RayBio® Cell-Based
Phosphorylation ELISA Kit - Preliminary

For the semi-quantitative detection of both phosphorylated and pan human, mouse or rat proteins in adherent whole cell lines.

User Manual
(Revised March 8th, 2017)

Cat#: CBEL-XXX-1 (1 plate kit)
Cat#: CBEL-XXX-2 (2 plate kit)
Cat#: CBEL-XXX-5 (5 plate kit)
5 plate kit = 3 total boxes (2 x 2 plate kits & 1 x 1 plate kit)

Please read manual carefully
before starting experiment

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the protein array pioneer company

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Cell-BasedPhosphorylation ELISA Kit - Preliminary

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I. INTRODUCTION

The RayBio® Cell-Based phosphorylation ELISA kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of protein phosphorylation and screening the effects of various treatments, inhibitors (such as siRNA or chemicals), or activators in cultured human, mouse and rat cell lines. By determining protein phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in preparing cell lysate and performing an analysis of Western Blot. In the Cell-Based phosphorylation ELISA kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatments, inhibitors or activators. After blocking, Anti-Phospho or Anti-pan antibodies are pipetted into the wells and incubated. The wells are washed, and HRP-conjugated anti-IgG is added to the corresponding wells. The wells are washed again, a TMB substrate solution is added to the wells and color develops in proportion to the amount of protein. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

See Figure 1 below for an illustration.

<u>What does it mean when a kit is listed as "Custom"?</u>

RayBiotech has a vast library of validated phoshpho antibodies, however not all of them have been developed for use with adherent cell lines. Thus, ELISA kits that are listed as "custom" require a 4-6 week development phase, which RayBiotech begins after receiving an order for the kit. The development only needs to occur once, after which the final kit will have passed RayBiotech's ELISA quality control tests, and will thereafter be a stock kit with normal lead times.

II. HOW IT WORKS

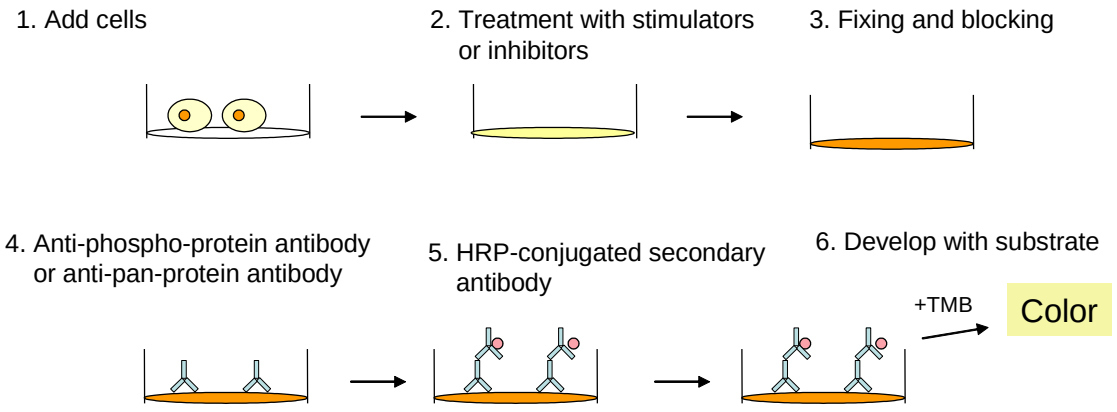

Fig.1. Cell-Based protein phosphorylation procedure

III. REAGENTS AND STORAGE

Store entire kit at ≤ -20 °C immediately upon arrival. Kit must be used within the 6 month expiration date. Avoid repeated freeze-thaw cycles.

ITEM	COMPONENT	1 PLATE KIT	2 PLATE KIT	STORAGE AFTER INITIAL THAW*
A	Uncoated 96-Well Microplate	1 plate	2 plates	Room Temperature
B	20X Wash Buffer A Concentrate	1 vial (30 ml)		2-8 °C
C	20X Wash Buffer B Concentrate	1 vial (30 ml)		
D	Fixing Solution	1 vial (30 ml)		
E	30X Quenching Buffer Concentrate	1 vial (2 ml)		
F	5X Blocking Buffer Concentrate	1 vial (20 ml)		2-8 °C (1 month)
G	Anti-phospho Concentrate	1 vial (7 µl)	2 vials (7 µl/ea)	-20 °C
H	Anti-pan Concentrate	1 vial (7µl)	2 vials (7µl/ea)	
I-1	HRP Conjugated Anti-IgG Concentrate	1 vial (10 µl)	2 vials (10 µl/ea)	
J	TMB Substrate	1 vial (12 ml)	2 vials (12 ml/ea)	2-8 °C
K	Stop Solution**	1 vial (14 ml)		

