



M30-Apoptosense[®] ELISA Kit

Manufactured by Peviva AB.

ALX-850-270-KI01



96 wells (~80 tests)

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For laboratory use only. Not for human or diagnostic use.

EXPLANATION OF SYMBOLS USED ON LABELS

 In Vitro Diagnostic Medical Device

 Catalogue number

 Contains sufficient for <n> tests

 Batch code

 Manufacturer

 Temperature limitation

 Use by

 Consult Instructions for Use

TRADEMARKS

M30-Apoptosense[®] and M65[®] are registered trademarks 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., British Journal of Cancer 92, 2005, 532.
7. Hägg et al., Molecular Cancer Therapeutics 3, 2004, 489.

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

INTENDED USE

M30-Apoptosense[®] ELISA is a one step *in vitro* immunoassay for the quantitative determination of the apoptosis-associated CK18Asp396 ("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 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-Apoptosense[®] 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-Apoptosense[®] 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 IgG_{2b} antibody. The M30-Apoptosense[®] ELISA has not been extensively tested for other species than human.

PRINCIPLE OF THE PROCEDURE

The M30-Apoptosense[®] ELISA is a solid-phase sandwich enzyme immunoassay. Standards, Controls and Samples react with a solid phase catcher antibody "M5" 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 color development is stopped and the absorbance is read. The resulting color 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 Liter (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). Donors do not need to be fasting prior to blood collection.

Serum: Collect blood by venipuncture, avoiding haemolysis, into plain tubes (without anti-coagulant) and separate the serum from the cells.

Plasma: The M30-Apoptosense[®] ELISA can also be used for plasma samples (EDTA, Heparin or Citrate).

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.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 recommend that repeated freeze-thawing should be avoided.

For dilution of samples see section: "Performance Characteristics".

COLLECTION AND PREPARATION OF SAMPLES FOR RESEARCH USE

a. Sample preparation from cell cultures

For many applications is it 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; 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 medium per well: add 10 µl 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 x 25 µl of the medium/lysate to the wells of a M30 Coated Microstrips

b. 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 (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.

WARNING AND PRECAUTIONS FOR USERS

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 sulfuric 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)

Deionized water

MATERIALS PROVIDED FOR 96 DETERMINATIONS

M30 Coated Microstrips: One Microplate, 96 dry wells (12 x 8).

The wells are coated with mouse monoclonal CK18 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 HRP Conjugate: Concentrate (**24 x conc**). One vial containing 0.4 mL mouse monoclonal M30 antibody (anti-CK18Asp396 neo-epitope) conjugated with horseradish peroxidase (HRP) in phosphate buffer with protein stabilizers. Should be diluted with M30 Conjugate Dilution Buffer (see Table 1). Preservative added.

Note! Do not expose to light!

M30 Conjugate Dilution Buffer: One vial containing 12 mL of phosphate buffer with protein stabilizers for dilution of the M30 HRP Conjugate. Preservative added. Blue colored. [Ready for use!](#)

M30 Standards A-G: Standard A containing 4 mL phosphate buffered FCS. Standards B-G, 0.5 mL each, containing standard material in phosphate buffered FCS. The values of the Standards A-G are 0, 75, 150, 250, 500, 750 and 1,000 U/L, respectively. Preservative added. Yellow colored. [Ready for use!](#)

Standard A can be used for dilutions of samples > 1,000 U/L.

M30 Control Low & High: Two 0.5 mL vials containing reactive components in phosphate buffered FCS. The values of the M30 Controls Low and High are stated on the respective vial. Preservative added. Yellow colored.

[Ready for use!](#)

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 8 mL of 1.0 M sulfuric acid. [Ready for use!](#)

Wash Solution: (10 x conc) One vial containing 50 mL of concentrated Wash Solution. Dilute with 450 mL of fresh distilled water before use. Diluted buffer consists of 0.014 M phosphate buffer with 0.15 M sodium chloride and 0.1 % Tween[®] 20. Preservative added.

Sealing Tape: One (1) sheet.

DILUTION OF M30 HRP CONJUGATE

Based on the number of strips needed, dilute the M30 HRP Conjugate in M30 Conjugate Dilution Buffer as follows:

Table 1

M30 Coated Microstrips (No of strips)	M30 HRP Conjugate (mL)	M30 Conjugate Dilution Buffer (mL)
3	0.1	2.3
6	0.2	4.6
9	0.3	6.9
12	0.4*	9.2

*dilute directly in M30 HRP Conjugate bottle

REAGENT STORAGE

The M30-Apoptosense[®] ELISA should be stored at 2 - 8°C.

