PRODUCT INFORMATION & MANUAL

Human Granzyme A Platinum ELISA

BMS2026 / BMS2026TEN

Enzyme-linked Immunosorbent Assay for quantitative detection of human Granzyme A.
For research use only.
Not for diagnostic or therapeutic procedures.

eBioscience®
An Affymetrix Company

Human Granzyme A Platinum ELISA

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1 Intended Use

The human Granzyme A ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Granzyme A. **The human Granzyme A ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

Granzymes are exogenous serine proteinases (enzymes) that are released from cytoplasmic granules of cytotoxic lymphocytes (CTLs) and NK cells.
The name “granzymes” is derived from: granules + enzymes. These granules contain next to granzymes other proteins including a pore-forming protein (Perforin). 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 in the intercellular space where after perforine will “perforate” the target cell membrane by forming transmembrane pores. Through these pores the granzymes can now enter the cytosol of the target cell. Granzyme B activates the intracellular cascade of caspases finally resulting in the killing of the target cells. Also Granzyme A is able to induce apoptosis in the target cell but the molecular mechanisms of the pathway involved need to be clarified.

Percentages of the Granzyme A and B positive CTLs can be determined by flow cytometry and immunocytochemical methods for many disorders.
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. These soluble granzymes can be measured by ELISAs.

Viral infections: Increased levels of soluble granzymes have been found with patients suspected of an increased NK cell and CTL-response caused by systemic viral infections such as EBV, HIV, CMV, hepatitis A and Dengue fever.

Lymphomas and carcinomas: It is shown that the presence of a high percentage of Granzyme B positive CTLs in glands of patients suffering from Hodgkin’s disease correlate with a severe prognosis.

Rheumatoid arthritis: Soluble Granzyme A and B is increased in synovial fluid from rheumatoid arthritis and significantly higher than levels in patients with osteoarthrosis.

Transplantation: Granzymes are likely involved in the acute rejection of kidney-transplants, as infiltrating lymphocytes in the rejected kidney strongly express granzymes. Increasing plasma levels of soluble granzymes in patients with a kidney transplants suggest a systemic viral infection, in particular an infection by CMV.

3 Principles of the Test

An anti-human Granzyme A coating antibody is adsorbed onto microwells.

Figure 1

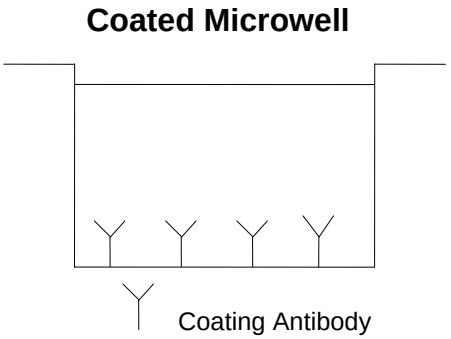


Figure 2

Human Granzyme A present in the sample or standard binds to antibodies adsorbed to the microwells.

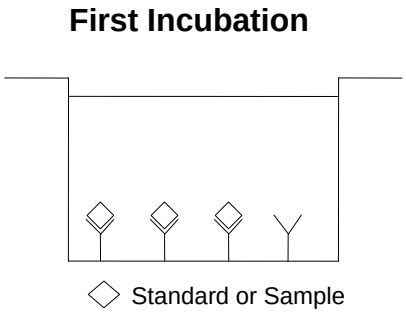


Figure 3

Following incubation unbound biological components are removed during a wash step. A biotin-conjugated anti-human Granzyme A antibody is added and binds to human Granzyme A captured by the first antibody.

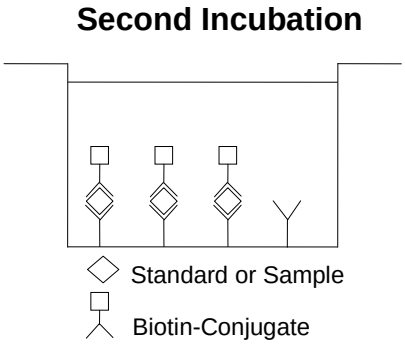
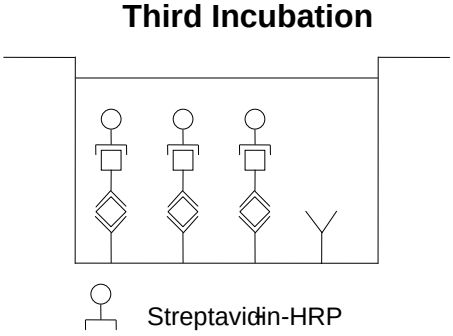


