

# Workflow for Determining the Apoptotic Effects of Caspase-8 Overexpression in HeLa Cells

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## 1 Introduction

Apoptosis, or programmed cell death, involves a large number of complex interacting cellular components. Prominent among those components is the highly conserved caspase family of proteases.

Caspases are involved in the initiation and execution of apoptosis (1). They are classified into two subgroups, initiator caspases (*e.g.*, caspase-2, -8, -9, and -10 in mammals) and effector caspases (*e.g.*, caspase-3, -6, and -7 in mammals) (2). These proteases are synthesized as inactive zymogens, which are selectively activated in response to changing environmental conditions. Of particular interest is the activation of the initiator caspase, caspase-8. Active caspase-8 cleaves and activates the effector caspase, caspase-3, starting a cascade of events that ends in cell death (3).

In the current study, we use a workflow involving both *in vitro* and *in vivo* assays to demonstrate that overexpression of caspase-8 alone is sufficient to induce apoptosis in HeLa cells.

## 2 Methods

### 1. Preparation and transfection of a caspase-8 expression plasmid

#### A) Plasmid preparation

The eukaryotic expression vectors pRK5 (BD Biosciences), pRK-Casp8 (containing the human caspase-8 gene cloned into pRK5), and pRK-GFP (containing the enhanced variant of the Green Fluorescent Protein EGFP gene cloned into pRK5) were purified from *E. coli* XL1-Blue using the Genopure Plasmid Maxi Kit (Roche). Three vials with 3 ml LB medium were each inoculated with 100 µl frozen glycerol stocks of XL1-blue bacteria containing the respective plasmids and grown at +37°C with vigorous shaking for 8 hours. Each of the 3 ml precultures was added to 200 ml LB medium containing 100 µg/ml ampicillin and the 200 ml cultures were incubated overnight at +37°C with vigorous shaking. The DNA preparation was performed as described in the Genopure Plasmid purification manual. Yields were 280 µg (pRK), 860 µg (pRK-GFP), and 480 µg (pRK-Casp8).

#### B) Cell culture and FuGENE® HD Transfection Reagent

HeLa cells (ATCC® CCL-2™) were grown in DMEM (without HEPES), supplemented with 10% fetal calf serum, 10 mM glutamine, and 50 µg/ml each of streptomycin and penicillin. Twenty-four hours prior to transfection, cells were trypsinized and seeded into 6-well plates at a density of  $2 \times 10^5$  cells/well. For each transfection mixture, 2 µg expression plasmid (pRK, pRK-GFP or pRK-Casp8) was mixed with 100 µl Opti-MEM, then 6 µl FuGENE® HD Transfection Reagent (Roche) was added. After a 15-minute incubation at room temperature, the transfection mixture was added dropwise to one well containing cells. Plates were incubated for up to 48 hrs at +37°C under 5% CO<sub>2</sub>.

### 2. Verification of gene expression after transfection

#### A) Estimate of GFP transfection efficiency by flow cytometry

Forty-eight hours after transfection, cells transfected with pRK-GFP were detached using trypsin, washed once with PBS and resuspended in 200 µl PBS. For control purposes, cells transfected with pRK5 were processed in parallel. Analysis for GFP-positive cells was performed on a FACSCalibur™ flow cytometer (BD Biosciences) using the 488 nm laser and the FL1 channel.

#### B) Detection of caspase-8 overexpression by western blotting

HeLa cells transfected with pRK-Casp8 were grown for 24 hours, then detached by trypsinization, washed once with PBS, and resuspended in 200 µl cComplete Lysis-M Reagent (Roche). For control purposes, untransfected cells and cells transfected with pRK5 were processed in parallel. After sedimentation of cell debris by centrifugation (5 min, 14000 rpm, +4°C), protein concentrations were determined using Coomassie Blue.

For each sample, 20 µg protein was separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane by blotting. After the membrane was blocked and washed 3x with PBS, it was incubated for 3 days at +4°C with a 1:1000 dilution of a mouse monoclonal anti-caspase-8 antibody (Cell Signaling Technologies) in PBS/5 % BSA. After 3 washes, the membrane was incubated with a POD-coupled rabbit anti-mouse antibody in PBS/5% BSA (antibody concentration, 0.008 µg/ml) for one hour at room temperature. After 5 washes, bound secondary antibody was detected by incubation with Lumi-Light<sup>PLUS</sup> Western Blotting Substrate (Roche) and subsequent exposure to photographic film, as described in the Lumi-Light<sup>PLUS</sup> Western Blotting manual.

