BACTERIAL PRODUCTION

PEF #	GENE NAME	EXPRESSION VECTOR	MOLECULAR WEIGHT
2015-XXXX	XXXX	pET-32a	50.9 kDa (full-length) 34.0 kDa (cleaved)

EXPRESSION METHOD OVERVIEW:

Plasmid DNA was transformed into BL21 (DE3) cells. A single colony was picked from the transformation plate to inoculate LB broth containing 50 μg/mL ampicillin, shaking overnight at 37ºC. This culture was used to inoculate a 1 L culture in a 2.5 L Tunair shake flask in Overnight Express^{TM} (Merck Millipore) at a ratio of 1:100, and incubated at 37ºC for 24 h. The cultures were harvested by centrifugation at 9,000 *g* at 4ºC and the cell pellet was stored at -80ºC until purification.

Table 1│ Expression Parameters

Construct	2015-XXXX
Total Culture Volume	1 L
Cell Strain	BL21 (DE3)
Media	Overnight Express^{TM} 50 μg/mL ampicillin
Growth Temperature	37˚C
Time of Harvest	24 h
OD_{600} at Harvest	8.0

PRIMARY PURIFICATION METHOD OVERVIEW:

PROTEIN EXTRACTION

Cell pellet from 1 L expression culture was resuspended in 200 mL lysis buffer and lysed using a Niro Soavi homogeniser. The lysate was centrifuged at 15,900 *g* for 30 min at 4˚C to obtain the soluble fraction (supernatant).

IMMOBILISED METAL AFFINITY CHROMATOGRAPHY (IMAC)

The soluble fraction was passed through a 0.45 μm filter prior to loading onto an IMAC column pre-equilibrated in wash buffer. Unbound protein was removed with 20 column volumes (CV) of wash buffer. Elution was performed using a gradient from 0 – 100% elution buffer over 5 CV and holding at 100% for a further 5 CV, collecting 2 mL fractions. All steps were performed on an AKTAxpress FPLC system (GE Healthcare) at a flow rate of 5 mL/min, 4˚C (Figure 1). Fractions corresponding to 2015-XXXX were pooled (Figure 2).

ANALYSIS

Purification samples were loaded onto a 4-12% Bis-Tris SDS-PAGE gel and run under denatured and reduced conditions (Figure 3). Analysis was performed using a Bio-Rad Chemi-Doc™ XRS+ imaging system. The molecular weight was calculated using ProtParam (http://web.expasy.org/protparam/).

Table 2 | IMAC Purification Details

Column	5 mL HisTrap Excel (GE Healthcare)
Lysis Buffer	20 mM Sodium Phosphate, 500 mM NaCl, 20 mM Imidazole, 1% Triton X-100, cOmplete Protease Inhibitors (Roche), pH 8.0
Wash Buffer	20 mM Sodium Phosphate, 500 mM NaCl, 20 mM Imidazole, pH 8.0
Elution Buffer	20 mM Sodium Phosphate, 500 mM NaCl, 400 mM Imidazole, pH 8.0

RESULTS:

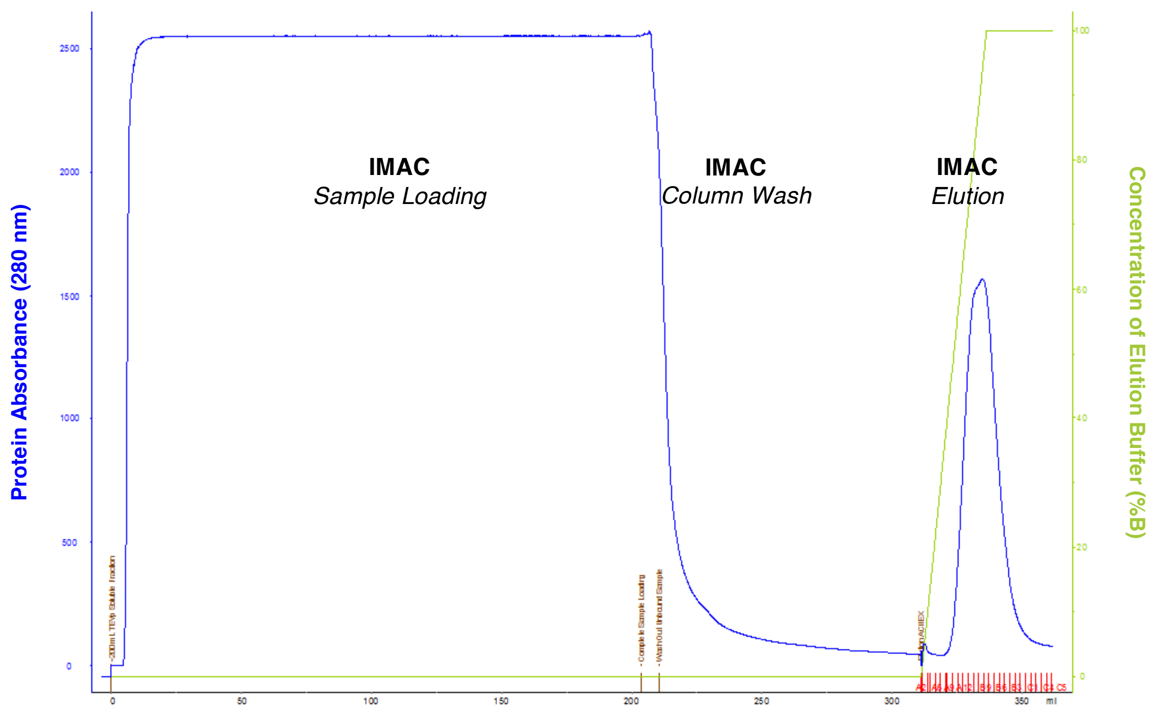

Figure 1 | 2015-XXXX IMAC purification chromatogram

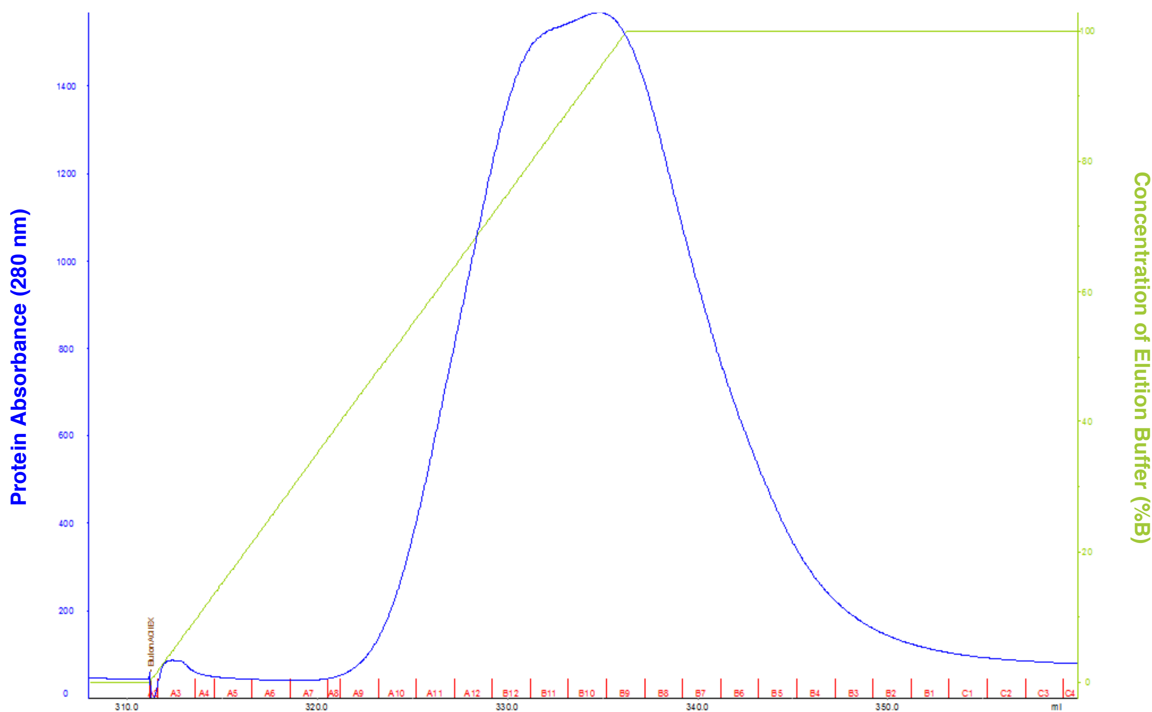

Figure 2 | 2015-XXXX IMAC purification chromatogram (elution)

