TECHNICAL MANUAL

Rat Cytochrome C ELISA Kit

- **SKU CODE:** RTFI00721
- **SIZE:** 96T/48T
- **DETECTION PRINCIPLE:** Sandwich (Double Antibody)
- **RUO:** Research-Use-Only

Revised: 07/26

Rat Cytochrome C ELISA Kit

Please read entire manual carefully before starting experiment. DO NOT mix reagents and use reagents from different kits or batches to prevent assay failure.

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1. Key Features

Detection Method:

Sandwich, Double Antibody

Sample Type:

Cell or Tissue Lysate, Other Liquid Samples

Reactivity:

Rat

Range:

0.391-25ng/ml

Sensitivity:

0.234ng/ml

2. Storage & Expiry

Assay Genie ELISA Kits are shipped on ice packs. Please store this ELISA Kit and/or components as described in section 4. Date of expiration is on the ELISA Box label.

3. Product Description

The Assay Genie Rat Cytochrome C ELISA Kit is a highly sensitive assay for the quantitative measurement of Cyt-C (Cytochrome C) in the following samples: Cell or Tissue Lysate, Other Liquid Samples.

This kit utilizes a sandwich enzyme-linked immunosorbent assay (ELISA) format. An antibody specific to the target protein is pre-coated onto the wells of a 96-well microplate. Samples and standards are added to the wells, allowing the target to bind to the immobilized antibody. After incubation, unbound substances are removed through washing.

A biotinylated detection antibody is then added, which binds specifically to the captured target. Following a second wash to remove excess detection antibody, HRP-conjugated Streptavidin is introduced, forming a biotin-streptavidin-HRP complex. After a third washing step, TMB substrate is added to initiate a colorimetric reaction catalyzed by HRP. The reaction produces a blue product that turns yellow upon addition of the acidic Stop Solution.

The optical density (OD) is measured at 450 nm using a microplate reader. The OD450 value is directly proportional to the concentration of the target analyte in the sample, which can be determined by referencing a standard curve.

This dual function kit includes validated Bradford Reagent to quantify total protein concentration for accurate sample normalization.

4. Kit Contents

Each kit contains reagents for either 48 or 96 assays, please store the reagents per conditions below.

No	Component Name	Size 48T	Size 96T	Storage
1	ELISA Microplate (Dismountable)	8×6	8×12	Put the rest strips into a sealed foil bag with the desiccant. Stored for 1 month at 2-8°C; Stored for 12 months at -20°C
2	Lyophilized Standard	1vial	2vial	Put the rest standards into a desiccant bag. Stored for 1 month at 2-8°C; Stored for 12 months at -20°C
3	Biotin-labeled Antibody (Concentrated, 100X)	60ul	120ul	2-8°C (Avoid Direct Light)
4	HRP-Streptavidin Conjugate (SABC, 100X)	60ul	120ul	2-8°C (Avoid Direct Light)
5	TMB Substrate	5ml	10ml	2-8°C (Avoid Direct Light)
6	Sample Dilution Buffer	10ml	20ml	2-8°C
7	Antibody Dilution Buffer	5ml	10ml	2-8°C
8	SABC Dilution Buffer	5ml	10ml	2-8°C
9	Stop Solution	5ml	10ml	2-8°C
10	Wash Buffer (25X)	15ml	30ml	2-8°C
11	Bradford Reagent	1 vial	1 vial	4°C
12	Plate Sealer	3 pieces	5 pieces	
13	Technical Manual	1 copy	1 copy	

