Preface

Since their discovery some 20 years ago, Wnt signaling molecules have been shown to control key events in embryogenesis, maintain tissue homeostasis in the adult and, when aberrantly activated, promote human degenerative diseases and cancer. Elucidation of Wnt signaling mechanisms has relied on both biochemical methodologies and vertebrate and invertebrate model systems. Therefore, I felt that an issue dedicated to Wnt signaling had to include both assays (biochemical readout) and model systems (functional readout) of Wnt signaling. It is not an exhaustive catalog, but rather a point of reference to current molecular protocols and the diverse model systems employed to study this signaling pathway. The issue is divided into two volumes. The first volume includes assays to measure activation of the diverse Wnt signaling pathways as well as models and strategies used to study mammalian Wnt/FZD function (from protein–protein interaction and simple cell line models to organoid cultures and mouse models). The second volume is dedicated to the diverse vertebrate and invertebrate models that have shaped the Wnt signaling field. It provides an entry point for the novice and an overview of the unique properties of each organism with respect to studying Wnt/FZD function (for example asymmetric cell division in *Caenorhabditis elegans*, epithelial morphogenesis in *Dictyostelium* and so on). Given the collective expertise and knowledge of the contributors, I anticipate that this two-volume issue will be an invaluable resource.

The Wnt field advances at an exceptionally rapid rate for several reasons. First, diverse fields of research converge on this pathway. Second, the Wnt community is very generous: reagents, knowledge, and ideas are shared freely. This is facilitated by informative web sites and regular Wnt meetings that are packed back-to-back with cutting-edge research. The "no-frills" approach to these meetings means that the whole community, including students, can participate. Equally important is the elusive nature of the Wnt pathway itself, which continues to intrigue and fascinate both novice and veteran researchers alike. This book is a testament to all these. It was steered by the generosity and enthusiasm of contributors from diverse fields. I thank them all. Special thanks to Randall Moon and Stefan Hoppler; their suggestions for authors and chapters helped shape this issue.

On a personal note, I would also like to take this opportunity to acknowledge Bill Boyle for being an inspirational mentor during my formative years; his infectious enthusiasm for research set me on this exciting and rewarding career path. I am indebted to Bob Thomas and Rob Ramsay for generously supporting my research into FZD7 function in colon cancer when funding in Australia for the Wnt field was scarce in the early years. Most importantly, I thank my very patient and accommodating children for allowing me to indulge myself!

I thank Tony Goodwin, Scott Bowden, and the University of Melbourne—without their assistance this book would not have been possible. John Walker and all at Humana Press, especially David Casey and Amina Ravi, for their generosity and for the opportunity to edit this issue—a truly rewarding experience.

Chapter 2

Isolation and Application of Bioactive Wnt Proteins

Karl H. Willert

Abstract

Wnt proteins and their signaling cascades are involved in a wide variety of developmental processes, and deregulation of this pathway is frequently associated with tumorigenesis. Unlike many other growth factors, Wnts long eluded biochemical purification, in large part because of their hydrophobic nature, which is imparted by one or more lipid modifications *(1–3)*. Here I describe a complete protocol that outlines the purification process for Wnt proteins. While this protocol has not been applied to all known Wnt proteins, it has been successfully applied to the purification of a large subset of Wnts, including the very divergent Wnt protein, Drosophila Wnt8 (Dwnt8 or WntD), indicating that this protocol is likely applicable to all Wnts.

Key words: Wnt, Wnt3A, β-catenin, Purification, Blue Sepharose, Immobilized metal affinity chromatography (IMAC), Gel filtration, Heparin cation exchange.

1. Introduction

The protocol described here, based on a previous publication *(3)*, outlines the purification protocol of Wnt proteins, starting with a crude and dilute Wnt sample, usually in the form of conditioned medium (CM) *(4)*, to the final purified protein. In addition, protocols to assay Wnt activity are described.

