Quantikine® ELISA

Human Granzyme B Immunoassay

Catalog Number DGZB00

For the quantitative determination of human Granzyme B concentrations in culture supernates.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION PAGE

Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

INTRODUCTION

Granzyme B is a member of the granzyme family of serine proteases found specifically in granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (1, 2). Granzyme B plays an essential role in granule-mediated apoptosis utilizing the substrates in this pathway, such as Caspase 3, Caspase 8 and Bid (3, 4). Recent research indicates expanded Granzyme B functionality to include extracellular roles along with its classical pro-apoptotic function. It has been found that Granzyme B is an important mediator of skin injury, repair and inflammation (4) through extracellular substrates including Laminin, VE-Cadherin, Fibronectin and the proteoglycans Aggrecan (3) and Decorin (4).

As one of the five Granzymes (A, B, H, K and M) identified in the human genome, Granzyme B (32kDa) (5) is the most widely researched in terms of its biological function and its utility in health and disease (4). It is synthesized as a precursor (247 residues) with a signal peptide (residues 1-18), a pro-peptide (residues 19-20), and a mature chain (residues 21-247) (6-8). Once inside granules, Granzyme B is fully processed into the mature chain and becomes an active protease when the pro-peptide, Gly-Glu is removed from the N-terminus by cleavage with Cathepsin C (9). The protease activity of Granzyme B is tightly controlled by Serpin B9/ Protease Inhibitor 9 (9). The amino acid sequence of human Granzyme B is 71%, 69%, and 68% identical to its canine, rat, and mouse counterparts, respectively.

Granzymes have been shown to modulate inflammation, and Granzyme B plasma levels have been found higher with atopic dermatitis and psoriasis when compared to healthy controls. This is in contrast to Granzyme A plasma levels which remain unchanged (10). Serum from patients with Crohn's disease have significantly higher Granzyme B levels than controls (11).

The Quantikine® Human Granzyme B Immunoassay is a 3.5 hour solid-phase ELISA designed to measure human Granzyme B in culture supernates. It contains NS0-expressed recombinant human Granzyme B and antibodies raised against the recombinant protein. Results obtained using natural human Granzyme B showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Granzyme B.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Granzyme B has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Granzyme B present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Granzyme B is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Granzyme B bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent, and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Granzyme B Microplate	899042	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Granzyme B.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Granzyme B Standard	899044	2 vials of recombinant human Granzyme B in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Human Granzyme B Conjugate	899043	21 mL of a monoclonal antibody specific for human Granzyme B conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards.
- Human Granzyme B Controls (optional; R&D Systems®, Catalog # QC252).

PRECAUTIONS

Granzyme B is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Cell culture supernates may need a dilution due to high endogenous levels. Multiple dilutions are recommended for unknown samples.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Concentrations of Granzyme B are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

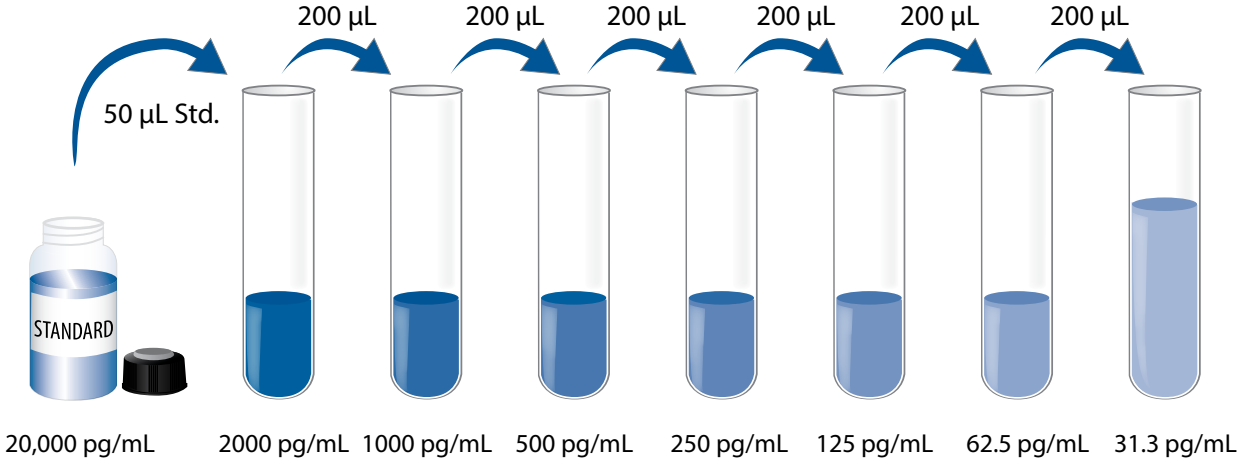
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 10 mL of Calibrator Diluent RD5P to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Granzyme B Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Granzyme B Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:5) into the 2000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *Concentrations of Granzyme B are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for **1 hour** at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Granzyme B Conjugate to each well. Cover with a new adhesive strip. Incubate for **2 hours** at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

