

Functional Analysis of Side Effects of Transfection Reagents in the Context of Bax-induced Apoptosis

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1 Introduction

Transfection is a way to transiently or permanently deliver nucleic acids into eukaryotic cells to study functional effects in response to altered gene expression and for protein production. Liposomal and non-liposomal transfection reagents are the most commonly used carriers for nucleic acid transfer into mammalian cells. However, most transfection reagents exhibit considerable cytotoxic and other side effects on cells. Such cellular off-target responses, if not carefully evaluated, may lead to misinterpretation of transfection results and inaccurate conclusions. Thus, selection and evaluation of transfection reagents providing high transfection efficiency with minimal side effects is an important step preceding transfection experiments (2).

Apoptosis is one of the major mechanisms of cell death. The key players that execute the apoptotic cascade are the initiator and the effector caspases (cysteinyI-aspartic acid proteases), which are activated early in apoptosis. Two major pathways, the intrinsic and the extrinsic pathway, of apoptosis converge on the effector caspases. The extrinsic pathway is induced by binding of cytokine-like molecules to so-called cell surface death receptors that directly activate the caspase cascade via an initiator caspase within a death-inducing signaling complex (4, 5).

In contrast, the intrinsic cell death pathway, also known as the mitochondrial apoptotic pathway, is initiated by a range of signals, including radiation, cytotoxic drugs, cellular stress, and growth factor withdrawal. It involves the release of proteins from the mitochondrial membrane space, such as cytochrome c. These released proteins trigger a cascade of caspase activation, culminating in cellular destruction by cleaving hundreds of proteins within the cell, resulting in the morphological and biochemical changes typically associated with apoptosis (4, 5). Outer membrane permeabilization is mediated by the pro-apoptotic Bcl-2 protein family member and is considered as a “point of no return” for apoptotic cell death. Accumulation of

bax-like molecules in the outer mitochondrial membrane leads to their oligomerization and activation, which subsequently leads to the formation of proteolipid pores and depolarization of the mitochondrial membrane potential (3).

In this study, we made use of three different commercially available transfection reagents in a workflow involving different *in vivo* and *in vitro* assays (summarized in Figure 8). We show that despite comparable transfection efficiencies, only the use of the FuGENE® HD Transfection Reagent shows minimal cytotoxic effects, allowing the specific monitoring and detection of bax-induced cell death.

2 Methods and Material

Cell culture and transfection procedure

HeLa cells (ATCC) were grown in Minimum essential medium (Gibco), supplemented with 10% Fetal calf serum (PAN), 1% Non-essential amino acids (PAN), and 2 mM Glutamine (PAN). About 26 hours prior to transfection, cells were trypsinized, counted and seeded in 100 µl culture medium at a density of 5,000 cells per well into either a 96-well standard microtiter plate or an E-Plate 96 (Roche).

Transfections with FuGENE® HD Transfection Reagent (Roche), Lipofectamine 2000 (Invitrogen) and Lipofectamine LTX including the PLUS reagent (Invitrogen) were optimized using titration studies and performed according to the manufacturers' instructions. Briefly, the pEGFP and pEGFP-BAX plasmids (7) were diluted in OPTI-MEM I reduced serum medium (Gibco). The transfection reagents were added directly (or pre-diluted in OPTI-MEM I reduced serum medium) to the DNA-medium mixtures, gently agitated and incubated for 20 minutes. Different transfection experiments were conducted in replicates (at least triplicates) with 100 ng DNA per well. Transfection reagent-DNA complexes were added to the cells at a ratio of 8:2 for FuGENE® HD, of 2:1 for Lipofectamine 2000 and of 3:1 (including 0.1 µl PLUS reagent per transfection) in case of Lipofectamine LTX. Complexes were added to cells in the E-Plate 96 and rocked back and forth for 30 seconds. Cells were incubated at +37°C and 5% CO₂ in a humidified incubator.

Verification of protein overexpression by Western Blotting

About 45 hours post transfection, cells were harvested by trypsinization, pelleted and lysed in cComplete Lysis-M (Roche) for 5 minutes at room temperature. Lysates were cleared by centrifugation and supernatants stored at -80°C until further processing. Protein content of the supernatants was determined using the BCA protein assay kit (Pierce). Then 20 µg of protein samples were subjected to separation by electrophoresis on a 4 to 20% sodium dodecyl sulfate polyacrylamide gradient gel (Invitrogen), and subsequently analyzed by immunoblotting with a monoclonal primary antibody against GFP (Roche). Blocking material (skim milk powder), washing buffer (TBST), secondary antibody (anti-mouse-IgG-peroxidase) as well as peroxidase substrate solutions (stable peroxide solution and enhancer) were taken from the Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit) (Roche). The visualization of actin with a peroxide-linked anti-actin antibody (Abcam) served as loading control. The detection of EGFP, EGFP-bax fusion protein and actin occurred by means of a Lumi-Imager (Roche).

Immunofluorescence microscopy

About 45 hours post transfection, cells were evaluated for the extent of EGFP expression by immunofluorescence microscopy using a Zeiss Axiovert 200 M microscope (10x objective) to estimate the transfection efficiency of the different transfection conditions. Phase contrast and fluorescence images of the cells were taken and merged.

Impedance-based real-time cell analysis with the xCELLigence System

As indicated under “Cell culture and transfection procedure,” some of the cells were seeded in E-Plate 96, and the plate was mounted into a cradle of the station of an Real-Time Cell Analyzer (RTCA) MP Instrument (Roche) in a standard cell culture incubator. The attachment and logarithmic growth of the cells was followed for 26 hours by obtaining measurements once every 15 minutes. The cells were then transfected in replicates with vector constructs encoding EGFP or EGFP-bax fusion protein. DNA was complexed with one of the three different transfection reagents, either FuGENE® HD, Lipofectamine 2000 or Lipofectamine LTX. Cellular effects in response to the transfection were monitored for 45 hours as described above.

