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A Simple Method to Assess Abundance of the β -Catenin Signaling Pool in Cells

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Abstract

β -catenin (CTNNB1) is a dual-function cell–cell adhesion/transcriptional co-activator protein and an essential transducer of canonical Wnt signals. Although a number of established techniques and reagents are available to quantify the nuclear signaling activity of β -catenin (e.g., TCF-dependent reporter assays, nuclear accumulation of β -catenin, and generation of N-terminally hypophosphorylated β -catenin), there are cell-type and context-dependent limitations of these methods. Since the posttranscriptional stabilization of β -catenin outside of the cadherin complex appears universally required for β -catenin signaling, the following method allows for simple assessment of the cadherin-free fraction of β -catenin in cells, using a GST-tagged form of ICAT (Inhibitor of β -Ca tenin and Tcf) as an affinity matrix. This method is more sensitive and quantitative than immunofluorescence and may be useful in studies that implicate TCF-independent signaling events.

Keywords

Wnt; β -catenin; ICAT; TCF (T-cell factor); GST (glutathione S transferase); Fusion proteins; Affinity precipitation; Western blot

1 Introduction

As a key subunit of the cadherin/catenin cell–cell adhesive complex, β -catenin is abundantly found at the plasma membrane and intercellular junctions (Fig. 1a, arrows). During Wnt signaling, modest fold changes in a small fraction of the total β -catenin pool are known to be biologically meaningful [1–3], but the magnitude of such changes can be challenging to quantify using immunofluorescence or detergent-free cell fractionation methods. While luciferase-based reporter assays are very sensitive, they are limited to cells that are easily transfected or infected by viruses and are unreliable in cell types, such as macrophages, that are notoriously difficult to transduce. Moreover, currently available reporters for β -catenin signaling rely exclusively on TCF -family consensus DNA binding sites, while other nuclear targets of β -catenin have been suggested [4–7]. Although nuclear fractionation techniques can be well applied to the study of β -catenin signaling, this type of assay is more cumbersome than the method presented below, and is complicated by co-fractionation of cadherins with nuclei, greatly reducing sensitivity of the assay. Lastly, evidence that an antibody, which specifically recognizes an N-terminally unphosphorylated, “nuclear signaling” form of β -catenin [8, 9] also detects β -catenin associated with the cadherin

complex [10, 11], indicates that interpreting changes in the abundance of hypophosphorylated β -catenin as a surrogate for changes in nuclear signaling should be considered carefully. Thus, there remains the need for a simple, sensitive method to assess changes in the signaling pool of β -catenin, where most of the total cellular β -catenin serves an important structural function as part of the cadherin complex.

The scale of the problem is visually depicted in Fig. 1, where stimulating β -catenin signaling in HEK293 cells with the GSK3 inhibitor, LiCl [12] or Wnt3a (not shown) more often than not fails to show visible differences in cytoplasmic/nuclear abundance of β -catenin (Fig. 1a). However, when a parallel sample of cells are solubilized in detergent and the cadherin-free pool of β -catenin is affinity-captured through its ability to bind a GST-tagged version of ICAT (a 71 amino-acid negative regulator of β -catenin signaling [13, 14]), one can readily detect a Wnt3a- or LiCl-mediated increase in cellular β -catenin (Fig. 1b). As ICAT directly binds β -catenin [15, 16] and in a manner that is competitive with β -catenin binding to cadherins [17], the GST-tagged ICAT can be used in conjunction with glutathione-sepharose beads to perform an immunoprecipitation technique, which selectively extracts β -catenin that is not bound to cadherin at the membrane. The protein fraction enriched for soluble cadherin-free β -catenin is subsequently released from the GST beads and can then be detected for analysis as usual by Western blot using one of the many available antibodies to β -catenin (see Subheading 3 below).

