

Mouse Granzyme B ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of mouse Granzyme B

Catalog Numbers BMS6029 or BMS6029TEN

Pub. No. MAN0016880 **Rev.** A.0 (30)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

The Mouse Granzyme B ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of mouse Granzyme B.

Summary

Granzyme B, (CGL1,cathepsin G-like-1), is a member of the granzyme serine protease family found in the granules of cytotoxic T-cells after activation and NK cells. Granzyme B is one of eleven granzymes found in the mouse genome and synthesized as a precursor of 247 amino acid residues. It consists of a signal sequence, a pro peptide and a mature chain. The amino acid sequence of mouse Granzyme B is 69% and 80% identical to human and rat Granzyme B. The name 'granzymes' is derived from: granules + enzymes. These granules contain next to granzymes other proteins including a poreforming 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. Granzyme B is crucial for the rapid induction of target cell death by apoptosis, induced by interaction with cytotoxic T-cells. The receptor involved has been identified as mannose 6-phosphate receptor. This receptor functions as a death receptor for Granzyme B during cytotoxic T-cell-induced apoptosis.

Measurement of release of Granzyme B in response to the appropriate target is useful for evaluating cell-mediated cytotoxicity.

For literature update, refer to our website.

Principles of the test

An anti-mouse Granzyme B coating antibody is adsorbed onto microwells.

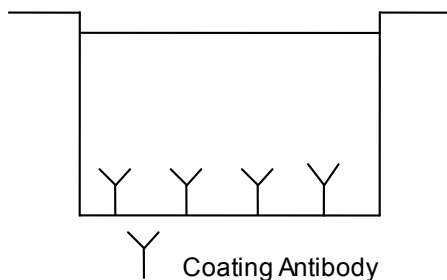


Fig. 1 Coated microwell

Mouse Granzyme B present in the sample or standard binds to antibodies adsorbed to the microwells and a biotin-conjugated anti-

mouse Granzyme B antibody is added and binds to mouse Granzyme B captured by the first antibody.

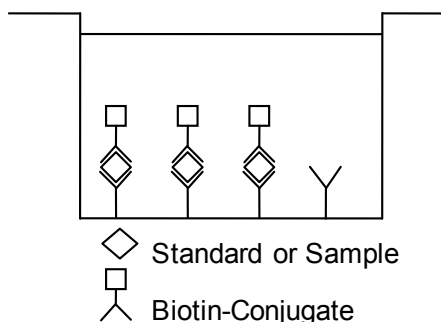


Fig. 2 First incubation

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

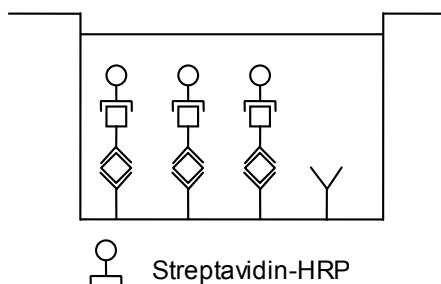


Fig. 3 Second incubation

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

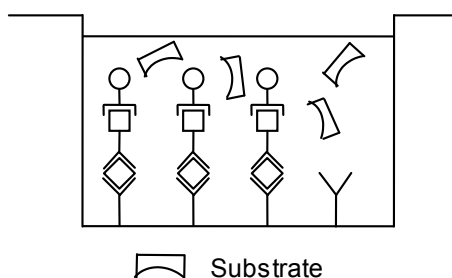


Fig. 4 Third incubation

A colored product is formed in proportion to the amount of mouse Granzyme B 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 mouse Granzyme B standard dilutions and mouse Granzyme B sample concentration determined.

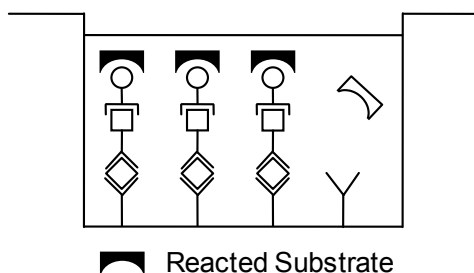


Fig. 5 Fourth incubation

Reagents provided

Reagents for mouse granzyme B ELISA BMS6029 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to mouse Granzyme B

1 vial (70 μ L) Biotin-Conjugate anti-mouse Granzyme B antibody

1 vial (150 μ L) Streptavidin-HRP

2 vials mouse Granzyme B Standard lyophilized, 1000 pg/mL upon reconstitution

1 bottle (12 mL) Sample Diluent

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween[™] 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween[™] 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

4 Adhesive Films

Reagents for mouse granzyme B ELISA BMS6029TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to mouse Granzyme B

10 vials (70 μ L) Biotin-Conjugate anti-mouse Granzyme B antibody

10 vials (150 μ L) Streptavidin-HRP

10 vials mouse Granzyme B Standard lyophilized, 1000 pg/mL upon reconstitution

7 bottles (12 mL) Sample Diluent

2 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween[™] 20, 10% BSA)

6 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween[™] 20)

10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (100 mL) Stop Solution (1M Phosphoric acid)

20 Adhesive Films

Storage instructions – ELISA kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 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.

