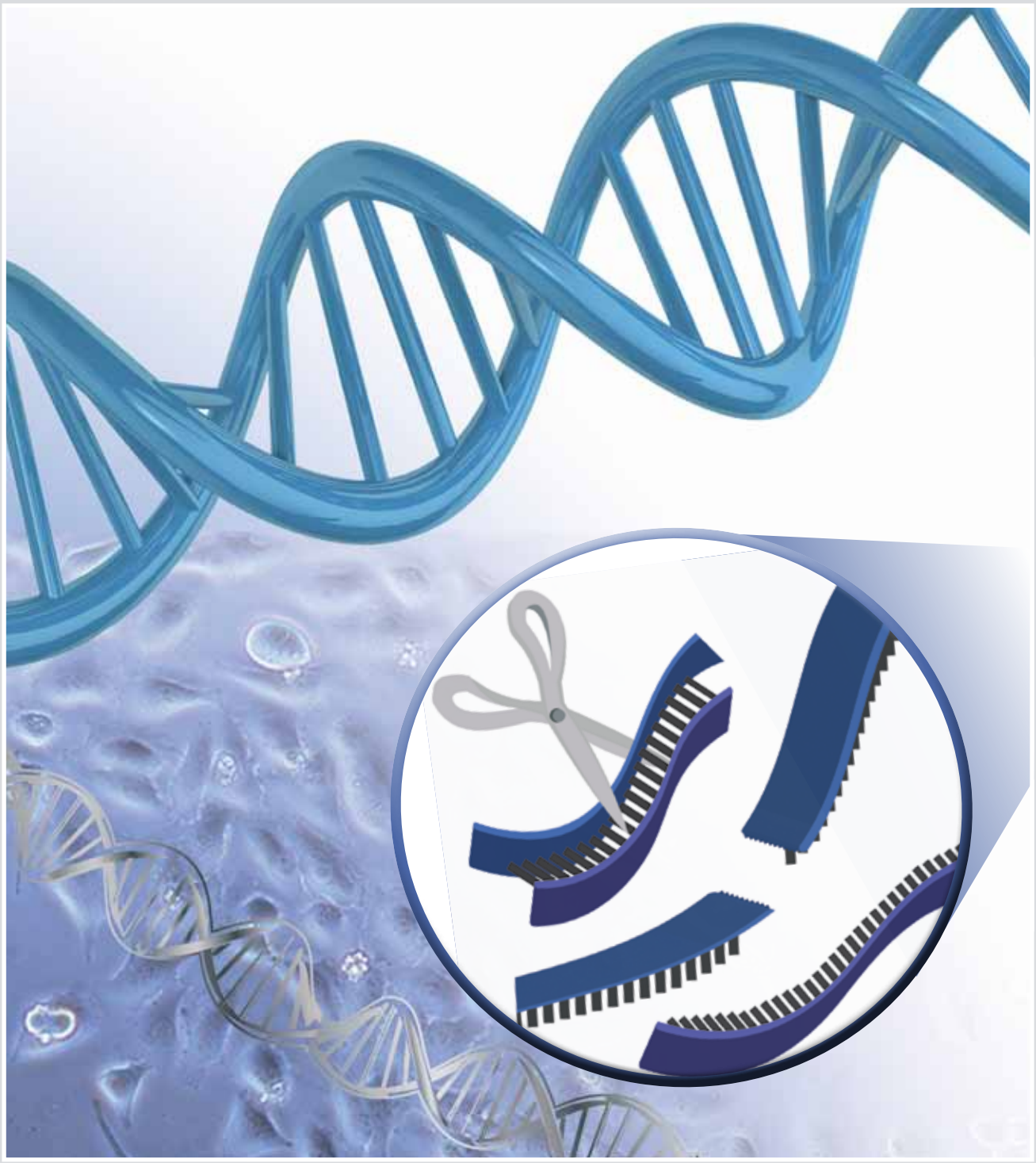
Application Note

siRNA dependent gene silencing in HeLa cells cultivated on various cell culture surfaces



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1 Introduction

1.1 RNA interference (RNAi)

One of the most important advances in biology in the last decades has been the discovery of RNA interference in nematodes by Fire and Mello in 1998 [1]. This ground breaking finding revolutionised the view of the role of RNA in the cell. Formerly known for its two main functions – as single stranded messenger RNA in transmitting information encoded on the DNA into a protein sequence and as ribosomal and transfer RNA having structural, catalytic and information decoding roles – the double stranded siRNA (small interfering RNA) plays a pivotal role in the regulation of gene expression [2].

