Protocol

pIMAGO-biotin Phosphoprotein Detection on 96-well Plate using Avidin-HRP

Components provided:

Phosphorylated protein (β-casein) as a control (mix 5 µL with 95 µL Binding buffer; store at 4ºC);

Blocking buffer (store at 4ºC);

pIMAGO buffer (store at 4 to 25ºC);

Binding buffer (store at 4ºC);

pIMAGO reagent (store at 4ºC);

avidin-HRP (store at 4ºC);

Colorimetric Detection Substrates A and B (store at 4ºC);

Stop Solution (store at 4 to 25ºC);

96-well plate for colorimetric detection;

Need to prepare: 1x TBST (Tris-buffered saline with 0.1% Tween 20)

Protocol

1) Binding of samples to the 96-well plate

Prepare a protein solution of your sample (phosphoprotein or substrate of interest) in **Binding buffer**. A dephosphorylated (e.g. alkaline phosphatase-treated or without ATP) form of the sample should also be prepared as a reference. If protein amount is known, use 10 to 500 ng of the protein or mixture of proteins per 100 µl of **Binding buffer** per well. Add 100 µl of the mix into each well on a 96-well plate. Incubate overnight at 4°C at 400-600 rpm to bind the proteins to the plate. As a positive control, in a separate well mix 5 µL of the provided phosphoprotein with 95 µL of Binding buffer.

2) Blocking the wells

Remove solution from wells, add 150 µl of the **Blocking buffer** into each well and incubate 2-3 minutes. Remove the solution and add 150 µl of the **Blocking buffer** again and incubate for 30 min while shaking at 400-600 rpm at room temperature.

*At this stage, any additional manipulations can be carried out (e.g. kinase/phosphatase assay, inhibitor screening, etc.). Make sure to wash the wells 3x with the **1x TBST** after each manipulation.*

3) pIMAGO incubation

In a clean tube, prepare a 1 to 100 mixture of the **pIMAGO reagent** in the **pIMAGO buffer** (1 µl of the reagent for every 100 µl of buffer). Empty the wells and add 100 µl per well of the prepared pIMAGO mix. Incubate 1 hour at 400-600 rpm at room temperature.

3) Washing the wells

Empty the wells and add 150 µl of the **pIMAGO buffer** into each well; incubate 2-3 minutes at 400-600rpm. Remove the buffer and repeat the washing step two more times with the **pIMAGO buffer** for a total of 3 washes. Remove the solution and incubate the wells with 150 µl of the **Blocking buffer** for 15 min at 400-600 rpm at room temperature.

4) Incubation with avidin-HRP

In a clean tube, prepare 1 to 100 mixture of **avidin-HRP** in the **Blocking buffer** (1 µl of **avidin-HRP** in 100 µl of **Blocking buffer**). Empty the wells and add 100 µl per well of the prepared avidin-HRP in blocking solution. Incubate the plate for 1 hour at 400-600 rpm at room temperature.

5) Washing the wells

Empty the wells and add 150 µl of **1x TBST** into each well; incubate 2-3 minutes at 400-600 rpm and remove the solution. Repeat the washing step with TBST two more times for a total of three washes. Empty the wells.

6) Signal detection

For normal and high concentrations of the proteins (majority of *in vitro* samples), use the provided colorimetry-based detection system. Prepare 9 to 1 mixture of the **Colorimetric Substrates A and B** (has to be made fresh each time before detection), and add 100 µl to each well. Shake the plate until satisfied with signal – solution will turn green if signal is present (usually 1-2 min), then add 150 µl of the **Stop solution** to stop the HRP-substrate reaction. Read the plate at 415 nm in a plate reader.

* Alternatively, any other peroxidase substrate can be used (chromogenic or chemiluminescent). However, for chemiluminescence-based detection, a different plate with non-transparent walls must be used. *