



M65[®] ELISA

96 test units (12 x 8 wells)

Prod. No. 10020

Instructions

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

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1. Name and Intended Use

M65[®] is an enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of soluble cytokeratin18 (CK18). CK18 is an intracellular protein expressed at high levels by many cell types of epithelial origin including the most common human carcinomas, but is absent in cells of the hematopoietic system or in neuronal cells. After disintegration of the cellular plasma membrane CK18 is eventually released from dying cells into the extracellular compartment.

The M65[®]ELISA is primarily intended to be used for research applications designed to detect and quantify total cell death of CK18-positive cells (e.g. carcinoma) in human serum and plasma samples.

The M65[®]ELISA is intended to be used in combination with the M30-Apoptosense[®]ELISA (PEVIVA Prod. No. 10010) as both cell death assays use identical reference material for standardization (U/L) which allows the calculation of "M30:M65 ratios". These ratios reflect cell death modes (apoptosis and necrosis; see Kramer et al., 2004).

2. Summary and Explanation of the Test

Tumor cells die by apoptosis and necrosis *in vivo*. The mode of cell death depends on the type and strength of the cytotoxic stimulus and various properties of the tumor and tumor microenvironment. An apoptotic stimulus may under conditions of insufficient cellular ATP generation fail to induce a complete apoptotic program and cells will undergo necrosis instead (Leist et al., 1997). During necrosis, loss of cell membrane integrity will result in the release of intracellular proteins (e.g. CK18) into the extracellular environment. Apoptosis, in contrary to necrosis, represents an active form of cell death that initially preserves plasma membrane integrity. Apoptosis is commonly followed by "secondary necrosis" that leads to the release of intracellular components.

The M65[®]ELISA uses two mouse monoclonal antibodies (clone "M6" and "M5", both IgG2b) specific for conventional epitopes on

CK18. The M65[®]ELISA assay measures total soluble CK18 protein (both caspase-cleaved and un-cleaved) released into the extracellular medium. Values obtained from cell culture supernatants or human serum or plasma samples by the M65[®]ELISA will therefore represent the total epithelial cell death "by any cause".

The M30-Apoptosense[®] assay (PEVIVA Prod. No. 10010) uses the mouse monoclonal antibody "M30" and can therefore specifically detect an apoptotic "finger-print" on CK18 in form of a "neo-epitope" only formed upon caspase-cleavage of CK18 at position Asp396 (CK18Asp396)(Leers et al., 1999). The M30-Apoptosense[®] assay will quantify exclusively caspase-cleaved CK18 present only in the extracellular compartment when released from previously apoptotic cells (Hägg et al., 2002; Bivén et al., 2003).

The benefit for the researcher using the M65[®]ELISA in addition to the M30-Apoptosense[®]ELISA is to determine the relative proportion of caspase-cleaved CK18 protein fragments present in the extracellular compartment. Induction of apoptosis in cells of epithelial origin will result in the release of caspase-cleaved CK18 protein fragments (detected by the M30-Apoptosense[®]ELISA AND the M65[®]ELISA) into the extracellular compartment and thereby will give comparatively higher "M30:M65 ratios". Induction of necrosis, however, will almost exclusively result in the release of CK18 molecules that are not caspase-cleave (detected ONLY by the M65[®]ELISA) and consequently in comparatively lower "M30:M65 ratios".

The "M30:M65 ratio" therefore represents a valuable parameter to determine the primary mode of cell death of CK18 positive cells. The M65[®]ELISA is the recommended tandem assay partner for the M30-Apoptosense[®]ELISA.

The M65[®]ELISA in combination with the M30-Apoptosense[®]ELISA has recently been used successfully with serum samples from patients to differentiate between death modes of epithelial carcinomas (Kramer et al., 2004).

3. Principle of the Procedure

The M65[®]ELISA is a solid-phase, two-site immunosorbent assay. Samples are reacted with a mouse monoclonal antibody "M6" against CK18, which has been immobilized to the polystyrene wells and, simultaneously, with the HRP (Horseradish Peroxidase) conjugated monoclonal antibody "M5" directed against a different epitope on CK18.

Following the formation of the solid phase/antigen/labeled antibody sandwich, excess unbound conjugate is removed by a washing step. TMB substrate is added and color develops in proportion to the bound analyte. The color development is then stopped and the intensity of the color is measured in a microplate reader at 450 nm.

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).