This outstanding discovery not only provides a powerful tool to study gene function in basic research, but also opens up therapeutical approaches in diseases caused by misguided gene activity like cancer, autoimmune diseases and dominant genetic disorders [3].

Double stranded RNA primarily suppresses gene expression by a post-transcriptional mechanism. It is triggered by exogenous or endogenous, long double stranded RNA molecules (dsRNA) (**Fig. 1A**), which are processed by RNase III-like enzymes – called Dicer – resulting in the formation of short dsRNAs comprising ~21 nucleotides short (**Fig. 1B**). In the next step, the 'sense' strand of the siRNA is removed leaving the antisense strand, which is complementary to the target mRNA (**Fig. 1C**). The antisense strand assembles with the RNAi effector complex to form the RNA-induced silencing complex (RISC). Activated RISC consists of an Argonaute protein with endonuclase activity and the single stranded siRNA. Latter guides the complex to complementary sequences within mRNAs (messenger RNA) (**Fig. 1D**) to destroy the target mRNA (**Fig. 1E**) [4].The translation into the protein sequence is therefore inhibited.

In the following experiment HeLa cells were transfected with Cy3-conjugated GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) siRNA on Tissue Culture (TC), Advanced TC™ (AdvTC), Poly-D-Lysine (PDL) and Collagen type I-surfaces to evaluate the impact of the cultivation surface on the siRNA transfection efficiency. This correlation was already proved for transfection with pcDNA3 plasmid encoding GFP (Green fluorescent protein) and luciferase respectively in HEK293 cells in former studies [5].

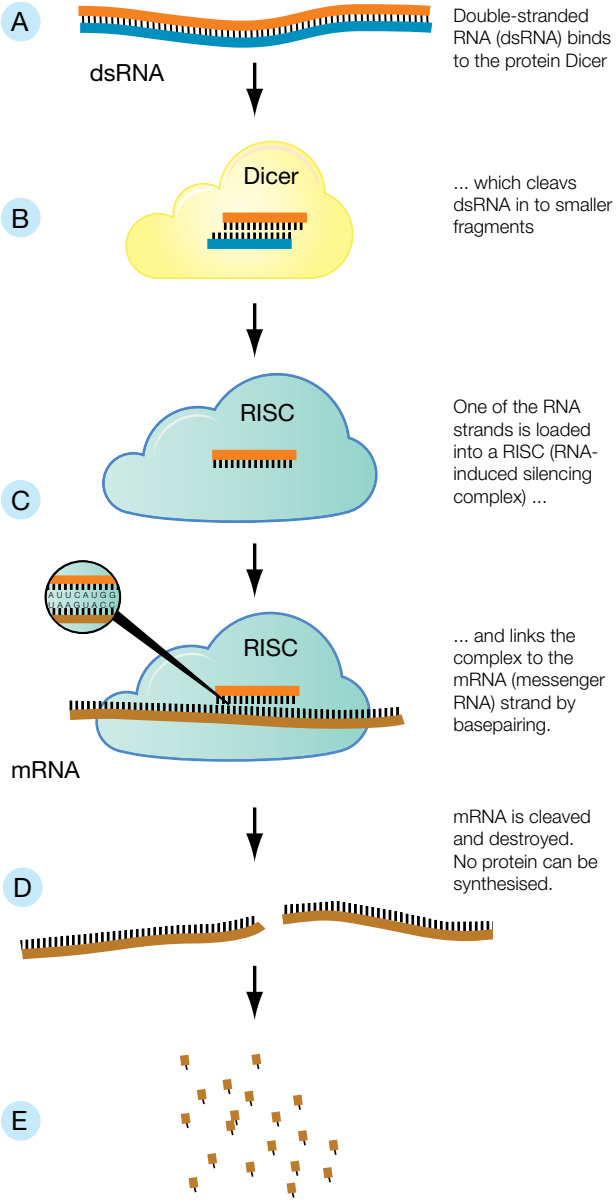

Figure 1: The RNAi mechanism [6]

