pIMAGO-based detection on ELISA plate

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1) Binding of samples to the 96-well plate

Prepare a protein solution of your sample (phosphoprotein or substrate of interest) in **binding buffer** (carbonate buffer: 3.03g sodium carbonate, 6.0g sodium bicarbonate, to 1L with water, pH 9.6). Use 1-500 ng of the protein or mixture of proteins per 100 ul of **binding buffer** per well. Add 100 ul of the mix into each well on a 96-well plate (use clear plate for normal protein amount, and black-wall plate for low protein concentration of < 10 ng to be used with chemiluminescence detection). Incubate overnight at 4°C at 500 rpm to bind the proteins to the plate.

*It is recommended to use at least one well for a known phosphoprotein (positive control) and one for a non-posphoprotein or dephosphorylated protein (negative control). For detection of *in vivo* phosphorylation, at least one well should be used to dephosphorylate the sample with a general phosphatase, to ensure the signal is specific to phosphorylation.*

2) Blocking the wells

Remove solution from wells using multi-channel pipet, and add 200 ul of the **blocking buffer** (currently, 1%BSA in Thermo blocking buffer) into each well and pipet up-and-down a few times; remove **blocking buffer**. Repeat 3 more times. Add 200 ul of the **blocking buffer** into each well again and leave to incubate for 30 min while shaking at 500 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 with the **blocking buffer** after each manipulation.*

3) pIMAGO incubation

In a clean tube, prepare a 1 to 200 mixture of the **pIMAGO reagent** in the **pIMAGO buffer** (0.5 ul of the reagent for every 100 ul of buffer; current pIMAGO buffer - 500mM glycolic acid, 1%TFA). Remove the **blocking buffer** from the wells and add 100 ul per well of the prepared pIMAGO mix. Incubate 1 hour at 500 rpm at room temperature.

3) Wash the wells

Remove **pIMAGO reagent** and add 200 ul of the **pIMAGO buffer** into each well; pipet up-and-down a few times to wash the wells from excess reagent. Remove the buffer and repeat the washing step three more times with the **pIMAGO buffer** and four more times with the **blocking buffer**. Incubate the wells with 200 ml of the **blocking buffer** for 30 min at 500 rpm at room temperature.

4) Incubation with avidin-HRP

In a clean tube, prepare 1 to 2,000 mixture of **avidin-HRP** in the **blocking buffer** (0.5 ml of **avidin-HRP** in 1 ml of **blocking buffer**). Remove the **blocking buffer** from the wells and add 100 ul per well of the prepared avidin-HRP in blocking solution. Incubate the plate for 1 hour at 500 rpm at room temperature.

5) Washing of the wells

Remove the avidin-HRP solution from the wells and add 200 ul of the **wash buffer** (currently, Thermo blocking buffer) into each well; pipet up-and-down a few times to wash the wells. Remove the **wash buffer** and repeat this washing step three more times. Remove the **wash buffer**.

6) Signal detection

For normal and high concentrations of the proteins (majority of *in vitro* samples), use colorimetry-based detection system. Prepare 9 to 1 mixture of the **colorimetric substrates A and B**, and add 100 ul 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 ul of the **stop solution** (2% oxalic acid) to stop the HRP-substrate reaction. Read the plate at 415 nm in a plate reader.

* If the concentation of the sample is low (< 10 ng) or colorimetry assay did not yield any signal, use chemiluminescent-based substrate. In this case, mix **chemiluminescence solutions A and B** at 1 to 1 ratio and add 100 ul of the mixture to each well. Incubate the plate for 5 min and read using the chemiluminescence setting on a plate reader. Make sure to use black-walled plate for this method of detection to prevent cross-lumination of signal between wells.*