Sample collection and storage instructions

Cell culture supernatant, serum and plasma (citrate, EDTA) were tested with this assay. Other biological samples might be suitable for use in the assay.

Pay attention to a possible *Hook Effect* due to high sample concentrations (see “Calculation of results” on page 4)

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 mouse Granzyme B. If samples are to be run within 24 hours, they may be stored at 2°C to 8°C (refer to “Sample stability” on page 6).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 μ L to 1000 μ L adjustable single channel micropipettes with disposable tips
- 50 μ L to 300 μ L 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)
- Microplate shaker
- 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

Precautions for use

- All reagents 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 pipet 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 samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that 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 samples and all potentially contaminated materials as if 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.

Preparation of reagents

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

Wash buffer (1x)

1. 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.
2. Transfer to a clean wash bottle and store at 2°C to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
3. 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

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2°C to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

Note: The Biotin-Conjugate should be used within 30 minutes after dilution.

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

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Streptavidin-HRP

Note: The Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.015	5.985
1 - 12	0.03	11.97

Mouse granzyme B standard

1. Reconstitute mouse Granzyme B standard by addition of distilled water.
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 1000 pg/mL). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 2-fold serial dilutions for the standard curve as follows: Pipette 250 µL of Sample Diluent into each tube.
3. Pipette 250 µL of reconstituted standard (concentration = 1000 pg/mL) into the first tube, labeled S1, and mix (concentration of S1 = 500.0 pg/mL).
4. Pipette 250 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).

Sample Diluent serves as blank.

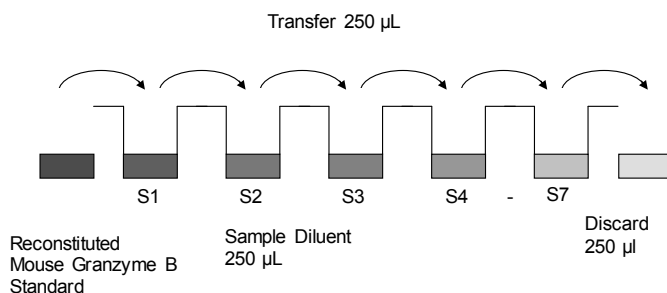


Fig. 6 Dilute standards - tubes

Test protocol

1. 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°C to 8°C sealed tightly.
2. Prepare Biotin-Conjugate (see "Biotin-Conjugate" on page 3).
3. 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.

- Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes – see “External standard dilution” on page 3): Add 100 µL of Sample Diluent in duplicate to all standard wells. Pipette 100 µL of prepared standard (see “Mouse granzyme B standard” on page 3, concentration = 1000 pg/mL), in duplicate, into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1 S1 = 500 pg/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of mouse Granzyme B standard dilutions, ranging from 500 pg/mL to 7.8 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

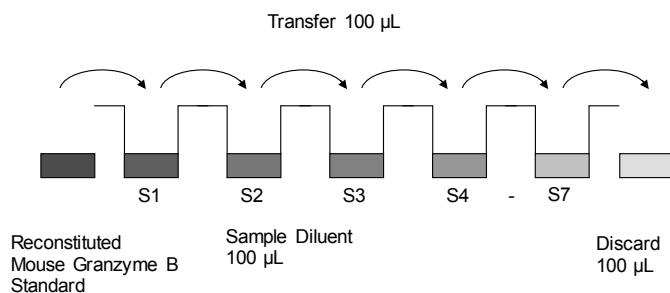


Fig. 7 Dilute standards - microwell plate

Table 1 Example of the arrangement of blanks, standards and samples in the microwell strips.

	1	2	3	4
A	Standard 1 500.0 pg/mL	Standard 1 500.0 pg/mL	Sample 1	Sample 1
B	Standard 2 250.0 pg/mL	Standard 2 250.0 pg/mL	Sample 2	Sample 2
C	Standard 3 125.0 pg/mL	Standard 3 [125.0 pg/mL]	Sample 3	Sample 3
D	Standard 4 62.5 pg/mL	Standard 4 (62.5 pg/mL)	Sample 4	Sample 4
E	Standard 5 31.3 pg/mL	Standard 5 31.3 pg/mL	Sample 5	Sample 5
F	Standard 6 15.6 pg/mL	Standard 6 15.6 pg/mL	Sample 6	Sample 6
G	Standard 7 7.8 pg/mL	Standard 7 7.8 pg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

In case of an external standard dilution (see “External standard dilution” on page 3) pipette 100 µL of these standard dilutions (S1 – S7) in the standard wells according to Table 1.