Two key observations have made the purification of Wnts possible: 1) inclusion of detergent to facilitate solubility of the highly hydrophobic Wnt protein, and 2) fractionation over Blue Sepharose, which binds Wnts with high selectivity. The purification consists of four chromatography steps: Blue Sepharose,

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immobilized metal affinity chromatography (IMAC), gel filtration, and heparin cation exchange. Throughout the purification, samples are assayed for both the presence of the Wnt protein (using a Wnt immunoblot or a Coomassie- or silver-stained gel) and activity (β-catenin stabilization or activation of Wnt reporter constructs) to ensure optimal recovery of protein and associated activity.

This protocol describes the purification of Wnt3A specifically; modifications for other Wnt proteins are necessary (e.g., elution profiles may vary; for detection use the appropriate Wnt antibody; not all Wnts stabilize β-catenin; etc.). The resulting purified product is useful in a variety of assays, including signaling studies in established cell lines, explant manipulations, and in vivo experiments (for examples, see refs. *(3, 5–15)*).

2. Materials

2.1. Production of Wnt3A CM

1. Cell line: L-Wnt3A (ATCC, Manassas, VA; ATCC# CRL-2647) (*see* **Note 1**).
2. Cell culture medium: Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1:100 dilution of penicillin–streptomycin solution with 10,000 U penicillin (base)/mL and 10,000 µg streptomycin (base)/mL in 0.85% NaCl (liquid) (*see* **Note 2**).
3. Dulbecco's phosphate-buffered saline (PBS).
4. Trypsin (0.25% liquid trypsin).
5. Cell culture dishes: 10- or 15-cm dishes or large surface area culture dishes (e.g., Corning® CellSTACK®).
6. CO_2 incubator and biosafety cabinet.
7. Filter bottle, 0.5–1 L, 0.2-µm pore size (Corning or equivalent).

2.2. Preparation of Wnt3A CM for Fractionation

1. 20% (v/v) Triton X-100.
2. 1 M Tris-HCl, pH 7.5.
3. 10% (w/v) NaN_3.
4. Filter bottle, 0.5–1 L, 0.2-µm pore size (Corning or equivalent).

2.3. Fractionation of Wnt3A CM

2.3.1 Step 1: Blue Sepharose

1. Sample: 1–4 L Wnt3A CM as prepared in **Section 2.2**.
2. Column: Blue Sepharose HP (Cibacron Blue F3G-A coupled to Sepharose), ~100 mL packed into an empty column with 100–200 mL of bed volume.

3. Binding Buffer: 1% (w/v) CHAPS, 150 mM KCl, and 20 mM Tris-HCl, pH 7.5, sterile filtered.
4. Elution Buffer: 1% (w/v) CHAPS, 1.5 M KCl, and 20 mM Tris-HCl, pH 7.5, sterile filtered.
5. Syringe (30–50 mL) with 0.2-μm pore size filter.

2.3.2. Step 2: IMAC

1. Sample: Pooled Wnt3A containing fractions from **Section 2.3.1**.
2. HiTrap™ Chelating, 1-mL column (GE Healthcare, Piscataway, NJ), loaded with Cu^{2+}.
3. Binding Buffer: 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, and 1% (w/v) CHAPS.
4. Elution Buffer: Binding Buffer with 100 mM imidazole, pH 7.5.
5. Syringe (10 mL) with 0.2-μm pore size filter.

2.3.3. Step 3: Gel Filtration

1. Sample: 5–10 mL pooled Wnt3A-containing fractions from **Section 2.3.2**.
2. HiLoad 26/60 Superdex 200 preparative grade.
3. Buffer: 1× PBS, 1% (w/v) CHAPS.
4. Syringe (30 mL) with 0.2-μm pore size filter.

2.3.4. Step 4: Heparin Cation Exchange

1. Sample: 20–40 mL pooled Wnt3A-containing fractions from **Section 2.3.3**.
2. 1 mL HiTrap heparin (GE Healthcare).
3. Binding Buffer: 1× PBS, 1% (w/v) CHAPS.
4. Elution Buffer: Binding Buffer, 1 M NaCl (adjust pH to that of Binding Buffer if necessary).
5. Syringe (10 mL) with 0.2-μm pore size filter.

