Store at 2 – 8 °C

For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

M30 CytoDEATH™ *Fluorescein*

200 tests (Peviva Prod. No. 10800)
Mouse monoclonal antibody (Clone M30)
For the detection of caspase-cleaved keratin 18 neo-epitope M30

1. Product Description

Name: M30 CytoDEATH™ Fluorescein
Clone: M30
Isotye: IgG2b
Immunogen: Keratin 18 (K18) fragments purified from supernatant from human carcinoma cell line WiDr CCL218.
Epitope: K18 fragment aa284 – 396

1.1 Formulation

Clear solution. 10 µg of M30 CytoDEATH™ Fluorescein monoclonal antibody provided in 200 µl PBS containing 0.1 % BSA, PEG, sucrose and 0.09 % sodium azide.

1.2 Specificity

M30 CytoDEATH™ Fluorescein antibody is recommended for the detection of the formalin-resistant M30 neo-epitope on human, monkey and bovine caspase-cleaved keratin 18 (K18) cytoskeletal protein. M30 CytoDEATH™ Fluorescein does not recognise intact K18.

1.3 Recommended applications

- Immunocytochemistry (ICC)
- Flow cytometry (FACS)

Only if used in combination with additional secondary signal enhancing reagents:

- immunohistochemistry (IHC) including formalin-fixed and paraffin-embedded tissue sections (PS) and cryostat sections FS).

1.4 Working solution

M30 CytoDEATH™ Fluorescein is provided in a convenient ready-to-use stock solution. Use a dilution 1:100 in Incubation Buffer (final concentration 0.5 µg/ml).

1.5 Storage and stability

The M30 CytoDEATH™ Fluorescein antibody is provided in a ready-to-use format and is stable at 2 – 8 °C through the expiration date printed on the label.

Alternatively, it can be stored in aliquots at -20 °C.

The antibody is shipped at ambient temperature.

Note: Repeated freezing and thawing should be avoided.

1.6 Quality control

The M30 CytoDEATH™ Fluorescein antibody is function tested using a celluar model: HeLa cells treated with recombinant TRAIL and CHX analysed by immunocytochemistry and flow cytometry.

M30 CytoDEATH™ – Key advantages

Benefits	Features
Early and specific detection of apoptosis	Detects caspase-cleaved keratin 18; caspase activity is one of the earliest and most common markers for apoptosis.
Sustained signal from cells at early to later stages of apoptosis	In contrast to measuring i. e. active caspase-3, where the signal is defined to a limited time window and decreases at later stages of apoptosis, the caspase-generated K18 neo-epitope can still be detected even after apotopic cells have disintegrated.
Superior sensitivity	The keratin 18 neo-epitope is an accumulating substrate generated by few activated caspase molecules.
Assay is independent of the activation of a single caspase	Keratin 18 is cleaved *in vivo* by several effector caspases, including caspase-3, 6, 7 and 9.
Clear results	Apoptotic cells are clearly distinguishable from viable cells or necrotic cells.
Apoptosis specific No false positive results in circumstances of DNA damage	Whereas TUNEL analysis can give rise to false positive results under conditions when DNA double-strand breaks occur, detection of the K18 neo-epitope using the M30 CytoDEATH™ antibody shows superior specificity for apoptotic cells compared to TUNEL.
Specificity for epithelial (i.e. carcinoma or liver tissue) apoptosis	Expression pattern of K18 is restricted to cells of epithelial origin. Lymphoid and neuronal cell apoptosis is not detected by M30 CytoDEATH™ antibody.

M30 CytoDEATH™ Fluorescein – additional advantages

Benefits	Features
Added convenience for flow cytometry and immunocytochemistry	One-step tool for the detection of apoptosis in epithelial cells by flow cytometry and immunocytochemistry. No additional anti-mouse IgG fluorochrome-conjugated secondary antibodies required.

