



**Sanquin**

**PeliSPOTä**  
**human GRANZYME B kit**

288 tests

An enzyme immunoassay for the quantitative determination  
of the frequency of human GRANZYME B secreting cells

PRODUCT INFORMATION

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**PROTOCOL SUMMERY AND CHECKLIST****PLEASE READ “IMPORTANT INFORMATION” ON PAGE 8 FIRST!**Day 1:

- Bring coating antibody to room temperature.
- Prepare PBS.
- Dilute coating antibody 1:100 in PBS.
- Activate PVDF-bottomed-well microtiter plate with 70% or 96% ethanol for 10 minutes at room temperature.
- Rinse once with distilled water and wash once with PBS.
- Remove all residual buffer by repeated tapping on absorbent paper.
- Add 50 µl per well of diluted coating antibody.
- Cover the plate with lid and incubate overnight at 2-8°C.

Day 2:

- Prepare PeliSPOT buffer.
- Prepare washing buffer.
- Prepare cell culture medium.
- Empty wells by flicking the plate over a sink and tapping it on absorbent paper.
- Wash the plate five times with wash buffer.
- Add 200 µl PeliSPOT buffer per well.
- Cover the plate with lid and incubate for one hour at room temperature.
- Prepare cell suspensions and solutions with antigen and mitogen in cell culture medium.
- Dilute positive assay control 1: 8 in cell culture medium.
- Empty wells by flicking the plate over a sink and tapping it on absorbent paper.
- Add 50 µl of the cell suspension containing the appropriate number of cells according to the plate plan (see plate plan example).

**TMB SUBSTRATE**

Empty the microtiterplate by flicking over a sink. Rinse the underside of the membrane with distilled water and wash plate five times with wash buffer as described on page 8. Tap plate gently on absorbent paper.

Directly add 50 µl ready to use TMB substrate per well.

Cover the plate with lid and incubate 10 – 15 minutes at room temperature (18-25°C).

Note: The speed of the enzymatic colour development is influenced by many factors including temperature, therefore monitor the development by eye.

Empty the microtiterplate by flicking over a sink. Rinse the underside of the membrane with distilled water and wash plate five times with distilled water to stop the the staining. Tap plate gently on absorbent paper.

Dry the plate overnight at room temperature (in the dark) or place the plate in an airflow in front of a fan until the membranes are completely dry.

**ANALYSE THE SPOTS**

Count the number of spots preferably using the A.EL.VIS automated spot analyser. Store the plates in the dark to prevent fading of the spots.

- Subsequently add to the wells 50 µl of the antigens of interest in the appropriate concentrations.
- Add for each cell sample 50 µl of a mitogenic stimulator (e.g. PMA / Ionomycin) as a positive secretion control as well as 50 µl cell culture medium as a negative secretion control.
- Add 100 µl of the positive assay control per plate to one well.
- Cover the plate with the lid and incubate overnight (16 – 24 hours) at 37°C in a CO<sub>2</sub> incubator.

**During this period make sure that the plate is completely horizontal and do not agitate or move the plate.**

Day 3

- Bring all required reagents, with the exception of streptavidin-poly-HRP, to room temperature.
- Dilute biotin conjugate 1:100 in PeliSPOT buffer.
- Empty wells, rinse underside of the membrane with distilled water, wash the plate five times with wash buffer and tap plate on absorbent paper.
- Add 100 µl of the diluted biotin conjugate per well, cover the plate with lid and incubate for one hour at room temperature.
- Dilute the streptavidin-poly-HRP 1:6,000 in PeliSPOT buffer.
- Empty wells, rinse underside of the membrane with distilled water, wash the plate five times with wash buffer and tap plate on absorbent paper.
- Add 100 µl of the diluted streptavidin-poly-HRP per well, cover plate with lid and incubate for one hour at room temperature.
- Empty wells, rinse underside of the membrane with distilled water, wash the plate five times with wash buffer and tap plate on absorbent paper.
- Add 50 µl TMB substrate per well, cover the plate with lid and incubate for 10-15 minutes at room temperature (monitor spot intensity by eye).
- Rinse underside of the membrane and wash the plate with an excess of distilled water.
- Remove all residual water by repeated tapping on absorbent paper
- Dry the plate overnight at room temperature (in dark) or place the plate in an air flow in front of a fan until the membranes are completely dry.
- Count the number of spots preferably using the A.EL.VIS automated spot analyzer.