### 3. Assays of metabolic changes in HeLa cells transfected with a caspase-8 expression plasmid

#### A) Determination of cellular metabolism with WST-1

HeLa cells ( $1 \times 10^4$  cells per well) were seeded on 96-well plates. Triplicate wells were either left untransfected or were transfected with one of the three expression plasmids (pRK5, pRK-GFP or pRK-Casp8; 0.1  $\mu$ g plasmid and 0.25  $\mu$ l FuGENE<sup>®</sup> HD Transfection Reagent per well). Four, 24, or 48 hrs post-transfection, 10  $\mu$ l WST-1 Cell Proliferation Reagent (Roche) was added to one well from each set of wells. Incubation continued for an additional 60 minutes at +37°C, then the amount of soluble WST-1 formazan formed in the test wells was determined spectrophotometrically as described in the WST-1 package insert.

#### B) Caspase-3 assay

HeLa cells transfected with pRK-Casp8 were grown for 24 hours, then detached by trypsinization, washed once with PBS, and resuspended in 200  $\mu$ l lysis buffer supplied in the Caspase 3 Activity Assay Kit (Roche). For control purposes, untransfected cells and cells transfected with pRK5 were processed in parallel. After the protein concentration of the lysates was determined using Coomassie Blue, 250  $\mu$ g protein was removed from each lysate and assayed fluorimetrically for caspase-3 activity as described in the Caspase Activity Assay manual. Positive control lysate and two dilutions of AFC (fluorescent assay product), all supplied in the assay kit, were also included as controls.

### 4. Apoptosis assays for HeLa cells transfected with a caspase-8 expression plasmid

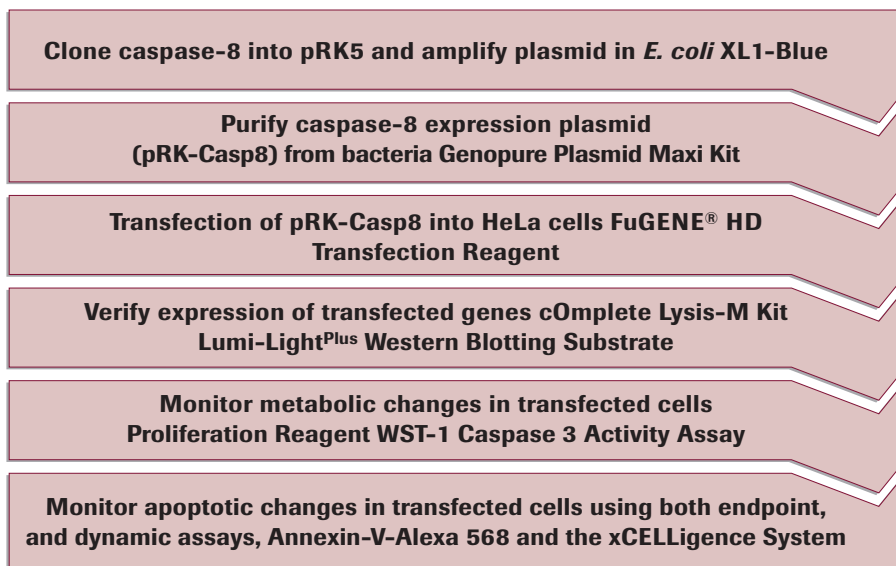
#### A) Flow cytometric detection of apoptosis with Annexin-V-Alexa<sup>™</sup> 568

HeLa cells transfected with either pRK-Casp8 or pRK5 were grown for 48 hours, then detached by trypsinization, washed once with PBS, and resuspended in 100  $\mu$ l incubation buffer (Hepes-NaCl-CaCl<sub>2</sub>), prepared as described in the package insert of the Annexin-V-Alexa 568 Kit (Roche). After resuspended cells were stained with Annexin-V-Alexa 568 (2  $\mu$ l/sample), they were analyzed using FACSCalibur<sup>™</sup> flow cytometer, with the 488 nm laser and the FL3 channel.

