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M30 CytoDeath™ ELISA

REF 10900

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

Instructions for Use of the M30 CytoDeath™ ELISA

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Contents

Explanation of Symbols Used on Labels

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

Trademarks

M30®, M30 Apoptosense®, M65®, EpiDeath® and PEVIVA® are registered trademarks of VLVbio (Vivalavida AB).

Patents

U.S. patents number 6,296,850 and 6,716,968 and 6,706,488.
European patent number EP 1 019 438.
Japanese patent number 4372340
Canadian patent number 2305681.

Shipping and Storage

The M30 CytoDeath™ ELISA is shipped in cooled conditions and should be stored at 2 – 8 °C. ***Note!*** Do not freeze!

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Assay Description

Intended Purpose

The M30 CytoDeath™ ELISA is a one step *in vitro* immunoassay for the quantitative determination of the apoptosis-associated K18Asp396 (*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 keratin 18 (K18). The M30 antibody recognises a neo-epitope exposed after caspase cleavage of K18 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 caspase-cleaved K18 (ccK18) fragments containing the K18Asp396 neo-epitope. After induction of apoptosis of epithelial cells, ccK18 increases 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 ccK18 increase during apoptosis is inhibited by the caspase-inhibitor zVAD-fmk.

The M30 CytoDeath™ ELISA can be used in combination with the M65 EpiDeath® ELISA (PEVIVA Prod. No. 10040) which measures total K18. Combining the two assays is useful for assessment of cell death mode (ref. 3).

The 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).

Principle of the Method

The M30 CytoDeath™ ELISA is a solid-phase sandwich enzyme immunoassay. Standards and samples react with a solid phase capture antibody M6 directed against K18 and the HRP- (horseradish peroxidase) conjugated M30 antibody directed against the K18Asp396 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).

Materials Provided for 96 Determinations

M6 Coated Microstrips: One microplate, 12 strips with 8 wells each, 96 dry wells in total. The wells are coated with mouse monoclonal K18 antibody M6. The microplate is sealed in an aluminium bag, which contains a desiccating device. If not all the strips are used, reseal the bag and keep the desiccating device inside. ***Ready for use!***

M30 CytoDeath HRP Conjugate: Concentrate (24 × conc.). One vial containing 0.4 mL of mouse monoclonal M30 antibody (anti-K18Asp396 neo-epitope) conjugated with horseradish peroxidase (HRP) in a phosphate buffer with protein stabilizers. Preservative added. Should be diluted with M30 CytoDeath Conjugate Dilution Buffer (see section "Component Preparation" on page 9). ***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 M30 CytoDeath HRP Conjugate. Preservative added. Green coloured.

M30 CytoDeath Standards: Standard Zero containing 0.5 mL of phosphate buffer with foetal calf serum (FCS). Standards Low, Medium and High, 0.5 mL each, containing standard material in a phosphate buffer with 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 prepared wash solution. Dissolve the Wash Tablet in 500 mL of fresh deionised water.

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

Stop Solution: One vial containing 7 mL of 1.0 M sulphuric acid. ***Ready for use!***

Sealing Tape: One (1) sheet.

Instructions for Use.

Certificate of Analysis.

Materials Required but not Provided

- Microplate reader (wavelength 450 nm; linear 0 – 3 OD)
- Microplate shaker (oscillation: 600 rpm; orbit: 1.5 – 4 mm)
- 96-well microtiter plate washer or multichannel pipette (volume 250 µL)
- Vortex mixer
- Precision pipettes: 25, 50, 75 and 200 µL
- Cylinder (500 mL)
- Deionised water

Assay Protocol

Warnings and Precautions

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 will cause irritation of the 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.com or by request.

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 keratin 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.com for further information.

Collection and Preparation of Samples

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

Note! The M30 CytoDeath™ ELISA cannot be used for blood samples. Consider the M30 Apoptosense® ELISA (Peviva prod. no. 10011) for measuring caspase-cleaved K18 in serum or plasma.

Note! The same type of material (lysate or supernatant) 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.com.

Store samples at 2 – 8 °C up to 4 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".