Note that the GST-ICAT-precipitable form of β -catenin is minor relative to the total cellular pool of β -catenin, as input lanes for this pull-down show no quantifiable differences in total β -catenin (Fig. 1b). Thus, cellular β -catenin is mostly cadherin-bound, and Wnt or Wnt-like (e.g., LiCl) signals affect changes on a proportionally small fraction of total cellular β -catenin. Since β -catenin binds to cadherins with nanomolar to picomolar affinity [18] and removing β -catenin from the cadherin complex requires harsh denaturing conditions (e.g., urea [19]), the GST-ICAT affinity precipitation method largely captures the pool of β -catenin stabilized by Wnt or Wnt-like signals. While in principle, any high-affinity β -catenin-binding protein could be fused to GST and used similarly to ICAT [17], the latter's small size and structural stability make it well suited to amplification and purification from bacteria (Fig. 2), as well as storage at 4 °C for weeks.

This method may not capture β -catenin already bound to components of the destruction complex (e.g., APC and Axin) or TCF-family members. However, a recent study suggests that stable accumulation of β -catenin within the destruction complex is unlikely [20]. Additionally, we and others (e.g., Xi He, Boston Children's Hospital, personal communication) have been unable to apply this technique to the quantification of β -catenin signaling from tissue homogenates, even using detergent washing conditions that increase stringency, for reasons that remain unknown. Thus, the method below is currently only validated for cells in culture.

2 Materials

All solutions should be prepared using ultrapure distilled deionized water (18 M Ω cm at 25 °C) and analytical grade reagents. Store and use all solutions at temperatures indicated. Dispose of waste materials according to local waste regulations.

2.1 Equipment and Disposables

1.5 mL microcentrifuge tubes, microcentrifuge tube rotator at 4 °C, refrigerated microcentrifuge, aspirator flask, pipettors and tips, cell scraper, sonicator, gel electrophoresis apparatus and power supply, electroblotter, blotting pads, nitrocellulose membrane, blot boxes, film cassette, film, film developer, heated water bath, petri dishes, 37 °C bacterial incubator, shaking incubator, 1000 mL flask, 15 and 50 mL sterile conical tubes, 17×100 mm sterile round-bottom tubes with cap, bacterial loop, cell spreader, 30 mL centrifuge tubes, spectrophotometer, 250 mL centrifuge bottles, refrigerated centrifuge, dialysis tubing or cartridges, stir bar, magnetic stir plate.

2.2 Reagents for GST-ICAT Fusion Protein Purification

1. pGEX GST-ICAT Plasmid DNA.
2. Glutathione sepharose beads.
3. BL21 (DE3pLysS) Competent Cells.
4. LB (Luria Broth) Miller.
5. LB Agar Miller.
6. Ampicillin (100 mg/mL stock in water).
7. SOC medium.
8. IPTG (100 mM stock in water).
9. Protease inhibitor tablets.
10. Lysis/Wash Buffer A (Non-ionic detergent buffer): 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100, 5% glycerol. For 500 mL: Add 400 mL water to 1 L beaker and add 15 mL of 5 M NaCl, 5 mL of 0.5 M EDTA, 25 mL of 1 M Tris pH 7.5 stock. Mix and adjust pH to 7.5 if necessary with HCl. Add 5 mL of 100% Triton-X100 and 25 mL of 100% glycerol. Bring final volume to 500 mL with water and transfer to storage bottle. Store at 4 °C.
11. Wash Buffer B (Non-ionic detergent “wash” buffer): same as Buffer A with the exception that the Triton X-100 concentration is reduced to 0.1% v/v (0.5 mL) (see **Note** ¹).
12. Protease inhibitor tablets.

¹This buffer is used mainly for the final washes of immunoprecipitations. Note that excess non-ionic detergent binding to proteins is thought to inhibit SDS present in the sample buffer from binding/denaturing proteins for PAGE analysis.

