

ab119506 – Bcl-2 Human ELISA Kit

Instructions for Use

For the quantitative measurement of Human Bcl-2 concentrations in cell lysates, serum and plasma.

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Abcam's Bcl-2 Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Human Bcl-2 concentrations in cell lysates, serum and plasma.

Bcl-2 specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells along with a biotin-conjugated Bcl-2 detection antibody then incubated at room temperature. Following washing, a Streptavidin-HRP conjugate is added to each well, incubated at room temperature and washed. TMB is added and then catalyzed by HRP to produce a blue product that changes to yellow after the addition of acidic stop solution. The density of yellow coloration is directly proportional to the amount of Bcl-2 captured on the plate.

Bcl-2 initiates a new gene family involved in the regulation of cell death and survival without affecting cell proliferation. This precise regulation and maintenance of balance between cell proliferation and cell death in multicellular organisms is critical for tissue homeostasis. Bcl-2, encoded by a proto-oncogene, is an intracellular membrane-associated protein that functions to block programmed cell death. Expression of Bcl-2 has been reported in a wide range of hematopoietic cells, non-neoplastic epithelia and epithelial malignancies. Bcl-2 levels are low or undetectable in endothelial cells.

The Bcl-2 gene has a unique function among mammalian oncogenes as a negative regulator of apoptosis. It was first discovered because of its involvement in chromosomal translocations commonly found in lymphomas. Bcl-2 is furthermore associated with stem cells committed to differentiation and morphogenesis.

A decrease in Bcl-2 levels leads to cell death by apoptosis. Overexpression of Bcl-2 on the other hand protects cells from death,

but it is neither able to immortalize normal cells, nor to cause tumorigenic transformation of immortalized cells.

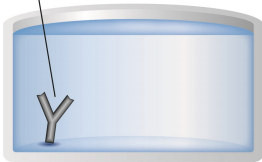
Heterogeneous expression of Bcl-2 in malignancies suggests that the gene is differentially regulated. Furthermore, its expression in association with precancerous lesions suggests a role in the early stage of tumorigenesis.

Several homologs of Bcl-2 have recently been discovered, some of which function as inhibitors of cell death and others as promoters of apoptosis that oppose the function of the Bcl-2 protein.

Bcl-2 protein plays a critical role in oncogenesis as well as resistance to therapy of cancer. Furthermore prognostic significance of Bcl-2 expression has been shown for several malignancies such as Non-Hodgkin's Lymphoma, squamous cell carcinomas, breast carcinoma, carcinoma of the gallbladder, and thymoma. Dysregulated Bcl-2 expression has been shown in Multiple Myeloma patients and in acute Myeloid leukemia. Bcl-2 has been suggested as a useful marker for adequate IL-2 therapy e.g. in AIDS patients.

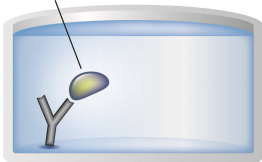
2. ASSAY SUMMARY

Primary Capture Antibody



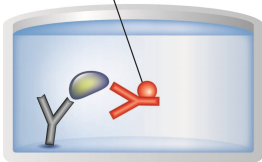
Prepare all reagents, samples and standards as instructed.

Sample



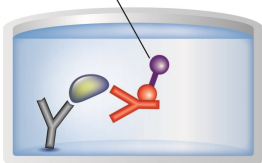
Add standard or sample to each well used.

Biotinylated Antibody



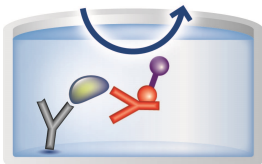
Add prepared biotin antibody to each well. Incubate at room temperature.

Streptavidin-Peroxidase Conjugate



Wash and add prepared Streptavidin-HRP Conjugate. Incubate at room temperature.

Substrate Colored Product



Wash and add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9 Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Microplate coated with monoclonal antibody to Bcl-2 (12 x 8 wells)	96 wells	2-8 °C
Biotin-Conjugate anti-Human Bcl-2 monoclonal antibody	70 µL	2-8 °C
Streptavidin-HRP	150 µL	2-8 °C
Human Bcl-2 Standard lyophilized (64 ng/mL upon reconstitution)	2 Vials	2-8 °C
Sample Diluent	12 mL	2-8 °C
20X Assay Buffer Concentrate	5 mL	2-8 °C
20X Wash Buffer Concentrate	50 mL	2-8 °C
10X Lysis Buffer	15 mL	2-8 °C
TMB Substrate Solution	15 mL	2-8 °C
Stop Solution (1M Phosphoric acid)	15 mL	2-8 °C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 5 mL and 10 mL graduated pipettes
- 5 μ L to 1000 μ L adjustable single channel micropipettes with disposable tips
- 50 μ L to 300 μ L adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radio immunotherapy has significantly increased the number of patients with Human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

9.1 1X Wash Buffer

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Wash Buffer should be stored at 2-8 °C and is stable for 30 days.