- Add 100 µL of Sample Diluent in duplicate to the blank wells.
- Add 50 µL of Sample Diluent to the sample wells.
- Add 50 µL of each sample in duplicate to the sample wells
- Add 50 µL of diluted Biotin-Conjugate to all wells, including the blank wells.
- Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 2 hours on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
- Prepare Streptavidin-HRP (see “Streptavidin-HRP” on page 3).
- Remove adhesive film and empty wells. Wash microwell strips 4 times according to point 3. of the test protocol. Proceed immediately to the next step.
- Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
- Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 1 hour on a microplate shaker set at 300 rpm. (Shaking is absolutely necessary for an optimal test performance.)
- Remove adhesive film and empty wells. Wash microwell strips 4 times according to point 3. of the test protocol. Proceed immediately to the next step.
- Pipette 100 µL of TMB Substrate Solution to all wells.

- Incubate the microwell strips at room temperature (18°C to 25°C) for 30 minutes. Avoid direct exposure to intense light.

The color development on the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable. Determination of the ideal time period for color 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 color. Alternatively the color 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.

- 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°C to 8°C in the dark.
- 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.

Shaking is absolutely necessary for an optimal test performance.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mouse Granzyme B 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 mouse Granzyme B 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 mouse Granzyme B concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent) and 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 will result in incorrect, low mouse Granzyme B levels (Hook Effect). Such samples require further external predilution according to expected mouse Granzyme B values with Sample Diluent in order to precisely quantitate the actual mouse Granzyme B level.
- It is suggested that each testing facility establishes a control sample of known mouse Granzyme B 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 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

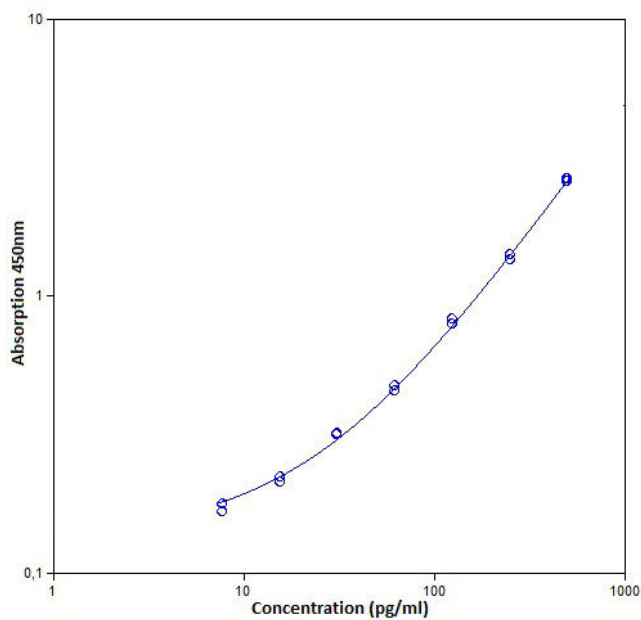


Fig. 8 Representative standard curve for mouse granzyme B ELISA. Mouse granzyme B was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

Table 2 Typical data using the mouse granzyme B ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Mouse Granzyme B Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	500.0	2.574 2.642	2.608	1.3
2	250.0	1.347 1.402	1.374	2.0
3	125.0	0.787 0.822	0.804	2.2
4	62.5	0.451 0.473	0.462	2.3
5	31.3	0.313 0.316	0.315	0.4
6	15.6	0.212 0.221	0.216	2.0
7	7.8	0.165 0.177	0.171	3.7
Blank	0.0	0.116 0.123	0.119	3.0

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 color intensity. Values measured are still valid.

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.

Performance characteristics

Sensitivity

The limit of detection of mouse Granzyme B 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 1.6 pg/mL (mean of 4 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum, plasma, cell culture supernatant samples containing different concentrations of mouse Granzyme B. Two standard curves were run on each plate. Data below show the mean mouse Granzyme B concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.5%.

Table 3 The mean mouse granzyme B concentration and the coefficient of variation for each sample

Sample	Experiment	Mean mouse Granzyme B Concentration (pg/mL)	Coefficient of Variation (%)
1	1	773.7	4.0
	2	763.2	1.7
	3	784.1	1.3
2	1	446.3	1.5
	2	430.9	2.1
	3	458.6	1.8
3	1	271.9	2.5
	2	272.8	3.4
	3	284.3	2.8
4	1	143.8	2.5
	2	142.8	2.8
	3	145.6	2.0
5	1	88.6	3.4
	2	89.8	6.6
	3	84.3	6.3
6	1	108.5	2.3
	2	108.9	4.7
	3	99.3	7.1
7	1	68.7	3.9
	2	65.6	3.2
	3	62.5	3.2
8	1	32.9	3.3
	2	27.6	6.1
	3	26.8	6.1

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 and plasma samples containing different concentrations of mouse Granzyme B. Two standard curves were run on each plate. Data below show the mean mouse Granzyme B 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 4.1%.

