# *Human Cytochrome c ELISA*

***650 070 096*** **1 x 96 tests**
***650 070 192*** **2 x 96 tests**

## INTENDED USE

The Cytochrome c ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human cytochrome c in cell culture lysates, human whole blood or serum. **The cytochrome C ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

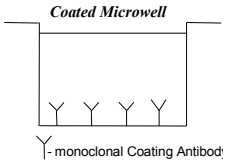
## SUMMARY

Apoptotic cell death is a fundamental feature of virtually all cells (5). It is an indispensable process during normal development, tissue homeostasis, development of nervous system and 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 induced cell death suggests that cells activate a common death program, towards which diverse signal – transducing pathways converge (2,17,18).
The mitochondria turned out to participate in the central control or executioner phase of the death cascade (1). Cytochrome c was identified as a component required for crutial 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).
Mitochondrial cytochrome c is a water – soluble protein of 15 kDa with a net positive charge, residing loosely attached in mitochondrial intermenbrane space. Cytochrome c functions in respiratory chain by interaction with redox partners. It is highly conserved during evolution. Like most mitochondrial proteins cytochrome c is encoded by a nuclear gene and synthesized as cytoplasmic precursor molecule, apocytochrome c, which becomes selectively imported into the mitochondrial intermembran 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 cytosol leads to an activation of an apopototic program via activation of a caspase dependent pathway (12,15,13,4). 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 (10,11,3). Bcl-2 on the other hand was shown to be able to prevent apoptosis by blocking the release of cytochrome c from mitochondria (18).

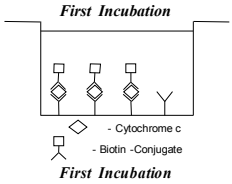
Measurment of cytochrome c release from mitochondria is tool to detect the first early steps for initiating apoptosis in cells. Cytochrome c release in cytosol occurs prior to the activation of caspases and DNA fragmentation which is considered the hallmark of apoptosis.
Detection of cytochrome c release from the mitochondria to the cytoplasm can be achieved by selective lysis of cell membrane.
Very recently it has been shown that the mitochondria dwelling molecule can be detected in medium already 1 h after apoptosis. Moreover, elevated cytochrome c levels were observed in serum from patients with hematological malignancies. In course of cancer chemotherapy, the serum-cytochrome c level grew rapidly and it decreased gradually as the patient was cleared from malignant cells. Thus, serum- cytochrome c monitoring might serve as a clinical marker indicating the onset of apoptisis and cell turn-over in vivo (9)

## PRINCIPLES OF THE TEST

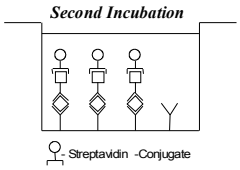
An anti-cytochrome c monoclonal coating antibody is adsorbed onto microwells.



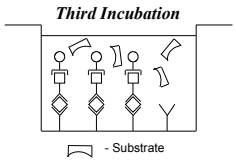

Cytochrome c present in the sample or standard binds to the antibodies adsorbed to the microwells; a biotin-conjugatemonoclonal anti-Cytochrome c antibody is added and binds to cytochrome c captured by the first antibody.

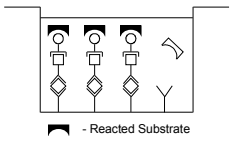



Following incubation unbound biotin conjugated anti-cytochrome c is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-cytochrome c. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.




A coloured product is formed in proportion to the amount of cytochrome c present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven cytochrome cstandard dilutions and cytochrome c sample concentration determined.