*For up to 3 months (unless otherwise stated) or until expiration date.
**Contains 0.2 M Sulfuric Acid

III. ADDITIONAL MATERIALS REQUIRED

1. A model cell line, protein tyrosine kinase inhibitors, growth factors or cytokines
2. Microplate reader capable of measuring absorbance at 450 nm
3. 37 ^{o}C incubator
4. Precision pipettes to deliver 2 µl to 1 ml volumes
5. Adjustable 1-25 ml pipettes for reagent preparation
6. 100 ml and 1 liter graduated cylinders
7. Absorbent paper
8. Distilled or deionized water
9. Orbital shaker or oscillating rocker

IV. REAGENT PREPARATION

NOTE: *Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.*

NOTE: *Briefly centrifuge (~1,000g) ITEMS G, H, and I before opening to ensure maximum recovery.*

	ITEM	COMPONENT	PREPARATION	EXAMPLE
	A	Uncoated 96-Well Microplate	No Preparation	N/A
	B	20X Wash Buffer A Concentrate	Dilute **each** 20-fold with distilled or deionized water	25 ml of concentrate + 475 ml of water = 500 ml of 1X working solution
	C	20X Wash Buffer B Concentrate		
	D	Fixing Solution	No Preparation	N/A
	E	30X Quenching Buffer Concentrate	Dilute 30-fold with 1X Wash Buffer A	1 ml of concentrate + 29 ml of wash buffer = 30 ml of 1X working solution
	F	5X Blocking Buffer Concentrate	Dilute 5-fold with distilled or deionized water	20 ml of concentrate + 80 ml of water = 100 ml of 1X working solution
PRIMARY ANTIBODY	G	Anti-phospho Concentrate	Will be determined after final QC	Will be determined after final QC
PRIMARY ANTIBODY	H	Anti-pan Concentrate	Will be determined after final QC	Will be determined after final QC
SECONDARY ANTIBODY	I-1	HRP Conjugated Anti- IgG Concentrate	Will be determined after final QC	Will be determined after final QC
	J	TMB Substrate	No Preparation	N/A
	K	Stop Solution		

V. ASSAY PROCEDURE:

NOTE: *ALL incubations and wash steps must be performed under gentle rocking or rotation (~1-2 cycles/sec).*

