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Kinase-activity tagged (KAT) western blotting

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1 Works for me dx.doi.org/10.17504/protocols.io.bhpzj5p6

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MATERIALS TEXT

- 10 %-formaldehyde neutral buffer: Nacalai Tesque Inc. #37152-51
- Anti-phospho (P)-Tyr antibody: Clone PY20, Zymed Laboratories Inc
- Anti-phospho (P)-PHI-1 (Thr57) antibody: Affinity-purified custom made antibody, Aves Labs Inc
- HRP-labeled anti-chicken IgY: Jackson ImmunoResearch Laboratories, Inc
- HRP-labeled anti-mouse IgG: Jackson ImmunoResearch Laboratories, Inc
- ECL solution: Pierce Supersignal WestPico plus®.
- Coomassie Brilliant Blue solution: Takara CBB Protein Safe Stain®
- Equipment used: Chemiluminescence imager: GE Amersham Imager 680
- Abbreviation: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; Gu, guanidine; PTM, posttranscriptional modification; TCEP, tris(2-carboxyethyl)phosphine); HRP, horse radish peroxidase
- Buffered 2-propanol: 20 % 2-propanol, 50 mM Tris-HCl, pH 8.0
- 6M Gu-HCl buffer: 6 M guanidine HCl, 10 mM MgCl2, 50 mM Tris-HCl, pH 8.0, 0.5 mM TCEP
- 3M Gu-HCl buffer: 3 M guanidine HCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 0.5 mM TCEP
- 0.1M Gu-HCl buffer: 0.1 M guanidine HCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 0.5 mM TCEP

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- Renaturation buffer: 10 mM $MgCl_2$, 0.05 % Tween20, 50 mM Tris-HCl, pH 8.0, 0.5 mM TCEP
- Phosphorylation buffer: 10 mM $MgCl_2$, 1 mM EGTA, 20 mM MOPS-NaOH, pH 7.0, 0.5 mM TCEP
- 0.1M ATP, pH 7.0
- Washing solution: PBS plus 0.02 % Tween20
- Blocking solution: PBS plus 1.0 % bovine serum albumin and 0.1 % Tween20
- Antibody dilution solution: PBS plus 0.1 % bovine serum albumin and 0.1 % Tween20

PROCEDURE

1 Experiment is conducted at room temperature unless noted.

2 Preparation of Laemmli Gel and Laemmli samples using a conventional method.

3 Running the SDS-PAGE with the samples (10-50µg of total lysates) and colored Mw marker and run electrophoresis.

4 Preparation of the blotting using PVDF membrane by a standard western blotting protocol.

5 Washing the membrane for 5 min with 10mL of Buffered 2-propanol.

6 Denaturation for 15min with 10 ml of 6 M Gu-HCl buffer.

7 Serial incubation each for 10 min with 10 mL of 3 M Gu-HCl buffer and then 10 mL of 0.1 M Gu-HCl buffer.

8 Washing for 3x 5min with 10 ml of Renaturation buffer.

9 Place the membrane in Hybri-bag

10 Incubation overnight with 2 ml of 0.2mg/mL substrate protein in Renaturation buffer at 4°C.

11 Rinse with 10 ml of Phosphorylation Buffer.

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12 Incubation for 1h with 2 mL of Phosphorylation Buffer including 1mM ATP using 37°C shaker.

13 Rinse with PBS.

14 Incubation for 20 min with 10 mL of 10 % buffered formalin.

15 Rinse with PBS.

16 Quenching for 10 min with 10 mL of 0.1 M glycine in PBS.

17 Blocking for 30 min with Blocking solution.

18 Washing for 5 min x4 with 20 mL of Washing solution.

19 Incubation for overnight at 4 °C with primary antibody diluted with Antibody dilution solution.

20 Washing for 5 min x4 with 20 mL of Washing solution.

21 Incubation for 45 min with secondary antibody conjugated with HRP (diluted at 1:5,000 with Antibody dilution solution)

22 Washing for 5 min x4 with 20 mL of Washing solution.

23 Incubation for 5 min with 2 mL of ECL solution.

24 Imaging using chemiluminescence imager

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25 Rinse the blot with distilled H2O twice

26 Staining the blot with Coomassie Brilliant Blue solution or others.

27 Troubleshooting
- For the first assay, a negative control blot, such as no ATP treatment, is highly recommended.
- Synthetic peptides conjugated with a carrier protein may be used as substrates.
- Renaturation efficiency may vary among kinases.

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