

Detection of Wnt5 in Media Conditioned by Mouse Embryonic Fibroblast

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[Abstract] This protocol describes the procedure of visualizing secreted Wnt5 protein in serum free media via western blotting. This procedure can also be used to visualize other secreted proteins larger than 10,000 daltons. The work presented in this paper visualizes Wnt5 secreted by mouse embryonic fibroblast (MEF), but can be adapted to other cell lines including those transiently transfected by plasmids.

Materials and Reagents

1. 10 cm Petri dishes
2. 50 ml Beckel centrifuge tubes
3. 1.5 ml microcentrifuge tubes
4. Amicon Ultra-15 centrifugal filter
5. 0.45 μ m immobilon-FL PVDF (EMD Millipore, catalog number: IPFL00010)
6. Wild type MEF and Rab8a^{-/-} MEF cell lines
7. MEF isolation and phenotypic analysis was previously described (Das *et al.*, 2015)
8. DEME with glucose, L-glutamine and sodium pyruvate (Mediatech, catalog number: 10-013)
9. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F2442-500ML)
10. Pen-strep (Thermo Fisher Scientific, Gibco™, catalog number: 15140-122)
11. Lactalbumin hydrolysate solution (50x) (Sigma-Aldrich, catalog number: 58901C-100ML)
12. NaN₃
13. NaF
14. Na₃VO₄
15. PMSF
16. DTT
17. Nonidet® P40 substitute (Sigma-Aldrich, catalog number: 74385-1L)
18. Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
19. Tween® 20 (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP337-500)
20. Sodium chloride (NaCl)
21. Potassium chloride (KCl)
22. Disodium hydrogen phosphate (Na₂HPO₄)
23. Potassium dihydrogen phosphate (KH₂PO₄)

24. Protease inhibitor cocktail tablets (Sigma Aldrich, catalog number: 11873580001)
25. Skim milk powder (EMD Millipore, catalog number: 1.15363.0500)
26. Amersham ECL Western blotting detection reagents (GE Healthcare, catalog number: RPN2209)
27. Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (GE Healthcare, catalog number: NA934V-1ML)
28. Wnt5a/b (C27E8) Rabbit mAb (Cell Signaling Technology, catalog number: 2530S)
29. Histone H3 (D1H2) XP[®] Rabbit mAb (Cell Signaling Technology, catalog number: 4499S)
30. 10% FBS DMEM (see Recipes)
31. 1x LAH DMEM (see Recipes)
32. Non-denaturing lysis buffer (see Recipes)
33. 0.1% Tween 20 in PBS (PBST) (see Recipes)
34. 5% skim milk in PBST (see Recipes)
35. 1x running buffer (see Recipes)
36. 1x transfer buffer (see Recipes)

Equipment

1. Thermo IEC Centra CL3R Bench-model, Refrigerated centrifuge
2. Avanti J-26 XP centrifuge (Beckman Coulter, model: Avanti J-26XP)
3. Beckman JA-25.50 rotor (Beckman Coulter, model: JA-25.50)
4. Bio-Rad Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, model: 1658005EDU)
5. XCell SureLock[®] Mini-Cell and XCell II blot module (Thermo Fisher Scientific, Novex[™], catalog number: EI0002)
6. QSonica XL-2000 (Qsonica, model: XL-2000)
7. Ultrospec 2100[®] UV-Visible spectrophotometer (Biochrom, model: 80-2112-21)

Procedure

A. Cell culture/transfection

1. Culture wild type MEF and Rab8a^{-/-} MEF cell lines in DMEM + 10% FBS until 90-95% confluent in a 10 cm Petri dish.
2. Replace DMEM + 10% FBS with DMEM supplemented with 12 ml of 1x lactalbumin hydrolysate solution (LAH).
3. Monitor the morphology of cells over the next 48 h.
4. After 48 h, collect the conditioned media in a 50 ml centrifuge tube and spin down at 10,000 x g at 4 °C for 10 min. Transfer the supernatant into a clean tube and immediately proceed with step B1 for maximum yield. Supernatant can be frozen and used after thawing but is generally not recommended.

5. Wash Petri dish with 1 ml of 1x PBS. Repeat wash.
6. Subsequently add 1 ml of 1x lysis buffer to Petri dish and lay flat on ice for 15 min.
7. Keeping the Petri dish on ice, collect the cell lysate using a cell scraper and transfer to a 1.5 ml microcentrifuge tube.
8. Sonicate cell lysate briefly (no more than 1 or 2 sec at a time for 3 cycles) and then centrifuge at 13,300 x g at 4 °C for 10 min.
9. Transfer supernatant to a clean microcentrifuge tube and store at -80 °C.