13. l-Glutathione.
14. Elution Buffer: 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 8, 5% glycerol, 30 mM l-glutathione (*see Note* ²).
15. Dialysis Buffer: 15 mM Tris-HCl pH 7.5, 300 mM NaCl, 5% glycerol, store at 4 °C.
16. Protein Assay Reagent (BioRad Cat#500-006). Use as per manufacturer's instructions.
17. Coomassie Blue: 50 mL acetic acid (10% w/v), 150 mL methanol (30% w/v), 1.25 g Coomassie Brilliant Blue, stir to mix and filter through Whatman paper, store at room temperature.
18. Destain solution: Same as solution above without Coomassie Blue.

2.3 Reagents for GST-ICAT Pull-Down

1. GST-ICAT purified protein (*see Note* ³).
2. Glutathione Sepharose 4B beads.
3. PBS with calcium and magnesium.
4. Protease inhibitor tablets.
5. Lysis/Wash Buffer A (Non-ionic detergent buffer): 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100, 5% glycerol. For 500 mL: Add 400 mL water to 1 L beaker and add 15 mL of 5 M NaCl, 5 mL of 0.5 M EDTA, 25 mL of 1 M Tris pH 7.5 stock. Mix and adjust pH to 7.5 if necessary with HCl. Add 5 mL of 100% Triton-X100 and 25 mL of 100% glycerol. Bring final volume to 500 mL with water and transfer to storage bottle. Store at 4 °C.
6. Wash Buffer B (Non-ionic detergent "wash" buffer): Same as Buffer A with the exception that the Triton X-100 concentration is reduced to 0.1% v/v (0.5 mL) (*see Note* ¹).
7. 2× SDS sample loading buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl pH 6.8. Store at room temperature.

2.4 Reagents for Western Blot

1. 10% Tris-HCl polyacrylamide gel.
2. 10× Tris-Glycine-SDS Running Buffer.
3. Protein Ladder.

²Add glutathione just before use to an aliquot of non-detergent lysis buffer and adjust the pH back to 8. It will be too acidic if you do not re-pH and will not work.

³The concentration of purified GST-ICAT protein will vary with preparation, assay for accuracy.

4. Transfer Buffer: Add to beaker in this order: 1.6 L water, 28.8 g glycine, 6.06 g Tris Base, then stir solution to mix and add 400 mL (20%) methanol (makes 2 L), store at room temperature.
5. TBS-Tween: Add to beaker 8 g NaCl, 0.2 g KCl, 3 g Tris Base, 800 mL water, adjust pH with HCl to 7.4, bring volume up to 1 L with water, add 1 mL/L Tween-20, store at room temperature.
6. 5% dry-milk TBS-Tween: Add to beaker 5 g dry milk per 100 mL TBS-T and stir to mix, store at 4 °C.
7. Ponceau S: Add to beaker 1 g Ponceau S (0.1% w/v), 50 mL acetic acid (5% w/v), and 950 mL water, mix with stir bar, store at room temperature.
8. C-terminal β -catenin mouse monoclonal antibody (BD Cat #610154) or N-terminal β -catenin rabbit polyclonal antibody (Millipore Cat #06-734).
9. ECL chemiluminescence reagent.

3 Methods

3.1 Transforming Protein-Competent Bacterial Cells with GST-ICAT

1. Thaw frozen BL21 cells on ice, flick tube gently to mix well.
2. Pipet 100 μ L of BL21 cells to sterile pre-chilled 14 mL round-bottom tube on ice.
3. Add 2 μ L (1–50 ng, volume not greater than 10 μ L) pGEX GST-ICAT plasmid DNA to BL21 cells, gently flick tube to mix, and incubate on ice for 30 min.
4. Heat pulse transformation for 45 s at 42 °C in water bath.
5. Immediately place tube on ice for 2 min.
6. Add 900 μ L pre-warmed SOC medium to the tube.
7. Incubate transformation at 37 °C in shaking incubator (225 rpm) for 1 h.
8. Pipet and spread 100 μ L of transformation to LB/Agar plate (*see Note* ⁴).
9. Incubate inverted plate in bacterial culture incubator at 37 °C overnight (12–14 h).
10. Check plate for colonies (*see Note* ⁵), pick a single colony with a bacterial loop and inoculate 5 mL of LB containing 100 μ g/mL of ampicillin, incubate culture with shaking at 37 °C overnight.