4. Warning and Precautions for Users

- The M65[®]ELISA kit is for research use only, not for use in diagnostic procedures.
- The components contain 0.1 % Kathon[™] CG as a preservative.
- The Stop Solution contains 1.0 M sulfuric acid (causes burns).
- Do not use reagents beyond their expiration dates.
- Do not mix or substitute components with those from other lots of kits.
- Handle all components and samples as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, U.S. Government Printing Office; Washington, DC, 1988, pages 11-12. Follow universal precautions when handling samples as established by your institution.

5. Storage

The M65[®]ELISA kit should be stored at 2° - 8°C. **Do not freeze!**

If not all of the kit is used at the same time, the reagents should be stored in their original containers and returned to cold storage. Keep the desiccating device in the aluminum bag containing the Coated Microstrips.

The **TMB Substrate** and the **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!

All reagents should be adapted to room temperature (20±4°C) prior to use.

6. Materials Supplied

Each kit contains reagents for 96 tests. The expiry date is printed on the external label. The following components are supplied with the M65[®]ELISA kit:

A. Coated Microstrips: ready to use!

One Microplate (dry) containing 12 strips with 8 wells for 96 determinations. The wells are coated with a mouse monoclonal antibody “M6” to CK18. The Microplate is sealed in an aluminum bag, which contains a desiccating device. If not all the strips are used at one occasion, cover the remaining strips with the provided plastic sealing tape. Reseal the bag and keep the desiccating device inside.

B. HRP Conjugate: concentrate!

One vial containing 0.4 ml mouse monoclonal antibody “M5” to CK18 conjugated with horseradish peroxidase (HRP) in phosphate buffer with protein stabilizers. Should be diluted 24 times with the Conjugate Dilution Buffer (see Table 1).

Note: Do not expose to light!

C. Conjugate Dilution Buffer: ready to use!

One vial containing 12 ml of phosphate buffer with protein stabilizers for dilution of the HRP Conjugate. Blue color for easy identification.

D. TMB Substrate Solution: ready to use!

One bottle containing 22 ml of TMB (3,3',5,5'-Tetramethylbenzidine) Solution.

E. Stop Solution: ready to use!

One vial containing 8 ml of 1.0 M sulfuric acid as Stop Solution.
Caution: H₂SO₄ causes burns!

F. Standards A-F: ready to use!

Six 0.5 ml vials containing standard material in Diluent. The values of the Standards A-F are 250, 750, 1500, 2500, 3750 and 5000 U/L, respectively. Yellow color for easy identification.

G. Control Low & High: ready to use!

Two 0.5 ml vials containing reactive components in phosphate buffered FCS. The values of the Controls Low and High are 500±50 U/L and 3,000±300 U/L, respectively. Yellow color for easy identification.

H. Diluent: ready to use!

One vial containing 8 ml of phosphate buffered FCS. Yellow color for easy identification.
Extra Diluent (PEVIVA Prod. No. 20220) can be ordered separately.

I. Wash Solution: concentrate!

One vial containing 50 ml of concentrated (10X) 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.

J. Plastic Sealing Tape

For sealing remaining strips, if the Microtiter plate is not used up at once.

K. Instructions

L. Certificate of Standards and Controls

Coated Microstrips No. Strips	HRP Conjugate (ml)	Conj. Dilution Buffer (ml)
3	0.1	2.3
6	0.2	4.6
9	0.3	6.9
12	0.4*	9.2

* (dilute in HRP Conjugate bottle)

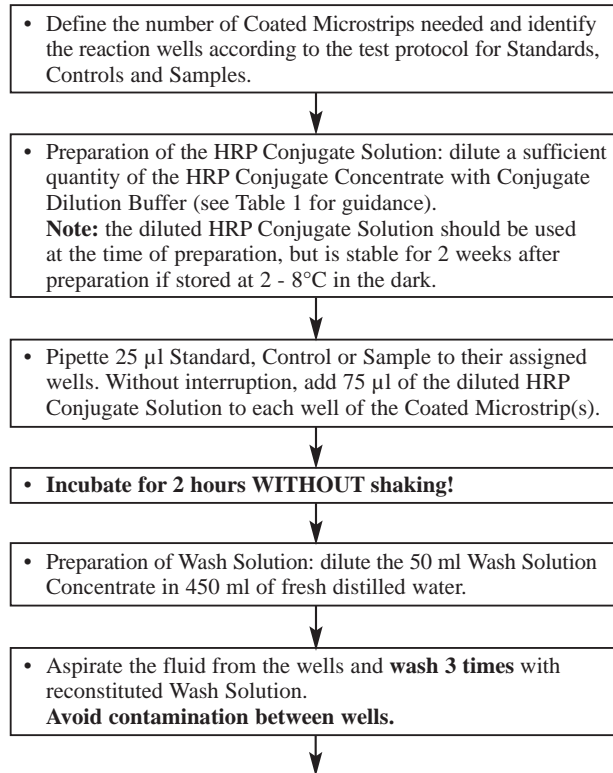
Table 1. Dilution scheme for HRP Conjugate and Conjugate Dilution Buffer.