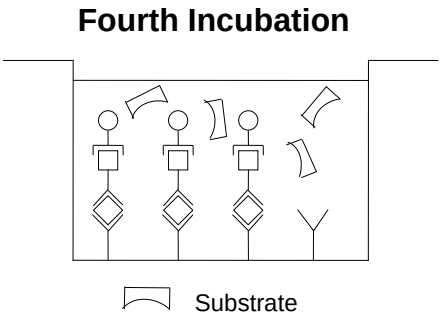
Figure 4

Following incubation unbound biotin-conjugated anti-human Granzyme A antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Granzyme A antibody.



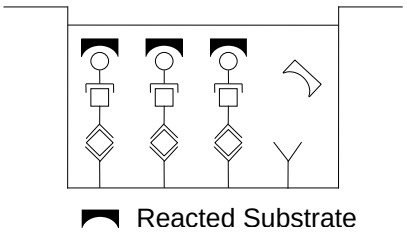
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 5



A coloured product is formed in proportion to the amount of human Granzyme A present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Granzyme A standard dilutions and human Granzyme A sample concentration determined.

Figure 6



4 Reagents Provided

4.1 Reagents for human Granzyme A ELISA BMS2026 (96 tests)

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human Granzyme A

2 vials (70 µl) **Biotin-Conjugate** anti-human Granzyme A monoclonal antibody

1 vial (150 µl) **Streptavidin-HRP**

2 vials human Granzyme A **Standard Concentrate**, 600 pg/ml upon dilution

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 bottle (7 ml) **Dilution Buffer Concentrate** 5x

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

1 vial (0.4 ml) **Red-Dye**

4 **Adhesive Films**

4.2 Reagents for human Granzyme A ELISA BMS2026TEN (10x96 tests)

10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human Granzyme A

20 vials (70 µl) **Biotin-Conjugate** anti-human Granzyme A monoclonal antibody

10 vials (150 µl) **Streptavidin-HRP**

10 vials human Granzyme A **Standard Concentrate**, 600 pg/ml upon dilution

8 bottles (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

10 bottles (7 ml) **Dilution Buffer Concentrate** 5x

10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)

10 vials (12 ml) **Stop Solution** (1M Phosphoric acid)

6 vials (0.4 ml) **Blue-Dye**

6 vials (0.4 ml) **Green-Dye**

6 vials (0.4 ml) **Red-Dye**

20 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store standard and Biotin-Conjugate or whole kit at -20°C. The standard and Biotin-Conjugate can also be removed, stored at -20°C, remaining kit reagents stored at 2-8°C. Immediately after use, remaining reagents should be returned to cold storage (standard and Biotin-Conjugate at -20°C; remaining kit reagents at 2-8°C). Expiry of the kit and reagents is stated on labels.
Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum and plasma were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Granzyme A. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.
Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Dilution Buffer

Mix the contents of the bottle well. Add contents of **Dilution Buffer Concentrate** (7.0 ml) to 28 ml distilled or deionized water and mix gently to avoid foaming. Store at 2º to 8ºC. Please note that the Dilution Buffer is stable for 30 days.

Dilution Buffer may be prepared as needed according to the following table:

Number of Strips	Dilution Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	3.5	14.0
1 - 12	7.0	28.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Dilution Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Dilution Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Dilution Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Dilution Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.5 Human Granzyme A Standard

It is recommended to spin vial in microcentrifuge to collect reagent at the bottom.

Prepare **human Granzyme A standard** by addition of Dilution Buffer as stated on the label and mix gently (concentration of standard = 600 pg/ml).

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.

