CytoFluor™

SNAI1 (Phospho-Ser246)
Fluorometric Cell-Based ELISA Kit

Catalog #: FLUO-CBP1479
Reactivity: H:S246, M:S246

Storage: 4°C
Expiration: 6 months from receipt

For Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

Manual Version: 45.7.1527

CONTENTS PAGE

INTRODUCTION

Fluorometric Cell-Based ELISA

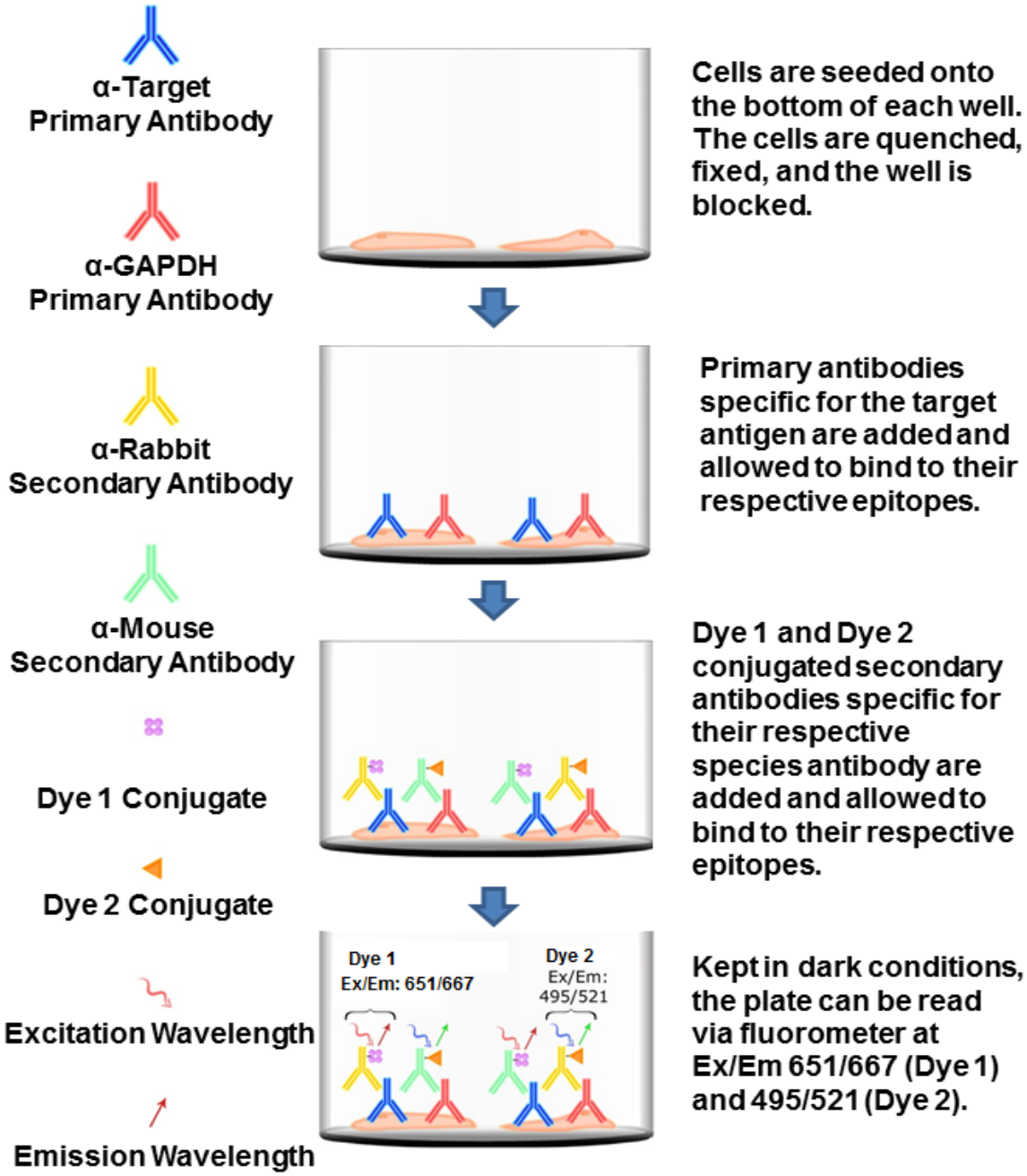
The Fluorometric Cell-Based ELISA Kit allows for the detection of various target proteins and the effects that certain stimulation conditions have on target protein expression in different cell lines. Qualitative determination of target protein concentration is achieved by an indirect ELISA format. In essence, the target protein is captured by target-specific primary (1°) antibodies while Dye 1-conjugated and Dye 2-conjugated secondary (2°) antibodies bind the Fc region of the 1° antibody. Through this binding, the dye conjugated to the 2° antibody can emit light at a certain wavelength given proper excitation, hence allowing for a fluorometric detection method.