## REAGENTS PROVIDED

| REAGENTS (store at 2-8°C) | QUANTITY 1 plate | QUANTITY 2 plates | RECONSTITUTION |
|---|---|---|---|
| 96-wells precoated microtiter plate coated with monoclonal antibody (murine) | 1 | 2 vials | Ready-to-use |
| Plate covers | 4 | 8 | |
| Cytochrome c Standard: 10 ng /ml | 2 vials | 4 vials | Reconstitute with the volume of distilled water indicated on the vial |
| Biotin-Conjugate anti cytochrome c monoclonal (murine) antibody* | 1 vial | 2 vials | (0.1ml) Dilute 100 times in Assay Buffer |
| Streptavidin-HRP* | 1 vial | 2 vials | (150 µl) Dilute 200 times in Assay Buffer |
| Assay Buffer Concentrate* | 1 vial | 2 vials | (5 ml) 20X concentrate. Dilute in distilled water |
| Wash Buffer Concentrate* | 1 bottle | 2 bottles | (50 ml) 20X concentrate. Dilute in distilled water |
| Lysis buffer | 1 bottle | 2 bottles | (15 ml), 10X. |
| Substrate Solution | 1 vial | 2 vials | (15 ml) Ready-to-use |
| Stop Solution (1 M Phosphoric acid) | 1 vial | 2 vials | (12 ml) Ready-to-use |
| Green Dye | 1 vial | 2 vials | (0.4 ml) Make a 1/100 dilution in the appropriate diluent |
| Red Dye | 1 vial | 2 vials | (0.4 ml) Make a 1/250 dilution in the appropriate diluent |

* Reagents contain preservative

## MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

## SAFETY

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents.The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on box front labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

## SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture lysates, human whole boold and human serum will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separed rapidly before storing at -20°C to avoid loss of bioactive cytochrome c. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

## PREPARATION OF REAGENTS

Prepare Wash Buffer (reagent A) and Assay Buffer (reagent B) before starting with the test procedure.

***1. Wash Buffer***

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table.

| Number of Strips | Wash Buffer Concentrate (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 25 | 475 |
| 1 - 12 | 50 | 950 |

***2. Assay Buffer***

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

| Number of Strips | Assay Buffer Concentrate (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 2.5 | 47.5 |
| 1 - 12 | 5.0 | 95.0 |

***3. Preparation of Cytochrome c Standards***

Reconstitute **Cytochrome C Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Shake or swirly gently to ensure solubilisation. Store reconstituted standard promptly at -20°C after use. Discard after one week.

***4. Preparation of Biotin Conjugate***

Make a 1:100 dilution with **Assay Buffer** (reagent B) in a clean plastic tube as needed according to the following table :

| Number of Strips | Biotin-Conjugate (µl) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 30 | 2.97 |
| 1 - 12 | 60 | 5.94 |

***5. Preparation of Streptavidin-HRP***

Make a 1:200 dilution with Assay Buffer of the concentrated **Streptavidin-HRP** solution as needed according to the following table:

| Number of Strips | Streptavidin-Conjugate (µl) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 30 | 5.97 |
| 1 - 12 | 60 | 11.94 |

***6. Preparation of Lysis Buffer***

Add contents of Lysis buffer concentrate (15ml) to 135 ml distilled or desionized water and mix gently. Store at room temperature.

***7. Addition of Colour-giving Dyes***

In order to help our customers to avoid any mistakes in pipetting, DIACLONE now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.
This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.
Alternatively, the dye solutions from the stocks provided (***Green-Dye, Red Dye***) can be added to the reagents according to the following guidelines:

**A. Biotin-Conjugate:**

Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-conjugate.

| | |
|---|---|
| 3 ml Assay Buffer | 30 µl ***Green-Dye*** |
| 6 ml Assay Buffer | 60 µl ***Green-Dye*** |
| 12 ml Assay Buffer | 120 µl ***Green-Dye*** |

**B. Streptavidin-HRP:**

Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

| | |
|---|---|
| 6 ml Assay Buffer | 24 µl ***Red-Dye*** |
| 12 ml Assay Buffer | 48 µl ***Red-Dye*** |

# TEST PROTOCOL

I. Cell Lysis procedure (cell culture sample)

a. Spin down cells for 15 minutes at 1200 rpm
b. Wash cell pellet once in cold PBS
c. Re-suspend cells in Lysis buffer to a concentration of 1.5 x $10^6$ cells/ml.
d. Incubate for 1 hour at room temperature with gentle shaking.
e. Centrifuge cells at 200 x g for 15 minutes.
f. Dilute the supernatant in assay buffer for the assay at least 50 –fold ( 5µl supernatant + 245 µl Assay Buffer) for assay. Aliquot and store supernatant not used immediately at -70°C

II. Cell Lysis procedure (whole blood samples)

a. Spin down 1 ml of whole blood for 15 minutes at 1200 rpm.
b. Remove plasma (supernatant) carefully.
c. Resuspend cell pellet in 3 ml Lysis buffer.
d. Incubate for 1 hour at room temperature with gently shaking.
e. Spin down for 15 minutes at 200 x g.
f. Dilute the supernatant at least 10 –fold in Assay Buffer and assay immediately. Aliquot supernatant not needed and store at -70°C.

