

The antibody validation experiments were essentially performed as described by Stadler et al (1), as briefly described below.

PREPARATION OF siRNA TRANSFECTION PLATES

A solid-phase transfection protocol (2) was used with Silencer Select siRNA from Ambion. For all genes, a scrambled siRNA sequence (Life Technologies, AM4635) was used as negative control.

1. For each target, specific siRNA 1.75 μ l Lipofectamine 2000 (Life Technologies) was mixed with 1.75 μ l H₂O.
2. To this mix, 3.5 μ l sucrose/Opti-MEM solution (13.7% (w/v)) and 5 μ l Silencer Select siRNA (3 μ M) were added.
3. The solution was gently mixed and incubated for 20 min at RT before 7 μ l 0.2% gelatin solution was added.
4. The siRNA transfection mix was stepwise diluted to a final concentration of 13 nM and subsequently transferred into the designated well of the 96-well plates (Greiner, SensoPlate).
5. The plates were dried in a speed-vac, GeneVac MiVac Quattro, initially for 90 min, then for 45 min, and thereafter for 30 minute until no solvent remained.

After complete lyophilization, the plates were stored with drying pearls in sealed boxes at room temperature for up to six months.

CELL CULTIVATION, TRANSFECTION & LYSIS

The U-2 OS human osteosarcoma cell line (ATCC-LGC Promochem, Borås, Sweden) was used. Cells were grown in McCoys growth media supplemented with 10% fetal bovine serum (FBS).

6. The cells were seeded (3500 cells per well) and incubated at 37°C in humidified air with 5.2% CO₂, for 72 h.
7. The growth medium was removed and 40 μ l lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS) was added to each well. The plate was then incubated for 30 min on ice. During the incubation the plate was vortexed repeatedly.

SDS-PAGE & TRANSFER

8. After centrifugation of the plate, the lysate was mixed with reducing buffer and boiled at 95°C for 5 min.
9. Molecular weight marker (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific) and siRNA and negative control sample preparations were loaded on a gel (4-20% Criterion™ TGX Stain-Free™ Precast Gels, Bio-Rad Laboratories) for SDS-PAGE.
10. The gel was imaged using a ChemiDoc MP and Image Lab 5.2 (Bio-Rad Laboratories) to obtain an image of the total amount of protein loaded prior to transfer.
11. The protein transfer was performed using the High MW program on Trans-Blot Turbo Blotting System (Bio-Rad Laboratories). The membrane was thereafter incubated in blocking buffer (1 mM Tris pH 7.5, 150 mM NaCl, 5% skim milk, 0.5% Tween20) for 45 min at RT.

ANTIBODY PROBING

12. Rabbit polyclonal antibody, specific for each target, was diluted to 0.9 µg/ml in blocking buffer and the membrane was incubated with the antibody solution for 1 h at RT, followed by washing (1 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20).

13. The membrane was thereafter incubated with a 1:3000 dilution of HRP-conjugated swine anti-rabbit antibody (Dako) for 45 min at RT, followed by washing.

IMAGING & DATA ANALYSIS

14. A substrate for chemiluminescence (Immobilon Western Chemiluminescence HRP Substrate, Millipore) was used for detection and the image was acquired using a ChemiDoc MP and Image Lab 5.2.

15. The relative signal intensity of the band in the siRNA lane was compared to the band in the negative control lane to measure protein downregulation. To verify protein downregulation, the image previously taken of the total amount of protein loaded was taken into consideration.

References

1. Stadler et al. Systematic validation of antibody binding and protein subcellular localization using siRNA and confocal microscopy. *J Proteomics*. 2012 Apr 3;75(7):2236-51.
2. Erfle H et al. Work flow for multiplexing siRNA assays by solid-phase reverse transfection in multiwell plates. *J Biomol Screen* 2008;13:575-80.