



PeliKine Compact™

human GRANZYME B ELISA kit

288 tests

An enzyme immunoassay
for the quantitative determination of human GRANZYME B

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I. INTRODUCTION

Granzymes are exogenous serine proteinases (enzymes) that are released from cytoplasmic granules of cytotoxic lymphocytes (CTLs) and NK cells.

Granzymes; 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 granzyme A and B positive CTL's 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 now be measured by ELISA's, which were developed by Sanquin.

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.

Lymphoma's and carcinoma's

It is shown that the presence of a high percentage of granzyme B positive CTL's 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 osteo arthrosis.

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.

USE

This PeliKine compact™ human GRANZYME B ELISA kit has been developed for fast, reproducible and specific quantification of human GRANZYME B (huGRANZYME B) in plasma and serum as well as in cell-culture supernatant. Also suitable for synovium fluid and BAL fluid.

II. PRINCIPLE OF THE TEST

The PeliKine compact™ human GRANZYME B ELISA kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-huGRANZYME B antibody is bound onto polystyrene microtiter wells. Human GRANZYME B, present in a measured volume of sample or standard is captured by the antibody on the microtiter well, and non-bound material is removed by washing. Subsequently, a biotinylated second monoclonal antibody to huGRANZYME B is added. This antibody binds to the huGRANZYME B-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 huGRANZYME B sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of huGRANZYME B present in the sample or standard. 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 standard curve, the concentration of huGRANZYME B can be determined by interpolation with the standard curve.

III. STORAGE AND STABILITY

The PeliKine compact™ human GRANZYME B ELISA kit should be stored at -18 to -32°C. The performance of the kit is guaranteed until the expiration date shown on the case label.

IV. CONTENTS OF THE KIT

The PeliKine compact™ human GRANZYME B ELISA kit contains material sufficient for 288 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume	Cap colour
1 vial	coating antibody	100-fold concentrated	375 µL	red
1 vial	Positive control	Range 420 to 570 units/mL	200 µL	transparent
1 vial	GRANZYME B standard	24,000 units/mL	100 µL	black
1 vial	biotinylated antibody	100-fold concentrated	375 µL	yellow
1 vial	streptavidin-poly-HRP conjugate	10,000-fold concentrated	20 µL	brown
1 bottle	Granzyme kit buffer	5- fold concentrated	60 mL	
3 pcs	microtiter plate + lid	-	-	
10 pcs	plate seals	-	-	

V. PRECAUTIONS FOR USE

- 1) Only use the reagents and microtiter plates supplied with the kit, do not mix reagents from different kit lots.
- 2) Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.

- 3) Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
- 4) All reagents contain merthiolate (0.001 % w/v).
- 5) Centrifuge all vials before use (1 minute 3000 x g).
- 6) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

VI. ADDITIONAL BUFFERS & SOLUTIONS REQUIRED

Coating buffer and substrate buffer : 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) 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. merthiolate, sodium azide), since this may affect the quality of the enzymatic colour development.

The buffer must be prepared fresh.

PBS stock solution [20 x]: 0.2 M Phosphate Buffered Saline (PBS)

Dissolve	32 g	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$
	6 g	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
	164 g	NaCl

in 900 mL distilled water

(intensive stirring and some heating will speed dissolution).

Bring the temperature of the solution back to 18-25°C and check pH; if necessary adjust pH to 6.8 - 6.9 with concentrated HCl or NaOH, and add distilled water to a volume of 1 liter (when diluted 20 times the obtained buffer will have a pH of 7.2 - 7.4).

Add 20 mg merthiolate as preservative. Do not use sodium azide (NaN_3) since this preservative reduces the quality of the enzymatic label.

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

Note: in the concentrated buffer salt crystals may appear when stored at 2-8°C. Before preparing the working-strength buffer, first warm the concentrated buffer briefly to 37°C to dissolve the precipitate.

Washing buffer: PBS with 0.02 % TWEEN 20

Make 1 liter of working-strength PBS by diluting the PBS stock solution [20x] (see above) with distilled water.

Add 200 µl TWEEN 20. The prepared buffer can be stored up to one month at 2-8°C.

Substrate buffer: 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) in 800 mL distilled water.
Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of liter.
Do not add any preservative (e.g. merthiolate, sodium azide) since this may affect the quality of the enzymatic colour development.

The prepared buffer can be stored up to two weeks at 2-8°C.

3,5,3',5'-tetramethylbenzidine (TMB) stock solution: 6 mg/mL TMB in DMSO

Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 mL dimethylsulfoxide (DMSO).

The prepared stock solution can be stored up to 1 month at 18-25°C and protected against light.

Hydrogen peroxide stock solution: 3% H_2O_2 solution in distilled water.

