Invitrogen® Cytochrome c ELISA Kit

Cat. No. 99-0040
Size: 96 Tests
For Research Use Only
Lot No. ??????????

INTENDED USE

Invitrogen® Cytochrome c ELISA Kit is an enzyme-linked immunosorbent sandwich assay for quantitative detection of human Cytochrome c in cell lysates, supernatants, whole blood, and serum.

INTRODUCTION

Apoptotic cell death, a fundamental feature of all cells[3], is process during normal development, tissue homeostasis, the nervous system development and immune system regulation. Insufficient or excessive cell death can contribute to human disease, including cancer or degenerative disorders.[6] The highly coordinated and stereotyped manner of this induced cell death suggests that the cells activate a common death program, towards which diverse signal transduction pathways converge.[2] Cytochrome c was shown to redistribute from mitochondria to cytosol during apoptosis in intact cells.[4, 5] Mitochondria participates in the central control of the cell death cascade.[1]

Cytochrome c can be detected by ELISA in cell culture medium as early as one hour from induction of apoptosis[7] in a non-radioactive lymphocyte killing assay, which is 3 hours faster than ^{51}Cr release assay and 8 hours faster than DNA degradation tunnel assays. Cytochrome c is released from apoptotic cells and detectable in serum of anti cancer-drug treated tumor patients.[8] Monitoring of cytochrome c in the serum of patients with tumors might serve as a useful clinical marker for the detection of the onset of apoptosis and cell turnover in vivo.[8]

REAGENTS AND MATERIALS PROVIDED

1 Coated Microwell Plate (coated with Monoclonal Antibody (murine) to human Cytochrome c)
1 vial Biotin-Conjugate anti-Cytochrome c (100 µl)
1 vial Streptavidin-HRP (150 µl) *
2 vials Cytochrome c Standard, lyophilized; 10 ng/ml upon reconstitution
1 bottle 20x Wash Buffer (50 ml) (PBS with 1 % Tween 20)*
1 vial 20x Assay Buffer (5 ml) (PBS with 1 % Tween 20 and 10 % BSA)*
1 bottle 10x Lysis Buffer (15 ml)
1 vial Substrate Solution (15 ml)
1 vial Stop Solution (12 ml) (1M Phosphoric acid)
1 vial each Green-Dye, Red-Dye (0.4 ml each)
4 adhesive Plate Covers
* Reagents containing thimerosal as preservative may be toxic if ingested

MATERIALS REQUIRED BUT NOT PROVIDED

5 ml and 10 ml graduated pipettes, beakers, flasks, and cylinders
10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
50 µl to 300 µl adjustable multichannel micropipette, disposable tips, and reservoir
Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

STORAGE

2° - 8°C

PRINCIPLE OF TEST

Protein of interest in the sample and standards binds to the antibody coated on the plate. A biotin-conjugated antibody is added and binds to protein captured by the first antibody. Streptavidin-HRP is added and binds to the biotin-conjugated antibody. The substrate solution is added to the wells to form the colored products. The reaction is then terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared to determine the protein concentration.

PREPARATION OF REAGENTS

A. Wash Buffer: If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved. Add 50 ml of the 20x Wash Buffer into 950 ml distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should be adjusted to 7.4. Store at 2° to 25°C. The Wash Buffer is stable for 30 days.

B. Assay Buffer: Mix the Assay Buffer well before use. Add 5 ml of 20x Assay Buffer to 95 ml distilled or deionized water. Mix gently to avoid foaming. Store at 2° to 8°C. The Assay Buffer is stable for 30 days.

C. Preparation of Biotin-Conjugate: Make 1:100 dilution with Assay Buffer as needed (50 ul/reaction).

D. Preparation of Standard: Reconstitute standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl gently. Store reconstituted Standard promptly at - 20°C after use. Discard after one week.

E. Preparation of Streptavidin-HRP: Make 1:200 dilution with Assay Buffer as needed (100 ul/reaction).

F. Preparation of Lysis Buffer: Add 15 ml of 10x Lysis Buffer to 135ml distilled or deionized water and mix gently. Store at room temperature.

G. TMB Substrate Solution: Use clean pipettes and containers known to be metal free.. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect the product performance. A blue color in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. Avoid direct exposure of TMB reagent to intense light and oxidizing agents during storage or incubation.

H. Addition of color-giving reagents: Green-Dye, Red-Dye

1. Biotin-Conjugate: Before dilution of the concentrated conjugate, add the *Green-Dye* at a dilution of 1:100.
2. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP; add the *Red-Dye* at a dilution of 1:250.

SAMPLE PREPARATION

I. Cell Lysis procedure (cell culture samples)

a. Spin down cells at 1200 rpm for 15 minutes.
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 1000x g for 15 minutes.
f. Dilute the supernatant in Assay Buffer at least 50-fold (5µl supernatant + 245µl Assay Buffer) for the assay.

II. Cell Lysis procedure (whole blood samples)

a. Spin down 1ml of whole blood at 1200 rpm for 15 minutes.
b. Remove plasma (supernatant) carefully.
c. Re-suspend cell pellet in 3ml Lysis Buffer.
d. Incubate for 1 hour at room temperature with gently shaking.
e. Spin down at 1000 x g for 15 minutes.
f. Dilute the supernatant at least 10-fold in Assay Buffer and assay immediately.

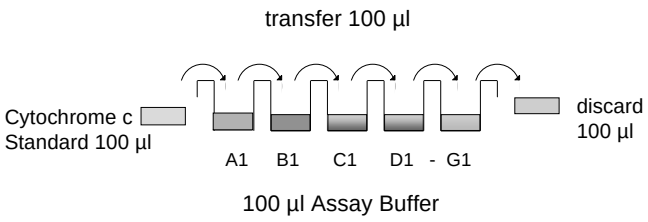
III. Serum samples

Dilute serum samples before assaying 1:2 in assay buffer (e.g. 150µl serum sample + 150µl assay buffer)

PROCEDURE

1. Mix all reagents thoroughly without foaming before use.
2. Wash the microwell strips twice with approximately 300 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Take caution 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.
3. Add 100 µl of Assay Buffer in duplicate to all standard wells and to the blank wells. Prepare standard (1:2 dilution) in duplicate ranging from 0.08ng/ml to 5ng/ml, as shown in Figure 1.

Figure 1. Preparation of Cytochrome c standard dilutions:



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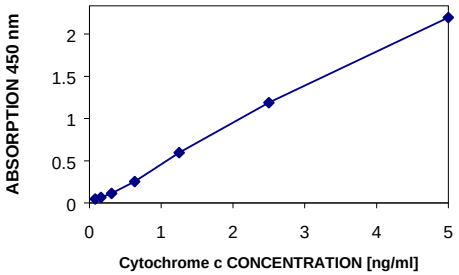
4. Add 100 µl of each prediluted sample, in duplicate, to the designated wells.
5. Add 50 µl of diluted biotin-conjugate to all wells, including the blank wells.
6. Cover with a plate cover and incubate at room temperature for 2 hours.
7. Remove plate cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol.
8. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.
9. Cover with a plate cover and incubate at room temperature for 1 hour.
10. Remove plate cover and empty the wells. Wash microwell strips 3 times as in step 2. Proceed to the next step.
11. Pipette 100 µl of mixed TMB Substrate Solution to all wells, including the blank wells.
12. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light. The point, at which the substrate reaction is stopped, is often determined by the ELISA reader. Many ELISA readers record absorbance only up to 2.0 O.D. Therefore the color development within individual microwells must be watched by the person running the assay and the substrate reaction stopped before positive wells are no longer properly detectable.
13. 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.
14. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable).

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
2. Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the protein concentration on the abscissa. Draw a best-fit curve through the points of the graph.
3. The protein concentration for each sample can be determined using the standard curve.
4. For the 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.
5. It is suggested that a control sample of known concentration is run in each assay.
6. A representative standard curve is shown in Figure 2. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 2. Representative standard curve for Cytochrome c ELISA



PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of Cytochrome c defined as the analyte concentration resulting in the absorption significantly higher than that of the dilution medium (mean plus three standard deviations) was less than 0.08 ng/ml.

B. Reproducibility

I. Intra-assay

Assay was carried out with 6 replicates of 8 samples containing different concentrations of Cytochrome c. The overall intra-assay coefficient of variation was calculated to be 6.0 %.

Table 2. Intra-assay

Sample	Average (ng/ml)	CV (%)
1	70.8	3.7
2	21.5	6.2
3	74.6	5.6
4	204.7	7.1
5	10.0	11.1
6	85.8	4.8
7	244.3	6.4
8	156.1	4.6

II. Inter-assay

Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Cytochrome c. The overall inter-assay coefficient of variation was calculated to be 3.96 %.

Table 2. Inter-assay

Sample	Average (ng/ml)	CV (%)
1	70.8	3.6
2	21.5	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

C. Spike Recovery

The spike recovery was evaluated by spiking three levels of Cytochrome c into cell lysates. Recoveries ranged from 78% to 88 % with an overall mean recovery of 82 %.

D. Specificity

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

REFERENCES:

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RELATED PRODUCTS

Product Name	Clone/PAD*	Cat. No.
Mouse anti-Cytochrome c	6H2B4	33-8200
Mouse anti-Cytochrome c	7H8.2C12	33-8500

*PAD: Polyclonal Antibody Designation

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