Sandwich KIRA-ELISA Protocol

Umed Boltaev,Yves Meyer

Abstract

KIRA-ELISA is used for quantitave detection of phosphorylation level of tyrosine kinase receptors. The protocol could be used for many different receptors. In our work, this protocol has been successful for TrkB and FGFR1 receptors. The underlying principle of the assay is capturing the receptor and detecting phosphorylated tyrosine on the intracellular domain of the receptor using pan phospho-tyrosine antibody. Since tyrosine kinase receptor are phosphorylated at multiple tyrosine residues, pan phospho-tyrosine antibody detects any possible antigen on the receptor, resulting in more sensitive assay. Our assay is based on previously published bioassay by M. Sadick et. al. and M. Cazorla et. al.

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Guidelines

Solutions and Buffers:

Note: Do not use sodium azide in any buffers or solutions, as sodium azide inactivates the horseradish-peroxidase enzyme.

Phosphate Buffered Saline (PBS):

80.0 g NaCl
14.4 g Na2HPO4
2.4 g KH2PO4
2.0 g KCl
Add ddH2O up to 10 L, pH to 7.2 with HCl

PBS/Tween:

0.5 ml of Tween-20 in 1 L PBS

Blocking Solution:

10% fetal bovine serum or 1% BSA in PBS. Filter before use to remove particulates.

General References:

1. Davies, C. 1994. The Immunoassay Handbook. D. Wild, Ed. Stockton Press, New York.
2. Abrams, J.S. 1995. Immunoenzymetric assay of mouse and human cytokines using NIP-labeled anti-cytokine antibodies. Current Protocols in Immunology (J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, W. Strober, Eds). John Wiley and Sons, New York. Unit 6.20.
3. Sander, B., et al. 1993. J. Immunol. Meth. 166:201.
4. Abrams, J.S., et al. 1992. Immunol. Rev. 127:5.

Cytokine ELISA Troubleshooting Tips

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Poor signal-to-noise ratio

• Try Capture Antibody at 1 – 10 µg/ml (generally 2 µg/ml).
• Try Detection Antibody at 0.25 – 2 µg/ml (generally 1 µg/ml).
• Titrate against each other to obtain optimal dilutions.

Low Sensitivity

• Try overnight incubations of standards and samples at 4°C.

Poor Signal

• If using HRP, avoid sodium azide in wash buffers and diluents, as sodium azide inhibits HRP.
• Verify that appropriate antibody pairs were used and the activity of the samples and/or standards.
• Check the activity of enzyme and substrate by coating with Detection Antibody (1 µg/ml), adding biotinylated avidin and revealing with the appropriate substrate. If the enzyme/substrate is active, a strong signal should be observed.

Poor Standard Curve

• Handling Instructions for standards are lot-specific. Refer to product information for proper handling.
• Recombinant protein vials should be quick-spun for maximum recovery.
• BioLegend suggests that cytokines be stored in a concentrated format (>100 ng/ml) and in the presence of a protein carrier.

High Background

• Increase stringency of washing steps by soaking plates for ~1 minute during washes.
• Determine optimum Capture and Detection Antibody dilutions.
• Increase the dilution of Detection Antibody and/or increase the number of washes after Av-HRP incubation.

Protocol

Coat the Plate

Step 1.

Dilute unlabeled capture antibody to a final concentration of 0.5 – 8 µg/ml in PBS and transfer 100 µl to each well of a high affinity, protein-binding ELISA plates (e.g., NUNC Immulon 4 HBX).

Prepare 2 plates: '**pY Plate**' and '**Total Protein**'. Use Sino Ab for pY plate (1/1000 dilution), R&D Ab for total protein plate (1/500 dilution).

AMOUNT

100 µl Additional info: per well

AMOUNT

10 ml Additional info: per plate

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REAGENTS

Anti-TrkB Antibody 10047-RP02 by Sino Biological

TrkB Goat anti-Human, Polyclonal, R&D Systems™ AF397 by Thermo Fisher Scientific

Coat the Plate

Step 2.

Seal plate to prevent evaporation. Incubate at 4°C overnight, or 2-3h at RT.

Block the Plate

Step 3.

Bring the plate to room temperature, flick off the capture antibody solution.

Block the Plate

Step 4.

Wash with PBS/Tween (5x).

AMOUNT

150 µl Additional info: per well

Block the Plate

Step 5.

Add 1% BSA PBS solution.

AMOUNT

100 µl Additional info: per well

Block the Plate

Step 6.

Seal plate and incubate at room temperature for ≥ 1 hour.

DURATION

01:00:00

Block the Plate

Step 7.

Wash with PBS/Tween (5x).

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Block the Plate

Step 8.

Firmly blot plate against clean paper towels.

Add Standards and Samples

Step 9.

Transfer lysate from experimental plate (where cells were treated).

AMOUNT

80 µl Additional info: per well for pY plate

AMOUNT

20 µl Additional info: per well for total protein (receptor) plate

Add Standards and Samples

Step 10.

Seal the plate and incubate at room temperature at 4°C overnight.

Add Standards and Samples

Step 11.

Wash with PBS/Tween (5x).

NOTES

Washes can be effectively accomplished by filling wells with either a squirt bottle, carboy, manifold dispenser, multi-channel pipettor or automatic plate washer. For increased stringency, after each wash, let the plate stand briefly, flick off the buffer, and blot plates on paper towels before refilling.

Perform **at least 3** washes.

Add Detection Antibody

Step 12.

Dilute the detection antibody to 0.25 – 2 µg/ml in Blocking Solution. Add 100 µl of diluted antibody to each well.

NOTES

For pY plate: prepare 1/2500 dilution (4 ul of Ab stock to 10 ml of 1% BSA PBS/Tween). For primary cultures, reduce the dilution to 1/1250 (8 ul of Ab stock to 10 ml of 1% BSA PBS/Tween).

For total protein (receptor) plate: prepare working dilution of detecting Ab. **Capturing Ab and detecting Ab MUST be from different host animals.**

Add Detection Antibody

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Step 13.
SIncubate at room temperature for 1 hour.

DURATION

01:00:00

Add Detection Antibody

Step 14.
Wash with PBS/Tween (5x).

NOTES

Perform **at least 3** washes.

Total protein plate: Add Horseradish Peroxidase Labeled Secondary Antibody

Step 15.
Dilute the Av-HRP conjugate (Cat. No. 405103) or other enzyme conjugate to its pre-determined optimal concentration in Blocking Buffer (usually between 1/500 – 1/2000). Add 100 µl per well.

REAGENTS

Anti-rabbit IgG, HRP-linked Antibody #7074 by Cell Signaling Technology

Total protein plate: Add Horseradish Peroxidase Labeled Secondary Antibody

Step 16.
Seal the plate and incubate at room temperature for 30 minutes.

DURATION

00:30:00

Total protein plate: Add Horseradish Peroxidase Labeled Secondary Antibody

Step 17.
Wash with PBS/Tween (5x).

NOTES

Perform **at least 5** washes.

pY Plate Color Development

Step 18.
Add TMB One solution and incubate for 30 min at RT

AMOUNT

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100 µl Additional info: per well

REAGENTS

TMB One Solution, 100ml G7431 by Promega

pY Plate Color Development

Step 19.

Add 1N HCl solution

AMOUNT

100 µl Additional info: per well

pY Plate Color Development

Step 20.

Read the optical density (OD) for each well with a microplate reader set to 405 nm.

Total Protein Plate Color Development

Step 21.

Add TMB One solution and incubate for 5-30 min at RT

AMOUNT

100 µl Additional info: per well

REAGENTS

TMB One Solution, 100ml G7431 by Promega

Total Protein Plate Color Development

Step 22.

Add 1N HCl solution

AMOUNT

100 µl Additional info: per well

Total Protein Plate Color Development

Step 23.

Read the optical density (OD) for each well with a microplate reader set to 405 nm.

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