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Homogeneous Caspases Assay, fluorimetric

Content version: February 2019

Cellular Assay for the quantitative *in vitro* determination of caspases activity in microplates

Cat. No. 03 005 372 001 — 100 tests on 96-well plates, 400 tests on 384-well plates

Cat. No. 12 236 869 001 — 1,000 tests on 96-well plates, 4,000 tests on 384-well plates

Store the kit at −15 to −25°C

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1. What this Product Does

Cat. No.
03 005 372 001

Bottle/ Cap	Label	Content including function	Storage temperature
1 green	Substrate stock solution	1 ml (500 μM) DEVD-R110 stock solution in DMSO, clear colorless	−15 to −25°C
2 violet	Positive control (10×)	250 μl lysate from apoptotic U937 cells (treated with camptothecin)	−15 to −25°C
3 yellow	R110 standard	250 μl (1 mM) stock solution in DMSO, yellow solution	−15 to −25°C
4 color-less	Incubation buf-fer		+2 to +8°C

Cat. No.
12 236 869 001

Bottle/ Cap	Label	Content including function	Storage temperature
1 green	Substrate stock solution	10 × 1 ml (500 μM) DEVD-R110 stock solution in DMSO, clear col-orless	−15 to −25°C
2 violet	Positive control (10 ×)	250 μl lysate from apoptotic U937 cells (treated with camptothecin)	−15 to −25°C
3 yellow	R110 standard	250 μl (1 mM) stock solution in DMSO, yellow solution	−15 to −25°C
4 color-less	Incubation buf-fer	100 ml, clear solution, foaming possible	+2 to +8°C

DEVD-R110 = Asp-Glu-Val-Asp-Rhodamine 110; R110 = Rhodamine 110

Additional Required Equipment

To perform assays with this kit, the following equipment is needed:
- Pipettes
- Cell culture grade black microplate with clear bottom
- Microplate fluorescence reader
- Calculation software recommended

Safety Information

Rhodamine 110 (vial 1 and vial 3) is an irritant. Wear gloves when handling!

2. How to Use this Product

2.1 Product Overview

Test Principle

The homogeneous caspase assay is a fluorimetric assay for the quantitative in vitro determination of caspases activity in microplates, which is especially useful for high throughput screening.

Test Principle-Basic Steps

❶	Cells are cultured in microplates and apoptosis is induced, causing an activation of caspases.
❷	Caspase substrate, prediluted in Incubation buffer, is added and incubated for 2 h at 37°C. The incubation buffer is lysing the cells during this incubation.
❸	Free R110 is determined fluorimetrically at $\lambda_{max} = 521$ nm. The developed fluorochrome is proportional to the concentration of activated caspases and could be quantified by a calibration curve.

Application

During apoptosis, the activation of specific proteases, called Caspases (cysteinyl-aspartic-acid-proteases), is one of the initial intracellular biochemical events. The Homogeneous Caspases Assay can be used to detect caspase activity in life-science research applications:

- for sensitive quantification of activated caspases
- for screening of caspase inhibitors as an automatable one-step assay for high-throughput screening

Sample Material

- Cells cultured in microplates
- Recombinant caspases

Assay Time

The total assay time is about 1.5 h excluding cell culturing.

Number of Tests

Cat. No. 12 236 869 001 is designed for 1,000 tests on 96-well plates or for 4,000 tests on 384-well plates.	Cat. No. 03 005 372 001 is designed for 100 tests on 96-well plates or for 400 tests on 384-well plates.

Stability

This product is stable at −15 to −25°C until the expiration date printed on the label.

Sensitivity

Activated caspases from approximately 10^2 – 10^4 apoptotic cells can be analyzed.

Specificity

- Enzyme activity of natural and recombinant caspase is detected by this homogeneous caspases assay.
- Activated caspases 2, 3, 6, 7, 8, 9 and 10 could be analyzed with different sensitivity

Quality Control

The kit is function tested using a cellular model (U937 cells treated with camptothecin).

Background Information

Programmed cell death:
Apoptosis is a major form of cell death, which was initially characterized by a series of stereotypic morphological changes. Several molecular events have been described as characteristic of apoptosis or programmed cell death (PCD). The most common characteristics of PCD is the fragmentation of DNA into ~180 bp fragments, which is a relatively late event during the process. As an earlier, almost unique event, the exposure of phosphatidylserine on the outer leaflet of the plasma membrane bilayer can be analyzed. A third characteristic is the activation of specific proteases called caspases (cysteinyl-aspartic-acid-proteases) (1), which is one of the initial biochemical events. Activated caspases cleave several cellular components related to DNA-repair and regulation (2, 3). The recognition sequences and cleavage sites of the target proteins have been described for the different caspases (4, 5, 6).