#### B) Real-time monitoring of apoptosis induction with the xCELLigence System

Approximately 4000 HeLa cells were seeded into each well of an E-Plate 96. The plate was mounted into the cradle of an xCELLigence Real-Time Cell Analyzer (RTCA) SP Instrument, and cells were allowed to grow for 26 hours at +37°C under 5% CO<sub>2</sub>. Cells were then transfected in triplicate with one of three expression vectors (pRK5, pRK-GFP, and pRK-Casp8) directly on the E-Plate 96, as described above for the WST-1 assay (see section 3.A), and incubated for an additional 48 hours. Cell impedance measurements were performed once every 15 minutes for the entire 72-hour course of the experiment. The resulting Cell Index (CI) values were produced by the RTCA software (see Figure 1).

**Figure 1:** Workflow to determine the apoptotic effects of caspase-8 overexpression in HeLa cells. Schematic representation of the steps for determining what happens when a caspase-8 expression plasmid is introduced into HeLa cells.



### 3 Results

#### 1. Efficient introduction of a human caspase-8 gene into HeLa cells using the FuGENE® HD Transfection Reagent

Expression plasmids carrying either EGFP (pRK-GFP) or the caspase-8 (pRK-Casp8) gene were prepared using the Genopure Plasmid Maxi Kit and transfected into HeLa cells with FuGENE® HD Transfection Reagent.

To estimate the transfection efficiency, pRK-GFP-transfected cells were analyzed by FACS (see Figure 2). More than 88% of transfected cells were GFP-positive 48 hours after transfection.

Successful transfection and activation of caspase-8 was established by western blot analysis (see Figure 3). In untransfected and pRK5-transfected cells, the two splice variants of endogenous, inactive caspase-8 (molecular weight, approx. 55 kDa) were seen. None of the cleavage products typically detected after caspase-8 activation were visible in these two transfectants. However, in pRK-Casp8-transfected cells, a protein slightly larger than endogenous caspase-8 was seen, corresponding to the transfected caspase-8 (which was fused to the FLAG® peptide). More significantly, 43 kDa and 18 kDa bands were strongly visible in the pRK-Casp8 transfectants, corresponding to the cleavage products of caspase-8 that typically occur after activation of that enzyme.

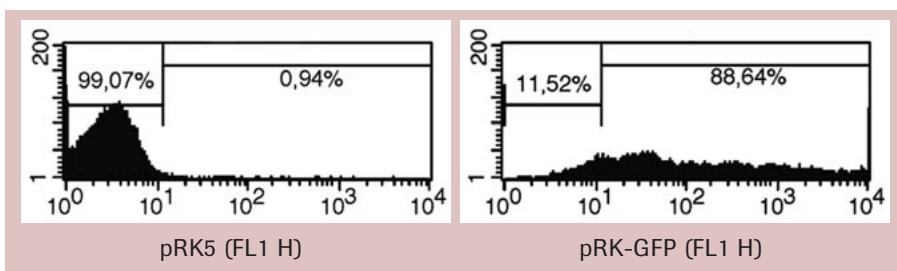
These results demonstrate that the FuGENE® HD Transfection Reagent can be used to introduce the EGFP and caspase-8 expression vectors into HeLa cells with very high efficiency. Moreover, the western blot findings indicate that caspase-8 is activated in the transfectants. The amounts of the activation products are consistent with overexpression of caspase-8 in the transfected cells.

#### 2. Evidence of metabolic changes in HeLa cells after introduction of the human caspase-8 gene

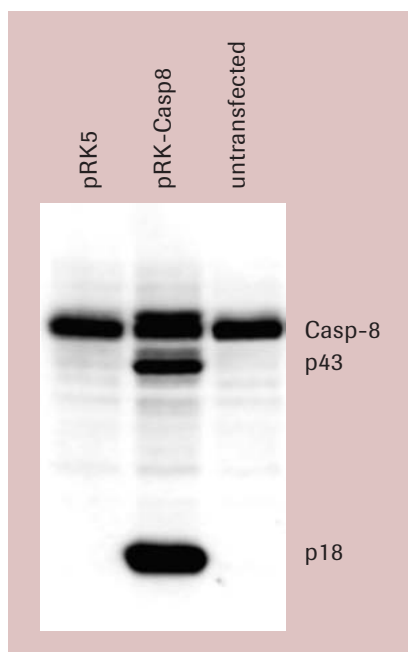
Two tests were used to detect metabolic changes caused by the overexpression of the caspase-8 gene.