Detection of cell viability by the WST-1 Assay

At the end of the real-time cell analysis experiment, the viability of cells transfected by the various reagents was determined by adding 15 µl Cell Proliferation Reagent WST-1 (Roche) to each well of the E-Plate 96. The cells were incubated with the reagent at +37°C in humidified atmosphere and 5% CO₂ for 1.5 hours. One hundred microliters of the supernatants was transferred into a standard microtiter plate and subjected to absorbance measurements against medium as blank with a microplate reader (infinite M200, TECAN) at 437 nm. The reference wavelength was 690 nm. The background-corrected values were expressed in percent cell viability with respect to the result for non-transfected cells (control) set to 100%.

Quantification of cytotoxic effects by the Cytotoxicity Detection Kit^{PLUS} (LDH)

After finishing the real-time cell analysis experiment, several wells with non-transfected cells were defined as positive control and received 5 µl lysis solution from Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche). The plate was placed back into the cell culture incubator for another 15 minutes. Then 100 µl cell supernatant of each E-Plate 96 well was transferred into a normal microtiter plate. To determine LDH activity, 100 µl freshly prepared LDH Kit reaction mixture was added; samples were incubated, protected from light, for 20 minutes at room temperature. Afterwards, 50 µl stop solution were pipetted on top of all samples, and the absorbance was determined at 490 nm with a corresponding reference wavelength of 690 nm.

Determination of cell death by the Cell Death Detection ELISA^{PLUS} Kit

At the end of real-time cell monitoring, the E-Plate 96 was centrifuged for 10 min at 200 x g. Supernatants were aspirated, 200 µl lysis buffer was added to each well, and cells lysed for 30 minutes at room temperature. The plate was spun again at 200 x g for 10 minutes. Twenty microliters of the lysates were transferred into the streptavidin-coated microtiter plate included in the Cell Death Detection ELISA^{PLUS} Kit (Roche). To each well of this plate, 80 µl freshly prepared immunoreagent was pipetted. The plate was covered by an adhesive foil and gently shaken at room temperature for 2 hours. Afterwards, the solutions were carefully removed and wells washed 3 times with 300 µl incubation buffer. Following the addition of 100 µl ABTS solution, the plate was gently agitated for another 20 minutes. To finish the color development, 100 µl ABTS stop solution was added to each of the wells. Photometric analysis was performed using a microplate reader at a wavelength of 405 nm with a reference wavelength of 490 nm. Samples were measured and normalized against 100 µl ABTS solution plus 100 µl ABTS stop solution as background control.

Detection of apoptosis by the Homogenous Caspases Assay

Following the real-time cell analysis experiment, 100 µl freshly prepared substrate working solution from the Homogenous Caspases Assay (Roche) was added to each well of the E-Plate 96. Samples were incubated for 2 hours in the cell culture incubator. Then 200 µl of all the cell supernatants were transferred into a black 96-well microtiter plate. Fluorescence was determined using a standard microplate fluorescence reader (Spectra FLUOR, TECAN) with an excitation filter of 500 nm and an emission filter of 550 nm. Measurements were corrected by the value for 150 µl culture medium plus 100 µl substrate working solution as background control.

3 Results

Overexpression of EGFP and the EGFP-tagged pro-apoptotic protein bax in HeLa cells

pEGFP and pEGFP-BAX plasmids (7) were introduced into HeLa cells using the liposomal transfection reagents FuGENE® HD, Lipofectamine 2000, or Lipofectamine LTX. To verify the delivery of the DNA constructs into cells, 45 hours after transfection, cells were lysed, resolved on a polyacryl amide gel, and analysed by immunoblotting for the overexpression of EGFP. Figure 1 verifies the successful introduction of the EGFP (lane 2, 4, 6) and EGFP-BAX genes (lane 3, 5, 7) into the cells and the overexpression of the corresponding proteins in comparison to non-transfected control cells (lane 1). Independent of the transfection reagent, all transfection conditions resulted in a nearly comparable production of EGFP or EGFP-bax fusion protein.

Comparison of transfection efficiencies

Forty-five hours after transfection of HeLa cells with pEGFP or pEGFP-BAX using either FuGENE® HD, Lipofectamine 2000 or Lipofectamine LTX, cells were

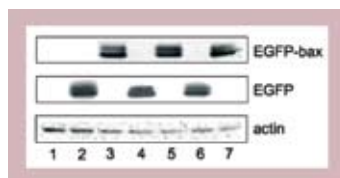


Figure 1: Immunoblotting for the amounts of EGFP or EGFP-bax in different transfectants. HeLa cells transiently transfected with pEGFP (lane 2, 4, 6) or pEGFP-BAX (lane 3, 5, 7), using either FuGENE® HD (lane 2, 3), Lipofectamine 2000 (lane 4, 5), or Lipofectamine LTX (lane 6, 7), were lysed in cOmplete Lysis M 45 hours after transfection. Lysates were cleared, resolved by sodium dodecyl sulfate polyacryl amide gel electrophoresis and analyzed by immunoblotting with an anti-GFP antibody. Further detection was performed with the Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit). Note: Equal loading of each lane was validated by Western Blot detection for actin.

