ELISA using antibodies or IR-pIMAGO

1) Coating: binding of protein or antibody of interest onto the 96-well polystyrene microplate

Prepare sample solution (phospho-protein or antibody of interest) in **coating buffer** (carbonate buffer: 3.03g sodium carbonate, 6.0g sodium bicarbonate, to 1L with water, pH 9.6). Recommend 1-500 ng of the protein or mixture of proteins per 100 ul of **coating buffer** per well (for the coating of capture antibody, 200-500 ng per 100 ul per well recommended). Add 100 ul of the mix into each well on a 96-well plate (use clear plate for normal colorimetric detection, black-wall plate for low amount chemiluminescent detection, or round-bottom clear plate for IR-pIMAGO detection). Incubate overnight at 4°C at 500 rpm (shaker) to coat the proteins or antibody to the plate or 2h at room temperature.

*It is recommended to use at least one well for a known phosphoprotein (positive control) and one for a non-phosphoprotein 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 (e.g. CIAP), to ensure the signal is specific to phosphorylation.*

2) Blocking

Remove solution from wells and add 200 ul of the **blocking buffer** (1% BSA in Thermo blocking buffer or TBS-T) into each well and pipet up-and-down 5-10 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, etc.). Make sure to thoroughly wash the wells with the **blocking buffer** after each manipulation.*

3) Primary antibody incubation

In a clean tube, prepare **primary antibody** solution in **blocking buffer.** For 100ng of antigen coated each well, 150-200 ng of the primary antibody is recommended for the first trial. Incubate 1 hour at 500 rpm at room temperature.

3)* IR-pIMAGO incubation
In a clean tube, prepare a 1 to 200 mixture of the **IR-pIMAGO reagent** in the **IR-pIMAGO buffer** (0.5 ul of the reagent for every 100 ul of buffer; IR-pIMAGO buffer - 200mM 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.

4) Washing (antibody)

Remove **primary antibody solution** and add 200 ul of the **blocking buffer** into each well; pipet up-and-down 5-10 times to wash the wells from excess reagent. Remove the buffer and repeat the washing step two more times with the **blocking buffer**.

4)* Washing (IR-pIMAGO)

Remove **IR-pIMAGO reagent** and add 200 ul of the **IR-pIMAGO buffer** into each well; pipet up-and-down a few time to wash the wells from excess reagent. Remove the buffer and repeat the washing step two more times with the **pIMAGO buffer** and three more times with the **blocking buffer**. Finally, wash once with **methanol** to clean the wells and then remove the solution and let the wells dry. **Ready for detection (Step 7)**.

5) Secondary antibody incubation

In a clean tube, prepare **secondary antibody (IR-Dye labeled)** solution in **blocking buffer.** For 100ng of antigen coated each well, 150-200 ng of the secondary antibody is recommended for the first trial. Incubate 1 hour at 500 rpm at room temperature.

6) Washing (antibody)

Remove **secondary antibody solution** and add 200 ul of the **blocking buffer** into each well; pipet up-and-down 5-10 times to wash the wells from excess reagent. Remove the buffer and repeat the washing step two more times with the **blocking buffer**. Finally, wash once with **methanol** to clean the wells and then remove the solution and let the wells dry. **Ready for detection (Step 7)**.

7) Detection

Use LI-COR scanning machine for plate scanning and signal reading (see Li Pan for instructions.)