A monoclonal antibody specific for human GAPDH is included to serve as an internal positive control in normalizing the target RFU values. If a phosphorylated target is being detected, an antibody against the non-phosphorylated counterpart will be provided for normalization purposes. The RFU values obtained for the non-phosphorylated target can be used to normalize the RFU value for the phosphorylated target.

SNAI1 (Phospho-Ser246) Fluorometric Cell-Based ELISA

The SNAI1 (Phospho-Ser246) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor SNAI1 protein phosphorylation and expression profile in cells. The kit can be used for measuring the relative amounts of phosphorylated SNAI1 in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on SNAI1 phosphorylation.

ASSAY FORMAT



MATERIALS INCLUDED

Component	Quantity	Storage/Stability after first use
10x TBS	24 ml	1 month at 4°C
Quenching Buffer	24 ml	
Blocking Buffer	50 ml	
15x Wash Buffer	50 ml	
Primary Antibody Diluent	12 ml	
100x Anti-SNAI1 (Phospho-Ser246) Antibody	60 µl	
100x Anti-SNAI1 Antibody	60 µl	
100x Anti-GAPDH Antibody	110 µl	
Dye-1 Conjugated Anti-Rabbit IgG Antibody	6 ml	
Dye-2 Conjugated Anti-Mouse IgG Antibody	6 ml	
96-Well Black Cell Culture Clear-Bottom Microplate	2 Plates	-
Adhesive Plate Seals	2 Seals	-

ADDITIONAL MATERIALS REQUIRED

The following materials and equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Fluorescent plate reader with two channels at Ex/Em: 651/667 and 495/521
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- **37% paraformaldehyde stock concentration or 8% paraformaldehyde from other sources**
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent papers or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Orbital shaker
- **Poly-L-Lysine or Poly-D-Lysine for suspension or loosely attaching cells**

ANTIBODY SPECIFICITY

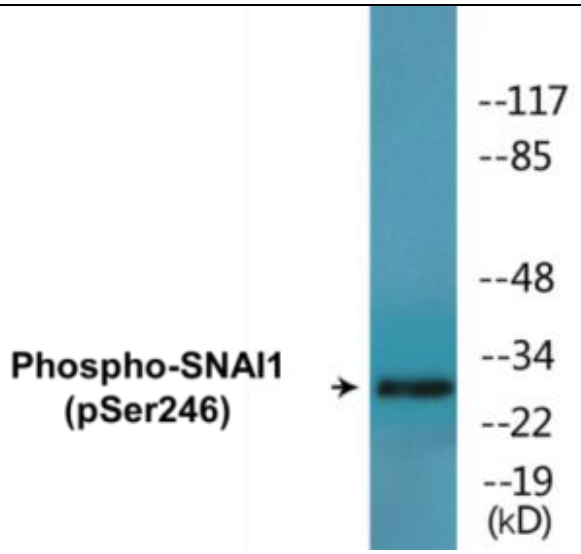


Figure 1. ***Anti-SNAI1 (Phospho-Ser246)***

Western blot analysis of extracts from HT29 cells, using SNAI1 (Phospho-Ser246) Antibody.