III. Serum samples dilute serum samples before assaying 1:2 in assay buffer (e.g 150 µl serum sample 150 µl assay buffer).

IV.a Mix all reagents thoroughly without foaming before use.

b Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal antibody** (murine) to human cytochrome c from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d Add 100 µl of Assay buffer in duplicate to all standard wells and to the blank well. Prepare standard dilutions by pipetting 100 µl of **reconstituted cytochrome c Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of Cytochrome C standard dilutions ranging from 5 to 0.08 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of Cytocrome C standard dilutions:

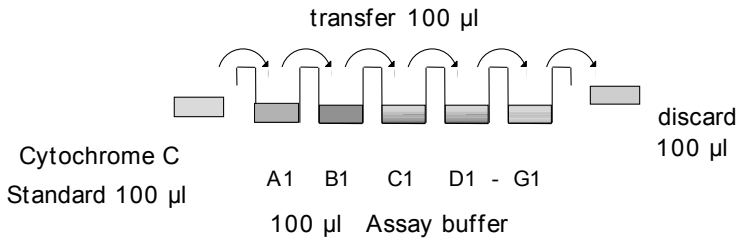


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

| | Standard Concentrations ng/mL | | Sample wells | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 5 | 5 | | | | | | | | | | |
| B | 2.5 | 2.5 | | | | | | | | | | |
| C | 1.25 | 1.25 | | | | | | | | | | |
| D | 0.63 | 0.63 | | | | | | | | | | |
| E | 0.32 | 0.32 | | | | | | | | | | |
| F | 0.16 | 0.16 | | | | | | | | | | |
| G | 0.08 | 0.08 | | | | | | | | | | |
| H | Blank | Blank | | | | | | | | | | |

e. Add 100 µl of each prediluted **Samples,** in duplicate, to the designated wells.

f. Prepare **Biotin-Conjugate**.

g. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.

h. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours

i. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

j. Prepare **Streptavidin-HRP**

k. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

l. Cover with a **Plate Cover** and incubate at room temperature (18º to 25ºC) for 1 hour.

m. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

n. Pipette 100 µl **TMB Substrate Solution** to all wells, including the blank wells.

o. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.
**The colour development on the plate should be monitored and the substrate reaction stopped (see point q. of this protocol) before positive wells are no longer properly recordable**.
It is recommended to add the stop solution when the highest standard has developed a dark blue colour.
Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped ad soon as an OD of 0.6 – 0.65 is reached.

p. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

q. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the Cytochrome c standards.

Note: In case of incubation without shaking the obained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

## CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the cytochrome C concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating Cytochrome C for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding Cytochrome c concentration.

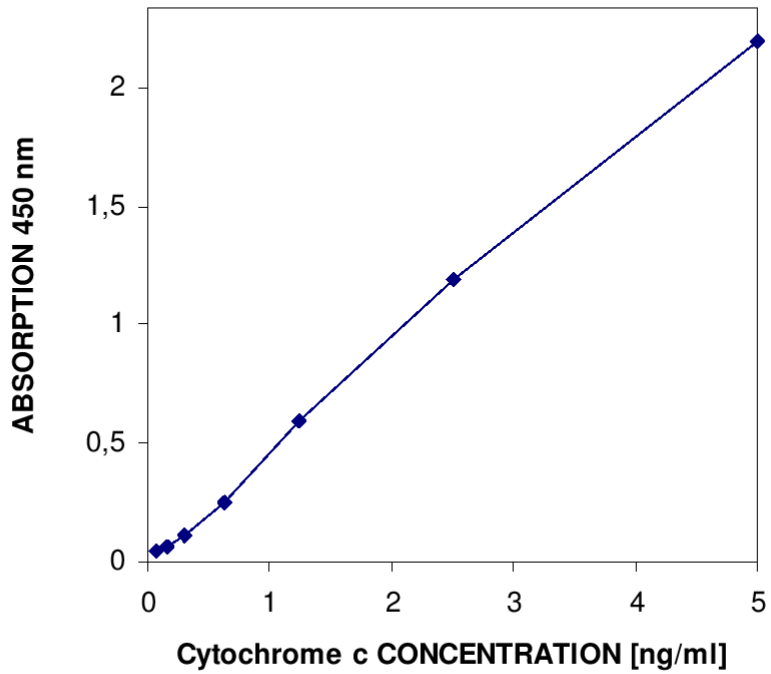
- **For samples which have been diluted according to the instructions given in this manual, the concentration read from the standard curve must be multiplied by the dilution factor .**

  **Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low cytochrome c levels. Such samples require further dilution with assay buffer in order to precisely quantitate the actual cytochrome C level.**

- It is suggested that each testing facility establishes a control sample of known cytochrome C concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for Cytochrome C ELISA. Symbols represent the mean of three parallel titrations.
Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the Cytochrome C ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

| Standard | Cytochrome C Concentration (ng/ml) | O.D. (450 nm) | O.D. Mean | C.V. (%) |
|---|---|---|---|---|
| 1 | 5.00 | 2.105 | 2.196 | 5.8 |
| | 5.00 | 2.286 | | |
| 2 | 2.50 | 1.205 | 1.187 | 2.2 |
| | 2.50 | 1.168 | | |
| 3 | 1.25 | 0.613 | 0.594 | 4.6 |
| | 1.25 | 0.574 | | |
| 4 | 0.63 | 0.264 | 0.254 | 5.5 |
| | 0.63 | 0.244 | | |
| 5 | 0.31 | 0.122 | 0.112 | 12.6 |
| | 0.31 | 0.102 | | |
| 6 | 0.16 | 0.063 | 0.066 | 5.3 |
| | 0.16 | 0.068 | | |
| 7 | 0.08 | 0.047 | 0.048 | 1.5 |
| | 0.08 | 0.048 | | |
| Blank | 0.00 | 0.029 | | |
| | 0.00 | 0.026 | | |

## LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins (serum, ascetic fluid, or monoclonal antibodies of irrevelant specifity) are added to the assay buffer.

## PERFORMANCE CHARACTERISTICS

***Sensitivity***

The limit of detection of cytochrome C defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be less than 0.05 ng/ml (mean of 6 independent assays).

***Reproducibility***

**a. Intra-assay**

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of cytochrome c. Two standard curves were run on each plate. Data below show the mean Cytochrome C concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 6.0 %.

| Positive Sample | Experiment | Cytochrome c Concentration (ng/ml) | Coefficient of Variation (%) |
|---|---|---|---|
| 1 | 1 | 68.5 | 5.7 |
| | 2 | 73.6 | 4.1 |
| | 3 | 70.4 | 1.2 |
| 2 | 1 | 22.1 | 11.4 |
| | 2 | 20.6 | 5.3 |
| | 3 | 21.7 | 1.9 |
| 3 | 1 | 74.1 | 9.5 |
| | 2 | 77.6 | 5.5 |
| | 3 | 72.1 | 1.8 |
| 4 | 1 | 209.6 | 9.0 |
| | 2 | 210.7 | 5.0 |
| | 3 | 193.7 | 7.2 |
| 5 | 1 | 10.1 | 8.5 |
| | 2 | 10.3 | 15.4 |
| | 3 | 9.5 | 9.5 |
| 6 | 1 | 88.2 | 7.9 |
| | 2 | 90.0 | 2.3 |
| | 3 | 79.3 | 4.1 |
| 7 | 1 | 247.6 | 9.9 |
| | 2 | 243.9 | 5.5 |
| | 3 | 241.4 | 3.8 |
| 8 | 1 | 151.0 | 9.6 |
| | 2 | 161.8 | 3.6 |
| | 3 | 155.6 | 0.5 |

**b. Inter-assay**

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of cytochrome c. Two standard curves were run on each plate. Data below show the mean Cytochrome C concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 3.96 %.

| Sample | Cytochrome c Concentration (ng/ml) | Coefficient of Variation (%) |
|---|---|---|
| 1 | 70.8 | 3.6 |
| 2 | 21.4 | 3.7 |
| 3 | 74.6 | 3.8 |
| 4 | 204.7 | 4.7 |
| 5 | 10.0 | 4.5 |
| 6 | 85.8 | 6.7 |
| 7 | 244.3 | 1.3 |
| 8 | 156.1 | 3.4 |

***Spike Recovery***

The spike recovery was evaluated by spiking three levels of cytochrome c into cells lysates. Recoveries were determined in three independent experiments with 6 replicates each. Recoveries ranged from 78 % to 88 % with an overall mean recovery of 82 %.

***Dilution Parallelism***

Four cell lystates with different levels of cytochrome c were assayed at four serial two-fold dilutions with 4 replicates each. In the table below the percent recovery of expected values is listed. Recoveries ranged from 78 % to 119 % with an overall mean recovery of 98 %.

| | | Cytochrome C Concentration (ng/ml) | | |
|---|---|---|---|---|
| Sample | Dilution | Expected Value | Observed Value | % Recovery of Exp. Value |
| 1 | 1:50 | -- | 74 | -- |
| | 1:100 | 37 | 33 | 89 |
| | 1:200 | 19 | 15 | 81 |
| | 1:400 | 9 | 7 | 78 |
| 2 | 1:50 | -- | 81 | -- |
| | 1:100 | 40 | 36 | 89 |
| | 1:200 | 20 | 20 | 97 |
| | 1:400 | 10 | 10 | 100 |
| 3 | 1:50 | -- | 240 | -- |
| | 1:100 | 120 | 133 | 111 |
| | 1:200 | 60 | 59 | 98 |
| | 1:400 | 30 | 27 | 91 |
| 4 | 1:50 | -- | 262 | -- |
| | 1:100 | 131 | 146 | 112 |
| | 1:200 | 66 | 75 | 115 |
| | 1:400 | 33 | 39 | 119 |

***Sample stability***

**a. Freeze-Thaw Stability**

Aliquots of serum samples (unspiked or spiked) were stored frozen at -20°C and thawed up to 5 times, and cytochrome c levels determined. There was a significant loss of cytochrome c by freezing and thawing up to 5 cycles freezing and thawing

**b. Storage stability**

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the cytochrome c level determined after 24 h. There was a significant loss of cytochrome C immunoreactivity during storage at above conditions.

***Specificity***

The interference of circulating factors of the immune systems was evaluated by spiking several of these proteins at physiologically relevant concentrations into a Cytochrome c positive serum. There was no detectable cross reactivity.

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## REAGENT PREPARATION SUMMARY

**A. Wash Buffer** Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

**B. Assay Buffer**

| Number of Strips | Assay Buffer Concentr. (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 2.5 | 47.5 |
| 1 - 12 | 5.0 | 95.0 |

**C. Standard** Reconstitute **Standard** by addition of distilled water. Reconstitution volume is stated on label of the standard vial.

**D. Biotin-Conjugate** Make a 1:100 dilution according to the table.

| Number of Strips | Biotin-Conjugate (µl) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 30 | 2.97 |
| 1 - 12 | 60 | 5.94 |

**E. Streptavidin-HRP**

| Number of Strips | Streptavidin-HRP (µl) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 30 | 5.97 |
| 1 - 12 | 60 | 11.94 |

**F. Lysis Buffer** Add Lysis Buffer Concentrate 10X (15 ml) to 135 ml distilled water

**G. TMB Substrate Solution**

| Number of Strips | Substrate Solution I (ml) | Substrate Solution II (ml) |
|---|---|---|
| 1 - 6 | 3.0 | 3.0 |
| 1 - 12 | 6.0 | 6.0 |

## TEST PROTOCOL SUMMARY

- Prepare and predilute samples
- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Assay buffer**, in duplicate, to standard wells
- Pipette 100 µl **Cytochrome c Standard** into the first wells and create standard dilutions ranging from 5 to 0.08 ng/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Assay buffer**, in duplicate, to the blank wells
- Add 100 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature
- Prepare **Streptavidin-HRP solution**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18º to 25ºC)
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 15 minutes at room temperature (18°to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

**Note: For samples which have been diluted according to the instruction given in this manual, the concentration read from the standard curve must be multiplied by the dilution factor. Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low Cytochrome C levels. Such samples require further dilution of with assay buffer in order to precisely quantitate the actual Cytochrome c level.**