DuoSet® IC

Rat/Mouse Total Cytochrome c

Catalog Number DYC897-2
DYC897-5

Short Format for High Throughput Screening

For the development of semi-quantitative sandwich ELISAs to measure rat or mouse Cytochrome c release from mitochondria.

Note: The reconstitution method has changed. Read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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PRINCIPLE OF THE ASSAY

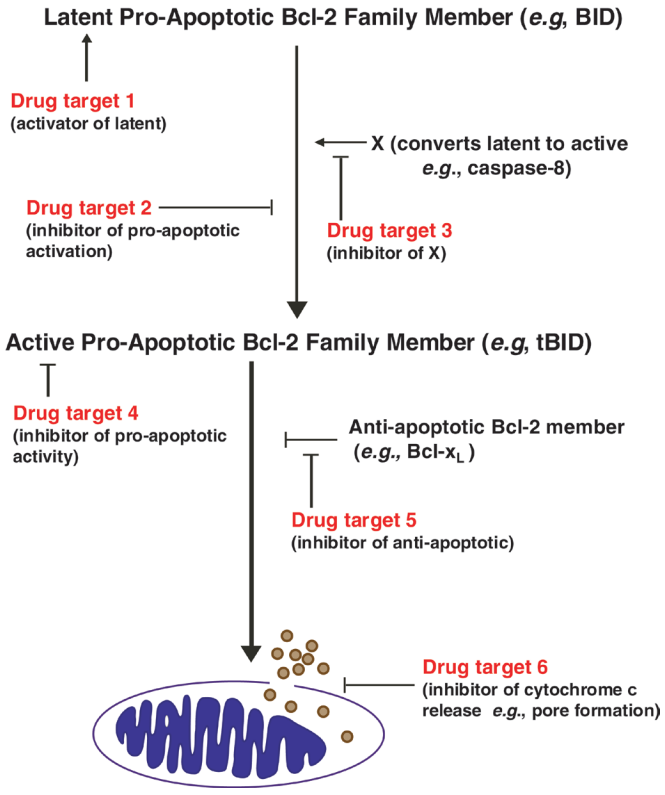
The Rat/Mouse Total Cytochrome c DuoSet® IC ELISA is an endpoint assay designed for use in high throughput drug screening. The assay can identify agonists and antagonists of the Bcl-2 protein family, as well as other proteins or chemical compounds that affect the permeability of the outer mitochondrial membrane to Cytochrome c (see Figure 1). Enriched mouse or rat liver mitochondria are used in the assay and released Cytochrome c is measured when it is "sandwiched" between a capture and detection antibody. Cytochrome c retained in the mitochondria does not have access to the capture and detection antibodies and is removed during washing.

The Rat/Mouse Total Cytochrome c DuoSet® IC ELISA does not depend on equilibrium binding of the antibodies to Cytochrome c . Therefore, it is semi-quantitative and should be used primarily to identify lead compounds. Lead compounds can be subsequently characterized by using the human or rat/mouse Cytochrome c ELISA (R&D Systems®, Catalog # DCTC0 or MCTC0, respectively) that has been developed as a quantitative assay.

The Rat/Mouse Total Cytochrome c DuoSet® IC ELISA provides the user with flexibility in several areas, including:

- Choosing an automated, robotic platform.
- Screening many different proteins or chemical compounds simultaneously.
- Choosing an appropriately sized microplate (96 or 384 wells).

Figure 1



MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	CATALOG # DYC897-2	CATALOG # DYC897-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Rat/Mouse Total Cytochrome c Capture Antibody	840773	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C or ≤ -70 °C for up to 3 months in a manual defrost freezer.*
Rat/Mouse Total Cytochrome c Detection Antibody	840774	1 vial	2 vials	Store for up to 3 months at 2-8 °C.* **DO NOT FREEZE.**
Rat/Mouse Total Cytochrome c Control	840775	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh control for each assay.**

* Provided this is within the expiration date of the kit.