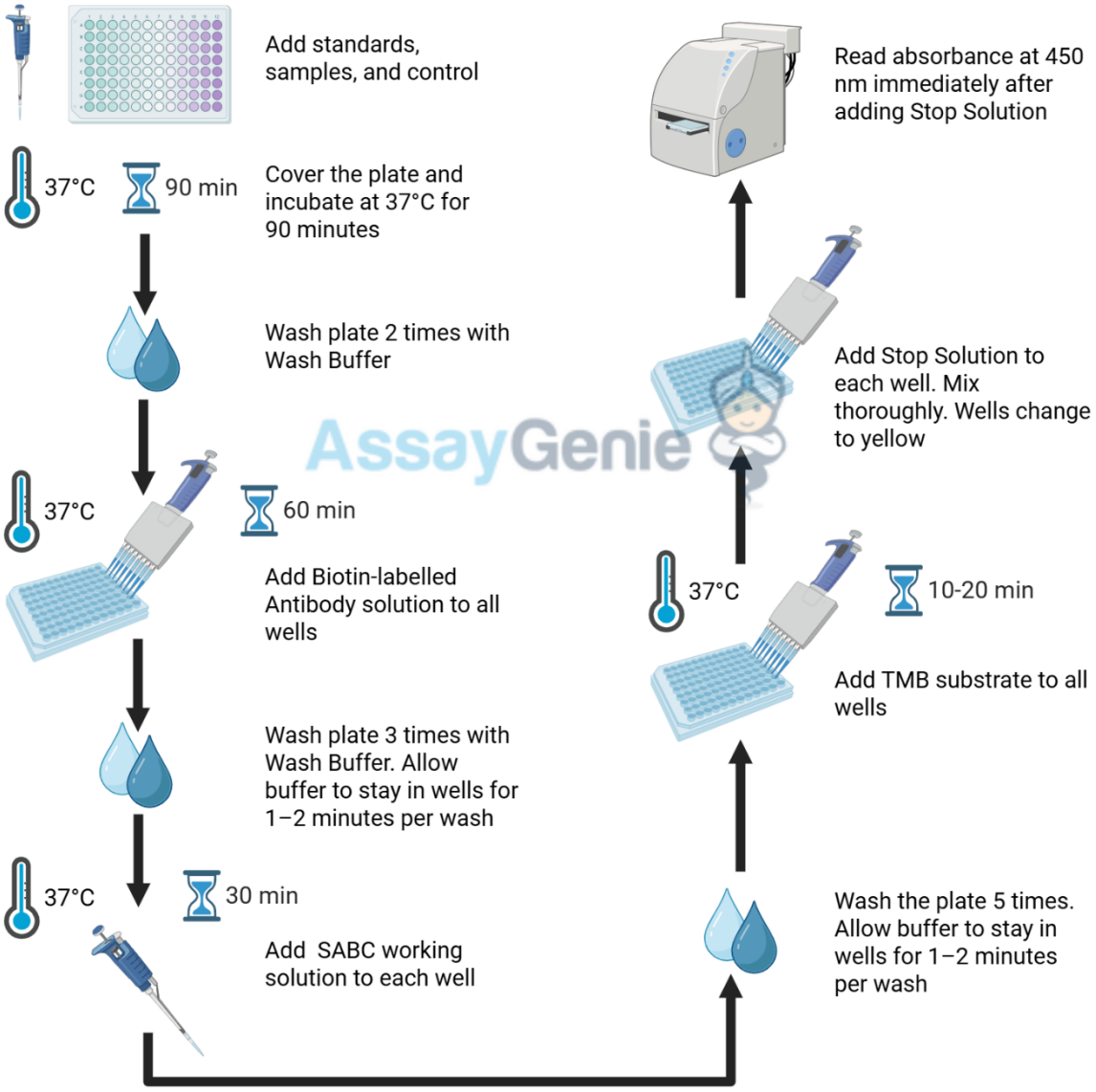
Additional materials required:

1. 37°C incubator.
2. Plate Reader with 450nm filter.
3. Precision pipettes and disposable pipette tips.
4. Distilled water.
5. Disposable tubes for sample dilution.
6. Absorbent paper.

5. Precautions

1. This kit is for research purposes only and not for diagnostics or therapeutic uses.
2. Store all components as listed in this manual. Do not use the ELISA Kit after its expiration date.
3. Allow all reagents and samples to reach room temperature before use.
4. Ensure unopened and unused plates are kept dry to avoid contamination.
5. Before using the kit, centrifuge tubes to spin down standard and/or antibody.
6. Prepare all reagents, samples and standards as directed in this manual.
7. Duplicate wells are recommended for both standard and sample testing.
8. Do not let the microplate wells dry during the assay.
9. Maintain consistent incubation times and temperatures as variations can affect results.
10. Do not reuse tips and tubes to avoid cross contamination.
11. Avoid using the reagents from different batches together.

6. Assay Summary



7. Sample Preparation

The procedures outlined in this document are provided as general recommendations for sample preparation in ELISA assays. Due to the variability of biological samples and specific assay requirements, users are advised to optimize protocols based on their own experimental conditions.

***Note**: For information regarding validation data in specific samples, please contact our Technical Support Team at techsupport@assaygenie.com.*

General Considerations

To prevent denaturation or degradation of target proteins, it is recommended to process samples promptly and store them under appropriate conditions.