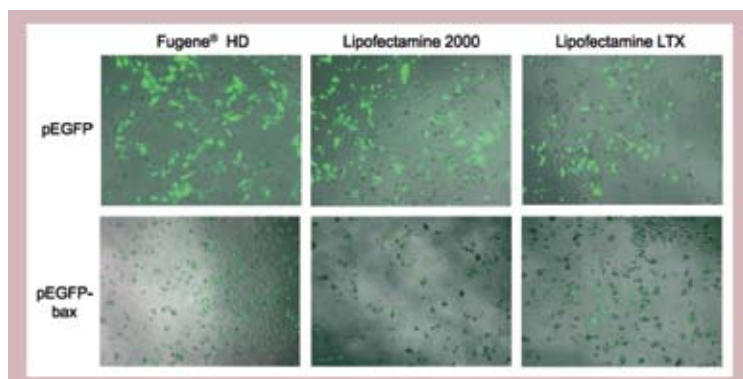


Figure 2: Estimation of the transfection efficiency and determination of protein localization by immunofluorescence microscopy. pEGFP (upper panels) or pEGFP-BAX plasmids (lower panels) were introduced into HeLa cells by means of either FuGENE® HD (left-hand panels), Lipofectamine 2000 (middle panels) or Lipofectamine LTX (right-hand panels). Forty-five hours after transfection, cells were viewed using immunofluorescence microscopy with a Zeiss Axiovert 200 M, 10x objective, and phase contrast and immunofluorescence images were merged. Merged images verified EGFP expression, localization of the protein, and permitted an estimation of the transfection efficiency after the different transfection procedures.

analyzed by immunofluorescence microscopy for the extent, location and distribution of EGFP expression. Merges of phase contrast and immunofluorescence images are represented (see Figure 2).

For all different conditions, the efficiency of transfection was appropriately high and estimated at about 80% for EGFP and 65–70% for EGFP-BAX. Whereas EGFP is present throughout the cytoplasm (see Figure 2, upper panels), the evaluation of bax expression was more difficult because the protein localizes to mitochondria. The fusion protein is evident as several microdot-like EGFP-positive structures within the same cell that were hard to discriminate from each other (see Figure 2, lower panel). In general, transfection efficiencies were in the range of what is stated in the manufacturer manual for each of the three different transfection reagents, however, somewhat lower in the case of Lipofectamine reagents compared to FuGENE® HD.

Analysis of transfection-dependent cellular effects by real-time cell analysis

Growing HeLa cells were transfected in replicates with pEGFP or pEGFP-BAX directly within the E-Plate 96, twenty-six hours after seeding. Monitoring of cell attachment, growth, as well as cellular responses to transfection were recorded using the xCELLigence System every 15 minutes. Cell index (CI) values were normalized to the last measured time point prior to transfection and plotted as means with standard deviations against the time, using the integrated RTCA Software (Figure 3A). Non-transfected control cells (red line) continue to grow exponentially, nearly reaching the plateau phase at the end of the experiment. Similarly, cells transfected with pEGFP based on FuGENE® HD (light green line in Panels 3A, 3B) were hardly affected by the transfection event.

