PRODUCT INFORMATION

human Total Bcl-2

Enzyme Immunometric Assay Kit

Catalog No. IB09621

96 Well Kit

Table of Contents

Description

The IBL-America human Total Bcl-2 Enzyme Immunometric Assay (EIA) kit is a complete kit for the determination of Bcl-2 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human Bcl-2 immobilized on a microtiter plate to bind the Bcl-2 in the standards or sample. A recombinant human Bcl-2 Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to Bcl-2 is added. This antibody binds to the Bcl-2 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 Bcl-2 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 Bcl-2 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

Bcl-2 (B-cell lymphoma 2) is an oncogenic protein that inhibits apoptosis. It is located within the mitochondrial membrane, endoplasmic reticulum and nuclear envelope[3]. Bcl-2 is encoded by a 230 kb gene that results in a 24-26 kDa protein[4]. Bakhshi first identified Bcl-2 as an apoptotic proto-oncogene while studying chromosomal translocations in human follicular lymphomas[5]. At approximately the same time Tsujimoto and Croce made the same observation. The function of Bcl-2 was first described by Vaux when it was demonstrated that it promoted the survival of factor dependent cells[6]. Bcl-2 overexpression has been implicated in a wide variety of malignancies including (but not limited to) breast, prostate, skin, colon and pancreatic cancers[7]. Related anti-apoptotic proteins include Bcl-xL, Mcl-1 and Bcl-w. Pro-apoptotic proteins in the Bcl-2 family include Bax, BAD, Bak, Blk and Bid[8].

Precautions

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

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 high levels of interfering substances may cause variation in assay results. **For best results, samples should be prepared in the buffers recommended and included in this kit**.
4. The human Bcl-2 Standard provided should be stored at or below -20°C and handled with care because of the known and unknown effects of Bcl -2.
5. Standards and samples must be prepared in polypropylene tubes. **Preparation in glass will result in decreased protein stability.**

Materials Supplied

1. **human Bcl-2 Microtiter Plate, One Plate of 96 Wells**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to human Bcl-2.
2. **Total Bcl-2 EIA Antibody, 10 mL**
   A yellow solution of biotinylated monoclonal antibody to Bcl-2.
3. **Assay Buffer 13, 110 mL**
   Tris buffered saline containing proteins, detergents and protease inhibitor.
4. **Total Bcl-2 EIA 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 Bcl-2 Standard, 2 vials**
   Two vials each containing 1,200 pg of lyophilized recombinant human Bcl-2.
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, 10 mL**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **Cell Lysis Buffer 2, 100 mL**
   1 mM EDTA, 6 M Urea, 0.5% Triton X-100, 0.005% Tween 20 in Phosphate Buffered Saline.
10. **human Total Bcl-2 Assay Layout Sheet, 1 each**
11. **Plate Sealer, 3 each**

Storage

All components of this kit, except the human Bcl-2 Standard, are stable at 4 °C until the kit's expiration date. The human Bcl-2 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.

