

# Transfection-to-PCR without extraction

## Cultivation of cells, transfection and quantitative gene expression analysis using the Advalytix AmpliCell.

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Preparation of cells for gene expression analysis can induce a stress response in cells during extraction and handling processes, and such stress may affect gene expression patterns. In addition, current methods employing high volume culture flasks and spin-column extractions are time-consuming.

With the innovative AmpliCell system, it is now possible to cultivate, treat and analyze cells by PCR on a single platform, minimizing cellular stress. We show a quantitative gene expression analysis on HeLa cells after cultivation and transfection of siRNA offering a simple and seamless workflow.

### Introduction

The Advalytix AmpliCell – cell culture & PCR system is based on the AmpliGrid 1  $\mu\text{L}$  reaction slide. The 48 hydrophilic reaction sites of the AmpliCell are treated with fibronectin to create an optimal surface for attachment of adherent cells. The hydrophobic regions outside the reactions sites are covered with a special foil that is easily removed for downstream PCR analysis. Disposable chambers for cell culture medium are pre-mounted on the slide to allow optimal growth conditions for the cells of interest.

In the current example, we are focusing on siRNA targeting the viral E6 anti apoptotic oncogene in HeLa cells (HPV18 positive cervical carcinoma epithelial cells). E6 is known to degrade the tumor suppressor p53 via the ubiquitin pathway leading to the inhibition of apoptosis in peculiar cells by p53.

HeLa cells are deposited on the AmpliCell and grown in a monolayer to allow for proper proliferation. Cells are subsequently transfected with siRNA using a commercial transfection reagent. Reverse transcription takes place on the AmpliCell in a 1  $\mu\text{L}$  reaction volume followed by quantitative analysis using the Stratagene® Mx3005P® real-time PCR instrument. Knockdown of viral E6 expression normalized with the housekeeping gene Calm is shown in a time course.

### Experiment

Seed  $3 \times 10^3$  HeLa cells in D-MEM® supplemented with 10% FCS (fetal calf serum) and supplemented antibiotics kanamycine and gentamycine on each AmpliCell chamber (figure 1 A).

1 Figure 1: AmpliCell and AmpliSpeed slide cyclers ASC200D



Incubate for 24 hours at 37°C and 5% CO<sub>2</sub> with 100% humidity. Then transfect cells by using Lipofectamine™ RNAiMAX transfection reagent with control siRNA or siRNA according to manufacturer instructions (40 pmol siRNA) at a confluency of 60%. After transfection let cells grow for a time course of 6, 14, 24 and 48 h. Afterwards wash the cells by dipping the cells without chamber in 1x PBS, second 0.05x PBS and H<sub>2</sub>O. Dry the chambers by air and remove the foil for molecular analysis.

Deposit 1  $\mu\text{L}$  of reverse primer for amplification of E6 and Calm on the AmpliCell reactions sites in a concentration of 0.6  $\mu\text{M}$  each and let air-dry. Afterwards prepare the reverse transcription master mix using the Qiagen One-Step RT-PCR kit (see table A). Transfer 1  $\mu\text{L}$  master mix to each of the reaction sites and immediately cover with 5  $\mu\text{L}$  sealing solution.

A Table A: reverse transcription master mix

Component	1 reaction	30 reaction
5x Qiagen RT-Buffer	0.2 $\mu\text{L}$	6 $\mu\text{L}$
dNTP-mix (10 mM each)	0.04 $\mu\text{L}$	1.2 $\mu\text{L}$
Enzyme mix	0.04 $\mu\text{L}$	1.2 $\mu\text{L}$
RNase inhibitor (40 U/ $\mu\text{L}$ )	0.02 $\mu\text{L}$	0.6 $\mu\text{L}$
Q-Solution, 5x	0.16 $\mu\text{L}$	4.8 $\mu\text{L}$
Nuclease free water	0.54 $\mu\text{L}$	16.2 $\mu\text{L}$
<b>Total volume</b>	<b>1 <math>\mu\text{L}</math></b>	<b>30 <math>\mu\text{L}</math></b>

Transfer the AmpliCell slide to the preheated AmpliSpeed slide cycler (figure 1 B) and incubate 10 min at 42°C and 30 min at 58°C.

During incubation for reverse transcription prepare two separate master mixes for both Calm and E6 according to table B.