DYC897-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC897-5 contains sufficient materials to run ELISAs on at least five 96 well plates.†

† Provided the following conditions are met:
- The reagents are prepared as described in this package insert.
- The assay is run as described in the Assay Procedure on page 7.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Tocris® # 4139)
- Leupeptin (Tocris® # 1167)
- Pepstatin A (Tocris® # 1190)
- Phenylmethylsulfonyl Fluoride (PMSF) (Sigma # P7626)
- Percoll® (Sigma # P1644)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Triton™ X-100 (Sigma # T9284)
- Centrifuge with a rotor capable of reaching 40,000 x g
- Centrifuge tubes capable of withstanding 40,000 x g
- 7.0 mL Tenbroeck ground glass homogenizer
- Dounce homogenizer
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems®, Catalog # DY990)
- Plate sealers (R&D Systems®, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems®, Catalog # DY006).

Block Buffer - 1% BSA* in PBS with 0.05% NaN_3.

Percoll Buffer - 320 mM Sucrose, 1 mM EGTA and 25 mM HEPES. Adjust pH to 7.2-7.4 with 1 M KOH.

Percoll Solutions - 26% and 60% (v/v) Percoll in Percoll Buffer.

Conjugate Buffer - 300 mM KCl, 40 mM HEPES, 3.2 mM $MgCl_2$, 20 mM Succinic Acid, 20 mM Glutamic Acid, 2.5% BSA*. Adjust pH to 7.2-7.4 with 1 M KOH.

Protein Diluent (Wash Buffer) - 100 mM KCl, 10 mM HEPES. Adjust pH to 7.2-7.4 with 1 M KOH.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems®, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems®, Catalog # DY994).

REAGENT PREPARATION

Rat/Mouse Total Cytochrome c Capture Antibody (Part 840773) - Each vial contains 360 µg/mL of mouse anti-rat/mouse Cytochrome c when reconstituted with 200 µL of PBS.

Rat/Mouse Total Cytochrome c Detection Antibody (Part 840774) - Each vial contains 20 µg/mL of mouse anti-rat/mouse Cytochrome c conjugated to horseradish peroxidase. Prepare only as much detection antibody as required to run each assay. Immediately before use, dilute the detection antibody to a working concentration of 2.0 µg/mL in Conjugate Buffer with the following amounts of protease inhibitors:
100 µg/mL Leupeptin, 100 µg/mL Pepstatin A, 16 µg/mL Aprotinin, and 400 µM Phenylmethylsulfonyl Fluoride (PMSF).

Rat/Mouse Total Cytochrome c Control (Part 840775) - **Refer to the vial label for the stock concentration of natural rat Cytochrome c when reconstituted with 500 µL of Percoll Buffer.**

*The use of Reagent Diluent Concentrate 2 (R&D Systems®, Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8 °C.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet® IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in techniques, plasticware, and water sources.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Protein Diluent should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Protein Diluent by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all controls and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8 °C or be prepared fresh daily.

PLATE PREPARATION

A plate layout is provided to record controls and samples assayed.

1. Dilute the capture antibody to a working concentration of 2.0 µg/mL in PBS. Immediately coat a 96 well microplate with 100 µL per well of the diluted capture antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Protein Diluent, repeating the process two times for a total of 3 washes. Wash by filling each well with Protein Diluent (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Protein Diluent by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

ISOLATION OF MITOCHONDRIA

All solutions and equipment for the isolation of mitochondria should be kept at 2-8 °C. Enriched mitochondria can be stored on ice for 4 hours with no effect on the Cytochrome c assay.

A. Homogenization of Mouse or Rat Liver - All steps and reagents are at 2-8 °C unless noted otherwise.

1. Rinse liver tissue twice with chilled PBS. Homogenize 0.5 g of tissue in 5.0 mL of chilled Percoll Buffer using ten strokes in a chilled 7.0 mL Tenbroeck ground glass homogenizer.
2. Homogenize the resulting slurry with 30 strokes of a tight fitting pestle in a chilled Dounce homogenizer.
3. Dilute the homogenate with 5.0 mL of chilled Percoll Buffer.
4. Centrifuge the homogenate for 10 minutes at 600 x g at 2-8 °C.
5. Save the liver homogenate supernate.

B. Enrichment of Mitochondria Pre-chill solutions and rotor to 2-8 °C.

1. Pipette 20 mL of chilled 26% Percoll solution into a 50 mL centrifuge tube.
2. Carefully insert the pipette tip through the 26% Percoll and pipette 10 mL of chilled 60% Percoll **into the bottom of the tube**. A distinct interface between the 26% and the 60% Percoll solutions should be apparent. Keep on ice.
3. Layer 5.0 mL of liver homogenate supernate from step A5 onto the top of the 26% Percoll.