2.4. Assays for Wnt Proteins

2.4.1. Wnt3A Immunoblots

1. Sample: Any fraction containing the Wnt protein of interest.
2. Protein Sample Loading Dye (4×): 250 mM Tris-HCl, pH 6.8, 8% (w/v) sodium dodecyl sulfate (SDS), 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, pinch of bromo phenol blue.
3. Suitable SDS-polyacrylamide gel electrophoresis (PAGE) and transfer setup (e.g., the BioRad Criterion Precast Gel System or equivalent).
4. Nitrocellulose or polyvinyl difluoride (PVDF) membrane.
5. Wnt3A antibody (available from R&D Systems, Minneapolis, MN).
6. Appropriate conjugated secondary antibody for detection.

2.4.2. β-Catenin Stabilization

1. Mouse L-cells (ATCC CCL-1.3 or CRL-2648).
2. Cell culture medium: Same as **Section 2.1.2**.
3. 96-well tissue culture plate (flat well).
4. Wnt3A samples (CM, fractions from **Sections. 2.3** and **2.4**).
5. PBS.
6. Lysis Buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% (v/v) Triton X-100.
7. Suitable SDS-PAGE and transfer setup as in **Section 2.4.1**.
8. Protein Sample Loading Dye (4×) as in **Section 2.4.1**.
9. Nitrocellulose or PVDF membrane.
10. Mouse anti-β-catenin antibody (available from various vendors, including BD Transduction Laboratories, Franklin Lakes, NJ; R&D Systems; and Santa Cruz Biotechnology, Santa Cruz, CA).
11. Appropriate conjugated secondary antibody for detection.

3. Methods

3.1. Production of Wnt3A CM

1. Grow L-Wnt3A cells to confluency.
2. Wash with warm (37°C) PBS, trypsinize, and divide the L-Wnt3A cells into plates with a surface area 20 times greater than the original dish(es). Use about 10 mL of cell culture medium per 75-cm^2 surface area).
3. Incubate cells in a humidified CO_2 incubator at 37°C for 4 days.
4. Remove CM and filter through 0.2-µm filter. Add fresh media to all plates and incubate another 3 days.
5. Harvest a second batch of CM. Discard dishes. Filter the second batch and combine with first batch of CM. The CM media can be stored at 4°C for several months without appreciable reduction in activity, as assessed by β-catenin stabilization in L-cells, over the course of 1 year. However, a sensitive and quantitative assay may detect a change in specific activity.

3.2. Preparation of Wnt3A CM for Fractionation

1. To the filtered CM from **Section 3.1**, add Triton X-100 to 1% (v/v), Tris-HCl, pH 7.5, to 20 mM, and NaN_3 to 0.01% (w/v) (e.g., to 930 mL CM, add 50 mL of 20% (v/v) Triton X-100, 20 mL of 1 M Tris-HCl, pH 7.5, and 1 mL of 10% (w/v) NaN_3).
2. Filter through a 0.2-µm filter.

3.3. Fractionation of Wnt3A CM

This fractionation can be performed with any chromatography setup, such as the Äkta FPLC (GE Healthcare). If such an instrument is not available, the purification can also be performed using a peristaltic pump that can accurately control flow rates of 1–5 mL/min. The purification involves four steps: Blue Sepharose, IMAC, gel filtration, and heparin cation exchange, each of which is described in detail below. All buffers and the sample should be sterile filtered through a 0.2-μm filter (*see* **Note 3**).

3.3.1. Step 1: Blue Sepharose

Purpose: This step serves to recover a large fraction of Wnt3A protein from the CM. In an optimal experiment, the Wnt protein can be enriched 2,000- to 2,500-fold (*see* **Notes 4** and **5**).

