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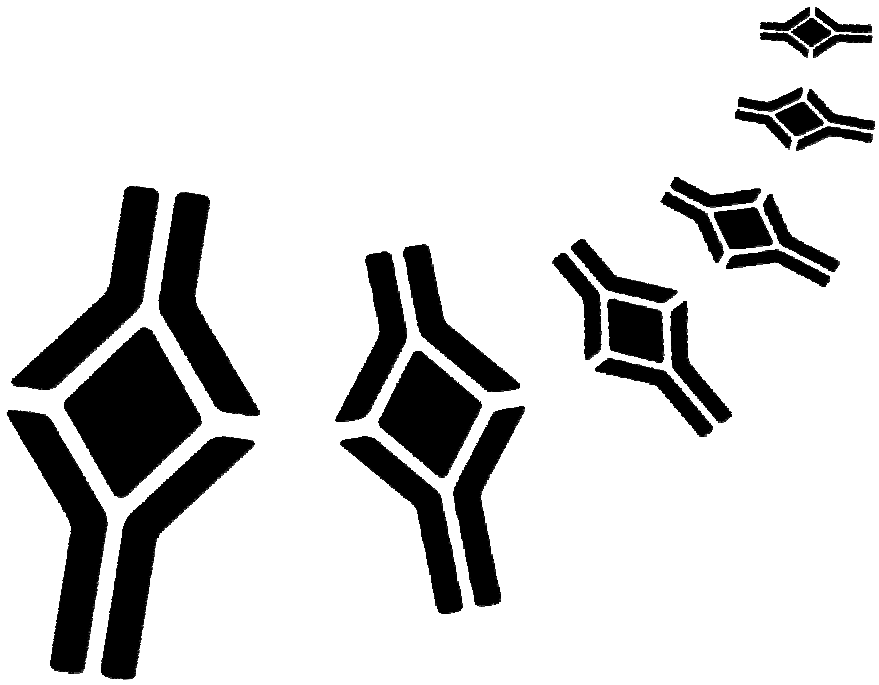


HUMAN Cytochrome c ELISA

Product Data Sheet

Cat. No.: RBMS263R

For Research Use Only

CONTENTS

This kit is manufactured by:
BioVendor – Laboratorní medicína, a.s.

Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The human Cytochrome c ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Cytochrome c. **The human Cytochrome c ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

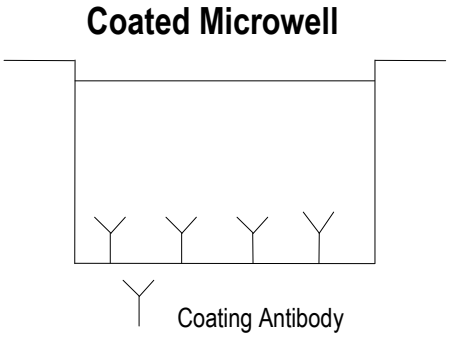
2 SUMMARY

Apoptotic cell death is a fundamental feature of virtually all cells (5). It is an indispensable process during normal development, tissue homeostasis, development of 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 turned out 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, 19).
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 (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).
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 already 1h after apoptosis. Moreover, elevated cytochrome c levels were observed in serum from patients with hematological malignancies. In the 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 apoptosis and cell turn-over in vivo (9).

3 PRINCIPLES OF THE TEST

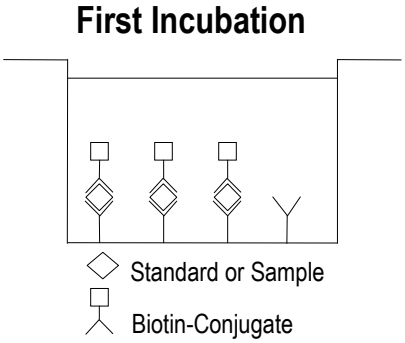
An anti-human Cytochrome c coating antibody is adsorbed onto microwells.

Figure 1



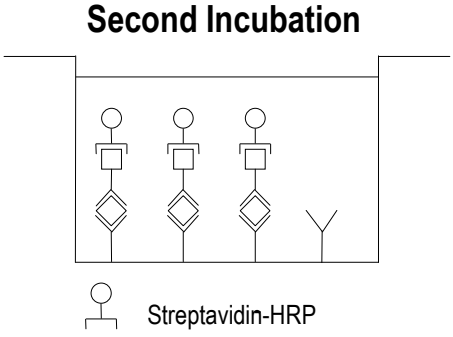
Human Cytochrome c present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human Cytochrome c antibody is added and binds to human Cytochrome c captured by the first antibody.