9.2 1X Assay Buffer

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer, combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Assay Buffer should be stored at 2-8 °C and is stable for 30 days.

9.3 1X Lysis Buffer

Prepare 1X Lysis Buffer by diluting the 10X Lysis Buffer Concentrate with distilled or deionized water. To make 150 mL 1X Lysis Buffer, combine 15 mL 10X Lysis Buffer Concentrate with 135 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Lysis Buffer should be stored at 2-8 °C and is stable for 30 days.

9.4 1X Biotin Conjugated Antibody

To prepare the Biotin Conjugated Antibody, dilute the anti-Human Bcl-2 monoclonal antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Biotin Conjugated Antibody as needed by adding the required volume (μL) of the Biotin Conjugated Antibody to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Biotin-Conjugated Bcl-2 antibody (μL)	1X Assay Buffer (mL)
1 - 6	30	2.97
7 - 12	60	5.94

Note: The 1X Biotin-Conjugated Antibody should be used within 30 minutes after dilution.

9.5 1X Streptavidin-HRP Conjugate

To prepare the Streptavidin-HRP Conjugate, dilute the anti-Streptavidin-HRP Conjugate 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Streptavidin-HRP Conjugate as needed by adding the required volume (μL) of the Streptavidin-HRP Conjugate to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Streptavidin-HRP (μL)	1X Assay Buffer (mL)
1 - 6	60	5.94
7 - 12	120	11.88

Note: The 1X Streptavidin-HRP should be used within 30 minutes after dilution.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

- 10.1 Prepare a 64 ng/mL **Stock Standard** by reconstituting one vial of the Human Bcl-2 standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 64 ng/mL **Stock Standard** cannot be stored for later use.
- 10.2 Label eight tubes with numbers 1 - 8.
- 10.3 Add 225 μ L 1X Assay Buffer into all tubes
- 10.4 Prepare a 32 ng/mL **Standard 1** by adding 225 μ L of the 64 ng/mL Stock Standard into tube 1. Mix thoroughly and gently.
- 10.5 Prepare **Standard 2** by transferring 225 μ L from Standard 1 to tube 2. Mix thoroughly and gently.
- 10.6 Prepare **Standard 3** by transferring 225 μ L from Standard 2 to tube 3. Mix thoroughly and gently.
- 10.7 Using the table below as a guide, repeat for tubes 4 through 7.
- 10.8 **Standard 8** contains no protein and is the Blank control

ASSAY PREPARATION

Standard	Sample to Dilute	Volume to Dilute (μL)	Volume of Diluent (μL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	225	225	64	32
2	Standard 1	225	225	32	16
3	Standard 2	225	225	16	8
4	Standard 3	225	225	8	4
5	Standard 4	225	225	4	2
6	Standard 5	225	225	2	1
7	Standard 6	225	225	1	0.5
8	None	-	225	-	0



11. SAMPLE COLLECTION AND STORAGE

- Cell lysates, serum and plasma were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.
- Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.
- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Human Bcl-2. If samples are to be run within 24 hours, they may be stored at 2° to 8°C
- Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.
- Aliquots of samples were stored at -20°C and thawed 5 times, and the Human Bcl-2 levels determined. There was no significant loss of Human Bcl-2 immunoreactivity detected by freezing and thawing.
- Aliquots of samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the Human Bcl-2 level determined after 24 h. There was no significant loss of Human Bcl-2 immunoreactivity detected during storage at -20°C. A significant loss of Human Bcl-2 immunoreactivity was detected during storage at 4°C (30%), at RT (45%) and 37°C (70%) after 24 h.

12. SAMPLE PREPARATION

Cell Lysate Protocol

- Numerous extraction protocols can be used. The following protocol is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures to find the optimal method.
- **For suspension cells:** Pellet by centrifugation, remove supernatant and proceed to Addition of Lysis Buffer.
- **For attached cells:** Remove supernatant from cells, wash cells once with PBS, harvest cells by scraping and gentle centrifugation, aspirate PBS, leaving an intact cell pellet (at this point the cell pellet can be frozen at -80°C and lysed at a later date) and proceed to Addition of Lysis Buffer.
- **Addition of Lysis Buffer:** Resuspend the pellet in 1x Lysis Buffer (we recommend a concentration of 5 x10⁶cells/mL.), incubate 60 minutes at room temperature with gentle shaking and transfer extracts to microcentrifuge tubes and centrifuge at 1000 x g for 15 minutes. Aliquot the cleared lysate to clean microfuge tubes and continue the test procedure (Alternatively lysates can be stored at -80°C and assayed at a later time. Divide lysates into small aliquots to avoid multiple freeze/thaw cycles.).

13. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use.
- Unused well strips should be returned to the plate packet and stored at 2-8°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

14. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
 - **It is recommended to assay all standards, controls and samples in duplicate.**
- 13.1. Prepare all reagents, working standards, and samples as directed in the previous sections. Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.
 - 13.2. Wash the microplate twice with approximately 400 μL 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.
 - 13.3. After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
 - 13.4. Add 100 μL of prepared standards (including the standard blank control) to the appropriate wells.
 - 13.5. Add 80 μL of Sample Diluent to all sample wells.
 - 13.6. Add 20 μL of samples to appropriate wells.
 - 13.7. Add 50 μL of 1X Biotin Conjugated Antibody to all wells.
 - 13.8. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).
 - 13.9. Remove adhesive film and empty wells. Wash microplate strips 3 times according to step 13.2. Proceed immediately to step 13.10.

- 13.10. Add 100 μ L of 1X Streptavidin-HRP to all wells, including the blank wells.
- 13.11. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).
- 13.12. Remove adhesive film and empty wells. Wash microplate strips 3 times according to step 13.2. Proceed immediately to the next step.
- 13.13. Pipette 100 μ L of TMB Substrate Solution to all wells.
- 13.14. Incubate the microplate strips at room temperature (18 to 25°C) for 10 minutes. Avoid direct exposure to intense light.

Note: The color development on the plate should be monitored and the substrate reaction stopped (see step 13.15) before the signal in the positive wells becomes saturated. Determination of the ideal time period for color development should be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 - 0.95.

- 13.15. Stop the enzyme reaction by adding 100 μ L of Stop Solution into each well.

Note: It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

- 13.16. Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader

according to the manufacturer's instructions by using the blank wells (standard 8). Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

15. CALCULATIONS

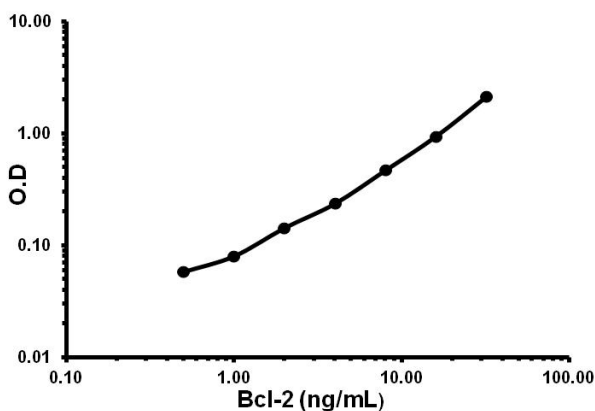
Average the duplicate readings for each standard, sample and control blank. Subtract the no protein control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A five parameter algorithm (5PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 5-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

If samples have been diluted 1:5, as stated in step 13.6, the concentration obtained from the standard curve must be multiplied by the dilution factor ($\times 5$) to obtain an accurate value, in addition to any initial sample dilution factor.

Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Bcl-2 levels. Such samples require further external predilution according to expected human Bcl-2 values with Sample Diluent in order to precisely quantitate the actual human Bcl-2 level.

16. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc.	O.D. 450 nm		Mean
(ng/mL)	1	2	O.D.
0.0	0.018	0.018	0.018
0.5	0.060	0.056	0.058
1.0	0.075	0.084	0.080
2.0	0.141	0.145	0.143
4.0	0.231	0.240	0.236
8.0	0.453	0.483	0.468
16.0	0.937	0.940	0.939
32.0	2.248	2.007	2.128

Figure 1. Example of Human Bcl-2 protein standard curve.

17. TYPICAL SAMPLE VALUES

SENSITIVITY -

The limit of detection of Human Bcl-2 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be < 0.5 ng/mL (mean of 6 independent assays).

RECOVERY –

The spike recovery was evaluated by spiking 4 levels of Human Bcl-2 into lysis buffer. Recoveries were determined in 3 independent experiments with 4 replicates each.

The overall mean recovery was 71%.

PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Human Bcl-2.

	Intra-Assay	Inter-Assay
n=	8	6
%CV	8.6	12.0

LINEARITY OF DILUTION –

4 samples with different levels of Human Bcl-2 were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 85% to 123% with an overall recovery of 102%

Sample	Dilution	Expected Human Bcl-2 Concentration. (ng/mL)	Observed Human Bcl-2 Concentration (ng/mL)	Recovery of Expected Human Bcl-2 Concentration (%)
1	1:5	-	81.7	-
	1:10	40.9	34.5	85
	1:20	20.4	22.4	110
	1:40	10.2	11.3	111
2	1:5	-	50.3	-
	1:10	25.2	23.3	92
	1:20	12.6	12.8	102
	1:40	6.3	6.4	102
3	1:5	-	138.0	-
	1:10	69.0	75.6	110
	1:20	34.5	42.4	123
	1:40	17.3	19.6	113
4	1:5	-	113.5	-
	1:10	56.8	53.3	94
	1:20	28.4	25.7	91
	1:40	14.2	13.0	92

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

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