2. Background Information

2.1 Caspase substrate K18 and apoptosis in epithelial cells

Apoptosis induced by either death-inducing receptors or other stimuli leads to activation of specific caspases [1, 2]. Subsequently, apoptotic cells are eliminated by an intrinsic suicide program, resulting in DNA fragmentation, nuclear condensation, cytoskeletal reorganisation, plasma membrane blebbing and loss of cell adhesion.

Keratin 18 (K18) is a type I intermediate filament protein and the major component of single-layer and glandular epithelial cells. It is expressed in most types of carcinomas such as lung, liver, prostate, breast and colon, and abundantly present in liver cells, whereas K18 is absent in lymphoid and neuronal cells and tissues. During apoptosis after initiation of effector caspases 3, 6, 7 and 9, K18 is cleaved into proteolytic fragments liberating neo-epitopes (NE) at the cleavage sites [3 – 6].

2.2 M30 CytoDEATH™ antibody for the specific detection of apoptosis

K18 is cleaved by capases, liberating a neo-epitope (M30) that is specifically recognised by the M30 CytoDEATH™ monoclonal antibody. Specific proteolytic cleavage of K18 is an event taking place before disruption of membrane asymmetry and induction of DNA strand breaks. Numerous studies confirm that M30 CytoDEATH™ antibody detects only apoptotic but not viable or necrotic cells. Reactivity of M30 CytoDEATH™ antibody in immunohistochemistry correlates to the apoptosis measured by TUNEL and shows superior reliability in conditions when DNA double-strand breaks occur independent of apoptosis [7].

The capacity of M30 CytoDEATH™ antibody in flow cytomery and immunohistochemistry studies to distinguish between necrotic and apoptotic epithelial cells has been verified in several disease entities. Consequently, M30 CytoDEATH™ antibody represents a unique tool for easy and reliable determination of apoptosis from very early until well advanced stages in single cells and tissue sections of epithelial origin [8].

Moreover, there are two M30 CytoDEATH™ antibody-based ELISAs available:

- M30 CytoDeath™ ELISA (PEVIVA Prod. No.: 10900) is suggested to serve as a high-throughput assay for functional screening and *in vitro* characterisation of effective pro-apoptotic drugs using cell culture supernatants, and spheroid or tissue lysates.
- The CE-marked M30 Apoptosense® ELISA (PEVIVA Prod. No: 10010) has been successfully used to determine elevated K18 neo-epitope levels in blood samples from patients as a useful biomarker to monitor response to treatment or disease staging [9 – 12].

3. Procedures and Materials Required

3.1 Procedure for immunofluorescence and flow cytometry

3.1.1 Introduction

The following procedure describes the detection of apoptosis with M30 CytoDEATH™ Fluorescein antibody in immunofluorescence and flow cytometry.

If using other detection methods or sample material, the conditions may vary and have to be adapted.

Additional reagents required

- PBS, Methanol and BSA

Preparation of working solutions

Incubation Buffer: PBS containing 1 % BSA
Washing Buffer: PBS

Preparation of M30 CytoDEATH™ Fluorescein antibody working solution

Dilute the M30 CytoDEATH™ Fluorescein antibody stock solution 1:100 in Incubation Buffer (final concentration 0.5 µg/ml).

Note: The antibody solutions should be free of precipitate. If necessary, centrifuge the solution at high speed prior to use.

3.1.2 Immunofluorescence and flow cytometry protocol

Step	Action
1	Wash cells in PBS.
2	Fix cells in ice-cold pure methanol at -20 °C for 30 min.
3	Wash cells with Washing Buffer twice.
4	Remove Washing Buffer.
5	Incubate with 100 µl M30 CytoDEATH™ Fluorescein antibody for 30 min at 15 – 25 °C.
6	Wash cells with Washing Buffer twice.
7	Examine the cells on a slide under the fluorescence microscope, or, dilute cells in 0.5 ml PBS and store samples in the dark until analysis by flow cytometry.