7. Materials Required but not Supplied

- 25 µl pipette, precision 8-channel pipette to deliver 50, 75 and 200 µl.
- Microplate shaker for agitation of the Microplate to ensure complete mixing of the TMB Substrate and the Stop Solution.
- Washing system for microplates.
- Microplate Reader for measuring absorbance at 450 nm.

8. Assay Procedure

The M65[®]ELISA assay should be performed at room temperature (20±4°C). Allow all reagents and samples to adapt to room temperature before performing the assay.



• Add 200 μl of TMB Substrate Solution 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 Solution and the Stop Solution, agitate the Microplate on a microplate shaker for 5-10 seconds.
Caution: The Stop Solution causes burns!
• Leave the Microplate for 5 minutes before starting the reading of the optical density (OD).



• Determine the absorbance at **450 nm** in a microplate reader within 30 minutes and record the results.



• Calculate the results as described in Section 10:

9. Collection and Preparation of Samples

The methods described in the following have been successfully evaluated by PEVIVA and can therefore serve as guidelines when collecting samples.

A. Serum and plasma specimens

Sufficient blood should be collected before, during and after exposure to the cytotoxic stimulus. The volume should be sufficient for 2x25 μl serum (duplicates) at each assay. Donors do not need to be fasting prior to blood collection and no special preparations are necessary.

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

The M65[®]ELISA can also be used for plasma samples. M65[®] values are comparable for serum and plasma samples when appropriate dilution is accounted for.

If the assay will be performed within 48 hours, the sample should be refrigerated at 2 - 8°C. If measurements will be performed at a later time (more than 48 hours), the sample should be divided into aliquots and stored frozen at -20°C or lower.

Frozen samples should be thawed only once for 1 hour at 20 \pm 4°C and sera thoroughly mixed after thawing. Avoid repeated freeze-thawing. Do not use samples that are contaminated.

B. Sample preparation from cell culture supernatants

The M65[®]ELISA can be used to assess total cell death of epithelial cells in vitro by measuring release of CK18 protein into the culture medium. The M65[®]ELISA and M30-Apoptosense[®]ELISA can be used to assess cell death mode by calculation of an “M30:M65 ratio” (see Section 2). This 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).

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 recommended as a starting point.

Day 2: Wash the cells once with PBS and add fresh medium (150-200 μl /well). Expose the cells to the desired agent(s).

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.

2x25 μl cell-free supernatant samples are used for each assay.

If the assay will be performed within 24 hours, the samples should be stored at 2 - 8°C. Samples to be analyzed later should be stored at -20°C or lower. Avoid repeated freeze-thawing.

10. Calculation of Results

The M65[®]ELISA assay results may be calculated manually or by using computer-assisted methods.

A. Computer assisted method

Evaluate the values of Controls and Samples using a suitable computer program for handling ELISA type data.

Fitting algorithm: Spline smoothed / Linear regression

x-axis: Concentration (U/L), log scale

y-axis: Absorbance at 450 nm (A450), log scale

B. Manual method

The M65[®]ELISA standard curve may be constructed manually on log-log graph 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. If the sample was diluted prior to assaying, the observed concentration must be multiplied by the dilution factor.

x-axis: Concentration (U/L), log scale

y-axis: Absorbance at 450 nm (A450), log scale

An example of typical values of the Standards can be seen in Table 2. Not to be used in any calculations!

Test Number (triplicate)	1	2	3	CV %
Standards (A: 450nm)				
Diluent 0 U/L	0.055	0.058	0.061	
Std A 250 U/L	0.086	0.103	0.099	9.3
Std B 750 U/L	0.299	0.306	0.319	3.3
Std C 1,500 U/L	0.601	0.643	0.625	3.4
Std D 2,500 U/L	1.114	1.060	1.071	2.6
Std E 3,750 U/L	1.597	1.616	1.657	1.9
Std F 5,000 U/L	2.159	2.144	2.072	2.2
Samples (U/L)				
Positive sample #1	475	506	512	4.0
Positive sample #2	3,074	2,925	3,106	3.2

Table 2. Examples of performance of standards and samples. No calculations can be based upon these values, the standard curve must be performed in every run. Depending upon assay conditions such as temperature, etc. values might vary substantially from these.

11. Performance Characteristics

Measuring range

The M65[®]ELISA assay has a working range of 250 - 5,000 U/L.