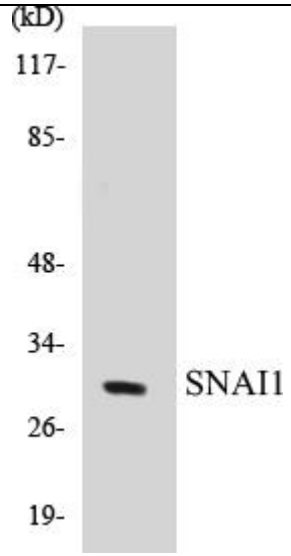


Figure 2. ***Anti-SNAI1 Antibody WB***

Western blot analysis of extracts from HT29 cells

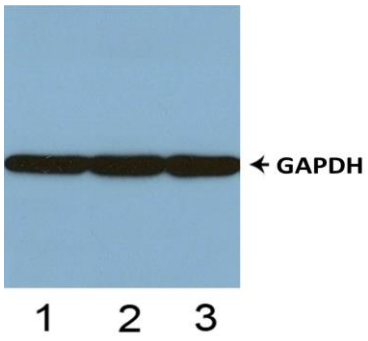
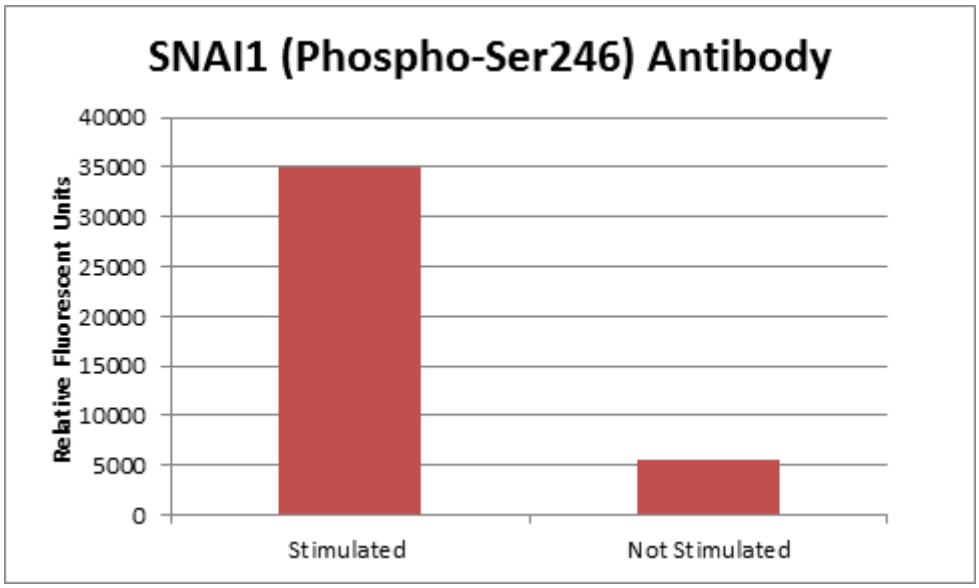


Figure 3. ***Anti-GAPDH Antibody WB***

Western blot analysis of tissue lysates from human (1), mouse (2) and rat (3).

ELISA SPECIFICITY

The Anti-SNAI1 (Phospho-Ser246) and Anti-SNAI1 Antibody were used to measure the effects of stimulation. Data in Figure 5 shows upregulation of the phosphorylated target protein detected via fluorometric reaction.