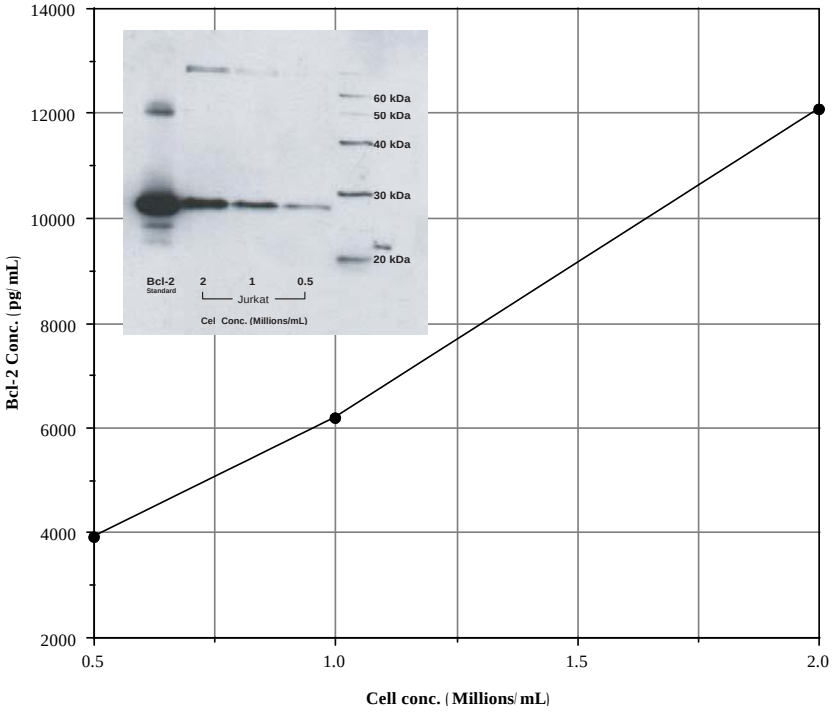
Sample Handling

This assay kit is compatible with Bcl-2 samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 13 plus Inhibitors (see Reagent Preparation, page 5, # 2) 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 2 modified by the addition of PMSF and PIC immediately prior to use (See Reagent Preparation, page 6, # 4). Samples lysed in this modified Cell Lysis Buffer 2 must be diluted at least 1:40 with Assay Buffer 13 plus Inhibitors prior to assaying. Note that this dilution is based on the lysis of 2 million Jurkat cells per mL. The 1:40 dilution contained 50,000 cells per mL with a calculated recovery of 93.6% .

If the end user chooses to use another lysis buffer or a greater number of cells, 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 plus Inhibitors should be used to calculate the concentration of Bcl-2. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive Bcl-2. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed.

Jurkat Cell Lysis Experiment

The number of Jurkat cells used in this experiment were: 2.0,1.0, and 0.5 million per mL. The total protein concentrations for the 2.0, 1.0 and 0.5 million cells per mL are 0.50 mg/mL, 0.30 mg/mL and 0.25 mg/mL, respectively. Cells were centrifuged at 1,500 rpm for 5 minutes and the supernatant discarded. The cell pellets were resuspended and washed with PBS. Cells were pelleted at 1,500 rpm for 5 minutes and the supernatant dicarded. The cell pellets were resuspended with modified Cell Lysis Buffer 2, vortexed and placed on ice for 5 minutes. The lysates were vortexed and centrifuged at 16,000 rpm for 15 minutes. The supernantants were split for the Western blot and EIA to generate the data illustrated. Note that 24 µL of sample was used per lane in the Western blot. The exposure time for development was 20 minutes.



Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in polypropylene 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. Assay Buffer 13 plus Inhibitors

Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail (PIC) # 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. **This modified Assay Buffer 13 must be used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of human Bcl-2. Fresh Assay Buffer 13 plus Inhibitors must be made for each assay.**

3. human Bcl-2 Standards

Allow the lyophilized human Bcl-2 Standard to warm to room temperature. Add 1 mL of Assay Buffer 13 plus Inhibitors to the lyophilized Bcl-2 vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard # 1. Label six 12 x 75 mm polypropylene tubes # 2 through # 7. Pipet 500 µL of Assay Buffer 13 plus Inhibitors into tube # 2 through # 7. Add 500 µL of reconstituted standard # 1 to tube # 2 and vortex. Add 500 µL of tube # 2 to tube # 3 and vortex thoroughly. Continue this for tubes # 4 through # 7.

The concentrations of human total Bcl-2 in standard vial # 1 and tubes # 2 through # 7 will be 1,200, 600, 300, 150, 75, 37.5 and 18.8 pg/mL, respectively. See human Total Bcl-2 Assay Layout Sheet for dilution details.

Reconstituted and diluted standards should be used within 60 minutes of preparation.

Discard any unused reconstituted standard and subsequent dilutions.

