

M65[®] ELISA

REF 10020

Instructions for Use

Bruksanvisning

Gebrauchsanweisung

Mode d'emploi

Istruzioni per l'uso

Instrucciones de uso

In USA, Canada and Japan

For research and laboratory use only.

Not for human or diagnostic use.

Instructions for Use of the M65® ELISA

Contents

Instructions for Use of the M65® ELISA	2
Explanation of Symbols Used on Labels	3
Trademarks	3
Shipping and Storage	3
Assay Description	4
Intended Purpose	4
Summary and Explanation of the Test	4
Principle of the Method	4
Materials Provided for 96 Determinations	5
Materials Required but not Provided	6
Assay Protocol	6
Warnings and Precautions	6
Collection and Preparation of Blood Samples	6
Collection and Preparation of <i>in vitro</i> Samples for Research Use Only	7
Component Preparation	7
Storage and Shelf Life After First Opening	8
Assay Procedure	9
Flow Chart	10
Calculation of Analytical Results	10
Assay Performance	11
Performance Characteristics	11
Traceability of Standard	11
Internal Quality Control	11
Limitations of the Method	12
Literature References	12
Warranty	12

Explanation of Symbols Used on Labels



Catalogue number



Contains sufficient for <n> tests



Batch code



Manufacturer



Temperature limitation



Use by



Consult Instructions for Use

Trademarks

M65® and M30 Apoptosense® are registered trademarks of PEVIVA AB. Tween® 20 is a registered trademark of ICI America, Inc.

Shipping and Storage

The M65® ELISA is shipped in cooled conditions and should be stored at 2–8 °C. *Note!* Do not freeze!

Assay Description

Intended Purpose

The M65[®] ELISA is a one-step *in vitro* immunoassay for the quantitative determination of soluble keratin 18 (K18) in serum and plasma.

Summary and Explanation of the Test

Extracellular K18 can be used as a marker for epithelial cell death. During necrosis, loss of cell membrane integrity will result in the release of intracellular proteins, including K18, into the extracellular compartment. Apoptosis represents an active form of cell death that initially preserves plasma membrane integrity but which is commonly followed by secondary necrosis where intracellular components are released. The M65[®] ELISA assay measures total soluble K18 released from dead cells (necrotic and apoptotic). Measurements from cell culture supernatants or human serum/plasma samples by the M65[®] ELISA will therefore represent the total epithelial cell death by any cause (ref. 1).

K18 is cleaved by caspases during apoptosis. The M30 Apoptosense[®] ELISA assay (PEVIVA prod. no. 10010; ref. 2) specifically measures the level of caspase-cleaved K18 fragments (ccK18) containing the K18Asp396 neo-epitope. The combination of the M30 Apoptosense[®] ELISA and the M65[®] ELISA therefore facilitates the determination of cell death mode *in vitro* and in serum or plasma from patients or experimental animals with human tumour xenografts (ref. 1, 3, 4).

The M65[®] ELISA uses two mouse monoclonal antibodies (clone M5, IgG2b, and M6, IgG2a) specific for conventional epitopes of K18. The M5 antibody detects human K18, but does not react to mouse K18 (ref. 4). The M65[®] ELISA will specifically detect tumour cell death in mice carrying human tumour xenografts (ref. 4).

M65[®] ELISA is used for research and clinical trials in the fields of oncology, hepatology, transplantation and sepsis.

Principle of the Method

The M65[®] ELISA is a solid-phase sandwich enzyme immunoassay. Standards, controls and samples react with a solid phase capture antibody M6 directed against K18 and the HRP (horseradish peroxidase) conjugated M5 antibody directed against a different epitope of K18. 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).

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Materials Provided for 96 Determinations

M65 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!*

M65 HRP Conjugate: Concentrate (24 × conc). One vial containing 0.4 mL of mouse monoclonal M5 antibody (anti-K18) conjugated with horseradish peroxidase (HRP) in a phosphate buffer with protein stabilizers. Preservative added. Should be diluted with M65 Conjugate Dilution Buffer. *Note!* Do not expose to light!

M65 Conjugate Dilution Buffer: One vial containing 12 mL of phosphate buffer with protein stabilizers for dilution of the M65 HRP Conjugate. Preservative added. Blue coloured. *Ready for use!*

M65 Standard A – G: Standard A containing 4 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, 125, 250, 500, 750, 1 200 and 2 000 U/L, respectively. Preservative added. Yellow coloured. *Ready for use!* Serum/plasma samples > 2 000 U/L can be diluted 1 + 1 with Standard A, but dilution with pooled human serum is recommended (see section “Performance Characteristics”).

M65 Control Low & High: Two vials containing 0.5 mL of reactive components in phosphate buffer with FCS. The values of M65 Control Low and High are stated on the respective vials. Preservative added. Yellow coloured. *Ready for use!*

Wash Solution: One vial containing 50 mL of concentrated (10 × conc) Wash Solution. Dilute with 450 mL of fresh deionised water before use. Phosphate buffer with Tween® 20. Preservative added.

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 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. The M65® 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.se or by request.

Collection and Preparation of Blood Samples

The sample volume should be sufficient for measuring each sample in duplicate (test volume $2 \times 25 \mu\text{L}$). 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 M65® 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 M65® ELISA using different types of samples, please consult www.peviva.se.

