Cloning/Expression of TGF-β signal antagonist: Smad6/Smad7

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Biochemistry C500 Report

Content

Introduction

Section 1. TGF-β

Transforming growth factor-β (TGF-β) proteins regulate cell function, and have many key roles in physiological development and carcinogenesis.[1] TGF-β and related polypeptides constitute the largest cytokine family that regulates a diverse array of cellular processes including cell proliferation, differentiation, adhesion and apoptosis.[2] More than 60 TGF-β family members have been identified in multicellular organisms. Among these 60, there are three TGF-βs, five activins, and at least eight bone morphogenetic proteins (BMPs) encoded by different genes.[3] The TGF-β family comprises many structurally related differentiation factors that act through a heteromeric receptor complex at the cell surface and an intracellular signal transducing Smad complex (composite name from Sma (*Caenorbabditis elegans*) and Mad (*Drosophila melanogaster*), intracellular proteins that mediate signaling from receptors for extracellular TGF β-related factors). The TGF-β receptor complex (TβR) consists of two type I and two type II transmembrane serine/threonine kinases. After phosphorylation by the receptors, Smad complexes translocate into the nucleus where they cooperate with sequences-specific transcription factors to regulate gene expression (figure 1).[4] The TGF-β-Smad signaling pathway plays a role in a number of disease states involving inflammation, angiogenesis, Alzheimer's disease, hypertension and cancer.[2]

1.1 Smads

Downstream signal transduction is mediated by the TβR kinase domain through the phosphorylation of Smad proteins, which as oligomeric complexes translocate to the nucleus and regulate gene expression via association with DNA transcription factors

(figure 1). There are eight vertebrate Smad proteins: Smad1 to Smad8 that are divided into three functional classes: (1) the receptor-regulated Smads (R-Smads), including Smad1, Smad2, Smad3, Smad5, and Smad8, which are each involved in a specific receptor kinase binding and phosphorylation; (2) the co-mediator Smad (co-Smad), Smad4, in conjunction with all R-Smads, (3) the inhibitory Smads, Smad6 and Smad7, which negatively regulate TGF-β pathways.[1] Smads act as ligand-induced transcription regulators of TGFβ responses. As the regulatory DNA sequences of genes, Smads activate transcription through assembly of a large nucleoprotein complex consisting of Smad-binding DNA elements, DNA-binding transcription factors, and the transcriptional coactivators.[3]

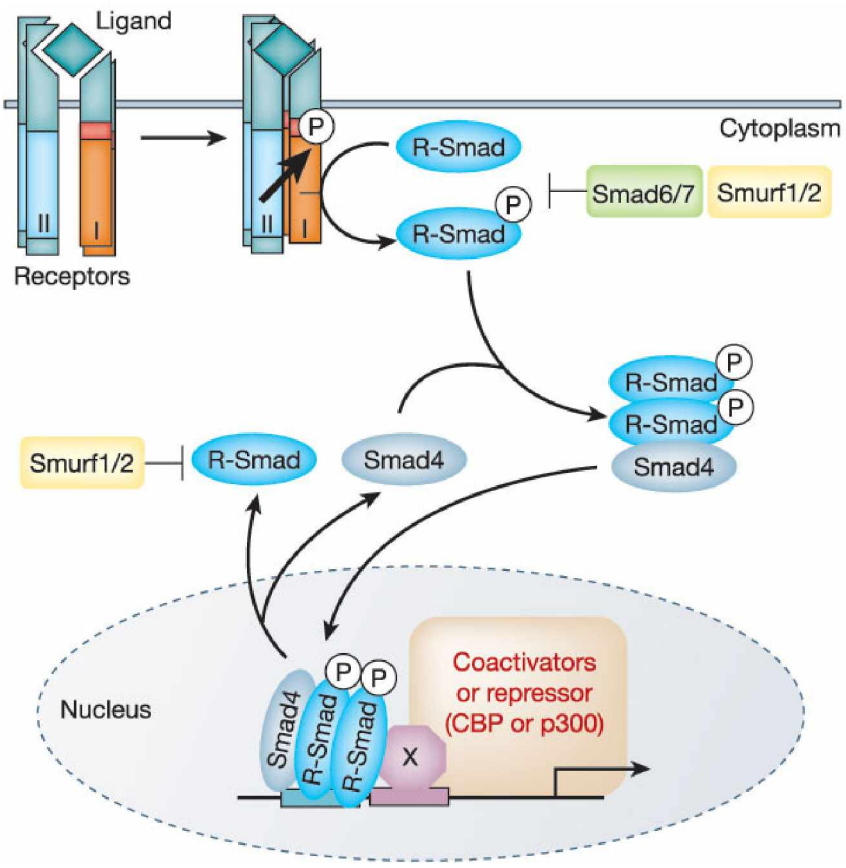

Figure 1. General mechanism of TGF-β receptor and Smad activation. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7.[1]

