KAMIYA BIOMEDICAL COMPANY

Human Granzyme A ELISA

For the quantitative determination of human granzyme A in plasma, serum, cell culture supernatant, urine, synovial fluid and BAL fluid.

Cat. No. KT-079

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION

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PRODUCT
The **K-*ASSAY*®** Human Granzyme A ELISA is an enzyme immunoassay for the quantitative determination of human granzyme A in plasma, serum, cell culture supernatant, urine, synovial fluid and BAL fluid.

INTRODUCTION
Granzymes are exogenous serine proteinases that are released from cytoplasmic granules of cytotoxic lymphocytes (CTLs) and NK cells. These granules contain granzymes and other proteins including perforin, a pore-forming protein. 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 into the intercellular space where perforin 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 A is also capable of inducing apoptosis in the target cell but the molecular mechanisms of the pathway involved need to be clarified.

Not all of the granzymes enter the target cell. A portion of the protein also "leaks" into the peripheral blood and other biological fluids. Detectable amounts of granzymes have been found in circulation in healthy volunteers. These soluble granzymes can now be measured by this ELISA assay.

PRINCIPLE
The **K-*ASSAY*®** Human Granzyme A ELISA is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-human granzyme A antibody is bound onto polystyrene microtiter wells. Human granzyme A present in a measured volume of sample or calibrator is captured by the antibody on the microtiter well, and non-bound material is removed by washing. A biotinylated second monoclonal antibody to human granzyme A then binds to the human granzyme A-antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by the addition of horseradish peroxidase (HRP)-conjugated streptavidin, which binds to the biotinylated side of the human granzyme A sandwich. After removal of non-bound HRP conjugate by washing, a chromogenic substrate is added to the wells. A colored product is formed in proportion to the amount of granzyme A present in the sample or calibrator. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of a calibration curve, the concentration of granzyme A can be determined by interpolation with the calibration curve.

STORAGE
Store at -20℃. The expiration date is shown on the box label.

COMPONENTS
The kit contains material sufficient for 288 tests including calibrators and control.

Quantity	Kit component		Volume	Cap color
1 vial	Coating Monoclonal Antibody (Ab)	100X concentrate	375 µL	red
1 vial	Positive Control	Range 550 to 850 units/mL*	200 µL	transparent
1 vial	Granzyme A Calibrator	22,750 units/mL	100 µL	black
1 vial	Biotinylated Monoclonal antibody (mAb)	100X concentrate	375 µL	yellow
1 vial	Streptavidin-HRP Conjugate	10,000X concentrate	20 µL	brown
1 bottle	Assay Buffer	5X concentrate	60 mL	-
3 pcs	Microtiter Plates + Lid	-	-	-
10 pcs	Plate Seals	-	-	-

* One unit/mL is approximately 1 pg/mL.

PRECAUTIONS

1) The **K-*ASSAY*®** Human Granzyme A ELISA is intended *for research purposes only, not for in vivo use*.
2) Use only the reagents and microtiter plates supplied with the kit. Do not mix reagents from different kit lots.
3) Handle all plasma and serum samples with care to prevent transmission of blood-borne pathogens.
4) Sodium azide inactivates HRP. Do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
5) All reagents contain thimerosal (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.
6) Centrifuge all vials before use (1 minute 3,000 x g).
7) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

ADDITIONAL BUFFERS AND SOLUTIONS REQUIRED

■ **Coating buffer and substrate buffer**: 0.11 M acetate buffer pH 5.5
- Dissolve 15.0 g sodium acetate ($CH_3COONa.3H_2O$) in 800 mL distilled water.
- Adjust pH to 5.5 with glacial acetic acid. Add distilled water to a volume of 1 liter.

Do not add any preservative (e.g. thimerosal**,** sodium azide), since this may affect the enzymatic color development. The buffer must be prepared fresh.

■ **PBS stock solution [20 x]**: 0.2 M PBS
- Dissolve 32 g $Na_2HPO_4.2H_2O$
6 g $NaH_2PO_4.2H_2O$
164 g NaCl
in 900 mL distilled water

(intensive stirring and some heating will speed dissolution).
Bring the temperature of the solution back to room temperature (RT, 18-25°C) and check pH; if necessary adjust pH to between 6.8 and 6.9 with concentrated HCl or NaOH, and add distilled water to a volume of 1 liter (when diluted 20 times, the working-strength buffer will have a pH between 7.2 and 7.4).
- Add 20 mg thimerosal as preservative. Do not use sodium azide (NaN_3) since it affects the HRP activity.
- The prepared buffer can be stored up to three months at 4°C.

Note: In the concentrated buffer salt crystals may appear when stored at 4°C. Before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C to dissolve the precipitate.