4. **Cell Lysis Buffer 2**
   Allow buffer to come to room temperature. Ensure that it is completely in solution prior to use. Immediately prior to use in assay, PMSF and PIC must be added to buffer. If using Sigma Protease Inhibitor Cocktail (PIC) # 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. **Fresh modified Cell Lysis Buffer 2 must be made for each assay.**

Assay Procedure

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

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. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 13 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of standards # 1 through # 7 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 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 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 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.
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 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.
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 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 Bcl-2 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 Bcl-2 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 human Bcl-2 concentration in each standard. Approximate a straight line through the points. The concentration of Bcl-2 in the unknowns can be determined by interpolation.

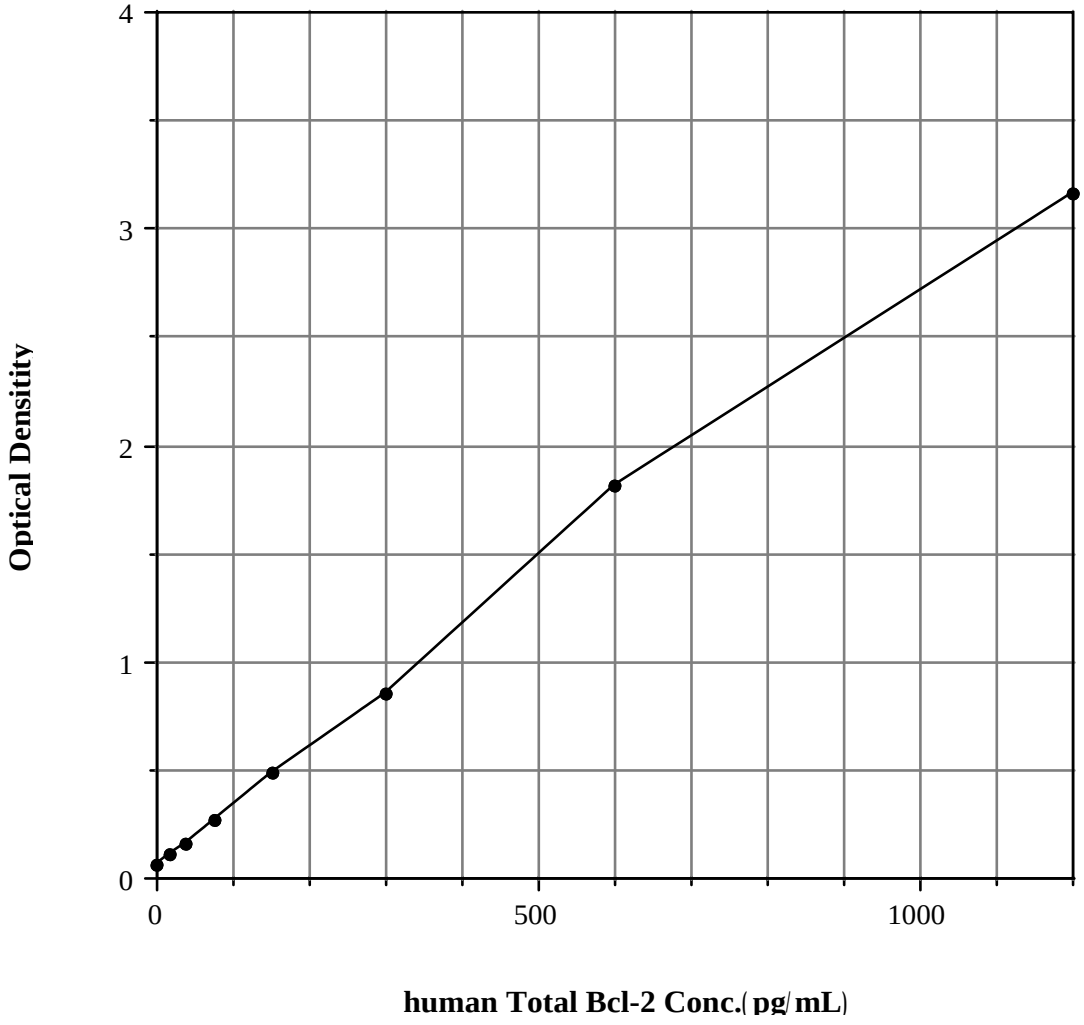
Typical Results

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

Sample	Average OD	Net OD	**human Bcl-2 (pg/mL)**
Blank	(0.078)		
S0	0.138	0.060	**0**
S1	3.245	3.167	**1,200**
S2	1.886	1.808	**600**
S3	0.931	0.853	**300**
S4	0.565	0.487	**150**
S5	0.340	0.262	**75**
S6	0.234	0.156	**37.5**
S7	0.191	0.113	**18.8**
Unknown 1	2.367	2.289	**795.1**
Unknown 2	0.442	0.364	**118.3**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Bcl-2 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[9].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average optical density for sixteen (16) wells run with Standard # 7. The detection limit was determined as the concentration of Bcl-2 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 = 0.054 ± 0.005 (9.9%)
Mean OD for Standard # 7 = 0.103 ± 0.013 (12.9%)

Delta Optical Density (18.8 - 0 pg/mL) = 0.103 - 0.054 = 0.049

2 SD's of 0 pg/mL Standard = 2 x 0.005 = 0.010

Sensitivity = $\frac{0.010}{0.049}$ x 18.8 pg/mL = **3.8 pg/mL**

Linearity