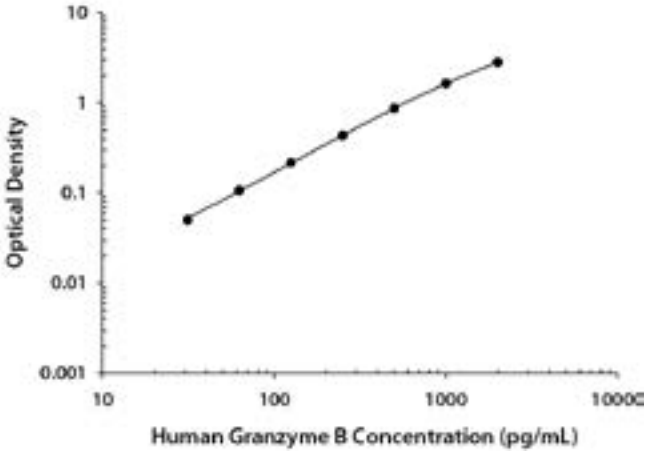
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Granzyme B concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.008 0.008	0.008	—
31.3	0.057 0.059	0.058	0.050
62.5	0.114 0.115	0.115	0.107
125	0.221 0.228	0.225	0.217
250	0.433 0.455	0.444	0.436
500	0.867 0.888	0.878	0.870
1000	1.639 1.666	1.653	1.645
2000	2.800 2.843	2.822	2.814

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	312	630	1298	311	615	1208
Standard deviation	8.04	11.0	28.7	27.5	56.1	77.2
CV (%)	2.6	1.7	2.2	8.8	9.1	6.4

RECOVERY

The recovery of human Granzyme B spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	93-114%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Granzyme B were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell culture supernate (n=4)
1:2	Average % of Expected	104	95
	Range (%)	101-106	94-96
1:4	Average % of Expected	107	94
	Range (%)	103-111	92-96
1:8	Average % of Expected	110	95
	Range (%)	108-113	91-99
1:16	Average % of Expected	112	93
	Range (%)	110-114	92-94

SENSITIVITY

Twenty-one assays were evaluated and the minimum detectable dose (MDD) of human Granzyme B ranged from 0.332-4.80 pg/mL. The mean MDD was 1.59 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of the zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Granzyme B produced at R&D Systems®.

SAMPLE VALUES

Human peripheral blood mononuclear cells (PBMC) (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum. Cells were cultured unstimulated or stimulated with 10 µg/mL of PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human Granzyme B.

Condition	pg/mL
Unstimulated	ND
Stimulated with PHA for 1 day	1534
Stimulated with PHA for 5 days	149,482

SPECIFICITY

This assay recognizes natural and recombinant human Granzyme B.