⁴It may be helpful to also plate transformation reaction at dilutions of 1:10 and 1:100 to ensure individual colony growth. Spread solution evenly over agar surface using sterile cell spreader.

⁵Agar plate with bacterial colonies may be wrapped with Parafilm and stored at 4 °C for later processing.

11. Inoculate 500 mL of LB containing 100 µg/mL of ampicillin in 1000 mL flask with the 5 mL overnight culture, incubate with shaking (225 rpm) for 2–3 h until OD reaches ~0.8 (*see Note* ⁶).
12. Add 500 µL (1 µL/mL) of stock IPTG to culture, continue incubation at 37 °C for additional 3 h.
13. Transfer culture to 2 × 250 mL centrifuge bottles and centrifuge to pellet bacteria at 1400 × *g*/RCF (Floor Sorvall 3000rpm F14S rotor), 15 min, 4 °C. Decant supernatant. Bacterial pellet may be stored at –80 °C until further processing.

3.2 Purification of GST-ICAT Fusion Protein

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Add 10 mL Lysis Buffer A with protease inhibitor (1 tablet per 10 mL solution) to bacterial pellet and pipet up and down gently with serological pipet until pellet is fully resuspended (*see Note* ⁷).
2. Transfer suspension to a 50 mL conical tube on ice.
3. Sonicate lysate five times for 15 s each with rest period of 30 s between sonications (*see Note* ⁸).
4. Transfer lysate to 30 mL polypropylene centrifuge tube and centrifuge for 20 min at 12,000 × *g*/RCF (Floor Sorvall 10000rpm SS-34 rotor) and at 4 °C.
5. Transfer cleared lysate (supernatant) to 15 mL conical centrifuge tube (*see Note* ⁹).
6. Prepare glutathione beads by washing three times in Lysis/Wash buffer A (*see Note* ¹⁰), and resuspend beads in Lysis/Wash buffer A to a 50:50 bead:buffer slurry.
7. Add 200 µL resuspended beads (100 µL of packed beads) to cleared lysate and tumble tube on tube rotator for 2 h at 4 °C.
8. Centrifuge tube of lysate for 5 min at 2100 × *g*/RCF (Tabletop Beckman 3000rpm) and 4 °C to pellet beads.

⁶Set aside an aliquot of clean LB to serve as blank for spectrophotometer reading.

⁷Allow cell pellet to thaw on ice if previously stored at –80 °C.

⁸We use Branson Sonifier 250, at output level 2, constant duty cycle. Keep lysate tube on ice during sonication to prevent protein overheating and degradation.

⁹A 20 µL aliquot of cleared lysate may be saved for gel analysis.

¹⁰Glutathione-sepharose beads come as a suspension containing 20% ethanol, so calculate the amount of beads to wash to allow for 25 µL of packed beads per sample. A preparation for *n* + 1 samples works best. Invert the vial of beads to resuspend before pipetting as they settle to the bottom. Resuspend packed beads in Buffer B (0.1% TX-100) to give a final slurry volume. Example: for ten samples, 350 µL beads equals ~280 µL packed beads, so wash and resuspend beads to 560 µL total slurry with buffer, and pipet 50 µL to each tube.