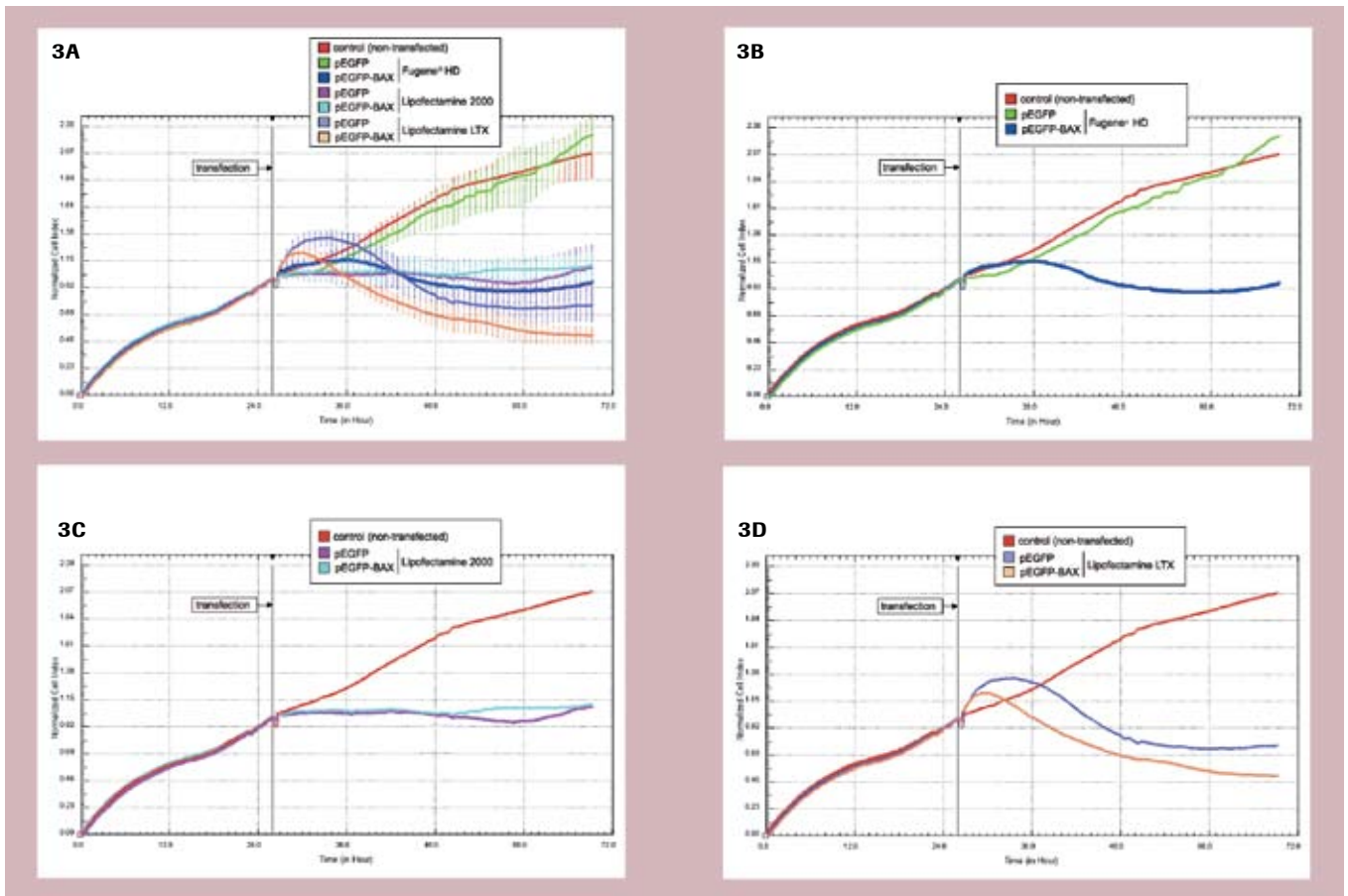


Figure 3: xCELLigence System real-time cell analysis of HeLa cell responses to transient transfections. HeLa cells (5,000/well) were seeded into an E-Plate 96, and cell attachment and growth were monitored using real-time cell monitoring every 15 minutes for 26 hours. Cells were then transfected in replicates in the E-Plate 96 with pEGFP or pEGFP-BAX plasmids using either FuGENE® HD Transfection Reagent, Lipofectamine 2000, or Lipofectamine LTX. Cell Index (CI) values were normalized to the last measurement before the transfection by the RTCA Software. Mean values with corresponding standard deviations were calculated by RTCA Software. All samples were run on the same plate (Panel 3A). To illustrate the differential effects of each reagent, the CI curves were separated out for cells transfected with FuGENE® HD Transfection Reagent (Panel 3B), Lipofectamine 2000 (Panel 3C), or Lipofectamine LTX (Panel 3D).

In contrast, the proliferation curve for cells transfected with the pEGFP-BAX plasmid and FuGENE® HD (blue line in Panel 3A, 3B) resulted in a clear separation from the control curve, six hours post transfection. From this time point onwards, CI values remained diminished, indicating successful overexpression of the pro-apoptotic EGFP-bax fusion protein. Increased concentrations of functional bax in cells are known to activate the mitochondrial apoptotic pathway without other factors (5). The morphological changes accompanying apoptosis, as well as the detachment of cells during apoptotic cell death, reduce cell contact to the gold microelectrodes on the bottom of the wells of the E-Plate 96, leading to a strong decline in impedance and corresponding CI values.

For the delivery of DNA constructs with Lipofectamine 2000, CI values remained at the same levels as at the time point of transfection. For the entire course of the experiment, no

curve separation could be detected (magenta and turquoise line in Panels 3A, 3C). Not even the introduction of the pEGFP control vector alone allowed the transfected cells (turquoise line in Panels 3A, 3C) to recover and proliferate again, indicating a general side effect of the transfection reagent itself. Transfection conditions applying Lipofectamine LTX gave similar results. Shortly after transfection, the proliferation curves are even higher compared to controls (violet and beige line in Panels 3A, 3D). For a short time period, the curves clearly separate from each other with lower CI values in case of pEGFP-BAX transfection (beige line in Panels 3A, 3D). Although this difference to the control plasmid is maintained until the end of the experiment, both curves show a sudden decline in CI values, well below values found in the Lipofectamine 2000- transfection experiments.

Reduction of cell viability

In order to verify that the observed CI values correlate with the total number of viable cells attached to the gold electrodes in the E-Plate 96 (1), and that in this particular study reduced CI values are due to cell death, an endpoint viability assay was conducted after completion of the real-time cell analysis experiment. The tetrazolium salt WST-1 was added directly to each well of the E-Plate 96 and incubated with the transfectants for 1.5 hours in a tissue culture incubator. In metabolically active cells, the WST-1 reagent is cleaved by succinate-tetrazolium reductase into a colored formazan product. Supernatants were analyzed for the color intensity using a standard spectrophotometer. Absorbance values were corrected by the background control (culture medium plus WST-1 reagent) and expressed in percent viability with respect to the non-transfected control sample, which was set as 100%. With the exception of cells expressing pEGFP using the FuGENE[®] HD Transfection Reagent, which only exhibited a moderate decrease in cell viability, all other cell samples show a strong reduction in viability of at least 50% compared to non-transfected control cells (see Figure 4). That means, in this study, that endpoint CI values are an accurate reflection of the number of viable cells in contact with the microelectrodes in the wells of an E-Plate 96. The xCELLigence System thus permits the continuous monitoring of the cellular responses to transfection, determining the appropriate time point for the performance of other functional assays.

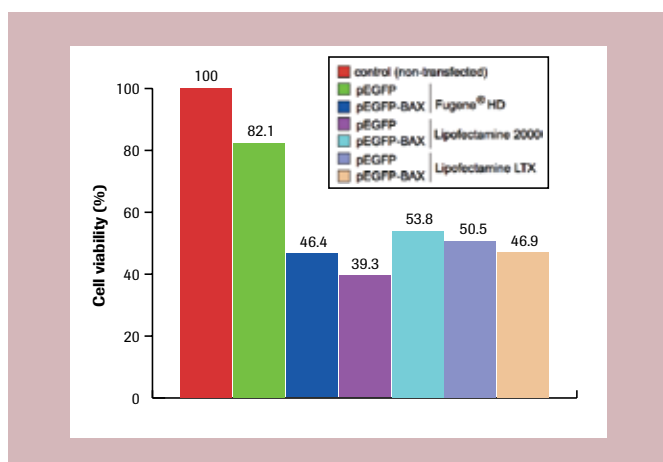


Figure 4: Relative cell viability determination using the WST-1 assay in the different transfected cell populations. WST-1 reagent was added to the culture medium of both transfected and non-transfected HeLa cells 45 hours post transfection. Color development was enhanced by incubating samples at +37°C. Color intensity was measured 1.5 hours later using a standard spectrophotometer. Absorbance values directly correlate with cell viability in the transfected populations. Results are represented in percent viability relative to non-transfected cells set at 100%.

Determination of the type of cell death – discrimination of apoptosis and necrosis

Endpoint viability assays and the xCELLigence System do not provide insight into the molecular mechanisms of cell death. There are two different ways by which eukaryotic cell death has been shown to occur: apoptosis and necrosis (6). To define which way the transfected HeLa cells died, they were subjected to additional functional assays.