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. 3, 5), but it is recommended that repeated freeze-thawing should be avoided. For dilution of samples see section “Performance Characteristics”.

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

The M65® ELISA can be used to assess total cell death of epithelial cells *in vitro* by measuring release of K18 protein into the culture medium. The M30 Apoptosense® ELISA and the M65® ELISA can be used to assess cell death mode by calculation of an M30:M65 ratio (ref. 1, 6). 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) (ref. 1).

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: 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 of 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 M65 HRP Conjugate

Dilute the M65 HRP Conjugate with M65 Conjugate Dilution Buffer. The M65 HRP Conjugate vial contains exactly 0.4 mL. Add 9.2 mL of M65 Conjugate Dilution Buffer directly to the M65 HRP Conjugate vial and mix.

Dilution of Wash Solution

The Wash Solution is a 10 × concentrate. Dilute the Wash Solution (50 mL) with 450 mL of fresh deionised water and mix.

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 M65 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 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 M65 HRP Conjugate in the vial at 2–8 °C. Do not expose to light. The diluted M65 HRP Conjugate solution is stable for 3 weeks.

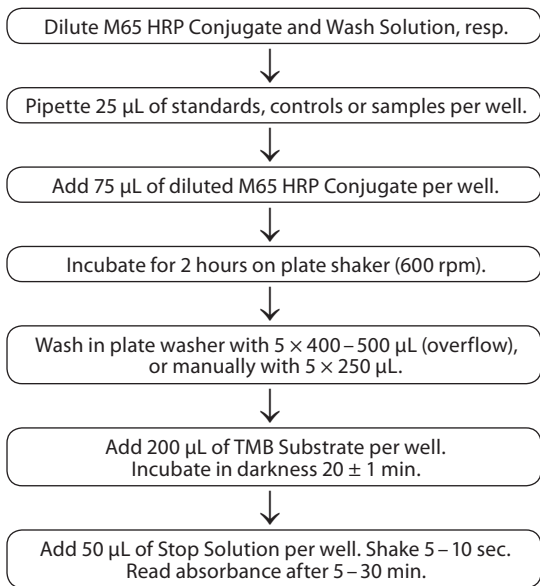
The diluted Wash Solution is stable for 5 weeks when stored at 2–8 °C.

Assay Procedure

The M65® 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 the Wash Solution with fresh deionised water (see “Component Preparation”).
3. Dilute the M65 HRP Conjugate with M65 Conjugate Dilution Buffer (see “Component Preparation”) and mix.
4. Pipette 25 µL of M65 Standard (A–G), M65 Control Low, M65 Control High or sample per well (duplicates are recommended).
5. Add 75 µL of the diluted M65 HRP 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 2 hours. Speed setting: 600 rpm.
8. Wash the plate in a plate washer 5 times with 400–500 µL of diluted 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 diluted 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”.

Flow Chart



Calculation of Analytical Results

The M65[®] 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 M65 concentration (U/L) must be accounted for.

Assay Performance

Performance Characteristics

Measuring range: The measuring range is 0–2 000 U/L.

High Dose Effect: No High Dose effect occurs up to 50 000 U/L.

Reproducibility: Within assay (WA % CV) variation is < 10 % and between assay (BA % CV) variation is < 10 % for samples > 200 U/L.

Sensitivity: The minimum detectable concentration of K18 in M65® ELISA is 11 U/L, defined as the concentration of K18 that corresponds to the absorbance being two standard deviations from the absorbance of the Standard A (0 U/L).

Spiking Recovery: The Standard provided with the kit contains recombinant material that behaves differently from the K18 protein in blood samples and is therefore not considered adequate for spiking recovery tests.

Linearity/Dilution: Recovery of human sera when diluted in M65 Standard A (0 U/L): 126 % (average) and 116–139 % (range). Recovery of human sera when diluted in blood donor serum: 120 % (average) and 101–133 % (range). Serum/plasma samples > 2 000 U/L can be diluted 1 + 1 with Standard A, but dilution with pooled human serum is recommended.

Reference range: In serum from 222 Swedish blood donors, the median was 264 U/L and the 95th percentile was 413 U/L. It is recommended that each laboratory establish its own reference range.

Traceability of Standard

The units measured by the M65® ELISA are defined against a synthetic peptide containing the M6 and M5 epitopes. 1 U/L = 1.24 pM (ref. 1).

Internal Quality Control

The supplied M65 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 K18 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 ($\leq 1\ 250$ mg/dL), icteric (≤ 12.5 mg/dL) or haemolysed (≤ 100 mg/dL) samples do not interfere in the assay.

Literature References

1. Kramer *et al.*, Cancer Res 64, 2004, 1751.
2. Hägg *et al.*, Invest New Drugs 20, 2002, 253.
3. Olofsson *et al.*, Clin Cancer Res 13, 2007, 3198.
4. Olofsson *et al.*, Cancer Biomarkers 5, 2009, 117.
5. Greystoke *et al.*, Ann Oncol 19, 2008, 990.
6. Linder *et al.*, Expert Rev Mol Diagn 10, 2010, 353.

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

Warranty

The performance data presented here were obtained using the procedure indicated. Any change or modification in this procedure as 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.

Products from PEVIVA

Assays

M30 Apoptosense® ELISA

Prod. No. 10010

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
- Red Prod. No. 10850

M5 Keratin 18

Prod. No. 10600

M6 Keratin 18

Prod. No. 10650



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