1.2 Principle of the GAPDH assay

To study the siRNA dependent protein knockdown, intracellular levels of GAPDH were determined.
GAPDH is a tetrameric enzyme, which catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate (G-3-P) to bisphosphoglycerate (BPG). It is ubiquitously expressed at relative constant level in cells and is therefore an ideal siRNA target to study the influence of the cultivation surface on transfection efficiency of cells.
The used assay measures the conversion of NAD^+ to NADH by GAPDH in the presence of phosphate and G-3-P. The rate of NADH production is proportional to the amount of GAPDH enzyme present [7].

$$G\text{-}3\text{-}P + PO_4^{2-} + NAD^+ \xrightarrow{GAPDH} BPG + NADH + H^+$$

The efficiency of siRNA delivery was monitored by measuring the fluorescence signal from Cy3-labelled siRNA as well as by the reduction in GAPDH protein levels in cells transfected with GAPDH siRNA relative to cells transfected with a negative control siRNA. Furthermore a non transfected control permits conclusion about the cytotoxic effects of the transfection process when compared to the cells transfected with the negative control siRNA.

2 Material

Item	Manufacturer	Cat.-No.
Silencer®Cy™3 labelled GAPDH siRNA	Applied Biosystems/ Ambion	AM4649
Silencer®FAM™ labelled Negative control #1 siRNA	Applied Biosystems/Ambion	AM4650
siPORT™ NeoFX™ Transfection Agent	Applied Biosystems/Ambion	AM4511
KDalert™ GAPDH Assay Kit	Applied Biosystems/Ambion	AM1639
MEM Earle's	Biochrom AG	1073T
OptiMEM I Medium	Invitrogen	51985-026
Fetal calf serum	Biowest S.A.S	S1810-500
L-Alanyl-L-Glutamine	Biochrom AG	K0302
MEM-Amino acids	Biochrom AG	K0363
Filter tips, 100-1000 µL	Greiner Bio-One GmbH	750 288
Filter tips, 10-200 µL	Greiner Bio-One GmbH	772 288
96 well microplate, Advanced TC™, µclear, black	Greiner Bio-One GmbH	655 986
96 well microplate, TC, µclear, black	Greiner Bio-One GmbH	655 090
96 well microplate, TC, clear	Greiner Bio-One GmbH	655 160
96 well microplate, µclear, black, CELLCOAT® Collagen Type I	Greiner Bio-One GmbH	655 946
96 well microplate, µclear, black, CELLCOAT® Poly-D-Lysine	Greiner Bio-One GmbH	655 956
Polypropylene tubes, 15 ml	Greiner Bio-One GmbH	188 261
Light protection tubes, 15 ml	Greiner Bio-One GmbH	188 283

3 Methods

3.1 Transfection of HeLa cells with siRNA

A reverse high-throughput screening transfection protocol [8] was used to introduce siRNA into HeLa cells. First, siRNA was pipetted into the wells, followed by complexation with transfection reagent. Finally, siRNA complexes were overlayed with cells.

Cells were trypsinised before transfection using the standard procedure and resuspended in growth medium (Earle's MEM, 10 % FCS, 2 % Glutamine, 2 % non essential amino acids) at a density of 50,000 cells/mL.
0.3 µL of 10 µM of GAPDH and negative control siRNA was pipetted in triplets into the wells of the 96 well microplates (Tissue Culture, Advanced TC™, Poly-D-Lysine and Collagen Type I surface). Using a sterile tube a dilution of 0.5 µL transfection agent and 19.5 µL OptiMEM medium per well was prepared, incubated for 10 min and pipetted onto the siRNA. After 10 min of incubation at room temperature, 80 µL of cell suspension containing 4,000 cells were pipetted onto the siRNA and incubated for 48 h at 37 °C in the tissue culture incubator (**Fig. 2**).