2.2 Procedures and Material Required

Solution	Content	Reconstitution/ Preparation of working solution	Stability of solution	For use as
1	Substrate stock solution (bottle 1)	For working concentration: Dilute 1 ml Substrate stock solution with 9 ml incubation buffer (bottle 4). *Result*: 10 ml clear, colorless solution, enough for 1 MP	unstable, prepare immediately before use	Substrate working solution
2	Positive control (bottle 2)	Dilute 20 µl cellular lysate with 180 µl Incubation buffer *Result*: 200 µl positive control	unstable, prepare immediately before use	Positive control
3	R110 standard solution (bottle 3)	Dilute 10 ml 1 mM R110 standard solution with 990 ml cell culture medium (see 2.4.2). *Result*: 10 µM stock	unstable, prepare immediately before use	Procedure for setup of a calibration curve (section 2.4.2)

2.3 Before You Begin

Reagents

- All reagents necessary to perform the assay are supplied with this kit.
- Bring all reagents to +15 to +25°C before use

Required equipment

- Use only pipettes that are carefully calibrated to their target volume.
- Microplate fluorescence reader with excitation filter 470 - 500 nm and emission filter 500 – 560 nm (maxima $\lambda_{ex} = 499$ nm and $\lambda_{em} = 521$ nm)
- Calculation software is suggested for calibrated sample evaluation.

Handling Instructions

- Pipet thoroughly to ensure accurate transfer of low volumes.
- Perform a separate calibration curve simultaneously with each test series, when calibrated values are needed.
- All measurements should be performed in duplicates.

Controls

Two negative controls and two positive controls should be included in each experimental set up.

Negative control	Cells in diluent only, without apoptosis inducing agent.
Positive control	Diluted lysate from apoptotic U937 cells (treated with camptothecin).

Pipetting Scheme for the Microplate

We recommend to use the pipetting scheme shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	P0	P0	P1	P1	...							
B	Sa	Sa	...	...	...							
C	Sb	Sb										
D	Sc	Sc										
E	Sd	Sd										
F	Se	Se										
G	Sf	Sf								...	...	...
H	Bl	Bl								...	P40	P40

Legend:

Bl = Blank (cell culture medium)

Sa - Sf = standards:
a) 1 µM R110, b) 0.5 µM R110, c) 0.25 µM R110,
d) 0.125 µM R110, e) 0.0625 µM R110, f) 0.03125 µM R110

P0 = positive control

P1 - P40 = samples 1-40, including negative control

2.4 Assay Procedure

2.4.1 Procedure for Cellular Analysis of Caspase Activity

Procedure

Follow these steps to detect caspase activity:

Step	Action
❶ Preparation of apoptosis inducing agent	Dispense double concentrated apoptosis inducing agent into microplate. Include negative control (diluent only). Volume should be 50 µl (96 well plate) or 12.5 µl (384 well plate).
❷ Seeding of cells and induction of apoptosis	• Onto prediluted apoptosis inducing agents, seed cells (4×10^4 per well, volume 50 µl on 96 well plate or 10^4 cells per well, volume 12.5 µl on 384 well plate) and incubate for desired interval for induction of apoptosis. • Do not seed into wells for BLANK, standard and positive control! BLANK should contain 100 µl (96-wells) or 25 µl (384-wells) cell culture medium only.
❸ Substrate cleavage	• Add 100 µl (96-wells) or 25 µl (384-well) prediluted positive control • Add 100 µl (96-wells) or 25 µl (384-well) prediluted standard solutions (prepared as described under 2.4.2) • Add 100 µl (96-wells) or 25 µl (384-well) substrate working solution (1), freshly prepared • Cover the microplate with a lid and incubate more than 1 h at 37°C.
❹ Fluorimetric measurement	Measure with an excitation filter 470-500 nm and emission filter 500 – 560 nm (maxima $\lambda_{ex} = 499$ nm and $\lambda_{em} = 521$ nm)

Comment

- If only low activation could be achieved, over night incubation at 37°C can be performed to increase sensitivity.
- Keep in mind, that seeding density depends on cell type and apoptosis induction interval. Above mentioned cell densities are optimized for our internal model
(U937 induced with 4 µg/ml camptothecin for 3 h).

2.4.2 Procedure for Calibrating the Caspase Activity

Calibration is not needed when comparisons with non-induced cellular lysates are
performed. Calibration is recommended when data from different microplates will be compared.

For setup of a calibration curve, follow the steps listed below:

Using 384-well plates, ¼ of all volumes are needed only.

