

M30 CytoDeath™ ELISA








REF 10900

Instructions for Use

**For research and laboratory use only.
Not for human or diagnostic use.**

Date of issue 2009-11-12

Explanation of Symbols used on Labels

-  Catalogue number
-  Batch code
-  Contains sufficient for <n> tests
-  Manufacturer
-  Temperature limitation
-  Use by
-  Consult Instructions for Use

Trademarks

M30-Apoptosense® and M65® are registered trademarks of PEVIVA AB. M30 CytoDeath™ is a trademark of PEVIVA AB.

Tween® 20 is a registered trademark of ICI America, Inc.

Patents

U.S. Patent No. 6,296,850 - U.S. Patent No. 6,716,968 - U.S. Patent No. 6,706,488.

References

1. Leers et al., J Pathol. 187, 1999, 567.
2. Schutte et al., Exp Cell Research 297, 2004, 11.
3. Kramer et al., Cancer Res 64, 2004, 1751.
4. Hägg et al., Invest New Drugs 20, 2002, 253.
5. Erdal et al., PNAS 102, 2005, 192.
6. Cummings et al., Br Journal of Cancer 92, 2005, 532.
7. Lakshmiathan et al., Int J Cancer 119, 2006, 221.

For further references and information, please consult www.peviva.se.

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APOPTOSIS DETERMINATION

Intended Use

M30 CytoDeath™ ELISA is a one step in vitro immunoassay for the quantitative determination of the apoptosis-associated CK18Asp396 (M30) neo-epitope in cultured human, monkey or bovine cells.

Summary and Explanation of the Test

Caspases cleave various cellular proteins during apoptosis. In epithelial cells, one of those substrates is the intermediate filament protein cytokeratin 18 (CK18). The M30 antibody recognizes a neo-epitope exposed after caspase cleavage of CK18 after the aspartic acid residue 396 (Ref. 1). Cleavage at this position occurs early during apoptosis by caspase-9 and during the execution phase by caspase-3 and caspase-7 (Ref. 2).

The M30 CytoDeath™ ELISA measures the levels of soluble CK18 fragments containing the CK18Asp396 neo-epitope. After induction of apoptosis of epithelial cells, increases in CK18Asp396 are first observed in cell extracts. Release of antigen into the extracellular compartment occurs later and is due to secondary necrosis of apoptotic bodies. The increase of CK18Asp396 during apoptosis is inhibited by the caspase-inhibitor zVAD-fmk.

The M30 CytoDeath™ ELISA can be used in combination with the M65® ELISA (PEVIVA Prod. No 10020) which measures total CK18. Combining the two assays is useful for assessment of cell death mode (Ref. 3). M30 is a mouse monoclonal IgG2b antibody. The M30 CytoDeath™ ELISA is suitable for human, monkey and bovine cells (murine cells **can not** be used).

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Principle of the Procedure

The M30 CytoDeath™ ELISA is a solid-phase sandwich enzyme immunoassay. Standards and Samples react with a solid phase catcher antibody *M6* directed against CK18 and the HRP- (Horseradish Peroxidase) conjugated M30 antibody directed against the CK18Asp396 neo-epitope. Unbound conjugate is removed by a washing step. TMB Substrate is added. The colour development is stopped and the absorbance is read.

The resulting colour is directly proportional to the concentration of the analyte. By plotting a standard curve from known concentrations versus measured absorbance, the amount of antigen in the sample can be calculated. The concentration of the antigen is expressed as Units per Litre (U/L).

Collection and Preparation of the Samples

The sample volume should be sufficient for measuring each sample in duplicates (test volume 2 x 25 µL).

Note! The M30 CytoDeath™ ELISA **can not** be used for blood samples. Consider the M30 Apoptosense® ELISA for measuring caspase cleaved CK18 in serum or plasma.

Note! The same type of material collected by one method should be used for a specific project. For further information on the performance of the M30 CytoDeath™ ELISA using different types of samples, please consult www.peviva.se.

Store samples at 2 – 8 °C up to 48 hours. For longer periods store samples frozen at -20 °C or lower. Samples can be freeze-thawed without loss of activity but it is recommended that repeated freeze-thawing should be avoided.

For dilution of samples see section “Performance Characteristics”.

Preparation for the assay

A detailed description of the materials provided is found on page 13. All reagents are ready to use except the HRP Conjugate and the Wash Tablet. Very high samples can be diluted before measurement in the assay.

Dilution of HRP Conjugate

Dilute the HRP Conjugate by adding 9.2 mL of the Conjugate Dilution Buffer directly to the HRP Conjugate bottle. Vortex before use.