1. Design your experiment. For example, see Figure 2 below.

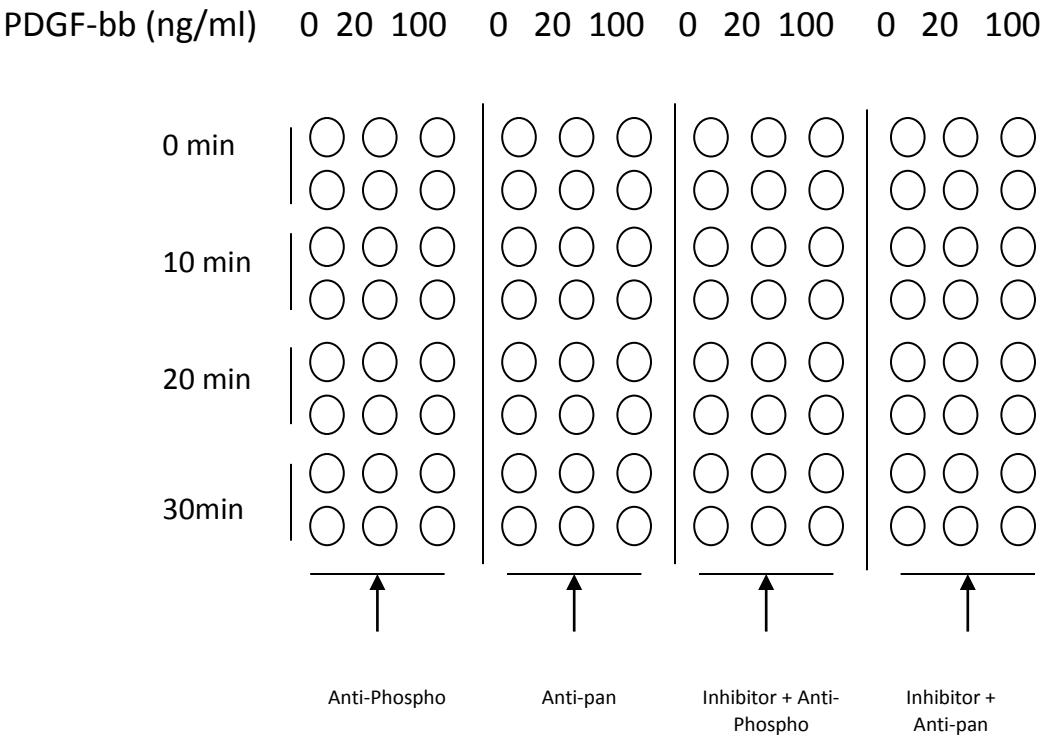

Fig. 2. Example of plate layout for RayBio® cell-based assay

OPTIONAL: *If seeding HUVECs, HMEC-1 or other loosely attached cells, coat the Uncoated 96-Well Microplate (ITEM A) by adding 100 µl poly-L-Lysine (Recommended Sigma Aldrich, Cat#: P4832) into each well and then follow manufacturer's instructions. A pre-coated CellBIND® microplate or other poly-lysine treated tissue culture plate may be used in place of Item A.*

2. Seed 100 μl of 30,000 cells into each well of the Uncoated 96-Well Microplate (ITEM A) provided and incubate overnight at 37°C with 5% CO_2.

NOTE: *The optimal cell number used will vary on the cell line and the relative amount of protein phosphorylation. More or less cells may be used but this must be determined empirically.*

NOTE: *The cells can be starved ~4-24 hours (depending on cell line) prior to treatment with inhibitors or activators.*

3. Apply various treatments, inhibitors (such as siRNA or chemicals) or activators according to manufacturer's instructions and incubate for the desired time points.

NOTE: *It is recommended to dissolve inhibitors or activators into serum-free cell culture medium before treating the cells (unless otherwise stated in the manufacturer's instructions.)*

4. Discard the cell culture medium by flipping the microplate upside down and **gently** tapping the bottom of the microplate over a sink.

5. Wash by pipetting 200 μl of the **prepared 1X** Wash Buffer A (ITEM B) into each well. Discard the wash buffer (same as step 4) and wash 2 more times for a total of 3 washes using fresh wash buffer each time. After the final wash, gently blot the microplate onto a paper towel to remove any excess/remaining buffer.

NOTE: *To avoid cell loss, do not pipette directly onto the cells. Instead, gently dispense the liquid down the wall of cell culture wells. Also avoid the use of vacuum suction or too forcefully tapping the microplate when discarding any solution.*

6. Add 100 μl of Fixing Solution (ITEM D) into each well and incubate for 20 minutes at room temperature.

NOTE: *The fixing solution is used to permeabilize the cells.*

7. Repeat wash step 5.

8. Add 200 μl of the **prepared 1X** Quenching Buffer (ITEM E) into each well and incubate 20 minutes at room temperature.

NOTE: *The quenching buffer is used to minimize the background response.*

9. Wash **4** times with 1X Wash Buffer A.

10. Add 200 μl of the **prepared 1X** Blocking Buffer (ITEM F) into each well and incubate for 1 hour at 37°C.

11. Wash **3** times with the **prepared 1X** Wash Buffer B (ITEM C).

NOTE: *If needed, the microplate may be stored at -80°C for several days after this wash.*

12. Add 50 μl of the **prepared 1X** primary antibody (ITEM G or H) into each corresponding well and incubate for 2 hours at room temperature.

13. Wash **4** times with 1X Wash Buffer B.

14. Add 50 μl of **1X HRP Conjugated secondary antibody** (ITEM I-1) into each well and incubate for 1 hour at room temperature.

15. Wash **4** times with 1X Wash Buffer B.

16. Add 100 μl of the TMB Substrate (ITEM J) into each well and incubate for 30 minutes at room temperature **in the dark**.

17. Add 50 μl of the Stop Solution (ITEM K) into each well. Read at 450 nm immediately.

VI. ASSAY PROCEDURE SUMMARY

1. Seed 30,000 cells into each well and incubate overnight.

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2. Apply various treatment, inhibitors or activators according to manufacturer's instructions.

⇩

3. Add 100 μl of Fixing Solution into each well and incubate for 20 minutes at room temperature.

⇩

4. Add 200 μl of prepared 1X Quenching Buffer and incubate for 20 minutes at room temperature.

⇩

5. Add 200 μl of prepared 1X Blocking Buffer and incubate for 1 hour at 37°C.

⇩

6. Add 50 μl of prepared 1X primary antibody to each well and incubate for 2 hours at room temperature.

⇩

7. Add 50 μl of prepared 1X HRP Conjugated secondary antibody and incubate for 1 hour at room temperature.

⇩

8. Add 100 μl TMB Substrate and incubate 30 minutes at room temperature.

⇩

9. Add 50 μl Stop Solution to each well. Read at 450 nm immediately.

VII. QUALITY CONTROL DATA - Representative

Representative results of CBEL-AKT are shown below:

1. Seeded 30,000 3T3 cells into appropriate wells of the microplate. Cells were incubated at 37°C in 5% CO_2 overnight.

2. Added 50 μl of different concentrations of stimulators (rhPDGF-bb concentration for 3T3 cells: 0, 50 ng/ml in serum free DMEM) to appropriate wells (shown below). Then incubated for 10 min at 37°C.

