Cell-Based ELISA

Human Phospho-Progesterone R/NR3C3 (S294) Immunoassay

Catalog Number KCB5955

An ELISA-based assay using fluorogenic substrates to measure phosphorylated Progesterone R (PR) in whole cells.

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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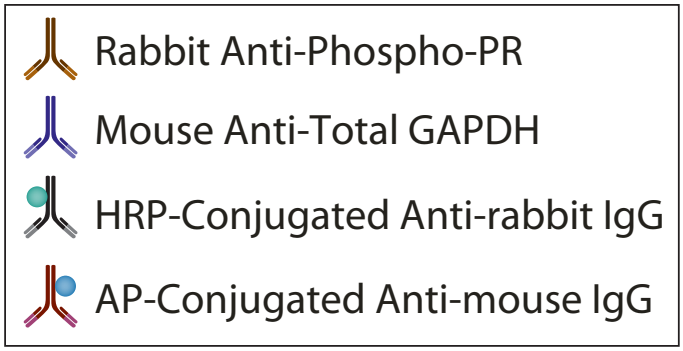
PRINCIPLE OF THE ASSAY

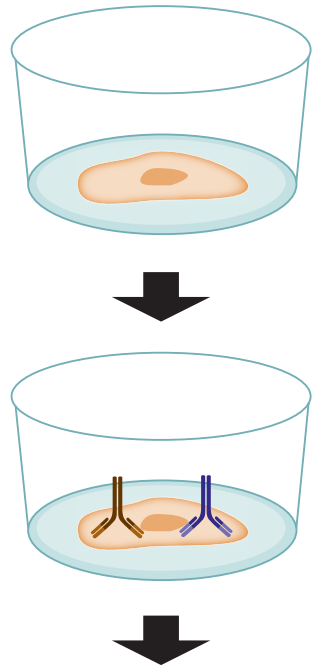
This Cell-Based ELISA contains the components required to run an ELISA using fluorogenic substrates to measure Progesterone Receptor (PR) phosphorylated at S294 in whole cells. This simple and efficient assay eliminates the need to prepare cell lysates and can be used to investigate signaling pathways and the effects of inhibitors on cells. Cells are grown in 96-well plates and stimulated with ligands. Following stimulation, cells are fixed and permeabilized in the wells. The target protein phosphorylation is measured using a double immunoenzymatic labeling procedure. The cells are simultaneously incubated with two primary antibodies: a phospho-specific antibody and a normalization antibody that recognizes GAPDH, a house keeping protein. The primary antibodies are derived from different species. Two secondary antibodies recognizing the different species are labeled with either horseradish-peroxidase (HRP) or alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for either HRP or AP are used for detection. The fluorescence of the phosphorylated protein is normalized to that of GAPDH in each well for the correction of well-to-well variations. This two-wavelength assay results in precise analysis of protein phosphorylation with good reproducibility.

TECHNICAL HINTS AND LIMITATIONS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based ELISA 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.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- A 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.
- To avoid cross-contamination, change pipette tips between additions of each reagent and/or sample. Also, use separate reservoirs for each reagent.
- It is recommended that all samples and controls be assayed in duplicate.
- 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.

