

# **ab110172 – Cytochrome C ELISA Kit**

## Instructions for Use

For the measurement of Cytochrome C in Human, mouse, rat and bovine samples of purified mitochondria, tissue and cell extracts.

This product is for research use only and is not intended for diagnostic use.

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## 1. BACKGROUND

Abcam's Cytochrome C *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the measurement of Cytochrome C in Human, mouse, rat and bovine samples of purified mitochondria, tissue and cell extracts.

Abcam's Cytochrome C ELISA kit is used to determine the amount of the cytochrome c in a sample. Cytochrome c is immunocaptured within the wells and the amount is determined by adding a cytochrome c specific antibody conjugated with horseradish peroxidase (HRP). This peroxidase changes the substrate from colorless to blue. This reaction takes place in a time dependent manner proportional to the amount of protein captured in the wells. The rate of development of blue color can be followed at 600 nm or the reaction can be stopped, at a user defined time, by the addition of 100  $\mu$ L of 1.5 N HCl to each well (not supplied) and read at 450 nm.

The permeabilization of the mitochondrial outer membrane and the consequent release of cytochrome c and other apoptogenic proteins from the mitochondrial intermembrane space into cytoplasm is considered hallmark of many apoptotic pathways leading to cell death. Therefore assaying these proteins in mitochondrial and cytoplasmic fractions is a prime interest of many researchers. Most of the current biochemical methods of quantification of cytoplasmic and mitochondrial cytochrome c involve mechanical disruption of cells to obtain mitochondria-enriched and cytosolic fractions. These methods are time-consuming and limited to small number of samples.

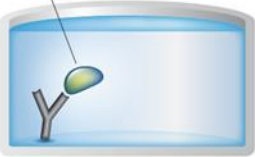
This cytochrome c detection assay was developed in conjunction with Abcam's ab109719 Cell Fractionation Kit - Standard and ab109718 Cell Fractionation Kit - HT, and the results were validated with ab110417 ApoTrack™ Cytochrome c Apoptosis Cocktail and with ab110415 ApoTrack™ Apoptosis Detection Kit for Immunocytochemistry.

## 2. ASSAY SUMMARY

### Primary capture antibody



### Sample



### Detector Antibody



### HRP Label



### Substrate **Colored product**



Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.

Add standard or sample to each well used. Incubate at room temperature.

Aspirate and wash each well. Add prepared Detector Antibody to each well. Incubate at room temperature.

Aspirate and wash each well. Add prepared HRP label. Incubate at room temperature.

Aspirate and wash each well. Add HRP Development Solution to each well. Immediately begin recording the color development. Alternatively add a Stop solution at a user-defined time.

### 3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

### 4. STORAGE AND STABILITY

Store kit at temperature stated in Section 5 immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Reagent and Sample Preparation sections.

### 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
20X Buffer (Tube 1)	1 x 15 mL	+2-8°C
10X Blocking Buffer (Tube 2)	1 x 15 mL	+2-8°C
20X Wash Buffer (Tube 3)	1 x 15 mL	+2-8°C
Development Solution	1 x 20 mL	+2-8°C
Detergent (contains 10% SDS)*	1 x 1 mL	+2-8°C
20X Detector Antibody (Tube A)	1 x 1 mL	+2-8°C
20X HRP Label (Tube B)	1 x 1 mL	+2-8°C
96 – Well microplate (12 strips)	1 x 96 wells	+2-8°C

\* Referred to as 10% SDS in the protocol.

### **6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 600 or 450 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi and single channel pipettes.
- PBS (1.4 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , 140 mM NaCl, 2.7 mM KCl, pH 7.3).
- Tubes for standard dilution.
- Stop solution (optional) – 1N Hydrochloric acid (HCl).
- Plate shaker for all incubation steps (optional).
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors) and/or phosphatase inhibitors.

### **7. LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

### 8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 10).
- All samples should be mixed thoroughly and gently.
- Avoid multiply freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps (optional).
- When generating positive control samples, it is advisable to change pipette tips after each step.
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use.

### 9.1 1X Wash Buffer

Prepare 1X Wash Solution by adding 15 mL 20X Wash Buffer (Tube 3) to 285 mL deionized water. Mix gently and thoroughly.

### 9.2 Incubation Solution

Prepare Incubation Solution by adding 5 mL of 10X Blocking Buffer to 45 mL of 1X Wash Buffer in a clean bottle or tube. Store prepared Incubation Solution at 4°C.

### 9.3 1X Cytochrome C Detector Antibody (Solution A)

Prepare 1X Cytochrome C Detector Antibody by diluting the 20X Cytochrome C Detector Antibody 20-fold with Incubation Buffer immediately prior to use. When using all 12 strips add 1 mL of 20X Detector Antibody (Tube A) to 20 mL of Incubation Solution. Mix gently and thoroughly.

### 9.4 1X HRP Label (Solution B)

Prepare 1X HRP Label by diluting the 20X HRP Label 20-fold with 1X Incubation Buffer immediately prior to use. When using 12 strips, add 1 mL of 20X HRP Label (Tube B) to 20 mL of Incubation Solution. Mix gently and thoroughly.

### 9.5 Solution 1

Prepare Solution 1 by adding 15 mL 20X Buffer (Tube 1) to 285 mL deionized H<sub>2</sub>O. Store prepared Solution 1 at 4°C.

### 9.6 Solution 2

Prepare the Solution 2 by adding 10 mL of 10X Blocking Buffer (Tube 2) to 90 mL of Solution 1. Mix gently and thoroughly. Store prepared Solution 2 at 4°C.



## 10. SAMPLE PREPARATION

### TYPICAL SAMPLE LINEAR RANGE -

Typical linear ranges	
Sample Type	Range
Cultured Cell Extracts	0.5 – 50 µg/mL (3 x 10 <sup>4</sup> – 3 x 10 <sup>5</sup> cells/mL)
Tissue Extracts	1 – 10 µg/mL
Pure cytochrome c	1 ng – 100 ng/mL

**Table 1.** Typical intra-assay variations (same day, same sample) <10%. NOTE: the range of the assay can be extended by using a dilution series of normal sample and a curve fit.

This assay is designed for use with purified mitochondria, tissue and cell extracts. Samples should be solubilized with the supplied detergent. The protein concentration should be measured and diluted within the linear range as described below. Control or normal samples should always be included in the assay as a reference. Also include a null or buffer control to act as a background reference measurement.

#### 10.1 Preparation of extracts from fractions generated by Abcam's ab109719 Cell Fractionation Kit - Standard

10.1.1. Thoroughly mix each sample that was prepared as per ab109719. To allow comparison between cell treatments, as per ab109719, samples were re-suspended to the same concentration (3.3 x 10<sup>6</sup> cells/mL, approximately 0.5-1 mg/mL depending on cell type). These samples were then separated into three fractions, cytosolic (C), mitochondrial (M) and nuclear (N) by the ab109719 procedure.

10.1.2. Add 1/10 volume of the provided 10% SDS Detergent to each sample (fraction), so that the final concentration of SDS is 1%. For example, if the total sample volume is 500 µL, add 50 µL of 10% SDS Detergent. If the sample becomes excessively viscous see Section 15 FAQ. Mix immediately. Keep

at room temperature - do not chill. The solution may become cloudy.

- 10.1.3. Add to each sample 9 volumes of Solution 2, so that the final concentration of Detergent in the sample is 0.1% SDS.
- 10.1.4. Spin the samples in a tabletop microfuge at maximum speed (~20,000 g) for 20 minutes.
- 10.1.5. Carefully collect the supernatant and save as sample. Discard the pellet (note that there may be no observable pellet). Keep the samples at room temperature. Proceed to Assay Method.
- 10.1.6. If samples are to be further diluted before being used in the assay, then the samples must be brought back to a final concentration of 0.1% SDS, using the supplier 10% SDS Detergent.

*Note: Since samples (fractions) were diluted 10X in step 10.1.3, they are now derived from  $3.3 \times 10^5$  cells/mL (approx. 50  $\mu$ g/mL).*

### 10.2 Preparation of extracts from fractions generated by Abcam's ab109718 Cell Fractionation Kit – HT

- 10.2.1 Thoroughly mix each sample prepared as per ab109718. To allow comparison between cell treatments, each cytosolic (C), mitochondrial (M) and nuclear (N) fractions were prepared from cells seeded at 15,000/well by the ab109718 procedure. Thus the fractions are derived from  $3 \times 10^5$  cells/mL.
- 10.2.2 Add 1/5 volume of the supplied 10% SDS Detergent Solution to the C and M fractions. Add 1/5 volume of deionized H<sub>2</sub>O to the N fractions. Mix immediately.

*Note: The N fractions already contain Detergent. The Detergent (from kit ab110172) is two times*

*more concentrated than the Detergent HTIII (from kit ab109718)*

For example, if the fraction sample volume is 40  $\mu\text{L}$ , add 10  $\mu\text{L}$  of 10% Detergent Solution or water, as appropriate.

If the sample becomes excessively viscous see Section 15 FAQ. Keep at room temperature – do not chill. The solution may become cloudy.

- 10.2.3 Add to each sample 4 volumes of Solution 2. For example, if the sample volume (after the addition of 10% SDS Detergent or water) is 50  $\mu\text{L}$ , add 200  $\mu\text{L}$  of Solution 2.

The final concentration of SDS in these samples will be 0.1%. Keep the samples at room temperature. Proceed to Assay Method.

- 10.2.4 If samples are to be further diluted before being used in the assay, then the samples must be brought back to a final concentration of 0.1% SDS, using the supplied 10% SDS Detergent.

*Note: Since samples (fractions) were diluted 5X in step 10.2.3, they are now derived from about  $6 \times 10^4$  cells/mL (approx. 10  $\mu\text{g/mL}$ ).*

### 10.3 Preparation for all other samples – e.g. mitochondria, cultured cells and tissues.

- 10.3.1 Measure protein concentration by BCA protein assay. To measure protein concentration, remove a small aliquot of your sample, eg 100  $\mu\text{L}$  and solubilize by adding appropriate volume of supplied 10% SDS Detergent, to achieve a final concentration of 1% SDS.

*Note: If original sample concentration is not at least 10 fold greater than the desired plate loading concentration, the sample should be centrifuged and*

*resuspend in a smaller volume prior to proceeding with the next step.*

- 10.3.2 Dilute the sample to 10X the desired concentration in Solution 1.

Table 1 shows suggested final sample concentrations. For tissue samples see Section 15 FAQ.

- 10.3.3 Add 1/10 volume of 10% SDS Detergent, so that the final concentration of Detergent in the sample is 1% SDS. For example, if the total sample volume is 100  $\mu$ L, add 10  $\mu$ L of 10% SDS Detergent. Mix immediately. Keep at room temperature – do not chill. If the sample becomes excessively viscous see Section 15 FAQ.

- 10.3.4 Add to the sample 9 volumes of Solution 2. so that the final concentration of Detergent in the sample is 0.1% SDS

*Note: The sample is now at the desired concentration.* Spin the sample in a tabletop microfuge at maximum speed (~20,000 g) for 20 minutes.

- 10.3.5 Carefully collect supernatant and save as sample. Discard the pellet (note that there may be no observable pellet). Keep the samples at room temperature. Proceed to Assay Method.

*Note: If samples are to be further diluted before being used in the assay, then the samples must be brought back to a final concentration of 0.1% SDS, using the supplied 10% SDS Detergent.*

### 10.4 Preparation of pure cytochrome c samples

- 10.4.1 Dilute samples in Solution 2 to desired concentration.

10.4.2 To each sample add appropriate volume of 10% SDS Detergent, so that a final concentration of 0.1% is obtained.

10.4.3 Proceed to Assay Method.

### **11. PLATE PREPARATION**

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of 2 wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay.

## **12. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all controls and samples in duplicate.**
  - 12.1 Prepare all reagents and samples as directed in the previous sections.
  - 12.2 Add 200  $\mu\text{L}$  of each sample into individual wells on the plate. Add replicates for accuracy. Also, include a buffer control (200  $\mu\text{L}$  Solution 2) as a null or background reference.
  - 12.3 Incubate the microplate for 3 hours at room temperature.
  - 12.4 The bound monoclonal antibody has immobilized the cytochrome c in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining liquid.
  - 12.5 Add 300  $\mu\text{L}$  of Wash Solution to each well used.
  - 12.6 Empty wells and add 200  $\mu\text{L}$  of Solution A to each well used.
  - 12.7 Incubate the plate for 1 hour at room temperature.
  - 12.8 Empty the wells of the plate and add 300  $\mu\text{L}$  of Wash Solution to each well used.
  - 12.9 Empty the wells and then add 200  $\mu\text{L}$  of Solution B to each well used.
  - 12.10 Incubate the plate for 1 hour at room temperature.
  - 12.11 Empty the wells again and now add 300  $\mu\text{L}$  of Wash Solution to each well. Repeat this step 4 more times.
  - 12.12 Empty the wells and add 200  $\mu\text{L}$  of Development Solution to each well used. Rapidly pop any bubbles that form with a needle.
  - 12.13 Measure the absorbance of each well at 600 nm at room temperature using a kinetic program. Make a measurement every 1.5 minutes for 20 measurements for a total time of

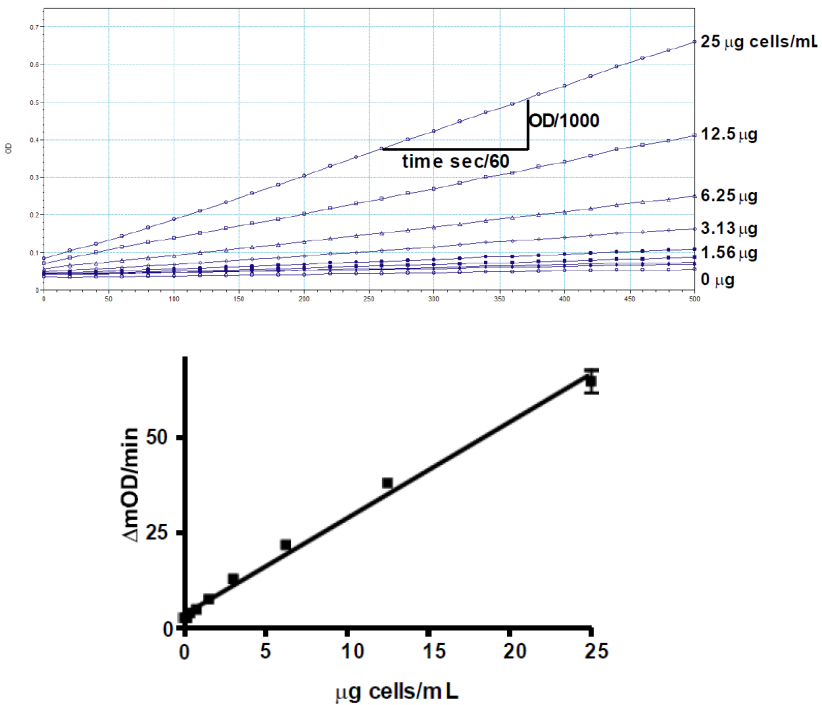
## ASSAY PROCEDURE

30 minutes, with a 3 second auto-mixing step between reads.

*NOTE: If necessary the user can stop the reaction at a desired time by the addition of 100  $\mu$ L of 1.5 N HCl to each well and then measure the endpoint OD 450 nm. Analyze data as described in Section 13 Typical Data.*

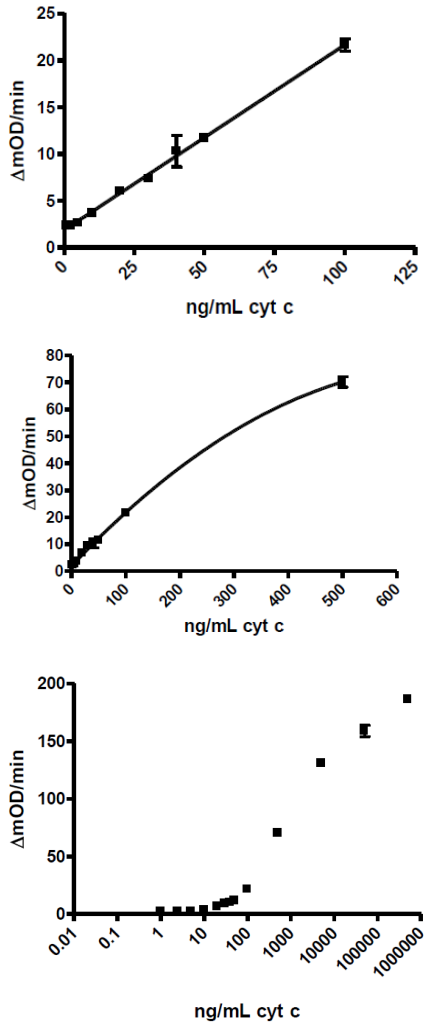
## 13. TYPICAL DATA

- The quantity of cytochrome c is expressed as the amount relative to a normal or control sample.
- When using the kinetic program at OD 600 nm, examine the color development and ensure that the rates are linear as shown below.
- Subtract the initial absorbance reading from the final absorbance reading to determine the relative quantity of cytochrome c captured in each well.
- Shown below (Figure 1 & 2) are rates obtained with extracts from a dilution series of cultured cells (HepG2).



**Figure 1.** The change in absorbance is expressed as change in milliOD/min. In the example above the change OD is divided by 1,000 and the time in seconds is divided by 60 to obtain minutes.



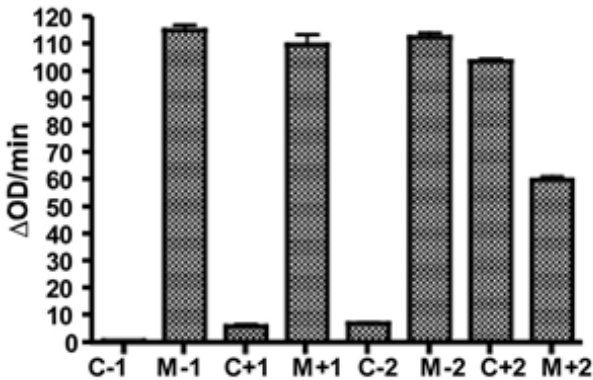


**Figure 2.** Standard curves showing Cytochrome C Concentration (ng/mL) versus the change in absorbance ( $\Delta\text{mOD}/\text{min}$ ).

## EXAMPLE EXPERIMENT #1

Shown below are the relative amounts of cytochrome c in samples derived from the abcam's ab109719 (MS861) Kit (see website for specific details of this kit).

Cytochrome c is detected in a sample only after apoptosis has been initiated and the cell outer membrane has been permeabilized using ab109719 Cell Fractionation Kit Detergent I.



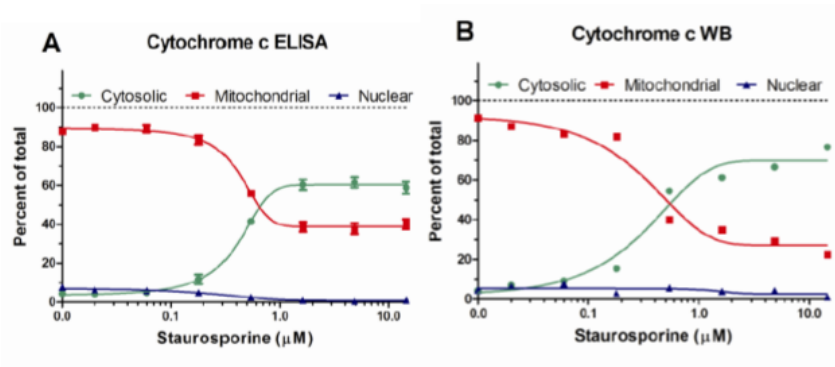
### Key:

- C Cytoplasmic fraction,
- M Pellet containing the mitochondria
- No cell membrane semi-permeabilization
- + Cell membrane permeabilization by addition of ab109719 Cell Fractionation Kit Detergent I.
- 1 No apoptotic induction
- 2 Apoptosis induced with 1  $\mu\text{M}$  staurosporine

## EXAMPLE EXPERIMENT #2

Shown below are the relative amounts of cytochrome c in cytosolic, mitochondrial and nuclear fractions of HeLa cells induced to undergo apoptosis by Staurosporine treatment. The fractions, each derived from one well of a 96-well plate, were prepared with the use of abcam's ab109718 Kit. The amounts of cytochrome c were determined in each fraction by using the ab110172 Kit as described in this Protocol (A) or by Western blot analysis using Abcam's ab110415 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (B). Data (in A) represent mean  $\pm$  standard error of the mean,  $n=4$ .

Staurosporine induces the release of cytochrome c from mitochondria into the cytosol with EC50 0.41 mM.



### **14. SPECIES REACTIVITY**

This kit detects Cytochrome C in Human, mouse, rat and bovine samples.

Other species have not been tested. Cytochrome c is highly conserved among species, therefore it is anticipated that this kit will work with a broad range of species.

Please contact our Technical Support team for more information.

## **15. FREQUENTLY ASKED QUESTIONS**

*What is the minimum amount of cells or tissue needed to accurately measure mitochondrial cytochrome c quantity?*

A signal of 2X background was measured when samples were diluted to 1 µg/mL of cell extract, approximately  $3.3 \times 10^3$  cells (i.e. 0.2 µg/well or 660 cells/well). However, accuracy and reproducibility is not guaranteed when starting with samples of very low concentrations. For tissues and mitochondria it is anticipated that sensitivity will be even greater.

*Is it possible to speed up this assay?*

Antigen-antibody reactions are dependent on many conditions such as temperature and movement of molecules. The higher the temperature and the faster the movement of molecules, the sooner the saturation of binding sites occur. This assay can be performed in about half the time if sample, detector, and label incubations are carried out at 37°C on a rotating platform. However, it is crucial to be consistent with all assays for cross-comparisons. Under this specified conditions, samples can be incubated for 1.5 hours and detector and label steps for 35 minutes each.

*Can I use this plate to determine mitochondrial cytochrome c quantity in tissues from rodents or other animal models?*

This plate can be used with mouse and rat samples. Cross reactivity with other species has not been tested with other species. However due to the very high level of sequence homologies of cytochrome c it is anticipated that this assay might function with a wide range of species.

*Which immunogen was used to develop the antibodies used in this kit?*

Bovine heart cytochrome c.

*What evidence do you have that the captured protein is in fact pure cytochrome c?*

Immunoprecipitations using these antibodies were performed on a large scale and analyzed by SDS-PAGE and mass spectrometry for purity.

Which is the exact epitope that binds to the capture (attached to the plate) and the detector antibodies provided with this kit?

The exact epitopes are unknown.

My sample is extremely viscous. What should I do?

Samples extracted from high concentrations of cells may become very viscous due to the presence of DNA. To avoid pipetting errors it is important to mechanically shear the DNA or digest the DNA by a nuclease. The shearing is best done using a probe sonicator. Alternatively the DNA may be sheered by very vigorous vortexing for about two minutes. The nuclease digestion can be done by addition of Benzonase Nuclease at 25 U/mL.

How do I analyze tissue samples?

Tissue samples must be homogenous before detergent extraction. Ensure homogenous tissue samples thoroughly by using a dounce homogenizer (see Abcam's ab110169 Isolation Kit) or microtissue grinder. Measure the protein concentration by BCA protein assay. Dilute to the desired protein concentration in Solution 1, then proceed with detergent extraction Section 10, step 10.3.2.

16. NOTES

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