The prepared stock solution can be stored up to one month at 2-8°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 just before use and has to be at 18-25°C for optimal reproducible results.

Stop solution: 1.8 M H_2SO_4 solution in distilled water.

VII. ADDITIONAL INFORMATION

Additional materials required

- Pipetting devices for accurate delivery of 1-10 μL , 50 μL , 100 μL and 1 mL volumes.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Device for delivery of washing buffer (wash bottle / automated plate washer).
- Microtiter plate reader.
- Horizontal plate shaker

Sensitivity

2 x MEAN calculated zero signal: < 10 units/mL.

Note: the sensitivity is dependent upon the type and quality of enzymatic substrate.

Expected values

GRANZYME B values in fresh serum and plasma of healthy individuals are below 40 units/mL.

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.

References

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

VIII. ASSAY PROCEDURE

Day 0

- Bring coating antibody to 18-25°C.
- Prepare coating buffer as described in section VI of the information leaflet.

1. MICROTITER PLATES

Coating antibody

Coating

The kit contains three micotiter plates for 96 tests each, including the standard curve samples.

Per microtiter plate add 120 μ L of coating antibody (red-capped vial) to 12 mL coating buffer. Add 100 μ L to all wells, cover microtiter plate(s) with lid and **incubate overnight at 18-25°C**.

Day 1

2. *BRING ALL REAGENTS TO 18-25°C*, with the exception of the streptavidin-HRP conjugate which has to be kept at -18 to -32°C to ensure stability. Centrifugate all vials before use (1 minute 3000 x g).
For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.
3. **GRANZYME KIT BUFFER**
The kit contains one bottle with 5-fold concentrated Granzyme kit buffer.
For optimal assay results, dilute samples and standards in working-strength Granzyme kit buffer.

Calculate the quantity of dilution buffer required (approximately 15 mL undiluted Granzyme kit buffer per microtiter plate) and prepare a working-strength solution by diluting the opalescent concentrated buffer 5 times in distilled water before use. The working-strength Granzyme kit buffer can be stored for up to one week at 2-8°C

Washing procedure

Prepare working-strength PBS (1:20 dilution of stock PBS as described in section VI of the information leaflet).

Aspirate supernatants from wells and completely fill the wells (> 300 μ L) with working-strength PBS and aspirate. Repeat this four times, after the final aspiration the wells should be dry.

Blocking procedure

Add 150 µl working-strength Granzyme kit 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, shaken, for 30 minutes at 18-25°C**.

4. GRANZYME B STANDARD and positive control

Standard curve preparation

HuGRANZYME B standard is from natural origin.

The kit contains one black-capped vial of huGRANZYME B.

1 unit approximately meets 1 pg/mL.

Label 7 tubes, one tube for each dilution: 960, 320, 106.7, 35.6, 11.9, 4.0 and 0 units/mL.

Pipette 240 µL of working-strength Granzyme kit buffer into the tube labelled 960 units/mL and 120 µL of working-strength Granzyme kit buffer into the other tubes

Transfer 10 µL of the GRANZYME B standard into the first tube labelled 960 units/mL, mix well and transfer 60 µL of this dilution into the second tube labelled 320 units/mL.

Repeat the serial dilutions four more times by adding 60 µL of the previous tube of diluted standard to the 120 µL of working-strength Granzyme kit buffer. The last tube (0 pg/mL) contains only working-strength Granzyme kit buffer.

It is recommended to prepare two separate series for each assay. Add 100 µL to the wells.

The positive control has to be diluted 1:5. Add 25 µL of the positive control to 100 µL of working-strength Granzyme kit buffer. Add 100 µL to the wells

Avoid repeated freeze-thawing of the standard and control, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the huGRANZYME B levels of the standard and control. Thaw on 18-25°C, do not use a waterbath for this purpose.

Immediately store standard and control after use at -18 to -32°C.

5. SAMPLES

Body fluids, serum, heparin or EDTA-anti-coagulated plasmas, and tissue culture fluids are suitable for use in the assay. Do not use grossly haemolyzed or lipemic specimens. If samples are to be run within 24 hours, they may be stored at 2-8°C; otherwise samples should be stored frozen (-18 to -32 °C).

Up to 3 freeze-thaw cycles have no effect on the GRANZYME B 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 18-25°C

It is recommended to dilute the test samples at least 1:5 in working-strength kit buffer. If high levels of GRANZYME B are expected in the test samples, additional dilutions of sample i.e. 1:25 should also be prepared.

Samples from R.A. and S.L.E. patients can cause false positive results. These samples should be treated with the IMX system from Abbott Laboratories (cat.nr. 1A14: IgM R.F. neutralisation reagent).

