PEVIVA

CE

M30 Apoptosense® ELISA

REF 10011

Instructions for Use

In USA, Canada and Japan
For research and laboratory use only.
Not for human or diagnostic use.

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

Patents

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

Shipping and Storage

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

Assay Description

Intended Purpose

The M30 Apoptosense® ELISA is a one-step *in vitro* immunoassay for the quantitative determination of the apoptosis-associated caspase-cleaved keratin 18 (ccK18, K18Asp396 or M30 neo-epitope) in serum and plasma.

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 Apoptosense 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 (ref. 3).

The M30 Apoptosense ELISA can be used in combination with the M65® ELISA (PEVIVA Prod. No. 10020) which measures total K18. Combining the two assays is useful for assessment of cell death mode (ref. 4).

The M30 Apoptosense ELISA detects human caspase-cleaved K18, but does not detect caspase-cleaved mouse, rat or canine K18 (ref. 5). The M30 Apoptosense ELISA will specifically detect tumour apoptosis in mice or rats carrying human tumour xenografts (ref. 5).

M30 Apoptosense ELISA is intended for use in research, clinical diagnostics and clinical trials in the fields of oncology, hepatology and transplantation.

Principle of the Method

The M30 Apoptosense ELISA is a solid-phase sandwich enzyme immunoassay. Standards, controls and samples react with a solid phase capture antibody M5 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

M5 Coated Microstrips: One microplate, 12 strips with 8 wells each, 96 dry wells in total. The wells are coated with mouse monoclonal K18 antibody M5. 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 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 phosphate buffer with protein stabilizers. Preservative added. Should be diluted with M30 Conjugate Dilution Buffer. ***Note!*** Do not expose to light!

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

M30 Standard A – G: Standard A containing 2 mL of phosphate buffer with foetal calf serum (FCS). Standard B – G, 0.5 mL each, containing standard material in phosphate buffer with FCS. The values of Standard A – G are 0, 75, 150, 250, 500, 750 and 1 000 U/L, respectively. Preservative added. Yellow coloured. ***Ready for use!*** Standard A can be used for dilutions of samples > 1 000 U/L.

M30 Control Low & High: Two vials containing 0.5 mL of reactive components in phosphate buffer with FCS. The values of M30 Control Low and M30 Control High are stated on the respective vials. Preservative added. Yellow coloured. ***Ready for use!***

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) Solution. ***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.

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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 Apoptosense ELISA kit is intended for *in vitro* use only.
2. Do not mix reagents from different kit lots.
3. All patient specimens 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.

Collection and Preparation of Blood Samples

The sample volume should be sufficient for measuring each sample in duplicate (test volume 2 × 25 µL). Donors do not need to be fasting prior to blood collection.

Serum: Collect blood by venipuncture, avoiding haemolysis, into plain tubes (without anti-coagulant), allow blood to clot and collect serum after centrifugation.

Plasma: The M30 Apoptosense ELISA can also be used for plasma samples (EDTA or heparin).

Note! The same type of material, i.e. serum or plasma collected by one method, should be used for a specific project. For further information on the performance of the M30 Apoptosense 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 (ref. 6, 7), but it is recommended that repeated freeze-thawing should be avoided. For dilution of samples see sections "Component Preparation" and "Performance Characteristics".

Collection and Preparation of *in vitro* Samples for Research Use Only

M30 Apoptosense ELISA has been used for cell-culture applications in a number of published studies (see www.peviva.com for references). Peviva has developed a specific product for *in vitro* cell cultures, ***M30 CytoDeath™ ELISA*** (PEVIVA Prod. No. 10900). This product has a dynamic range and sensitivity suitable for *in vitro* work.

The following protocols can be used for detection of apoptosis of cultured epithelially derived cells using the M30 Apoptosense ELISA.

Sample preparation from cell cultures

For many applications, it is advantageous to measure total M30 reactivity (ccK18) at a single, late time point. Such measurements reflect an integrated assessment of apoptosis. To assay total ccK18 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; 5 000–10 000 cells per well in a 96-well plate is usually adequate.

Day 2: Wash the cells once with PBS and add fresh medium (200 µL/well). Expose the cells to the desired agent(s).

Day 2 – 4: For 96-well plates containing 200 µL of medium per well, add 10 µL of 10 % NP 40 per well. Allow lysis to occur on a rotatory 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 M5 Coated Microstrips.

Sample preparation from cell culture supernatants

The M30 Apoptosense 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.

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.

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

Component Preparation

Dilution of M30 Conjugate

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

Dissolving of Wash Tablet

Dissolve one Wash Tablet in 500 mL of fresh deionised water.

Dilution of Samples

Samples higher than Standard G (1 000 U/L) should be diluted with Standard A or blood donor serum. Since dilution in the assay is linear, the original concentration is calculated by multiplying the measured concentration with the dilution factor. In case blood donor serum/plasma was used as sample diluent, its concentration (U/L) must be accounted for.

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 Conjugate are sensitive to light and 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 at several occasions, store the diluted M30 Conjugate in the vial at 2 – 8 °C. Do not expose to light. The diluted M30 Conjugate solution is stable for 3 weeks.