1. Detection of LDH activity in cell-free culture supernatants (*i.e.*, measurement of necrosis)

A typical indicator of necrosis is cytolysis accompanied by the release of cytoplasmic molecules into the cell culture medium (6). The Cytotoxicity Detection Kit^{PLUS} (LDH) offers a simple way to measure plasma membrane damage, based on the release of lactate dehydrogenase (LDH), a stable enzyme present in most cells. LDH participates in a coupled reaction that converts a yellow tetrazolium salt into a red, formazan-class dye. The amount of formazan produced is directly proportional to the amount of LDH in the culture medium and is in turn directly proportional to the number of dead or damaged cells.

To determine if necrosis was the mechanism of cell death, the Cytotoxicity Detection Kit^{PLUS} (LDH) was run. At the end of the real-time cell analysis experiment, cell-free culture supernatants were taken from the E-Plate 96 and analyzed for the amount of LDH. The colorimetric assay was conducted using a standard spectrophotometer. Absorbance values are presented in a column diagram with respect to a positive control (detergent-lysed HeLa cells) in Figure 5A. In comparison to the positive control, the culture supernatant of non-transfected control cells hardly exhibits LDH activity. The same is the case for the FuGENE[®] HD transfectants. In contrast, cells transfected with Lipofectamine 2000 or Lipofectamine LTX show a 2- to 3-fold increase in LDH activity in their culture supernatants. Lipofectamine 2000 appeared to be a stronger plasma membrane-damaging reagent than Lipofectamine LTX. Figure 5B summarizes the extent of cytotoxicity (in percent of control) induced by the different transfection experiments, showing the average absorbance value for each of the different transfectants, relative to the positive control and non-transfected control.

2. Quantification of nucleosomes in the cytoplasm (*i.e.*, measurement of apoptosis)

During apoptosis, various endonucleases are activated and cleave the chromatin into nucleosome-sized fragments of 180 base pairs and multiples thereof (4). Nucleosomes are elementary units of chromatin formed by an octamer of histones and 146 base pairs DNA wrapped around it. The nucleosomes are connected by linker DNA of about 15 to

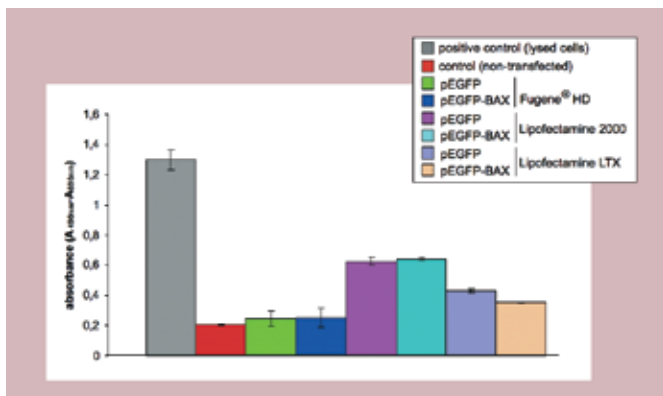


Figure 5A: Quantification of LDH release using the Cytotoxicity Detection Assay (LDH). HeLa cells were transfected in an E-Plate 96 as indicated. Forty-five hours later, replicates of non-transfected cells were lysed for 15 min in the tissue culture incubator, serving as positive controls. All cell-free culture supernatants were incubated with reaction mixture to induce the color production. Color intensity was determined using a spectrophotometer, and absorbance values show levels of LDH activity in the culture medium and are thus a direct measure of cell damage, cytolysis and necrosis.

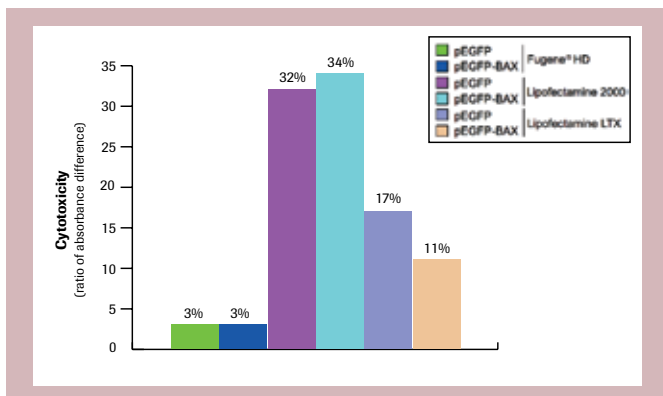
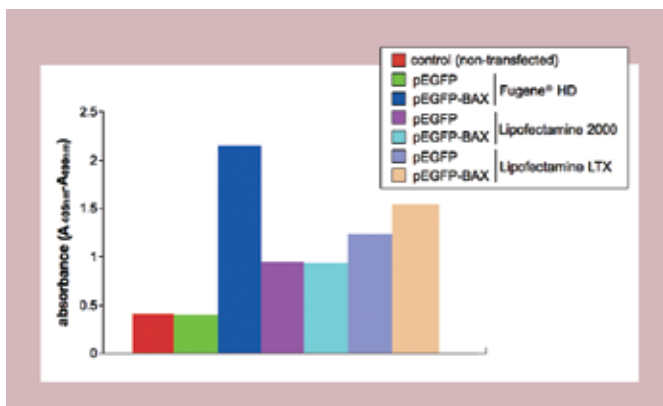


Figure 5B: Calculation of cytotoxicity based on LDH release, using data shown in Figure 5A. The extent of cytotoxicity was calculated from the ratio of the absorbance difference between transfected and non-transfected cells and the absorbance difference between the positive control and non-transfected cells. This calculation measures the cytotoxicity of each of the transfection reagents.



100 base pairs that are the preferential binding sites of the endonucleases. The Cell Death Detection ELISA^{PLUS} is a test to detect the enrichment of nucleosomes in the cytoplasm as a hallmark of apoptosis. The test is a quantitative sandwich enzyme immunoassay.

