PRODUCT INFORMATION

human Bax
Enzyme Immunometric Assay Kit

Catalog No. IB09623

96 Well Kit

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Description

The IBL-America human Bax Enzyme Immunometric Assay (EIA) kit is a complete kit for the determination of Bax in human cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to Bax immobilized on a microtiter plate to bind the Bax in the standards or sample. A recombinant human Bax-α Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to Bax is added. This antibody binds to the Bax captured on the plate. After a short incubation, the excess antibody is washed out and Streptavidin conjugated to Horseradish peroxidase is added, which binds to the biotinylated monoclonal Bax antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of Bax in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

Many physiological processes, including cellular development and homeostasis, are regulated by a balance between cell proliferation, differentiation and apoptosis[3]. Several gene products play a significant role in regulating apoptosis. The Bcl-2 gene family is comprised of both pro-apoptotic (e.g. Bax, Bid) and anti-apoptotic (e.g. Bcl-2, Bcl-XL) members, with membership dependent on the presence of at least one Bcl-2 homology (BH) domain[4]. These proteins form homo- and heterodimers, with the ratio between pro- and anti-apoptotic levels an important determinant of cell survival. Bax is a 21 kDa protein that accelerates apoptosis by binding to, and antagonizing the death repressor activity of Bcl-2 in vivo[3]. Bax normally resides in the cytoplasm, but following an apoptotic stimuli, Bax undergoes a conformational change and translocates to mitochondrial membranes, where it inserts and mediates the release of cytochrome c from the intermembrane space into the cytosol, in this manner activating caspase 3 and perpetuating the apoptotic cascade[5]. Protein levels of Bax and other Bcl-2 family members are altered in various human malignancies[6,7], with changes in expression levels implicated in diseases involving apoptosis such as cancer, autoimmune diseases and neurodegenerative disorders.

Precautions

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1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The Bax-α Standard provided should be handled with care because of the known and unknown effects of Bax.
5. The Bax-α standard should be stored at or below -20 °C.
6. Standards and samples must be prepared in polypropylene tubes. Preparation in glass will result in decreased protein stability.

Materials Supplied

1. **Bax Microtiter Plate, One Plate of 96 Wells**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to Bax-α. This antibody, aa 55-178, was generated from human Bax-α and reacts with human and dog.
2. **Bax Antibody, 10 mL**
   A yellow solution of biotinylated monoclonal antibody to Bax-α. This antibody, aa 3-16, reacts with human. It does not react with mouse or rat.
3. **Assay Buffer 13, 1x, 100 mL**
   Tris buffered saline containing proteins and detergents.
4. **Bax Conjugate, 10 mL**
   A blue solution of Streptavidin conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 100 mL**
   Tris buffered saline containing detergents.
6. **human Bax-α Standard, 1 vial**
   A solution of 20,000 pg/mL recombinant human Bax-α.
7. **TMB Substrate, 10 mL**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
8. **Stop Solution 2 , 0 mL**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **Cell Lysis Buffer 4, 100 mL**
   100 mM PBS, pH 7.3, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 0.005% Tween 20.
10. **Bax Assay Layout Sheet, 1 each**
11. **Plate Sealer, 3 each**

Storage

All components of this kit, **except the Bax-α Standard,** are stable at 4 °C until the kit's expiration date. The Bax-α Standard **must** be stored at or below -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma # P7626 or equivalent.
3. Protease Inhibitor Cocktail (PIC), Sigma # P8340 or equivalent.
4. Precision pipets for volumes between 100 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 at 450 nm, preferably with correction between 570 nm and 590 nm.
11. Graph paper for plotting the standard curve.
12. Polypropylene test tubes.

Sample Handling

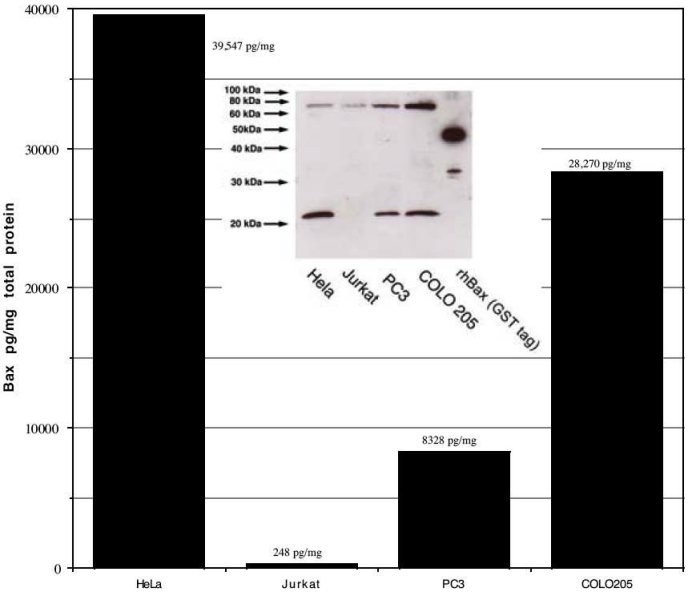
This assay kit is compatible with Bax samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 13 can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

It is recommended that all samples be lysed with the provided Cell Lysis Buffer 4 modified by the addition of PIC and PMSF (see Reagent Preparation, page 5, #3) immediately prior to use. Samples lysed in this Cell Lysis Buffer 4 must be diluted at least 1:2 with Assay Buffer 13 prior to assaying. Note that this dilution is based on the lysis of 62,500 HeLa cells per mL. The 1:2 dilution contained 31,250 cells per mL with a calculated recovery of 98.6%.

If the end user chooses to use another lysis buffer, a greater number of cells, or varies from the stimulation procedure noted below, it is up to the end user to determine the appropriate dilution of samples and assay validation. Only standard curves generated in Assay Buffer 13 should be used to calculate the concentration of Bax. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive Bax. Excessive freeze/thaw cycles should be avoided. Prior to running the assay, frozen samples should be brought to 4 °C slowly and gently mixed. **Samples must be prepared in polypropylene tubes.**

Lysates Experiment

HeLa, Jurkat, PC3 and COLO205 cells were all used at 4 million cells per mL. The total protein concentrations for the HeLa, Jurkat, PC3 and COLO205 are 1.17 mg/mL, 0.43 mg/mL, 1.68 mg/mL and 1.08 mg/mL, respectively. Cells were centrifuged at 1,600 rpm for 7 minutes and the supernatant discarded. The cell pellets were resuspended and washed with PBS. Cells were pelleted at 1,600 rpm for 7 minutes and the supernatant dicarded. The cell pellets were resuspended with modified Cell Lysis Buffer 4, vortexed and placed on ice for 15 minutes. The lysates were vortexed and centrifuged at 16,000 rpm for 15 minutes. The supernatants were split for the Western blot and EIA to generate the data illustrated. The exposure time for development was 6 minutes.



Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents, with the exception of the human Bax-α standard, to warm to room temperature for at least 30 minutes before opening.
3. **Standards and samples must be made up in plastic tubes.**
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. human Bax-α Standards

Allow the human Bax-α 20,000 pg/mL standard to warm for no more than 0 minutes.

Label six polypropylene tubes #1 through #6. Pipet 900 µL of Assay Buffer 13 into tube #1. Pipet 500 µL of Assay Buffer 13 into tubes #2 through #6. Add 100 µL of the 20,000 pg/mL standard into tube #1 and vortex. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

The concentration of human Bax-α in standards #1 through #6 will be 2,000, 1,000, 500, 250, 125 and 62.5 pg/mL respectively. See Bax Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

Discard any unused standard dilutions.

3. Cell Lysis Buffer 4

Allow to come to room temperature. Ensure buffer is completely in solution prior to use.

Immediately prior to use for cell lysis, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

Assay Procedure

Bring all reagents, except the human Bax-α Standard, to room temperature for at least 30 minutes prior to opening.

The human Bax-α Standard solution should not be left at room temperature for more than 10 minutes.

All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of Assay Buffer 13 into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 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 100 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 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.
11. Add 100 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 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.
14. Pipet 100 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 100 µL Stop Solution 2 to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

Calculation of Results

Several options are available for the calculation of the concentration of Bax in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Bax can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound 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. Using linear graph paper, plot the Average Net OD for each standard versus Bax concentration in each standard. Approximate a straight line through the points. The concentration of Bax in the unknowns can be determined by interpolation.

