ELISA PRODUCT INFORMATION & MANUAL

GRP78/HSPA5

NBP2-62145

Enzyme-linked Immunosorbent Assay for quantitative detection of Human, Mouse, Rat GRP78/HSPA5.

For research use only.

Not for diagnostic or therapeutic procedures.

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Novus Biologicals Technical Support if necessary.

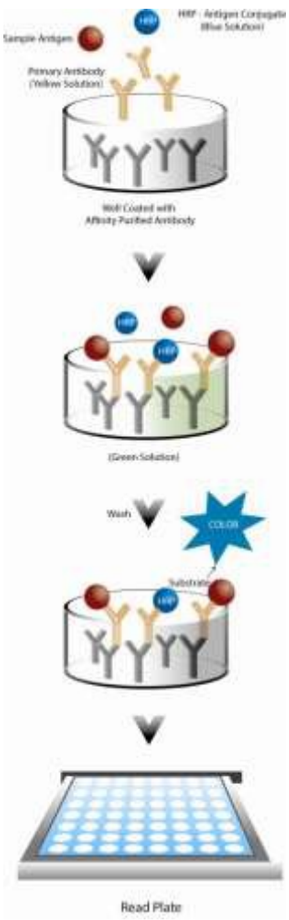
BACKGROUND

The Novus Biologicals GRP78/HSPA5 Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete, competitive kit for the quantitative determination of GRP78/HSPA5 in cell lysates or serum. Please read the complete kit insert before performing this assay.

Glucose-regulated protein (GRP78/HSPA5) also known as binding immunoglobulin protein or BiP, is a resident molecular chaperone of the ER involved in the folding and assembly of proteins, transport of newly synthesized polypeptides across the ER membrane, regulation of calcium homeostasis and targeting misfolded proteins for degradation.[1-2] GRP78 also regulates the transmembrane proteins, PERK, IRE1 and ATF6 by binding the N-terminal domains in the lumen of the ER preventing these signal transducers from initiating the unfolded protein response (UPR), an adaptive response to changes in the intracellular environment meant to restore normal ER function.[3]

When protein misfolding occurs, exposed hydrophobic residues on the protein are bound by GRP78/HSPA5 preventing protein aggregation, further transit, and secretion. Intracellular stress such as glucose deprivation and viral infection can lead to a rapid accumulation of unfolded proteins.[4] As unfolded proteins accumulate, more available GRP78/HSPA5 is required to bind the hydrophobic regions causing it to dissociate from the transmembrane signaling proteins thus initiating the UPR.

After dissociation from GRP78/HSPA5, activated PERK attenuates general translation to prevent further protein synthesis, ATF6 and IRE1 upregulate the production of ER folding and chaperone proteins including GRP78/HSPA5, and proteins that promote degradation of misfolded proteins through ER- associated protein degradation (ERAD). The overexpression of GRP78/HSPA5 as a result of UPR activation is believed to contribute to the pathology of metabolic disease, inflammatory disease, neurodegenerative disorders, and cancer.[5-8]



PRINCIPLE

1. Standards and samples are added to wells coated with a donkey anti-sheep IgG antibody. A solution of goat polyclonal antibody to GRP78/HSPA5 is also added to each well and the plate is incubated.
2. A solution of GRP78/HSPA5 conjugated to horseradish peroxidase is then added to each well and incubated. During this incubation the antibody binds, in a competitive manner, the GRP78/HSPA5 in the sample or conjugate. The plate is washed leaving only bound GRP78/HSPA5.
3. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
4. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is inversely proportional to the level of GRP78/HSPA5 in the sample.

MATERIALS SUPPLIED

1. **Donkey anti-Sheep IgG Microtiter Plate**
   One plate of 96 wells
   A clear plate of break-apart strips coated with a Donkey anti-sheep polyclonal antibody
2. **Assay Buffer 28**
   50 mL
   Tris buffered saline containing BSA and detergents
3. **GRP78/HSPA5 Conjugate**
   5 mL
   A blue solution of GRP78/HSPA5 conjugated to horseradish peroxidase
4. **GRP78/HSPA5 Antibody**
   5 mL
   A yellow solution of goat polyclonal antibody specific for GRP78/HSPA5

Do not mix components from different kit lots or use beyond the expiration date of the kit.

5. **GRP78/HSPA5 Standard**
   Two vials each containing 4.5 µg of recombinant GRP78/HSPA5
6. **Extraction Reagent #2**
   10 mL
   5x Tris buffered solution containing detergent
7. **Wash Buffer Concentrate**
   100 mL
   20x Tris buffered saline containing detergents
8. **TMB Substrate**
   2x10 mL
   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide

Protect substrate from prolonged exposure to light.

