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STEM CELLS RESEARCH & THERAPEUTICS

# Cell-Based ELISA

Catalog Number K36102-29E

## Cell-Based ELISA to Characterize Human Breast Cancer Stem Cell in culture.

**This package insert must be read in its entirety before using this product.**

**FOR RESEARCH USE ONLY.**
**NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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# I. PRINCIPLE OF THE ASSAY

The Human Breast Cancer Stem Cells have CD133 which is a predominantly surface biomarker for Breast Cancer Stem Cells. This Cell-Based ELISA Kit contains components required to measure one protein (CD133) in whole cells to determine whether the cell has Breast Cancer Stem Cell status or not. Utilizing this Cell Based assay format, stem cells can be differentiation into Breast Cancer Cells and Breast Cancer Stem Cells biomarker CD133 can be determined. This simple and efficient assay eliminates the need to prepare cell lysates and is broadly applicable for drug discovery and drug screening. This assay format may be used to assess stem cell differentiation status or effect of stimulation and/or inhibition of compounds and ligands on target molecules directly associated with stem cell differentiation into Breast Cancer Stem Cells.

Cells are grown in 96-well plates pre-coated with extracellular matrix and may be stimulated with ligands and/or incubated with inhibitors. Cells are then fixed and dehydrated in the wells. The status of target protein in response to treatment is analyzed using an immunoenzymatic labeling procedure. The cells are incubated with one primary antibody: a rabbit or mouse monoclonal antibody specific for the protein of interest. After washing away unbound antibodies,anti-rabbit and or anti-mouse secondary antibodies labeled with horseradish-peroxidase (HRP) or alkaline phosphatase (AP), respectively, two spectrally distinct fluorogenic substrates for either HRP or AP may be used for detection.

# II. LIMITATIONS OF THE PROCEDURE

**FOR RESEARCH USE ONLY.**
**NOT FOR USE IN DIAGNOSTIC PROCEDURES**.
This Cell-Based ELISA Kit should not be used beyond the expiration date on the kit label.
Do not mix or substitute reagents with those from other lots or sources.
For assay optimization, HRP is the more robust enzyme, it is recommended that the HRP-conjugated secondary antibody may be used with the primary antibody detecting the less abundant protein. The AP-conjugated secondary antibody should be used with the primary antibody detecting the more abundant protein.
Suitability of the primary antibodies for the assay and the final concentrations used are to be determined by individual laboratories.

# TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.

2 Individual results may vary due to differences in technique, plastic-ware, water sources, and cell density.

3. Thorough and consistent wash technique is essential for proper assay performance. To minimize cell loss during the wash steps, avoid dispensing liquid directly onto the cell surface. Instead, gently dispense the liquid down the wall of the cell culture wells, always using the same side of the wells. Empty the wells by decanting and remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.

4. Avoid cross-contamination, change pipette tips between additions of each reagent and/or sample. Also, use separate reservoirs for each reagent.

5. It is recommended that all samples and controls be assayed in duplicate at the very minimum but triplicates readings are highly recommended.

6. Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2 - 8° C.

# MATERIALS PROVIDED

**Store the unopened kit at 2 - 8°C.**
**Do not use kit, past the kit expiration date.**
**This kit contains sufficient materials to run ELISAs on two 96-well plates.**

**Microplates** (Cat# E36102B) - Two 96-well cell culture clear-bottom black microplates and covers for use as vessels in the assay.

**Primary antibody (CD133)** (Cat# 136102B) - One vial (5µl /vial)
*use 2.5µl Antibody per reaction (5ml)*

**Secondary Antibody (HRP-conjugated anti-rabbit IgG)** (Cat# 236102B) – One vial (5µl /vial). *use 2.5µl Antibody per reaction (5ml)*

**Substrate MN1 Concentrate** (Cat# 336102B) - One vial (130µl /vial) of a fluorogenic substrate for horseradish-peroxidase (HRP). *use 64µl substrate per reaction (5ml)*

**Substrate MN1 Diluent (bottle A)** (Cat# 436102B) - One bottle (10 ml/bottle) of a solution for diluting the Substrate MN1 Concentrate.

**Blocking Buffer** (Cat# 536102B) - One bottle (10 ml/bottle) of diluted animal serum in Wash Buffer.

**Wash Buffer** (Cat# 636102B) - One Bottle (100 ml/bottle) of a buffered surfactant with preservatives.

**Fixing solution** (Cat#036102B) One Bottle (10ml /bottle).

**Dehydrating solution** (Cat#136102B) One Bottle (10 ml / bottle).

## OTHER SUPPLIES REQUIRED

Deionized or distilled water.
Pipettes and pipette tips.
Multi-channel pipette for washing.
Cell culture incubator.
Microfuge tubes.
Orbital shaker.
Fluorescence plate reader with two channels: excitation 540 nm / emission 600 nm and excitation 360 nm / emission 450 nm.

## REAGENT PREPARATION

**Primary Antibody Mixture**** - Optimal concentrations of primary antibodies used in the assay should be determined by each laboratory. Immediately before use, dilute 2.5µl of the primary antibody in 5 ml of Wash Buffer for one plate. For two full plates, 5µl of primary antibody is diluted in 10 ml Wash Buffer. **If both plates are not being assayed, adjust volumes accordingly.**

**Secondary Antibody Mixture**** - Immediately before use, dilute 2.5µl of the HRP-conjugated in 5ml Wash Buffer for one plate. For two full plates, 5µl of the HRP-conjugated antibody and 10mL of Wash Buffer. **If both plates are not being assayed, adjust volumes accordingly.**

**Substrate MN1** - Add the contents of Substrate MN1 Concentrate vial (64µl) to 5 ml of MN1 Diluent (bottle A) for one plate. For two full plates, 128µl to 10mL of MN1 Diluent (bottle A).Store Substrate MN1 at 2 - 8° C for up to 60 days.*

**Wash Buffer** – 100 ml Wash Buffer. Store at 2 - 8° C for up to 60 days.*

*Provided this is within the expiration date of the kit.
**Once prepared, the primary and secondary antibody mixtures cannot be stored. Prepare only enough as needed to run the assay.

## GENERAL ASSAY PROCEDURE

**A. Culture, Stimulate, Fix, and Block Cells**
1. Seed 100µl of 10,000 - 20,000 adherent cells into each well of the black 96-well microplates provided and incubate overnight at 37° C in a cell culture incubator.
**Note:** The cell number used is dependent upon the cell line and the relative amount of target protein. Optimal cell numbers should be determined by each laboratory for each assay.
2. Grow and treat the cells as desired.
3. Fix cells by replacing 100% medium with 50µl of fixing solution. Add the plate covers and incubate for 20 minutes at room temperature. For maximum sensitivity, it is recommended that the assay be performed immediately after cell fixation. Alternatively apply plate sealers and store the plates containing the fixed cells at 2 - 8° C for up to 2 weeks.
4. Remove 100% Fixing solution and wash the cells 3 times with 50µl of Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
5. Remove 100% Wash Buffer and add 50µl Cell Dehydration solution to each well and incubate at room temperature for 5 minutes.
5. Remove 100% Cell Dehydration solution and wash 3 times with wash buffer for 5 minutes with gentle shaking.
6. Remove 100% Wash Buffer and add 50µl of Blocking Buffer. Add the plate covers and incubate for 60 minutes at room temperature.
7. Remove 100% Blocking Buffer and wash the cells 3 times with 50µl of Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
8. Remove 100% Wash Buffer, and add 50µl of Blocking Buffer. Add the plate covers and incubate for 1 hour at room temperature.

**B. Binding of Primary and Secondary Antibodies** Optimized for this Assay System Based on the Recommended Cell Type and Cell Density
1. Remove 100% Blocking Buffer and wash the cells 3 times with 50µl of Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

2. Add 50µl of the Primary Antibody Mixture to each well. Cover with plate sealers and incubate at room temperature for 1-2 hours. In cells known to generate high amounts of target protein, 2 hour incubation is sufficient; however, for maximum sensitivity, an Overnight incubation is recommended. For overnight incubation store at 2-8°C.

**Note:** Depending on the experimental design, some wells may be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative controls (secondary antibody alone).

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 50µl of Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
4. Add 50µl of the Secondary Antibody Mixture to each well. Cover with plate sealers and incubate for 2 hours at room temperature.
**Note:** The Secondary Antibody Mixture is added into each well including the negative control wells.