1. Column Packing: Pour a 1:1 slurry of the Blue Sepharose into a clean and empty column, such as XK26-20 or XK50-20 (GE Healthcare). Allow resin to settle overnight; close the column from the top by lowering and tightening the plunger so that no air is trapped and no gap exists between the top of the resin bed and the plunger.
2. Column Equilibration: Using a suitable pump, wash the column with 2–3 column volumes (CV) of filtered and distilled water or 20 mM Tris-HCl, pH 7.5, at a flow rate of 1–5 mL/min, being sure not to exceed the allowable back pressure for the Blue Sepharose resin. Wash the column with 2–3 CV of binding buffer. If using a UV monitor, adjust it to zero. The column is now ready for sample application.
3. Sample Application: At a flow rate of 1–5 mL/min, apply the entire volume of the Wnt3A CM. The flow rate can be adjusted so that this step can be performed within the span of a few hours or overnight (e.g., a 1 L sample can be applied at 5 mL/min in 3 hours and 20 minutes or at 1 mL/min in 16 hours and 40 minutes). Collect the flow-through material, which can be examined for the presence of Wnt protein by immunoblotting to ensure depletion (*see* **Note 6**). During this loading step, the UV monitor may exceed its detection limit, which is in part due to the high protein content of the sample (10% FBS) and in part due to the Triton X-100, a nonionic detergent containing an aromatic group that exhibits intense UV absorbance.
4. Washing Column: Wash the column with Binding Buffer until the UV reading has stabilized near baseline. This may take four to five CVs. Washing can be done at a flow rate of up to 5 mL/min, as long as the maximal allowed back pressure tolerated by the resin is not exceeded.
5. Elution: Once the UV reading has established a stable baseline, start the elution by switching the buffer to Elution Buffer. Start collecting 10-mL fractions. A large protein peak

will emerge immediately coincident with the increase in salt concentration. Continue to collect fractions for about 100 to 200 mL beyond the main protein peak. Assay all fractions for Wnt3A protein by immunoblotting (*see* **Section 3.4.1**). It is important to note that a large portion of the Wnt3A protein trails behind the main protein peak. These trailing Wnt fractions (Pool 2 in **Fig. 2.1**) contain very little of the other proteins that bind to Blue Sepharose under these conditions. If this separation is not achieved and all the Wnt protein co-elutes with the main protein peak, the following fractionation steps will serve to remove the majority of contaminating proteins. Combine the fractions containing the highest amounts of Wnt protein and sterile filter through a 0.2-µm syringe filter. Depending on the amount of starting material and the size of the column, the total volume of pooled eluate fractions will vary from 40 to 100 mL. Store the samples at 4°C before proceeding to the next step. Do not freeze the fractions.

6. Regeneration of column: Wash the column into distilled water and then into 20% (v/v) ethanol for long-term storage. To clean the column rigorously, wash the column in reverse flow