ASSAY OVERVIEW

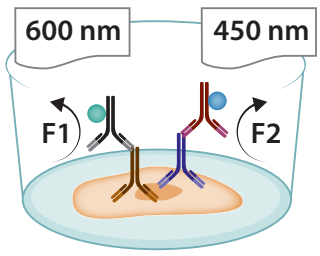




1. Seed cells in a 96 well plate. Stimulate cells with ligands. Fix, permeabilize, and block cells.

2. Add primary antibodies (rabbit anti-phospho-PR (S294) and mouse anti-total GAPDH).

3. Add secondary antibodies (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG).



4. Add fluorogenic substrates F1 and F2 and measure fluorescence.

MATERIALS PROVIDED

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-PR (S294) Antibody	967387	1 vial of lyophilized rabbit anti-phospho-PR (S294) antibody.	Store at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 2 months.*
Total GAPDH (Ms) Antibody	967346	1 vial of lyophilized mouse anti-total GAPDH antibody.	
HRP-conjugated anti-rabbit IgG	893230	110 µL of HRP-conjugated goat anti-rabbit IgG secondary antibody.	May be stored at 2-8 °C for up to 2 months.*
AP-conjugated anti-mouse IgG	893231	110 µL of AP-conjugated goat anti-mouse IgG secondary antibody.	
Substrate F1 Concentrate	893232	50 µL of a sensitive and stable fluorogenic substrate for horseradish-peroxidase (HRP).	
F1 Diluent	893233	10 mL of a solution for diluting the Substrate F1 Concentrate.	
Substrate F2	893234	10 mL of a sensitive and stable fluorogenic substrate for alkaline phosphatase (AP).	
Blocking Buffer	893235	35 mL of 10% fetal bovine serum in buffer with preservatives.	
Wash Buffer (5X)	893236	60 mL of a buffered surfactant with preservatives.	
Microplate	607582	96-well cell culture clear-bottom black microplate and cover for use as a vessel in the assay.	Store at room temperature.
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- 37% formaldehyde (Molecular Biology Grade; Sigma, Catalog # F8775). Refer to the MSDS prior to use.
- 30% H_2O_2 (Sigma, Catalog # H1009). Refer to MSDS prior to use.
- 1X PBS (Irvine Scientific, Catalog # 9240).
- Deionized or distilled water.
- 500 mL graduated cylinder.
- 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

Phospho-PR (S294) Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X PBS.

Total GAPDH (Ms) Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X PBS.

Primary Antibody Mixture* - Immediately before use, add 100 µL of the Phospho-PR (S294) Antibody and 100 µL of the Total GAPDH Antibody to 9.8 mL of Blocking Buffer. 100 µL of the Primary Antibody Mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Secondary Antibody Mixture* - Immediately before use, add 100 µL of the HRP-conjugated antibody and 100 µL of the AP-conjugated antibody to 9.8 mL of Blocking Buffer. 100 µL of the Secondary Antibody Mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Substrate F1 - Add the contents of the Substrate F1 Concentrate vial (50 µL) to the 10 mL of F1 Diluent in the brown bottle. Store Substrate F1 at 2-8 °C for up to 2 months.

1X Wash Buffer - Add 60 mL of Wash Buffer (5X) to 240 mL of 1X PBS to prepare 1X Wash Buffer.

SOLUTION PREPARATION

4% Formaldehyde (for adherent cells) - Add 1.3 mL of 37% formaldehyde to 10.7 mL of 1X PBS to prepare 4% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

8% Formaldehyde (for non-adherent cells) - Add 2.6 mL of 37% formaldehyde to 9.4 mL of 1X PBS to prepare 8% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

Quenching Buffer - Add 200 µL of 30% H_2O_2 to 9.8 mL of 1X Wash Buffer to prepare 0.6% H_2O_2. **If a full plate is not being assayed, adjust volumes accordingly.**

*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 100 µL of 10,000-20,000 adherent cells into each well of the black 96-well microplate with clear bottom 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 the medium with 100 µL of 4% formaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the cells in fixing solution at 2-8 °C for up to 2 weeks.

   Caution: *Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical. Refer to MSDS prior to use.*

4. Remove the formaldehyde solution, and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

5. Remove the Wash Buffer and add 100 µL of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.

6. Remove the Quenching Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.

B. Binding of Primary and Secondary Antibodies

1. Remove the Blocking Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

2. Add 100 µL of the Primary Antibody Mixture to each well. Cover with a plate sealer and incubate for 16 hours at 2-8 °C. In cells known to generate high amounts of phosphorylated PR, a 2-3 hour primary antibody incubation is sufficient; however, for maximum sensitivity, an overnight incubation is recommended.

   Note: *Depending on the experimental design (refer to the Calculation of Results section), some wells should be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative control (secondary antibody alone).*

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

4. Add 100 µL of the Secondary Antibody Mixture to each well. Cover with a plate sealer 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 2 times with 200 µL of 1X Wash Buffer, followed by 2 washes with 200 µL of 1X PBS. Each wash step should be performed for 5 minutes with gentle shaking.

2. Remove the 1X PBS from the plate and add 75 µL of Substrate F1 to each well. Incubate for 20-60 minutes at room temperature. Protect the plate from direct light. A pink or rosy color should develop in the wells.

   Note: *It is critical to add Substrate F1 into each well and incubate for 20-60 minutes for fluorescence development prior to the addition of Substrate F2. Adding Substrate F1 and Substrate F2 simultaneously into the wells will result in the inhibition of fluorescence development.*

3. Add 75 µL of Substrate F2 to each well and incubate for an additional 20-40 minutes at room temperature. Protect the plate from direct light.

4. Read the plate using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm. Then read the plate with excitation at 360 nm and emission at 450 nm. The readings at 600 nm represent the amount of phosphorylated PR in the cells, while readings at 450 nm represent the amount of total GAPDH in the cells.