■ **Wash buffer**: PBS with 0.02 % TWEEN 20
- Make 1 liter of working-strength PBS by diluting the 20X PBS stock solution (see above) with distilled water.
- Add 200 µL TWEEN 20.
- The prepared buffer can be stored up to one month at 4°C.

■ **3,5,3',5'-tetramethylbenzidine (TMB) stock solution**: 6 mg/mL TMB in dimethylsulfoxide (DMSO)
- Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 mL DMSO.
- The prepared stock solution can be stored up to 1 month at RT in the dark.

■ **Hydrogen peroxide stock solution**: 3% H_2O_2 solution in distilled water.
- The prepared stock solution can be stored up to one month at 4°C.

■ **Substrate solution**

- For each plate mix the following reagents:

12 mL	Substrate buffer
200 µL	TMB stock solution
12 µL	H_2O_2 stock solution

The substrate solution should be prepared immediately before use and has to be at RT for optimal reproducible results.

■ **Stop solution**: 1.8 M H_2SO_4 solution in distilled water.

ADDITIONAL INFORMATION

Additional materials required

- Pipettes for accurate delivery of 1-10 µL, 50 µL, 100 µL and 1 mL volumes.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Automated plate washer or wash bottle.
- Microtiter plate reader.

Sensitivity

2 x MEAN calculated zero signal (shaken incubation): < 20 units/mL.
Note: the sensitivity is dependent upon the type and quality of enzyme substrate.

Expected values

Granzyme A values in fresh serum and plasma samples of healthy individuals are below 60 units/mL.

Specificity

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

ASSAY PROCEDURE

1. *BRING ALL REAGENTS TO RT*, with the exception of the Streptavidin-HRP Conjugate, Positive Control and Calibrator which have to be kept at -20°C to ensure stability. Centrifuge all vials before use (1 minute 3,000 x g).

   For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this package insert.

2. **Assay Buffer**
   The kit contains one bottle with 5X concentrated Assay Buffer.

   Calculate the quantity of assay buffer required (approximately 15 mL undiluted buffer per Microtiter Plate) and prepare a working-strength solution by diluting the opalescent concentrated buffer 5 times in distilled water before use. Shake gently. The working-strength Assay Buffer can be stored for up to one week at 4°C.

   For optimal assay results, dilute samples, Calibrator and Positive Control in working-strength Assay Buffer.

3. **Microtiter Plates**
   Coating antibody
   The kit contains three Microtiter Plates for 96 tests each (including the calibration curve and control samples).
   - Prepare coating buffer as described on page 3. For each Microtiter Plate, add 120 µL of Coating Monoclonal Antibody (red-capped vial) to 12 mL of coating buffer.
   - Add 100 µL to all wells, cover Microtiter Plate(s) with lid and **incubate overnight at RT.**

   Washing procedure
   - Prepare working-strength PBS (1:20 dilution of stock PBS as described on page 3). Add Tween 20 to 0.02%.
   - Aspirate contents from wells and completely fill the wells (> 300 µL/well) with working-strength PBS/TWEEN and aspirate.
   - Repeat this four times. Place plate upside down on a paper towel for a while to remove excess buffer. After the final aspiration the wells should be dry.

   Blocking procedure

   - Add 150 µL Assay Buffer to all wells, cover Microtiter Plate(s) with adhesive seal, gently agitate the Microtiter Plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate, <u>shaken</u>, for 30 minutes at RT**.

4. **Granzyme A Calibrator and Positive Control**
   Calibration curve preparation
   Human Granzyme A Calibrator is from a natural origin. The kit contains one black-capped vial of 22,750 units/mL human Granzyme A. <u>1 unit is approximately 1 pg/mL</u>.

- Label 7 tubes, one tube for each calibrator dilution: 1,200, 400, 133.3, 44.4, 14.8, 4.9 and 1.6 units/mL.
- Pipette 180 µL of Assay Buffer into the tube labeled 1,200 units/mL and 120 µL of Assay Buffer into the other tubes.
- Transfer 10 µL of the Granzyme A Calibrator (22,750 units/mL) into the first tube labeled 1,200 units/mL, mix throughly and transfer 60 µL of this dilution into the second tube labeled 400 units/mL.
- Repeat the serial dilution five more times by transfering 60 µL of the previous tube of diluted calibrator to the 120 µL of Assay Buffer.
- It is recommended to prepare two separate series for each assay.

Positive control

- The positive control has to be diluted 1:5. Add 25 µL of the positive control to 100 µL of Assay Buffer.

Avoid repeated freeze-thawing of the calibrator and control, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the human granzyme A levels of the calibrator and control. Thaw at RT. Do not use a waterbath for this purpose. Store calibrator and control immediately after use at -20°C.