**** Results may vary based on stimulations and fluorometer used****

Figure 5. Jurkat cells were starved for 24hrs. And stimulated with 1mM of NaF 30 mins

REAGENT PREPARATION

Ensure that all supplied solutions are at ambient temperature before use. It is recommended to conduct assays for all controls and samples in duplicate. Sufficient reagents are provided to assay 192 wells; therefore, on the day of the experiment, prepare only the required amount. During incubation steps, use an orbital shaker set at 200 rpm to ensure proper equilibration of solutions in the cell culture plate wells. **All other provided solutions are ready to use.**

1. Dilute the 10x TBS to 1x TBS by combining 9 volumes of ddH2O and 1 volume of 10x TBS.

2. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH2O and 1 volume of 15x Wash Buffer.

3. Prepare the Fixing solution to 4% paraformaldehyde (for adherent cell lines) or 8% paraformaldehyde (for suspension or loosely attaching cell lines). Dilute 37% paraformaldehyde with 1x PBS to desired paraformaldehyde percentage.

4. For suspension or loosely attached cells: Prepare Poly L Lysine to working concentration as stated in manual. (Not included, recommended Sigma Cat# P4832).

   If the cell line is known to digest Poly-L-Lysine, Poly-D-Lysine may be an alternative for this step. Follow the manufacturer's instructions if using a different Poly-L-Lysine or Poly-D-Lysine.

5. Immediately before use, add 50 ul of 100x Anti-SNAI1 (Phospho-Ser246) Antibody and 50 ul of 100x Anti-GAPDH Antibody to 4,900 ul of Pirmary Antibody Diluent (for one plate, 96 wells). Gently mix and label the tube as "Primary Antibody Mixture Phospho".

6. Immediately before use, add 50 ul of 100x Anti-SNAI1 Antibody and 50 ul of 100x Anti-GAPDH Antibody to 4,900 ul of Primary Antibody Diluent (for one plate, 96 wells). Gently mix and label the tube as "Primary Antibody Mixture Non-Phospho".

7. Immediately before use, for one plate, mix 3 ml of Dye-1 Conjugated Anti-Rabbit IgG Antibody and 3 ml of Dye-2 Conjugated Anti-Mouse IgG Antibody. Gently mix and label tube as "Secondary Antibody Mixture". This solution is light sensitive. Please store and handle in the dark.

ASSAY PROTOCOL

1. Seed 100 µl of 20,000 adherent cells into each well in a 96-well plate.
   Note: Optimal seeding concentrations should be determined by investigator and experimental goals.

   For suspension cells and loosely attached cells:

   a. Coat plates evenly with 20 uL of Poly-L-Lysine (Sigma Cat# P4832).
   b. After 5 minutes, remove solution by aspiration and rinse once with tissue culture grade water.
   c. Allow 2 hours to dry prior to seeding cells.

2. Incubate cells overnight or at least 6 hours at 37°C, 5% CO_2.

3. Treat the cells as desired.

4. Remove the cell culture medium *gently* and fix cells with 100 µl of 8% Paraformaldehyde per well. Incubate for 20 minutes at room temperature (RT).

   Note: 8% Paraformaldehyde solution is volatile. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

5. Aspirate the solution and add 300 µl of 1x Wash Buffer into each well being used and gently shake for 2-3 mins on an orbital shaker. Repeat this process 3 times. After the last wash ensure no liquid remains by inverting the plate and tapping it against clean paper towels.

   Note: The plate can be stored at 4°C for a week after this step.

6. Add 100 µl Quenching Buffer to each well and incubate for 20 mins at RT.

7. Repeat step 5.

8. Add 200 µl of Blocking Buffer and incubate for 1 hour at RT.

9. Add 50 µl of 1x Primary Antibody Mixture solutions into each well and incubate overnight at 4°C. If target concentration is known to be high, incubate for 2 hours at RT. Designate 2 wells for negative control by omitting the 1x Primary Antibodies.

10. Repeat step 5.

11. Add 50 µl of Secondary Antibody Mixture solutions into each well and incubate for 1.5 hrs.

12. Repeat step 5.

13. Read the plate(s) at Ex/Em: 651/667 (Dye 1) and 495/521 (Dye 2). Shield plates from direct light exposure.

DATA ANALYSIS

Background subtraction
Average the duplicate or triplicate absorbance readings for each control and sample, subtracting them from the averaged absorbance for wells where primary antibodies have been omitted.