Dilution of Wash Tablet

Dilute the Wash Tablet in 500 mL fresh distilled water. Let the tablet dissolve in the water and shake before use.

Dilution of Samples

Samples higher than Standard High should be diluted with cell culture medium. Since dilution in the assay is linear, original concentration is calculated by multiplying the measured concentration with the used dilution factor.

Reagent Storage

The M30 CytoDeath™ ELISA should be stored at 2 – 8 °C.

Do not freeze!

Store reagents in their original containers at 2 – 8 °C. The TMB Substrate and the M30-HRP Conjugate are sensitive to light and metal ions and should be stored in the original amber bottles at 2 – 8 °C at all times. TMB Substrate can not be used after exposure to light.

Assay Procedure

See page 8 – 9 for a flow chart.

The M30 CytoDeath™ ELISA should be performed at room temperature (24 ± 3 °C).

1. Allow all reagents to reach room temperature before performing the assay. Vortex all reagents prior to use.

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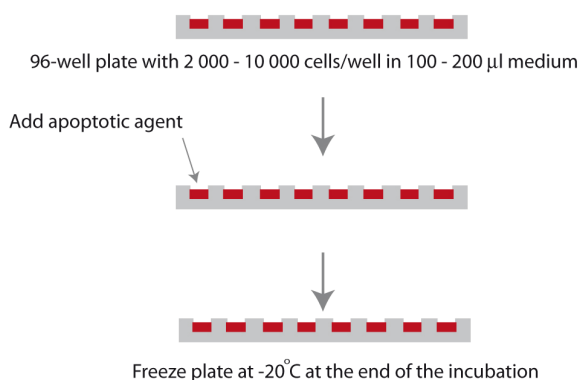
- Dilute HRP Conjugate with Conjugate Dilution Buffer and mix thoroughly (see page 6).
- Dilute the Wash Tablet in 500 mL fresh distilled water.
- Pipette 25 µL of Standards and Samples per well (duplicates are recommended).
- Add 75 µL of the diluted HRP Conjugate Solution to each well. Note that steps 4 and 5 should be performed sequentially without interruption within 15 minutes.
- Cover the wells with sealing tape.
- Incubate on shaker for four (4) hours. Optimal speed setting: ≈ 600 rpm on a plate shaker (other shakers: make sure the liquid is moving while staying in the well).
- Aspirate and wash the wells five (5) times with 250 µL Wash Solution. Avoid contamination between wells.
- Add 200 µL of TMB Substrate to each well. Incubate in darkness at room temperature for 20 ± 1 minutes.
- Add 50 µL of the Stop Solution to each well. To ensure complete mixing of the TMB Substrate and the Stop Solution, shake the Microplate for 5 – 10 seconds. Leave the Microplate for 5 minutes before reading of the absorbance.
- Determine the absorbance at 450 nm in a Microplate reader within 30 minutes and record the results.
- Calculate the results as described in Section “Processing of results”.

Processing of Results

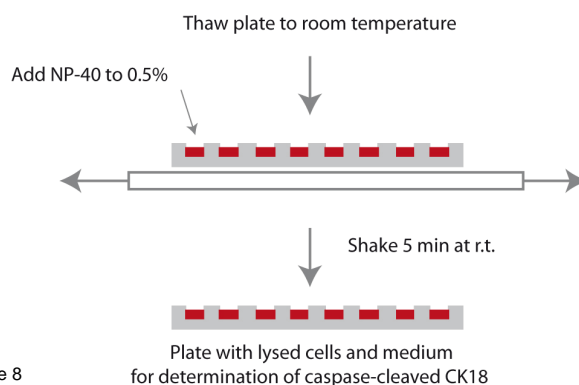
The M30 CytoDeath™ ELISA results may be calculated manually or, preferably, by using computer-assisted methods. Evaluate the values of samples using a suitable program for handling ELISA type data. Fitting algorithm: Cubic Spline or 4 PL function. x-axis: Concentration (U/L); y-axis: Absorbance at 450 nm (A450).