A sample containing 868.5 pg/mL of human Bcl-2 was serially diluted 5 times 1:2 in the Assay Buffer 13 plus Inhibitors and measured in the assay. The data was plotted graphically as actual Bcl-2 concentration versus measured Bcl-2 concentration.

The line obtained had a slope of 0.967 with a correlation coefficient of 1.00.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Bcl-2 and running these samples multiple times (n= 24) in the same assay. Inter-assay precision was determined by measuring two samples with low and high concentrations of Bcl-2 in multiple assays (n= 10).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Bcl-2 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	human Bcl-2 (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	112	2.9	
Medium	403	2.4	
High	764	4.2	
Low	112		4.2
Medium	413		3.4
High	783		3.6

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving cross reactants in assay buffer at a concentration of 12,000 pg/mL. Theses samples were then measured in the assay.

Compound	Cross Reactivity
human Bcl-2	100%
Bad	< 0.01%
Bax	< 0.01%
Bid	< 0.01%
Bcl-X L	< 0.01%
cIAP1	< 0.01%
total JNK	< 0.01%
pTEN	< 0.01%
Smac/DIABLO	< 0.01%
β-catenin	< 0.01%

Sample Recoveries

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

Bcl-2 concentrations were measured in modified Cell Lysis Buffer 2 and cell lysates. Bcl-2 was spiked into the undiluted modified Cell Lysis Buffer 2. The cells were run unspiked. These samples were then diluted with Assay Buffer 13 plus Inhibitors and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Cell Lysis Buffer 2	97.6%	≥1:40
2 million Jurkat cells per mL	93.6%	≥1:40

WARNING: If the end user chooses to not use the provided Cell Lysis Buffer 2, 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. J. Reed, Nature, (1997) 387: 773-776.
4. D.M. Hockenbery, Bioessays, (1995) 17: 631-638.
5. A. Bakhshi, et al., Cell, (1985) 41(3): 899-906.
6. D.L. Vaux, et al., Nature, (1988) 335(6189): 440-442.
7. D.M. Hockenbery, Proceedings of the National Academy of Sciences, (1991) 88: 6961-6965.
8. P. Bouillet and A. Strasser, Journal of Cell Science, (2002) 115: 1567-1574.
9. 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.

IBL-America must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if IBL-America 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.

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.

Immuno-Biological Laboratories, Inc.
8201 Central Ave NE
Minneapolis, MN 55432

Telephone: 1 (763) 780-2955

Fax: 1 (763) 780-2988
E-mail: ibl@ibl-america.com
Website: www.ibl-america.com

August 3, 2010