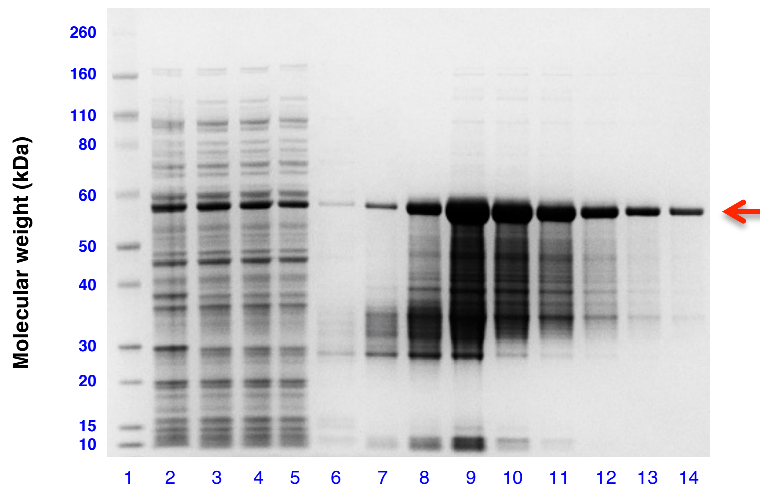

Figure 3 | 2015-XXXX IMAC purification analysed by SDS-PAGE

Lane	Sample	Lane	Sample
1	Novex® Sharp Pre-stained MW ladder	8	IMAC Elution Fraction 2
2	Lysate*	9	IMAC Elution Fraction 3
3	Soluble Fraction*	10	IMAC Elution Fraction 4
4	IMAC Load (Filtered)*	11	IMAC Elution Fraction 5
5	IMAC Flow Through*	12	IMAC Elution Fraction 6
6	IMAC Wash	13	IMAC Elution Fraction 7
7	IMAC Elution Fraction 1	14	IMAC Elution Fraction 8

*Samples diluted 1:20

Tag Removal Method Overview:

Tag Removal Scouting

A small aliquot of the IMAC purified 2015-XXXX was used to scout for optimal tag removal conditions, to generate the native protein sequence. Samples were either buffer exchanged into cleavage buffer using a PD SpinTrap G-25 spin column (GE Healthcare), or stored in IMAC elution buffer. Protease was incubated with 2015-XXXX at a ratio of 0.01%, 0.003%, 0.001%, 0.0003% or 0.0001% (w/w) at room temperature. Samples were taken for analysis after 16 h (Figure 4).

Table 3 | Tag Removal Scouting Details

IMAC Elution Buffer	20 mM Sodium Phosphate, 500 mM NaCl, 200 mM Imidazole, pH 8.0
Cleavage Buffer	20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0
Temperature	Room temperature
Protease to Substrate Ratio	0.01%, 0.003%, 0.001%, 0.0003%, 0.0001% (w/w)
Incubation Time	16 hours

Buffer Exchange and Large-scale Cleavage

The remaining IMAC elution sample was passed through a 0.45 μm filter and buffer exchanged into cleavage buffer using a desalting column. All steps were performed on an AKTAxpress FPLC at a flow rate of 7 mL/min, 4˚C. The buffer exchanged sample was incubated with protease at the optimal conditions determined from tag removal scouting (0.001% protease to substrate (w/w), 16 hours at room temperature).

Table 4 | Buffer Exchange Details

Column	HiPrep 26/10 Desalting Column (GE Healthcare)
Cleavage Buffer	20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0

Protease Removal

Immediately following tag removal, the sample was filtered and applied to a benzamidine column pre-equilibrated in wash buffer. Cleavage fragments were collected in reverse mode in the flow through sample, while protease was bound to the column. 10 CV of a high salt buffer (wash buffer #2) was passed through the column to wash out non-specifically bound proteins. All steps were performed on an AKTAxpress FPLC system at a flow rate of 2 mL/min, 4˚C (Figure 5). Samples from the purification process were analysed by SDS-PAGE (Figure 6).