9. Remove supernatant (*see Note*¹¹) and wash beads twice with ice-cold Lysis/Wash Buffer A (*see Note*¹²) with centrifugations between as in **step** 8.
10. Repeat **step** 9 with non-detergent Wash Buffer B.
11. Remove supernatant and add 1 mL elution buffer (*see Note*²) to bead pellet, resuspend beads and transfer bead-buffer slurry to 1.5 mL microcentrifuge tube.
12. Tumble tube on tube rotator for 1 h at 4 °C.
13. Pre-equilibrate dialysis tubing or cassette in cold dialysis buffer at least 1 h before use.
14. Centrifuge tube 5 min at $11,000 \times g$ /RCF (Microcentrifuge 10,000rpm) and 4 °C to pellet beads.
15. Collect eluent protein supernatant and transfer to prepared dialysis tubing or cassette (*see Note*¹³), place into beaker containing 2 L cold dialysis buffer and magnetic stir bar.
16. Place beaker on magnetic stir plate and dialyze protein overnight with stirring at 4 °C.
17. Repeat the dialysis the next morning with 2 L fresh dialysis buffer for an additional 4 h.
18. Collect eluent protein and aliquot 50 µL or smaller portions to microcentrifuge tubes (*see Notes*¹⁴ and ¹⁶) and store at –80 °C.

3.3 Lysing Cells

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Aspirate media and rinse cells three times in PBS containing calcium and magnesium.
2. For a 100 mM culture dish of cells, add 0.5 mL Buffer A (1% TX-100) with protease inhibitor added (1 tablet per 10 mL solution), then use a cell scraper to detach the cells.
3. Transfer the cell suspension to a 1.5 mL microcentrifuge tube.
4. Sonicate cells on ice at the lowest power with pulse setting, ten pulses (*see Note*¹⁵).

¹¹A 20 µL aliquot of unbound fraction supernatant may be saved for gel analysis.

¹²Invert tube quickly 20 times to ensure adequate bead suspension and washing.

¹³We have used SpectraPor dialysis membrane (MWCO 12-14,000) Cat # 132676, and Thermo Slide-A-Lyzer G2 dialysis cassette (10K MWCO) Cat # 88250.

¹⁴Set aside two 10 µL aliquots of dialyzed protein for assay and gel analysis with BSA standard curve. Avoid multiple freeze/thaw of purified protein.

¹⁶We used commercially available Bradford assay kit. Use as per manufacturer's instructions with BSA standard.

¹⁵We use Branson Sonifier 250, at output level 1, 10% duty cycle. Keep lysate tube on ice during sonication to prevent protein overheating and degradation.

5. Centrifuge lysate at $21,000 \times g$ /RCF (Microcentrifuge 14,000rpm) for 10 min at 4 °C.
6. Transfer supernatant to a fresh microcentrifuge tube.
7. Assay protein using standard Bradford Method to obtain concentration (*see Note* ¹⁶).

3.4 Preparing Glutathione-Sepharose Beads

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Use 50 μ L of a 50:50 glutathione-sepharose bead:buffer slurry per sample. Pre-wash the required quantity of glutathione-sepharose beads to remove ethanol in 1 mL (or more) of Buffer B (0.1% TX-100), centrifuge at $21,000 \times g$ /RCF (Microcentrifuge 14,000rpm) briefly to pellet the beads, aspirate the wash buffer and resuspend beads in required volume of Buffer B (*see Note* ¹⁰).

3.5 GST-ICAT Pull-Down

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Pipet 1000 μ g cell lysate (*see Notes* ¹⁷ and ¹⁸) plus Buffer B (0.1% TX-100) up to 900 μ L total volume into a 1.5 mL microcentrifuge tube.
2. Add 50 μ L of glutathione-sepharose bead slurry per tube (*see Note* ¹⁰).
3. Add 10 μ g GST-ICAT per tube (*see Notes* ³ and ¹⁸).
4. Tumble tubes on tube rotator at 4 °C, 2 h.
5. Centrifuge tubes at 4 °C to pellet beads at $21,000 \times g$ /RCF (Microcentrifuge 14,000rpm), 2 min.
6. Aspirate supernatant (*see Note* ¹⁹).
7. Wash beads with 1 mL Buffer B (0.1% TX-100, centrifuge at $21,000 \times g$ /RCF (Microcentrifuge 14,000rpm), 2 min and aspirate off supernatant three times (*see Notes* ¹² and ²⁰).
8. Add 35 μ L 2 $\times$ SDS loading buffer to final bead pellet (*see Note* ²¹).
9. Heat protein/bead pellet at 95 °C for 5 min and centrifuge tubes to pellet beads before loading supernatant to acrylamide gel (*see Note* ²²).