- **Storage Conditions**:
    - **Short-term**: 2-8 °C for up to 5 days.
    - **Medium-term**: -20 °C for up to 6 months.
    - **Long-term**: -80 °C or cryopreservation in liquid nitrogen.
- **Thawing Protocol**: Frozen samples should be thawed rapidly in a 15-25 °C water bath to minimize ice crystal-induced damage. Thawed samples can be analyzed immediately or stored temporarily at 2-8 °C.
- **Freeze-Thaw Cycles**: Repeated freeze-thaw cycles should be strictly avoided due to their detrimental effect on protein stability.

A. Blood-Derived Samples

- **Serum:** Allow whole blood to coagulate at room temperature (2 h) or 2-8 °C overnight. Centrifuge at 1000 × g for 20 min and collect the supernatant. Store or use immediately.
- **Plasma:** Collect in anticoagulant tubes (EDTA, citrate, or heparin), mix gently, and centrifuge within 30 min at 1000 × g, 2-8 °C for 15 min. Store or assay as needed.

B. Tissue Homogenates

Tissue samples should be homogenized prior to use. Avoid buffers containing NP-40, Triton X-100, or DTT, as these strongly inhibit the assay. We recommend using 50 mM Tris + 0.9% NaCl + 0.1% SDS, pH 7.3.

The recommended protocol is as follows:

- **Sample Collection and Washing**
    - Place the target tissue on ice.
    - Rinse the tissue with pre-cooled PBS buffer (0.01 M, pH 7.4) to remove residual blood.
    - Weigh the tissue for further processing.
- **Homogenization**
    - Homogenize the tissue on ice using an appropriate lysis buffer.
    - The lysate volume should correspond to the tissue weight; typically, 9 mL PBS is used per 1 g of tissue. It is recommended to add protease inhibitors to the PBS (e.g., 1 mM PMSF). ***Note:*** *PBS buffer or mild RIPA lysis buffer can be used for homogenization. When using RIPA, adjust pH to 7.3.*
- **Cell Disruption**
    - Further disrupt the tissue using ultrasonic homogenization or freeze–thaw cycles.
        - Ultrasonic homogenization: Keep samples on an ice bath during sonication to avoid overheating.
        - Freeze–thaw cycles*:* Repeat twice for effective lysis.
- **Centrifugation and Storage**
    - Centrifuge the homogenate at 5000 × g for 5 minutes.
    - Collect the supernatant for immediate analysis, or aliquot and store at –20°C or –80°C for future assays.
- **Protein Concentration Measurement**
    - Determine total protein concentration using the Bradford Reagent included in this kit.

- For ELISA assays, the total protein concentration should generally be 1–3 mg/mL.
- Tissues with high endogenous peroxidase levels (e.g., liver, kidney, pancreas) may react with TMB substrate, causing false positives. If this occurs, treat samples with 1% H_2O_2 for 15 minutes before repeating the assay.

Note: *Liver, kidney, and pancreas samples often contain high levels of endogenous peroxidase, which may react with the chromogenic substrate at elevated sample concentrations, potentially resulting in false positive signals.*

If analysis of these tissues is required, a gradient dilution assay is recommended. A proportional decrease in signal with increasing dilution typically indicates minimal interference and supports the accuracy of the results.

To further minimise potential interference, samples can be pre-treated with 1% hydrogen peroxide (H_2O_2) for 15 minutes prior to testing. To prepare the treatment solution, add 1 µl of pure H_2O_2 to 100 µl of sample (1% v/v).

C. Cell Culture Supernatant

Centrifuge the sample at 2500 rpm for 5 minutes at 2–8°C. Carefully collect the clarified cell culture supernatant for immediate analysis, or aliquot and store it at –80°C for future assays.

D. Cell Lysates

- **Suspension Cell Lysate:** Centrifuge the cell suspension at 2500 rpm for 5 minutes at 2–8°C and collect the cell pellet. Wash the pellet with pre-cooled PBS (0.01 M, pH 7.4) and mix gently. Repeat centrifugation and discard the supernatant. Add 0.5–1 mL of cell lysis buffer containing an appropriate protease inhibitor (e.g., PMSF, final concentration: 1 mM). Lyse the cells on ice for 30–60 minutes or disrupt them using ultrasonic homogenization.

- **Adherent Cell Lysate:** Remove the supernatant and wash the cells three times with pre-cooled PBS. Add 0.5–1 mL of cell lysis buffer supplemented with an appropriate protease inhibitor (e.g., PMSF at a final concentration of 1 mmol/L). Scrape the adherent cells using a cell scraper and transfer the cell suspension to a centrifuge tube. Lyse the cells on ice for 30–60 minutes, or disrupt the cells by ultrasonic treatment.