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STEP 1: PREPARE CELLS

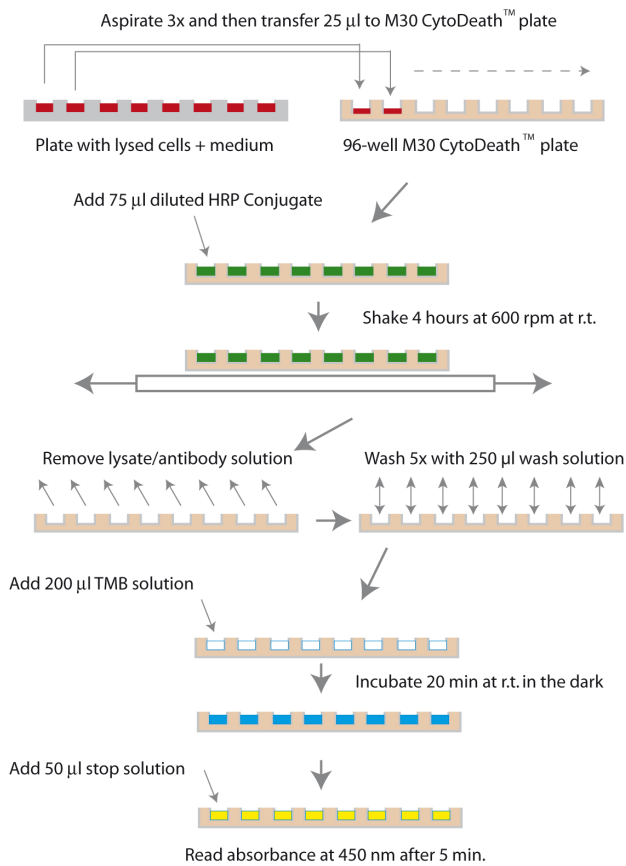


STEP 2: PREPARE SAMPLES



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STEP 3: DETERMINE CASPASE-CLEAVED CK18



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for 5 minutes at room temperature. Mix gently by pipetting up and down, careful not to create air bubbles and transfer 2 x 25 µL of the medium/lysate to the wells of the Coated Microtiter Plate.

b. Sample Preparation from Cell Culture Supernatants

The M30 CytoDeath™ ELISA and M65® ELISA can be used to assess **cell death mode** by calculation of an **M30:M65 ratio** (Ref. 3). Such measurements should be performed using medium supernatants! The ratio should be calibrated for each carcinoma cell line using appropriate controls; i.e. agents known to induce apoptosis (e.g. genotoxic agents, Staurosporine) and/or mainly necrosis (e.g. oligomycin/glucose starvation or hydrogen peroxide).

Day 1/Day 2: Seed the cells, wash and add agents as described above (a).

Day 2 - 4: Collect the sample medium from each well. To avoid drying out effects, it is not recommended to collect multiple samples from the same wells. Centrifuge the medium and collect the cell-free supernatant. **Note!** Avoid collecting cells. 2 x 25 µL cell-free supernatant samples are used for each assay.

If the assay is to be performed the same day, the samples can be stored at 2 – 8 °C. Samples to be analyzed later should be stored at -20 °C or lower. Avoid repeated freeze-thawing.

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EXPERIMENT DESCRIPTION

Suitable Cell Lines

The M30 CytoDeath™ ELISA detects apoptosis in *in vitro* cell cultures. The assay is specific for human, monkey and bovine cells. Since the quantification of apoptosis is due to a measurement of Cytokeratin 18, make sure that the used cell line expresses this protein.

Many different cell lines have been used successfully with the M30 CytoDeath™ ELISA. Visit the Peviva webpage on www.peviva.se for further information.

Collection and Preparation of Samples

a. Sample Preparation from Lysed Cell Cultures

For many applications it is advantageous to measure total M30-reactivity (CK18Asp396) at a single, late time point. Such measurements reflect an **integrated assessment of apoptosis**. To assay total CK18Asp396 fragments in cell culture media and cell extracts, add non-ionic detergent directly to the cells in the tissue culture medium.

Day 1: Seed the cells. The seeding density needs to be determined for the specific cell type and the type of cytotoxic agent; 2 000 – 10 000 cells per well in a 96-well plate is usually adequate. Recommended cell medium volume in the well is 100 – 200 µL.

Day 2: Remove the old medium, wash the cells once with PBS and add fresh medium (100 – 200 µL/well). Expose the cells to the desired agent(s). Negative and positive controls (e.g. treated with Staurosporine) are recommended.

Day 2 - 4: Stop treatment by freezing the plate to -20 °C or lower.