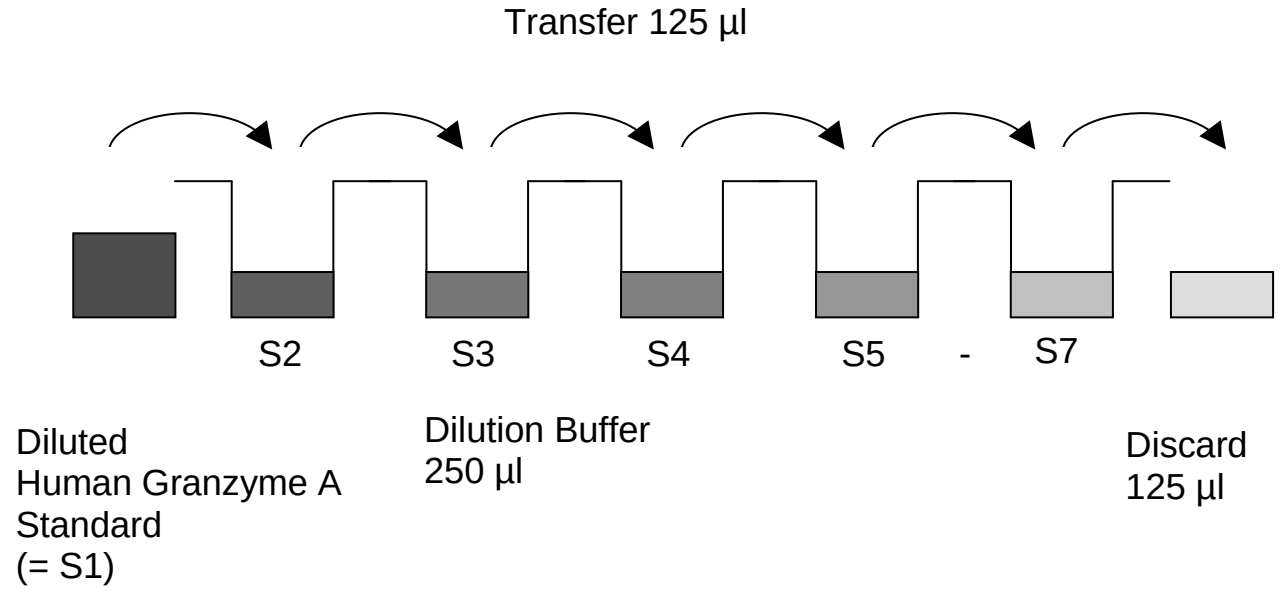
S1, S2, S3, S4, S5, S6, S7

Then prepare 1:3 serial dilutions for the standard curve as follows:
Pipette 250 µl of Dilution Buffer into tubes S2 – S7.

Pipette 125 µl of diluted standard (serves as the highest standard S1, concentration of standard 1 = 600 pg/ml) into the first tube, labelled S2, and mix (concentration of standard 2 = 200 pg/ml).
Pipette 125 µl of this dilution into the second tube, labelled S3, and mix thoroughly before the next transfer.
Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 7).

Dilution Buffer serves as blank.

Figure 7



9.6 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye, Red-Dye

In order to help our customers to avoid any mistakes in pipetting Platinum ELISAs, eBioscience offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent: Before standard and sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Dilution Buffer	20 µl ***Blue-Dye***
12 ml Dilution Buffer	48 µl ***Blue-Dye***
50 ml Dilution Buffer	200 µl ***Blue-Dye***

2. Biotin-Conjugate: Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Dilution Buffer used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of Biotin-Conjugate.

6 ml Dilution Buffer	60 µl ***Green-Dye***
12 ml Dilution Buffer	120 µl ***Green-Dye***

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to Dilution Buffer used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet: Preparation of Streptavidin-HRP.

6 ml Dilution Buffer	24 µl ***Red-Dye***
12 ml Dilution Buffer	48 µl ***Red-Dye***