If values are still above normal value after treatment, the signal obtained is not expected to be false positive, but specific for GRANZYME B.

6. FIRST WASH STEP

Prepare washing buffer as described in section VI of this leaflet.

Wash the required microtiter plates five times with washing buffer in a plate washer. In case of manual washing, completely fill the wells ($> 300 \mu\text{L}$) with washing buffer and aspirate, repeat this four times. After the final aspiration the wells should be dry.

7. FIRST INCUBATION STEP

Standards and samples

Leaving the substrate blank wells empty, transfer $100 \mu\text{L}$ of the prepared standards 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 18-25°C**.

8. SECOND WASH STEP

Aspirate supernatant from wells and wash the microtiter plate(s) as described in section VII, point 6 above.

9. SECOND INCUBATION STEP

biotinylated antibody

The kit contains one yellow-capped vial with concentrated biotinylated antibody.

Per microtiter plate, add $120 \mu\text{L}$ biotinylated antibody to 12 mL working-strength Granzyme kit buffer just before use.

Leaving the substrate blank wells empty, add $100 \mu\text{L}$ 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 18-25°C**.

10. WASH STEP

Aspirate supernatant from wells and wash the microtiter plate(s) as described in section VII, point 6 above.

11. THIRD INCUBATION STEP

Streptavidin-HRP conjugate

The kit contains one brown vial of concentrated streptavidin-HRP conjugate, which must be stored at -18 to -32°C to maintain maximal stability. The contents of the vial will not be frozen at this temperature.

Per microtiter plate, add $3\ \mu\text{L}$ streptavidin-HRP conjugate to $30\ \text{mL}$ of working-strength Granzyme kit buffer just before use. *Do not prepare in advance of assay.*

Leaving the substrate blank wells empty, add $100\ \mu\text{L}$ of streptavidin-HRP conjugate to all wells.

Cover the microtiter plate(s) with adhesive seal, 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 $18-25^{\circ}\text{C}$.**

12. FOURTH WASH STEP

Aspirate supernatant from wells and wash the microtiter plate(s) as described in section VII, point 6 above.

13. FOURTH INCUBATION STEP

Enzymatic colour development

Approximately 10 minutes before use, prepare the substrate solution as described in section VI of this leaflet.

The substrate solution should be at $18-25^{\circ}\text{C}$ for optimal reproducible results.

Add $100\ \mu\text{L}$ of substrate solution to all wells, **including the substrate blank wells.**

Cover microtiter 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 $18-25^{\circ}\text{C}$ in the dark.**

Do not cover the plate with aluminium foil.

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

14. STOP ENZYMATIC REACTION

Add 100 μ L of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

15. PLATE READ-OUT

Read at 450 nm in an ELISA reader.

IX. RESULTS

Substrate blank

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

Standard curve

- Record the absorbance at 450 nm for each well containing standard and average the duplicate values.
- Calculate the net average absorbance's by subtracting the average of the substrate blank wells.
- Plot the net average absorbance's (ordinate) versus the GRANZYME B concentration in units/mL (abscissa) on log-linear paper and draw the best fitting curve. An example of a standard curve is given on the next page.