1. • Important: diluted free R110 is photostable for 2 h only - prepare dilutions prior to analysis and pipet shortly before reading on the fluorescence reader
   • Dilute R110 (solution 3) as follows:
   First, predilute the 1 mM stock solution to 10 µM using cell culture medium (see 2.2, preparation of working solutions)
   • Prepare serial dilution of standards for the calibration curve using the 10 µM prediluted stock:

Final Concentration	**Volume of R110 Stock**	**Volume of Cell Culture Medium**
10 µM stock	10 µl	990 µl

   • Prepare serial dilution of standards for the calibration curve using the 10 µM prediluted stock:

Final Concentration	**Prediluted solution**	**Cell culture medium**
2 µM	200 µl (10 µM)	Add 800 µl
1 µM	300 µl (2 µM)	Add 300 µl
0.5 µM	300 µl (1 µM)	Add 300 µl
0.25 µM	300 µl (0.5 µM)	Add 300 µl
0.125 µM	300 µl (0.25 µM)	Add 300 µl
0.0625 µM	300 µl (0.125 µM)	Add 300 µl

2. Add 100 µl of each serial dilution into wells (duplicates), as shown in the pippetting scheme (section 2.3)

3. Add 100 µl substrate solution per well, resulting in R110 standard concentrations of 1 µM, 0.5 µM, 0.25 µM, 0.125 µM, 0.0625 µM, 0.03125 µM

4. Measure with an excitation filter 470-500 nm and emission filter 500 – 560 nm (maxima $\lambda_{ex} = 499$ nm and $\lambda_{em} = 521$ nm)

3. Results

3.1 Calculation

Plotting the Standard Curve

Use the diluted R110-standards to prepare a six-point calibration curve. Follow these steps to plot a standard curve.

1. Correct each fluorescence value of all standards by subtracting the value of the reagent blank (Bl = substrate only),
2. Calculate the mean fluorescence value for each standard from the duplicates.
3. Prepare a plot correlating the mean fluorescence values of the standards to the analyte concentrations of the standards.
4. The plot may be prepared in either of two ways:

IF you want to ...	THEN...
measure automatically	enter the fluorescence values and the analyte concentrations into a suitable data analysis software program. To achieve best results make sure the software is able to calculate the standard curve with a four-parameter Rodbard-function. Several data analysis programs perform additional statistical analysis (*e.g.,* recalculation, mean, SD, CV, regression analysis) on the absorbance values entered.
measure manually	plot on double logarithmic graph paper, the mean fluorescence values on the y-axis against the analyte concentrations on the x-axis

Determination of the Analyte Concentration

Follow these steps to determine the analyte concentration.

1. Correct the fluorescence value of all samples (and controls) by subtracting the value of the reagent blank (Bl = substrate only).
2. Calculate the mean fluorescence values for each sample of the duplicates.

   If the fluorescence of the sample duplicates is above the fluorescence of the highest standard, do not use those values to determine analyte concentration in the diluted sample (next step). Repeat the assay for those samples as outlined in the next section, "Handling very concentrated samples".

❸	Determine the analyte concentration in the diluted sample in either of two ways:

IF you want to ...	THEN...
measure automatically	enter the mean of the sample fluorescence values into the same data analysis program used to prepare the standard curve (as described in the previous section). The data analyte program will automatically determine the analyte concentration by comparing the fluorescence of the sample to the standard curve.
measure manually	locate the mean sample fluorescence on the y-axis of the standard curve drawn, and read (from the x-axis) the analyte concentration that corresponds to that fluorescence value.

3.2 Typical Results

Typical Standard Curve

A standard curve is shown as an example (Fig.1).

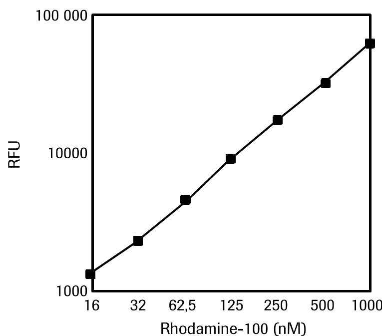

Fig. 1: Standard curve, generated by different dilutions of R110 standard included in the kit.

Typical Fluorescence Values

Fig. 2 shows the release of rhodamine induced by caspase activation in U937 cells in a 384-well plate and a 96-well plate.