To determine if apoptosis was the mechanism of cell death, the Cell Death Detection ELISA^{PLUS} was run upon completion of the real-time cell analysis experiment. Cells were briefly pelleted and lysed in a mild lysis buffer that opens up the plasma membrane, but keeps the nucleic membranes intact. The cleared lysates were transferred into a streptavidin-coated microtiter plate and incubated with a mixture of biotinylated anti-histone antibodies and peroxidase-labeled anti-DNA antibodies. The antibodies bind to the histones and DNA of the nucleosomes, respectively, and the immunocomplexes are fixed to the coated microtiter plate by a streptavidin-biotin interaction. Bound peroxidase-linked complexes were subjected to a peroxidase substrate solution, resulting in color development proportional to the number of nucleosomes captured in the antibody sandwich.

Figure 6 shows the quantification of nucleosomes using spectrophotometric determination of the absorbance. Whereas control (red) and FuGENE[®] HD-pEGFP-transfected cells (light green) contained hardly any nucleosomes, the overexpression of EGFP-bax resulted in an about 6-fold increase of nucleosomes 45 hours post transfection. This clearly verifies the bax-dependent initiation of apoptosis in a specific manner. In contrast, the Lipofectamine transfections produced a moderate enrichment of nucleosomes in the cytoplasm of the cells. They exhibit both the criteria of necrotic cell death (e.g., cytolysis), and apoptotic cell death (e.g., DNA fragmentation). Interestingly, for these transfectants, the levels of LDH activity in the culture supernatant and the amounts of nucleosomes in the cytoplasm are proportionally reversed (see Figure 6).

Figure 6: Quantification of apoptosis by detecting nucleosomes using the Cell Death Detection ELISA^{PLUS}Kit. HeLa cells transfected using the three different transfection reagents (see text) were analyzed for the extent of apoptosis-induced nucleosome release. The abundance of nucleosomes in the cytoplasm was detected using anti-histone and anti-DNA antibodies in an enzyme-based colorimetric biotin-streptavidin binding immunoassay. Color intensity directly correlates with the amount of detected nucleosomes as determined by absorbance measurements using a spectrophotometer.

3. Determination of caspases activity

DNA fragmentation is a relatively late event during apoptosis. One of the earliest biochemical hallmarks of apoptosis is the activation of caspases that cleave several cellular proteins related to DNA repair and regulation (4, 6). The Homogeneous Caspases Assay is a fluorimetric one-step assay for the quantitative *in vitro* determination of caspase activity. The assay was performed in triplicates on cells derived from the real-time cell analysis experiment. Forty-five hours after transfection, the caspases substrate DEVD-R110 (Aspartic acid-Glutamic acid-Valine-Aspartic acid-Rhodamine 110) was added to each well of the E-Plate 96. During this incubation period, the cells were lysed to allow close proximity and interaction of the proteases with the substrate. DEVD-R110 is cleaved in proportion to the amount of activated caspases, forming free fluorescent R110 that is easily fluorometrically determined.

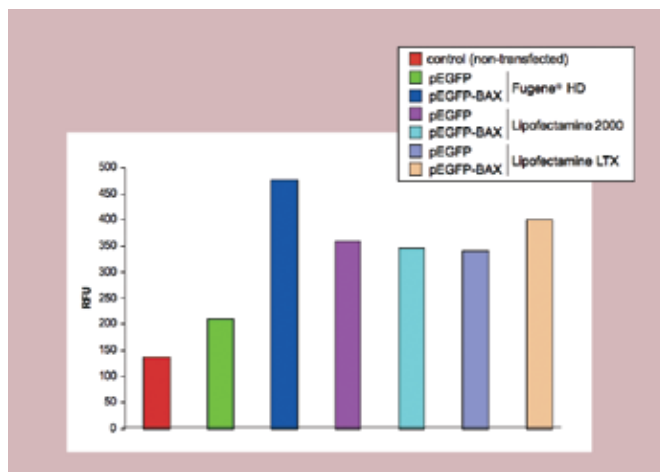


Figure 7A: Quantification of apoptosis by measuring caspase activation using the fluorimetric Homogenous Caspases Assay. HeLa cells transfected with pEGFP or pEGFP-BAX using three different liposome-based transfection reagents were analyzed 45 hours post-transfection for the activation of caspases, the characteristic apoptotic effector proteases. Caspase cleavage was detected by adding the appropriate caspase substrate, and its subsequent conversion into a fluorescent product. The latter was detected using a standard fluorescence reader. Caspase activity is expressed in relative fluorescent units (RFU).

The column diagram in Figure 7A summarizes the caspase activity in the different transfectants. The overexpression of EGFP by FuGENE® HD produced a minimal increase in caspase activity. In contrast, FuGENE® HD-based introduction of the pEGFP-BAX construct led to a 2.5-fold elevation of caspase activation, suggesting a strictly bax-dependent induction of the apoptotic pathway. In comparison to the control, all Lipofectamine-transfected samples showed a much higher activation of caspases. In contrast to FuGENE® HD Transfection Reagent, this up-regulation appears not to be due to a specific induction of apoptosis based on the overexpression of the EGFP-bax fusion protein, as the pEGFP alone appears to significantly increase caspase activation. Figure 7B also shows the strength of apoptosis initiation. The induction factor is defined as the ratio of the RFU (RFU = relative fluorescence units) signal of a transfectant and the RFU signal of the non-transfected control cells.

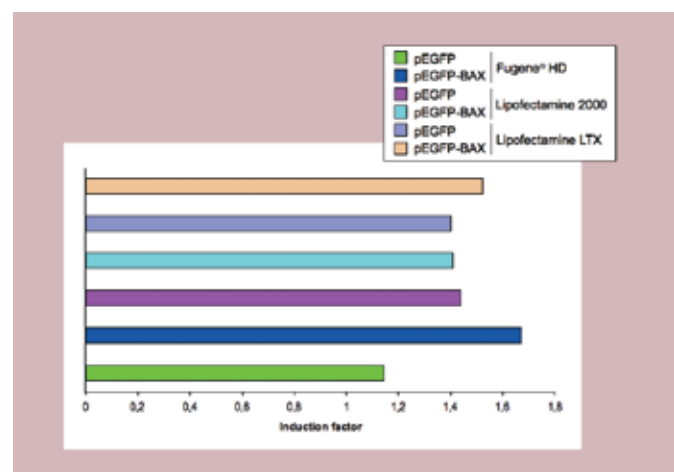


Figure 7B: Calculation of the apoptosis induction factor, using data shown in Figure 7A. The induction factor is expressed as the ratio of caspase activity (in RFU) for a certain transfectant and the caspase activity (RFU) in non-transfected control cells. It is a measure of the intensity of apoptosis induction.

