

## User Guide

# Human HCC-4 ELISA Kit

For Serum, Plasma, Cell Culture Supernatants

**RAB0049**

## Introduction

The Human HCC-4 ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human HCC-4 in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for human HCC-4 coated on a 96-well plate. Standards and samples are pipetted into the wells and HCC-4 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human HCC-4 antibody is added. After washing away unbound biotinylated 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 HCC-4 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 date of shipment. Avoid repeated freeze thaw cycles. The kit may be stored at 4°C for up to 6 months. For extended storage, it is recommended to store at -80°C.

## Components

- Human HCC-4 Microplate - RAB0049A-EA: 96 wells (12 strips x 8 wells) coated with anti-Human HCC-4. Stable in storage 1 month at 4 °C once opened. Return unused wells to the pouch containing desiccant pack, reseal along the edge.
- Human HCC-4 Standard Protein - RAB0049C-1VL: 2 vials of Human HCC-4. 1 vial is enough to run each standard in duplicate. Stable in storage 1 week at -80 °C once opened.
- Human HCC-4 Detection Antibody - RAB0049D-1VL: 2 vials of biotinylated anti-Human HCC-4. Each vial is enough to assay half the microplate. Stable in storage 5 days at 4 °C once opened.
- Wash Buffer - RABWASH4: 25 mL of 20X concentrated solution. Stable in storage 1 month at 4 °C once opened.
- HRP-Streptavidin - RABHRP5: 200 µL 300X concentrated HRP-conjugated streptavidin. Do not store and reuse.
- TMB One-Step Substrate Reagent - RABTMB3: 12 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution.
- Stop Solution - RABSTOP3: 8 mL of 0.2 M sulfuric acid.
- Assay Diluent A - RABELADA-30ML: 30 mL of diluent buffer, 0.09% sodium azide as preservative.
- 5X Assay Diluent B - RABELADE-15ML: 15 mL of 5X concentrated buffer. Stable in storage 1 month at 4°C once opened.

## Additional Materials Required (But Not Provided)

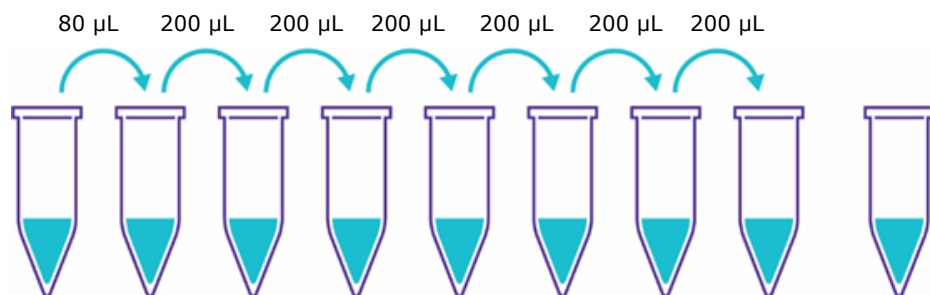
- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 2 µl to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and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

## Reagent Preparation

1. Bring all reagents and samples to room temperature (18-25 °C) before use.
2. 5X Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
3. Sample dilution: Assay Diluent A should be used for dilution of serum and plasma samples. 1X Assay Diluent B should be used for dilution of cell culture supernatant samples. The suggested dilution for normal serum/plasma is 2 - 20-fold.

**Note:** Levels of HCC-4 may vary between different samples. Optimal dilution factors for each sample must be determined by the investigator.

4. Preparation of standard: Briefly spin a vial of Standard Protein. Add 400 µL Assay Diluent A (for serum/plasma samples) or 1X Assay Diluent B (for cell culture medium) into the Standard Protein vial to prepare a 50 ng/mL standard. Dissolve the powder thoroughly by a gentle mix. Add 80 µL HCC-4 standard from the vial of Standard Protein, into a tube with 586.7 µL Assay Diluent A or 1X Assay Diluent B to prepare a 6000 pg/mL stock standard solution. Pipette 400 µL Assay Diluent A or 1X Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent A or 1X Assay Diluent B serves as the zero standard (0 pg/mL).



|                       |              | Std 1      | Std 2      | Std 3       | Std 4       | Std 5       | Std 6       | Std 7      | 0 Std   |
|-----------------------|--------------|------------|------------|-------------|-------------|-------------|-------------|------------|---------|
| <b>Diluent volume</b> | Std + 400 µL | 586.7 µL   | 400 µL     | 400 µL      | 400 µL      | 400 µL      | 400 µL      | 400 µL     | 400 µL  |
| <b>Concentration</b>  | 50 ng/mL     | 6000 pg/mL | 2000 pg/mL | 666.7 pg/mL | 222.2 pg/mL | 74.07 pg/mL | 24.67 pg/mL | 8.23 pg/mL | 0 pg/mL |

5. If the Wash Buffer (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. Briefly spin the Detection Antibody vial before use. Add 100  $\mu$ L of 1X Assay Diluent B 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). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B and used in step 5 of the assay procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted 300-fold with 1X Assay Diluent B.

For example: Briefly spin the HRP-Streptavidin vial and pipette up and down to mix gently. Add 50  $\mu$ L of HRP-Streptavidin concentrate into a tube with 15 mL 1X Assay Diluent B to prepare a final 300-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

## Assay Procedure

1. Bring all reagents and samples to room temperature (18-25 °C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100  $\mu$ L of each standard (see Reagent Preparation step 4) and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300  $\mu$ L) 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.
5. Add 100  $\mu$ L of 1X prepared biotinylated antibody (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  $\mu$ L of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100  $\mu$ L 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  $\mu$ L of Stop Solution to each well. Read at 450 nm immediately.

## Assay Procedure Summary

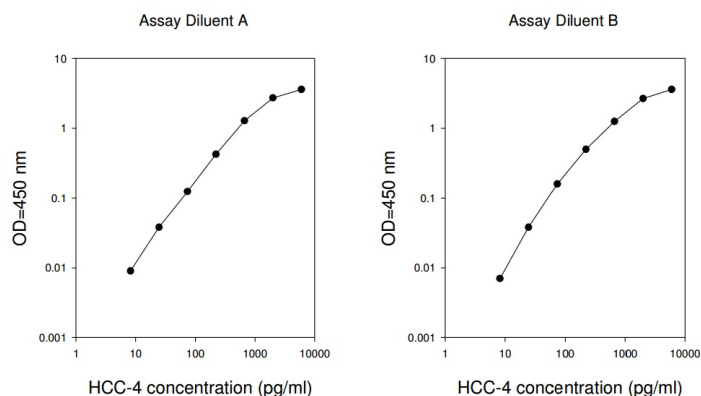
1. Prepare all reagents, samples and standards as instructed.
2. Add 100  $\mu$ L standard or sample to each well. Incubate 2.5 hours at room temperature.
3. Add 100  $\mu$ L prepared biotin antibody to each well. Incubate 1 hour at room temperature.
4. Add 100  $\mu$ L prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100  $\mu$ L TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50  $\mu$ L Stop Solution to each well. Read at 450 nm immediately.

## Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

## Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



## Sensitivity

The minimum detectable dose of Human HCC-4 was determined to be 8 pg/mL.

Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluent buffer).

## Spiking & Recovery

Recovery was determined by spiking various levels of Human HCC-4 into the sample types listed below. Mean recoveries are as follows:

| Sample Type               | Average % Recovery | Range (%) |
|---------------------------|--------------------|-----------|
| Serum                     | 95                 | 84-103    |
| Plasma                    | 95                 | 83-103    |
| Cell Culture Supernatants | 96                 | 85-104    |

## Linearity

| Sample Type |                             | Serum  | Plasma | Cell Culture Supernatants |
|-------------|-----------------------------|--------|--------|---------------------------|
| 1:2         | Average % of Expected       | 93     | 94     | 92                        |
|             | Range (%)                   | 82-102 | 83-103 | 82-102                    |
| 1:4         | Average % of Expected Range | 95     | 95     | 94                        |
|             | Range (%)                   | 83-103 | 84-103 | 82-102                    |

## Reproducibility

Intra-Assay CV%: <10%

Inter-Assay CV%: <12%

## Specificity

This ELISA kit shows no cross-reactivity with any of the cytokines tested: Human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, I-309, IP-10, G-CSF, GM-CSF, IFN-gamma, Leptin, MCP-1, MCP-2, MCP-3, MDC, MIP-1 alpha, MIP-1 beta, MIP-1 delta, PARC, PDGF, RANTES, SCF, TARC, TGF-beta, TIMP-1, TIMP-2, TNF-alpha, TNF-beta, TPO, VEGF.

## Troubleshooting Guide

| Problem             | Cause                                                         | Solution                                                                                                                                                                     |
|---------------------|---------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Poor standard curve | Inaccurate pipetting                                          | Check pipettes                                                                                                                                                               |
|                     | Improper standard dilution                                    | Briefly centrifuge the standard protein and dissolve the powder thoroughly by gently mixing                                                                                  |
| Low signal          | Improper preparation of standard and/or biotinylated antibody | Briefly spin down vials before opening. Dissolve the powder thoroughly.                                                                                                      |
|                     | Too brief incubation times                                    | Ensure sufficient incubation time. Assay procedure step 3 may be done overnight at 4 °C with gentle shaking.<br><br>Note: may increase overall signals including background. |
|                     | Inadequate reagent volumes or improper dilution               | Check pipettes and ensure correct preparation                                                                                                                                |
| Large CV            | Inaccurate pipetting                                          | Check pipettes                                                                                                                                                               |
|                     | Air bubbles in wells                                          | 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 standard 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                                                                                                                          |

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