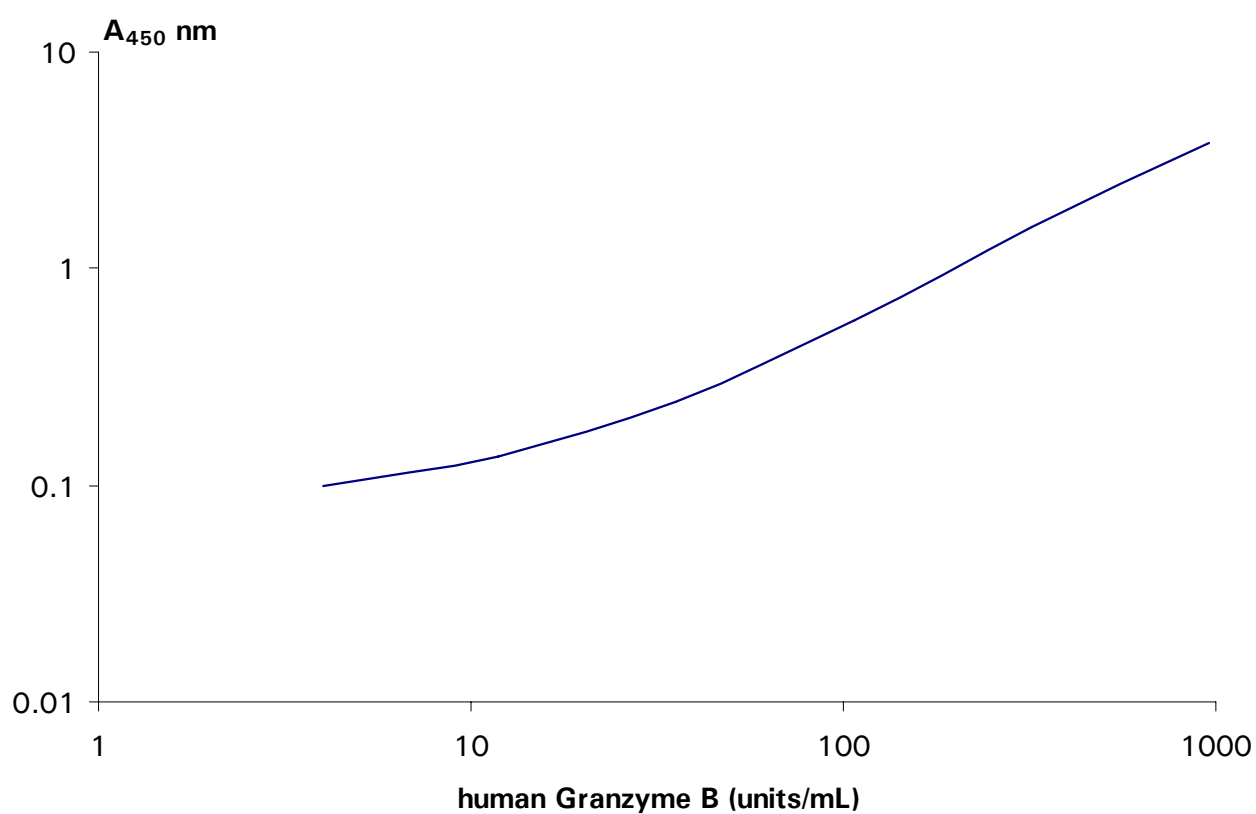
Samples

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

X. INCREASED SENSITIVITY

The assay sensitivity can be increased by a small adaptation of the incubation methodology. Just follow all the instructions as stated in the assay procedure (chapter VIII), but do all incubations of day 1 including the enzymatic colour development on a horizontal plate shaker at 700 ± 100 rpm.

This will result in an increase in assay sensitivity, with small effects on the background levels.



Typical standard curve for the PeliKine compact™ human GRANZYME B ELISA kit

	Calculated mean absorbance at 450 nm
Substrate blank	0
0 units/mL	0.073
4.0 units/mL	0.098
11.9 units/mL	0.137
35.6 units/mL	0.245
106.7 units/mL	0.583
320 units/mL	1.574
960 units/mL	> 3.000

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

Plate plan proposed for the PeliKine compact™ human GRANZYME B ELISA kit:

	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	B	B										

Key: B: substrate blank S1-S7: GRANZYME B standards 0-960 units/mL
Empty: samples and positive control

Protocol summary and checklist PeliKine compact™ human GRANZYME B ELISA kit

Day 0:

- Bring coating antibody to 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 18-25°C.

Day 1:

- Bring all reagents, with the exception of streptavidin-HRP, positive control and standard, to 18-25°C. Positive control and standard should be thawed prior for diluting.
- Prepare working-strength Granzyme kit buffer and washing buffer (PBS / TWEEN 0,02%)
- Wash the plate(s) five times with washing buffer.
- Add 150 µl working-strength Granzyme kit buffer to all wells and incubate, shaken, for 30 minutes at 18-25 °C.
- Thaw standard and positive control, prepare dilutions and restore directly at -18 to -32 °C after preparation. Prepare sample dilutions.
- Wash the plate(s) five times with washing buffer.
- Leaving the substrate blank wells empty, add 100 µl of standard dilutions, positive control and sample dilutions to the appropriate wells, cover the plate(s) and incubate, shaken, for one hour at 18-25°C.
- Dilute biotinylated antibody 1:100 in working-strength Granzyme kit buffer.
- Wash the plate(s) five times with washing 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 18-25°C.
- Dilute the streptavidin-HRP conjugate 1:10,000 in working-strength Granzyme kit buffer.
- Wash the plate(s) five times with washing buffer.
- Leaving the substrate blank wells empty, add 100 µL of the streptavidin-HRP conjugate to all wells, cover the plate(s) and incubate, shaken, for 30 minutes at 18-25 °C.
- Just before use, prepare substrate solution.
- Wash the plate(s) five times with washing buffer.
- Add 100 µL substrate solution to all wells, including the substrate blank wells, incubate static for 30 minutes at 18-25 °C in the dark.
- Add 100 µL stop solution to all wells and read the plate at 450 nm.
- Calculate the amount of GRANZYME B in the samples.