9. **Stop Solution 2**
   10 mL
   A 1N solution of hydrochloric acid in water

Stop solution is caustic. Keep tightly capped.

10. **GRP78/HSPA5 Assay Layout Sheet**
    1 each
11. **Plate Sealer**
    3 each

STORAGE

All kit components should be stored at 4ºC except the GRP78/HSPA5 Conjugate which needs to be store at -80ºC upon receipt. Avoid repeated freeze thaw cycles of the GRP78/HSPA5 Conjugate. Shipping conditions may not reflect storage conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Phenylmethylsulfonyl fluoride (PMSF)
3. Protease Inhibitor Cocktail (PIC)
4. Precision pipets for volumes between 5 µL and 1,000 µL
5. Repeater pipet for dispensing 100 µL
6. Disposable beakers for diluting buffer concentrates
7. Graduated cylinders
8. A microplate shaker
9. Adsorbent paper for blotting
10. Microplate reader capable of reading a 450 nm
11. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

If buffers other than those provided are used, the end-user must determine the appropriate dilution and assay variation.

SAMPLE HANDLING

The assay is suitable for the measurement of GRP78/HSPA5 in cell lysates and human serum. Prior to assay, frozen samples should be brought to 4°C and centrifuged, if necessary, to remove residual debris.

A minimum dilution of 1:5 in assay buffer is required to remove matrix and or lysis buffer interference with most cell lysate samples. It is necessary to validate the use of any lysis buffer other than that provided in this kit.

Due to variation in samples, a different dilution may be required. Users must determine the optimal dilutions for their particular experiments.

Sample handling procedures should be completed prior to reagent preparation.

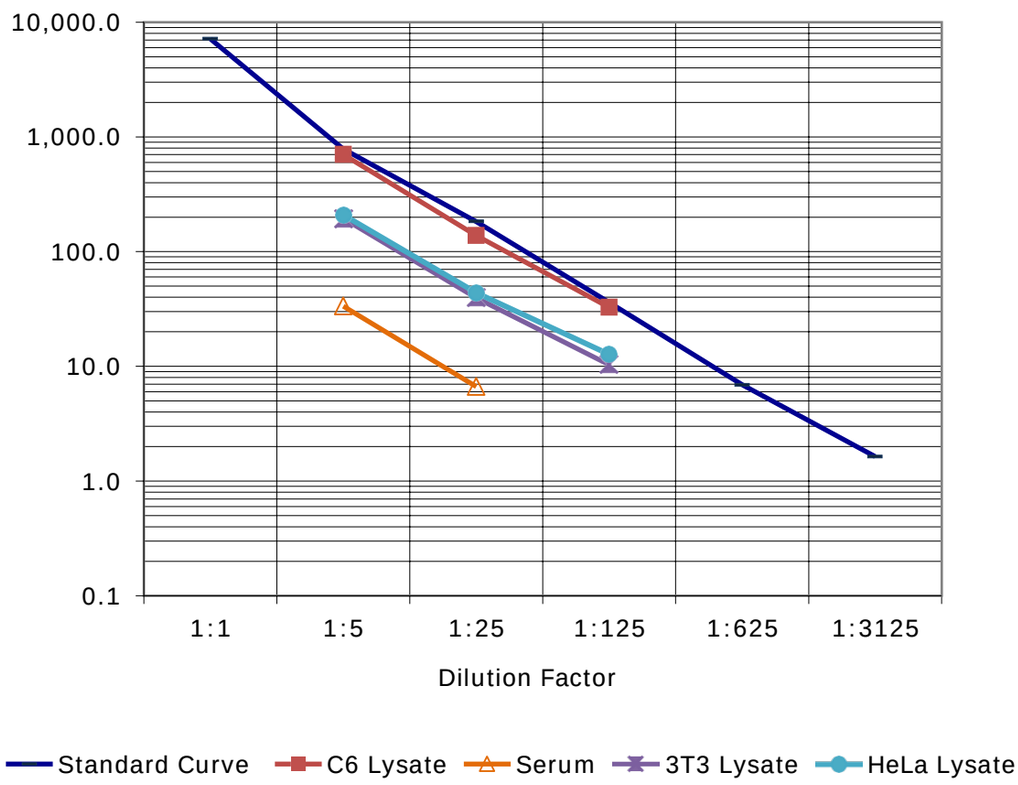
Linearity

The minimum required dilution for common lysates and serum was determined by serially diluting samples into the provided assay buffer and identifying the dilution at which linearity was observed.