¹⁷GST-ICAT pull-down may also be done proportionally without assay, saving 10% of input lysate to run on a duplicate gel.

¹⁸The amount of protein needed to pull-down detectable β -catenin may vary greatly between cell types, so you may require much less than this. We have successfully used as little as 200 μ g protein. The volume of GST-ICAT purified protein added will depend on its concentration. Use GST-ICAT at a minimum of 10% in proportion to the total quantity of protein.

¹⁹Use an aspirator flask with the vacuum turned down to a low level, with a glass Pasteur pipet covered by a 200 μ L plastic pipet tip so that the supernatant can be removed gently and slowly without losing beads. The 200 μ L pipet tip can be quickly changed between samples. You may prefer to do this manually with a pipettor. The supernatant can be saved for future evaluation by gel electrophoresis of unbound fraction.

²⁰Invert tubes ten times to make sure beads have been resuspended in buffer and washed well before centrifuging.

²¹Bead pellet may be stored at -20 °C until gel run.

3.6 Western Blot for β -Catenin

1. Place gel into electrophoresis apparatus and add running buffer diluted to 1 $\times$ in distilled deionized water, remove comb.
2. Load samples to 10% polyacrylamide-Tris-SDS gel with protein standard, run gel at 100 V until the lowest standard is at bottom of gel and/or dye front runs off.
3. Pre-soak gel, blotting pads and nitrocellulose membrane in transfer buffer for 15 min and assemble components according to the manufacturer's instructions and transblot proteins to nitrocellulose at 18 V for 1 h.
4. To view protein transfer quality, stain membrane in Ponceau S for 10 min at room temperature with rocking, rinse membrane in distilled H₂O.
5. Place membrane in blotting box and block membrane in 5% milk-TBS-T at room temperature for 1 h with rocking.
6. Remove blocking solution and replace with β -catenin antibody diluted at 1:1000 in 5% milk-TBS-T, incubate with rocking overnight at 4 °C.
7. Wash blot three times, 15 min each with TBS-T.
8. Dilute goat-anti-mouse-HRP secondary antibody 1:3000 in 5% milk-TBS-T, incubate with rocking at room temperature 1 h.
9. Wash blot three times, 15 min each with TBS-T.
10. Cover membrane with premixed ECL reagent for 1 min, wrap in plastic film and place in film cassette. Expose membrane to film up to 15 min and develop.

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²²Heating in sample loading buffer will release the protein from glutathione beads. Set pipettor to pull off volume of sample loading buffer added (35 μ L). Do not load beads to gel wells.

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Fig. 1.

Assessment of cadherin-free β -catenin by immunofluorescence and GST-ICAT affinity precipitation analysis. **(a)** Immunofluorescence images of HEK293 cells fixed and stained for β -catenin (green) after GSK3 inhibition by 30 mM LiCl (6 h). Media supplemented with 30 mM NaCl serves as a control. Arrow indicates β -catenin staining at cell-cell contacts. Scale bar = 10 μ m. **(b)** Immunoblots from HEK293 cells (60 mm plate) treated 6 h with Wnt3a (R&D Biosystems), 30 mM LiCl or NaCl (control). Cells were solubilized, clarified and lysates were normalized by Bradford analysis. A small fraction was used for input lanes (*top blot*), while the remaining lysate was used for affinity precipitation by GST or GST-ICAT as described (*lower blot*)

Fig. 2.

Purification of GST-ICAT from bacteria. GST (control) and GST-ICAT were purified from bacteria as described and fractionated by standard SDS-PAGE. Gel was fixed in methanol/acetic acid and stained with Coomassie Brilliant Blue to visualize proteins. Molecular weight standards are shown