Measurement: Thaw the plate to room temperature. For 96-well plates containing 200 µL medium per well: add 10 µL 10% NP-40 per well. Allow lysis to occur on a plate shaker

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ASSAY DESCRIPTION

Warning and Precautions for Users

1. M30 CytoDeath™ ELISA kit is intended for *in vitro* use only.
2. Do not mix reagents from different kit lots.
3. All samples should be regarded as contagious and handled and disposed of according to appropriate regulations.
4. Do not use samples that are contaminated.
5. The Stop Solution contains 1.0 M sulphuric acid, which might cause irritation on skin and is harmful to the eyes. In case of contact, flush with plenty of water and seek medical advice.
6. Material Safety Data Sheets (MSDS) are available on www.peviva.se or by request.

Materials Required But Not Provided

- Microplate reader (wavelength 450 nm)
- Microplate shaker (oscillation ≈ 600 rpm)
- 96-well microtiter plate washer or multichannel pipette (volume 250 µL)
- Vortex mixer
- Precision pipettes: 25; 50; 75 and 200 µL
- Graduated cylinder (500 or 1 000 mL)
- Deionised water

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Materials Provided For 96 Determinations

M30 CytoDeath™ Coated Microtiter Plate: One Microplate, 96 dry wells. The wells are coated with mouse monoclonal CK18 antibody M6. The microplate is sealed in an aluminium bag, which contains a desiccating device. **Ready for use!**

M30 CytoDeath™ HRP Conjugate: Concentrate (24 x conc.). One vial containing 400 µL mouse monoclonal M30 antibody (anti-CK18Asp396 neo-epitope) conjugated with horseradish peroxidase (HRP) in phosphate buffer with protein stabilizers. Should be diluted with Conjugate Dilution Buffer (see page 6). Preservative added. **Note!** Do not expose to light!

M30 CytoDeath™ Conjugate Dilution Buffer: One vial containing 11 mL of phosphate buffer with protein stabilizers for dilution of the HRP Conjugate. Preservative added. Green coloured.

M30 CytoDeath™ Standards: Standard Zero containing 0.5 mL phosphate buffered FCS. Standards Low, Medium and High: 0.5 mL each, containing standard material in phosphate buffered FCS. The values of the Standards are 0 U/L (Zero), 250 U/L (Low), 1 000 U/L (Medium) and 3 000 U/L (High). Preservative added. Yellow coloured. **Ready for use!** Standard Zero can be used for dilutions of samples > 3 000 U/L.

Wash Tablet: One tablet for 500 mL of Wash Solution. Dilute in 500 mL of fresh distilled water.

TMB Substrate: One bottle containing 22 mL of TMB (3,3',5,5'-Tetramethylbenzidine) Solution. **Note!** Do not expose to light! **Ready for use!**

Stop Solution: One vial containing 6 mL of 1.0 M sulphuric acid. **Ready for use!**

Performance Characteristics

Measuring range: The measuring range is 0 – 3 000 U/L;

High Dose Effect: No High Dose effect occurs until 26 000 U/L.

Sensitivity: Detection Limit is 60 U/L (calculated as Standard Z + 3 standard deviations); Lowest Limit of Quantification (LLOQ) is 250 U/L.

Reproducibility: Within assay (WA % CV) reproducibility is < 7 %, between assay (BA % CV) reproducibility is < 10 % and total reproducibility < 10 % for samples over LLOQ.

Spiking Recovery: 80 – 120 %.

Linearity/Dilution: 80 – 120 %, dilutions in cell culture medium or Standard Zero.

Calibration

The Units measured by the M30 CytoDeath™ ELISA are defined against a synthetic peptide standard containing the M30 and M6 epitopes. 1 U/L = 1.24 pM (Ref. 3).

Warranty

The performance data presented here were obtained using the procedure indicated. Any change or modification in this procedure, recommended by PEVIVA AB, may affect the results. In such event PEVIVA AB disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and the fitness for use. PEVIVA AB and its authorized distributors, in such event, shall not be liable for damages indirect or consequential.

Other products from PEVIVA

ASSAYS

M30-Apoptosense® ELISA

Prod. No. 10010

M65® ELISA

Prod. No. 10020

M6 EpiDeath™ ELISA

Prod. No. 10040

ANTIBODIES

M30 CytoDeath™

Unlabeled Prod. No. 10700

Biotin Prod. No. 10750

Fluorescein Prod. No. 10800

Orange Prod. No. 10830

Red Prod. No. 10850

M5 CytoKERATIN 18

Prod. No. 10600

M6 CytoKERATIN 18

Prod. No. 10650



PEVIVA AB

Strömkarlsvägen 82, SE-167 62 Bromma, Sweden
Phone: +46 (0)8 122 053 00; Fax: +46 (0)8 730 16 10
Website: www.peviva.com; E-mail: info@peviva.com