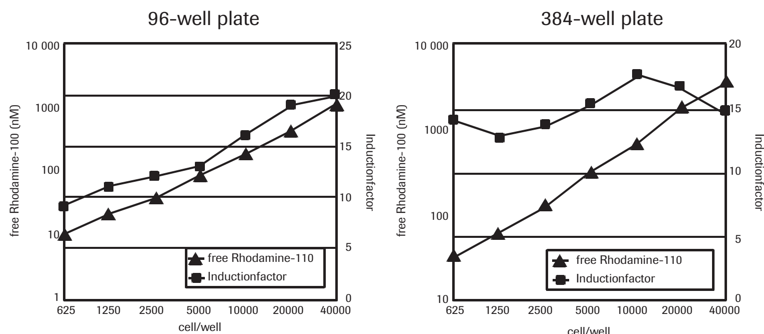

Fig. 2: Different concentrations of U937 cells per well were exposed to 4 mg/ml camptothecin at 37°C. The cells were incubated in a 96-well plate (volume 100 ml) or in a 384-well plate (25 ml). After 4 h the cells were incubated for 2 h with the substrate solution. The relative fluorescence units (RFU) signal is converted to nM free Rhodamine-110 via the standard curve.

The induction factor of caspase activity is calculated as the ratio of the RFU signal of the induced cells devided by the RFU signal of the uninduced cells.

The increase of caspase activity is analyzed as the difference of the RFU signal of the induced cells minus the RFU signal of non-induced cells (Δ RFU).

Typical Calibrated Values

Fig. 3 shows caspase activation by exposure of U937 cells to different concentrations of camptothecin (dose-response curve), performed in 384-well plates.

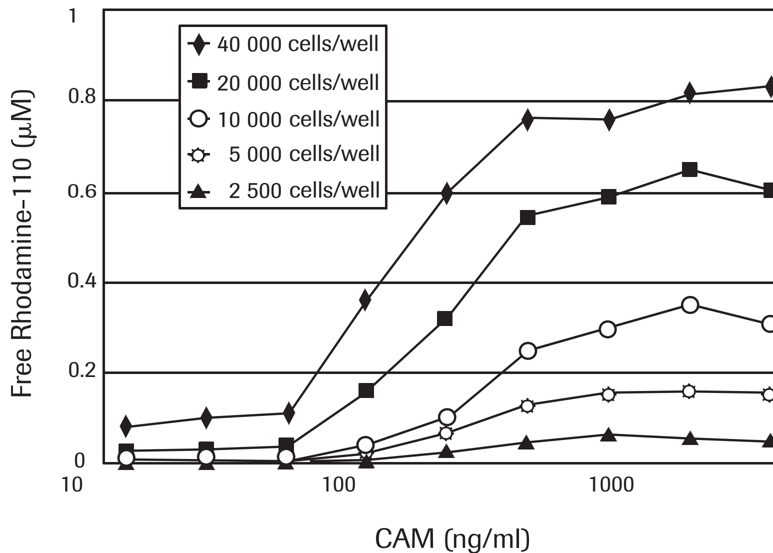

Fig. 3: U937 cells were exposed to different concentrations of camptothecin for 4 h at 37°C. Subsequently, the cells were directly incubated with the substrate solution for 2h. The RFU signal is converted to nM free Rhodamine-110 via the standard curve. The increase of the caspase activity is calculated as difference of the RFU signal of the induced cells to the RFU signal of the non-induced cells (Δ RFU).

4. Troubleshooting

Problem	Possible Cause	Recommendation
Unexpected high fluorescence development	Gain of fluorescence reader is too high	Decrease gain.
Weak or no signal	Inadequate incubation time and temperature	Ensure that incubation intervals are correct, and that all reagents achieve the adequate temperature before testing.
	Caspases are digested nonspecifically by other proteases	Decrease apoptosis induction interval.
	Activation of caspase is too low	Induce more apoptosis by longer exposure of inducer, or apply a higher concentration of inducer. Incubation over night could be performed, if low activation is achieved only.
	Caspases are not involved in this specific pathway of cell death	Ensure that apoptosis is induced by other methods (TUNEL, Annexin-V, PARP cleavage). If no, use another model/inducer.
	Non-suitable optical filters have been used.	Check the optical filters in the microplate reader for the correct wavelength.
	Gain of fluorescence reader is too low	Increase gain.
Drift	Evaporation of fluids	Use adhesive cover foils for reduction of evaporation.
Poor precision	Non-homogeneous sample after addition of substrate solution	Mix sample well after addition of substrate solution.
	Turbidity, particles or high lipid or DNA content within the sample	Reduce cell number and concentration of fetal calf serum, respectively.
	Carry over occured between samples/standards	Change pipette tips between each pipetting step.
	Unequal volumes added to the wells	Check pipette function, and recalibrate if necessary.
Questionable readings	Non-suitable optical filters have been used.	Check the optical filters in the microplate reader for the correct wavelength.