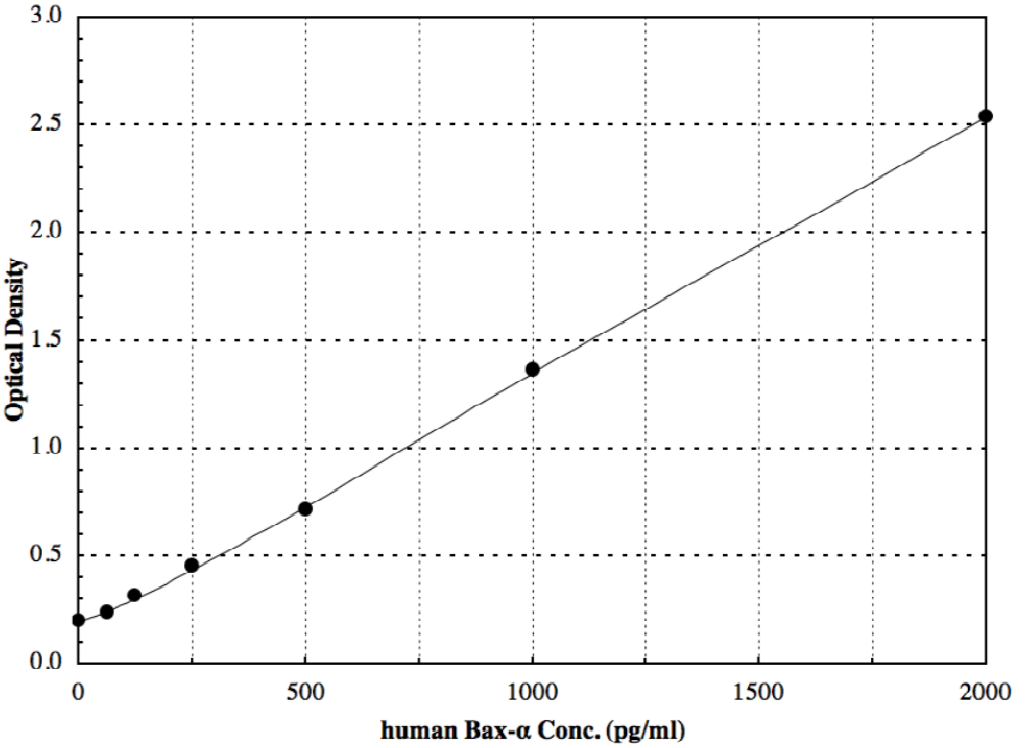
Typical Results

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

Sample	Average OD	Net OD	**Bax (pg/mL)**
Blank	(0.044)		
S0	0.243	0.199	**0**
S1	2.586	2.542	**2,000**
S2	1.407	1.363	**1,000**
S3	0.757	0.713	**500**
S4	0.499	0.455	**250**
S5	0.357	0.313	**125**
S6	0.284	0.240	**62.5**
Unknown 1	1.191	1.147	**833**
Unknown 2	0.360	0.316	**136**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Bax concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[8].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty (24) wells run with 0 pg/mL Standard, and comparing to the average optical density for twenty (24) wells run with Standard #6. The detection limit was determined as the concentration of Bax measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 = 0.193 ± 0.009 (4.84%)
Mean OD for Standard #6 = 0.239 ± 0.005 (2.14%)
Delta Optical Density (62.5 - 0 pg/mL) = 0.239 - 0.193 = 0.046

2 SD's of 0 pg/mL Standard = 2 x 0.009 = 0.018

$$\text{Sensitivity} = \frac{0.018}{0.046} \times 62.5 \text{ pg/mL} = \mathbf{24.5\ pg/mL}$$

Linearity

A sample containing 1,541 pg/mL Bax was serially diluted 4 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual Bax concentration versus measured Bax concentration.

The line obtained had a slope of 0.95 with a correlation coefficient of 0.994.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Bax and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Bax in multiple assays (n=10).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Bax determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Bax (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	136.7	5.0	
Medium	389.8	6.3	
High	789.7	4.9	
Low	114		17.0
Medium	429		6.3
High	800		5.7

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant in the kit assay buffer. These samples were then measured in the human Bax assay and the measured Bax concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
human Bax-α	100%
p21	<0.7%
Bcl-2	<0.1%
Bcl-XL	<0.1%
CDK4	<0.1%
p27	<0.1%
PCNA	<0.1%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Bax concentrations were measured in modified Cell Lysis Buffer 4. Bax was spiked into the undiluted sample, which was then diluted with Assay Buffer 13 and assayed in kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
modified Cell Lysis Buffer 4	100.6%	none required

For the lysis of 62,500 HeLa cells per mL, the following results were obtained:

Sample	% Recovery*	Recommended Dilution*
62,500 cells per mL	98.6%	≥1:2

WARNING: If the end user chooses to not use the provided Cell Lysis Buffer 4, it is up to the end user to determine the appropriate dilution of samples and assay validation for their chosen cell lysis buffer.

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard, "An Introduction to Radioimmunoassay & Related Techniques, 4th Ed.", (1990) Amsterdam: Elsevier.
2. P. Tijssen, "Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. Z.N. Oltvai, et al., Cell, (1993) 74: 609-619.
4. S. Willis, et al., J. Cell Sci., (2003) 116: 4053-4056.
5. S. Shimizu, et al., Nature, (1999) 399: 483-487.
6. A. Bettaieb, et al., Curr. Med. Chem. Anti-Canc. Agents, (2003) 3(4): 307-18.
7. V. Kirkin, et al., Biochim. Biophys. Acta., (2004) 1644(2-3): 229-49.
8. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

LIMITED WARRANTY

IBL-America warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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For more details concerning the information within this kit insert, or to order any IBL-America products, please call (763) 780-2955 between 8:00 a.m. and 5:00 p.m. CST.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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