B. Centrifugation of conditioned media

1. Take the conditioned media and add it to the Amicon Ultra-15 centrifugal filter.
2. Centrifuge the filtration device at 4,000 x g at room temperature for 90 min.
3. Use a 200 µl pipette to recover the concentrate. For maximum recovery, collect the concentrate immediately. Expect roughly, 180 µl of concentrated media from collection.
4. Save the concentrate at -80 °C. Aliquot can remain frozen for up to a year.

C. Western blot detection

1. Measure protein concentrate using Bradford assay (Bio-Rad reagent protocol). Use lysis buffer as a blank for protein measurements.
2. Prepare 30 µg of total protein samples in 1x LDS sample buffer.
3. Run samples in 10% SDS-PAGE gel at 80 V and immediately stop when dye runs off gel.
4. Transfer protein onto a 0.45 µm PVDF membrane. Setup a transfer apparatus and run at 300 mA for 90 min.
5. After transferring, take the PVDF membrane and block using 5% skim milk in PBST. Leave the membrane for blocking at room temperature on a shaker for 1 h.
6. Subsequently, take the membrane and incubate overnight in a 1:1,000 dilution of anti-mWnt5a/b antibody at 4 °C. Dilute antibody in 5% skim milk in PBST.
7. After 16 h of incubation in primary antibody, wash membrane in PBST and place on a shaker for 10 min. Repeat two more times for a total of three washes.
8. Incubate with anti-rabbit antibody at a 1:2,000 dilution at room temperature for 1 h. Dilute antibody in 5% skim milk in PBST.
9. Wash membrane in PBST and place on a shaker for 10 min. Repeat two more times for a total of three washes.
10. Apply ECL reagent to membrane for 5 min.
11. Proceed to expose x-ray film for 30 sec and develop.

Representative data

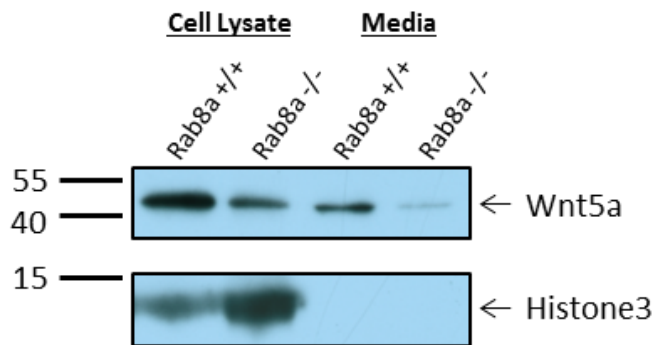


Figure 1. Detection of Wnt5a in conditioned media. Cell lysates and conditioned media from Rab8a^{+/+} and Rab8a^{-/-} MEF visualized using anti-mWnt5a primary antibody. Histone3 was used as a control to determine contamination from cell lysate in media samples.

Recipes

1. 10% FBS DMEM
 - 950 ml DMEM with 4.5 g/L glucose, L-glutamine & sodium pyruvate
 - 50 ml fetal bovine serum
 - 5 ml pen-strep
2. 1x LAH DMEM (50 ml)
 - 49 ml DMEM with 4.5 g/L glucose, L-glutamine & sodium pyruvate
 - 1 ml lactalbumin hydrolysate solution (50x)
3. Non-denaturing lysis buffer
 - 50 mM Tris-HCl (pH 7.5)
 - 150 mM NaCl
 - 10 mM EDTA
 - 0.02% NaN₃
 - 50 mM NaF
 - 1 mM Na₃VO₄
 - 0.5% Np40 substitute
 - 1 mM PMSF
 - 0.5 mM DTT
 - Protease inhibitor cocktail tablets (1tablet for 10mL)
4. 0.1% Tween 20 in PBS (PBST) (1 L)
 - 1 ml Tween[®] 20
 - 8 g NaCl
 - 0.2 g KCl
 - 1.44 g Na₂HPO₄

0.24 g KH_2PO_4

Dissolved in 1,000 ml MilliQ water, and pH adjusted to 7.4 using NaOH.

5. 5% skim milk in PBST

5 g skim milk powder dissolved in 100 ml PBST.

6. 1x running buffer

3.0275 g Tris base

14.4 g glycine

10 g sodium dodecyl sulfate

Dissolved in up to 1,000 ml of MilliQ water.

7. 1x transfer buffer

14.4 g glycine

3.02 g Tris base

Dissolved in 700 ml of MilliQ water

Add 200 ml methanol once completely dissolved.

Acknowledgments

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References

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