3. Discarded the solution and wash 3 times with 1X Wash Buffer A (200 μl each) immediately. Then flipped the plate upside down and tapped to remove all of excess wash buffer. The protocol was then followed as stated.

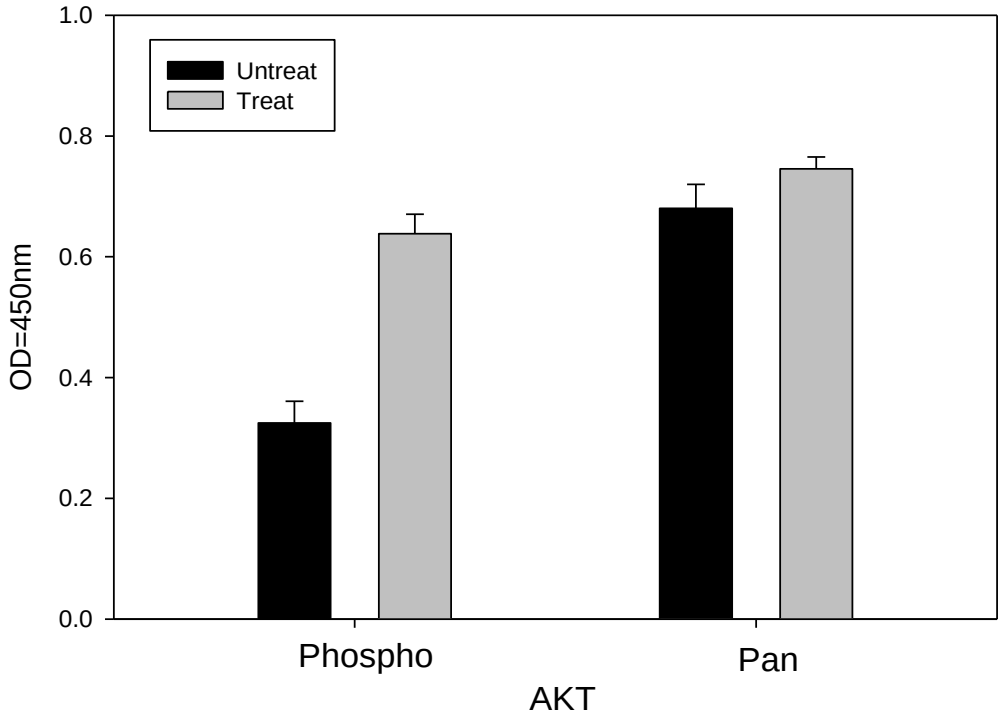

Fig. 3. NIH3T3 cells were stimulated by 50ng/ml of recombinant human PDGFbb for 10 minutes at 37°C

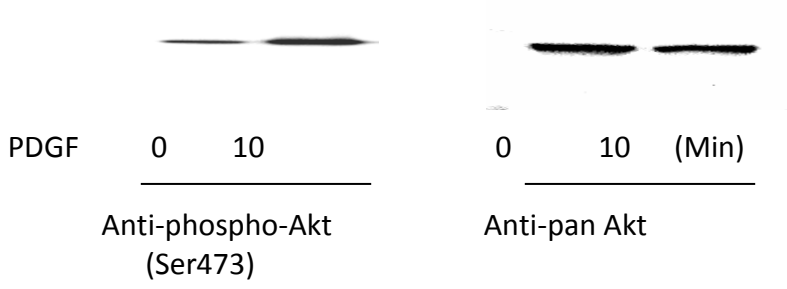

Fig. 4. NIH3T3 cells were treated or untreated with PDGFBB for 10 min. Cell lysates were analyzed using Western Blot.

IX: TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1. Improper storage of the ELISA kit	1. Store the kit according to manual instructions. Keep substrate solution in dark.
	2. Improper dilution	2. Ensure correct preparation of antibody and reagents.
	3. Cells drop off from the wells	3. Some of treatments may make cells drop off the wells. Reduce inhibitor or activator concentration.
2. High background	1. Inadequate washing	1. Be sure to remove all of washing solution and follow the recommendation for washing.
	2. Too much cells	2. Reduce the cell number.
3. Large CV	1. Inaccurate pipetting	1. Check pipette.
	2. Remaining wash buffer in the well	2. Remove all of wash buffer.
	3. Cells drop off from the wells	3. Please don't directly face the cells with tips when adding reagents or wash buffer.

This product is for research use only.