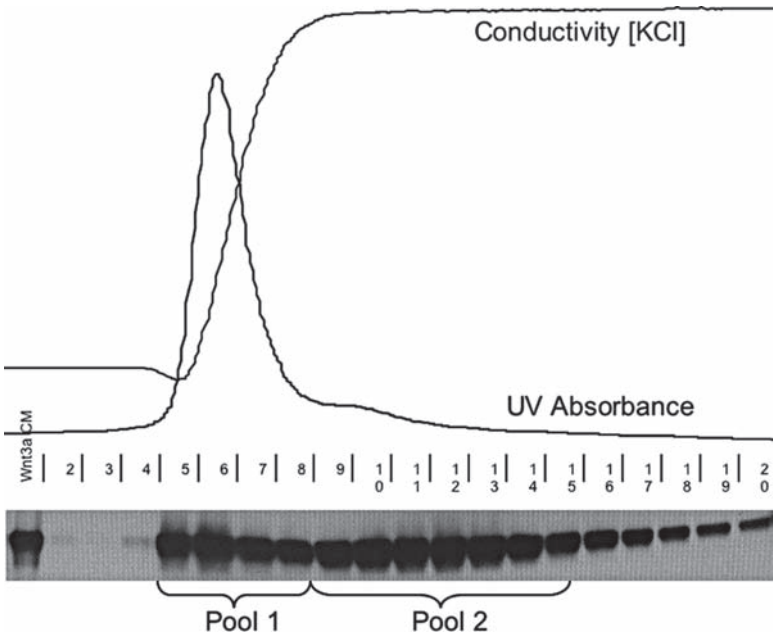

Fig. 2.1. Blue Sepharose fractionation of Wnt3a CM. This step leads to an approximate enrichment of the Wnt protein of 2,000- to 2,500-fold. A large proportion of Wnt3A, detected by immunoblotting, trails behind the major protein peak and consequently contains a higher specific activity of Wnt protein. While the eluate fractions containing Wnt3A can be combined and used for further purification, separating Pool 2 from Pool 1 will yield a purer final Wnt preparation. The Wnt3A protein continues to elute for quite a while, suggesting that in this elution buffer Wnt3A is sticking to Blue Sepharose.

with two to three CVs of 0.1–0.5 N NaOH. This treatment will remove some of the immobilized Cibacron Blue dye from the resin, so contact time should be minimized (*see* **Note 4**).

3.3.2. Step 2: IMAC

Purpose: This step serves to remove contaminating proteins and to concentrate the volume of Wnt3A-containing fractions.

1. Column Equilibration: At a flow rate of 1 mL/min, wash the column with 5 mL distilled and filtered water, and then with 5 mL Binding Buffer. The column is now ready for sample application.
2. Sample Application: Apply the pooled and filtered fractions from Step 1 at a flow rate of 1 mL/min, using a Superloop™ (GE Healthcare) or a sample pump. Collect the flow-through material. Once the entire sample has been applied, wash the column with 5 to 10 CVs of Binding Buffer or until the UV absorbance has established a stable baseline.
3. Elution: Bound protein is eluted by a combination of a step followed by a linear gradient elution (*see* **Fig. 2.2**): 1) step to 5% Elution Buffer/95% Binding Buffer and collect 1-mL fractions for 10 CV (note: a large protein peak should elute as shown in **Fig. 2.2**); 2) elute with a gradient from 5% Elution Buffer/95% Binding Buffer to 100% Elution Buffer over

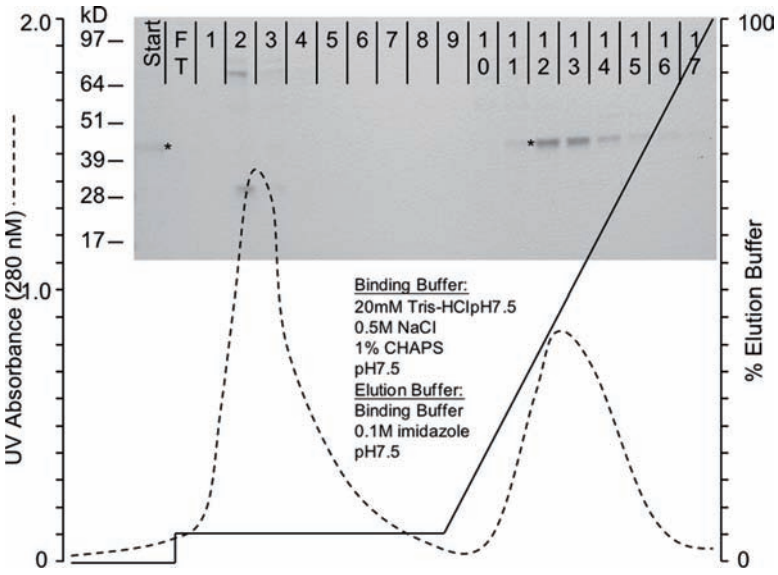

Fig. 2.2. IMAC fractionation of Wnt3A. This step separates Wnt3A from some of the contaminants that co-purify in the Blue Sepharose step. In addition, this step acts to concentrate the samples significantly. After this fractionation step, Wnt3A can be readily detected on a Coomassie-stained gel, which also serves to indicate the purity of the Wnt protein samples. The starting material (*Start*) is Wnt3A eluted from Blue Sepharose; the flow-through material is indicated by *FT*. The *asterisk* indicates the position of the Wnt3A band.