% Dilutional Linearity				
Dilution	**HeLa Lysate**	**C6 Lysate**	**3T3 Lysate**	**Serum**
Neat	---	---	---	---
1:5	96	101	99	101
1:25	100	100	100	100
1:125	116	118	103	---

Parallelism

To assess parallelism, HeLa, 3T3, and C6 lysates and serum were serially diluted into assay buffer and run in the assay. The GRP78/HSPA5 concentration in each sample was assigned using the standard curve. Assigned concentrations were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted samples of human, mouse, and rat origin.



Spike and Recovery

After diluting each individual sample to read within the dynamic range of the assay, recombinant GRP78/HSPA5 was spiked at high, medium and low concentrations. Endogenous GRP78/HSPA5 was subtracted from the spiked values and the recovery in each of the spiked matrices was compared to the recovery of identical spikes in the assay buffer. The percent recovery at the three concentrations is indicated below for each matrix.

Sample Matrix	Dilution	Spike Concentration	Recovery of Spike
Hela Cell Lysate	1:25	1000 ng/mL	109%
		300 ng/mL	108%
		13 ng/mL	90%
C6 Cell Lysate	1:125	1000 ng/mL	82%
		300 ng/mL	93%
		13 ng/mL	101%
3T3 Cell Lysate	1:25	1000 ng/mL	94%
		300 ng/mL	97%
		13 ng/mL	98%
Serum	1:5	1000 ng/mL	120%
		300 ng/mL	110%
		13 ng/mL	113%

Cell Lysate Preparation

1. Centrifuge at 1700 x g for 10 minutes at room temperature to pellet cells and/or cellular debris.
2. Prepare Extraction Reagent
    a. Calculate the amount of 1X Extraction Reagent that will be required. For every $1x10^6$ to $1x10^7$ cells, use 1 mL of 1X Extraction Reagent.
    b. Dilute an appropriate amount of 5X Extraction Reagent with cold deionized or distilled water to generate the required volume of 1X Extraction Reagent.
    c. Add 0.5 µL of protease inhibitor cocktail (PIC) per mL of 1X Extraction Reagent and add PMSF to a final concentration of 1mM. Inhibitors must be added fresh just prior to lysis. Extraction Reagent containing inhibitors should not be stored for later use.
3. Resuspend cell pellet in 1X Extraction Reagent with inhibitors and incubate on ice for 30 minutes. Vortex occasionally.
4. Pellet cellular debris via centrifugation at 10,000 x g for 10 minutes in a 4ºC refrigerated centrifuge.
5. Collect the supernatant (lysate) without disturbing the cell pellet. Divide the lysates into aliquots and store at or below -20°C, or use immediately in the assay.
6. Avoid repeated freeze-thaw cycles.

Serum Preparation

1. Collect whole blood in appropriate serum tubes.
2. Blood samples should be incubated in an upright position at room temperature for 30-45 min (no longer than 60 min) to allow clotting.
3. Centrifuge at 1000 x g for 15 minutes at room temperature.
4. Remove serum to a clean plastic tube.
5. Divide serum into aliquots and store at or below -20°C, or use immediately in the assay.
6. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

1. **Wash Buffer**

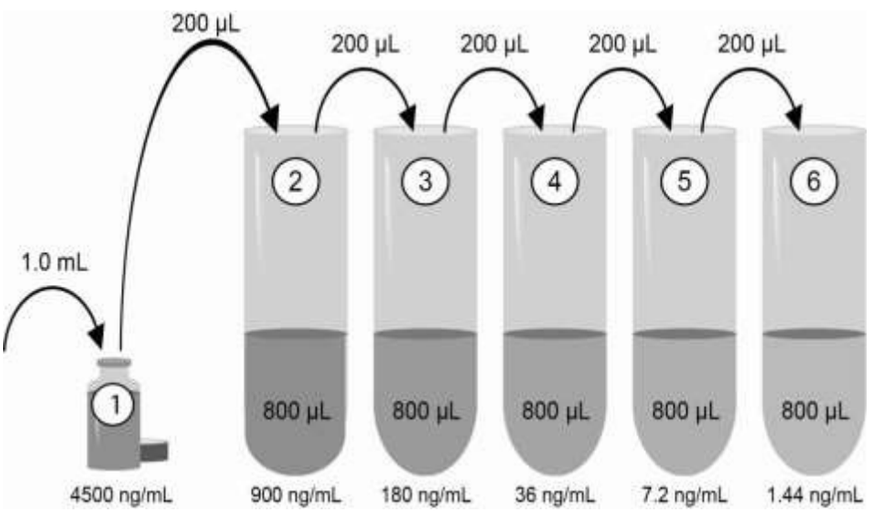
   Prepare Wash buffer by diluting 30 mL of the supplied Wash Buffer concentrate with 570 mL of deionized water. Store the diluted wash buffer at room temperature. Diluted wash buffer should be used within 3 months.

2. **GRP78/HSPA5 Standard**

   Allow the GRP78/HSPA5 standard to warm to room temperature. Reconstitute one vial of GRP78/HSPA5 standard with 1.0 mL Assay Buffer 28 for a 4500ng/mL standard concentration. Vortex thoroughly, wait 5 minutes and vortex again prior to use. Label this vial #1.

   Label five 12 x 75mm tubes #2 through #6. Pipet 800 µL of assay buffer into each tube. Remove 200 µL from the reconstituted standard vial and add to tube #2 and vortex thoroughly. Remove 200 µL from tube #2 and add to tube #3. Vortex thoroughly. Continue this for tubes #4 through #6.

Polypropylene tubes may be used for standard preparation. Avoid polystyrene.



Diluted standards should be used immediately. Do not store remaining standard.

All other kit components should be brought to room temperature prior to use in the assay.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

All standards and samples should be run in duplicate.

1. Pipet 100 µL of Assay Buffer 28 into the B_0 (0 ng/mL standard) wells.
2. Pipet 100 µL of standards #1 through #6 to the bottom of the appropriate wells.
3. Pipet 100 µL of the samples into the appropriate wells.
4. Pipet 50 µL of yellow Antibody solution into each well, except the blanks.
5. Seal the plate. Incubate for 1 hour with mixing on a plate shaker at room temperature*. **Do not wash at this step.**
6. Add 50 µL of blue conjugate to each well except the blank. Seal the plate. Incubate for 1 hour with mixing on a plate shaker at room temperature.
7. Empty the contents of the wells and wash with an automated washer by adding 300 µL of 1X Wash Buffer to every well. Repeat 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 200 µL of TMB solution into each well.
9. Seal the plate. Incubate for 30 minutes with shaking on a plate shaker at room temperature.
10. Pipet 50 µL of Stop Solution into each well.
11. After blanking the plate reader against the substrate, read optical density at 450 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Do Not Wash wells after step 5.

Pipet the reagents to the sides of the wells to avoid possible contamination.

*** Note:** The optimal speed for each shaker will vary and may range from 120-700 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.

CALCULATION OF RESULTS

Be sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of GRP78/HSPA5 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program. The concentration of GRP78/HSPA5 can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (B_0), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net } B_0 \text{ OD}} \times 100$$

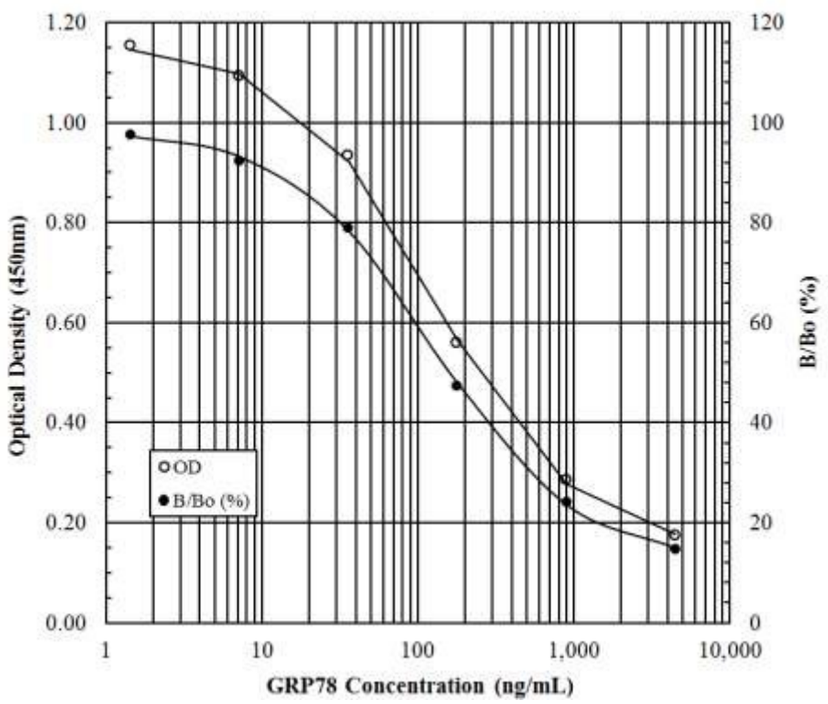
3. Plot the Percent Bound (B/B_0) versus concentration of GRP78/HSPA5 for the standards. Approximate a straight line through the points. The concentration of GRP78/HSPA5 in the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average Net OD	Percent Bound	GRP78/HSPA5 (ng/mL)
Blank	0.044	---	---
B_0	1.182	100%	0
S1	0.174	14.7%	4500
S2	0.285	24.1%	900
S3	0.560	47.4%	180
S4	0.933	79.0%	36
S5	1.092	92.4%	7.2
S6	1.120	97.7%	1.44