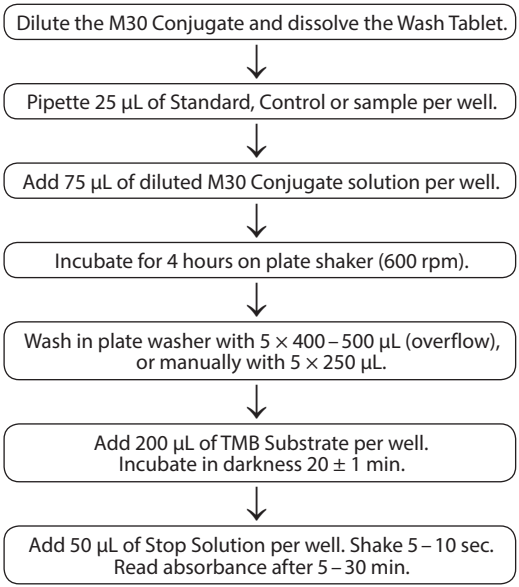
The Wash Tablet solution is stable for 5 weeks when stored at 2 – 8 °C.

Assay Procedure

The M30 Apoptosense 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 "Component Preparation").
3. Dilute the M30 Conjugate with M30 Conjugate Dilution Buffer (see "Component Preparation") and mix.
4. Pipette 25 µL of M30 Standard (A – G), M30 Control Low, M30 Control High or sample per well (duplicates are recommended).
5. Add 75 µL of the diluted M30 Conjugate solution to each well.
   Note! *Steps 4 and 5 should be performed sequentially without interruption within 20 minutes.*
6. Cover the wells with sealing tape or a microtiter plate lid.
7. Incubate on shaker for four (4) hours. Speed setting: 600 rpm.
8. Wash the plate in a plate washer five (5) times with 400 – 500 µL/well of Wash Tablet solution (overflow wash)
   or
   Wash the plate manually, discarding the incubation solution and washing the wells five (5) times with 250 µL of Wash Tablet 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".

Flow Chart



Calculation of Analytical Results

The M30 Apoptosense ELISA results are calculated using computer-assisted methods. Evaluate the values of controls and 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, and in case blood donor serum/plasma was used as sample diluent, its concentration (U/L) must be accounted for.

Assay Performance

Performance Characteristics

Measuring range: The measuring range is 0–1 000 U/L.

High Dose Effect: No High Dose effect occurs until 195 000 U/L.

Reproducibility: Within assay (WA %CV) variation is ≤ 10 %, between assay (BA %CV) variation is ≤ 10 % and total variation is ≤ 10 % for samples over 200 U/L.

Sensitivity: The minimum detectable concentration of K18Asp396 neo-epitope in the M30 Apoptosense ELISA is 20 U/L, defined as the concentration of ccK18 that corresponds to the absorbance being two standard deviations from the absorbance of the Standard A (0 U/L).

Lower Limit of Quantification: The lowest concentration at which an analyte in the sample matrix can be measured with acceptable level of accuracy and precision is 40 U/L.

Spiking Recovery: Recovery of high standard when spiked into human blood samples: 109 % (average) and 98 – 120 % (range).

Linearity/Dilution: Recovery of human sera when diluted in M30 Standard A (0 U/L): 107 % (average) and 99 – 122 % (range).

Reference range: In serum from 200 Swedish blood donors, the median was 94 U/L and the 95th percentile was 251 U/L. It is recommended that each laboratory establishes its own reference range.

Traceability of Standard

The units measured by the M30 Apoptosense ELISA are defined against native antigen spiked into serum. Native antigen is calibrated against a recombinant protein standard. 1 U/L = 1.24 pM. ***Note!*** Due to different assay buffers, standard material cannot be exchanged between different Peviva kits.

Internal Quality Control

The supplied M30 Control Low and High with their given concentrations should be sufficient to secure the assay performance and should be used, at least, in duplicate each time the assay is performed.

If this procedure is not sufficient, each laboratory needs to establish its own controls by the guidelines in section "Collection and Preparation of *in vitro* Samples for Research Use Only" or by individual laboratory routine. These controls should be frozen in aliquots and treated in the same way each time the assay is performed.

Limitations of the Method

The clinical utility of ccK18 measurement in human blood samples as a prognostic indicator and in the management of patients on therapy regimens has not been fully established.

Grossly lipemic (≤ 1 250 mg/dL), icteric (≤ 12.5 mg/dL) or haemolysed (≤ 50 mg/dL) samples do not interfere in the assay.

Literature References

1. Leers *et al.*, J Pathol. 187, 1999, 567.
2. Schütte *et al.*, Exp Cell Res. 297, 2004, 11.
3. Hägg *et al.*, Invest New Drugs 20, 2002, 253.
4. Kramer *et al.*, Cancer Res 64, 2004, 1751.
5. Olofsson *et al.*, Cancer Biomarkers 5, 2009, 117.
6. Greystoke *et al.*, Ann Oncol 19, 2008, 990.
7. Olofsson *et al.*, Clin Cancer Res 13, 2007, 3198.
8. Ueno *et al.*, Eur J Cancer 39, 2003, 769.

For further references, please consult www.peviva.com/literature.aspx.

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.

PEVIVA 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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