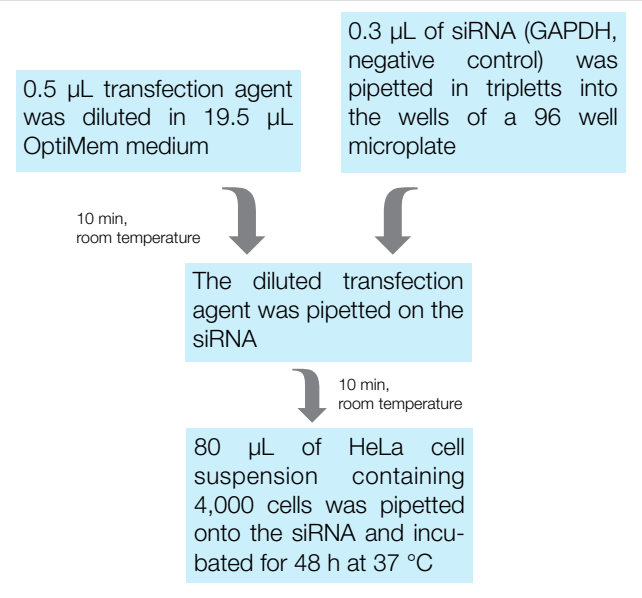

Figure 2: siRNA-transfection protocol

3.2 GAPDH assay

The efficiency of siRNA delivery and siRNA dependent gene silencing was monitored by measuring the reduction of the GAPDH protein levels of cells transfected with GAPDH siRNA relative to cells transfected with negative control siRNA.

GAPDH expression serves as a marker for cellular toxicity resulting from transfection. Putative cytotoxic effects can be identified by transfecting cells with negative control siRNA and evaluating whether the transfection leads to a decrease in endogenous GAPDH protein levels.

For the detection of GAPDH, medium was removed from the cells and replaced with 200 µL of lysis buffer. After 20 min incubation at 4 °C the lysate was homogenised by pipetting up and down. 10 µL were then transferred to a new transparent 96 well microplate. 90 µL of freshly prepared substrate were pipetted onto the cell lysate. Immediately afterwards a kinetic readout was performed for a period of 3 minutes using a fluorescence reader at 560/590 nm. The activity of GAPDH was calculated by subtracting the fluorescence at $t_{0\ min}$ from the endpoint measurement at $t_{3\ min}$ (see Figure 3).

After 48 h of incubation, siRNA containing medium was removed and 200 µL lysis buffer was pipetted on the cells

20 min, 4 °C

After homogenising cell lysate by pipetting up and down, 10 µL of the lysate was pipetted into a new 96 well plate

90 µL of substrate was mixed with the 10 µl cell lysate

Fluorescence at 560/590 nm was measured immediately ($t_{0\ min}$) and 3 min ($t_{3\ min}$) after mixing cell lysate and substrate. The increase in fluorescence correlated with the activity of GAPDH enzyme.

Figure 3: GAPDH assay

4 Results

4.1 Transfection efficiency in HeLa cells cultivated on various surfaces detected by Cy3 labelled siRNA

Cy3 labelled GAPDH siRNA was transfected in HeLa cells cultivated on tissue culture (TC), Advanced TC™, Poly-D-Lysine and Collagen Type I microplates according to a high-throughput screening (HTS) protocol. The transfection efficiency was detected based on Cy3 driven fluorescence via fluorescence microscopy after 48 h. On all surfaces a confluent monolayer of HeLa cells could be observed after transfection (Fig. 4 A-D). According to the fluorescence signal obtained using Cy3 labelled siRNA, an efficient transfection of GAPDH siRNA into HeLa cells occurred with all tested surfaces (Fig. 4 E-H). A comparison of the microscopic images obtained with the different cultivation surfaces indicates, that the transfection was most efficient with cells cultivated on the Advanced TC™ and Poly-D-Lysine surfaces. However, fluorescence was also detected with all other surfaces.