Follow next steps for protein extraction and supernatant collection:

- **Protein Release and DNA Disruption**
    - During lysis, pipette gently or intermittently shake the tube to enhance protein extraction.
    - Mucilaginous material formed during lysis is DNA, which can be broken down by ultrasonic disruption (3–5 mm probe, 150–300 W, 3–5 seconds per cycle, with 30-second intervals for 1–2 minutes total).
- **Supernatant collection**
    - After lysis or ultrasonic treatment, centrifuge the lysate at 10,000 rpm for 10 minutes at 2–8°C. Collect the supernatant for immediate use or aliquot and store at –80°C for future assays.

Notes: *Refer to the "Tissue Sample Notes" for additional buffer and inhibitor recommendations.*

E. Other Sample Types

For more information about how to process other sample types, (e.g., body fluids, breast milk & more), please contact our Tech Support Team at techsupport@assaygenie.com.

7.1. Protein Quantification (Optional)

To quantify total protein levels, use the Bradford Reagent included in this kit. Visit Bradford Protein Assay Protocol to view the full protocol.

8. Standard and Reagent Preparation

Manual Washing

Discard the solution in the plate without touching the side of the wells. Clap the plate on absorbent filter paper or other absorbent material. Fill each well completely with 350 µl wash buffer and soak for 1 to 2 mins, then aspirate contents from the plate, and clap the plate on absorbent filter paper or other absorbent material. Repeat this procedure for the designated number of washes.

Automated Washing

Aspirate all wells, then wash plate with 350 µl wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter paper or other absorbent material. It is recommended that the washer is set for a soaking time of 1 minute.

Note: *Set the height of the needles; be sure the fluid can be taken up completely.*

Sample Dilution Guidelines

Determine the concentration of the target protein in the test sample and then select the optimal dilution factor to ensure the target protein concentration falls within the optimal detection range of the kit. Dilute the samples with the dilution buffer provided with the kit. Several dilution tests may be required to achieve the optimal results. The test samples must be well mixed with the dilution buffer. Standard and sample dilution should be performed before starting the experiment.

Note: *Dilution may be necessary to minimize matrix effects. However, if the target concentration in the sample is very low, the pre-treated sample can be added directly to the assay without dilution.*

Reagent Preparation

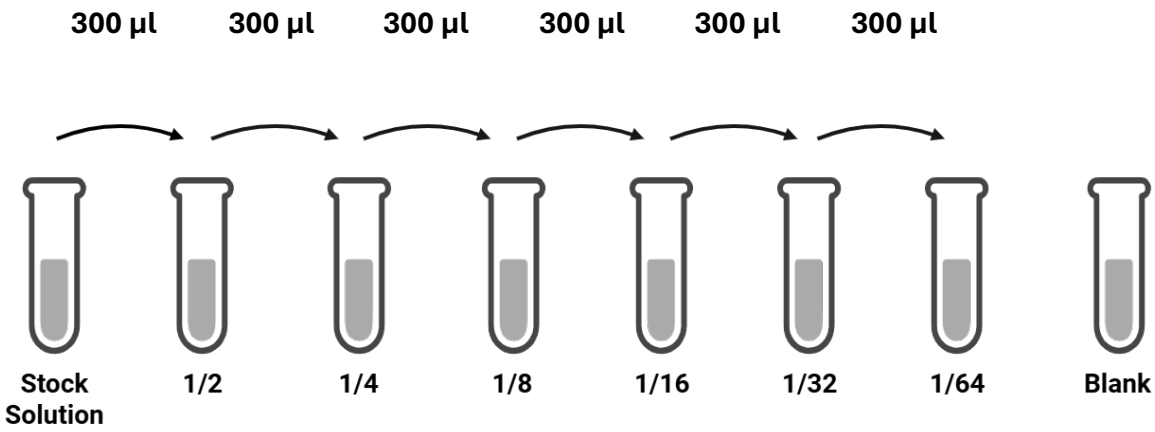
Bring all reagents and samples to room temperature 20 minutes before use (18 - 25°C). For repeated assays, please use only strips and standards required and store remaining reagents at the appropriate temperatures.

A. Wash Buffer:

Dilute 30 ml (15 ml for 48T) of Concentrated Wash Buffer into 750 ml (375 ml for 48T) of Wash Buffer with deionized or distilled water (recommended resistivity of ultrapure water is 18MΩ). Store unused solution at 4°C. If crystals have formed in the concentrate, warm at 40°C in water bath (Heating temperature should not exceed 50°C) and mix gently until crystals have completely dissolved. The solution should be cooled to room temperature before use.

