RayBio® Human Phosphotyrosine FGFR1 ELISA Kit

For Measuring Phosphorylated FGFR1 (phosphotyrosine protein) in Human Cell Lysates

User Manual (Revised June 7th, 2016)

RayBio® Phospho-FGFR1 ELISA Kit Protocol

(Cat#: PEL-FGFR1-Y)

RayBiotech, Inc.

ISO 13485 & GLP Certified

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I. INTRODUCTION

RayBio® Phospho-FGFR1 ELISA kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cell lysates. By determining phospho-FGFR1 in your experimental model system, you can verify pathway activation in your cell lysates. You can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western Blot analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human phospho-FGFR1. An anti-FGFR1 antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and unphosphorylated FGFR1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-phosphotyrosine antibody is used to detect only tyrosine-phosphorylated protein. After washing away unbound antibody, HRP-conjugated streptavidin is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of phospho-FGFR1 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. MATERIAL PROVIDED

1. FGFR1 Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-FGFR1.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Assay Diluent (Item E2): 15 ml of 5x concentrated buffer. For diluting cell lysate sample, detection antibody (Item C) and HRP-Streptavidin Concentrate (Item G).
4. Biotinylated anti-phosphotyrosine (Item C): 2 vial of biotinylated anti-phosphotyrosine (each vial is enough to assay half microplate).
5. HRP-Streptavidin Concentrate (Item G): 1 vials, 200 µl/vial, 300x concentrated HRP-conjugated streptavidin.
6. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
7. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
8. Cell Lysate Buffer (Item J): 5 ml 2x cell lysis buffer (not including protease and phosphatase inhibitors).
9. Positive Control K5PVS001-1 (Item K): 1 vial of lyophilized powder from cell lysates.

III. STORAGE

Upon receipt, the kit should be stored at –20 °C. Please use within 6 months from the date of shipment. After initial use, Wash Buffer Concentrate (Item B), Assay Diluent (Item E2), TMB One-Step Substrate Reagent (Item H), HRP-Streptavidin (Item G), Stop Solution (Item I) and Cell Lysate Buffer (Item J) should be stored at 4 °C to avoid repeated freeze-thaw cycles. Return unused wells to the pouch containing desiccant pack, reseal along entire edge and store at –20 °C. Reconstituted Positive Control (Item K) should be stored at -70 °C.

IV. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Protease and Phosphatase inhibitors.
3. Shaker.
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. Distilled or deionized water.
8. Tubes to prepare sample dilutions.

V. SAMPLE PREPARATION

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4 x 10^7 cells/ml in 1x Lysis Buffer (we recommend adding protease and phosphatase inhibitors to lysis buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8° C for 30 minutes. microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8° C, and transfer the supernates into a clean test tube. Lysates should be used immediately or aliquoted and stored at –70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, we recommend to do a serial dilution testing such as 5-fold and 100-fold dilution for your cell lysates with Assay Diluent (Item E2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

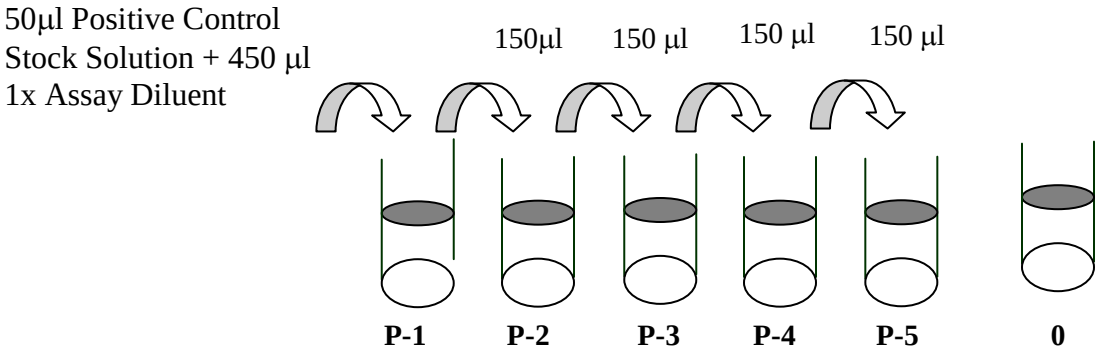
Cell lysate buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

VI. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.

2. Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use.

3. Preparation of Positive Control: Briefly spin the Positive Control vial of Item K. Add 1000µl 1x Assay Diluent (Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use) into Item K vial to prepare a Positive Control Stock Solution. Dissolve the powder thoroughly by a gentle mix. Add 50 µl prepared Positive Control Stock Solution from the vial of Item K, into a tube with 450 µl 1x Assay Diluent to prepare P-1 (See i. Positive control of part IX. TYPICAL DATA for a typical result). Pipette 300 µl 1x Assay Diluent into each tube. Transfer 150 µl prepared P-1 into a tube with 300 µl 1x Asaay Diluent to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.



4. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.

5. Briefly spin the biotinylated antibody (Item C) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a biotinylated anti-phosphotyrosine antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The biotinylated phosphotyrosine antibody should be diluted 80x with 1x Assay Diluent and used in step 4 of Part VII Assay Procedure.

6. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 300 fold with 1x Assay Diluent.

   For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 40 µl of HRP-Streptavidin concentrate into a tube with 12 ml 1x Assay Diluent to prepare a 300-fold diluted HRP Streptavidin solution (don't store the diluted solution for next day use). Mix well.

7. Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

VII. ASSAY PROCEDURE:

1. Bring all reagents to room temperature (18 - 25°C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.

2. Add 100 µl of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or over night at 4°C with shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of prepared 1X biotinylated anti-phosphotyrosine antibody (Reagent Preparation step 5) to each well. Incubate for 1 hour at room temperature with shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 µl of prepared 1X HRP-Streptavidin solution (see Reagent Preparation step 6) to each well. Incubate for 45 minutes at room temperature with shaking.

7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with shaking.

9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 µl sample or positive control to each well. Incubate 2.5 hours at room temperature or over night at 4°C.

3. Add 100 µl prepared 1X biotinylated phosphotyrosine antibody to each well. Incubate 1 hour at room temperature.

4. Add 100 µl prepared 1X HRP-Streptavidin solution. Incubate 45 minutes at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

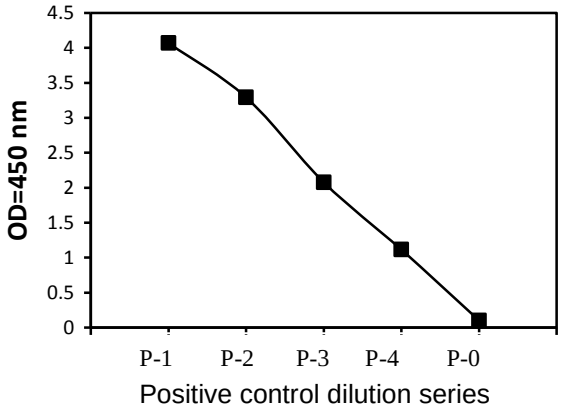
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

IX. TYPICAL DATA

ELISA data analysis: Average the duplicate readings for each sample or positive control then subtract the average blank optical density.

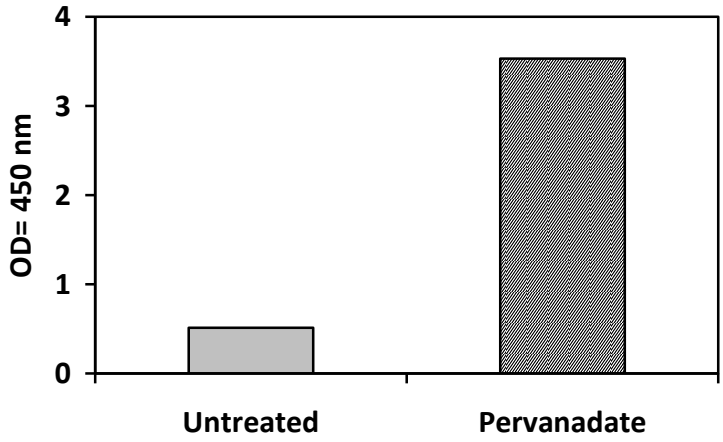
i. Positive Control

K562 cells were treated with Pervanadate at 37°C for 10 min. Solubilize cells at 4 x 10^7 cells/ml in lysis buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Part VI Reagent Preparation for detail.



ii. Pervanadate Stimulation of K562 Cell Line

K562 cells were treated or untreated with Pervanadate for 10 min at 37°C. Cell lysates were analyzed using this phosphoELISA:



X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Sample signals:		
a. Too low	a. Sample concentration is too low	a. Increasing sample concentration
b. Too high	b. Sample concentration is too high	b. Reducing sample concentration
2. Large CV	a. Inaccurate pipetting	a. Check pipettes
3. High background	a. Plate is insufficiently washed	a. Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	b. Contaminated wash buffer	b. Make fresh wash buffer
4. Low positive control signal	a. Improper storage of the ELISA kit	a. Upon receipt, the kit should be stored at -20^{o}C. Store the positive control at -70^{o}C after reconstitution.
	b. Stop solution	b. Stop solution should be added to each well before measurement and read OD immediately.
	c. Improper primary or secondary antibody dilution	c. Ensure correct dilution

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Notes:

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