

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

Components provided:

Phosphorylated protein (β -casein) as a control (mix 5 μ L with 95 μ L Binding buffer; store at 4°C);

Blocking buffer (store at 4°C);

pIMAGO buffer (store at 4 to 25°C);

Binding buffer (store at 4°C);

pIMAGO reagent (store at 4°C);

avidin-Fluor (store at 4°C);

96-well plate for fluorescence 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 μ l of **Binding buffer** per well. Add 100 μ l 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 μ L of the provided phosphoprotein with 95 μ L of Binding buffer.

2) Blocking the wells

Remove solution from wells, add 150 μ l of the **Blocking buffer** into each well and incubate 2-3 minutes. Remove the solution and add 150 μ l 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 μ l of the reagent for every 100 μ l of buffer). Empty the wells and add 100 μ l 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 μ l 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 μ l of the **Blocking buffer** for 15 min at 400-600 rpm at room temperature.

4) Incubation with avidin-Fluor

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

5) Washing the wells

Empty the wells and add 150 μ l 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

The signal can be detected using any fluorescence imager with an appropriate filter. The number listed with the provided avidin-Fluor reagent represents the absorption maximum (emission wavelength) for that fluorophore.