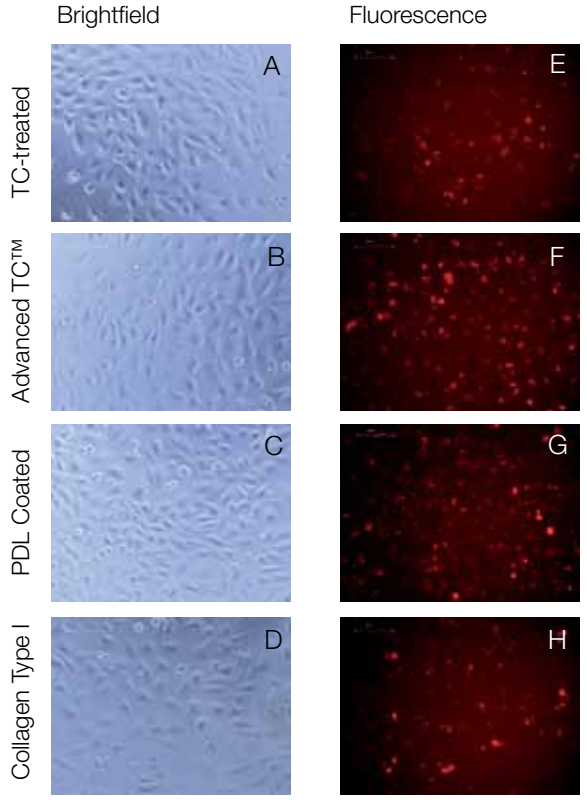

Figure 4: Transfection of Cy3-labeled GAPDH-siRNA in HeLa cells plated on tissue culture (A, E), Advanced TC™ (B, F), Poly-D-Lysine (C, G) and Collagen type I microplates (D, H). Brightfield images demonstrate the morphology of plated cells (A-D) while fluorescence pictures show the transfection rate of Cy3 labelled siRNA on different surfaces (E-H).

4.2 Evaluation of GAPDH knockdown caused by siRNA on the protein level

4.2.1 Influence of the cultivation surface on GAPDH mRNA silencing

The GAPDH protein knockdown of siRNA transfected HeLa cells was evaluated using a fluorescence based assay. Substrate formed by the oxidation of NAD^+ to NADH in the presence of G-3-P and phosphate by GAPDH was quantified based on its fluorescence at 560/590 nm over a 3 minute interval. GAPDH siRNA transfected cells were compared to cells transfected with a negative control siRNA and non-transfected cells. All transfections were conducted on Tissue Culture, Advanced TC™, Poly-D-Lysine and on Collagen type I microplates, respectively (Fig. 5).

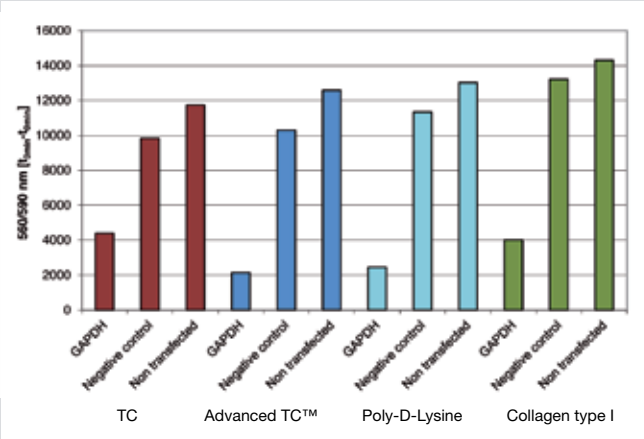

Figure 5: Evaluation of GAPDH knockdown by RNAi technology of HeLa cells plated on various surfaces. HeLa cells were transfected with siRNA against GAPDH mRNA and a negative control siRNA construct to show the influence of the surface on GAPDH protein knockdown. Non-transfected HeLa cells were used as a control to evaluate cytotoxicity of transfection itself. THE GAPDH activity was calculated by the increase of fluorescence at 560/590 nm at $t_{0\ min}$ and $t_{3\ min}$.

GAPDH knockdown was evaluated by comparing cells transfected with negative control siRNA and cells transfected with GAPDH siRNA. GAPDH knockdown was observed on all tested surfaces (for detailed results see table 1).

Table 1: Percentage of total GAPDH knockdown of HeLa cells transfected with GAPDH siRNA on different surfaces.

Surface	GAPDH knockdown [%]
Tissue culture	55.3
Advanced TC™	79.3
Poly-D-Lysine	78.4
Collagen Type I	69.6

Since the total knockdown reached 55.3 % for cells cultivated on the standard Tissue Culture surface, siRNA dependent protein knockdown was increased by 24 % to achieve a total knockdown of 79.3 % for cells cultivated on the Advanced TC™ surface. Similar results were obtained with the Poly-D-Lysine surface, where siRNA-dependent protein knockdown was increased by 23.1 % to achieve 78.4 % in total. Cells cultivated on Collagen Type I showed a 69.6 % total GAPDH knockdown response to siRNA, an increase of 14.3 % as compared to the standard TC surface (Fig. 6).