**Suggested plate plan**

	1	2	3	4	5	6	7	8	9	10	11	12
A	neg	neg	neg	pos	pos	pos	anti gen 1	anti gen 1	anti gen 1	anti gen 2	anti gen 2	anti gen 2
B												
C												
D												
E												
F												
G												
H												pos cont

**Key:**

neg :negative secretion control (cell suspension only).

pos :positive secretion control (polyclonal stimulator e.g. PMA / Ionomycin).

antigen :specific antigens (peptides) of interest.

pos cont :positive assay control (no cells).

Cover the plate with lid and incubate overnight (16 – 24 hours) at 37°C in a CO<sub>2</sub> incubator.

***During this period make sure that the plate is completely horizontal and do not agitate or move the plate.***

**DAY 3**

Bring the working strength PeliSPOT buffer, the biotin conjugate and the TMB substrate to room temperature.

**BIOTIN CONJUGATE**

Bring the biotin conjugate (yellow-capped vial) to room temperature.

Centrifuge vial before use (30 seconds 3,000 x g).

Dilute 120 µl of biotinylated antibody in 12 ml PeliSPOT buffer per microtiterplate.

Empty the microtiterplate by flicking over a sink. Rinse the underside of the membrane with distilled water and wash plate five times with wash buffer as described on page 8. Tap plate gently on absorbent paper.

Directly add 100 µl diluted biotin conjugate per well.

Cover the plate with lid and incubate 1 hour at room temperature (18-25°C).

**STREPTAVIDIN-POLY-HRP**

Keep the streptavidin-poly-HRP (brown capped vial) at -18 to -32 °C to maintain maximal stability. The contents of the vial will not freeze at this temperature.

Centrifuge vial before use (30 seconds 3,000 x g).

Dilute 2 µl of streptavidin-poly-HRP in 12 ml PeliSPOT buffer per microtiterplate.

***Do not prepare in advance of assay.***

Empty the microtiterplate by flicking over a sink. Rinse the underside of the membrane with distilled water and wash plate five times with wash buffer as described on page 8. Tap plate gently on absorbent paper.

Directly add 100 µl diluted streptavidin poly-HRP per well.

Cover the plate with lid and incubate 1 hour at room temperature (18-25°C).

**Prepare cell culture medium**

Supplement cell culture medium, e.g. IMDM or RPMI-1640, with:

- 5% FCS
- 100 U/ml penicillin
- 100 U/ml streptomycin
- 2 mM glutamin.

**Prepare cell suspension**

Isolate, thaw or harvest cells.

Centrifuge cells and wash them twice with cell culture medium. Resuspend the cell suspension to avoid cell clumping. Handle cells with care, do not vortex.

Determine the concentration of cells in the cell suspension.

Dilute the cell suspension to a concentration of e.g.:

- $4 \times 10^6$  cells/ml for a final concentration of  $2 \times 10^5$  cells per well, or
- $2 \times 10^6$  cells/ml for a final concentration of  $1 \times 10^5$  cells per well.

**Prepare cell culture medium with antigen or mitogen**

The concentration of the antigen and mitogen must be twice the final concentration.

Antigen, e.g.:

- 20  $\mu$ g/ml for a final concentration of 10  $\mu$ g/ml, or
- 10  $\mu$ g/ml for a final concentration of 5  $\mu$ g/ml.

PMA/Ionomycin

Add 1  $\mu$ l per ml cell culture medium from each stock solution (page 6) to obtain a final concentration of 1 ng/ml PMA and 0,5  $\mu$ g/ml Ionomycin.

**Positive assay control**

Bring the positive assay control (black-capped vial) to room temperature.

Centrifuge vial before use (30 seconds 3,000 x g).

Dilute 15  $\mu$ l of positive control in 105  $\mu$ l cell culture medium.

**Cell incubation**

Empty the microtiterplate by flicking over a sink and tap on absorbent paper.

Pipette the prepared solutions in triplicate according to the recommended plate plan on page 3:

- Resuspend the cell suspension carefully to obtain a homogeneous cell suspension and directly add 50  $\mu$ l to each well.
- Add 50  $\mu$ l cell culture medium with antigen, PMA / Ionomycin or without stimulus to the wells with cells.
- Add 100  $\mu$ l of the diluted positive assay control to one well (do not add cells).

**I. INTRODUCTION**

Cytotoxic T lymphocytes (CTL) and natural killer cells (NK) play an important role in the elimination of virus infected and malignant cells. One of the major mechanisms to trigger apoptosis in target cells is the so called granule exocytose pathway. Upon binding of the CTL to a target cell (by CTL-receptor and antigen-presenting MHC molecules on the target cell) the contents of the granules are released. These cytolytic granules contain a number of proteins: perforin, a pore forming protein, and granzymes A and B, serine proteases, are the main effectors of the granule mediated cell death. Initially it was proposed that granzyme B enters the target cell through channels formed by polymerised perforin. Recent studies reported receptor-mediated endocytosis (1,2).