10 CV and collect 1-mL fractions; 3) collect an additional ten 1-mL fractions at 100% Elution Buffer. Verify the presence of Wnt3A by immunoblotting (*see* **Section 3.4.1**). The vast majority of Wnt3A should be present in fractions collected during the gradient from 5 to 100% Elution Buffer. The bound Wnt protein elutes in a broad peak, totaling about 5 mL, at a concentration of approximately 10–40 mM imidazole. If sufficient Wnt3A CM is fractionated (>1 L), a Coomassie stained gel should be sufficient to allow detection of the Wnt protein in the eluate fractions (as shown in **Fig. 2.2**). Combine fractions containing highest amounts of Wnt protein and sterile filter through a 0.2-µm syringe filter. The sample can be stored at 4°C before continuing with the purification. Do not freeze the fractions.

3.3.3. Step 3: Gel Filtration

Purpose: This step serves to separate low molecular weight Wnt from high molecular weight Wnt and some contaminating proteins. Additionally, it serves to exchange the buffer from high (0.5 M) to physiological (1× PBS) salt concentrations, which is important to perform Step 4 of this purification.

1. Column Equilibration: At a flow rate of 1–2.5 mL/min (be sure not to exceed the maximal back pressure permissible for this column), wash the column with one CV distilled and filtered water, then with two CV Buffer. The column is now ready for sample application.
2. Sample Application and Fractionation: Load the sample using a Superloop™ at a flow rate of 1 to 2.5 mL/min. If the sample exceeds the maximum volume that can be efficiently fractionated on this column (consult the vendor's recommendations for details), split the sample and perform two identical fractionations. Once the entire sample has been applied, collect 10-mL fractions for an entire CV. The majority of the Wnt protein should emerge at the same position as a molecular weight standard of 50 kD (*see* **Fig. 2.3**). Occasionally a small amount of Wnt protein elutes with the void volume of the column suggesting that it is part of a large complex or aggregate (*see* **Note 7**). The Wnt protein can be readily detected on a Coomassie-stained gel. Combine all Wnt-containing fractions and pass through a 0.2-µm syringe filter.

3.3.4. Step 4: Heparin Cation Exchange

Purpose: This fractionation step serves to concentrate the Wnt protein and remove remaining contaminating proteins, such as bovine serum albumin (BSA).

1. Column Equilibration: Wash the column with 5 mL distilled and filtered water at a flow rate of 1 mL/min, then with 5 mL Binding Buffer. The column is now ready for sample application.

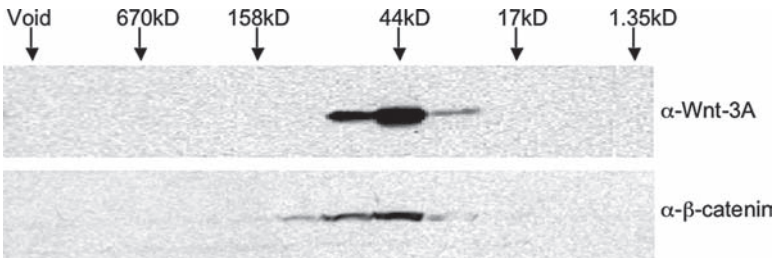

Fig. 2.3. Gel filtration of Wnt3A and β-catenin assay. The elution peak positions of molecular weight standards on the Superdex 200 column are indicated at the top. Wnt3A has an approximate molecular weight of 50 kD (*top panel*). Eluate fractions were diluted 1:100 in DMEM with 10% FBS and applied to mouse L cells for a β-catenin stabilization assay (bottom panel, *see* **Section 3.4.2**). Only fractions containing Wnt3A stimulate the stabilization of β-catenin.