5. Additional Information on this Product

5.1 References

1 Alnemri E.S. *et al*. (1996) Human ICE/CED-3 Protease Nomenclature. *Cell* 87, 171.
2 Casciola-Rosen *et al*. (1996) Apopain/CPP32 Cleaves Proteins That Are Essential for Cellular Repair: A Fundamental Principle of Apoptotic Death. *J. Exp. Med*. **183**, 1957-1964.
3 Alnemri E.S. (1997) Mammalian Cell Death Proteases: A Family of Highly Conserved Aspartate Specific Cystein Proteases. *J. Cell. Biochem*. **64**, 33-42.
4 Nicholson D.W. & Thornberry N.A. (1997) Caspases: killer proteases. *TIBS* **22**, 299-306.
5 Cohen G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.* **326**, 1-16.
6 Stroh C. & Schulze-Osthoff K. (1998) Death by a thousand cuts: an ever increasing list of caspase substrates. *Cell Death Differ*. **5**(12), 997-1000.

6. Supplementary Information

6.1 Conventions

6.1.1 Text Conventions

To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered stages labeled ①, ② etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷ etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Diagnostics.

6.1.2 Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
🔍	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

Editorial changes.

6.3 Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.4 Trademarks

All other product names and trademarks are the property of their respective owners.

6.5 Disclaimer of Licence

For patent license limitations for individual products please refer to:
List of biochemical reagent products

6.6 Ordering Information

Kits

Apoptosis - specific physiological change	Detection by	Products	Cat. No.
DNA fragmenta-tion	Gel Electrophoresis	Apoptotic DNA-Ladder Kit	11 835 246 001
	In situ assay	• In situ Cell Death Detection Kit, Fluorescein • In situ Cell Death Detection Kit, AP • In situ Cell Death Detection Kit, POD • In situ Cell Death Detection Kit, TMR red Single reagents for TUNEL and supporting reagents: • TUNEL AP • TUNEL POD • TUNEL Enzyme • TUNEL Label • TUNEL Dilution Buffer	11 684 795 910 11 684 809 910 11 684 817 910 12 156 792 910 11 772 457 001 11 772 465 001 11 767 305 001 11 767 291 910 11 966 006 001
	ELISA	• Cell Death Detection ELISAPLUS • Cell Death Detection ELISAPLUS, 10× • Cellular DNA Fragmentation ELISA	11 774 425 001 11 920 685 001 11 585 045 001
Cell membrane alterations	Annexin V	• Annexin-V-Alexa1) 568 • Annexin-V-Biotin • Annexin-V-FLUOS • Annexin V FLUOS Staining Kit50 tests • Annexin V FLUOS Staining Kit250 tests	03 703 126 001 11 828 690 001 11 828 681 001 11 858 777 001 11 988 549 001
Enzymatic activity	PARP cleavage	Anti-Poly(ADP-Ribose)Polymerase	11 835 238 001
	Caspase 3 activity	Caspase 3 Activity Assay	12 012 952 001
	Caspase 2, 3, 7 activity	• M30 CytoDEATH (formalin grade)250 tests • M30 CytoDEATH (formalin grade)50 tests • M30 CytoDEATH, fluorescein 250 tests	12 140 349 001 12 140 322 001 12 156 857 001
Expression of apoptosis- related proteins	Fas	Anti-Fas	11 922 432 001

7. Quick Reference Procedure

7.1 Preparation of Solutions

Solution	Content	Diluent	Details see	Used for
❶	Substrate working solution	Incubation buffer	2.2, p.5	7.2 step 3
❷	Positive control	Incubation buffer	2.2, p.5	7.2 step 2
❸	1 × Standards	Cell culture medium	2.4.2, p.7	7.2 step 2

7.2 Working Procedure: Flow Sheet

96-Well Plates

Step	Action	Volume/ well	Time/ Temp.
❶	Induce apoptosis with your model system	100 µl	various
❷	Pipet dilutet standard solutions and positive control	100 µl	-
❸	Add substrate working solution Cover the microplate tightly with a lid and incubate	100 µl	1 – 2 h / 37°C
❹	Fluorimetric measurement: Ex 499 nm, Em 521 nm	–	–

384-well plates

Step	Action	Volume/ well	Time/ Temp.
❶	Induce apoptosis with your model system	25 µl	various
❷	Pipet dilutet standard solutions and positive control	25 µl	–
❸	Add substrate working solution Cover the MTP tightly with a lid and incubate	25 µl	1 – 2 h / 37°C
❹	Fluorimetric measurement: Ex 499 nm, Em 521 nm	–	–

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany