ELISA Kit
Catalog #KHO1051

Cytochrome c

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INTRODUCTION

Apoptotic cell death is a fundamental feature of virtually all cells (5). It is an indispensable process during normal development, tissue homeostasis, development of the nervous system, and the regulation of the immune system. Insufficient or excessive cell death can contribute to human disease, including cancer or degenerative disorders (14). The highly coordinated and stereotyped manner of this induced cell death suggests that the cells activate a common death program, towards which diverse signal – transducing pathways converge (2,17,18).

The mitochondria was found to participate in the central control or executioner phase of the cell death cascade (1). Cytochrome c was identified as a component required for the crucial steps in apoptosis, caspase-3 activation, and DNA fragmentation (8). Cytochrome c was shown to redistribute from mitochondria to cytosol during apoptosis in intact cells (6a,b).

Mitochrondrial Cytochrome c is a water soluble protein of 15 kDa with a net positive charge, residing loosely attached in the mitochrondrial intermembrane space. Cytochrome c functions in the respiratory chain by interaction with redox partners. It is highly conserved during evolution. Like most mitrochondrial proteins, Cytochrome c is encoded by a nuclear gene and synthesized as a cytoplasmic precursor molecule, apocytochrome c, which becomes selectively imported into the mitochondrial intermembrane space. The molecular mechanisms responsible for the translocation of Cytochrome c from mitochondria to cytosol during apoptosis are unknown.

A reduction in mitochondrial transmembrane potential has been reported to accompany early apoptosis (7). The release of Cytochrome c into the cytosol leads to an activation of an apoptotic program via activation of a caspase dependent pathway (4,12,13,15). Cytochrome c achieves this goal by interaction with other cytosolic factors forming a complex (apoptosome) composed of Cytochrome c, Apaf-1, dATP and Apaf-3/caspase 9 (3,10,11). Bcl-2 on the other hand was shown to be able to prevent apoptosis by blocking the release of Cytochrome c from mitochondria (18).

Measurement of Cytochrome c release from the mitochondria is a tool to detect the first early steps for initiating apoptosis in cells. Cytochrome c release in the cytosol occurs prior to the activation of caspases and DNA fragmentation which is considered the hallmark of apoptosis. Detection of Cytochrome c released from the mitochondria to the cytoplasm can be achieved by a selective lysis of the cell membrane.

Very recently it has been shown that this mitochondria dwelling molecule can be detected in the medium as early as 1 hour after apoptosis.

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen Cytochrome c kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Cytochrome c has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing Cytochrome c, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the Cytochrome c antigen binds to the immobilized (capture) antibody. After washing, a biotinylated secondary antibody specific for Cytochrome c is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Cytochrome c protein captured during the first incubation. After removal of excess detection antibody, a Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Streptavidin-Peroxidase, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Cytochrome c present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
Cytochrome c Standard. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide, red dye*; 25 mL per bottle.	1 bottle
*Cytochrome c Antibody Coated Well*s, 12 x 8 Well Strips.	1 plate
Cytochrome c Biotin Conjugate (biotin-labeled anti-Cytochrome c antibody). Contains 0.1% sodium azide, blue dye*; 11 mL per bottle.	1 bottle
Streptavidin-HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
HRP Diluent. Contains 3.3 mM thymol, yellow dye*; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
*Stabilized Chromoge*n, *Tetramethylbenzidine (TMB);* 25 mL per bottle.	1 bottle
*Stop Solutio*n; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3
* In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored *Standard Diluent Buffer*, *Detection Antibody*, and *HRP Diluent* to help monitor the addition of solutions to the reaction wells. This does not in any way interfere with the test results.	

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Cell extraction buffer (see Recommended Formulation, p. 11).
4. Distilled or deionized water.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
6. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
7. Glass or plastic tubes for diluting and aliquoting standard.
8. Absorbent paper towels.
9. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.

3. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
4. If particulate matter is present, centrifuge or filter prior to analysis.
5. All standards, controls and samples should be run in duplicate.
6. Samples that are greater than the highest standard point should be diluted with *Standard Diluent Buffer* and retested.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. Cover or cap all reagents when not in use.
9. **Do not mix or interchange different reagent lots from various kit lots**.
10. Do not use reagents after the kit expiration date.
11. Read absorbances within 2 hours of assay completion.
12. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
13. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
14. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS

A. Recommended Formulation of Cell Extraction Buffer:

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)
Protease inhibitor cocktail (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 100 µL per 1 mL Cell Extraction Buffer.

This buffer is stable for 2 to 3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the Cell Extraction Buffer should be thawed on ice. This buffer (minus protease inhibitor cocktail and PMSF) can be obtained from Invitrogen Cat. # FNN0011. **Important:** add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

B. Protocol for Cell Extraction

This protocol has been successfully applied to several cell lines. Researchers should optimize the cell extraction procedures for their own applications.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80^{o}C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes, on ice, with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of Cytochrome c. For example, 5 x 10^{7} Jurket cells grown in RPMI-1640 plus 10% FBS can be extracted in 1 mL of Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in *Standard Diluent Buffer* (See **Assay Method**) is sufficient for the detection of Cytochrome c.
5. Transfer extract to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4^{o}C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80^{o}C. Avoid multiple freeze/thaw cycles.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Cytochrome c Standard

Note: This *Cytochrome c Standard* is prepared from natural Cytochrome c protein.

1. Reconstitute *Cytochrome c Standard* with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 5 ng/mL Cytochrome c. Use the standard within 1 hour of reconstitution.
2. Add 0.25 mL of *Standard Diluent Buffer* to each of 6 tubes labeled as 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 ng/mL Cytochrome c.
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Cytochrome c Standard

Standard:	Add:	Into:
5 ng/mL	Prepare as described in step 1	
2.5 ng/mL	0.25 mL of the 5 ng/mL std.	0.25 mL of the Diluent Buffer
1.25 ng/mL	0.25 mL of the 2.5 ng/mL std.	0.25 mL of the Diluent Buffer
0.625 ng/mL	0.25 mL of the 1.25 ng/mL std.	0.25 mL of the Diluent Buffer
0.312 ng/mL	0.25 mL of the 0.625 ng/mL std.	0.25 mL of the Diluent Buffer
0.156 ng/mL	0.25 mL of the 0.312 ng/mL std.	0.25 mL of the Diluent Buffer
0.078 ng/mL	0.25 mL of the 0.156 ng/mL std.	0.25 mL of the Diluent Buffer
0 ng/mL	0.25 mL of the Diluent Buffer	An empty tube

Remaining reconstituted standard should be discarded or frozen in aliquots at -80^{o}C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

C. Storage and Final Dilution of Streptavidin-HRP (100X)

Please Note: The *Streptavidin-HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards, samples or controls to the appropriate microtiter wells. Samples prepared in Cell Extraction Buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample into 90 µL buffer). While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:50 or 1:100 may be optimal. The dilution chosen should be optimal for

each experimental system. Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)

4. Cover wells with *plate cover* and incubate for **2 hours at room temperature.**
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
6. Pipette 100 µL of *Cytochrome c Biotin Conjugate* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover wells with *plate cover* and incubate for **1 hour at room temperature.**
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 µL of *Streptavidin-HRP* Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.

13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
14. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution.*
16. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
17. Read the Cytochrome c concentrations for unknown samples and controls from the standard curve plotted in step 16. **Multiply value(s) obtained for sample(s) by the appropriate dilution factor to correct for the dilution with *Standard Diluent Buffer*.**

(Samples producing signals higher than the highest standard [5 ng/mL] should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration by the appropriate dilution factor.)

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 5 ng/mL of Cytochrome c.

Standard Cytochrome c (ng/mL)	Optical Density (450 nm)
5	3.444
2.5	1.858
1.25	0.849
0.625	0.418
0.312	0.255
0.156	0.167
0.078	0.146
0	0.118

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 5 ng/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >5 ng/mL with *Standard Diluent Buffer;* reanalyze these and multiply results by the appropriate dilution factor.

The influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native Cytochrome c in various matrices has not been investigated. Although Cytochrome c degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <0.156 ng/mL of Cytochrome c This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 24 times.

PRECISION

1. Intra-Assay Precision

Samples of known Cytochrome c concentrations were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	2.45	1.21	0.55
SD	0.09	0.06	0.03
%CV	3.71	4.61	5.45

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	2.42	1.20	0.61
SD	0.15	0.06	0.05
%CV	6.20	5.25	8.77

SD = Standard Deviation
CV = Coefficient of Variation

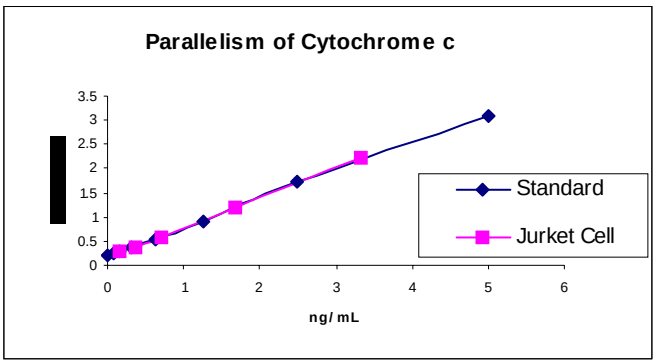
RECOVERY

To evaluate recovery, Cytochrome c Standard was spiked at 3 different concentrations into 10% Cell Extraction Buffer. The average recovery was 110%.

PARALLELISM

Natural Cytochrome c from Jurket cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the Cytochrome c standard curve. Parallelism demonstrated by the figure below indicates that the standard accurately reflects Cytochrome c content in samples.

Figure 2



LINEARITY OF DILUTION

Jurket cells were grown in tissue culture medium containing 10% fetal bovine serum and lysed with Cell Extraction Buffer. The lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for Cytochrome c. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

	Cell Lysate		
Dilution	**Measured (ng/mL)**	**Expected (ng/mL)**	**% Expected**
1/2	3.326	3.326	100
1/4	1.669	1.660	100.4
1/8	0.716	0.830	86.1
1/16	0.368	0.420	88.5

SPECIFICITY

Various human, rat, and mouse cell lines were tested on the Cytochrome c ELISA kit and it was found that the assay can detect human, rat, and mouse forms of the Cytochrome c protein.

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

Cytochrome c Assay Summary