B. Standard Dilution:

1. Centrifuge the standard tube for 1 min at 10,000 x g.
2. Add 1 ml of Sample dilution buffer into one Standard tube (labelled as Stock Solution), keep the tube at room temperature for 10 min. Invert the tube several times to mix (or use a low-speed vortex mixer for 3 – 5 seconds).
3. Finally, centrifuge for 1 min at 1,000 x g to collect liquid at the bottom of the tube and remove bubbles. ***Note:*** *If the concentration of the standard vial differs from the highest value in the standard curve range (see page 3), dilute it with sample buffer to match the highest range value and prepare the stock solution.*
4. Label 7 Eppendorf tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Aliquot 300 µl of the Sample dilution buffer into each tube. Add 300 µl of the above (Stock Solution) standard solution into 1st tube and mix thoroughly. Transfer 300 µl from 1st tube to 2nd tube and mix thoroughly. Transfer 300 µl from 2nd tube to 3rd tube and mix thoroughly, and so on. Sample dilution buffer is used as blank control.



Note: *The standard solutions are best used within 2 hours. The standard solution series should be kept at 4°C for up to 12 hours. Or store at -20 °C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

C. Preparation of biotin-labelled Antibody Working Solution

The antibody working solution should be prepared within 30 minutes of starting the assay and it cannot be stored for a long period of time.

1. Calculate the total volume of the working solution: 100 µl / well × quantity of wells (Allow 100-200 µl more than the total volume).
2. Centrifuge the biotin-labelled antibody for 1 min at 1,000 x g to collect all the liquid at the bottom of the tube.
3. Dilute the biotin-detection antibody with antibody dilution buffer at 1:100 and mix thoroughly (i.e. Add 10 µl of Biotin-labelled Antibody into 990 µl of Antibody Dilution Buffer.)

D. Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution:

1. The SABC working solution should be prepared within 30 minutes of starting the assay and it cannot be stored for a long period of time.
2. Calculate the total volume of the working solution: 100 µl / well × quantity of wells. (Allow 100-200 µl more than the total volume).

3. Centrifuge the concentrated SABC solution for 1 min at 1,000 x g to collect all the liquid at the bottom of the tube.
4. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly. (i.e. Add 1µl of SABC into 99 µl of SABC dilution buffer.)

9. Assay Procedure

1. **Reagent Preparation:** Before adding to the wells, equilibrate the TMB substrate for at least 30 mins at room temperature. When diluting samples and reagents, ensure they are mixed completely and evenly. It is recommended to plot a standard curve for each test.
2. **Plate Setup:** Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate.
3. **Standard Loading:** Aliquot 100 µl of standard solutions into the standard wells.
4. **Control (Blank) Loading**: Add 100 µl of Sample dilution buffer into the control (blank) well.
5. **Samples Loading**: Add 100 µl of properly diluted sample into test sample wells.
6. **First Incubation**: Seal the plate with a cover and incubate at 37 °C for 90 mins.
7. **Washing**: Remove the cover, aspirate the liquid from the plate and wash plate 2 times with 350 µl Wash Buffer. Do NOT let the wells dry completely at any time.
8. **Biotin-labelled Antibody Addition**: Add 100 µl of Biotin-labelled Antibody working solution to the bottom of each well (standard, test sample & zero wells) without touching the side walls.
9. **Second Incubation**: Seal the plate with a cover and incubate at 37°C for 60 mins.
10. **Washing**: Remove the cover, and wash plate 3 times with 350 µl Wash buffer. Let the Wash Buffer stay in the wells for 1-2 minutes for each wash.
11. **SABC Addition (HRP-Streptavidin Conjugate)**: Add 100 µl of SABC working solution into each well, cover the plate and incubate at 37°C for 30 mins.

12. **Wash**: Remove the cover and wash plate 5 times with 350 µl Wash buffer. Let the Wash Buffer stay in the wells for 1-2 minutes for each wash.
13. **TMB Substrate Addition and Colour Development**: Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark for 10-20 mins. (***Note**: This incubation time is for reference only, the optimal time should be determined by the end-user*.) As soon as a blue colour develops in the first 3-4 wells (with most concentrated standards) and the other wells show no obvious colour, terminate the reaction by moving to Step 14.
14. **Stop Reaction**: Add 50 µl of Stop solution into each well and mix thoroughly. The colour changes into yellow immediately.
15. **OD Measurement**: Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

10. Data Analysis

Average the duplicate readings for each standard, control, and sample, then subtract the mean optical density of the zero standard. Construct a standard curve by plotting Cyt-C (Cytochrome C) concentration on the x-axis against absorbance on the y-axis, and fit the data using an appropriate best-fit curve.