Anti-GAPDH Internal positive control
The OD450 values obtained ensure the assay is functioning, and cell densities between wells should be proportionate to seeding concentration. Stimulation of cells should impact target primary antibody absorbance values, with no effect on the anti-GAPDH absorbance.

Intra well Anti-GAPDH Normalization
The Anti-GAPDH normalization method enables intensity normalization within the same well. By using the ratio, the fluorescent Em.667 readings obtained can be normalized with the Em.521 values. This constitutes a within-well method of analysis.

a. Em.667 anti-SNAI1 (Phospho-Ser246)/Em.521 Anti-GAPDH.

b. Em.667 anti-SNAI1/Em.521 Anti-GAPDH.

By doing so, the expression levels of SNAI1 (Phospho-Ser246) and SNAI1 are adjusted to account for the cell density present in each well.

Phosphorylation to Non-Phosphorylation Comparison
After Anti-GAPDH Normalization, the resultant proportional values can be used to analyze the effects of stimulants in this assay.

A non-phospho specific primary antibody is included to normalize the absorbance values between phosphorylated to non-phosphorylated values.

Both antibodies utilize the proportion for analysis as follows:

Em.667 (Anti-GAPDH Normalized) (SNAI1 (Phospho-Ser246))/

Em.667 (Anti-GAPDH Normalized) (SNAI1)

SHORT PROTOCOL

Seed cells into wells and incubate overnight at 37°C, 5% CO_2

Apply desired treatment conditions

Add 100ul of Fixing Solution per well and incubate 20 minutes at room temperature

Add 100ul per well of Quenching Buffer and incubate 20 minutes at room temperature

Add 200ul per well of Blocking Buffer and incubate for 1 hour at room temperature

Add 50ul of "Primary Antibody Mixture P" and/or "Primary Antibody Mixture NP" per well and incubate overnight at 4°C

Add 50ul of "Secondary Antibody Mixture" per well and incubate for 1.5 hours at room temperature

Read the plate at Ex/Em: 651/667 (Dye 1) and 495/521 (Dye 2)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin.

- Fixing Solution contains formaldehyde. Formaldehyde is known to be a highly toxic reagent. Personal protection is strongly recommended while working with this chemical.

ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.

- Materials included in this kit should NOT be used past the expiration date on the kit label.

- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.