Do not freeze!

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 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 in between use. If a new plastic container is used it has to be protected from light! TMB Substrate can not be used after exposure to light.

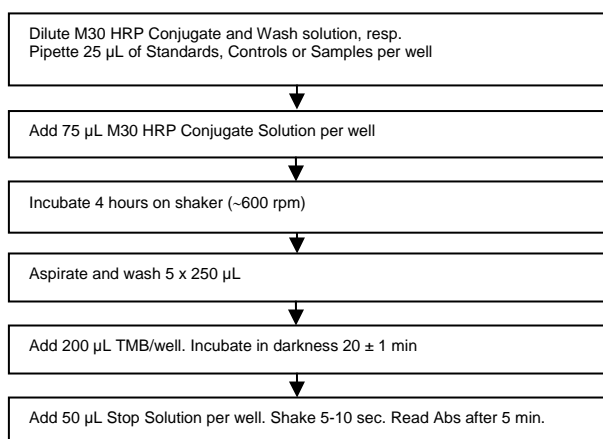
The diluted M30 HRP Conjugate Solution is stable for 2 weeks if stored at 2 - 8°C in the dark.

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. Dilute M30 HRP Conjugate with M30 Conjugate Dilution Buffer and mix thoroughly (see Table 1).
3. Pipette 25 µl of M30 Standards (A-G), M30 Control Low, M30 Control High and Samples per well (duplicates are recommended).
4. Add 75 µl of the diluted M30 HRP Conjugate Solution to each well.
Note that steps 3 and 4 should be performed sequentially without interruption within 15 minutes.
5. Cover the wells with sealing tape.
6. Incubate on shaker for four (4) hours. Optimal speed setting:
~ 600 rpm.
7. Aspirate and wash the wells five (5) times with 250 µl diluted Wash Solution. *Avoid contamination between wells.*
8. Add 200 µl of TMB Substrate to each well. Incubate in darkness at room temperature for 20 ± 1 minutes.
9. 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.
10. Determine the absorbance at 450 nm in a Microplate reader within 30 minutes and record the results.
11. Calculate the results as described in Section "Processing of results".

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PROCESSING OF RESULTS

The M30-Apoptosense[®] ELISA results may be calculated manually, or, preferably by using computer-assisted methods.

Computer-assisted methods: Evaluate the values of Controls and 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).

Manual method: The M30-Apoptosense[®] ELISA standard curve may be constructed manually on lin-lin paper by plotting the absorbance at 450 nm for each Standard on the y-axis versus the concentration of the Standard on the x-axis. The best fit curve should be drawn through the standard points. The concentration in a sample is determined from the constructed standard curve.

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, their “M30” concentration (U/L) must be accounted for.

LIMITATIONS OF THE PROCEDURE

The clinical utility of Asp396 neo-epitope 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, icteric or haemolysed (≤ 100 mG/dL) samples do not interfere in the assay.

PERFORMANCE CHARACTERISTICS

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

High Dose Effect: No High Dose effect occurs until 70,000 U/L.

Reproducibility: Within assay (WA % CV) reproducibility is < 10 % and between assay (BA % CV) reproducibility is < 10 %.

Sensitivity: The minimal detectable concentration of CK18 Asp396 neo-epitope in M30-Apoptosense[®] ELISA is 25 U/L, defined as the concentration of “M30” that corresponds to the absorbance being two standard deviations from the absorbance of the Standard 0 U/L.

Spiking Recovery: The Standard provided with the kit contains recombinant material that behaves differently from the CK18 fragment in blood samples and is therefore not considered adequate for spiking recovery tests.

Linearity/Dilution: Recovery of human sera when diluted in M30 Standard A (0 U/L): 116% (average) and 106-124 % (range).

Recovery of human sera when diluted in blood donor serum: 97% (average) and 81-106% (range).

Reference range: In serum from 236 Swedish blood donors, the 95th percentile was 260 U/L. It is recommended that each laboratory establishes its own reference range.

CALIBRATION

The Units measured by the M30-Apoptosense® ELISA are defined against a synthetic peptide standard containing the “M30” and “M5” epitopes. 1 U/L = 1.24 pM (Ref. 3).

INTERNAL QUALITY CONTROL

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

If this procedure is not sufficient, each laboratory would need to establish their own controls by the guidelines in Section: “Collection and Preparation of Samples” or by individual laboratory routine. These controls should be frozen in aliquots and treated in the same way each time the assay is performed.

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.