In the cytoplasm, Granzyme B can trigger apoptosis by cleaving a caspase substrate that initiates a cascade of other reactions. Activation of other substrates, which leads to cell death may also be relevant.

Granzyme A is also able to induce apoptosis in the target cell, but acts via a different pathway and with slower kinetics.

Granzyme B positive CTLs can be determined by flow cytometry and immunocytochemical methods used in different clinical applications.

Not all granzymes enter the target cell, part of them also "leak" in to the peripheral blood and other biological fluids. Detectable amounts of granzymes have been found to circulate in healthy volunteers (3). These soluble granzymes A and B can be measured by ELISA (catno.: M1935 and M1936 respectively). Using the ELISPOT assay granzyme B producing cells can now be enumerated on a single cell level, facilitating a sensitive method to study vaccine efficiencies, anti-tumor effects, etc.

The ELISPOT or ELISA SPOT technique is a method that has increased its significance in the last few years mainly in the field of anti-viral immunity and tumor-immunology. The ELISPOT assay for the detection of IFN $\gamma$  secreting T-cells was first described by Czerkinski (4). Compared to the ELISA technique the substantial improvement is the increased sensitivity, where ELISA requires at least 400 cells per well to produce sufficient granzymes to give detectable levels, in ELISPOT 1 positive cell per well can be detected resulting in a sensitivity of  $1 \times 10^5$  positive cells. Not only the increased sensitivity, but also the possibility to enumerate cytokine or Granzyme B producing T cells at the single cell level, is an enormous advantage. Since the total percentage of production can be evaluated by using an automated ELISPOT analyzer, which is correlated to levels in ELISA, two parameters can be measured in the same assay.

**II. INTENDED USE**

This PeliSPOT™ human GRANZYME B kit has been developed for reproducible and specific quantification of the frequency of human CTL and NK cells releasing granzyme B. The kit is for research use only.

### III. PRINCIPLE OF THE TEST

Cells are stimulated in a well coated with a high affinity monoclonal antibody. Secreted products of interest will bind locally to the antibody. Subsequently cells are washed away and a biotin labelled antibody is added. Signal amplification is established by adding a streptavidin-conjugated enzyme. The area is visualised as a spot by a precipitating blue/purple coloured substrate. Enumeration of spots can be reliably performed with the A.EL.VIS, or other automated plate reader system. The results can be expressed as the number of spot forming cells per million cells.

### IV. STORAGE AND STABILITY

The components of the humane Granzyme B PeliSPOT kit (except TMB substrate solution) should be stored at -18 to -32 °C. The TMB substrate solution should be stored at 2-8 °C. The performance of the kit is guaranteed until the expiration date shown on the case label.

### V. CONTENTS OF THE KIT

The PeliSPOT™ human GRANZYME B kit contains material sufficient for 288 tests. The reagents provided are:

Kit component	Volume	Storage	Cap colour	Handling
Coating antibody	190 µl	-18 to -32°C	Red	Dilute 1:100 in PBS
Biotin conjugate	375 µl	-18 to -32°C	Yellow	Dilute 1:100 in PeliSPOT buffer
Streptavidine-poly-HRP	20 µl	-18 to -32°C	Brown	Dilute 1:6,000 in PeliSPOT buffer
Positive assay control	100 µl	-18 to -32°C	Black	Dilute 1:8 in cell culture medium
PeliSPOT buffer stock solution	50 ml	-18 to -32°C		Dilute 1:5 in distilled water
TMB Substrate for PeliSPOT	18 ml	<b>2 to 8 °C</b>		Ready for use

### VI. PRECAUTIONS FOR USE

1. The PeliSPOT™ human GRANZYME B kit is intended for research purposes only, ingredients are not for *in vivo* use.
2. Only use the reagents supplied with the kit; do not mix reagents from different kit lots.
3. Handle all blood, tissue and cell samples with care to prevent transmission of infections.
4. Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.

Prepare PBS as described on page 7 of this leaflet.  
Bring the coating antibody (red-capped vial) to room temperature.  
Centrifuge vial before use (30 seconds 3,000 x g).  
Add 60 µl of coating antibody to 6 ml PBS per microtiterplate.