2. Sample Application: Load the entire sample from **Section 3.3.3** and collect the flow-through material. Upon loading, wash the column with 5 to 10 CV or until UV absorbance has established a stable baseline.
3. Elution: Elute the bound Wnt protein by applying a linear gradient from 0 to 100% Elution Buffer. The Wnt protein elutes in a tight peak at approximately 200 mM NaCl (i.e., 20% Elution Buffer = PBS + 200 mM NaCl). Alternatively, the Wnt protein can be eluted in a single step from 0 to 100% Elution Buffer. The maximum concentration of Wnt that can be achieved in these buffer conditions is approximately 100 µg/mL (2.5 µM for Wnt3A). The Wnt protein can be readily detected on a Coomassie-stained gel. Combine all Wnt-containing fractions, filter through a 0.2-µm syringe filter, and store (*see* **Note 8**).

3.4. Assays for Wnt Proteins

There are three simple assays for Wnt3A: 1) Wnt3A immunoblot, 2) β-catenin stabilization in L-cells, and 3) Super-TOP-Flash reporter assays. Here I describe the Wnt3A immunoblot and the β-catenin assay. For details on the reporter assay, *see* **Chapter 8 (Volume 1)** of this book. Wnt proteins that do not lead to β-catenin stabilization, such as Wnt5A, can be assayed for their ability to inhibit Wnt3A-stimulated Super-TOP-Flash reporter assays *(11)*. Additional biochemical assays to monitor Wnt activity(ies) are described elsewhere.

3.4.1 Wnt3A Immunoblotting

1. Prepare samples by combining a small amount of a Wnt sample with 4× loading dye and water to give a final concentration of the loading dye of 1× in a total volume of 20 to 40 µL. Use the following amounts of Wnt per 20 µL sample: 1) Wnt3A CM: 1 µL; 2) Blue Sepharose fractions: 1 µL; 3) IMAC fractions: 1 µL for Wnt3A immunoblot (or 10 µL for

Coomassie-stained gel); 4) gel filtration fractions: 2 µL for Wnt3A immunoblot (or 15 µL for Coomassie-stained gel); 5) heparin fractions: 1 µL for Wnt3A immunoblot or Coomassie-stained gel.

2. Boil all samples for 5 minutes.
3. Perform SDS-PAGE (10% acrylamide gel) followed by transfer to a suitable membrane, such as nitrocellulose or PVDF using standard protocols.
4. Detect Wnt3A protein using a Wnt3A-specific antibody according to manufacturer's recommendations.

3.4.2. β-Catenin Stabilization

1. One or 2 days prior to the assay, seed a 96-well plate with L-cells (*see* **Note 9**). Seeding one tenth of the cells from a confluent 10-cm dish of L-cells into all 96 wells will give the desired cell density to perform the assay. Most of the following manipulations can be performed with 12-well multichannel pipetters.
2. Prepare samples to be tested in a separate 96-well plate: To 100 µL of cell culture media, add 1–2 µL of the Wnt3A-containing fractions (*see* **Note 2**).
3. Aspirate media from L-cells in 96 well plates.
4. Transfer diluted Wnt samples to the cells. For a positive control, use 100 µL of Wnt3A CM per well and for a negative control use cell culture medium (or CM from L-cells prepared as described for Wnt3A CM in **Section 3.1**).
5. Incubate cells for 2 hours at 37°C in a humidified CO_2 incubator.
6. Aspirate all control and test wells and wash once with 100 µL of PBS.
7. Add 30 µL of Lysis Buffer, incubate 1–2 minutes, and transfer lysates (do not try to dislodge nuclei by scraping) to tubes containing 10 µL of Protein Sample Loading Dye.
8. Boil and resolve protein samples by SDS-polyacrylamide gel electrophoresis.
9. Transfer protein from the gel to a suitable membrane and perform a β-catenin immunoblot as recommended by manufacturer. An example of this β-catenin stabilization assay is shown in **Fig. 2.3.**

4. Notes

1. This purification can be applied to other Wnt proteins: to date, Wnt3A, 5A, 7A, 16, Wingless, and Dwnt8/WntD have been successfully purified using this scheme *(3, 11)*. Aside from the

cells producing Wnt3A, ATCC makes available a similar cell line overexpressing Wnt5A (ATCC# CRL-2814).