5. Samples

Body fluids, urine, serum, heparin or EDTA anti-coagulated plasmas, and tissue culture supernatants are suitable for use in the assay. Do not use grossly hemolyzed or lipemic samples. If samples are to be run within 24 hours, they may be stored at 4°C; otherwise samples should be stored frozen (< -20°C).
Up to 3 freeze-thaw cycles have no effect on the Granzyme A levels of serum or plasma samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay, frozen samples should be thawed **as quickly as possible** at RT.

It is recommended to dilute the test samples at least 1:5 in working-strength Assay Buffer. If high levels of granzyme A are expected in the test samples, additional dilutions of sample (i.e. 1:25) should also be prepared.

Research samples of R.A. and S.L.E. can cause false positive results. These samples should be treated with the IMX system from Abbott Laboratories (Cat No. 1A14: IgM R.F. neutralization reagent). If values are still above normal value after treatment, the signal obtained is not expected to be false positive, but specific for granzyme A.

6. First Wash Step

- Prepare wash buffer as described on page 3.
- Wash Microtiter Plates five times with wash buffer in a plate washer. In case of manual washing, completely fill the wells (> 300 µL/well) with washing buffer and aspirate, repeat this four times. Place plate upside down on a paper towel for a while to remove excess buffer. After the final aspiration the wells should be dry.

7. First Incubation Step: calibrators and samples

- Leaving the substrate blank wells empty, transfer 100 µL of the prepared calibrators, control and samples in duplicate into the appropriate wells (see recommended plate plan).
- Cover plate(s) with adhesive seal, gently agitate the Microtiter Plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate, shaken, for 1 hour at RT.**

8. Second Wash Step

- Aspirate contents from wells and wash the Microtiter Plate(s) as described in Step 6.

9. Second Incubation Step: **Biotinylated Antibody**

- For each Microtiter Plate, add 120 µL Biotinylated Antibody (yellow cap) to 12 mL working-strength Assay Buffer immediately before use.
- Leaving the substrate blank wells empty, add 100 µL of diluted Biotinylated Antibody to all wells.
- Cover plate(s) with adhesive seal, gently agitate the Microtiter Plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate, shaken, for 1 hour at RT.**

10. Third Wash Step

- Aspirate contents from wells and wash the Microtiter Plate(s) as described in Step 6.

11. Third Incubation Step: Streptavidin-HRP Conjugate

The kit contains one brown capped vial of concentrated Streptavidin-HRP Conjugate, which must be stored at -20°C to maintain maximum stability. The contents of the vial will not be frozen at this temperature.

- For each Microtiter Plate, add 3 µL Streptavidin-HRP Conjugate to 30 mL of working-strength Assay Buffer just before use. **Do not prepare in advance of assay.**
- Leaving the substrate blank wells empty, add 100 µL of Streptavidin-HRP Conjugate to all wells.
- Cover the Microtiter Plate(s) with adhesive seal, gently agitate the Microtiter Plate by tapping the edge of

the frame for a few seconds to mix contents of each well and **incubate, shaken, for 30 minutes at RT.**

12. Fourth Wash Step

Aspirate contents from wells and wash the Microtiter Plate(s) as described in Step 6.

13. Fourth Incubation Step: Enzymatic color development

- Approximately 10 minutes before use, prepare the substrate solution as described in Page 4.
Substrate solution should be at RT for optimal reproducible results.

- Add 100 µL of substrate solution to all wells, **including the substrate blank wells.**
- Cover Mictrotiter Plate(s) with lid, gently agitate the Microtiter Plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate, STATIC, for 30 minutes at RT in the dark.**
Do not cover the plate with aluminium foil.

Note: The speed of enzymatic color development is influenced by many factors including temperature and quality of the TMB and H_2O_2 used.

14. Stop Enzymatic Reaction

- Add 100 µL of stop solution to each well.

15. Plate Read-out

- Measure the absorbance at 450 nm in a Microtiter Plate reader. After adding stop solution, the color is stable for a maximum of 30 minutes.

RESULTS

Substrate blank

- Record the absorbance at 450 nm for the substrate blank wells and average the duplicate values.