Activate the PVDF-bottomed-wells by adding 100 µl of 70% or 96% ethanol.  
Cover the plate with the lid and incubate for 10 minutes at room temperature.

Empty the microtiterplate by flicking over a sink and completely fill the wells with distilled water, repeat this with PBS.  
Empty plate and tap plate gently on absorbent paper.

Directly add 50 µl of the diluted coating antibody per well.  
Cover the plate with lid and incubate overnight at 2-8 °C.

### DAY 2

Prepare wash buffer (PBS + 0.005% Tween-20) as described on page 7.

Prepare 50 ml PeliSPOT buffer per microtiterplate:  
10 ml PeliSPOT buffer stock solution  
40 ml Distilled water

### BLOCKING

Empty the microtiterplate by flicking over a sink and wash plate five times with wash buffer. Empty plate and tap plate gently on absorbent paper.

Directly add 200 µl PeliSPOT buffer per well.  
Cover the plate with lid and incubate 1 hour at room temperature (18-25°C).

Store the remaining working strength PeliSPOT buffer at 2-8 °C.

### CELLS

Please read the chapter PREPARATION OF CELLS on page 9 for recommendations regarding the best method for cell preparation.

Optimal concentrations for cells and stimuli and the optimal incubation period have to be determined experimentally.

**Adherence**

For monocyte removal pre-incubate 20 hours in a 24 well culture plate ( $2 \times 10^6$  cells/ml/well). After pre-incubation the non-adherent fraction is collected and washed once with pre-warmed cell culture medium.

**Magnetic cell separation**

Monocytes are depleted from the PBMC fraction with CD14 beads (e.g. MACS, Miltenyi Biotech GmbH) according to the manufacturer 's protocol. The CD14 negative fraction is collected and washed once with cell culture medium.

Results of a typical experiment with non-stimulated cells:

Cellfraction	Method	Spot forming cells per million cells	Reduction of spots
PBMC	-	1420	-
CD14 - cells	Macs	420	70-90 %
Non-adherent cells	20 h pre-incubation	624	35-65%
	44 h pre-incubation	206	70-90%

After the pre-treatment cells are pipetted in the assay plate at an appropriate concentration. Triplicates are recommended, see also the suggested plate-plan on page 3.

Add a mitogen as a positive cell secretion control, for example PMA (1 ng/ml) and Ionomycin (0.5  $\mu$ g/ml). Wells that exceed the number of  $2.5 \times 10^4$  mitogen stimulated cells per well are generally not countable due to high granzyme B production.

For antigen specific stimulation the optimal concentration of the antigen and the optimal number of cells per well have to be determined experimentally. Start with a cell concentration of e.g.  $2 \times 10^5$  cells per well and an antigen concentration between 1 and 10  $\mu$ g/ml.

**IX. ASSAY PROCEDURE**

Please read the chapter IMPORTANT INFORMATION on page 8 first.

For your convenience an easy reference protocol and a suggested plate plan are available on the first pages of this leaflet.

**DAY 1****COATING**

*Make sure not to scrape the membrane bottom of the wells during pipetting and do not let the membrane dry out, empty plate just before adding reagents.*

5. All reagents contain merthiolate (0.001% w/v) and may be toxic upon ingestion, inhalation or skin contact. Avoid contact of skin, eyes or clothing with the solutions. In case of contact, wash skin or eyes with water and consult a physician.

**VII. ADDITIONAL MATERIALS AND REAGENTS REQUIRED****Materials**

- 96-wells microtiterplate: preferably Millipore MAIPN45 ( PVDF membrane) in combination with a single cell culture tray , Millipore MAMC S01
- Device for delivery of wash buffer: automated or manual
- CO<sub>2</sub> Incubator
- A.EL.VIS or other immunospot analyser
- Pipetting devices for accurate delivery of 1-20  $\mu$ l, 50  $\mu$ l, 100  $\mu$ l and 1 ml volumes
- Beakers, flasks, cylinders, tubes necessary for preparation of reagents
- Device for counting of cells

**Reagents**

- Cell culture medium: IMDM or RPMI-1640
- Supplemented with:
  - FCS 5 %
  - Glutamin 2 mM
  - Penicillin 100 U/ ml
  - Streptomycin 100 U/ ml

Recommended for positive secretion control stimulation:

- PMA (phorbol 12-myristate 13-acetate); Sigma, P1585 stock solution: 2  $\mu$ g/ ml in ethanol, store at -18 to -32°C
- Ionomycin (Calcium ionophore); Sigma I0634 stock solution: 1  $\mu$ g/ml in ethanol, store at -18 to -32°C
- Ethanol 96% (v/v) (70 % is also suitable)
- Distilled water
- Tween-20
- PBS pH 7.2-7.4 (do not use PBS tablets as a component for the coating buffer)

