User Guide

# Human and Mouse Phosphotyrosine HCK ELISA Kit

**RAB0966**

## Introduction

The Phosphotyrosine HCK 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 phosphorylated HCK 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-based ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of Human and Mouse phospho-HCK. An anti-HCK antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and unphosphorylated HCK 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 to 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-HCK bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

## Storage

The entire kit may be stored at −20 °C for up to 1 year from the date of shipment. Avoid repeated freeze thaw cycles.

## Components

- Anti-Pan-HCK Microplate: 96 wells (12 strips x 8 wells) coated with anti-Anti-Pan-HCK. Stable in storage 1 month at 20 °C after preparation (Return unused wells to the pouch containing desiccant pack, reseal along entire edge).
- Positive Control: 1 vial of lyophilized powder from THP1 cell lysate. Stable in storage 1 week at −80 °C after preparation.
- Biotinylated anti-phosphotyrosine: 2 vials of Biotinylated anti-phosphotyrosine. Each vial is enough to assay half microplate. Stable in storage 5 days at 4 °C
- HRP-Streptavidin: 1 vial (200 µL) of 600X concentrated HRP-conjugated streptavidin Do not store and reuse.
- Wash Buffer: 25 mL of 20X concentrated solution. Stable in storage 1 month at 4 °C after preparation.
- Assay Diluent B: 15 mL of 5X concentrated assay diluent. Stable in storage 1 month at 4 °C after preparation.
- Lysis Buffer: 15 mL of 2X cell Lysis Buffer. Stable in storage 1 month at 4 °C after preparation.
- TMB One-Step Substrate 12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.
- Stop Solution: 8 mL of 0.2 M sulfuric acid.

## Additional Materials Required (But Not Provided)

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 2 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1-liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions

## Sample Preparation

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation step 2) 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.

## Reagent Preparation

1. Bring all reagents and samples to room temperature (18-25 °C) before use.
2. 5X Assay Diluent should be diluted 5-fold with deionized or distilled water before use.
3. Cell lysate buffer should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.
4. Preparation of Positive Control: Briefly spin the Positive Control Vial. Add 400 µL of prepared 1X Assay Diluent into Positive Control. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial, and then pipetting the supernatant only for the assay.
5. If the Wash Concentrate (20X) 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.
6. Preparation of Biotinylated anti-phosphotyrosine: Briefly spin the vial of Biotinylated anti-phosphotyrosine. Add 100 µL of 1X Assay Diluent into the vial to prepare a detection 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 concentrate should then be diluted 80-fold with 1X Assay Diluent and used in step 5 of the Assay Procedure.
7. Preparation of HRP-Streptavidin: Briefly spin the vial of HRP-Streptavidin concentrate before use. HRP-Streptavidin should be diluted 600x with 1X Assay Diluent and used in step 7 of the Assay Procedure.

## Assay Procedure

1. Bring all reagents and samples to room temperature (18-25 °C) before use. It is strongly recommended to run all positive controls and samples in at least duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µL of positive control (see Reagent Preparation step 4) or sample into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4 ° C with gentle shaking.
4. 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 auto-washer. Complete removal of liquid at each step is essential for 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.
5. Add 100 µL of prepared 1X biotinylated anti-phosphotyrosine (see Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µL of prepared HRP-Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 1 hour at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µL of Stop Solution to each well. Read at 450 nm immediately.

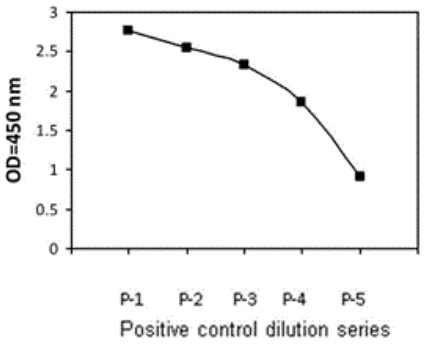
## Assay Procedure Summary

1. Prepare all reagents, samples, and positive control as instructed.
2. Add 100 µL positive control or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4 °C with gentle shaking.
3. Add 100 µL prepared detection antibody to each well. Incubate for 1 hour at room temperature with gentle shaking.
4. Add 100 µL prepared HRP-Conjugated solution. Incubate for 1 hour at room temperature with gentle shaking.
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.

# Typical Data

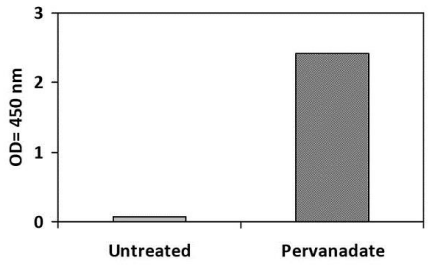
## Positive Control

THP1 cells were treated with Pervanadate for 10 min. Cells were solubilized at 4 x $10^7$ cells/mL in lysis buffer. Serial dilutions of lysates were analyzed in this ELISA.




## Pervanadate Stimulation of THP1 Cell Line

THP1 cells were untreated or treated with Pervanadate for 10 min. Cell lysates were analyzed using this phospho-ELISA:



## Troubleshooting Guide

| Problem | Cause | Solution |
|---|---|---|
| Low signal in samples | Sample concentration is too low | Increase sample concentration. Briefly spin down vials before opening. Dissolve the powder thoroughly |
| | Improper preparation of detection antibody | |
| | Too brief incubation times | Ensure sufficient incubation time; assay procedure step 3 may be done overnight. Check pipettes and ensure correct preparation |
| | Inadequate reagent volumes or improper dilution | |
| High signal in samples | Sample concentration is too high | Reduce sample concentration |
| Large CV | Inaccurate pipetting. Air bubbles in wells | Check pipettes. Remove bubbles in wells |
| High background | Plate is insufficiently washed | Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed |
| | Contaminated wash buffer | Make fresh wash buffer |
| Low sensitivity | Improper storage of the ELISA kit | Store your positive control at−70 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light. |
| | Stop solution | Add stop solution to each well before reading plate |
| | Improper primary or secondary antibody dilution | Ensure correct dilution |

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