4 Conclusion

The intention of the present study was to distinguish the cellular responses to transfection and subsequent protein overexpression from non-specific side effects induced by cytotoxicity of the transfection reagent (2). To do this, we used the xCELLigence System for cellular analysis and different functional assays in a workflow for the evaluation of three different commercially available liposomal transfection reagents. We transiently overexpressed the pro-apoptotic protein bax in HeLa cells, which localizes and accumulates in mitochondria, where it induces the apoptotic pathway by outer membrane permeabilization (5).

To verify the protein expression and determine transfection efficiencies, bax expressed as an EGFP-tagged fusion was compared to cells transfected with pEGFP only, which served as a control (7). HeLa cells were successfully transfected with pEGFP or pEGFP-BAX using either FuGENE[®] HD, Lipofectamine 2000, or Lipofectamine LTX. The extent of protein expression was nearly the same for the different transfection reagents. Transfection efficiencies were comparable for all transfection experiments, and proteins were appropriately localized within the cells.

Using the technology of the xCELLigence System for continuous, label-free impedance-based cell monitoring, it was possible to capture cellular responses non-invasively and in real-time (1). This allowed us to easily record the alterations in cell behavior due to transfection of the pEGFP or pEGFP-BAX plasmid with the three different gene delivery agents. In particular, the discrimination of cell death events due to the transfection procedure itself and reagent-based side effects due to specific bax-induced apoptotic cell death provided an illustrative example of the cytotoxic potential of the three different transfection reagents and a precise timeline of apoptosis. This helped to determine the optimal time points for the performance of additional *in vitro* assays for better defining the observed cell death events.

Our findings suggest that, independent of the vector, Lipofectamine 2000- and Lipofectamine LTX-transfectants were negatively affected by the transfection procedure itself. There was evidence for extensive cell death primarily due to the high cytotoxicity of both these transfection reagents. Cells transfected with pEGFP-BAX using the FuGENE[®] HD Transfection Reagent responded 6 hours after gene delivery, and their responses were clearly different from their EGFP-transfected control counterparts. The FuGENE[®] HD transfected EGFP cells proliferated with the same kinetics as non-transfected control cells, indicating that FuGENE[®] HD does not induce nonspecific side effects.

The WST-1 assay is a reliable way to quantify viable cells within a population and was therefore used to determine the extent of cell death after transfection. The results of this assay at 45 hours after transfection correlated with the other endpoint assay measurements (see below) and with the findings obtained using real-time cell monitoring. The reduction of CI values obtained using the xCELLigence System after transfections using the three different reagents was shown to be due to cell death leading to the detachment of dying cells from the E-Plate 96 microelectrodes. Interestingly, only FuGENE[®] HD-EGFP-transfectants showed a relatively high number of viable cells compared to non-transfected control cells.

To better characterize cell death in the different transfectants, we first used the Cytotoxicity Detection Kit^{PLUS} (LDH) to determine the extent of necrosis-based cell death. A typical feature of necrosis is plasma membrane disruption and cell lysis, leading to leakage of cytosolic proteins into the culture medium (6). The LDH assay quantifies cytosolic LDH in the cell-free supernatant. Our findings did not completely overlap with the WST-1 measurements, indicating that the cells transfected with the different transfection reagents did not die of the same type of cell death. Lipofectamine 2000- and Lipofectamine LTX-transfected cells contained significantly elevated levels of LDH activity in their culture supernatants, supporting the idea that these cells died of transfection reagent-induced necrosis (6).

The cell population transfected with pEGFP-BAX and the FuGENE[®] HD Transfection Reagent ultimately showed greatly diminished CI values and a large increase in non-viable cells. Since many of these cells did not die due to necrosis, all samples were screened using assays that are more specific for apoptosis. Both the Cell Death Detection ELISA^{PLUS} Kit and the fluorimetric Homogenous Caspases Assay verified that the FuGENE[®] HD BAX-transfectants died primarily due to apoptosis, and not necrosis. In contrast, only a fraction of the Lipofectamine-transfected cells appeared to have died by apoptosis. In particular Lipofectamine LTX transfectants appeared to have died largely due to necrosis. In conclusion, in contrast to our findings for Lipofectamine 2000 and Lipofectamine LTX, FuGENE[®] HD Transfection Reagent produced minimal cytotoxic side effects, while maintaining high transfection efficiency. As a result, FuGENE[®] HD Transfection Reagent enabled the study of the impact of the apoptosis-inducing bax protein, whereas the cytotoxicity and off-target effects of the Lipofectamine reagents made it impossible to distinguish the impact of the bax protein from the impact of the transfection reagent itself.

The workflow presented here (summarized in Figure 8) was straightforward and accurate in determining and characterizing the process of cell death after transfection of the bax gene into HeLa cells using three different commercially available transfection reagents. In principle, this workflow can be used for any study in which the

efficiency and the cytotoxicity of transfection reagents needs to be evaluated and compared. This workflow can be used and adapted to discriminate apoptosis and necrosis in response to overexpression of apoptosis genes as well as for testing external factors, such as experiments in compound- and cell-mediated cytotoxicity.