3.2 Procedure for immunohistochemistry

Application note for M30 CytoDEATH™ Fluorescein use for immunohistochemistry

Due to the limited sensitivity of the M30 CytoDEATH™ Fluorescein antibody in immunohistochemistry this application requires the use of additional secondary signal enhancing reagents as it does for the unconjugated M30 CytoDEATH™ antibody (PEVIVA Prod. No.:10700).

3.2.1 Introduction

The following procedure describes the detection of apoptosis with M30 CytoDEATH™ Fluorescein antibody in a three step method in immunohistochemistry (paraffin-embedded tissue) for maximal sensitivity.

Please note: A biotin-conjugated M30 CytoDEATH™ antibody is available from PEVIVA (Prod. No: 10750), which can be used in combination with sensitive amplification reagents. If using other detection methods or sample material, the conditions may vary and have to be adapted.

3.2.2 Recommended reagents

For preparation of samples:

- Xylol
- Ethanol 96 %
- Ethanol 70 %
- Methanol/H_2O_2 (3 %)
- Citric acid
- NaOH, 1 M
- Hematoxylin (i.e. from Merck)
- Mounting medium (i.e. Kaiser´s glycerine gelatine from Merck)

For the immunohistochemistry procedure:

- Anti-mouse-IgG biotin (i.e. from DAKO)
- Streptavidin-POD (i.e. from DAKO)
- DAB or AEC substrate (i.e. from Zymed)
- PBS
- BSA

Preparation of working solutions

The following table lists the working solutions needed to perform the immunohistochemistry staining procedure.

Working Solution	Composition	Stability/ storage	Use
Washing Buffer	PBS	4 weeks at 2 – 8 °C	Washing step
Incubation Buffer	PBS containing 1 % BSA	4 weeks at 2 – 8 °C	Preperation of antibody working solution
Citric Acid Buffer (0.01 M)	2 g/l citric acid, pH 6.0 adjusted with 1 M NaOH	4 weeks at 2 – 8 °C	Antigen retrival

Preparation of M30 CytoDEATH™ Fluorescein antibody working solution

Dilute the M30 CytoDEATH™ Fluorescein antibody stock solution 1:100 in Incubation Buffer (final concentration 0.5 µg/ml).

Note: The antibody solutions should be free of precipitate. If necessary, centrifuge the solution at high speed prior to use.

Preparation of sample material

Before starting the immunohistochemical protocol, dewax paraffin-embedded tissue sections as described in the following table.

Step	Action
1	Place paraffin-embedded sections into an incubator at 37 °C over night to air-dry.
2	To dewax formalin-fixed, paraffin-embedded tissue sections, process the sections as follows: • 2 coplin jars of xylol (2 – 5 min), • 2 coplin jars of ethanol (96 %) • 1 coplin jar of ethanol (70 %) • 1 coplin jar of methanol/H_2O_2 (3 %) for 10 min at 15 – 25 °C.
3	Rinse 10 min in PBS.

Note: The sections should not be allowed to dry during this procedure.

3.2.3 Immunohistochemistry protocol

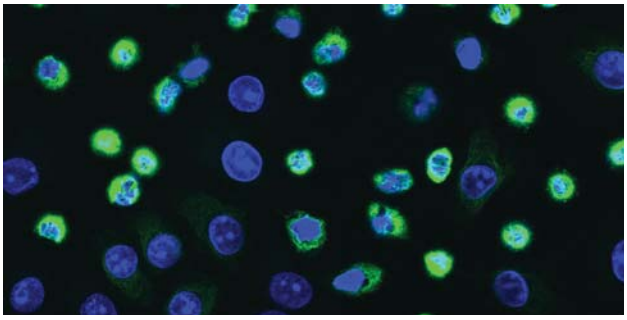
Note: For optimal results it is highly recommended to follow the below mentioned method for antigen retrieval.