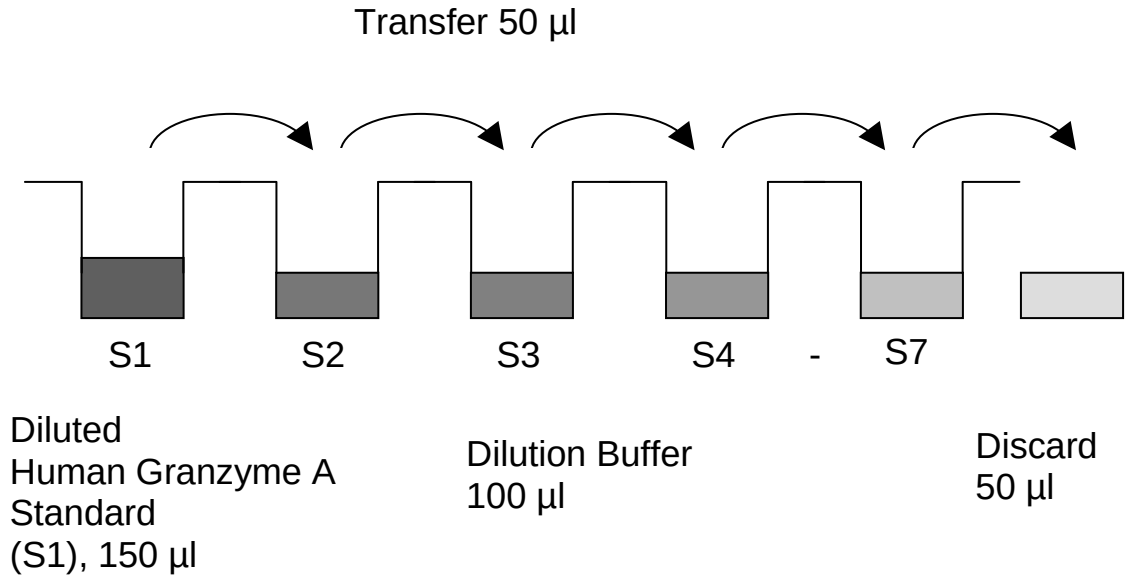
10 Test Protocol

a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.

c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1):
Add 100 µl of Dilution Buffer in duplicate to **standard wells** B1/2 - G1/2, leaving A1/A2 empty. Pipette 150 µl of prepared **standard** (see Preparation of Standard 9.5, concentration = 600 pg/ml) in duplicate into well A1 and A2 (see Table 1). Transfer 50 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection, and transfer 50 µl to wells C1 and C2, respectively. (see Figure 8). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human Granzyme A standard dilutions ranging from 600.0 to 0.8 pg/ml. Discard 50 µl of the contents from the last microwells (G1, G2) used.

Figure 8


Transfer 50 µl
S1
S2
S3
S4
-
S7
Diluted
Human Granzyme A
Standard
(S1), 150 µl
Dilution Buffer
100 µl
Discard
50 µl

In case of an **external standard dilution** (see 9.5.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (600.0 pg/ml)	Standard 1 (600.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (200.0 pg/ml)	Standard 2 (200.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (66.7 pg/ml)	Standard 3 (66.7 pg/ml)	Sample 3	Sample 3
D	Standard 4 (22.2 pg/ml)	Standard 4 (22.2 pg/ml)	Sample 4	Sample 4
E	Standard 5 (7.4 pg/ml)	Standard 5 (7.4 pg/ml)	Sample 5	Sample 5
F	Standard 6 (2.5 pg/ml)	Standard 6 (2.5 pg/ml)	Sample 6	Sample 6
G	Standard 7 (0.8 pg/ml)	Standard 7 (0.8 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

d. Add 100 µl of **Dilution Buffer** in duplicate to the **blank wells**.

e. Add 50 µl of **Dilution Buffer** to the **sample wells**.

f. Add 50 µl of each **sample** in duplicate to the **sample wells**.

g. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.

h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).

i. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point b. of the test protocol. Proceed immediately to the next step.

j. Add 100 µl of **Biotin-Conjugate** to all wells.

k. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.

l. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).

m. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point b. of the test protocol. Proceed immediately to the next step.

n. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

o. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 30 minutes, if available on a microplate shaker set at 400 rpm.

p. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point b. of the test protocol. Proceed immediately to the next step.

q. Pipette 100 µl of **TMB Substrate Solution** to all wells.

r. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol)

before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

t. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human Granzyme A concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human Granzyme A for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Granzyme A concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Dilution Buffer), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect human Granzyme A levels. Such samples require further external predilution according to expected human Granzyme A values with Dilution Buffer in order to precisely quantitate the actual human Granzyme A level.**
- It is suggested that each testing facility establishes a control sample of known human Granzyme A concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 9. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 9