Overall metabolic activity of the HeLa cells was determined with the colorimetric WST-1 assay (see Figure 4), an assay that indirectly monitors glycolysis in viable cells. In this assay, pRK5-transfected cells had slightly lower metabolic activity than untransfected cells both 24 and 48 hours post-transfection, suggesting that the transfection procedure slightly decreased the metabolic activity of transfected cells. However, cells transfected with pRK-Casp8 showed far more strongly reduced metabolic activity 48 hours post-transfection. These assays indicate that caspase-8 overexpression results in dramatic reductions in the metabolism of HeLa cells.

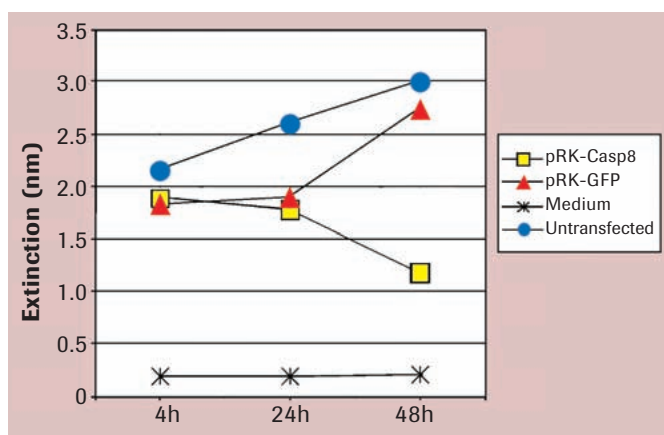
Since caspase-8 is a potent activator of caspase-3, the levels of caspase-3 in the cells were determined with the Caspase 3 Activity Assay Kit (see Figure 5). Lysates of HeLa cells transfected with pRK-Casp8 contained high caspase-3 activity. In contrast, the untransfected and pRK5-transfected cells showed practically no active caspase-3. This indicates that overexpression of caspase-8 alone activates caspase-3 in these cells without addition of any further apoptosis inducing agent.



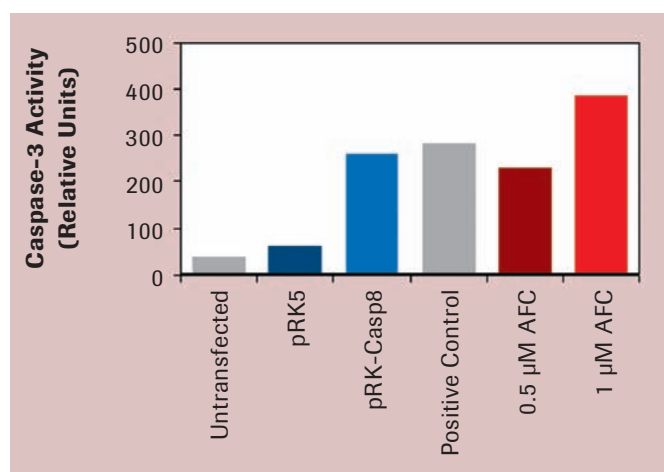
**Figure 2:** Estimate of transfection efficiency. Cells transfected with the expression plasmid pRK-GFP were examined by flow cytometry. Cells containing pRK5 alone were used as control. More than 88% of the pRK-GFP-transfected cells contained detectable levels of EGFP (emission peak, 509 nm).



**Figure 3:** Western blot comparison of HeLa cells transfected with various expression vectors. Proteins were prepared from pRK5-transfected and from pRK-Casp8-transfected cells, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Caspase-8-related proteins on the membrane were visualized using an anti-caspase-8 antibody and the chemiluminescent Lumi-Light<sup>PLUS</sup> Western Blotting Substrate. Endogenous caspase-8 (Casp-8, 55 kDa) was visible in the untransfected cells as well as in both strains containing expression plasmids. Only the pRK-Casp8-transfected cells contained the slightly larger caspase-8 protein expressed by pRK-Casp8 and the two cleavage products characteristic of activated caspase-8 (p43 and p18).



**Figure 4:** WST-1 assay of cell metabolism in transfected HeLa cells. Cell Proliferation Reagent WST-1 was added to cultures of both transfected and untransfected cells in order to monitor their total metabolic activity. Aliquots of the cultures were taken at the indicated time intervals after transfection and analyzed spectrophotometrically to determine the amount of WST-1 formazan formed. Higher amounts indicate higher metabolic activity. At the last time point measured (48 hours post-transfection), the metabolic activity of cells transfected with pRK-Casp8 was significantly less than that of untransfected cells and of cells transfected with either pRK5 or pRK5-GFP.