1.2 Inhibitory Smads

The structurally divergent Smad6 and Smad7 act as 'inhibitory' Smads. R-Smads and Smad4 contain a conserved MH1 and C-terminal MH2 domain, flanking a divergent middle linker segment. Inhibitory Smads lack the recognizable MH1 domain, but have a MH2 domain. The MH2 domain has limited structural similarity to the phosphopeptide-binding domain FHA. Both the MH1 and MH2 domains can interact with select sequence-specific transcription factors, whereas the C-terminus of the R-Smads interact with and recruits the related coactivators CREB-binding protein (CBP) or p300.[1] In contrast to R-Smad expression, expression of the inhibitory Smad6 or Smad7 is highly regulated by extracellular signals. Induction of Smad6 and Smad7 expression by BMP and THF-β represents an auto-inhibitory negative feedback mechanism for ligand-induced signaling.[1] Smad6 and Smad7 also regulate activation of R-Smads. Smad6 and Smad7 inhibit TGF-β family signaling through binding to the type I receptor, thus preventing recruitment and phosphorylation of effector Smads. Smad6 also interferes with the heteromerization of BMP-activated Smads with Smad4, preventing the formation of an effector Smad complex.[5] In addition to the competitive interference of Smad6 and Smad7 with R-Smad binding to type I receptors, Smad6 inhibits complex formation of BMP-activated Smad1 with Smad4. Smad6 and Smad7 also inhibit TGF-β family signaling by interacting directly with Smad ubiquitination regulatory factor E3 (SmurfE3) ubiquitin ligases and mobilizing these ligases to the type I receptors, leading to proteasomal degradation of the receptors.[6]

Several signaling pathways lead to a rapid induction of Smad6 and Smad7 expression, which constitutes a critical point for negative regulation of TGF-β signaling. TGF-β or BMP signaling induces Smad6 or Smad7 expression that can result in attenuation of ligand-induced Smad activation and gene expression.[7]

Questions that we want to answer about these inhibitory Smads (Smad6 and Smad7) are: (1) what are the structures of theses two Smads, (2) what is the mechanism of the antagonistic relationship of these Smads with the TβR kinase?

Section 2. Objective & Specific Aims

The objective of this C500 project is to successfully clone and express the Smad6 and Smad7 proteins. I will be cloning and expressing both the full length and MH2 domains of these two Smad proteins. The purification of the proteins and protein domains collected, and the eventual crystallization of these two TGF-β inhibitory proteins themselves and in complex with TβR kinase domain. Our specific aims are: (1) cloning, (2) expression, (3) purification, (4) crystallization of these two inhibitory Smad proteins.

Section 3. Procedures

3.1. Invitrogen™ Gateway® System

Cloning and expression of the Smad6 and Smad7 were preformed with the Invitrogen™ Gateway® System. This system was chosen because Gateway® Technology is a universal cloning technology that provides a highly efficient and rapid route to functional analysis, protein expression, and cloning/subcloning of DNA segments. Based on the well

characterized site-specific recombination system of phage l, Gateway® Technology allows you to transfer DNA segments between different cloning vectors while maintaining orientation and reading frame, effectively replacing the use of restriction endonucleases and ligase. The Gateway® Technology is also a powerful method for high efficiency directional cloning of PCR products.

3.2. Ligation-Independent Cloning System (LIC)

Another method of cloning and expression was attempted using the LIC cloning system, recombinants are generated between PCR products and a PCR-amplified plasmid vector. One of the features of this system is that it does not require the use of restriction enzymes, T4 DNA ligase or alkaline phosphatase. The 5´-ends of the primers used to generate the clonable PCR fragments contain an additional 12 nucleotide sequence lacking dCMP. Due to this nucleotide sequence the amplification products include 12 nucleotide sequence lacking dGMP at their 3´-ends.[8] The 3´-terminal sequence can be removed with the (3´→ 5´) exonuclease activity of the T4 DNA polymerase in the presence of dGTP, which leads to fragments with 5´-extending single-stranded tails of a defined sequence and length. The vector oligos have additional 12 nucleotide tails complementary to the tails used for the fragment amplification, allowing the creation of single-strand-ends with T4 DNA polymerase in the presence of dCTP. Circularization can occur between the vector and PCR fragments mediated by the 12 nucleotide cohesive ends. The resulting circular recombinant molecules do not require *in vitro* ligation for efficient bacterial transformation.[8]

3.3. PCR Reaction

In designing a PCR primer a CACC 4 bp sequence was needed on the 5' end before the ATG initiation codon. PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (TOPO cloning vector) (GTGG) invades the 5'-end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. This (GTGG) overhang is due to the protease Topoisomerase which cleaves the vector so a new gene can be entered into the vector. PCR will be preformed; production of blunt-end PCR product was made utilizing the properly designed PCR primers.[9]

3.4. TOPO® Cloning Reaction

Above described how the PCR product would be entered into the entry clone by mixing together the PCR product and the pENTER™ TOPO® vector. Once the entry cloning reaction has taken place, transformation of the vector construct into competent *E. coli* was preformed. One Shot® TOP10 competent *E. coli* are used to facilitate transformation. To confirm transformation PCR was preformed again and a gel will be used to determine if Smad6 was introduced into the vector. Once confirmed, QI Aprep Spin Miniprep Kit will be used to prepare the plasmid from a single clone, and a gel will be used to confirm entry into plasmid. PCR for sequencing will be preformed and the products will be given to the Indiana Molecular Biology Institute for sequencing to confirm that the total gene sequence in the plasmid is correct with no mutation.

