Product Information & ELISA Manual

Human Dfna5 ELISA Kit (Colorimetric)
NBP3-43461

Enzyme-linked Immunosorbent Assay
for quantitative detection.

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Novus kits are guaranteed for 6 months from date of receipt.

For research use only. Not for diagnostic or therapeutic procedures.

Table of Contents

1. Intended Use

The Human Dfna5 ELISA Kit (Colorimetric) is to be used for the *in vitro* quantitative determination of human Dfna5 in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

Programmed cell death is a key mechanism involved in several biological processes ranging from development and homeostasis to immunity. Apoptosis, pyroptosis and NETosis are three well-studied modes of cell death (1). Pyroptosis is a form of necrotic cell death that has emerged as an important innate immune mechanism against intracellular pathogens. All three modes of cell death cross-talk between them and converge onto the activation of the same cell death effector, the pore-forming protein Gasdermin D (GSDMD).

The inflammasome platform leads to activation of caspase-1 and also the recently characterized caspase-11 (non-canonical inflammasome pathway), leading to cleavage of the intracellular protein Gasdermin D (2). Upon caspase-1/11 cleavage of the Gasdermin N- and C-domain linker, the cleaved N-terminal fragment of Gasdermin D oligomerizes and forms pores on the host cell membrane, leading to a cell death called pyroptosis (3). The Gasdermin family is an important protein that mediates pyroptosis, it can induce cell death and promote the release of inflammatory factors. It is mainly composed of six members: Gasdermin A (GSDMA), Gasdermin B (GSDMB), Gasdermin C (GSDMC), Gasdermin D (GSDMD), Gasdermin E (GSDME; also called *deafness, autosomal dominant 5*, *DFNA5*), and DFNB58 (4). Recent studies found that GSDME features a caspase-3 /-7 cleavage motif in its linker region. Similar to GSDMD, cleavage of GSDME by caspases-3 /-7 liberates the N-terminal pyroptosis-inducing domain ($GSDME^{NT}$) from its autoinhibitory C-terminal regulatory domain to trigger membrane pores and pyroptosis (5). Gasdermin E is expressed in different tissues/cells such as placenta, brain, heart, kidney, cochlea, intestines and IgE-primed mast cells. Specific cells express GSDME and in such cells caspase-3 activation is switched from driving an apoptotic program to causing pyroptosis. Thus, certain cells are programmed to undergo pyroptosis instead of apoptosis. *GSDME*-knockout mice were shown to be resistant to the toxicity of chemotherapy drugs such as cisplatin (6). GSDME-N terminus domain also permeabilizes the mitochondrial membrane, releasing cytochrome c and activating the apoptosome (7). Recently, it has been reported that granzyme B from killer cells activates caspase-independent pyroptosis in target cells by directly cleaving GSDME at the same site as caspase-3 (8). Mutations in *GSDME* in human are associated with development of heritable, nonsyndromal deafness (9). *GSDME* expression is suppressed in many cancers and reduced GSDME is associated with decreased breast cancer survival. *GSDME* gene expression has been shown to be associated with favorable prognosis following chemotherapy, and it could therefore be a potential predictive biomarker (10).

The **Human Dfna5 ELISA Kit (Colorimetric)** detects the **C-terminal domain** of human Dfna5 as well as the full-length protein. Upon cleavage of Dfna5 and pore formation, the C-terminus fragment should be released in the extracellular space and found in serum/plasma. The role of the secreted C-terminus fragment of the Gasdermin protein family has not been characterized yet.

3. General References

1. The innate immune system and cell death in autoinflammatory and autoimmune disease: D.E. Place & T.D. Kanneganti; Curr. Opin. Immunol. **67**, 95 (2020)

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4. Activation of pyroptotic cell death pathways in cancer: an alternative therapeutic approach: K. Nagarajan, et al.; Transl. Oncol. **12**, 925 (2019)

5. The caspase-3/GSDME signal pathway as a switch between apoptosis and pyroptosis in cancer: M. Jiang, et al.; Cell Death Discov. **6**, 112 (2020)

6. Chemotherapy drugs induce pyroptosis through caspase -3 cleavage of a Gasdermin: Y. Wang, et al.; Nature **547,** 99 (2017)

7. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation: C. Rogers, et al.; Nat. Commun. **10**, 1689 (2019)

8. Gasdermin E–mediated target cell pyroptosis by CAR T cells triggers cytokine release syndrome: Y. Liu, et al.; Science Immunol. **5**, 43 (2020)

9. Further evidence for "gain-of-function" mechanism of DFNA5 related hearing loss: H. Wang, et al.; Sci. Rep. **8,** 8424 (2018)