**B** Table B: qPCR master mix

Component	1x
2x SYBR® Premix Ex Taq™ (TaKaRa)	5 µL
Primer mix forward and reverse (6 µM each)	1 µL
Nuclease free water	3 µL
<b>Total volume</b>	<b>9 µL</b>

Transfer 9 µL of each master mix into each MTP well as schematically described in table C.

**C** Table C: Schematic drawing of the microtiter plate for the quantitative analysis; previous position on AmpliCell in red color; analysed gene shown in green color. The knockdown of E6 is shown in a time course (6, 14, 24, 48 h) with 6 replicates and two samples each. The same experiment is also done using control siRNA (amplification of E6 or Calm fragments).

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	A1 E6	A3 E6	A5 E6	B2 E6	B4 E6	B6 E6	C1 E6	C3 E6	C5 E6	D2 E6	D4 E6	D6 E6
<b>B</b>	A1 E6	A3 E6	A5 E6	B2 E6	B4 E6	B6 E6	C1 E6	C3 E6	C5 E6	D2 E6	D4 E6	D6 E6
<b>C</b>	A1 CALM	A3 CALM	A5 CALM	B2 CALM	B4 CALM	B6 CALM	C1 CALM	C3 CALM	C5 CALM	D2 CALM	D4 CALM	D6 CALM
<b>D</b>	A1 CALM	A3 CALM	A5 CALM	B2 CALM	B4 CALM	B6 CALM	C1 CALM	C3 CALM	C5 CALM	D2 CALM	D4 CALM	D6 CALM

After reverse transcription, add 4 µL 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 µL, adding 4 µL does cause the layer of cover oil 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 aspirate 4 µL of the diluted sample. Distribute 1 µL to each of the microtiter plate wells with the pre-deposited master mixes for E6 and Calm (column by column).

Close the MTP with optical capable lids and run the instrument as shown in table D.

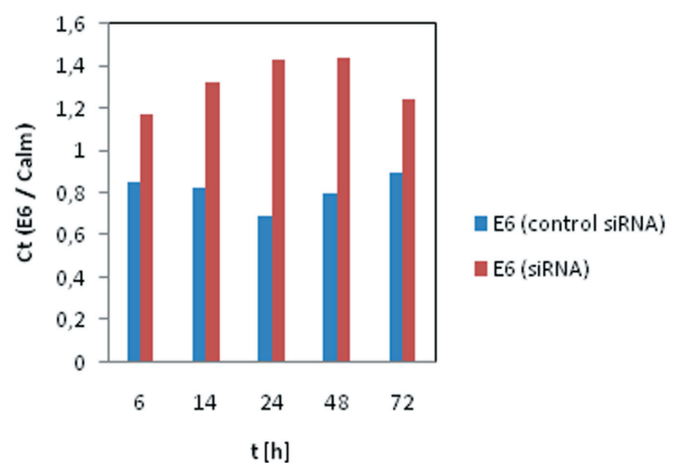
**D** Table D: Stratagene® Mx3005P® programme

Temperature	Time	Cycle
95°C	10 min	
94°C	30 sec	
62°C	60 sec	45 cycles
72°C	60 sec	
72°C	10 min	
25°C	1 min	

## Results:

The combination of the AmpliCell with the Stratagene qPCR platform opens the possibility to analyze gene expression in transfected cells minimizing cellular stress and time-consuming handling procedures. Figure 2 shows the  $C_t$  value ratio of the expression levels of human papillomavirus gene E6, which is stably integrated into the HeLa genome. The data are normalized for the housekeeping gene Calm. The gene expression of E6 is significantly reduced during the time course of 48 hours after transfection of the specific E6 siRNA – visible through the higher  $C_t$  value ratio.

**2** Figure 2:  $C_t$  value ratio of the expression levels of human papillomavirus oncogene E6 normalized for the housekeeping gene Calm. Each bar represents six replica with two samples each. The downregulation of E6 expression is shown (red bars); the control siRNA has no significant effect on the E6 expression (blue bars).



## Discussion:

The treatment of HeLa cells with E6 targeting siRNA causes a significant downregulation of E6 in the cells. The effect is visible already 6 hours after transfection and reaches a maximum of downregulation after 24 hours as expected. The data are normalized for the housekeeping gene Calm and the control siRNA shows no significant downregulation of E6.

This application report shows a convenient and seamless workflow including the cultivation of cells, transfection and quantitative gene expression analysis with the innovative platform AmpliCell.

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The AmpliGrid system is for research use only. Not for use in diagnostic procedures.