Table 4 The mean mouse Granzyme B concentration and the coefficient of variation of each sample

Sample	Mean mouse Granzyme B Concentration (pg/mL)	Coefficient of Variation (%)
1	773.7	1.3
2	445.2	3.1
3	276.3	2.5
4	144.1	1.0
5	87.6	3.3
6	105.6	5.2
7	65.6	4.7
8	29.1	11.3

Spike recovery

The spike recovery was evaluated by spiking 3 levels of mouse Granzyme B into serum, plasma (EDTA, citrate) and cell culture supernatant. Recoveries were determined with 2 replicates each. The amount of endogenous mouse Granzyme B in unspiked samples was subtracted from the spike values.

Sample matrix	Spike high		Spike medium		Spike low	
	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)
Serum	91	84-98	102	84-111	113	84-151
Plasma (EDTA)	82	78-86	87	78-95	119	117-121
Plasma (citrate)	77	-	75	-	78	-
Cell culture supernatant	85	-	104	-	106	-

Dilution parallelism

Serum, plasma (EDTA, citrate), cell culture supernatant samples with different levels of mouse Granzyme B were analyzed at serial 2-fold dilutions with 4 replicates each.

Sample matrix	Recovery of Exp. Val.		
	Dilution	Mean (%)	Range (%)
Serum	1:4	104	99-108
	1:8	105	88-123
	1:16	99	85-116
Plasma (EDTA)	1:4	101	99-102
	1:8	95	90-100
	1:16	65	62-67
Plasma (citrate)	1:4	85	82-88
	1:8	91	83-100
	1:16	81	76-86
Cell culture supernatant	1:4	78	
	1:8	108	
	1:16	90	

Sample stability

Freeze-Thaw stability

Aliquots of serum, plasma, cell culture supernatant samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the mouse Granzyme B levels determined.

There was no significant loss of mouse Granzyme B immunoreactivity detected by freezing and thawing.

Storage stability

Aliquots of serum, plasma, cell culture supernatant samples (spiked or unspiked) were stored at -20°C, 2°C to 8°C, room temperature, and at 37°C, and the mouse Granzyme B level determined after 24 hours.

There was no significant loss of mouse Granzyme B immunoreactivity detected during storage under above conditions.

Specificity

The assay detects both natural and recombinant mouse Granzyme B. The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a mouse Granzyme B positive sample.

There was no cross reactivity or interference detected.

Expected values

Panels of serum as well as plasma samples (EDTA, citrate) were tested for mouse Granzyme B. For detected mouse Granzyme B levels see Table 5.

Table 5 Expected values.

Sample matrix	Number of samples evaluated	Mean (pg/mL)	Range (pg/mL)	Standard deviation (pg/mL)
Serum	23	38	0-58	1
Plasma (EDTA)	2	95	79-112	24
Plasma (citrate)	4	30	19-41	10

Reagent preparation summary

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

Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

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

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Streptavidin-HRP

Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.015	5.985
1 - 12	0.03	11.97

Mouse granzyme B standard

Reconstitute mouse Granzyme B standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

Test protocol summary

1. Determine the number of microwell strips required.
2. Prepare Biotin-Conjugate.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 μ L Sample diluent (1x), in duplicate, to all standard wells. Pipette 100 μ L prepared standard into the first wells and create standard dilutions by transferring 100 μ L from well to well. Discard 100 μ L from the last wells.

Alternatively external standard dilution in tubes (see "External standard dilution" on page 3): Pipette 100 μ L of these standard dilutions in the microwells.
5. Add 100 μ L Sample Diluent, in duplicate, to the blank wells.
6. Add 50 μ L Sample Diluent to sample wells.
7. Add 50 μ L sample in duplicate, to designated sample wells.
8. Add 50 μ L diluted Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18°C to 25°C) on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 4 times with Wash Buffer.
12. Add 100 μ L diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18°C to 25°C) on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
14. Empty and wash microwell strips 4 times with Wash Buffer.
15. Add 100 μ L of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 30 minutes at room temperature (18°C to 25°C)

17. Add 100 μ L Stop Solution to all wells.
18. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 μ L sample + 50 μ L Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor ($\times 2$).

Customer and technical support

Visit thermofisher.com/support for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
 - Product FAQs
 - Software, patches, and updates
 - Training for many applications and instruments
- Order and web support
- Product documentation
 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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