Sample Preparation from Lysed Cell Cultures

For many applications is it advantageous to measure total M30-reactivity (K18Asp396) at a single, late time point. Such measurements reflect an integrated assessment of apoptosis. To assay total K18Asp396 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 for 5 minutes at room temperature. Mix gently by pipetting up and down, careful not to create air bubbles and transfer 2 × 25 µL of the medium/lysate to the wells of the M6 Coated Microstrips.

Sample Preparation from Cell Culture Supernatants

The M30 CytoDeath™ ELISA and M65 EpiDeath® 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 or staurosporine) and/or mainly necrosis (e. g. oligomycin treatment of glucose starved cells or treatment with hydrogen peroxide).

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

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

Component Preparation

Dilution of M30 CytoDeath HRP Conjugate

Dilute the M30 CytoDeath HRP Conjugate with M30 CytoDeath Conjugate Dilution Buffer. The M30 CytoDeath HRP Conjugate vial contains exactly 0.4 mL. Add 9.2 mL of M30 CytoDeath Conjugate Dilution Buffer directly to the HRP Conjugate vial and mix.

Dissolving of Wash Tablet

Dissolve one Wash Tablet in 500 mL of fresh deionised water. Let the tablet dissolve in the water and mix before use.

Dilution of Samples

Samples higher than Standard High should be diluted with cell culture medium or Standard Zero (0 U/L). Since dilution in the assay is linear, the original concentration is calculated by multiplying the measured concentration by the dilution factor.

Storage and Shelf Life After First Opening

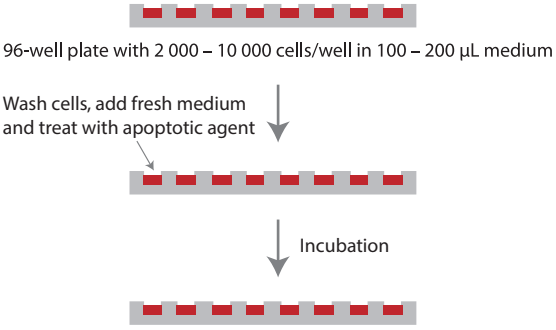
If the entire kit is not used, store reagents in their original containers at 2 – 8 °C. If not all strips are used, reseal the microstrips bag. Remember to include the desiccating device.

The TMB Substrate and the M30 CytoDeath HRP Conjugate are sensitive to light and to metal ions and should be stored in the original amber bottles at 2 – 8 °C at all times between uses. If a new container is used it has to be protected from light! TMB Substrate cannot be used after exposure to light.

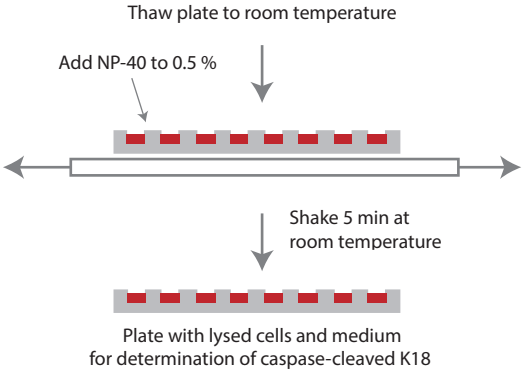
If the kit is used on several occasions, store the diluted M30 CytoDeath HRP Conjugate in the vial at 2 – 8 °C. Do not expose to light. The diluted M30 CytoDeath HRP Conjugate solution is stable for 3 weeks.

The prepared wash solution is stable for 5 weeks when stored at 2 – 8 °C.