Table 5│ Protease Removal Chromatography Details

Column	1 mL HiTrap Benzamidine FF (GE Healthcare)
Wash Buffer	20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0
Wash Buffer #2	20 mM Tris-HCl, 500 mM NaCl, 2 mM $CaCl_2$, pH 8.0

RESULTS:

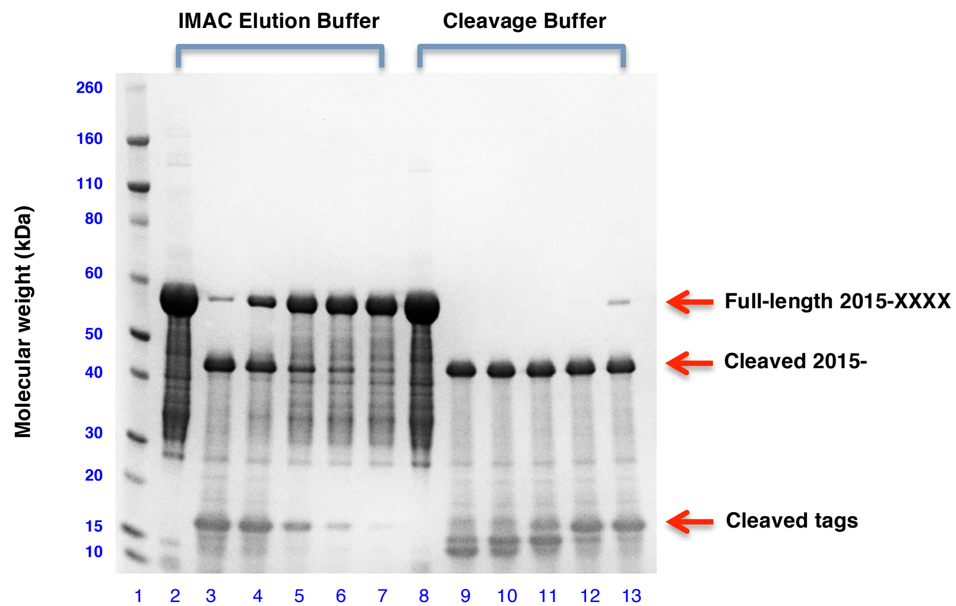

Figure 4 | 2015-XXXX tag removal scouting analysed by SDS-PAGE

Lane	Sample	Lane	Sample
1	Novex® Sharp Pre-stained MW ladder	8	Full-length (Cleavage Buffer)
2	Full-length (IMAC Elution Buffer)	9	0.01% Cleavage Ratio
3	0.01% Cleavage Ratio	10	0.003% Cleavage Ratio
4	0.003% Cleavage Ratio	11	0.001% Cleavage Ratio
5	0.001% Cleavage Ratio	12	0.0003% Cleavage Ratio
6	0.0003% Cleavage Ratio	13	0.0001% Cleavage Ratio
7	0.0001% Cleavage Ratio		

*All samples diluted 1:5

COMMENTS:

- Complete cleavage of 2015-XXXX was observed when a protease to substrate ratio of 0.01%, 0.003% and 0.001% was used in cleavage buffer (Figure 4, lanes 9-11). Cleavage efficiency was significantly reduced when conducted in IMAC elution buffer (Figure 4, lanes 3-7).
- Based on these results, the optimal cleavage condition is 0.001% ratio protease to substrate, 16 hours incubation time in cleavage buffer at room temperature.

RESULTS:

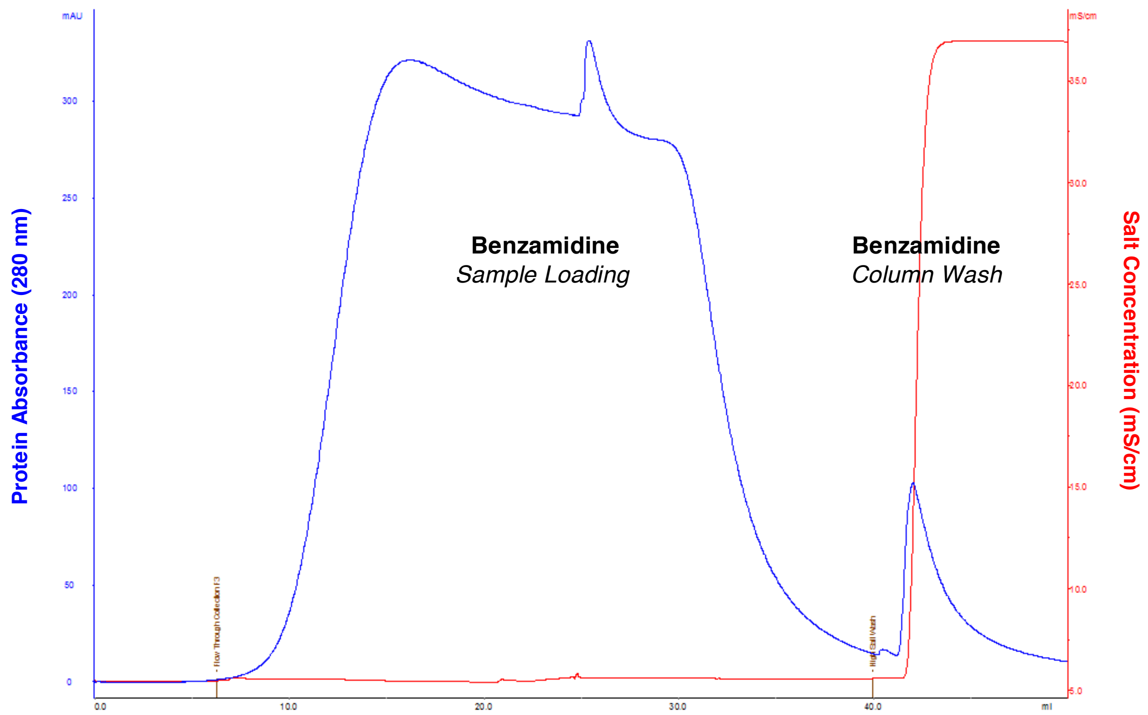

Figure 5 | 2015-XXXX benzamidine purification chromatogram

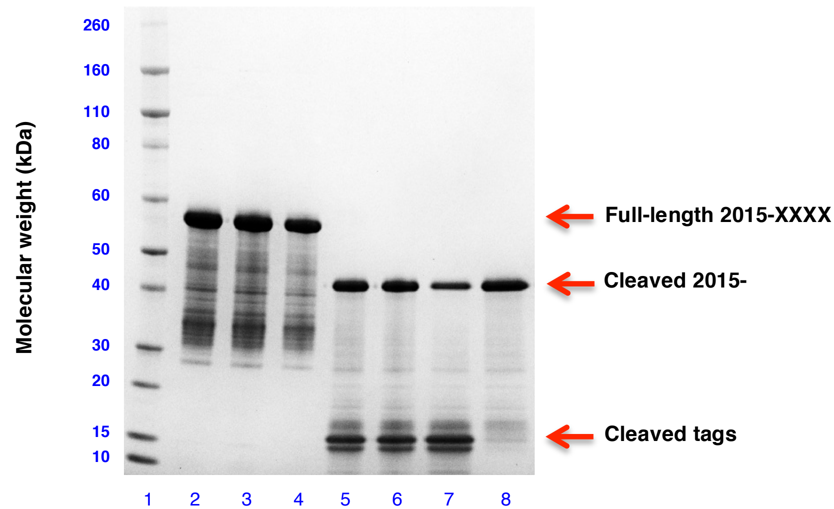

Figure 6 | 2015-XXXX large-scale tag removal and protease removal analysed by SDS-PAGE

Lane	Sample
1	Novex® Sharp Pre-stained MW ladder
2	IMAC Pooled Fractions*
3	Buffer Exchange Load (Filtered)*
4	Buffer Exchanged*
5	Protease Cleavage*
6	Protease Cleavage (Filtered)*
7	Benzamidine Flow Through**
8	Benzamidine Wash**

*Samples diluted 1:20
**Samples diluted 1:10

COMMENTS:

- Complete cleavage of 2015-XXXX was observed for scaled-up protease digestion (Figure 6, lanes 4 and 5).