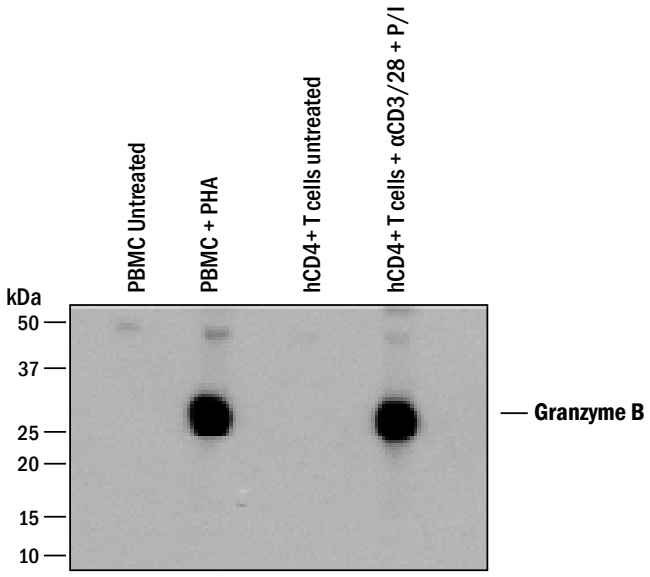
The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Granzyme B control were assayed for interference. No significant cross-reactivity or interference was observed.

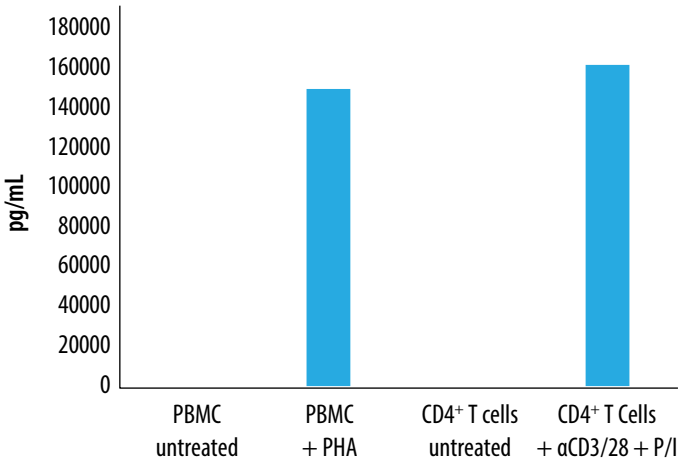
Recombinant human:

FGF R1
Granzyme A
Granzyme H
IFN-γ
Integrin αL/Integrin β2 Heterodimer
Myeloblastin
Notch-1
Perforin-1
Serpin B9
Serglycin

Other:

Bovine Chymotrypsin
Bovine Trypsin
Granzyme B
Recombinant Mouse Granzyme B





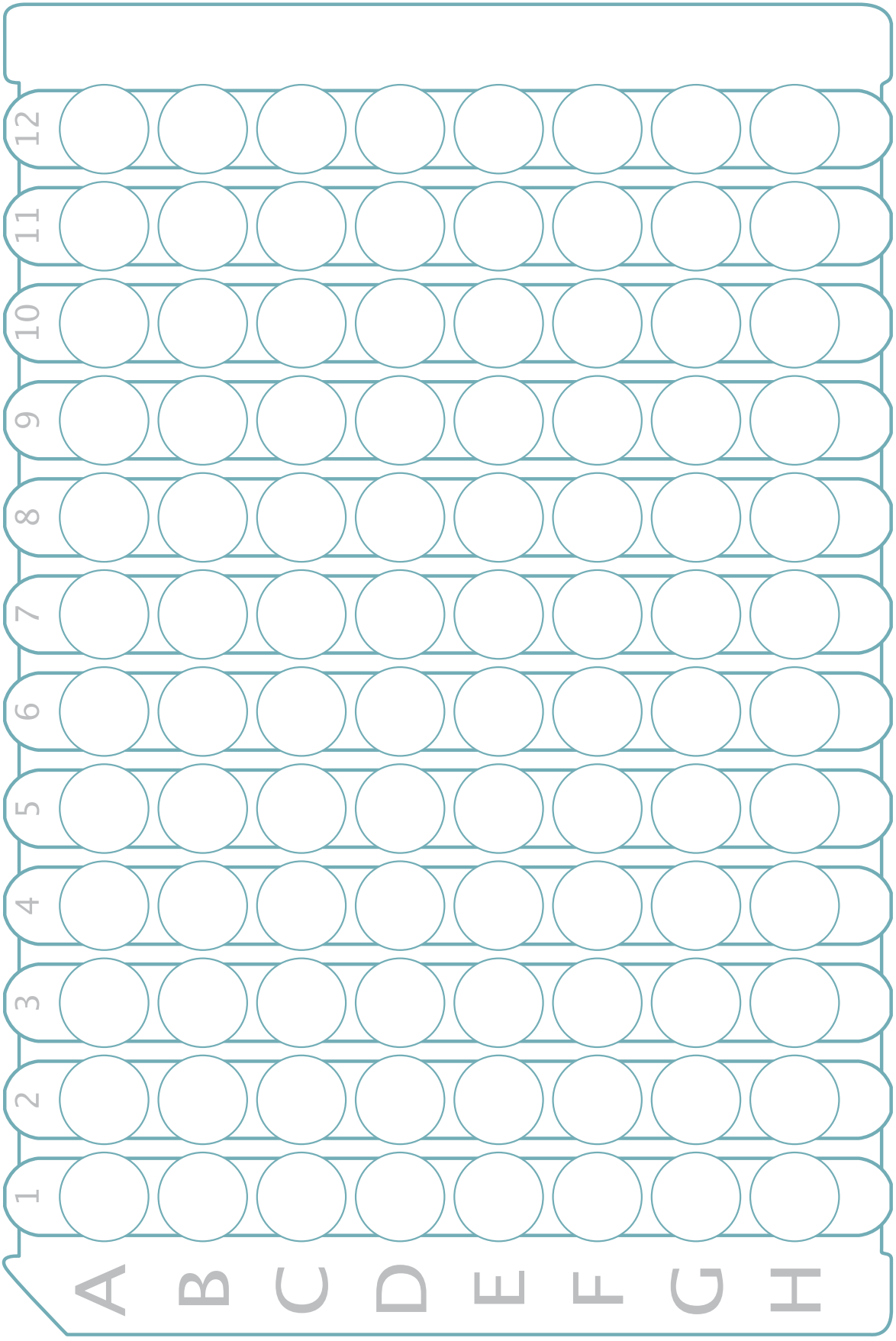
Conditioned media samples were analyzed by Western Blot. PBMCs were isolated via Ficoll density gradient and then left unstimulated or stimulated with 10 µg/mL PHA for 5 days. $CD4^+$ T cells were isolated from human PBMCs using the MagCellect Human $CD4^+$ T cell Isolation Kit (R&D Systems®, Catalog # MAGH102) and left unstimulated or stimulated with anti-CD3 (R&D Systems®, Catalog # MAB100) + anti-CD28 (R&D Systems®, Catalog # MAB342) for 5 days, followed by 10 ng/mL PMA and 500 ng/mL Ionomycin for an additional 24 hours (P/I). Conditioned media was harvested, resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with goat anti-human Granzyme B (R&D Systems®, Catalog # AF2906).

REFERENCES

1. Kam, C-M. *et al.* (2000) Biochim. Biophys. Acta **1477**:307.
2. Smyth, M.J. *et al.* (1996) J. Leukoc. Biol. **60**:555.
3. Froelich, C.J. (2004) in Handbook of Proteolytic Enzymes, A.J. Barrett, N.D. Rawlings, and J.F. Woessher (Eds), Academic Press, New York, pp. 1549.
4. Turner, C.T. *et al.* (2017) Matrix Biol. 1387.
5. Garcia-Sanz, J.A. *et al.* (1990) J. Immunol. **145**:3111.
6. Schmid, J. and C. Weissman (1987) J. Immunol. **139**:250.
7. Caputo, A. *et al*. (1988) J. Biol. Chem. **263**:6363.
8. Trapani, J.A. *et al*. (1988) Proc. Natl. Acad. Sci. USA **85**:6924.
9. Hlongwane, P. *et al*. (2018) Biomedicines **6**:72.
10. Kamata, Y. *et al*. (2017) J. Dermatological Sci. **86**:e10.
11. Kim, T.J. *et al*. (2018) J. Biomarkers **23**:161.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES