Quantikine® ELISA

Human Cytochrome c Immunoassay

Catalog Number DCTC0
SCTC0
PDCTC0

For the quantitative determination of human Cytochrome c concentrations 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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INTRODUCTION

Human somatic Cytochrome c is a 15 kDa, 104 amino acid (aa) polypeptide that participates in both oxidative phosphorylation and apoptosis (1). It exhibits 91% aa identity with mouse/rat Cytochrome c (1-3). The molecule is initially synthesized in the cytoplasm as an extended, 12 kDa apoprotein. This molecule is subsequently transported across the outer mitochondrial membrane into the intermitochondrial space via a receptor-independent process. Here, it associates with an inner mitochondrial membrane enzyme called CCHL (Cytochrome c heme lyase) which covalently attaches one heme molecule to the N-terminus, forming functional Cytochrome c (4-6). This covalent attachment causes Cytochrome c to undergo a conformational change to a globular molecule effectively trapping Cytochrome c in the intermitochrondrial space.

Within the intermitochondrial space, Cytochrome c is actively involved in the oxidative phosphorylation pathway. It transports electrons from the Cytochrome c reductase complex to the Cytochrome c oxidase complex (7-9). This transports excess electrons along the respiratory pathway and generates ATP for energy-dependent processes. Alternatively, and in response to apoptotic signals, Cytochrome c can be released from mitochondria into the cytosol. Here, it activates an apoptotic program via one of many possible caspase-driven cascades (8, 10-12). The events which trigger an apoptotic signal (such as growth factor withdrawal) are not well understood. The result of such a signal, however, has been suggested to be a translocation of cytosolic Bax and/or Bad to the outer mitochondrial membrane where they overcome a Bcl-2-mediated stabilization (13-15). Bax-mediated destabilization may take the form of channel creation or mitochondrial swelling, resulting in Cytochrome c release (15-18). Whatever the mechanism, released Cytochrome c participates in the formation of a cytosolic complex which is composed of 15 kDa Cytochrome c, 130 kDa Apaf-1 (apoptotic protease activating factor-1), dATP, and 46 kDa Apaf-3/caspase-9 (15, 18, 19). Within this complex, Apaf-3/caspase-9 is activated, leading to the downstream activation of caspases-3, -7 and -9, followed by additional caspases that ultimately lead to cellular apoptosis (10, 18, 20, 21).

The Quantikine® Human Cytochrome c Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Cytochrome c in cell lysates.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique and is designed to replace a Western Blot. A monoclonal antibody specific for human Cytochrome c has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Cytochrome c present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Cytochrome c is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Cytochrome c bound in the initial step. The color development is stopped and the intensity of the color is measured.

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.
- It is important that the calibrator diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, further 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

- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	CATALOG # DCTC0	CATALOG # SCTC0	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Cytochrome c Microplate	890070	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Cytochrome c.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Cytochrome c Conjugate	890071	1 vial	6 vials	21 mL/vial of a monoclonal antibody specific for human Cytochrome c conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Cytochrome c Standard	890072	1 vial	6 vials	Human Cytochrome c in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for standard reconstitution volume.*	
Calibrator Diluent RD5P Concentrate	895151	1 vial	6 vials	21 mL/vial of a concentrated buffered protein base with preservatives. *Use diluted 1:10 in this assay.*	
Cell Lysis Buffer 2	895347	2 vials	12 vials	21 mL/vial of a buffered solution with preservative.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DCTC0 contains sufficient materials to run an ELISA on one 96 well plate.
SCTC0 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDCTC0). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

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.
- 250 mL and 500 mL graduated cylinders.
- Centrifuge
- Cold PBS for cell lysis
- Test tubes for dilution of standards and samples.
- Human Cytochrome c Controls (optional; R&D Systems®, Catalog # QC75).

PRECAUTIONS

The Human Cytochrome c Standard contains Cytochrome c obtained from human hearts. The hearts have been certified by the supplier to be non-reactive for anti-HIV 1/2, Hepatitis B surface antigen, anti-HCV and HIV-1 antigen. Since no known test method can rule out the possibility of infection, this material should be handled as if it is capable of transmitting disease.

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.

CELL LYSIS PROCEDURE

Quantitation of total Cytochrome c in extracts of cultured cells:

1. Wash cells three times in cold PBS.
2. Re-suspend cells in Cell Lysis Buffer 2 to a concentration of 1.5 x 10^6 cells/mL.
3. Incubate for 1 hour at room temperature with gentle mixing.
4. Centrifuge cells at 1000 x g for 15 minutes.
5. Dilute the supernate at least 5-fold and assay immediately or aliquot and store at ≤ -70 °C. A suggested 5-fold dilution is 50 µL of sample + 200 µL Calibrator Diluent RD5P (diluted 1:10).*

Note: *Cell Lysis Buffer 2 will solubilize the Cytochrome c in the cytosol and in the mitochondria. To determine the distribution of Cytochrome c in apoptotic cells, subcellular fractionation must be performed prior to solubilization. A procedure for subcellular fractionation of apoptotic cells has not been tested or validated for this assay.*

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

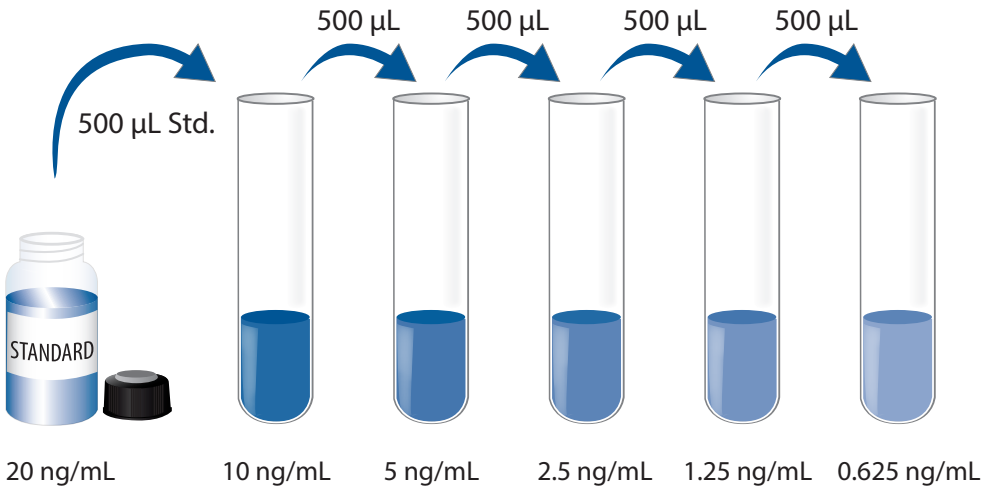
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:10) - Add 20 mL of Calibrator Diluent RD5P Concentrate to 180 mL of deionized or distilled water to prepare 200 mL of Calibrator Diluent RD5P (diluted 1:10).

Human Cytochrome c Standard - **Refer to the vial label for standard reconstitution volume.** Reconstitute the Human Cytochrome c Standard with deionized or distilled water. This reconstitution produces a stock solution of 20 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 µL of Calibrator Diluent RD5P (diluted 1:10) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:10) serves as the zero standard (0 ng/mL).



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, working standards, 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 Calibrator Diluent RD5P (diluted 1:10) to each well.
4. Add 100 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four 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 toweling.
6. Add 200 µL of Human Cytochrome c Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. 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.

*Samples require lysis and dilution. See Cell Lysis Procedure.

CALCULATION OF RESULTS

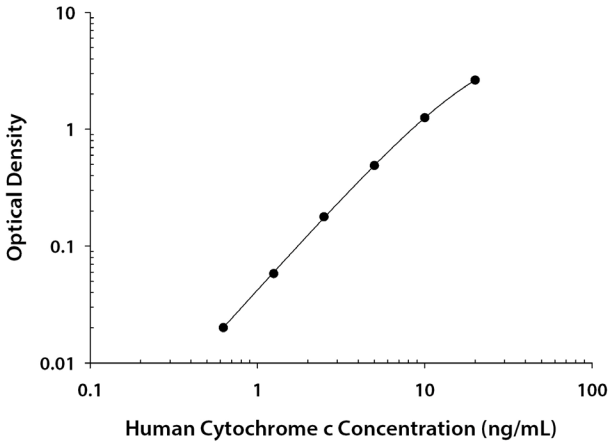
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Cytochrome c 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.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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	0.049 0.050	0.050	—
0.625	0.070 0.070	0.070	0.020
1.25	0.106 0.110	0.108	0.058
2.5	0.226 0.230	0.228	0.178
5	0.533 0.544	0.538	0.488
10	1.261 1.343	1.302	1.252
20	2.658 2.694	2.676	2.626

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 forty 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	40	40	40
Mean (ng/mL)	3.64	7.31	10.7	3.54	6.74	10.2
Standard deviation	0.15	0.30	0.42	0.18	0.34	0.51
CV (%)	4.1	4.1	3.9	5.1	5.0	5.0

SENSITIVITY

The minimum detectable dose (MDD) of human Cytochrome c is typically less than 0.31 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 the linearity of the assay, samples containing high concentrations of human Cytochrome c were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell lysates (n=6)
1:2	Average % of Expected	99
	Range (%)	97-101
1:4	Average % of Expected	100
	Range (%)	94-104
1:8	Average % of Expected	99
	Range (%)	89-105
1:16	Average % of Expected	98
	Range (%)	88-103

CALIBRATION

This immunoassay is calibrated using natural Cytochrome c purified from human hearts.

SAMPLE VALUES

MCF-7 human breast cancer cells (1 x 10^7 cells/mL) were cultured in 50% DMEM and 50% Kaigns F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 109 ng/mL.

TF-1 human erythroleukemic cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and 2.5 ng/mL of recombinant human GM-CSF. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 148 ng/mL.

HUVEC human umbilical vein endothelial cells (5 x 10^6 cells/mL) were cultured in EGM supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and bovine brain extract. Cells were grown to confluence and trypsinized. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 68.8 ng/mL.

KG-1 human acute myelogenous leukemia cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 102 ng/mL.

Raji human Burkitt's lymphoma cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 284 ng/mL.

COLO 205 human colorectal adenocarcinoma cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. 1.5 x 10^6 cells/mL were lysed, and the supernate was assayed and measured 660 ng/mL.

SPECIFICITY

This assay recognizes natural human Cytochrome c.

The factors listed below were prepared in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared in a mid-range human Cytochrome c control were assayed for interference.

Recombinants:

Apo Cytochrome c
bovine Cytochrome c
canine Cytochrome c
equine Cytochrome c
mouse Cytochrome c
porcine Cytochrome c
rat Cytochrome c

Recombinant rabbit Cytochrome c cross-reacts approximately 0.67% in this assay.

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