Calibration curve

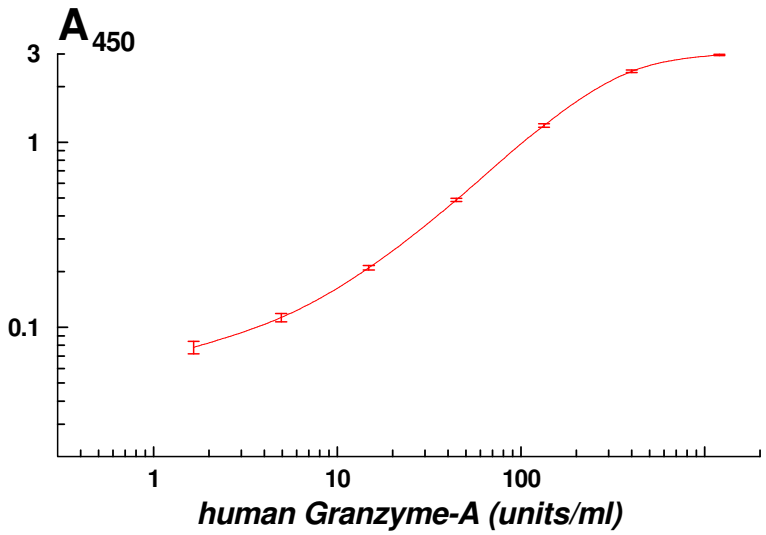
- Record the absorbance at 450 nm for each well containing calibrator and average the duplicate values.
- Calculate the net mean absorbance by subtracting the mean of the substrate blank wells.
- Plot the net mean absorbance (ordinate) versus the granzyme A concentration in units/mL (abscissa) on log-linear graph paper and draw the best fitting curve. An example of a calibration curve is given on page 7.

Samples

- Record the absorbance at 450 nm for each sample well, and average the duplicate values.
- Calculate the net mean absorbance by subtracting the mean of the substrate blank wells.
- Locate the net mean absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the calibration curve. At the point of intersection, read the granzyme A concentration (units/mL) from the horizontal axis.
- Multiply the obtained granzyme A concentration with the dilution factor of the sample.

SENSITIVITY

The assay sensitivity is dependent on the incubation methodology. Just follow all the instructions as stated in the assay procedure. Incubate at RT on a horizontal plate shaker at **500 ± 100 rpm**. All incubations, with exception of the enzymatic color development, have to be completed on the shaker. This will result in assay sensitivity, with small effects on the background levels (see the figure, page 7).



Typical calibration curve for The K-*ASSAY*® Human Granzyme A ELISA. The assay is completed shaken at RT.

	SHAKEN INCUBATION
	Calculated mean OD at 450 nm
Substrate blank	0
0 units/mL	0.037
1.6 units/mL	0.078
4.9 units/mL	0.113
14.8 units/mL	0.210
44.4 units/mL	0.487
133.3 units/mL	1.232
400 units/mL	2.414
1,200 units/mL	2.961

DO NOT USE THESE DATA TO CONSTRUCT A CALIBRATION CURVE FOR SAMPLE VALUE CALCULATIONS.

PLATE PLAN PROPOSED

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8									B	B

Key: B: substrate blank S1-S8: GRANZYME A Calibrators: 0 – 1,200 units / mL
Empty: samples and Positive Control.

PROTOCOL SUMMARY AND CHECKLIST

Day 0:

- ○ Bring coating antibody to RT (18-25°C).
- ○ Prepare coating buffer.
- ○ Dilute coating antibody 1:100 in coating buffer, add 100 µL to all wells, cover the plate(s) and incubate overnight at RT.

Day 1:

- ○ Bring all reagents, with the exception of Streptavidin-HRP Conjugate, Positive Control and Calibrator, to RT. Positive Control and Calibrator should be thawed prior to dilution.
- ○ Prepare Assay Buffer and wash buffer (PBS / TWEEN 0.02%).
- ○ Wash the plate(s) five times with wash buffer.
- ○ Add 150 µL Assay Buffer to all wells and incubate, shaken, for 30 minutes at RT.
- ○ Thaw Calibrator and Positive Control and prepare dilution and restore directly at -20°C after preparation. Prepare sample dilutions.
- ○ Wash the plate(s) five times with wash buffer.
- ○ Leaving the substrate blank wells empty, add 100 µL of Calibrator dilutions, Positive Control and sample dilutions to the appropriate wells, cover the plate(s) and incubate, shaken, for one hour at RT.
- ○ Dilute Biotinylated Antibody 1:100 in Assay Buffer.
- ○ Wash the plate(s) five times with wash buffer.
- ○ Leaving the substrate blank wells empty, add 100 µL of the diluted Biotinylated Antibody to all wells, cover the plate(s) and incubate, shaken, for one hour at RT.
- ○ Dilute the Streptavidin-HRP Conjugate 1:10,000 in Assay Buffer.
- ○ Wash the plate(s) five times with wash buffer.
- ○ Leaving the substrate blank wells empty, add 100 µL of the Streptavidin-HRP Conjugate to all wells, cover plate(s) and incubate, shaken, for 30 minutes at RT.
- ○ Immediately before use, prepare substrate solution.
- ○ Wash the plate(s) five times with wash buffer.

- Add 100 µL substrate solution to all wells, including the substrate blank wells, and incubate, static, for 30 minutes at RT in the dark.
- Add 100 µL of stop solution to all wells and read the plate(s) at 450 nm.
- Calculate the amount of granzyme A in the samples.

FOR RESEARCH USE ONLY

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