XIAP (human), ELISA kit

Catalog No. ADI-900-124

96 Well Kit

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Description

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

XIAP (X-linked Inhibitor of Apoptosis) is know by a variety of other names (baculoviral IAP repeat containing protein 4 (BIRC4), mammalian IAP homolog A (MIHA[3]), X-linked inhibitor of apoptosis protein 3, X-linked IAP, HILP, IAP Like protein and ILP[4]). XIAP belongs to the IAP family (inhibitors of apoptosis) which is characterized by one or more BIR (baculovirus IAP repeat) domains. These proteins have multiple biological functions that include binding and inhibiting caspases, regulating cell cycle progression, and modulating receptor-mediated signal transduction. XIAP is a broad-ranging suppressor of apoptosis[5]. XIAP functions by direct binding and inhibition of several caspases, including caspase-3, caspase-7 and caspase-9[6,7]. There are three BIR domains in XIAP: BIR1, BIR2 and BIR3. BIR2 has been implicated in the binding and inhibition of caspase-3 and caspase-7[8], and BIR3 has been reported to be a specific inhibitor of caspase-9[7].

Precautions

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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 XIAP Standard provided, Catalog No. 80-1176, should be handled with care because of the known and unknown effects of XIAP.
5. The XIAP standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.
6. Standards and samples must be prepared in polypropylene tubes. Preparation in glass will result in decreased protein stability.

Materials Supplied

1. **human XIAP Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1175**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to human XIAP.
2. **human XIAP Antibody, 10 mL, Catalog No. 80-1174**
   A yellow solution of biotinylated monoclonal antibody to human XIAP.
3. **Assay Buffer 21, 100 mL, Catalog No. 80-1519**
   Tris buffered saline containing proteins, detergents and phosphatase inhibitor.
4. **human XIAP Conjugate, 10 mL, Catalog No. 80-1180**
   A blue solution of Streptavidin conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **human XIAP Standard, 1 bottle, Catalog No. 80-1757**
   One bottle containing two vials of lyophilized recombinant human XIAP, 5,000 pg each.
7. **TMB Substrate, 10 mL, Catalog No. 80-0350**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
8. **Stop Solution 2, 10 mL, Catalog No. 80-0377**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**
   50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS.
10. **XIAP Assay Layout Sheet, 1 each, Catalog No. 30-0201**
11. **Plate Sealer, 3 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the XIAP Standard,** are stable at 4°C until the kit's expiration date. The XIAP 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. Polpropylene test tubes.

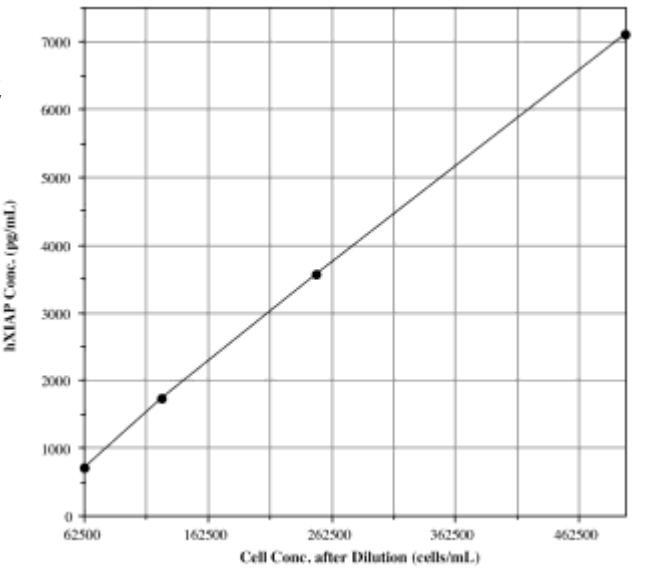
Sample Handling

The XIAP (human), ELISA is compatible with human XIAP samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 21 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 RIPA Cell Lysis Buffer 2 modified by the addition of PIC and PMSF (see Reagent Preparation, page 5, #3). Samples lysed in RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:2 with Assay Buffer 21 prior to assaying. Note that this dilution is based on the lysis of 0.5 million HeLa cells per mL. The 1:2 dilution contained 0.25 million cells per mL with a calculated recovery of 98.7%.

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 21 should be used to calculate the concentration of XIAP. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive XIAP. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed. **Samples must be prepared in polypropylene tubes.**

HeLa Cell Stimulation Experiment

The number of HeLa cells used in this experiment was ~16 million per mL, with a protein concentration of 9 mg/mL. 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 RIPA Cell Lysis Buffer 2. The lysates were vortexed and centrifuged at 14,000 rpm for 10 minutes. The supernantants were then diluted in modified RIPA Buffer to 0.5 million cells/mL for ELISA testing.



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 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 100 mL of the supplied concentrate with 1,900 mL of de-ionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. human XIAP Standards

Allow the lyophilized human XIAP standard to warm to room temperature. Add 0.5 mL of Assay Buffer 21 to the lyophilized XIAP vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five polypropylene tubes #2 through #6. Pipet 250 µL of Assay Buffer 21 into tubes #2 though #6. Add 250 µL of reconstituted standard #1 into tube #2 and vortex. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

The concentration of human XIAP in standards #1 through #6 will be 10,000, 5,000, 2,500, 1,250, 625 and 312.5 pg/mL respectively. See XIAP Assay Layout Sheet for dilution details.

Reconstituted and diluted standards should be used within 20 minutes of preparation. Discard any unused reconstituted standard and subsequent dilutions.

3. RIPA Cell Lysis Buffer 2 plus Inhibitors

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

Fresh RIPA Cell Lysis Buffer 2 plus Inhibitors must be made each time the cells are lysed.

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 the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 21 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 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 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 XIAP 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 XIAP 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. Plot the Average Net OD for each standard versus XIAP concentration in each standard. Approximate a straight line through the points. The concentration of XIAP in the unknowns can be determined by interpolation.

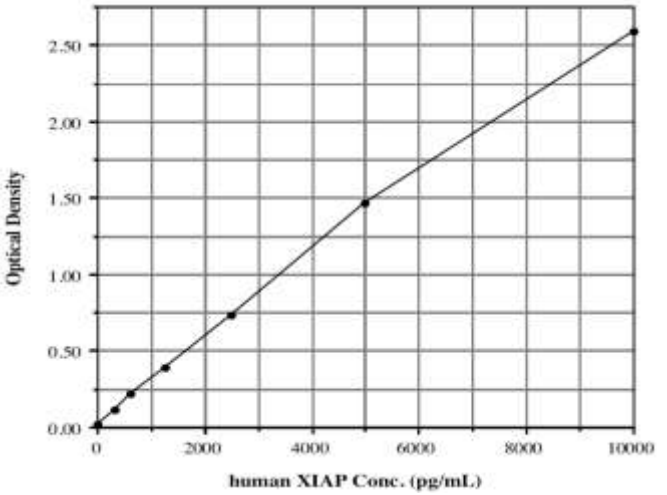
Typical Results

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

Sample	Average OD	Net OD	**XIAP (pg/mL)**
Blank	(0.078)		
S0	0.101	0.023	**0**
S1	2.666	2.588	**10,000**
S2	1.545	1.467	**5,000**
S3	0.810	0.732	**2,500**
S4	0.464	0.386	**1,250**
S5	0.300	0.222	**625**
S6	0.194	0.116	**312.5**
Unknown 1	0.426	0.348	**1123**
Unknown 2	0.251	0.173	**528**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate XIAP 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 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 XIAP measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 =	0.039 ± 0.010 (25.7%)
Mean OD for Standard #6 =	0.108 ± 0.009 (8.3%)
Delta Optical Density (312.5 - 0 pg/mL) = 0.108 - 0.039 =	0.069
2 SD's of 0 pg/mL Standard = 2 x 0.010 =	0.020

Sensitivity = $\frac{0.020}{0.069}$ x 312.5 pg/mL = **90.6 pg/mL**

Linearity

A sample containing 8,091 pg/mL XIAP was serially diluted 4 times 1:2 in the Assay Buffer 21 supplied in the kit and measured in the assay. The data was plotted graphically as actual XIAP concentration versus measured XIAP concentration.

The line obtained had a slope of 0.932 with a correlation coefficient of 0.9996.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of XIAP and running these samples multiple times (n=20) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of XIAP in multiple assays (n=11).

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

	XIAP (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	829	15.1	
Medium	1,342	5.7	
High	7,494	2.6	
Low	578		13.4
Medium	1,081		7.5
High	7,642		6.0

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 XIAP assay and the measured human XIAP 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 XIAP	100%
Survivin	3.2%
cIAP1	<0.1%
Smac/DIABLO	<0.1%
Caspase-3	<0.1%

Sample Recoveries

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

Human XIAP concentrations were measured in cells lysed in modified RIPA Cell Lysis Buffer 2, diluted with Assay Buffer 21 and assayed in kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
0.5 million HeLa cells/mL	98.7%	≥1:2

WARNING: If the end user chooses to not use the provided RIPA 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

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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