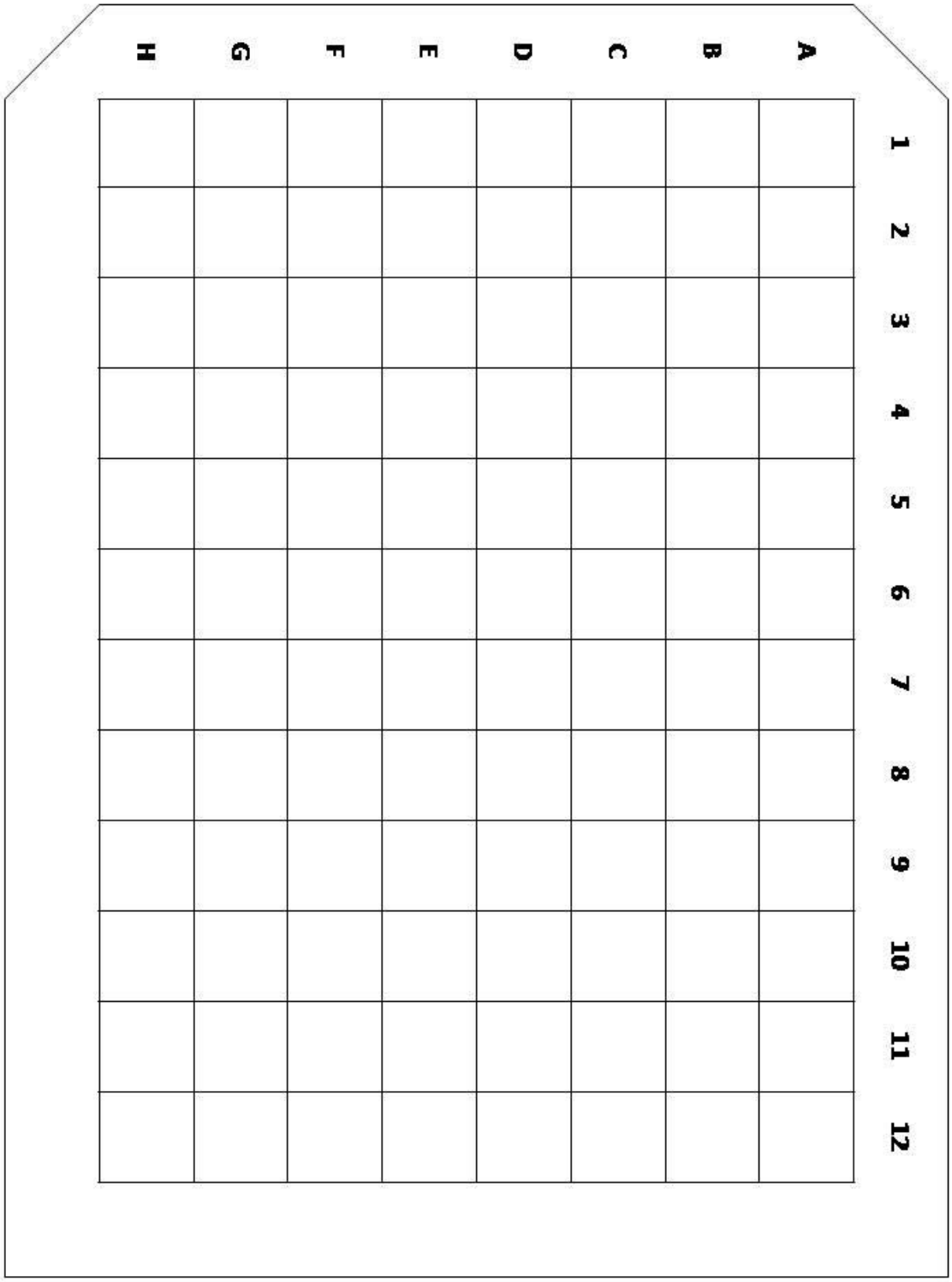
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.

- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.
- Individual results may vary due to differences in technique, plasticware and water sources.

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Low Signal	Storage and Expiration	Read Manual for storage condition and expiration.
	Antibody Dilution	Dilute antibody 1:100 with Antibody Diluent.
	Low Cell Number	Seed more cells.
	Cells Detach from Well-Bottoms	Add solutions slowly from the side wall of the wells. Use Poly-L-Lysine to coat the plate period seeding the cells, if necessary.
High Background	Washing	Remove Wash Buffer completely.
	High Cell Number	Reduce the amount of seeded cells.
Variation	Pipetting	Check and/or calibrate pipettes.
	Washing	Remove Wash Buffer completely.
	Cells Detach from Well-Bottoms	Add solutions slowly from the side wall of the wells. Use Poly-L-Lysine to coat the plate period seeding the cells, if necessary.

ELISA PLATE TEMPLATE



TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.assaybiotech.com or contact us at:

Assay Biotechnology Company, Inc.
47787 Fremont Blvd.
Fremont, CA 94538
United States of America

Email: tech@assaybiotech.com

Phone: (408) 747-0185
Toll-Free Phone: (877) 883-7988

Fax: (408) 747-0145
Toll-Free Fax: (877) 610-9758

Over 2,000 Assay Kits including Sandwich, Cell-Based and Transcription Factor ELISA Kits

Visit us at www.assaybiotech.com