PERFORMANCE CHARACTERISTICS

Specificity

The cross reactivities for related compounds were determined by diluting the cross reactant in the kit assay buffer at a concentration of ten times the high standard then measuring in the assay.

Analyte	Cross Reactivity
GRP94	≤0.01%
PDI	≤ 0.01%
Calreticulin	≤ 0.01%

Sensitivity

The sensitivity or limit of detection of the assay is 8.4 ng/mL, determined by interpolation at 2 standard deviations away from the mean signal of 24 replicates of zero standard using data from 8 standard curves.

Interference

Detergents commonly used in lysis buffers were analyzed for interference in the assay and the tolerance was determined.

Detergent	Assay Tolerance	
	(%)	molarity
SDS	≤ 0.03	≤ 1 mM
Tx100	≤ 1.0	≤ 15 mM
NP-40	≤ 6.0	≤ 98 mM
Sodium Deoxycholate	≤ 0.06	≤ 1 mM
Zwittergent	≤ 0.1	≤ 3.4 mM

Intra-assay precision was determined by assaying 24 replicates of two buffer controls containing GRP78/HSPA5 in a single assay.

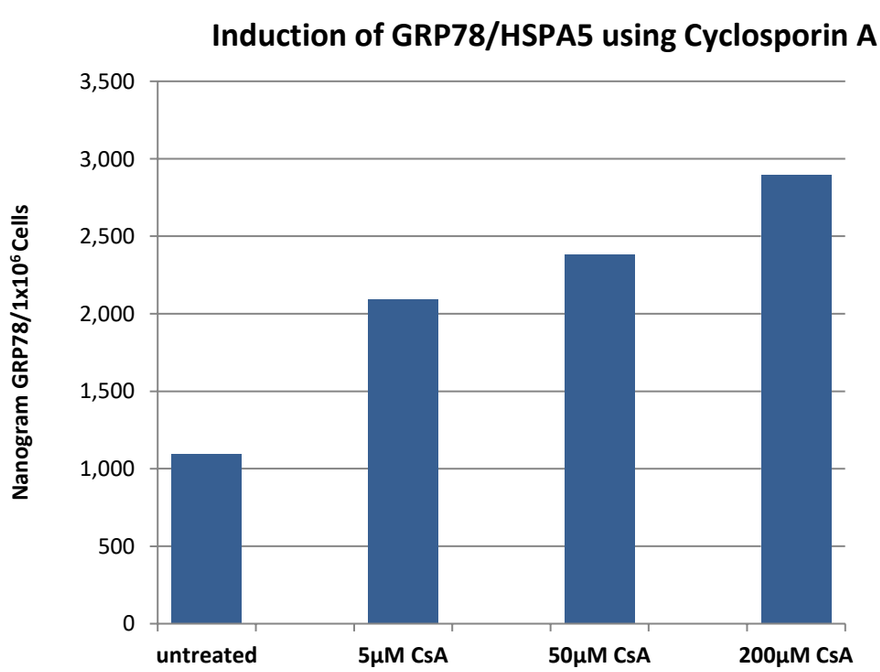
ng/mL	%CV
373.2	4.5
12.5	14.1

Inter-assay precision was determined by measuring buffer controls of varying GRP78/HSPA5 concentrations in multiple assays over several days.

ng/mL	%CV
352.8	4.8
13.0	13.1

Experimental Evaluation

HeLa cells were treated for 5 hours with 5 µM, 50 µM or 200 µM concentrations of cyclosporine A. Following the incubation, treated and untreated cells were lysed with Extraction Reagent #2 as described above. The lysates were then evaluated in the assay. This data agrees with the results reported in the literature, low concentrations of cyclosporine A lead to the induction of GRP78/HSPA5 in HeLa cells (Paslaru, L. et al. (1994)) and supports the claim that the Novus ELISA is able to detect and quantitate changes in native levels of GRP78/HSPA5.



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