Step	Action
1	• Prewarm citric acid buffer (0.01 M, pH 6.0) by incubation in a microwave oven at 750 W until solution boils. • When the solution is boiling, turn the setting of the microwave oven to "keep warm" (about 100 W). • Place tissue section slides in a slide rack and put them into the heated citric acid solution (approx. 90 °C). • Incubate at this setting for 20 min. ***Note:*** For optimal morphology it is recommended to keep the solution shortly below the boiling point to avoid gas formation under the sections.
2	Rinse 3 × in PBS and incubate 10 min in a separate jar of PBS to cool down.
3	Remove Incubation Buffer and add 100 µl M30 CytoDEATH™ Fluorescein antibody working solution. Incubate for 30 min at 15 – 25 °C in a humid chamber.
4	Wash slides in Washing Buffer (use 3 separate jars and dip 3 × into each jar).
5	Cover the section with 100 µl of anti-mouse-IgG biotin according to your established optimised procedure for the reagent from your selected supplier or use DAKO reagent at 1:400 dilution. Incubate for 30 min at 37 °C in a humid chamber.
6	Wash slides in Washing Buffer (use 3 separate jars and dip 3 × into each jar).
7	Cover the section with 100 µl of streptavidin-POD according to your established optimised procedure for the reagent from your selected supplier, or use DAKO reagent at 1:600 dilution. Incubate for 30 min at 15 – 25 °C in a humid chamber.
8	Wash slides in Washing Buffer (use 3 separate jars and dip 3 × into each jar).
9	Incubate slides in a freshly prepared substrate solution (i. e. AEC) at 15 – 25 °C until a clearly visible colour develops (1 – 5 min). A negative control should not show any development of colour during the incubation period.
10	Stop the reaction by extensive rinsing in double distilled water.
11	Subsequently, counterstain the preparation with hematoxylin and mount the section (i. e. Kaiser's glycerine gelatine when using AEC).

4. Results

Immunocytochemistry

A.



B.

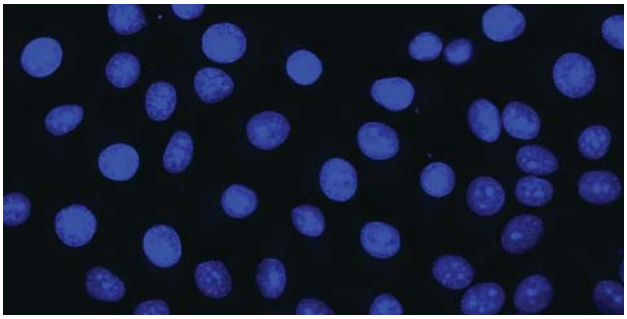


Figure: HeLa (human cervical cancer) cells were grown to 80 – 90 % confluence on coated cover slides in a 24-well plate and either treated with cycloheximide (CHX) at 20 µg/ml final concentration for 2.5 h alone or in the presence of recombinant human TRAIL (KillerTRAIL™, ALEXIS Corporation, Lausen, Switzerland) at 100 ng/ml final concentration for further 1.5 h.

Slides with cells were then fixed and stained with M30 CytoDEATH™ Fluorescein and analyzed by fluorescence microscopy. DNA of the nuclei was counterstained with DRAQ5™ (ALEXIS Corporation, Lausen, Switzerland).

A: HeLa cells that received combined treatment with CHX and TRAIL show a sustained and strong caspase activity and concomitant caspase-cleaved K18 accumulation as detected by the M30 CytoDEATH™ Fluorescein antibody compared to treatment with low non-toxic concentrations of CHX alone (**B**).

5. Appendix

K18 positive cell lines and tissues successfully analyzed with the M30 CytoDEATH™ antibody:

Human epithelial cell lines:

Breast cancer: MDA-MB-231, MCF-7, HBL100
Colon cancer: WiDr, HCT 116, HT29, SW620
Cervical cancer: HeLa
Kidney cancer: ACHN, A498
Head & neck cancer: SCC9, SCC25, FaDu
Prostate cancer: PC-3, LNCaP, DU 145
Bladder cancer: RT4, J82

Human epithelial tissues:

Breast, lung, liver, colon, pancreas, intestine, kidney, salivary gland, trophoblast, endometrium, bladder, oral epithelium.