Figure 2



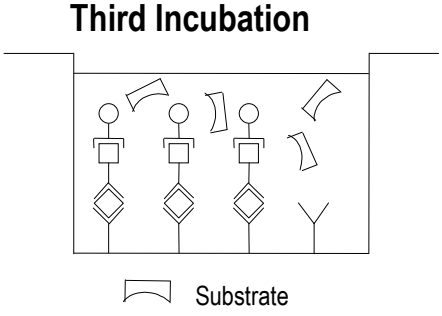
Following incubation unbound biotin-conjugated anti-human Cytochrome c antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Cytochrome c antibody.

Figure 3



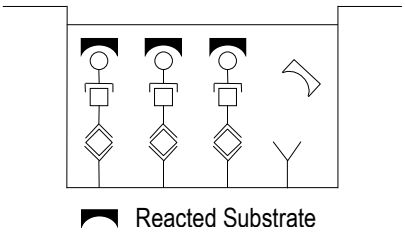
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 4



A coloured product is formed in proportion to the amount of human Cytochrome c present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Cytochrome c standard dilutions and human Cytochrome c sample concentration determined.

Figure 5



4 REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to human Cytochrome c
1 vial (100 µl) **Biotin-Conjugate** anti-human Cytochrome c monoclonal antibody
1 vial (150 µl) **Streptavidin-HRP**
2 vials human Cytochrome c **Standard** lyophilized, 10 ng/ml upon reconstitution
1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
1 bottle (15 ml) **Lysis Buffer** 10x
1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
1 vial (0.4 ml) **Green-Dye**
1 vial (0.4 ml) **Red-Dye**
4 **Adhesive Films**

5 STORAGE INSTRUCTIONS – ELISA KIT

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 labels.
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, this reagent is not contaminated by the first handling.

6 SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS

Cell culture lysates were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.
Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.
Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Cytochrome c. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 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 regression analysis

8 PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- 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.
- Avoid contact of substrate solution 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 reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as 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.

9 PREPARATION OF REAGENTS

Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

9.1 Wash Buffer

If crystals have formed in the **Wash Buffer Concentrate (20x)**, warm it gently until they have completely dissolved. Pour entire contents (50 ml) of the Wash Buffer Concentrate into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.
Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer is stable for 30 days. Wash Buffer may also 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

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
Assay Buffer (1x) may also 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

9.3 Lysis Buffer

Pour the entire contents (15 ml) of the **Lysis Buffer Concentrate** (10x) into a clean 150 ml graduated cylinder. Bring to final volume of 150 ml with distilled or deionized water and mix gently. Store at room temperature. Please note that the Lysis Buffer is stable for 30 days.

9.4 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate(ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

9.5 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP(ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

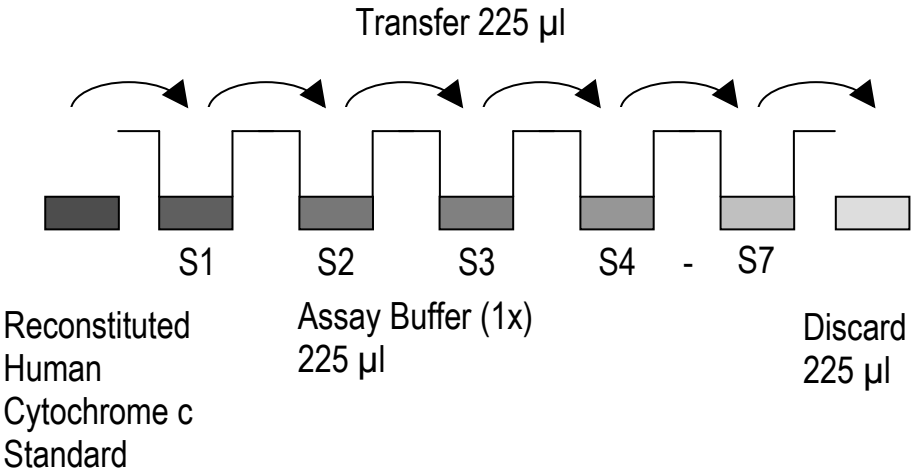
9.6 Human Cytochrome c Standard

Reconstitute **human Cytochrome c standard** by addition of distilled water.
Refer to the Quality Control Sheet for current volume of Distilled water needed for reconstitution of standard.
Swirl or mix gently to insure complete and homogeneous solubilisation
(concentration of reconstituted standard = 10 ng/ml).
Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
After usage remaining standard cannot be stored and has to be discarded.
Standard dilutions can be prepared directly on the microwell plate (see 10.e) or alternatively in tubes (see 9.6.1).

9.6.1 External Standard Dilution

Label 7 tubes, one for each standard point.
S1, S2, S3, S4, S5, S6, S7
Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Assay Buffer (1x) into each tube.
Pipette 225 µl of reconstituted standard (concentration = 10 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 5 ng/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see **Chyba! Nenalezen zdroj odkazů.**Figure 6).
Assay Buffer (1x) serves as blank.

Figure 6



9.7 Addition of Colour-giving Reagents: Green-Dye, Red-Dye

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:

1. Biotin-Conjugate: Before dilution of the concentrated Biotin-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.

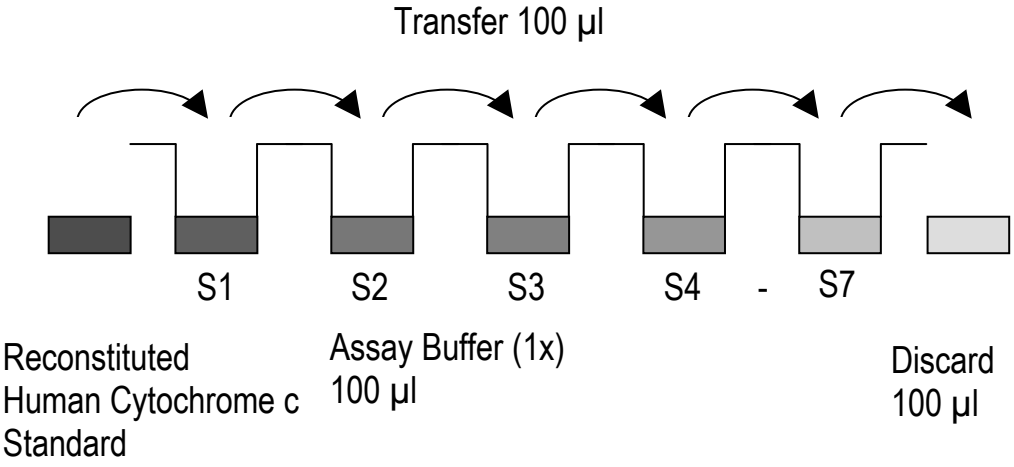
3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***

2. 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 (1x)	24 µl ***Red-Dye***
12 ml Assay Buffer (1x)	48 µl ***Red-Dye***

a. For cell lysis follow the cell lysate protocol
   Cell lysis procedure for cell culture samples:
   Spin down cells for 15 minutes at 1200 rpm. Wash cell pellet once in cold PBS. Re-suspend cells in Lysis Buffer to a concentration of 1.5 x 10^6 cells/ml. Incubate for 1 hour at room temperature with gentle shaking. Centrifuge cells at 200 x g for 15 minutes.
   Dilute the supernatant at least 50-fold in Assay Buffer (1x) (5µl supernatant + 245µl Assay Buffer (1x)) for the assay. Aliquot and store supernatant not used immediately at -70°C.
   Cell lysis procedure for whole blood samples:
   Spin down 1ml of whole blood for 15 minutes at 1200 rpm.
   Remove plasma (supernatant) carefully. Re-suspend cell pellet in 3ml Lysis Buffer.
   Incubate for 1 hour at room temperature with gently shaking. Spin down for 15 minutes at 200 x g. Dilute the supernatant at least 10-fold in Assay Buffer (1x) and assay immediately. Aliquot and store supernatant not used immediately at -70°C.
b. Predilute serum samples before starting with the test procedure 1:2 with Assay Buffer (1x) according to the following scheme:150 µl serum sample + 150 µl Assay Buffer (1x).
c. 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 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
d. Wash the microwell strips twice with approximately 400 µl **Wash** Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells
   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.
e. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.6.1): Add 100 µl of Assay Buffer (1x) in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.6, concentration = 10 ng/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 5 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human Cytochrome c standard dilutions ranging from 5.00 to 0.08 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7



In case of an **external standard dilution** (see 9.6.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (5.00 ng/ml)	Standard 1 (5.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (2.50 ng/ml)	Standard 2 (2.50 ng/ml)	Sample 2	Sample 2
C	Standard 3 (1.25 ng/ml)	Standard 3 (1.25 ng/ml)	Sample 3	Sample 3
D	Standard 4 (0.63 ng/ml)	Standard 4 (0.63 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.31 ng/ml)	Standard 5 (0.31 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.16 ng/ml)	Standard 6 (0.16 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.08 ng/ml)	Standard 7 (0.08 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

f. Add 100 µl of **Assay Buffer (1x)** in duplicate to the **blank wells**.
g. Add 100 µl of each prediluted **sample** in duplicate to the **sample wells**.
h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.4).
i. Add 50 µl of **Biotin-Conjugate** to all wells.
j. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours.
k. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 0).
l. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point d. of the test protocol. Proceed immediately to the next step.
m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

n. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour.
o. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point d. of the test protocol. Proceed immediately to the next step.
p. Pipette 100 µl of **TMB Substrate Solution** to all wells.
q. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.
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 as soon as Standard 1 has reached an OD of 0.9 – 0.95.
r. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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.
s. 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 standards.

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

11 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 value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human Cytochrome c concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human 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 human Cytochrome c concentration.
- **For samples which have been diluted according to the instructions given in this protocol, the concentration read from the standard curve must be multiplied by the dilution factor.**

- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Cytochrome c levels (Hook Effect). Such samples require further external predilution according to expected human Cytochrome c values with Assay Buffer (1x) in order to precisely quantitate the actual human Cytochrome c level.**
- It is suggested that each testing facility establishes a control sample of known human Cytochrome c concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8
Representative standard curve for human Cytochrome c ELISA. Human Cytochrome c was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed

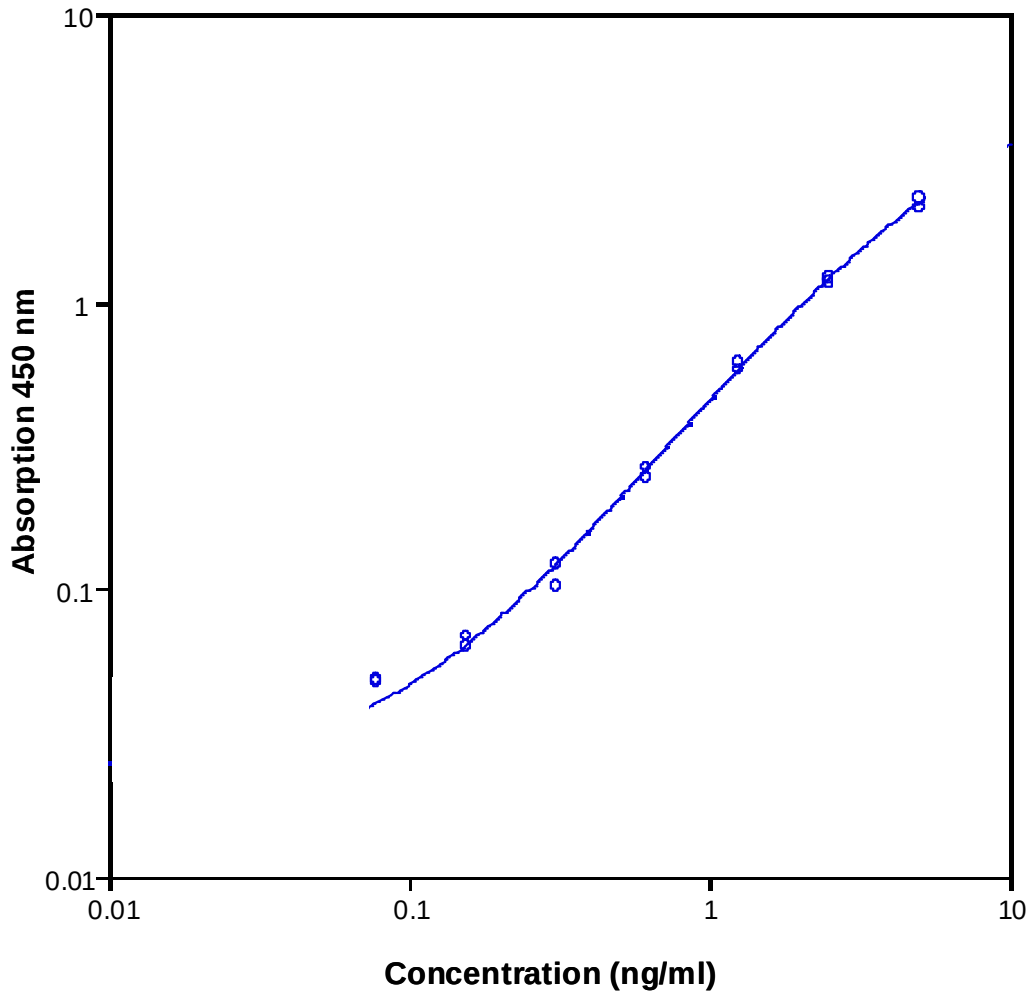


Table 2
Typical data using the human Cytochrome c ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human Cytochrome c Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5.00 5.00	2.105 2.286	2.196	5.8
2	2.50 2.50	1.205 1.168	1.187	2.2
3	1.25 1.25	0.613 0.574	0.594	4.6
4	0.63 0.63	0.264 0.244	0.254	5.5
5	0.31 0.31	0.122 0.102	0.112	12.6
6	0.16 0.16	0.063 0.068	0.066	5.3
7	0.08 0.08	0.047 0.048	0.048	1.5
Blank	0 0	0.029 0.026		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen 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. Empty wells completely before dispensing fresh wash solution, 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 antibodies (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 can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of human Cytochrome c defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.05 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of human Cytochrome c. 2 standard curves were run on each plate. Data below show the mean human Cytochrome c concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.0%.

Table 3
The mean human Cytochrome c concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human 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

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of human Cytochrome c. 2 standard curves were run on each plate. Data below show the mean human Cytochrome c concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.0%.

Table 4
The mean human Cytochrome c concentration and the coefficient of variation of each sample

Sample	Mean Human 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

13.3 Spiking Recovery

The spiking recovery was evaluated by spiking 4 levels of human Cytochrome c into cell lysates. Recoveries were determined in 3 independent experiments with 6 replicates each.
The amount of endogenous human Cytochrome c in unspiked cell lysates was subtracted from the spike values.
The recovery ranged from 78% to 88% with an overall mean recovery of 82%.

13.4 Dilution Linearity

4 cell lysates with different levels of human Cytochrome c were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 78% to 119% with an overall recovery of 98% (see Table 5).

Table 5

Sample	Dilution	Expected Human Cytochrome c Concentration (ng/ml)	Observed Human Cytochrome c Concentration (ng/ml)	Recovery of Expected Human Cytochrome c Concentration (%)
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

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human Cytochrome c levels determined. There was no significant loss of human Cytochrome c immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Cytochrome c level determined after 24 h. There was no significant loss of human Cytochrome c immunoreactivity detected during storage under above conditions.

13.6 Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human Cytochrome c positive sample. There was no crossreactivity detected.

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15 REAGENT PREPARATION SUMMARY

15.1 Wash Buffer

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

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

15.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

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

15.3 Lysis Buffer

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

15.4 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

15.5 Streptavidin-HRP

Make a 1:200 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

15.6 Human Cytochrome c Standard

Reconstitute lyophilized **human Cytochrome c standard** with distilled water. (Reconstitution volume is stated in the Quality Control Sheet.)

16 TEST PROTOCOL SUMMARY

1. Prepare cell extracts by addition of Lysiy Buffer.
2. Predilute serum samples with Assay Buffer (1x) 1:2.
3. Determine the number of microwell strips required.
4. Wash microwell strips twice with Wash Buffer.
5. Standard dilution on the microwell plate: Add 100 µl Assay Buffer (1x), in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells. Alternatively external standard dilution in tubes (see 9.6.1): Pipette 100 µl of these standard dilutions in the microwell strips.
6. Add 100 µl Assay Buffer (1x), in duplicate, to the blank wells.
7. Add 100 µl prediluted sample in duplicate, to designated sample wells.
8. Prepare Biotin-Conjugate.
9. Add 50 µl Biotin-Conjugate to all wells.
10. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
11. Prepare Streptavidin-HRP.
12. Empty and wash microwell strips 3 times with Wash Buffer.
13. Add 100 µl diluted Streptavidin-HRP to all wells.
14. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
15. Empty and wash microwell strips 3 times with Wash Buffer.
16. Add 100 µl of TMB Substrate Solution to all wells.
17. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
18. Add 100 µl Stop Solution to all wells.
19. Blank microwell reader and measure colour intensity at 450 nm.

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

NOTES

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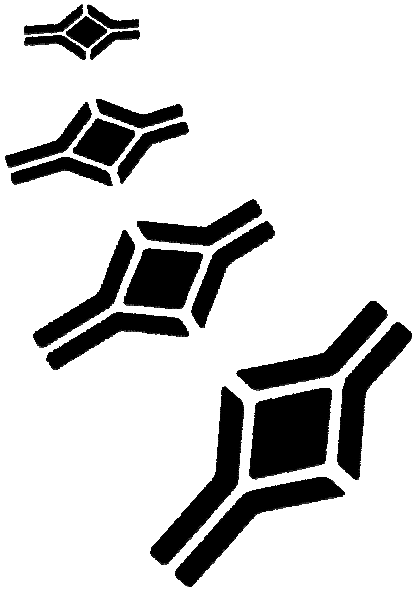

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