10. GSDME and its role in cancer: From behind the scenes to the front of the stage: E. De Schutter, et al.; Int. J. Cancer. (Epub ahead of print) (2020)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Dfna5 in cell culture supernatants, serum and plasma. An antibody specific for human GSDME has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, GSDME is recognized by the addition of a biotinylated antibody specific for human GSDME (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450nm after acidification and is directly proportional to the concentration of GSDME in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human GSDME Standard (lyophilized)	(100 ng)	(STD)
• 1 vial GSDME Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 1 bottle Sample Buffer 5X	(1 x 30 ml)	(Sample Buffer 5X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with GSDME Antibody	(6 x 16-well strips)	
• 2 plate Covers (plastic film)		
• 2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **Sample Buffer 5X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml Sample Buffer 5X + 80 ml water) to obtain Sample Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in Sample Buffer 1X (20 µl DET + 10 ml Sample Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of Sample Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of Sample Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human GSDME (STD)** has to be reconstituted with 100 µl of Sample Buffer 1X.
  - This reconstitution produces a stock solution of 1 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in Sample Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in Sample Buffer 1X is recommended.

  - Suggested standard points are:
    4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 ng/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of GSDME (STD) (1 µg/ml)	990 µl of Sample Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
4 ng/ml	320 µl of GSDME (10 ng/ml)	480 µl of Sample Buffer 1X
2 ng/ml	300 µl of GSDME (4 ng/ml)	300 µl of Sample Buffer 1X
1 ng/ml	300 µl of GSDME (2 ng/ml)	300 µl of Sample Buffer 1X
0.5 ng/ml	300 µl of GSDME (1 ng/ml)	300 µl of Sample Buffer 1X
0.25 ng/ml	300 µl of GSDME (0.5 ng/ml)	300 µl of Sample Buffer 1X
0.125 ng/ml	300 µl of GSDME (0.25 ng/ml)	300 µl of Sample Buffer 1X
0.0625 ng/ml	300 µl of GSDME (0.125 ng/ml)	300 µl of Sample Buffer 1X
0 ng/ml	300 µl of Sample Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma and Cell Culture Supernatant have to be diluted in Sample Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/2 dilution of serum or of plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with GSDME antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma, serum or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **2 hours at Room Temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 hour at Room Temperature.**
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at Room Temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at Room Temperature in the dark for 20-25 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding GSDME concentration (ng/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the GSDME concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human GSDME in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

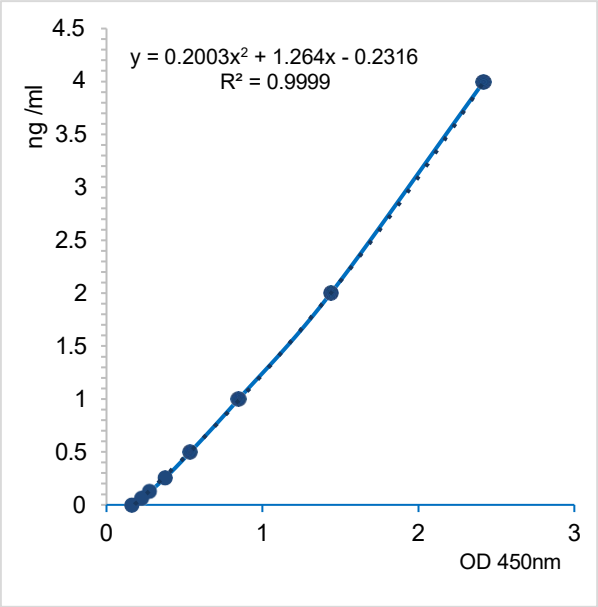

Figure: Standard curve

Standard GSDME (ng/ml)	Optical Density (mean)
4	2.42
2	1.439
1	0.848
0.5	0.538
0.25	0.374
0.125	0.275
0.0625	0.225
0	0.164

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of human GSDME that can be detected by this assay is **<0.05 ng/ml**.

NOTE: *The Limit of detection was measured by adding three standard deviations to the mean value of 30 zero standard replicates.*

B. Assay range: 0.0625 ng/ml – 4 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human GSDME.

D. Intra-assay precision:

Four samples of known concentrations of human GSDME were assayed in replicates 5 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	5.99	0.164	2.73	5
A2	1.1	0.085	7.72	5
A3	0.55	0.010	4.45	5
A4	2.90	0.11	3.79	5

E. Inter-assay precision:

Four samples of known concentrations of human GSDME were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	0.24	0.009	3.62	5
B2	4.02	0.068	1.69	5
B3	0.50	0.027	5.29	5
B4	0.33	0.026	7.91	5

F. Recovery:

When samples are spiked with known concentrations of human GSDME, the recovery averages range from 86% to 104%.

G. Linearity:

Different samples containing human GSDME were diluted several folds (1/2 to 1/8 for sera and plasmas) and the measured recoveries ranged from 86% to 119%.

12. Technical Hints and Limitations

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes