



**ab210575 –
Mouse/Rat Cytochrome
C SimpleStep ELISA[®]
Kit**

Instructions for use:

For the quantitative measurement of Cytochrome C in mouse and rat cell and tissue extract samples.

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 SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Cytochrome C protein in mouse and rat cell and tissue extracts.

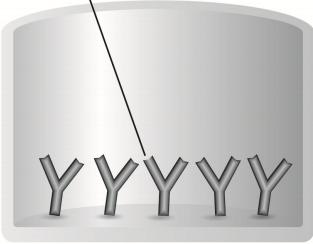
The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Cytochrome C is 11 kDa mitochondrial intermembrane space electron carrier protein. The oxidized form of the cytochrome C heme group can accept an electron from the heme group of the cytochrome c1 subunit of cytochrome reductase. Cytochrome C then transfers this electron to the cytochrome oxidase complex, the final protein carrier in the mitochondrial electron-transport chain.

Cytochrome C also plays a role in apoptosis. Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial outer membrane permeability resulting in release of cytochrome C into the cytosol. Binding of cytochrome C to Apaf-1 triggers the activation of caspase-9, which then accelerates apoptosis by activating other caspases.

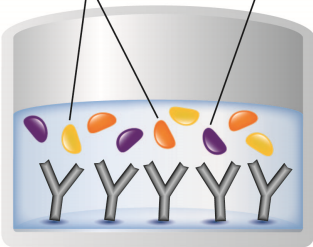
2. ASSAY SUMMARY

Immobilization Antibody



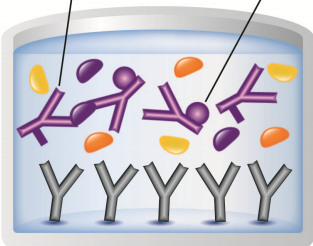
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.

Matrix Proteins Target Analyte



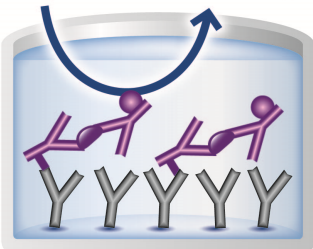
Add standard or sample to appropriate wells.

Capture Antibody Detector Antibody



Add Antibody Cocktail to all wells. Incubate at room temperature.

Substrate Color Development



TMB substrate addition.

Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after

GENERAL INFORMATION

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 2-8°C 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 Standard Preparation sections.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Mouse/Rat Cytochrome C Capture Antibody	600 µL	+2-8°C
10X Mouse/Rat Cytochrome C Detector Antibody	600 µL	+2-8°C
Mouse/Rat Cytochrome C Lyophilized Recombinant Protein	2 Vials	+2-8°C
Antibody Diluent 5BR	6 mL	+2-8°C
10X Wash Buffer PT	20 mL	+2-8°C
5X Cell Extraction Buffer PTR	10 mL	+2-8°C
50X Cell Extraction Enhancer Solution	1 mL	+2-8°C
Denaturant	500 µL	+2-8°C
TMB Substrate	12 mL	+2-8°C
Stop Solution	12 mL	+2-8°C
Sample Diluent NS	12 mL	+2-8°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8°C
Plate Seal	1	+2-8°C

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 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease 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.

GENERAL INFORMATION

- 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 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided 50X Cell Extraction Enhancer Solution and the Denaturant may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution and the 40X Denaturant can be stored at room temperature to avoid precipitation.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **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. The kit contains enough reagents for 96 wells. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer PTR

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 8 mL deionized water and 2 mL 5X Cell Extraction Buffer PTR. Mix thoroughly and gently. If required protease inhibitors can be added.

9.2 1X Cell Extraction Buffer PTR + Enhancer

Prepare 1X Cell Extraction Buffer PTRE by diluting 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTRE combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 μ L 50X Cell Extraction Enhancer Solution. Mix thoroughly and gently. If required protease inhibitors can be added.

9.3 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

9.4 **Antibody Cocktail**

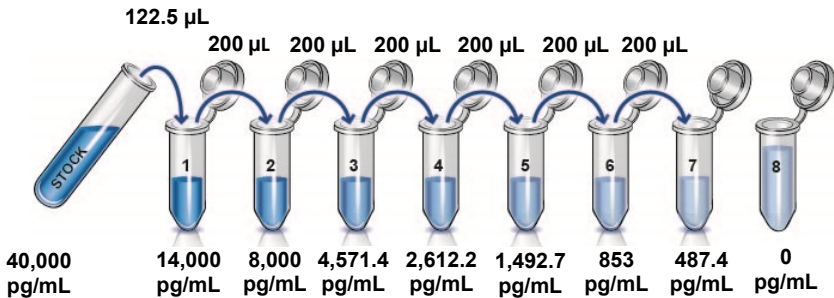
Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent 5BR. Mix thoroughly and gently.

10. STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following section describes the preparation of a standard curve for duplicate measurements (recommended).

- 10.1 **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the Cytochrome C standard by adding that volume of 1X Cell Extraction Buffer PTR + Enhancer indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Cytochrome C standard by adding 500 μL 1X Cell Extraction Buffer PTR + Enhancer. Hold at room temperature for 10 minutes and mix gently. This is the 40,000 pg/mL **Stock Standard** Solution.
- 10.2 Label eight tubes, Standards 1– 8.
- 10.3 Add 227.5 μL 1X Cell Extraction Buffer PTR + Enhancer into tube number 1 and 150 μL of 1X Cell Extraction Buffer PTR + Enhancer into numbers 2-8.
- 10.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
C2C12 Cell Extract	10-100 µg/mL
NIH/3T3 Cell Extract	4-65 µg/mL
C6 Cell Extract	4-100 µg/mL
Mouse Heart Tissue Extract	1-15 µg/mL
Rat Heart Tissue Extract	1-15 µg/mL
Human Heart Tissue Extract	2-50 µg/mL

11.1 Preparation of extracts from cell pellets

- 11.1.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.
- 11.1.2 Rinse cells twice with PBS.
- 11.1.3 Solubilize pellet at 2×10^7 cell/mL in chilled 1X Cell Extraction Buffer PTR + Enhancer.
- 11.1.4 Incubate on ice for 20 minutes.
- 11.1.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.1.6 Transfer the supernatants into clean tubes and discard the pellets.
- 11.1.7 At this point extract samples can be aliquoted and stored at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.1.8 To 39 volume parts of extract add 1 volume part of Denaturant. Mix thoroughly and gently.

ASSAY PREPARATION

- 11.1.9 Incubate samples at room temperature for 5 minutes.
- 11.1.10 Dilute samples 10 fold in 1X Cell Extraction Buffer PTR. Mix thoroughly and gently.
- 11.1.11 Dilute samples further to desired concentration in 1X Cell Extraction Buffer PTR + Enhancer.
- 11.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol)**
 - 11.2.1 Remove growth media and rinse adherent cells 2 times in PBS.
 - 11.2.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR + Enhancer directly to the plate (use 750 μ L - 1.5 mL 1X Cell Extraction Buffer PTR + Enhancer per confluent 15 cm diameter plate).
 - 11.2.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.
 - 11.2.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
 - 11.2.5 Transfer the supernatants into clean tubes and discard the pellets.
 - 11.2.6 At this point extract samples can be aliquoted and stored at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
 - 11.2.7 To 39 volume parts of extract add 1 volume part of Denaturant. Mix thoroughly and gently.
 - 11.2.8 Incubate samples at room temperature for 5 minutes.
 - 11.2.9 Dilute samples 10 fold in 1X Cell Extraction Buffer PTR. Mix thoroughly and gently.
 - 11.2.10 Dilute samples further to desired concentration in 1X Cell Extraction Buffer PTR + Enhancer.

11.3 Preparation of extracts from tissue homogenates

- 11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
- 11.3.2 Homogenize 100 to 200 mg of wet tissue in 500 μ L – 1 mL of chilled 1X Cell Extraction Buffer PTRE. For lower amounts of tissue adjust volumes accordingly.
- 11.3.3 Incubate on ice for 20 minutes.
- 11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.3.5 Transfer the supernatants into clean tubes and discard the pellets.
- 11.3.6 At this point extract samples can be aliquoted and stored at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.3.7 To 39 volume parts of extract add 1 volume part of Denaturant. Mix thoroughly and gently.
- 11.3.8 Incubate samples at room temperature for 5 minutes.
- 11.3.9 Dilute samples 10 fold in 1X Cell Extraction Buffer PTR. Mix thoroughly and gently.
- 11.3.10 Dilute samples further to desired concentration in 1X Cell Extraction Buffer PTRE.

12. 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 plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two 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).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

- 13.1. Prepare all reagents, working standards, and samples as directed in the previous sections.
- 13.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
- 13.3. Add 50 µL of all sample or standard to appropriate wells.
- 13.4. Add 50 µL of the Antibody Cocktail to each well.
- 13.5. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
- 13.6. Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
- 13.7. Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.

Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.

- 13.8. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

ASSAY PROCEDURE

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 μ L Stop Solution to each well and recording the OD at 450 nm.

13.9. Analyze the data as described below.

14. CALCULATIONS

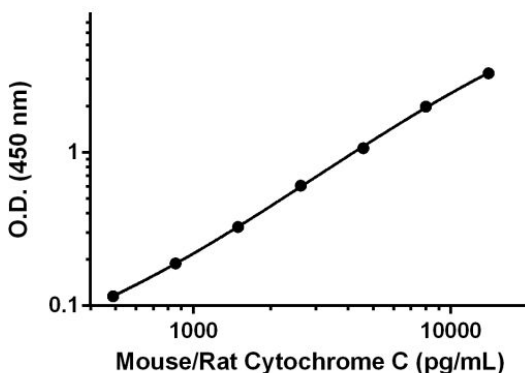
- 14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- 14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

- 14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
- 14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.116	0.114	0.115
487.4	0.237	0.228	0.232
853.0	0.314	0.297	0.306
1,492.7	0.452	0.434	0.443
2,612.2	0.749	0.696	0.722
4,571.4	1.196	1.168	1.182
8,000	2.171	2.039	2.105
14,000	3.317	3.459	3.388

Figure 1. Example of mouse/rat Cytochrome C standard curve. The Cytochrome C standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The calculated minimal detectable dose (MDD) is less than 44 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=27) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of mouse/rat Cytochrome C were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
50% Cell Culture Media Containing 10% Bovine Serum	111	106-116
2 µg/mL Mouse Heart Tissue Extract	108	106-112

DATA ANALYSIS

LINEARITY OF DILUTION –

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Cytochrome C was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTRE.

Dilution Factor	Interpolated value	15 µg/mL Mouse Heart Extract	7 µg/mL Rat Heart Extract	50 µg/mL Human Heart Extract	65 µg/mL NIH/3T3 Cell Extract	100 µg/mL C2C12 Cell Extract	100 µg/mL C6 Cell Extract
Undiluted	pg/mL	9,314	4,589	9,521	11,072	10,921	13,763
	% Expected	100	100	100	100	100	100
1.75	pg/mL	4,983	2,398	5,914	6,115	5,775	7,991
	% Expected	94	91	109	97	93	102
3.06	pg/mL	2,771	1,310	3,204	3,446	2,979	4,260
	% Expected	91	87	103	95	95	95
5.36	pg/mL	1,506	720	1,717	1,890	1,636	2,442
	% Expected	87	84	97	91	105	95
9.38	pg/mL	952	415	972	1078	897.7	1343
	% Expected	96	85	96	91	115	92
16.41	pg/mL	573	NL	583	595	NL	797
	% Expected	101	NL	100	88	NL	95

NL – Non-Linear

PRECISION –

Mean coefficient of variations of interpolated values of Cytochrome C in 3 concentrations of NIH/3T3 cell extract sample within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	5	3
CV (%)	2.7	5.3

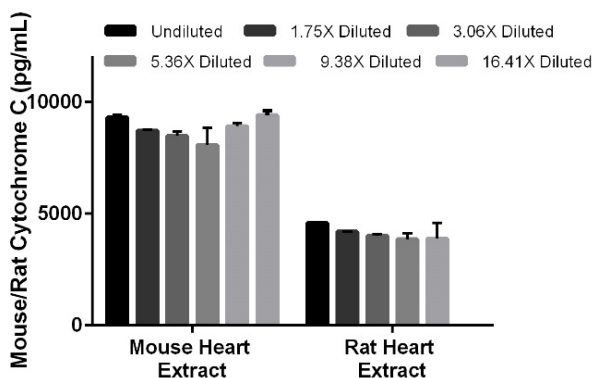


Figure 2. Interpolated concentrations of native Cytochrome C in mouse heart tissue extract samples based on 15 $\mu\text{g}/\text{mL}$ extract load and in rat heart tissue extract samples based on 7 $\mu\text{g}/\text{mL}$ extract load. The concentrations of Cytochrome C were measured in duplicate and interpolated from the Cytochrome C standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean \pm SD, $n=2$). The mean Cytochrome C concentration was determined to be 8,822 pg/mL in mouse heart extract and 4,109 pg/mL in rat heart extract samples.

DATA ANALYSIS

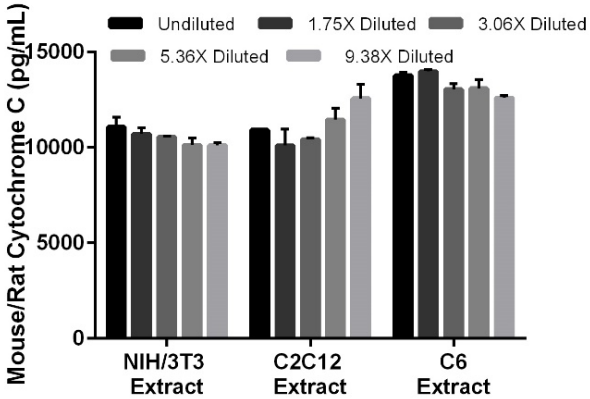


Figure 3. Interpolated concentrations of native Cytochrome C in mouse NIH/3T3 cell extract samples based on 65 $\mu\text{g/mL}$ extract load, and mouse C2C12 and rat C6 cell extract samples based on 100 $\mu\text{g/mL}$ extract load. The concentrations of Cytochrome C were measured in duplicate and interpolated from the Cytochrome C standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean \pm SD, $n=2$). The mean Cytochrome C concentration was determined to be 10,516 pg/mL in NIH/3T3 extract, 11,096 pg/mL in C2C12 extract, and 13,297 pg/mL in C6 extract.

17. ASSAY SPECIFICITY

This kit recognizes both native and recombinant mouse and rat Cytochrome C protein in cell and tissue extract samples only.

Serum, plasma, cell culture supernatants, urine and milk samples have not been tested with this kit.

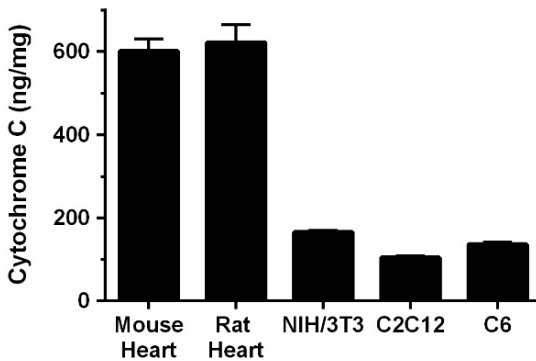


Figure 4. Interpolated concentrations of native Cytochrome C in mouse and rat extract samples. The concentrations of Cytochrome C were measured in three different dilutions in duplicate and interpolated from the Cytochrome C standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted in ng of Cytochrome C per mg of extract (mean \pm SD, n=3). Cytochrome C concentration was determined to be 601 ng/mg in mouse heart, 622 ng/mg in rat heart, 166 ng/mg in mouse NIH/3T3 cell, 105 ng/mg in mouse C2C12 cell and 136 ng/mg in rat C6 cell extract samples.

18. SPECIES REACTIVITY

This kit recognizes mouse and rat Cytochrome C protein.

Human species reactivity was determined by measuring 15 µg/mL human heart extract sample, interpolating the protein concentrations from the mouse/rat standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse heart extract assayed at the same concentration.

Level of reactivity:

Species	% Cross-reactivity
Human	31

Please contact our Technical Support team for more information.

RESOURCES

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. NOTES

RESOURCES

Technical Support

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For all technical or commercial enquiries please go to:

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www.abcam.co.jp/contactus (Japan)