SECONDARY PURIFICATION AND PROTEIN STORAGE METHOD OVERVIEW:

BUFFER STABILITY SCOUTING

A small volume of cleaved 2015-XXXX was buffer exchanged into PBS using a PD SpinTrap G-25 column (GE Healthcare). An aliquot of this sample was snap-frozen in liquid nitrogen and stored at -80˚C overnight, the remainder stored at 4˚C, to determine a suitable storage condition. Analysis of the samples indicates that the cleaved protein is soluble in PBS, and that it maintained solubility following storage at both 4˚C and -80˚C in the absence of any additives (results not shown).

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

2015-XXXX was concentrated using an Amicon Ultra-15 centrifugal filtration device with a 10 kDa molecular weight cut off (Millipore). The concentrated sample was filtered and loaded onto an SEC column pre-equilibrated in storage buffer, collecting 2 mL fractions. All steps were performed using an AKTAxpress FPLC at a flow rate of 2.5 mL/min, 4˚C. Bovine serum albumin (BSA) and blue dextran/acetone standards were also run under the same conditions to enable an estimation of protein molecular weight (Figure 7). Samples from the purification process were analysed by SDS-PAGE (Figure 8).

Table 6 | SEC Purification Details

Column	HiLoad 26/600 Superdex 200 (GE Healthcare)
Strorage Buffer	Phosphate buffered saline (PBS)

PROTEIN CONCENTRATION AND STORAGE

SEC fractions corresponding to 2015-XXXX were pooled and concentrated from 22 mL to 8.1 mL as described previously, then passed through a 0.2 µm filter. The filtered protein was snap-frozen in liquid nitrogen in 1 mL aliquots and stored at -80˚C.

RESULTS:

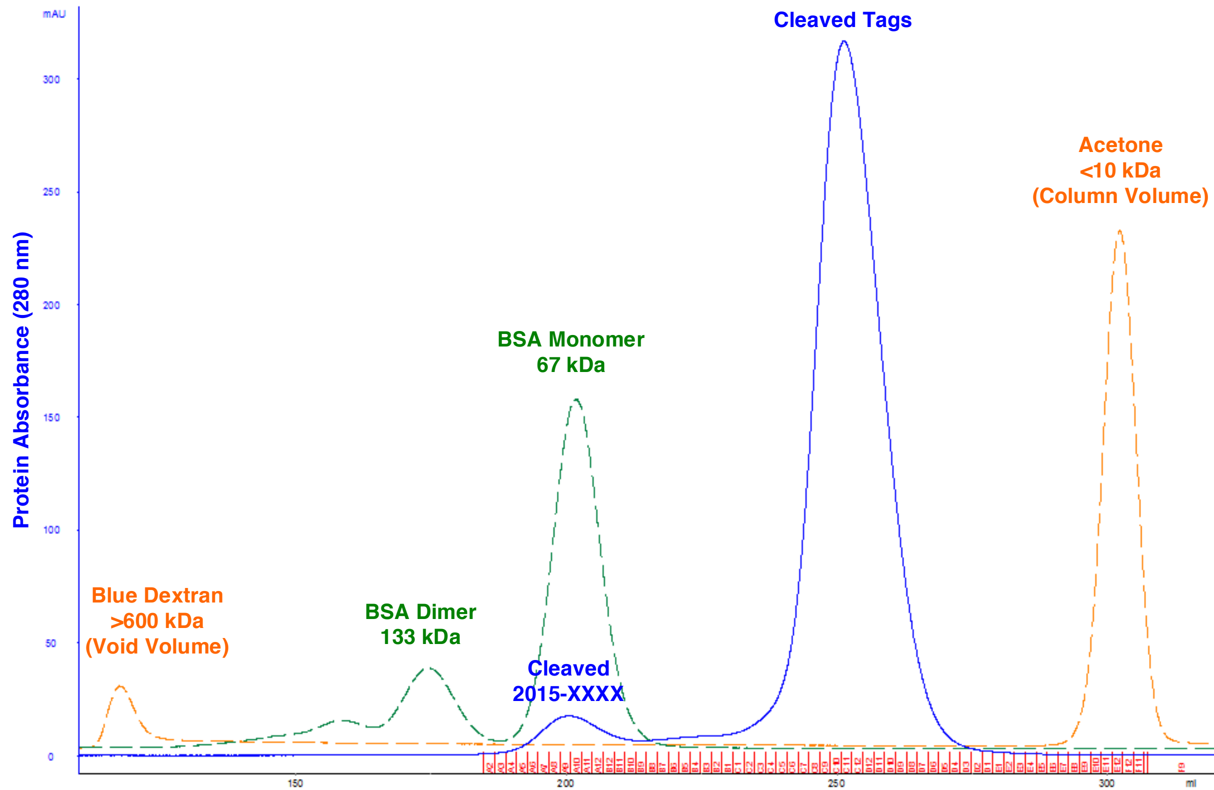

Figure 7│ 2015-XXXX SEC purification chromatogram. Overlayed with BSA (green) and blue dextran/acetone (orange) standards.

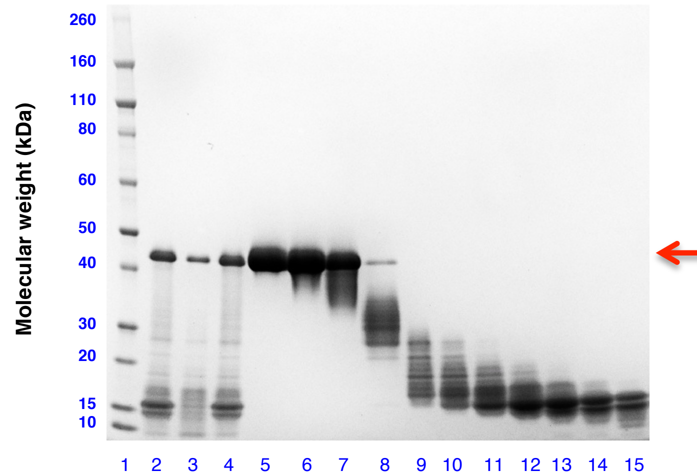

Figure 8│ 2015-XXXX SEC purification analysed by SDS-PAGE

Lane	Sample	Lane	Sample
1	Novex® Sharp Pre-stained MW ladder	9	SEC Elution Fraction 5
2	Concentrated*	10	SEC Elution Fraction 6
3	Concentrated Flow Through (Waste)	11	SEC Elution Fraction 7
4	SEC Load*	12	SEC Elution Fraction 8
5	SEC Elution Fraction 1	13	SEC Elution Fraction 9
6	SEC Elution Fraction 2	14	SEC Elution Fraction 10
7	SEC Elution Fraction 3	15	SEC Elution Fraction 11
8	SEC Elution Fraction 4		

*Samples diluted 1:40

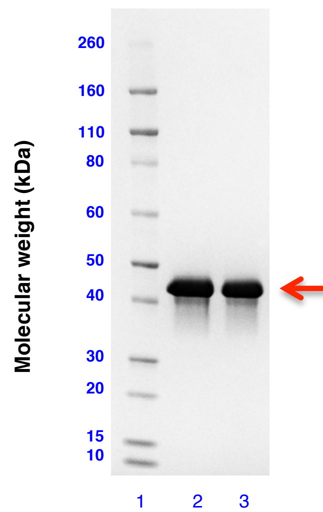

Figure 9│ 2015-XXXX final sample analysed by SDS-PAGE

Lane	Sample
1	Novex® Sharp Pre-stained MW ladder
2	SEC Pooled Fractions
3	Final Sample (Concentrated and Filtered)*

*Sample diluted 1:4

SAMPLE INFORMATION:

Table 7 | Purified Protein Information

Final Protein	2015-XXXX (Cleaved)
Volume	8 mL
Protein Purity	>99%
Target Protein Concentration	760 µg/mL
Target Protein Yield	6.08 mg
Buffer	Phosphate Buffered Saline (PBS)
Storage Conditions	-80˚C, 8 x 1 mL aliquots

COMMENTS:

- Target protein concentration was estimated by absorbance at 280 nm using a NanoDrop spectrophotometer, taking into consideration the protein's extinction coefficient and sample purity. Target protein purity was estimated by band intensity analysis using ImageLab software.

Research groups using the UQ Protein Expression Facility (PEF) must acknowledge the contribution provided by the Facility. Please acknowledge UQ PEF's provision of Services in your written publications and/or oral presentations relating to the Processed Material, thank you.