2. Lowering FBS concentrations in the media will lead to a reduction of soluble Wnt3A in the CM. Since Wnt proteins are extremely hydrophobic, it is likely that the high lipid content of serum promotes the solubility of the Wnt protein. Upon fractionation of the Wnt CM, the requirement for serum is displaced by the presence of detergent. If purified Wnt3A is diluted into an aqueous buffer, either detergent or 10% FBS should be included. In fact, dilution of purified Wnt into a buffer lacking either detergent or serum will lead to precipitation and loss of activity.
3. The purification has been successfully performed at 4°C and room temperature. However, I have not carefully examined whether one of these temperatures yields a superior purified Wnt protein preparation. Given common wisdom of protein stability and temperature, I recommend that the purification is performed at 4°C and all samples are stored in the cold. I recommend against freezing any samples, from the starting material to the final purified protein fractions, as this may result in protein denaturation and precipitation.
4. While Blue Sepharose binds all the Wnts tested so far, I recommend that a given column is dedicated to the purification of a single Wnt protein. Wnt proteins are extremely sticky and are likely present at low levels even after washing the column extensively.
5. During the Blue Sepharose fractionation, the detergent is exchanged from 1% Triton X-100 to 1% CHAPS. This is done because Triton X-100 is significantly less expensive than CHAPS. However, Triton X-100 is not a suitable detergent for the entire purification because of its low critical micelle concentration (0.0155% w/v) relative to CHAPS (0.492–0.615% w/v) and its cellular toxicity. In addition, in the presence of Triton X-100, Wnt3A is incorporated into high molecular weight complexes (potentially micelles) as assessed by gel filtration, and is significantly less active.
6. The concentration of Wnt3A in CM is approximately 200 ng/mL. Blue Sepharose has a binding capacity of approximately 0.64 mg Wnt3A protein per 100 mL Blue Sepharose HP. This estimation is derived from these observations: the maximal amount of Wnt3A CM loaded onto a 100-mL Blue Sepharose column has been 4 L (~0.8 mg Wnt3A) and 80% of Wnt3A was depleted, indicating that 0.64 mg of Wnt3A was bound. Flow-through fractions collected at the end of the loading step contained significantly more Wnt3A protein than flow-through fractions collected at the beginning, suggesting

that the column was approaching saturation with respect to Wnt binding.

7. By gel filtration, the majority of Wnt3A should emerge as a protein whose molecular size corresponds closely to its monomeric or slightly larger size. A high molecular weight complex containing Wnt3A is often observed. This high molecular weight Wnt is most likely aggregated protein, because 1) it is less active than low molecular weight Wnt3A, 2) it cannot be readily dissociated with detergent, and 3) it can be pelleted by centrifugation (50,000×*g*).
8. The stability and shelf life of purified Wnt protein, specifically Wnt3A, is not known. However, it is clear that purified Wnt protein is unstable and looses activity over time. I have found that after 6 months of storage at 4°C, only 10% activity will remain. Therefore, it is best to use the Wnt protein shortly after purification. Preliminary studies indicate that the purified Wnt protein can be flash frozen in liquid nitrogen and stored at –150°C without appreciable loss in activity. While it has not been tested experimentally, it is likely that stored under such conditions, the Wnt protein will retain maximal activity indefinitely. Avoid repeated freeze–thaws by freezing down multiple small volume aliquots. Some Wnt proteins (Wnt3a, 5a, 5b, and 7a) are provided by R&D Systems in a lyophilized form, suggesting that lyophilization does not adversely affect the activity of the Wnt protein.
9. Mouse L cells lack cadherins and consequently have very little to no membrane-associated β-catenin. As a result, the Wnt-stimulated cytoplasmic accumulation of β-catenin is readily detectable. If cells with cadherins are used in place of L cells, the Wnt-stimulated cytoplasmic accumulation of β-catenin may be masked by the large amounts of β-catenin at the membrane. In this case, cells should be fractionated to separate cytosolic proteins from membrane-bound proteins (e.g., by lysis in hypotonic buffer without detergent).

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