PROCEDURE FOR NON-ADHERENT CELLS

This protocol has been validated for use with non-adherent cells by culturing and fixing cells as follows.

1. Seed 100 µL of suspension cells into each well of the black 96-well microplate in serum-free or normal growth media at the desired cell density (seeding cells at a density of 0.2-1.0 x 10^6 cells/mL is recommended).

2. Incubate the plate at 37 °C. Depending on the cell line and treatment, the typical incubation time is 0.5-16 hours.

3. Treat the cells as desired. Prepare the treatment media containing a 5X final concentration of treatment. Add 25 µL of 5X treatment media to the wells and bring the total volume up to 125 µL. Mix solution by gently agitating the plate and incubate according to your treatment protocols.

4. Centrifuge the plate at 500 x g for 3 minutes at 2-8 °C and remove media.

5. Fix the cells by adding 100 µL of 8% formaldehyde in 1X PBS. Apply the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer 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 5.

CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The relative fluorescence units (RFUs) from these wells is the background fluorescence and is subtracted from all sample wells. Normalized results can be determined by dividing the phospho-PR fluorescence at 600 nm in each well by the total GAPDH fluorescence at 450 nm in each well. The normalized duplicate readings for each sample are then averaged.

KIT PERFORMANCE DATA

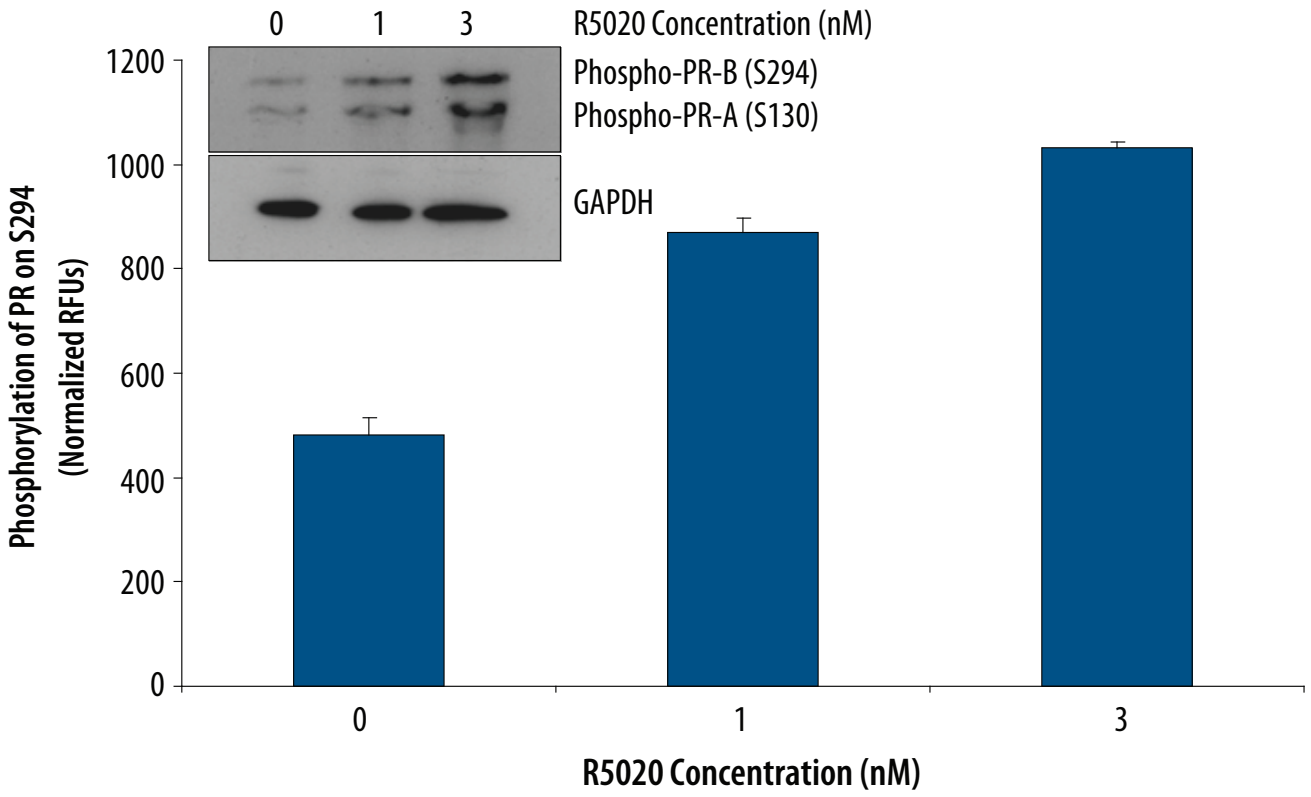


Figure 1: Dose response of R5020-induced phosphorylation of PR (S294) in human cells. T47D human breast cancer cells were cultured in 96-well plates and treated with the indicated concentrations of the synthetic progestin promegestone (R5020) for 60 minutes. After fixation of cells in the wells, phosphorylation of PR on S294 was determined using this Human Phospho-Progesterone R/NR3C3 (S294) Cell-Based ELISA. Values represent the mean ± the range of duplicate determinations. Analysis of PR phosphorylation and GAPDH (the normalization protein) by Western blotting using the antibodies supplied in this kit is also shown (inset).