6. PEVIVA Products

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

Keratin 18 antibodies

M5 Keratin 18 mAb	Prod. No. 10600
M6 Keratin 18 mAb	Prod. No. 10650

ELISA kits

M30 Apoptosense® ELISA	Prod. No. 10011
M65® ELISA	Prod. No. 10020
M65 EpiDeath® ELISA	Prod. No. 10040
M30 CytoDeath™ ELISA	Prod. No. 10900

7. References

1. Creagh EM, Conroy H, Martin SJ. (2003) Caspase-activation pathways in apoptosis and immunity. Immunol Rev. 193:10-21

2. Creagh EM, Martin SJ. (2001) Caspases: cellular demolition experts. Biochem Soc Trans. 29: 696-702.

3. Caulin C, Salvesen GS, Oshima G. (1997) Caspase Cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell Apoptosis. J Cell Biol. 138: 1379-1394.

4. Leers MP, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B, Björklund P, Ramaekers FCS, Björklund B, Nap M, Jörnvall H, Schutte B. (1999) Immunocytochemical detection and mapping of a Cytokeratin 18 neoepitope exposed during early apoptosis. J Pathol. 187: 567-572.

5. Schutte B, Henfling M, Kolgen W, Bouman M, Meex S, Leers MP, Nap M, Bjorklund V, Bjorklund P, Bjorklund B, Lane EB, Omary MB, Jornvall H, Ramaekers FCS. (2004) Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res. 297:11-26.

6. Dinsdale D, Lee JC, Dewson G, Cohen GM, Peter ME. (2004) Intermediate filaments control the intracellular distribution of caspases during apoptosis. Am J Pathol. 164: 395-407.

7. Walker JA and Quirke P. (2001) Viewing apoptosis through a "TUNEL". J Pathol. 95: 275-276.

8. Grassi A, Susca M, Ferri S, Gabusi E, D'Errico A, Farina G, Maccariello S, Zauli D, Bianchi FB, Ballardini G. (2004) Detection of the M30 neoepitope as a new tool to quantify liver apoptosis: timing and patterns of positivity on frozen and paraffin-embedded sections. Am J Clin Pathol. 121: 211-219.

9. Bivén K, Erdal H, Hägg M, Ueno T, Zhou R, Lynch M, Rowley B, Wood J, Zhang C, Toi M, Shoshan MC, Linder S. (2003). A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera. Apoptosis 8: 263-268.

10. Ueno T, Toi M, Bivén K, Bando H, Ogawa T, Linder S. (2003) Measurement of an apoptosis product in the sera of breast cancer patients. Eur J Cancer 39: 769-774.

11. Kramer G, Erdal H, Mertens H, Nap M, Mauermann J, Steiner G, Marberger M, Bivén K, Shoshan MC, Linder S. (2004) Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. Cancer Res. 64: 751-1756.

12. Linder S, Havelka AM, Ueno T, Shoshan MC. (2004) Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. Cancer Lett. 214: 1-9.

8. M30 CytoDEATH™ Application References

Immunocytochemistry (ICC)

MacFarlane M, Merrison W, Dinsdale D, Cohen GM. (2000) Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. J Cell Biol. 148: 1239-1254.

Barrett KL, Willingham JM, Garvin AJ, Willingham MC. (2001) Advances in cyto-chemical methods for detection of apoptosis. J Histochem Cytochem 49: 821-32.

Lee JC, Schickling O, Stegh AH, Oshima RG, Dinsdale D, Cohen GM, Peter ME. (2002) DEDD regulates degradation of intermediate filaments during apoptosis. J Cell Biology 158: 1051-1066.

Dinsdale D, Lee JC, Dewson G, Cohen GM, Peter ME. (2004) Intermediate filaments control the intracellular distribution of caspases during apoptosis. Am J Pathol. 164: 395-407.