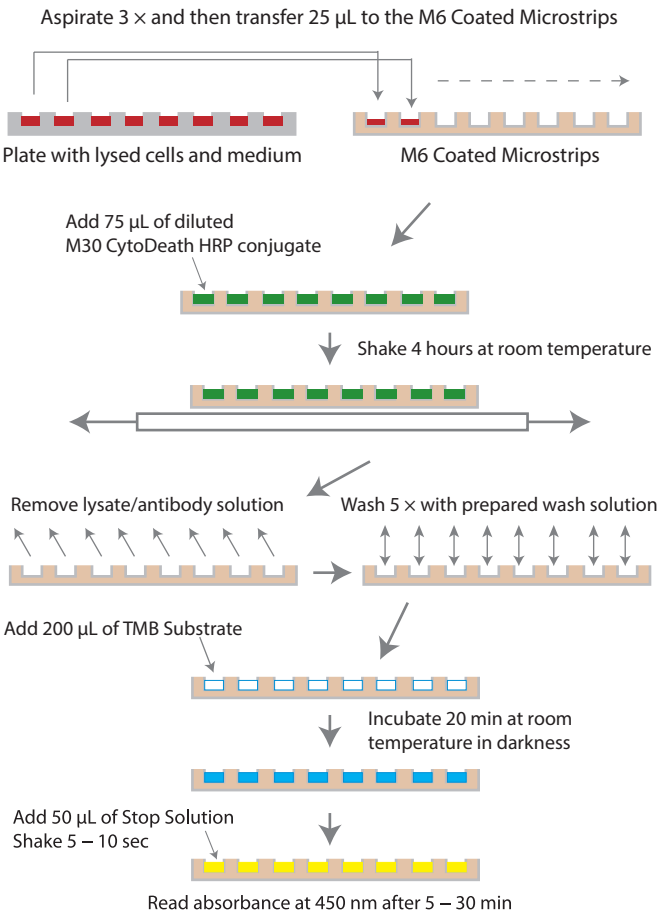
Step 1: Prepare Cells



Step 2: Prepare Cell Lysates



Step 3: Determine Caspase-Cleaved K18



Assay Procedure

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.
2. Dissolve the Wash Tablet in fresh deionised water (see section "Component Preparation" on page 9).
3. Dilute M30 CytoDeath HRP Conjugate with 9.2 mL of M30 CytoDeath Conjugate Dilution Buffer and mix (see section "Component Preparation" on page 9).
4. Pipette 25 µL of standard or sample per well (duplicates are recommended).
5. Add 75 µL of the diluted M30 CytoDeath HRP Conjugate solution per well.
   Note! *Steps 4 and 5 should be performed sequentially without interruption within 15 minutes.*
6. Cover the wells with sealing tape or a microtiter plate lid.
7. Incubate on shaker for 4 hours. Speed setting: 600 rpm on a plate shaker (other shakers: make sure the liquid is moving while staying in the well).
8. Wash the plate in a plate washer 5 times with 400 – 500 µL prepared wash solution per well (overflow wash)
   or
   Wash the plate manually, discarding the incubation solution and washing the wells 5 times with 250 µL of prepared wash solution. Avoid contamination between wells.
9. Add 200 µL of TMB Substrate to each well. Incubate in darkness at room temperature for 20 ± 1 minutes.
10. Add 50 µL of 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 the absorbance.
11. Determine the absorbance at 450 nm in a microplate reader within 30 minutes and record the results.
12. Calculate the results as described in section "Calculation of Analytical Results".

Calculation of Analytical Results

The M30 CytoDeath™ ELISA results are calculated using computer-assisted methods. Evaluate the values of samples using a suitable program for handling ELISA type data. Fitting algorithm: Cubic Spline. x-axis: concentration (U/L); y-axis: absorbance at 450 nm (A450).

Note! If samples have been diluted, the observed concentration must be multiplied by the dilution factor.

Assay Performance

Performance Characteristics

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

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

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

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

Spiking Recovery: 80–120 %.

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

Traceability of Standard

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

Literature

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2. Schutte *et al.*, Exp Cell Research 297, 2004, 11.
3. Hägg *et al.*, Invest New Drugs 20, 2002, 253.
4. Kramer *et al.*, Cancer Res 64, 2004, 1751.
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8. Herrmann *et al.*, J Biomol Screen. 13, 2008, 1.
9. Brnjic *et al.*, Mol Biosyst. 6, 2010, 767

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

Warranty

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

Products

Assays

M30 Apoptosense® ELISA
Prod. No. 10011

M65® ELISA
Prod. No. 10020

M30 CytoDeath™ ELISA
Prod. No. 10900

M65 EpiDeath® ELISA
Prod. No. 10040

Antibodies

M30 CytoDEATH™
– Unconjugated Prod. No. 10700
– Biotin Prod. No. 10750
– Fluorescein Prod. No. 10800
– Orange Prod. No. 10830

M5 Keratin 18
Prod. No. 10600

M6 Keratin 18
Prod. No. 10650

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