Quantikine® ELISA

Human Active Caspase-3 Immunoassay

Catalog Number KM300

For the quantitative determination of human* active Caspase-3 concentrations in cell extracts.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

*This assay also recognizes mouse active Caspase-3.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The active Caspase-3 ELISA measures active Caspase-3 in systems that contain Caspase-3 zymogen and other active caspases. Caspase-3 is a cysteine protease that cleaves substrates at the carboxyl terminus of aspartate residues. Active Caspase-3 has two active sites and consists of two identical large and two identical small subunits that are derived from two precursor Caspase-3 polypeptides. Each active site involves a cysteine located near the carboxyl terminal of the large subunit. Caspase-3 is proteolytically activated by other caspases. Cleavage of the Caspase-3 zymogen generates a pro-region, a large subunit, and a small subunit.

The active Caspase-3 ELISA uses a biotinylated caspase inhibitor to covalently modify the large subunit of Caspase-3. The Inhibitor is added directly to the culture medium where it enters apoptotic cells and forms a stable thio-ether bond with the cysteine on the active site. The Inhibitor does not covalently modify inactive Caspase-3, which is the basis for discrimination between active and inactive Caspase-3. Cells are then solubilized in a denaturing extraction buffer and diluted to reduce denaturant concentration. Caspase-3 specificity is achieved by using a Caspase-3 specific monoclonal antibody coated on the microplate. The monoclonal antibody captures both Caspase-3 zymogen and the large subunit. Detection is with HRP-streptavidin that binds the biotin on the inhibitor attached to the Caspase-3 large subunit. Because zymogen is not modified with biotinylated inhibitor, it is not detected by HRP-streptavidin. The ELISA measures the relative amount of Caspase-3 large subunit modified with biotin-ZVKD-fmk (fluoromethylketone). Since the modification requires that the large subunit is present in an active Caspase-3, the amount of active Caspase-3 is directly proportional to the amount of biotin-ZVKD-fmk-modified large subunit.

Cell lines tested in the Active Caspase-3 ELISA

The following human, mouse, and rat cell lines have been tested and the incubation times with 1 µM staurosporine (prior to the 1 hour incubation with 10 µM biotin-ZVKD-fmk) required for Caspase-3 activation are indicated. The shortest times indicate the start of Caspase-3 activation and the longer times indicate when active Caspase-3 levels are high.

Cell Line	Cell Line Description	Incubation Times
Jurkat	human acute T cell leukemia	1-3 hours
CEM-NKr	human Natural Killer cell resistant T-lymphoblastoid	4-6 hours
U937	human histiocytic lymphoma	3-7 hours
M07e	human megakaryocytic leukemic	3-6 hours
HL-60	human acute promyelocytic leukemia	3-5 hours
THP-1*	human acute monocytic leukemia	3-7 hours
CHP-100	human neuroblastoma	4-7 hours
SH-SY5Y	human neuroblastoma	1-4 hours
HeLa	human cervical epithelial carcinoma	1-3 hours
HT-29*	human colon adenocarcinoma	3-5 hours
HepG2	human hepatocellular carcinoma	overnight treatment with anti-Fas and 40 µM cycloheximide
DA-1a	mouse myeloid leukemia	1-6 hours
CH-1	mouse B cell lymphoma	1-4 hours
TS1	mouse helper T cell	1-6 hours
PC-12	rat adrenal pheochromocytoma	1-6 hours

*Based on measurement of active caspase-3 with the active Caspase-3 ELISA and by Western Blot with goat anti-Caspase-3 (R&D Systems®, Catalog # AF-605-NA), these cell lines contain less Caspase-3 than the other listed lines.

The Quantikine® Human Active Caspase-3 Immunoassay is a 3.5 hour solid phase ELISA designed to measure human active Caspase-3 in cell extracts. It contains biotinylated-inhibitor derivatized recombinant Caspase-3 and a monoclonal antibody raised against Caspase-3. Results obtained using natural active Caspase-3 covalently bound to biotin-ZVKD-fmk inhibitor in apoptotic cell extracts showed linear curves that were parallel to the Quantikine® kit standard. These results indicate that this kit can be used to determine relative mass values for human active Caspase-3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Caspase-3 has been pre-coated onto a microplate. Standards and cell extract samples containing covalently linked active Caspase-3-biotin-ZVKD are pipetted into the wells and any Caspase-3 present is bound by the immobilized antibody. Inactive Caspase-3 zymogen is not modified by the biotin-ZVKD-fmk inhibitor and therefore is not detected. Following a wash to remove any unbound substances, streptavidin conjugated to horseradish peroxidase is added to the wells and binds to the biotin on the inhibitor. Following a wash to remove any unbound Streptavidin-HRP, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of active Caspase-3 bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- This kit is validated for cell extracts only. Serum samples have not been validated in this kit.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- Cells must be washed and free of culture media and serum prior to assay.
- When storing samples, storage in Extraction Buffer (diluted 1:5) containing protease inhibitors at 2-8 °C is recommended. Dilute with calibrator diluent (diluted 1:5) immediately before assaying.
- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Active Caspase-3 Microplate	890819	96 well microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Caspase-3.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Active Caspase-3 Conjugate Concentrate	890820	0.75 mL of a 23-fold concentrated solution containing streptavidin conjugated to horseradish peroxidase (HRP) with preservatives.	May be stored for up to 1 week at 2 - 8° C.*
Human Active Caspase-3 Standard	890821	Caspase-3 derivatized with a biotinylated inhibitor in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated feeze-thaw cycles. *Biotin-ZVDK-fmk Inhibitor must be protected from moisture.*
Biotin-ZVDK-fmk Inhibitor	890065	400 µg of biotinylated ZVKD-fmk inhibitor; lyophilized.	
Type 12 Conjugate Diluent	895510	12 mL of buffer for diluting the conjugate concentrate with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Calibrator Diluent Concentrate RD5-20	895346	21 mL of a concentrated buffered protein solution with preservatives. *Use diluted 1:5 in this assay.*	
Extraction Buffer Concentrate, 5X	895287	21 mL of a concentrated buffered protein solution containing surfactants with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

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OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.

OTHER REAGENTS REQUIRED

- Urea (Ultra Pure Grade)
- Dimethylsulfoxide, DMSO (ACS grade or equivalent)
- Aprotinin (Sigma, Catalog # A6279)
- Leupeptin (Sigma, Catalog # L8511)
- Pepstatin (Sigma, Catalog # P4265)
- Phenylmethylsulfonylfluoride (PMSF; Sigma, Catalog # P7626)
- Isopropanol
- Phosphate Buffered Saline (PBS); pH 7.0

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 μL of the resultant mixture is required per well.

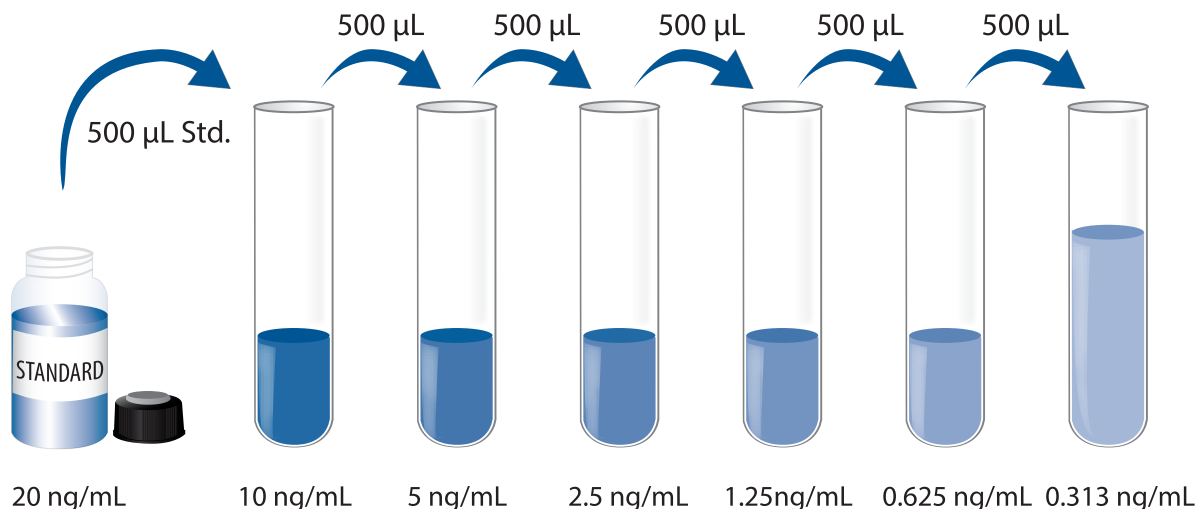
Calibrator Diluent RD5-20 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-20 Concentrate to 80 mL deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-20 (diluted 1:5). Mix well.

Human Active Caspase-3 Conjugate Concentrate - To prepare enough conjugate for the entire plate, add 0.5 mL Conjugate Concentrate to 11.0 mL of Conjugate Diluent. Use a sterile container.

Biotin-ZVKD-fmk Inhibitor - Add 92 μL of DMSO to 400 μg of inhibitor to make a 5 mM stock solution.

Human Active Caspase-3 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Active Caspase-3 Standard with Calibrator Diluent RD5-20 (diluted 1:5). Do not substitute other diluents. This reconstitution produces a stock solution of 20 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 500 μL Calibrator Diluent RD5-20 (diluted 1:5) into each tube. Use the 20 ng/mL standard to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human Active Caspase-3 Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5-20 (diluted 1:5) serves as the zero standard (0 ng/mL).



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REAGENT PREPARATION *CONTINUED*

Protease Inhibitors:

Leupeptin - Prepare a 25 mg/mL stock in DMSO. Store at ≤ -20 °C.

Pepstatin - Prepare a 25 mg/mL stock in DMSO. Store at ≤ -20 °C.

Aprotinin - Use as supplied by the vendor. Store at 2-8 °C.

PMSF - Prepare a 100 mM stock in isopropanol. PMSF is unstable in aqueous solution. Discard any remaining stock. Use freshly prepared stock for each assay.

Extraction Buffer (diluted 1:5) - To prepare 10 mL of Extraction Buffer (diluted 1:5), add 3.6 g Urea to 2 mL of Extraction Buffer Concentrate. Bring final volume to 10 mL with deionized or distilled water.

Immediately before use, add protease inhibitors to Extraction Buffer (diluted 1:5) as follows:

Protease Inhibitor	µL Protease Inhibitor/mL Extration Buffer (1X)
Leupeptin (25 mg/mL stock)	1 µL/mL
Pepstatin (25 mg/mL stock)	1 µL/mL
PMSF (100 mM stock)	1 µL/mL
Aprotinin	3 µg/mL

SAMPLE PREPARATION

Labeling of active caspases in cells

After induction of apoptosis, add 2 µL of 5 mM biotin-ZVKD-fmk per 1 mL of culture medium to obtain a final concentration of 10 µM. To conserve biotin-ZVKD-fmk, use a minimal amount of culture medium. Incubate cells with the biotin-ZVKD-fmk inhibitor for 1 hour.

CELL EXTRACT PREPARATION

A. Cells grown in suspension

1. Centrifuge cell culture sample at 1000 x g for 5 minutes.
2. Discard supernatant and resuspend cells in PBS.
3. Centrifuge cells at 1000 x g for 5 minutes. Discard the supernatant.
4. Add Extraction Buffer (diluted 1:5) containing Protease Inhibitors at 1 mL per 1 x 10^7 cells.
5. Vortex for 1 minute and allow to sit for 2 hours at room temperature or overnight at 2-8 °C. The extended time in Extraction Buffer (diluted 1:5) containing protease inhibitors ensures that maximum denaturation is achieved. Samples can be stored in Extraction Buffer (diluted 1:5) for up to 14 days at 2-8 °C.
6. Immediately prior to assay, dilute samples 10-20 fold with Calibrator Diluent RD5-20 (diluted 1:5). Diluted samples can be stored for up to 14 days at ≤ -20 °C.

B. Cells grown in monolayers-general procedure

1. Remove medium containing detached cells and save.
2. Gently wash attached cells with PBS. Collect wash and pool with medium.
3. Scrape attached cells into Extraction Buffer (diluted 1:5) containing protease inhibitors using 1 mL per 1 x 10^7 cells.
4. Centrifuge the combined PBS wash and medium at 1000 x g for 5 minutes to pellet detached cells. Discard supernate.
5. Suspend pelleted detached cells in PBS and centrifuge at 1000 x g for 5 minutes. Discard supernate.
6. Add extract from step 3 to the pellet from step 5.
7. Vortex for 1 minute and allow to sit for 2 hours at room temperature or overnight at 2-8 °C. The extended time in Extraction Buffer (diluted 1:5) containing protease inhibitors ensures that maximum denaturation is achieved. Samples can be stored in Extraction Buffer (diluted 1:5) for up to 14 days at 2-8 °C.
8. Immediately prior to assay, dilute samples 10-20 fold with Calibrator Diluent RD5-20 (diluted 1:5). Diluted samples can be stored for 14 days at ≤ -20 °C.

C. Monolayer cells grown in 6 well dishes (assumes 5 x 10^5 cells/well)

1. Gently rinse cells with PBS
2. Add 110 µL Extraction Buffer (diluted 1:5) containing protease inhibitors and scrape cells (a bent plastic 200 µL pipette tip works well).
3. Cover the dish and set at room temperature for 2 hours or at 2-8 °C overnight.
4. To obtain 1 x 10^6 cells per mL extract, add 400 µL of Calibrator Diluent RD5-20 (diluted 1:5) and rock plate to mix. Collect diluted extract and vortex for 1 minute. Diluted samples can be stored at ≤ -20 °C for up to 14 days.
5. To obtain 5 x 10^5 cells per mL extract, use 150 µL of Extraction Buffer in step 2 and 850 µL of calibrator diluent in step 4.

D. Monolayer cells grown in 24 well dishes (assumes 2.2 x 10^5 cells/well)

1. Gently rinse cells with PBS.
2. Add 70 µL Extraction Buffer (diluted 1:5) containing protease inhibitors and scrape cells (a bent plastic 200 µL pipette tip works well).
3. Cover the dish and set at room temperature for 2 hours or at 2-8 °C overnight.
4. To obtain 5 x 10^5 cells per mL extract, add 370 µL of Calibrator Diluent RD5-20 (diluted 1:5) and rock plate to mix. Collect diluted extract and vortex for 1 minute. Diluted samples can be stored at ≤ -20 °C for up to 14 days.

Note: *The final concentration of urea in samples should be less than 1.2 M.*

DILUTION OF CELL EXTRACTS

Cell extracts containing 1 x 10^7 cells/mL require a final 20-fold dilution in Calibrator Diluent RD5-20 (diluted 1:5) to obtain 5 x 10^5 cells/mL. A suggested 20-fold dilution is 25 µL cell extract + 475 µL Calibrator Diluent RD5-20 (diluted 1:5).

For apoptotic cells that contain low levels of active Caspase-3 (*e.g.* THP-1 and HT-29), a final 10-fold dilution in Calibrator Diluent RD5-20 (diluted 1:5) to obtain 1 x 10^6 cells/mL is recommended. A suggested 10-fold dilution is 50 µL cell extract + 450 µL Calibrator Diluent RD5-20 (diluted 1:5).

Note: *The Caspase-3 capture antibody binds both inactive zymogen and the Caspase-3 large subunit, linearity of the signal can be affected by adding too much cell extract. To ensure linearity of the sample, take diluted extracts from cells expressing the highest amount of active Caspase-3, dilute serially 2-fold and test the diluted samples for linearity.*

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, standard dilutions, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
4. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of Human Active Caspase-3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
8. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Cell extracts require dilution. See Sample Preparation Section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human active Caspase-3 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

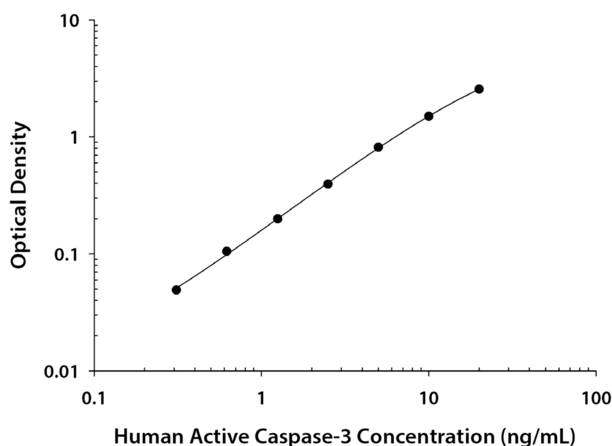
To determine the active Caspase-3 large subunit concentration (ng/mL) of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding active Caspase-3 concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

To determine the relative amount of active Caspase-3 large subunit in cells, divide the detected concentration (ng/mL) of the Caspase-3 large subunit by the concentration of the extract (cells/mL) in the diluted sample.

Note: *The relative levels of active caspase-3 or the amount of active caspase-3 large subunit detected should be reported.*

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.018 0.016	0.017	—
0.313	0.069 0.064	0.066	0.049
0.625	0.124 0.119	0.122	0.105
1.25	0.221 0.211	0.216	0.199
2.5	0.401 0.424	0.412	0.395
5	0.831 0.831	0.831	0.814
10	1.516 1.515	1.516	1.499
20	2.576 2.563	2.570	2.553

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	1.06	3.09	7.70	1.06	3.27	8.25
Standard deviation	0.05	0.16	0.20	0.07	0.19	0.50
CV (%)	4.7	5.2	2.6	6.6	5.8	6.1

RECOVERY

The recovery of human active human Caspase-3 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Calibrator Diluent RD5-20 (diluted 1:5)	99	98-100%
Extraction Buffer (1X)*	110	104-114%
Extraction Buffer (1X)*	98	97-101%
Cell extract sample from Jurkat cells treated with Staurosporine*	92	87-95%

*Samples were diluted prior to assay.

SENSITIVITY

The minimum detectable dose (MDD) of human active Caspase-3 is typically less than 0.1 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess linearity of the assay, samples spiked with high concentrations of human active Caspase-3 in each matrix were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Calibrator Diluent RD5-20 (diluted 1:5)* (n=5)	Extraction Buffer (diluted 1:5)* (n=5)	Cell extract samples from Jurkat cells treated with Staurosporine* (n=10)
1:2	Average % of Expected	100	102	109
	Range (%)	97-104	94-106	102-114
1:4	Average % of Expected	100	108	117
	Range (%)	90-108	102-113	107-132
1:8	Average % of Expected	100	109	121
	Range (%)	87-108	93-127	104-145
1:16	Average % of Expected	104	112	125
	Range (%)	87-112	107-117	108-153

*Samples were diluted prior to assay.

CALIBRATION

This assay is calibrated against a highly purified recombinant human caspase-3 treated with biotin-Asp-fmk. Caspase-3 precursor was expressed in *E. coli* and auto-processed active caspase-3 was purified. On SDS polyacrylamide gels, the recombinant active Caspase-3 migrated as two polypeptides of 18 kDa and 10 kDa. Only the 18 kDa large subunit which contains the active site cysteine is modified by biotin-Asp-fmk. The protein concentration of the recombinant human active Caspase-3-biotin-Asp-fmk was determined by the method of Bradford (1) using purified bovine serum albumin as a standard.

SAMPLE VALUES

The following treated cell lines (1 x 10^7 cells/mL) were incubated with biotin-ZVKD-fmk inhibitor. The cells were lysed with Extraction Buffer (diluted 1:5), diluted 20-fold in Calibrator Diluent RD5-20 (diluted 1:5) (final concentration of 5 x 10^5 cells/mL) and assayed for detectable levels of human active Caspase-3 large subunit.

Cell Line	Assay Value (ng/mL)	Calculated Value (ng/10^6 cells)
Jurkat cells (human acute T cell leukemia clone E6-1), treated for 3 hours with staurosporine	7.2	14.4
HepG2 cells (human hepatocellular carcinoma), treated with cycloheximide + anti-Fas antibody	2.3	4.6
CHP-100 cells (human neuroblastoma), treated for 6 hours with staurosporine	3.4	6.8

SPECIFICITY

Specificity of the active Caspase-3 ELISA has been evaluated by three independent methods (2).

1. Caspases captured on the microplate by the Caspase-3 monoclonal antibody were transferred to membranes and immunoblotted with antibodies against Caspases -2, -3, -7, -8, and -9. No Caspase-2, -7, -8, and -9 were detected. Immunoblotting with three different antibodies against Caspase-3 detected both precursor and large subunit of Caspase-3. Blotting with HRP-streptavidin detected the large subunit but not the precursor. Thus, the capture antibody is specific for Caspase-3 and the precursor caspase-3 is not modified with the biotinylated inhibitor.

2. MCF-7 cells, which do not express Caspase-3 were tested in this assay. No active Caspase-3 was detected in apoptotic MCF-7 cells.

3. This assay recognizes biotin-ZVKD-fmk modified natural and biotin-D-fmk modified recombinant active Caspase-3. Active Caspase-2, -7, -8 and -10 were prepared at 50 ng/mL in Calibrator Diluent RD5-20 (diluted 1:5) and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range active Caspase-3 control were assayed for interference. No significant cross-reactivity or interference was observed.

MOUSE ACTIVE CASPASE-3 CROSS-REACTIVITY

The assay also detects mouse active Caspase-3. The cross-reactivity of the kit with mouse active Caspase-3 is greater than 90%.

REFERENCES

1. Bradford, M.M. (1976) Anal. Biochem. **72**:248.
2. Saunders, P.A. *et al.* (2000) Anal. Biochem. **284**:114.

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