**Preparation of PBS (phosphate buffered saline):****Stock solution (20x)**

Dissolve in 900 ml distilled water

- |              |       |   |
|--------------|-------|---|
| 38 mM        | 6 g   | NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O |
| 18 mM        | 32 g  | Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O |
| 280 mM       | 164 g | NaCl  |
| 0.001% (w/v) | 20mg  | Merthiolate (do not use Na-azide as preservative!)  |

Check pH and adjust to 6.8- 6.9 with concentrated NaOH or HCl and add distilled water to a volume of 1 L.

The prepared buffer can be stored up to three months at 2-8°C.

#### Preparation of PBS pH 7.2- 7.4

50 ml PBS stock solution (20x)

950 ml Distilled water

#### Preparation of wash buffer

50 µl Tween-20

1 L PBS

### VIII. REFERENCES

1. Trapani, J.A. et al., Functional significance of the perforine/granzyme cell death pathway, *Nature Reviews*, 2: 735-747 (2002).
2. Pinkoski, M.J, et al., Lymphocyte apoptosis, refining the paths to perdition, *Current opinion in Hematology*, 9: 43-49 (2002).
3. Spaenij-Dekking, E.H.A., et al., Extracellular Granzyme A and B in humans: detection of native species during CTL response in vitro and in vivo, *J. Immunology*, 160, 3610-3616 (1998).
4. Czerkinsky C.C., et al., Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. *Journal of Immunological Methods*, 110(1): 29-36 (1988).
5. Rininsland, F.H., et al., Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity, *Journal of Immunological Methods*, 240: 143-155 (2000).

### IX. IMPORTANT INFORMATION

#### Microtiterplate

Millipore MAIP N45 (PVDF membrane) is recommended. Make sure not to scrape the bottom of the wells and do not let the membrane dry out, empty plate just before adding reagents. Best results are obtained after activating the membrane with ethanol before use.

#### Washing

A disadvantage of using PVDF membrane plates is the chance of leakage to the underside of the well. This can result in a high background or unequal staining of the well. To prevent this, remove the bottom of the plate and use a separate culture tray (Millipore MAMC S01). Rinsing the underside of the membrane with distilled water after incubation with the biotinylated antibody and streptavidin poly-HRP will further reduce eventual background staining.

Washing thoroughly will result in a low background and good spot development.

Washing can be carried out manually with a squirt bottle or with an automated washing device.

#### PeliSPOT buffer

The provided PeliSPOT buffer is 5-fold concentrated, dilute with distilled water to obtain

a working strength buffer.

The buffer can be used for the blocking procedure and as a dilution buffer for the biotinylated antibody and the streptavidin-poly-HRP.

#### Positive assay control

The human Granzyme B positive assay control is from natural origin. Dilute as described under ASSAY METHOD and add 100 µl to one well in the assay plate. If the assay is performed correctly it will result in a totally blue/purple well.

Avoid repeated freeze-thawing of the control, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the human GRANZYME B levels of the control. Thaw at room temperature (18-25°C), do not use a waterbath for this purpose. Immediately store control after use at -18 to -32°C.

#### Working conditions

Sterile conditions are not necessary for coating and blocking if cell incubation is not longer than 40 hours and the cell culture medium is supplemented with antibiotics. When working with human specimen use of a flow cabinet is recommended.

#### Specificity

No crossreactivity was observed with the following human proteins: Proteinase 2 (PR3), Tryptase, Cathepsin G (Cath G), Granzyme A, Human Neutrophil Elastase (HNE), Trypsin, Chymotrypsin.

### X. PREPARATION OF CELLS

As a source of cells freshly isolated or frozen peripheral blood mononuclear cells (PBMC), cell lines or cultured cells can be used.

After being drawn, anti-coagulated blood is kept at room temperature for a maximum of 24 hours. PBMC are isolated from venous blood by density centrifugation according to the manufacturers protocol. Wash cells twice with cell culture medium.

#### Depletion of monocytes

Non-stimulated PBMC from healthy donors can release Granzyme B when directly added to the assay plate. After depletion of monocytes this spontaneous release decreases dramatically.

Different methods are known to remove monocytes, e.g. density centrifugation, panning with anti-CD14, glass/wool filters, FACS sorting. Monocytes can also be removed by adherence or magnetic cell separation. These methods are described below.