### C. Fluorogenic Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 3 times with 50µl of Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
2. Remove the Wash Buffer from the plate and add 50µl of Substrate MN1 to each well. Incubate for 20 minutes at room temperature. Protect the plates from direct light. A pink or rosy color should develop in the wells.
**Note:** It is critical to add 64µl Substrate MN1 into 15 ml sterile conical tube and 5ml of Substrate Diluent (Bottle A). Mix well and then add 50µl to each well and incubate for 20 - 30 minutes for fluorescence development.
3. Read the plates using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm.

## PROCEDURE FOR NON-ADHERENT CELLS

The General Assay Procedure can be modified for use with non-adherent cells by culturing and fixing the cells as follows.
1. Seed the appropriate amount of cells for your cell line into each well.
2. Grow and treat the cells as desired.
3. Fix the cells by replacing the medium with 50µl of fixing solution. Add the plate covers and incubate for 20 minutes at room temperature. Alternatively, apply plate sealers and store the plate containing the fixed cells at 2 - 8° C for up to 2 weeks.
6. Continue with section A (step 4 of the General Assay Procedure on page 6).

# CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The fluorescence (RFUs) from these wells is the background fluorescence and is subtracted from all sample wells. If normalization is desired, the fluorescence at 600 nm derived from the target protein in each well is normalized to the fluorescence at 450 nm derived from normalization protein. The normalized duplicate readings for each sample are then averaged.

## Protocol Overview for Flurometric ELISA Assay

-Remove 100% Media, Wash 3 times with Wash Buffer

-Fix cells with 50µl of Fixing Solution per well. Incubate for 20 minutes at room temperature

-Wash 3 times with Wash Buffer

-Add 50µl of Dehydration Solution per well for 5 minutes

-Wash 3 times with Wash Buffer

-Add 50 µl Blocking Buffer per well for 1 hour at room temperature

-Wash 3 times with Wash Buffer

-Incubate with 50µl primary antibody mix per well for 1 hour at room temperature, or overnight at 2-8°C

-Wash 3 times with Wash Buffer

-Incubate with 50µl secondary antibody mix per well for 2 hours

-Wash 3 times with Wash Buffer

-Add 50µl of Substrate MN1 mix per well. Incubate for 20 minutes at room temperature away from light.

*Substrate MN1 mix: 64µl Substrate MN1 + 5ml Substrate MN1 diluent*

-Measure on plate reader with two channels Excitation 540/Emission 600
*read at desired times*

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# FREQUENTLY ASKED QUESTIONS (ELISA)

1. How do you get the antibody out of the vial?
    - Do a quick spin of the vial.
    - For 1 plate, add 100µl of wash buffer into the vial and pipette up and down several times to mix.
    - Pipette out 50µl of the mixture and add to 5mLs of Wash Buffer.
    - Freeze the other 50µl of antibody/Wash Buffer mix until ready to use.
    - For 2 plates, add 100µl of wash buffer into the vial and pipette up and down several times to mix.
    - Remove all 100µl of Antibody/Wash Buffer mix and add to 10mLs of Wash Buffer.

2. There is no color development. Why is that?
    - Make sure you added Substrate MN1 Concentrate (red cap) to the Substrate MN1 Diluent (Bottle A).
    - For 1 plate, use 64µl of Substrate MN1 Concentrate (red cap) and add to 5 mLs of MN1 Substrate Diluent (Bottle A) in a 15mL conical.
    - Mix gently and add 50µl of Substrate mix into each well.
    - For 2 plates, use 128µl of Substrate MN1 Concentrate (red cap) and add to 10 mLs of MN1 Substrate Diluent (Bottle A) in a 15mL conical.
    - Read at 20 minutes. A pink color should develop within 20 minutes.
    - Check cell density by viewing plate under a microscope.
    - If reagents are stored at 2-8˚C make sure they have reached room temperature before using.
    - Make sure you have utilized HRP secondary antibody after the primary antibody incubation.
    - Make sure you have utilized the substrate after the secondary antibody incubation.
    - Make sure you have not cross contaminated reagents.

    ***NOTE: Check cell density in between washes. Assay is very sensitive and may remove cells if not preformed gently. Before beginning assay, make sure there is a high cell density***.

3. What cells types has this assay been utilized for in Celprogen's lab?
    - Human Embryonic SC and Human Mesenchymal SC

    ***NOTE: This kit has been optimized for the provided reagents and only works with these reagents.***

## PLATE LAYOUT

Use this plate layout to record assayed samples.