Representative standard curve for human Granzyme A ELISA. Human Granzyme A was diluted in serial 3-fold steps in Dilution Buffer. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

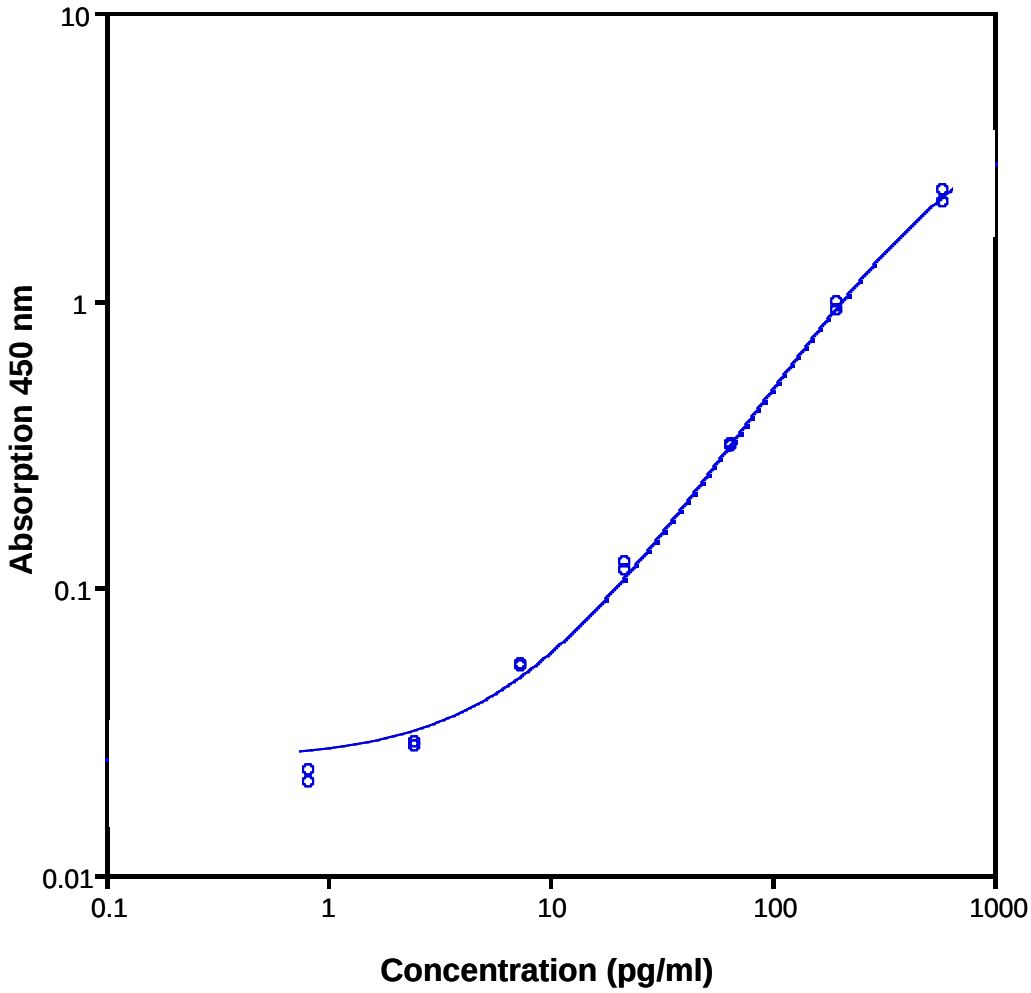


Table 2

Typical data using the human Granzyme A ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human Granzyme A Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	600	2.195	2.294	5.0
	600	2.393		
2	200	0.922	0.951	3.5
	200	0.979		
3	66.6	0.316	0.312	1.7
	66.6	0.307		
4	22.2	0.114	0.118	3.4
	22.2	0.121		
5	7.4	0.053	0.054	1.1
	7.4	0.054		
6	2.5	0.029	0.029	1.7
	2.5	0.028		
7	0.8	0.023	0.022	5.2
	0.8	0.021		
Blank	0	0.009	0.009	
	0	0.008		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human Granzyme A defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.4 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human Granzyme A. 2 standard curves were run on each plate. Data below show the mean human Granzyme A concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 8.7%.