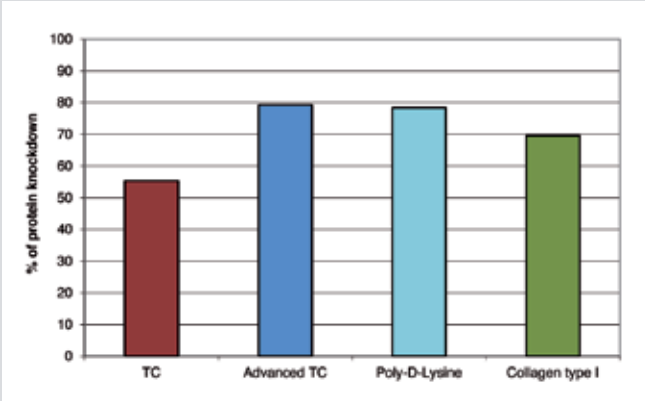

Figure 6: Graphic chart of the total GAPDH knockdown of HeLa cells transfected with GAPDH siRNA on Tissue Culture, Advanced TC™, Poly-D-Lysine and Collagen type I surfaces.

4.2.2 Cytotoxic effects of siRNA transfection on HeLa cells cultivated on different surfaces

By comparing GAPDH activity in non-transfected controls with cells transfected with negative control siRNA cytotoxic effects of the transfection procedure could be assessed. Cell viability is summarised in table 2.

Table 2: Cell viability of HeLa cells after transfection with siRNA measured by the GAPDH activity of transfected cells in comparison to untransfected cells.

Surface	Cell viability after transfection [%]
Tissue culture	83.8
Advanced TC™	87.7
Poly-D-Lysine	87.0
Collagen Type I	92.4

Cell viability of transfected HeLa cells varied from 83.8 % when cultured on Tissue Culture surface, 87.7 % on Advanced TC™ surface, 87.0 % on Poly-D-Lysine and 92.4 % on Collagen Type I surface (Fig. 7).

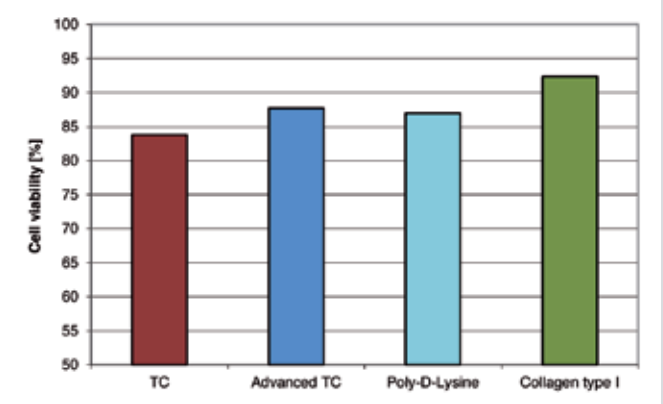

Figure 7: Cell viability of siRNA transfection in HeLa cells cultivated on different surfaces.

5 Conclusion

The influence of the culture surface on the efficiency of siRNA transfection and corresponding protein knockdown was evaluated in the performed experiment. For that reason GAPDH siRNA as well as negative control siRNA was transfected in HeLa cells cultured on Tissue Culture, Advanced TC™, Poly-D-Lysine and Collagen Type I surfaces.

Both transfection efficiency, measured using Cy3 labelled siRNA with fluorescence microscopy, as well as corresponding GAPDH knockdown, evaluated by protein assay, were determined to be the most effective when using Advanced TC™ and Poly-D-Lysine surfaces. Although the results indicate that these surfaces are the most suitable ones for siRNA transfection in HeLa cells, Tissue Culture and Collagen Type I surfaces also performed well in the described experiment.
Cytotoxic effects were further evaluated by comparing non-transfected HeLa cells to cells transfected with negative control siRNA. In general the cytotoxic effects of transfection were quite low on all tested surfaces. In summary, all tested cell culture surfaces were suitable for siRNA transfection in HeLa cells. An enhancement of transfection efficiency and protein knockdown was achieved with the use of the Poly-D-Lysine and the Advanced TC™ surface.

6 Literature

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4 Ameres er al. (2007): “Molecular basis for target RNA recognition and cleavage by human RISC”
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5 Application Note: “Enhanced transfection efficiency on protein coated microplates”
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7 Manual “KDalert™ assay”
Applied Biosystems/Ambion

8 Manual “siPORT™ NeoFX™ Transfection Agent“
Applied Biosystems/Ambion

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