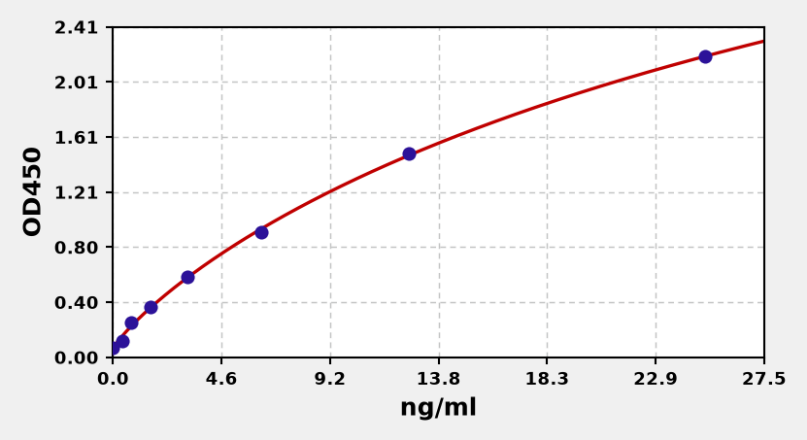
For diluted samples, multiply the concentration obtained from the standard curve by the corresponding dilution factor to determine the final concentration. Data analysis and curve fitting may be performed using suitable plotting software (e.g., CurveExpert).

***Note:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution*.

11. Typical Data

Standard Curve

Results of a typical standard run of an ELISA kit are shown below. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment.



Specificity

This assay has high sensitivity and excellent specificity for detection of Cyt-C (Cytochrome C). No significant cross-reactivity or interference between Cyt-C (Cytochrome C) and other targets was observed.

Note: *Limited by current skills and knowledge, it is difficult for us to complete the cross-reactivity detection between Cyt-C (Cytochrome C) and other analytes, therefore, cross reaction may still exist.*

Recovery

Matrices listed below were spiked with a certain level of Cyt-C and the recovery rates were calculated by comparing the measured value to the expected amount of Cyt-C in the samples.

Matrix	Recovery Range (%)	Average (%)
cell lysates (n=5)	90-105	95

Precision

- Intra-Assay: CV<8%
- Inter-Assay: CV<10%

Stability

The stability of the Cyt-C (Cytochrome C) is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage conditions.

Sample	37°C for 1 month	4°C for 12 months
Average (%)	80	95-100

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

12. ELISA Troubleshooting

Problem	Possible Causes	Solutions
Standard curve without signal	Incorrect reagent order; Mixed components from different kits; Missing reagents.	Ensure correct reagent order and use components from the same kit. Verify all reagents are added.
Overflow OD	Mixed components from different kits; Over-concentrated working solution.	Use correct components and prepare solutions at recommended concentrations.
Poor standard curve	Incorrect curve fitting model.	Try alternative curve fitting models.
Samples without signal	Sample concentration too low; Incompatible buffer; Incorrect preparation; Sample degradation or excessive freeze-thaw.	Reduce dilution or concentrate sample. Check buffer compatibility and follow proper preparation and storage.
High CV%	Precipitate formation; Unclean plate; Foaming; Uneven washing; Incomplete reagent mixing; Pipetting inconsistency.	Dilute samples if needed, avoid foaming, ensure uniform washing, mix reagents thoroughly, and use calibrated pipettes.
Low standard signal	Improperly reconstituted standards; Degraded standards; Incorrect pipetting; Expired kit; Improper storage; Over-dried wells.	Reconstitute standards properly, use fresh kits, follow storage recommendations, and prevent wells from drying.
Slow colour development	TMB not equilibrated; Incorrect microplate reader wavelength; Over-washing.	Pre-warm TMB (30 min at 37°C), confirm correct wavelength (450 nm), and follow recommended washing times.
High background	Insufficient washing; Contaminated wash buffer; Excess detection reagents; Delayed reading; TMB exposed to light.	Wash adequately, prepare fresh wash buffer, use correct reagent amounts, read results promptly, and incubate TMB in the dark.

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Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Manufacturers Statement: This final kit system is assembled and quality-released by Assay Genie Limited.