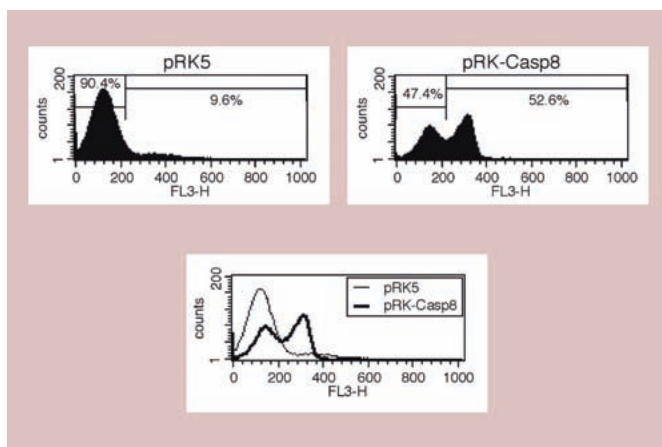


**Figure 5:** Caspase-3 activity in transfected HeLa cells. HeLa cells were transfected with either pRK5 or pRK-Casp8 and incubated for 24 hours. The cells were lysed and 250 μg protein from each culture was assayed with the Caspase 3 Activity Assay, a fluorometric immunosorbent enzyme assay. A positive control lysate and two dilutions of the fluorescent assay product, AFC (7-amido-4-trifluoromethylcoumarin) were also included in the assay. Both the untransfected and the pRK5-transfected cells showed very low levels of caspase-3 activity after 24 hours, but cells transfected with pRK-Casp8 had a high level of caspase-3 activity.

### 3. Evidence that overexpression of human caspase-8 induces apoptosis in HeLa cells

Increases in caspase-3 activity initiate a series of events that terminate in apoptotic cell death. To determine the apoptotic state of the transfected cells, they were exposed to Annexin-V-Alexa 568, a fluorescent stain that detects phosphatidylserine (PS) on the outside of the plasma membrane. Since translocation of PS across the cell membrane is one of the events occurring early in apoptosis (4), an increased binding of Annexin-V is indicative of cells are undergoing apoptosis.

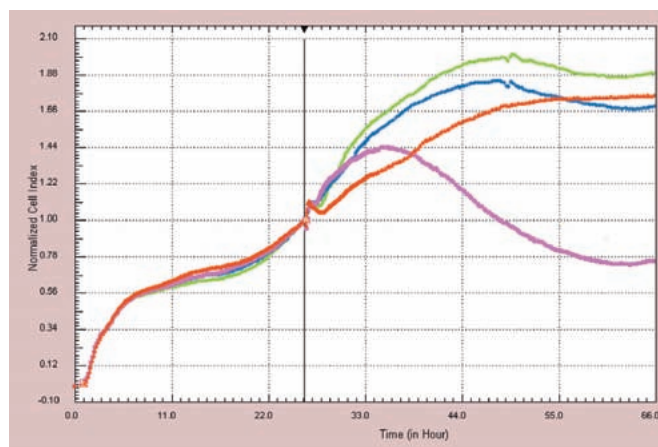
More than 50% of the pRK-Casp8-transfected cells were Annexin-positive (see Figure 6), indicating that the apoptosis-related changes had occurred in these cells just 48 hours after transfection. In contrast, less than 10% of the control, pRK5-transfected cells were Annexin-positive.



**Figure 6:** Flow cytometric detection of early stages of apoptosis in HeLa cells transfected with a caspase-8 expression vector, using Annexin-V-Alexa 568. HeLa cells were transfected with either pRK5 or pRK-Casp8 and incubated for 48 hours afterward. The cells were washed and resuspended in buffer containing Annexin-V-Alexa 568, a fluorescent stain which binds to phosphatidylserine (PS) on the outside of the plasma membrane. Stained cells were quantified flow cytometrically. Approx. 53% of the pRK-Casp8-transfected cells were stained with the Annexin-V reagent, while only about 10% of the pRK5-transfected cells were Annexin-positive. Since translocation of PS from the inside to the outside of the plasma membrane is one of the changes that occur in the early stages of apoptosis, Annexin-V staining is an indicator of cells undergoing apoptosis.

