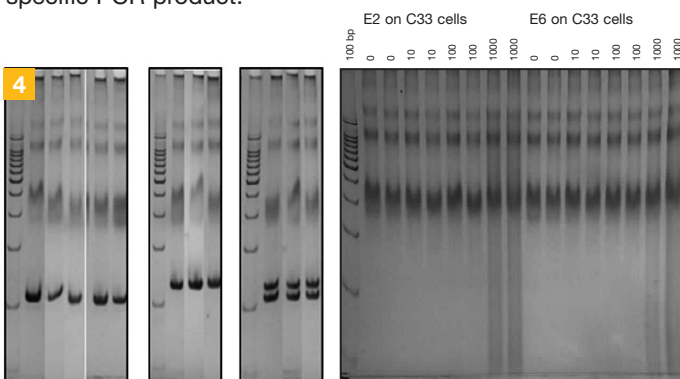


Figure 4: Results of HPV specific PCR on single SiHa cells; A: E2 specific amplification products; B: E6 specific amplification products; C: coamplification using combination of primer pairs; mol weight marker: 100bp ladder); D: negative control with different amounts of C33 cells, amplification of E2 or E6

In addition, single SiHa cells are amplified after spiking them into volumes containing various amounts of HPV negative C33 cells. The numbers of C33 cells are estimates based on limiting dilution. Results of spike in experiments are given in Fig. 5.

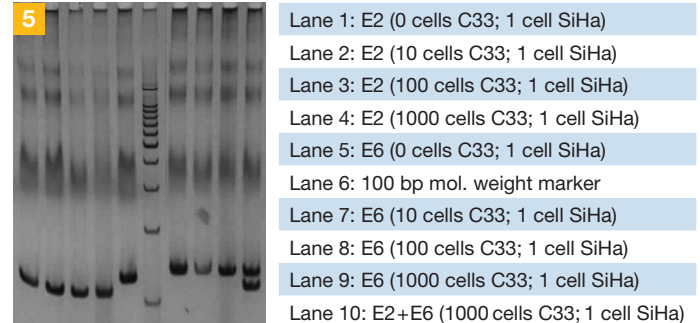


Figure 5: Results of spike in experiments

Clearly, the sensitivity of the AmpliGrid system allows the detection of single viral sequences in single cells even against a thousand fold background of non-infected cells.

Prospect:

HPV specific PCR on the AmpliGrid enables the detection of two viral genes, E2 and E6, from one or two HPV16 genomes in single SiHa cells in a background of up to 1000 non infected C33 cells. Using a convenient workflow on the AmpliGrid system the potential for a broad range of research applications is shown.

Contact:
norman.haefner@med.uni-jena.de; Matthias.Duerst@med.uni-jena.de

Patents:
Polymerase Chain Reaction (PCR) process is covered by patents which are owned by Hoffmann-La-Roche Inc. and F. Hoffmann-La-Roche Ltd.
ThinPrep®: ThinPrep® Pap Test PreservCyt® solution (Cytoc Corp, Germany)

For reseach use only. Not for use in diagnostic procedures.