ISOLATION OF MITOCHONDRIA *continued*

4. Centrifuge for 30 minutes at 40,000 x g at 2-8 °C. The mitochondria will accumulate at the interface between the 26% and 60% Percoll solutions.
   Note: *After centrifugation, the fractionated liver homogenate should have the following appearance*

   - Top layer is a translucent red solution containing the liver homogenate.
   - Buffy band between the homogenate and the 26% Percoll.
   - Partially cloudy 26% Percoll.
   - Buffy band containing mitochondria at the interface between the 26% and 60% Percoll.
   - Clear 60% Percoll often containing a gelatinous material (remove gelatinous material when removing 60% Percoll).
   - A small, firm pellet at the bottom of the tube (avoid collecting the pellet when collecting the 26%/60% Percoll interface).

5. Pipette off the translucent red solution and the 26% Percoll.

6. Quickly push the tip of a pipette through the interface and remove the 60% Percoll solution and gelatinous material in the 60% Percoll fraction until the 6/60% interface is at the bottom of the tube.

7. Collect the interface containing the mitochondria (approximately 1.5 mL).
   Note: *The protein concentration, determined by the Bradford method [Bradford, M.M. (1976) Anal. Biochem. **72**:248], in the 1.5 mL of enriched mitochondria is on average 4.5 mg/mL (average of 17 separate experiments with a range of 2.6-6.3 mg/mL).*

ASSAY PROCEDURE

I. Sample Preparation

A. Dilute proteins or chemical compounds to the desired concentration in the appropriate diluent (*e.g.*, DMSO).

B. Dispense 35 µL of sample/well.
Note: *Samples can be incubated to allow protein-protein or compound-protein interactions to come to equilibrium prior to addition of the detection antibody and mitochondria. This decreases consumption of proteins and chemical compounds. Alternatively, increasing the protein or chemical compound concentrations can eliminate the need for incubations. Users will need to determine the best approach that applies to their applications.*

II. Control Preparation

Controls should be assayed at the same time as experimental samples.

A. Assay controls and blank

1. Reconstitute the control in 500 µL of Percoll Buffer and let it sit for at least 15 minutes at room temperature. Dilute to working concentrations of 200 ng/mL, 50 ng/mL, and 12.5 ng/mL in Percoll Buffer.
2. Add 35 µL of Protein Diluent to the control wells and 45 µL of Protein Diluent to the blank wells.
3. Add 10 µL of each control dilution from step 1 to the control wells.
Do not add mitochondria to blank or control wells.

B. Rat/Mouse Total Mitochondrial Cytochrome c Sample
Rat/Mouse Total mitochondrial Cytochrome c is defined as the amount of Cytochrome c detected when mitochondria are lysed with 0.1% Triton X-100.

1. Add 10 µL of Protein Diluent to the wells that will be used to determine rat/mouse total mitochondrial Cytochrome c.
2. Add 20 µL of 0.25% Triton X-100 in Protein Diluent.
3. Add 5.0 µL of the diluent (*e.g.*, DMSO) used to prepare the samples that are being screened in this assay.

C. Background Cytochrome c in Untreated Mitochondria
Background Cytochrome c is defined as the spontaneous release of Cytochrome c when mitochondria are incubated under assay conditions without added proteins or chemical compounds.

1. Add 30 µL of Protein Diluent to the wells that will be used to determine background Cytochrome c.
2. Add 5.0 µL of diluent (*e.g.*, DMSO) used to prepare the samples.

III. Addition of Detection Antibody

Add 5.0 µL of diluted detection antibody into each well.

IV. Addition of Mitochondria

Enriched mitochondria should be diluted 25-fold in Percoll Buffer. Optimal dilutions for the desired application can be determined by the user. The objective is to minimize the amount of mitochondria in order to maximize the number of assays that can be run with one preparation of mitochondria and to minimize the consumption of target proteins and chemical compounds. At a 25-fold dilution, mitochondria isolated from 0.5 grams of mouse liver is sufficient for more than 3500 assays when 10 µL per well is used.

A. Dilute mitochondria to the appropriate concentration in Percoll Buffer.

B. Add 10 µL of the diluted mitochondria to the appropriate wells. Incubate at 30 °C for 30 minutes.

V. Color Development and Quantitation

A. Aspirate each well and wash by filling the well with Protein Diluent. Repeat four times for a total of five washes.
Note: *Complete removal of liquid at each step is essential for optimal performance.*

B. Add 100 µL of Substrate Solution to each well. Incubate at room temperature for 6 minutes. Avoid placing the plate in direct light.

C. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure mixing.

D. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

TYPICAL DATA

Activity of Human Bcl-2 Family Proteins in the Rat/Mouse Total Cytochrome c Short Format DuoSet® IC.

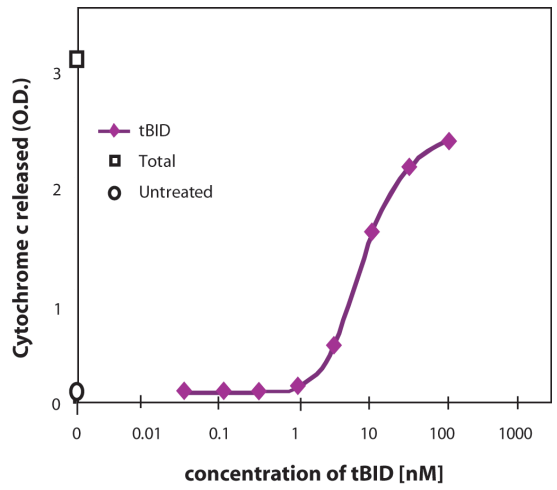


Figure 2: Increasing concentrations of tBID (R&D Systems®, Catalog # 882-B8) were incubated with mitochondria at 30 °C for 30 minutes. Rat/Mouse Total Cytochrome c in the mitochondria was determined by addition of 0.1% Triton X-100. No recombinant proteins were added to untreated mitochondria.

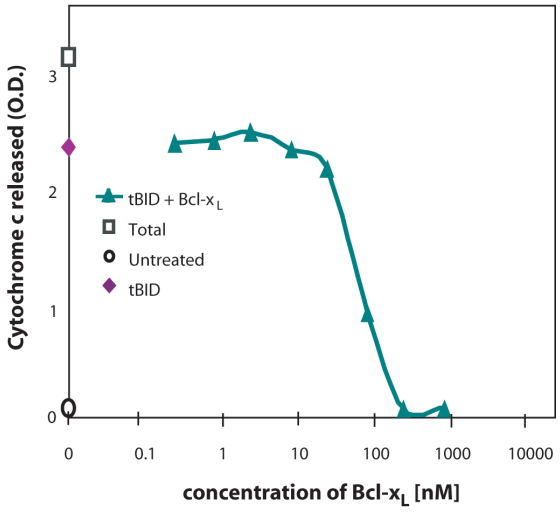


Figure 3: Increasing concentrations of Bcl-xL (R&D Systems®, Catalog # 878-BC) were incubated with 60 nM tBID for 60 minutes prior to addition of mitochondria and subsequent incubation at 30 °C for 30 minutes.

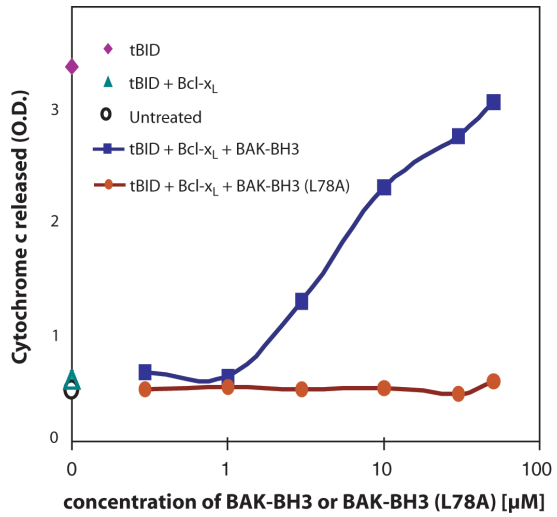


Figure 4: Increasing concentrations of a 22 amino acid wild type BAK-BH3 domain peptide (R&D Systems®, Catalog # 879-BK) or a mutant BAK-BH3 (L78A) domain peptide (R&D Systems®, Catalog # 881-BA) were incubated with Bcl-xL for 30 minutes. tBID was then added for an additional 60 minute incubation prior to addition of mitochondria and subsequent incubation at 30 °C for 30 minutes.

PLATE LAYOUT

Use this plate layout to record controls and samples assayed.