Since apoptosis occurs over time, no endpoint assay can give a complete picture of the progress of apoptosis. To provide a picture of apoptotic changes over time, HeLa cells were monitored continuously both before and after transfection with the xCELLigence Real-Time Cell Analyzer (RTCA) System (see Figure 7), which continuously measures cell impedance changes using a network of microelectrodes in contact with the HeLa cells, over the entire course of the experiment. These cell impedance changes are expressed in Cell Index (CI) units. CI values are directly correlated to changes in cell number and cell adhesion (5). Decreases in CI values are indicative of cell death (6), and thus measure the ongoing apoptosis.

In contrast to other transfectants, cells transfected with pRK-Casp8 show a decrease in Cell Index values as early as 12 hours post-transfection. This decrease continues throughout the 72-hour monitoring period.



**Figure 7:** Real-time analysis of apoptosis induction in HeLa cells transfected with a caspase-8 expression vector. HeLa cells transfected with one of three expression plasmids (pRK5, pRK5-GFP, pRK5-Casp8) were monitored continuously both before and after transfection using the xCELLigence RTCA SP Instrument. As a control, untransfected cells were also monitored throughout the experiment. Cells transfected with pRK-Casp8 show a strong decrease in Cell Index (CI) starting at about 12 hours post-transfection. In contrast, the CIs of cells transfected with control vectors pRK5 or pRK-GFP are similar to that of untransfected cells throughout the monitoring period (up to 46 hours post-transfection). Decreases in CI are strongly correlated to decreases in cell number.

## 4 Conclusion

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The aim of the current study was to design an easy to carry out workflow (see Figure 1) for analyzing the effects of caspase-8 overexpression on HeLa cells. As shown here, we were able to determine that overexpression of the caspase-8 gene alone, is sufficient to induce apoptosis in HeLa cells. This effect can be clearly seen as early as 24 hours after the caspase-8 gene is transfected into HeLa cells.

In principle, this same workflow can be used to monitor the effects of overexpression of other apoptosis-related genes. More significantly, a number of the steps in this workflow can be adapted to the study of many other complex cellular processes.

A case in point, FuGENE® HD Transfection Reagent has been used to efficiently introduce genes into many different types of eukaryotic cells. It is also gentle enough to allow high cell survival and high levels of protein production post-transfection.

The Cell Proliferation Reagent WST-1 can easily determine the number of metabolically active cells in a culture, even when the cell number in the culture is low. Thus, it could be adapted for sensitive, nonradioactive monitoring of cell proliferation, cell viability or cytotoxicity mediated by chemical compounds or cells.

The xCELLigence System comprises three instruments for low (RTCA DP Instrument with 3x16-well plates), medium (RTCA SP Instrument with 1x96-well plate), and high (RTCA MP Instrument with 6x96-well plates) throughput. These automated, impedance-based biosensors are ideal for validating a wide variety of cell-based assays, including those for cell proliferation and cell death, cytotoxic effects, cell adhesion, and activation of cell surface receptors. It allows monitoring of cellular changes in real time without cell labeling or other invasive assay procedures, pinpointing the times during *in vitro* experiments when significant cellular responses are taking place.

## References

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**Abbreviations:** AFC, 7-amido-4-trifluoromethylcoumarin; EGFP, enhanced Green Fluorescent Protein.

## Ordering Information

Product	Cat. No.	Pack Size
<b>Genopure Plasmid Maxi Kit</b>	03 143 422 001	1 kit (for up to 10 isolations)
<b>FuGENE® HD Transfection Reagent</b>	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
<b>cOmpete Lysis-M</b>	04 719 956 001	1 kit
<b>cOmpete Lysis-M, EDTA-free</b>	04 719 964 001	1 kit
<b>Lumi-Light<sup>PLUS</sup> Western Blotting Substrate</b>	12 015 196 001	100 ml (1,000 cm <sup>2</sup> membrane)
<b>Cell Proliferation Reagent WST-1</b>	05 015 944 001	8 ml
	11 644 807 001	25 ml (2,500 tests)
<b>Caspase 3 Activity Assay</b>	12 012 952 001	1 kit (96 tests)
<b>Annexin-V-Alexa 568</b>	03 703 126 001	500 µl (250 tests)
<b>RTCA SP Instrument</b>	05 229 057 001	1 instrument
	05 228 972 001	1 instrument
	05 229 014 001	1 instrument

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