Schutte B, Henfling M, Kolgen W, Bouman M, Meex S, Leers MP, Nap M, Bjorklund V, Bjorklund P, Bjorklund B, Lane EB, Omary MB, Jornvall H, Ramaekers FC. (2004) Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res. 297:11-26.

Flow cytometry (FACS)

Rupa JD, De Bruine AP, Gerbers AJ, Leers MP, Nap M, Kessels AG, Schutte B, Arends JW. (2003) Simultaneous detection of apoptosis and proliferation in colourectal carcinoma by multiparameter flow cytometry allows separation of high and low-turnover tumors with distinct clinical outcome. Cancer 97: 2404-2411.

Morsi HM, Leers MP, Radespiel-Troger M, Björklund V, Kabarity HE, Nap M, Jäger W. (2000) Apoptosis, bcl-2 expression, and proliferation in benign and malignant endometrial epithelium: An approach using multiparameter flow cytometry. Gynecol. Oncol. 77, 11-7.

Immunohistochemistry (IHC)

Morsi HM, Leers MP, Jäger W, Björklund V, Radespiel-Troger M, el Kabarity H, Nap M and Lang N. (2000) The patterns of expression of an apoptosis-related CK18 neoepitope, the bcl-2 proto-oncogene, and the Ki67 proliferation marker in normal, hyperplastic, and malignant endometrium. Int J. Gynecol. Pathol. 19: 118-126.

Bantel H, Ruck P, Gregor M, Schulze-Osthoff K. (2001) Detection of elevated caspase activation and early apoptosis in liver diseases. Eur J Cell Biol. 80: 230-239

Fukuda M, Tanaka A, Kitada M, Fukuda F, Suzuki S, Jiang Y, Kebusa Y, Ohno J, Yamamoto Y, Minato H, Nonomura A, Sakashita H, Kusama K. (2001) Immunohistochemical detection of cytokeratin 18 and its neo-epitope in Warthin's tumor (adenolymphoma) of the parotid glands. Anticancer Res 21:109-12.

Walker JA and Quirke P. (2001) Viewing apoptosis through a "TUNEL". J. Pathol 195: 275-276

Leers MP, Björklund V, Björklund B, Jörnvall H, Nap M. (2002) An immunohistochemical study of the clearance of apoptotic cellular fragments. Cell Mol Life Sci. 59: 1358-1365.

Grassi A, Susca M, Ferri S, Gabusi E, D'Errico A, Farina G, Maccariello S, Zauli D, Bianchi FB, Ballardini G. (2004) Detection of the M30 neoepitope as a new tool to quantify liver apoptosis: timing and patterns of positivity on frozen and paraffin-embedded sections. Am J Clin Pathol. 121: 211-219.

Koornstra JJ, Rijcken FE, De Jong S, Hollema H, De Vries E, Kleibeuker JH. (2004) Assessment of apoptosis by M30 immunoreactivity and the correlation with morphological criteria in normal colourectal mucosa, adenomas and carcinomas. Histopathol. 44: 9-17.

Takada M, Kataoka A, Toi M, Bando H, Toyama K, Horiguchi S, Ueno T, Linder S, Saji S, Hayashi Y, Funata N, Kinoshita J, Murakami S, Ohono S. (2004) A close association between alteration in growth kinetics by neoadjuvant chemotherapy and survival outcome in primary breast cancer. Int J Oncol. 25: 397-405.

Simopoulos C, Tsaroucha AK, Asimakopoulos B, Giatromanolaki A, Gavriilidis P, Polychronidis A, Karayiannakis A. (2008) Total and caspase-cleaved cytokeratin 18 in chronic cholecystitis: a prospective study. BMC Gasteroenterol. 8:14–18.

For further, up-to-date information and to order, please visit ***www.peviva.com****.*

VLVbio

VIVALAVIDA AB, Hästholmsvägen 32, 131 30 Nacka, Sweden
Website: www.vlvbio.com • E-mail: info@vlvbio.com
Phone: +46 8 122 053 00