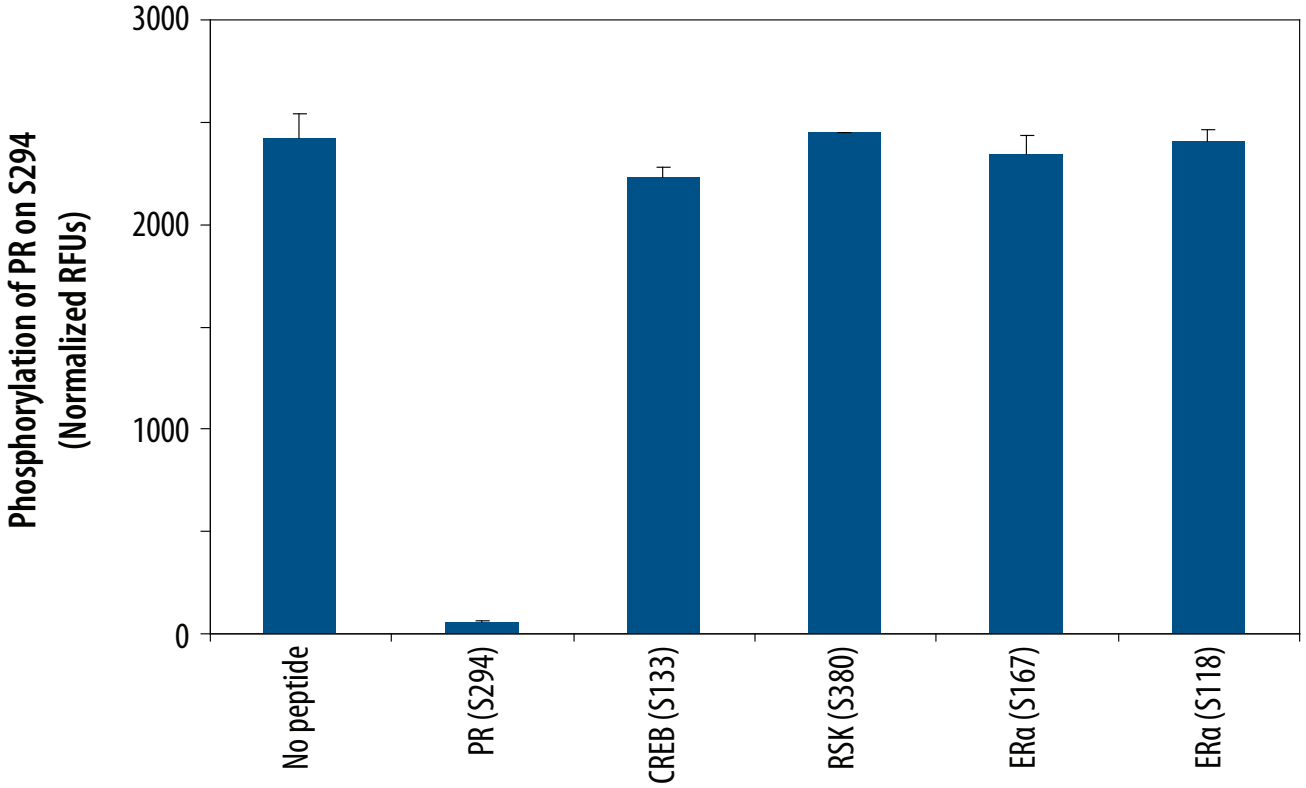
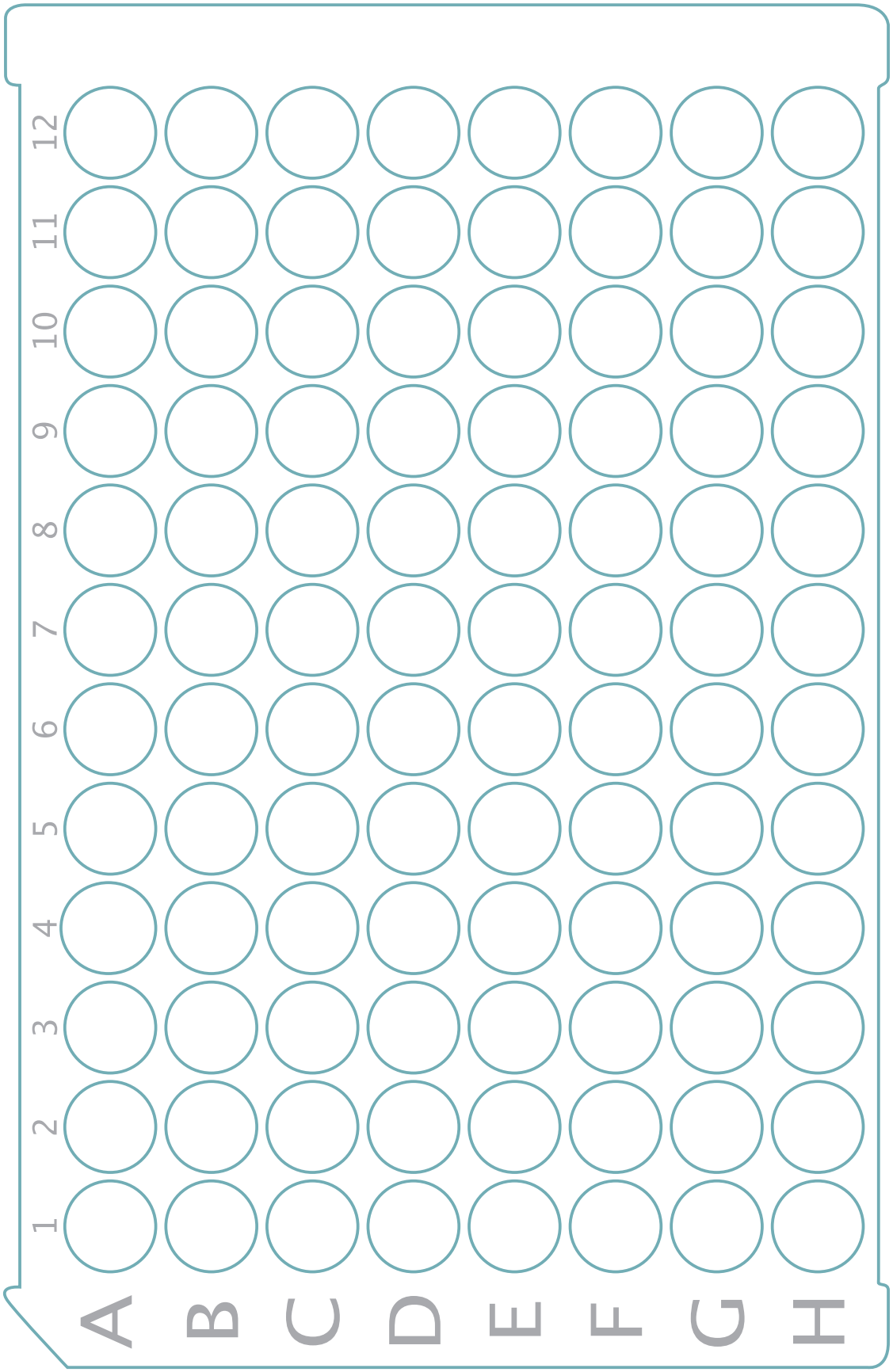


Figure 2: Peptide competition confirms the specificity of the Human Phospho-PR (S294) Cell-Based ELISA. T47D human breast cancer cells in 96-well plates treated with 3 nM R5020 for 60 minutes were analyzed with this Cell-Based ELISA. The Phospho-PR (S294) Antibody was either untreated (no peptide) or pre-incubated with phosphopeptide containing either the PR (S294), CREB (S133), RSK (S380), Estrogen Receptor alpha (ERα) (S167), or ERα (S118) phosphorylation sites. Peptides were used at 10 ng/mL. A significant reduction of the phospho-PR signal was observed only with the peptide containing the phospho-PR (S294), suggesting the specificity of this Cell-Based ELISA kit for PR phosphorylated at S294.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.



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