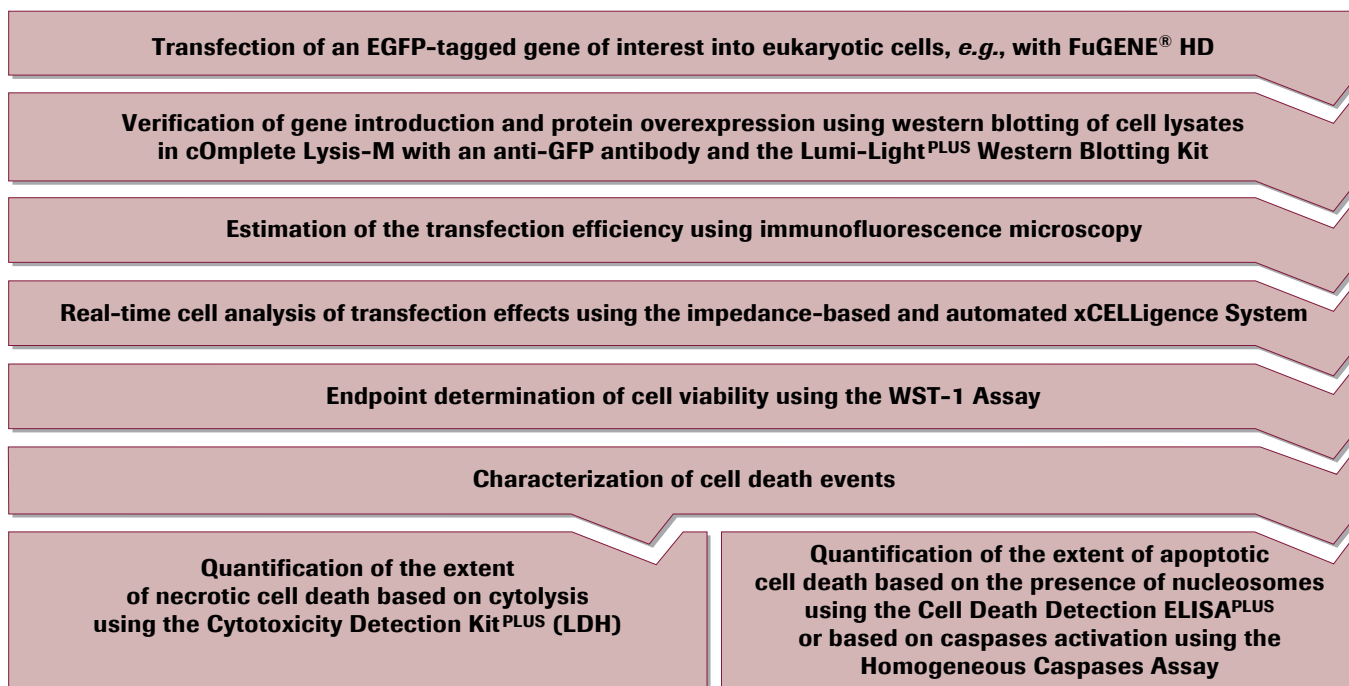


Figure 8: Workflow to determine the suitability of transfection reagents and their cytotoxic potential. Schematic representation of the different steps revealing the efficiency and eventual side effects of the three different transfection reagents used in the present study. The mechanism of cell death induced by each of the transfection reagents could be determined using the overexpression of BAX genes involved in cell cycle regulation, apoptosis and viability. Roche products are named and listed in the Ordering Information.

References

1. Abassi YA and Jackson JA. Real-time and dynamic monitoring of cell proliferation and viability for adherent cells. Application Note. 2008; 1:1-5
2. Calvin S, Wang J, Emch J, Pitz S, Jacobsen L. FuGENE® HD Transfection Reagent: Choice of a transfection reagent with minimal off-target effect as analyzed by microarray transcriptional profiling. Biochemica. 2006; 4: 22-25
3. Chipuk JE and Green RG. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 2008; 18(4):157-64.
4. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007; 35(4):495-516
5. Kang MH and Reynolds CP. Bcl-2 Inhibitors: Targeting Mitochondrial Apoptotic Pathways in Cancer Therapy. Clin Cancer Res. 2009;15(4):1126-32.
6. Leist M and Nicotera P. The shape of cell death Biochem Biophys Res Commun. 1997; 236(1):1-9
7. Neise D, Graupner V, Gillissen BF, Daniel PT, Schulze-Osthoff K, Jänicke RU, Essmann F. Activation of the mitochondrial death pathway is commonly mediated by a preferential engagement of Bak. Oncogene. 2008; 27(10):1387-96

Ordering Information

Product	Cat. No.	Pack Size
FuGENE® HD Transfection Reagent	04 709 691 001	0.4 ml
	04 709 705 001	1.0 ml
	04 709 713 001	5 x 1 ml
	05 061 369 001	10 ml
cOmplete Lysis-M	04 719 956 001	1 kit (200 ml lysis reagent, 20 cOmplete tablets)
Anti-GFP	11 814 460 001	200 µg
Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit)	12 015 218 001	1 kit (1,000 cm ² membrane)
RTCA SP Station	05 229 057 001	1 instrument
RTCA MP Station	05 331 625 001	1 instrument
RTCA Analyzer	05 228 972 001	1 instrument
RTCA DP Analyzer	05 469 759 001	1 instrument
RTCA Control Unit 1.1	05 454 417 001	1 notebook (with preinstalled RTCA Software)
Cell Proliferation Reagent WST-1	05 015 944 001	8 ml (1,000 tests)
	11 644 807 001	25 ml (2,500 tests)
Cell Death Detection ELISA^{PLUS}	11 774 425 001	96 tests
Cytotoxicity Detection Kit^{PLUS} (LDH)	04 744 926 001	1 kit (400 tests)
	04 744 934 001	1 kit (2,000 tests)
Homogeneous Caspases Assay, fluorimetric	03 005 372 001	1 kit (100 tests on 96-well plates, 400 tests on 384-well plates)
	12 236 869 001	1 kit (1,000 tests on 96-well plates, 4,000 tests on 384-well plates)

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