

1 DESCRIPTION

The Human Bax ELISA kit is a complete kit for the quantitative 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].

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.

3 PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution 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.

4 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**, 11 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, 1x**, 105 mL,
Tris buffered saline containing proteins and detergents.
4. Bax **Conjugate**, 11 mL,
A blue solution of Streptavidin conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate**, 105 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**, 12 mL,
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.
Protect from prolonged exposure to light.
8. **Stop Solution**, 11 mL,
A 1N solution of hydrochloric acid in water. Keep tightly capped. **Caution:** Caustic.
9. **Cell Lysis Buffer**, 105 mL,
100 mM PBS, pH 7.3, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 0.005% Tween20.
10. Bax **Assay Layout Sheet**, 1 each,
11. **Plate Sealer**, 3 each,

5 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.

6 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 μ L and 1,000 μ L.
5. Repeater pipet for dispensing 100 μ L.
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.

7 SAMPLE HANDLING

The ELISA is compatible with Bax samples in a wide range of cell lysates and buffers.

Samples diluted sufficiently into Assay Buffer can be read directly from a standard curve. Please refer to the *Sample Recovery recommendations* for details of suggested dilutions.

It is recommended that all samples be lysed with the provided Cell Lysis Buffer modified by the addition of PIC and PMSF (see *Reagent Preparation*, #3) immediately prior to use. Samples lysed in this Cell Lysis Buffer must be diluted at least 1:2 with Assay Buffer 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 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.**

8 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 discarded.

The cell pellets were resuspended with modified Cell Lysis Buffer, 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. The exposure time for development was 6 minutes.

9 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.

10 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 10 minutes.

Label six polypropylene tubes #1 through #6.

Pipet 900 μ L of Assay Buffer into tube #1.

Pipet 500 μ L of Assay Buffer into tubes #2 through #6.

Add 100 μ L of the 20,000 pg/mL standard into tube #1 and vortex.

Add 500 μ L of tube #1 to tube #2 and vortex thoroughly.

Add 500 μ L 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

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 μ L/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.

11 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 μ L of Assay Buffer into the S0 (0 pg/mL standard) wells.
3. Pipet 100 μ L of Standards #1 through #6 into the appropriate wells.
4. Pipet 100 μ L of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.

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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 μ L 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 μ L 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 μ L 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 μ L 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 μ L 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 μ L of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 100 μ L Stop Solution 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.

12 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.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

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.

13 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	(0.097)		
S0	0.188	0.091	0
S1	2.126	2.029	2,000
S2	1.174	1.077	1,000
S3	0.717	0.620	500
S4	0.440	0.343	250
S5	0.284	0.187	125
S6	0.246	0.149	62.5
Unknown 1	0.989	0.892	797
Unknown 2	0.318	0.221	134

14 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].

14.1 Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty (20) wells run with 0 pg/mL Standard, and comparing to the average optical density for twenty (20) 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.

$$\begin{aligned} \text{Mean OD for S0} &= 0.078 \pm 0.005 (6.02\%) \\ \text{Mean OD for Standard \#6} &= 0.140 \pm 0.009 (6.43\%) \\ \text{Delta Optical Density (62.5 - 0 pg/mL)} &= 0.140 - 0.078 = 0.062 \\ \text{2 SD's of 0 pg/mL Standard} &= 2 \times 0.005 = 0.010 \\ \text{Sensitivity} &= 0.010 / 0.062 \times 62.5 \text{ pg/mL} = \mathbf{10.1 \text{ pg/mL}} \end{aligned}$$

14.2 Linearity

A sample containing 1,541 pg/mL Bax was serially diluted 4 times 1:2 in the Assay Buffer 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.

14.3 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

14.4 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.

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

14.5 Sample Recoveries

Please refer to sections *Sample Handling* recommendations and *Standard preparation*.

Bax concentrations were measured in modified Cell Lysis Buffer. Bax was spiked into the undiluted sample, which was then diluted with Assay Buffer and assayed in kit.

The following results were obtained:



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<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
modified Cell Lysis Buffer	100.6%	none required

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

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

WARNING:

If the end user chooses to not use the provided Cell Lysis Buffer, 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 for details.

15 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.

16 LIMITED WARRANTY

DRG 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.






DRG must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if DRG is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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SYMBOLS USED WITH DRG ELISA 'S

Symbol	English	Deutsch	Francais	Espanol	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consultez le mode d'emploi	Consulte instrucciones de empleo	Consulti d'Istruzioni d'uso
CE	European Conformity	CE-Konfirmitätskennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Ussage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke			
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Numero de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Temperature de conservation	Temperatura de conservacion	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeitsdatum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Distributeur	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Instruções de uso	Brugermanual	Användar manual	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
RUO				
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilstrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevaringstemperatur	Förvaringstemperatur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..



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