Detection limit

The statistically defined minimal detectable concentration in the M65[®]ELISA is 184 U/L, defined as the concentration corresponding to the absorbance that is two standard deviations from the absorbance of the Diluent.

Precision study

Performed to determine individual measurements within (Intra) and between (Inter) assays and between operators (Precision-Reproducibility).

Intra-precision

5 samples were assayed in 12 replicates three times per day for 2 days. Presented are mean values from 6 assays.

Sample No.	A450	CV %	U/L	CV %
blank	0.052	–	0	–
1	0.214	3.4 (2.4-4.1)	470	4.5 (3.3-5.5)
2	0.865	4.9 (3.7-8.3)	2,350	5.3 (4.0-9.8)
3	1.937	5.1 (2.7-8.0)	4,700	4.3 (2.8-5.7)
4	0.240	6.7 (4.7-10.2)	648	7.8 (5.7-12.2)
5	1.054	6.0 (3.8-7.3)	2,625	7.4 (5.2-8.8)

Inter-precision

3 samples were assayed in 12 replicates twice a day for 3 days.

	Sample No. 1	Sample No. 2	Sample No. 3
Mean	648 U/L	822 U/L	2,624 U/L
Std. Dev.	51.0	58.3	97.1
CV %	7.9	7.1	3.7

Precision-Reproducibility

7 samples were assayed in 12 replicates twice by 2 different operators.

Sample No.	Operator 1	Operator 2	CV %
blank	0.056 / 0.058 (A450)	0.060 / 0.060 (A450)	–
1	0.115 / 0.117 (A450)	0.112 / 0.113 (A450)	2.1
2	0.855 / 0.819 (A450)	0.755 / 0.736 (A450)	8.1
3	1.792 / 1.683 (A450)	1.844 / 1.742 (A450)	2.2
4	803 / 738 (U/L)	844 / 817 (U/L)	5.3
5	1,064 / 1,042 (U/L)	1,100 / 1,062 (U/L)	1.9
6	1,357 / 1,345 (U/L)	1,334 / 1,367 (U/L)	0.1
7	1,804 / 1,837 (U/L)	1,921 / 1,919 (U/L)	3.8

Limitations

The M65[®]ELISA assay has not shown “high dose hook effect” (giving falsely low results) up to 126 900 U/L.

The working range for the assay is 250 - 5,000 U/L. Samples expected to have concentrations above this value should be appropriately diluted before assay (see Section 9: Collection and Preparation of Samples).

Specificity

The monoclonal antibodies “M6” and “M5” used in the M65[®]ELISA procedure are specific for epitopes on Cytokeratin 18. No cross reactivity to other proteins has been detected.

Recovery and Dilutions

Quantities of reactive components were added to human serum specimens. The recovery was 90-110 %.

Human serum specimens and positive tissue culture medium were diluted in the Diluent provided. For results, see Table 3.

Dilution	Sample 1 U/L	Sample 2 U/L	Sample 3 U/L
1/1	686	1,669	2,378
1/2	329	964	1,401
1/4	155	470	743
1/8	80	221	437

Table 3. Dilution of human serum (sample 1 and 2) and tissue culture medium (sample 3) samples in Diluent.

12. Observed Values in Human Healthy Individuals

The M65[®]ELISA was used to determine soluble CK18 in plasma from 126 human healthy individuals.

Mean +/- S.D.: 261.3 +/- 160.8 U/L.

Comparable values were obtained in serum samples. See Section 9:A

13. Interfering Substances

The results obtained from plasma are NOT affected by Heparin < 10 U/ml.

The assay is NOT sensitive to highly elevated hemoglobin levels, which means that grossly haemolyzed samples are acceptable although this is not recommended.

14. Critical Steps for a Successful Assay

- It is essential for the assay that the HRP Conjugate Solution is removed **COMPLETELY** before adding the TMB Substrate Solution. Make sure that the Wash Solution completely fills each well and is completely removed before the next filling.
Avoid contamination between wells.
- Dispense the Stop Solution with the same speed as the TMB Substrate Solution to obtain the **same incubation time in all of the wells.**

- Avoid non-homogenous samples. Ensure that all samples are sufficiently mixed and/or centrifuged before performing the assay. This is described in Section 9: Collection and Preparation of Samples.

15. Quality Control

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

If this procedure is not sufficient, each laboratory would need to establish their own controls by culturing cells either by the guidelines in Section 9: 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.

PEVIVA AB can not accept any responsibility for the performance of any control sample generated without its supervision. They will fall under the conditions stated in the warranty section.

16. 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.

17. References

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**For further references and information, please consult
Peviva's Website, which is frequently updated.**

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