DuoSet® IC

Human/Mouse Cleaved Caspase-3 (Asp175)

Catalog Number DYC835-2
DYC835-5

For the development of sandwich ELISAs to measure human and mouse Caspase-3 cleaved at Asp175 in cell lysates.

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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PRINCIPLE OF THE ASSAY

This DuoSet IC® ELISA contains the basic components required for the development of sandwich ELISAs to measure human and mouse Caspase-3 cleaved at aspartic acid 175 (Asp175) in cell lysates. An immobilized capture antibody specifically binds Caspase-3. After washing away unbound material, a biotinylated detection antibody specific for human/mouse Caspase-3 cleaved at Asp175 is used to detect the captured protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC835-2**	**Cat. # DYC835-5**
Human/Mouse Cleaved Caspase-3 (Asp175) Capture Antibody	843542	2-8° C	1	2
Human/Mouse Cleaved Caspase-3 (Asp175) Detection Antibody	843543	2-8° C	1	2
Human/Mouse Cleaved Caspase-3 (Asp175) Standard	843544	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC835-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC835-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Fluoride (NaF) (Sigma # 201154)
- Sodium Orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium Pyrophosphate ($Na_4P_2O_7$) (Sigma # P8010)
- Triton™ X-100 (Sigma # T9284)
- Urea
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates(R&D Systems Catalog # DY990)
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer.

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY006).

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA,* 0.05% NaN_3, in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 μm filtered.

IC Diluent #8** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF in PBS, pH 7.2-7.4

Note: *IC Diluent #8 is also the base buffer for IC Diluent #3, IC Diluent #7, and Lysis Buffer #6. Approximately 50 mL of this diluent is required to run the assay on one plate.*

IC Diluent #3** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea in PBS, pH 7.2-7.4.

IC Diluent #7** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea in PBS, pH 7.2-7.4

Lysis Buffer #6** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 100 μM PMSF, 3 μg/mL Aprotinin, 2.5 mM sodium pyrophosphate, and 1 mM activated sodium orthovanadate in PBS, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog DY994).

*The use of Reagent Diluent Concentrate 2 (R&D Systems, Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8° C.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human/Mouse Cleaved Caspase-3 (Asp175) Capture Antibody (Part 843542) - Each vial contains 360 µg/mL of mouse anti-human Caspase-3 antibody when reconstituted with 200 µL of PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Human/Mouse Cleaved Caspase-3 (Asp175) Detection Antibody (Part 843543) - Each vial contains 5.4 µg/mL of biotinylated rabbit anti-human cleaved Caspase-3 (Asp175) when reconstituted with 1.0 mL of IC Diluent #1. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Human/Mouse Cleaved Caspase-3 (Asp175) Standard (Part 843544) - Each vial contains 95 ng/mL of recombinant human cleaved Caspase-3 (Asp175) when reconstituted with 500 µL of IC Diluent #7. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** An initial 6-fold dilution should be made in IC Diluent #8. Further dilutions should be made in IC Diluent #3 immediately before use. A seven point curve using 2-fold serial dilutions and a high standard of 14,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Store at 2-8° C. **DO NOT FREEZE.**

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

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #6 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. For assaying, dilute lysates 6-fold with IC Diluent #8 and make further serial dilutions in IC Diluent #3.

Note: *The final concentration of urea in all samples and standards should be 1 M prior to addition to the plate.*

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

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

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

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

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8° C or be prepared fresh daily.

GENERAL ELISA PROTOCOL

A plate layout is provided to record standards and samples assayed.

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 2.0 μg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 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 for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in IC Diluent #3 per well. Use IC Diluent #3 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.

   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 14,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Dilute the Detection Antibody to a working concentration of 150 ng/mL in IC Diluent #1 before use. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 μL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, 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.

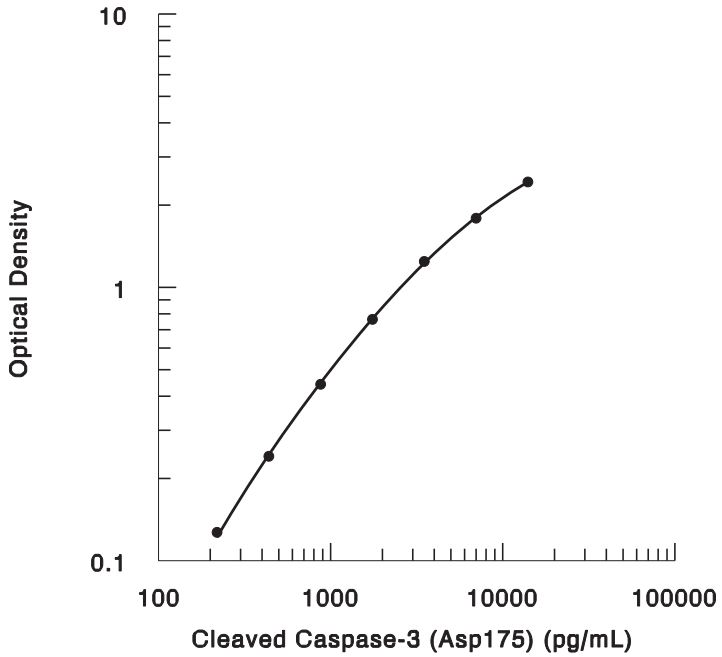
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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/mouse cleaved Caspase-3 (Asp175) 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.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human/Mouse Cleaved Caspase-3 (Asp175) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human/Mouse Cleaved Caspase-3 (Asp175) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human Cleaved Caspase-3 (Asp175) produced at R&D Systems. Samples containing natural Cleaved Caspase-3 (Asp175) showed linear dilution parallel to the standard curve obtained using the Cleaved Caspase-3 (Asp175) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the concentration of Cleaved Caspase-3 (Asp175) in natural samples.

SPECIFICITY

The Human/Mouse Cleaved Caspase-3 (Asp175) DuoSet IC ELISA specifically recognizes Caspase-3 cleaved at Asp175. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the ELISA.

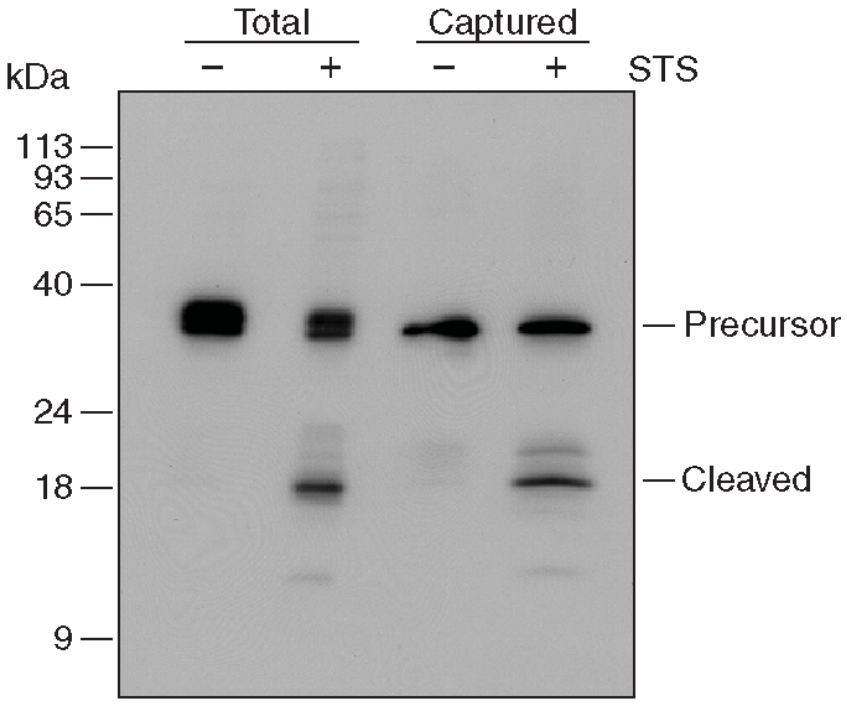


Figure 1: Lysates prepared from Jurkat human acute T cell leukemia cells, untreated or treated for 2 hours with 1 μg/mL staurosporine (STS), were incubated in wells coated with Human/Mouse Cleaved Caspase-3 (Asp175) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured protein were electrophoresed, transferred to a PVDF membrane, and immunoblotted with a caspase-3 polyclonal antibody (R&D Systems, Catalog # AF-605-NA) known to detect intact and cleaved caspase-3. The band corresponding to cleaved caspase-3 was detected in staurosporine-treated captured material.

Cross-reactivity experiments were performed with this DuoSet IC ELISA to further determine specificity. Recombinant human (rh) Caspase-2, rhCaspase-7, rhCaspase-8, rhCaspase-9, and rhCaspase-10 were assayed at 140 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of cleaved caspase-3, as quantified by the Human/Mouse Cleaved Caspase-3 (Asp175) DuoSet IC ELISA, are consistent with the relative amounts of cleaved caspase-3 determined by qualitative Western blot analysis.

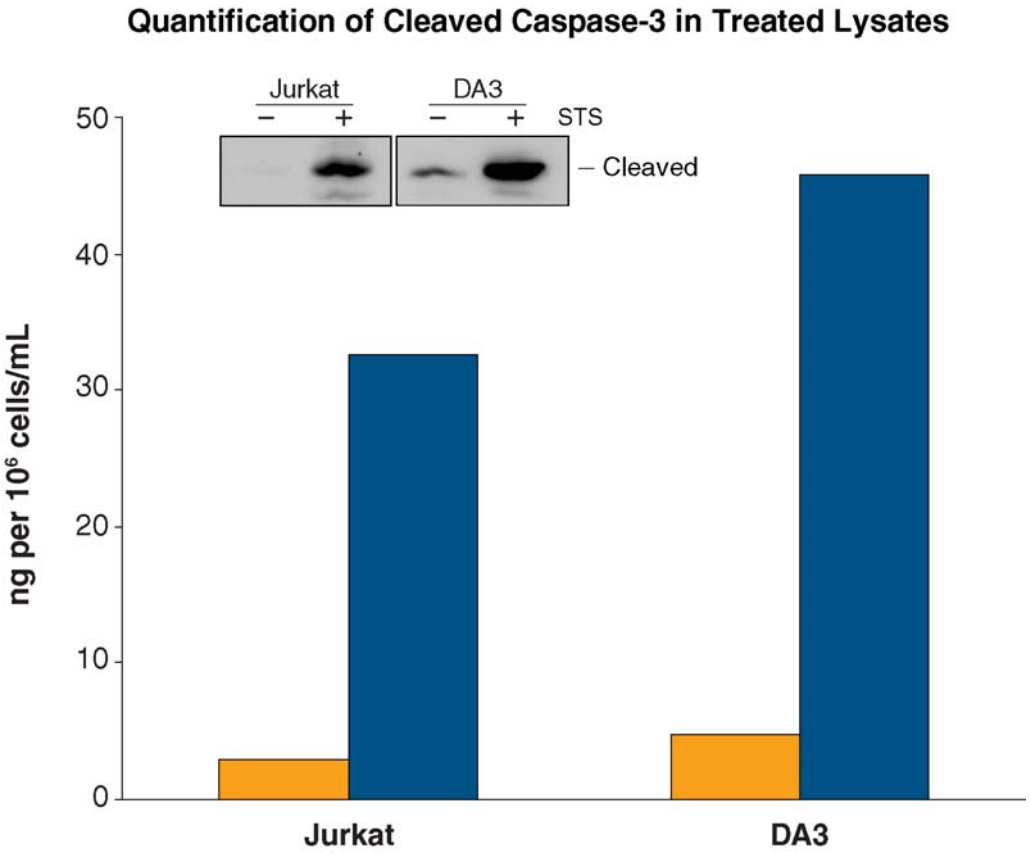


Figure 2: Lysates prepared from Jurkat human acute T cell leukemia cells and DA3 mouse myeloma cells, untreated or treated for 2 hours with 1 μg/mL staurosporine (STS), were quantified with this DuoSet IC ELISA. The same lysates were immunoblotted (inset) with an anti-cleaved caspase-3 monoclonal antibody (R&D Systems, Catalog # MAB835). The DuoSet IC ELISA results correlate well with the relative amounts of cleaved caspase-3 detected by Western blot.

Amounts of caspase-3 cleaved *in vitro* by the addition of cytochrome c and dATP as quantified by the Human/Mouse Cleaved Caspase-3 (Asp175) DuoSet IC ELISA are consistent with the relative amounts of cleaved caspase-3 determined by Western blot analysis.

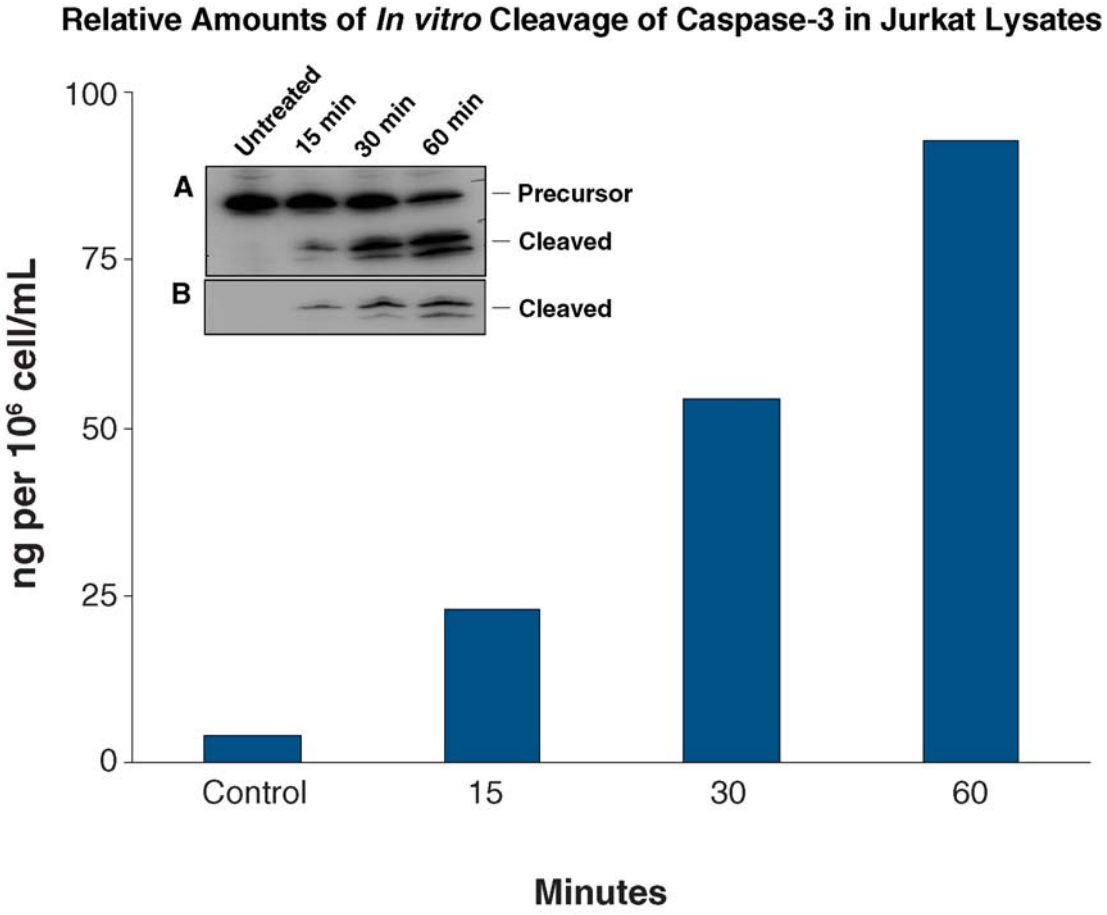


Figure 3: Jurkat human acute T cell leukemia cells were lysed in Extraction Buffer (50 mM HEPES-KOH (pH 7.5), 10 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 0.2% CHAPS, 0.2 mM DTT). Following the addition of cytochrome c and dATP, lysates were incubated at 30° C for the indicated times. The reaction was stopped by the addition of an equal volume of IC Diluent #7. The samples were diluted and quantified for cleaved caspase-3 with this DuoSet IC ELISA. The same lysates were immunoblotted (inset) with the following:

(A) Anti-caspase-3 polyclonal antibody (R&D Systems, Catalog # AF-605-NA)

(B) Anti-cleaved caspase-3 (Asp175) monoclonal antibody (R&D Systems, Catalog # MAB835).

The DuoSet IC ELISA results correlate well with the relative amounts of cleaved caspase-3 detected by Western blot.

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.