Table 3

The mean human Granzyme A concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human Granzyme A Concentration (pg/ml)	Coefficient of Variation (%)
1	1	93.2	4
	2	82.5	5
	3	86.0	4
2	1	39.6	5
	2	34.8	6
	3	30.0	6
3	1	7.0	16
	2	6.9	16
	3	6.2	5
4	1	1.5	10
	2	1.2	11
	3	1.4	15
5	1	49.9	5
	2	41.4	4
	3	46.6	4
6	1	19.3	8
	2	18.6	3
	3	18.5	14
7	1	6.2	8
	2	5.7	8
	3	5.5	17
8	1	1.9	20
	2	1.5	0
	3	1.9	16

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human Granzyme A. 2 standard curves were run on each plate. Data below show the mean human Granzyme A concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 8.6%.

Table 4
The mean human Granzyme A concentration and the coefficient of variation of each sample

Sample	Mean Human Granzyme A Concentration (pg/ml)	Coefficient of Variation (%)
1	87.2	6.3
2	34.8	13.9
3	6.7	6.8
4	1.4	13.0
5	46.0	9.3
6	18.8	2.4
7	5.8	5.8
8	1.8	11.4

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human Granzyme A into different serum samples. Recoveries were determined in 3 independent experiments with 6 replicates each.
The amount of endogenous human Granzyme A in unspiked serum was subtracted from the spike values.
The overall mean recovery was 78%.

13.4 Dilution Parallelism

4 serum samples with different levels of human Granzyme A were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 85% to 102% with an overall recovery of 93%.

13.5 Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human Granzyme A positive serum.
There was no crossreactivity detected, namely not with Proteinase 2 (PR3), Tryptase, Cathepsin G (Cath. G), Granzyme B, Human Neutrophil Elastase (HNE), Trypsin and Chymotrypsin.

13.6 Expected Values

A panel of 40 serum samples from randomly selected donors (males and females) was tested for human Granzyme A.
The detected human Granzyme A levels ranged between 9.1 and 83.3 pg/ml.

14 Bibliography

1) Hamann D., et al.. Phenotypic and functional separation of memory and effector human CD8+ T cells. J. Exp. Med., 186, 9, 1407-1418 (1997).

2) Spaenij-Dekking EHA., 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).

3) Wever PC, et.al.. The CD8+ granzyme B+ T-cell subset in peripheral blood from healthy individuals contains activated and apoptosis-prone cells. Immunology, 93, 383-389 (1998).

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16 Reagent Preparation Summary

16.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

16.2 Dilution Buffer

Add **Dilution Buffer Concentrate** 5x (7.0 ml) to 28 ml distilled water.

16.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Dilution Buffer:

Number of Strips	Biotin-Conjugate (ml)	Dilution Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

16.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Dilution Buffer:

Number of Strips	Streptavidin-HRP (ml)	Dilution Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

16.5 Human Granzyme A Standard

Prepare **human Granzyme A standard** by addition of Dilution Buffer as stated on the label and swirl or mix gently.

17 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Dilution Buffer, in duplicate, to all standard wells leaving the first wells empty. Pipette 150 µl prepared standard into the first wells and create standard dilutions by transferring 50 µl from well to well. Discard 50 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Dilution Buffer, in duplicate, to the blank wells.
5. Add 50 µl Dilution Buffer to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
8. Prepare Biotin-Conjugate.
9. Empty and wash microwell strips 5 times with Wash Buffer.
10. Add 100 µl Biotin-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
12. Prepare Streptavidin-HRP.
13. Empty and wash microwell strips 5 times with Wash Buffer.
14. Add 100 µl diluted Streptavidin-HRP to all wells.
15. Cover microwell strips and incubate 30 minutes at room temperature (18° to 25°C).
16. Empty and wash microwell strips 5 times with Wash Buffer.
17. Add 100 µl of TMB Substrate Solution to all wells.
18. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
19. Add 100 µl Stop Solution to all wells.
20. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Dilution Buffer), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).