3.5. LR Recombination Reaction

The Gateway® Technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences between vectors. The LR reaction facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. The reaction is catalyzed by a LR Clonase™ II enzyme mix.[10] In addition to the LR reaction a His-tag and GST-tag was also added to the gene, which will be utilized for protein purification. The plasmid from a single clone again was prepared with QI Aprep Spin Miniprep Kit. Transformation into BL21-AI™ One Shot® cells to be used to amplify the expression vector. At this point we will need to test for proper protein expression, by using SDS-PAGE (PolyAcrylamide Gel Electrophoresis). If there is proper protein expression a large scale culture will be made to gather enough protein for purification.

3.6. Protein Purification

At this point there should be two different proteins that need to be purified, one with a His-tag and one with a GST-tag. The protein with the His-tag will be passed through a Ni-column for the first stage of purification, and the protein with GST-tag passed through a Glutathione-column. After the protein is added to the Glutathione-column TEV protease will also be added cleaving the protein from the GST-tag. The tag will stay connected to the column as the protein passes through the column. The proteins will then be put through an ion-exchange column (separately), followed by a gel-filtration column. The purified protein will be crystallized under different screening conditions to optimize crystallization. The protein will be crystallized by itself and in complex with other proteins or cofactors (to be determined).

Section 4. General Considerations

Optical measurements were preformed with the Beckman DV® 640B spectrophotometer. All PCR reactions were preformed using the Eppendorf^{AG} Mastercycler gradient Thermal cycler. When specified that a tabletop centrifuge was used the Eppendorf centrifuge 5417R was used, for all other centrifugation the Beckman Avanti™ J-20 centrifuge or Beckman Coulter J2-HS centrifuge were used (both are almost identical). Culture plates were incubated at 37 °C in the Ficher Scientific Isotemp Incubator. All samples that were placed in incubator/shaker were placed in the New Brunswick Scientific Series 25 incubator shaker. All sonication was prformed with the Ficher Scientific 550 Sonic Dismembrator.

Section 5. Experimental

To date only the Smad6 full length and Smad6 MH2 protein and domain respectively have been cloned. As state above the Smad6 full length and MH2 domain primers were used (mixture in table 1). The PCR mixture was distributed into 4 tubes with 15 µl in each for both the full length and MH2 domain (total of 8 tubes). PCR was preformed at varied annealing temperatures (table 2) for each of the four different tubes (50 °C, 55 °C, 60 °C, 65 °C), with steps 2-3 repeated 30 times. Once the PCR was complete, a 0.8% agarose gel was ran with a current of 150 volts for approximately 35 minutes to analyze the products (results shown below in figure 2 (left gel)). Only the Smad6 MH2 domain was used for cloning and expression, but the gel indicated that there was no PCR product for the full length Smad6 protein. Tubes (4) from the Smad6 MH2

PCR product was selected (from lane 4 in figure 2 (left gel)) for TOPO cloning and transformation into One Shot® competent *E. coli* cells. Before TOPO cloning was preformed the concentration of the PCR product was found with the spectrophotometer to be 576.44 µg/ml.

Table 1. PCR Reaction mixture

Sample	**Volume (µL)**
Water	48.8
10x Herculase rxn Buffer	6.00
dNTP (10 mM)	1.20
DNA Template (PGEX-Smad6) (~10 ng)	1.00
Sense Primer (10 pmol/µl)*	1.20
Anti-Sense Primer (10 pmol/µl)	1.20
Hotstart PfuTurbo DNA Polymerase	0.6

***** Two sense primers were used one for the MH2 domain, and one for the Full length protein (hSmad6mh2topo-sense), (hSmad6topo-sense) respectfully.

The PCR product was diluted 100 fold to a concentration near 5 ng/µl for the TOPO cloning reaction (mixture presented in table 3). The PCR product was keep on ice, and the TOPO vector was stored at 4 °C in freezer and was added to the reaction mixture when needed. Reagents were mixed and incubated for 15 minutes at room temperature, then placed on ice before transformation into One Shot® cell. Transformation was preformed by mixing 2 µl of cold TOPO cloning reaction mixture, and 100 µl of competent *E. coli* cells. The mixture was incubated at 0 °C for 30 minutes, then heat-shocked for 30 seconds at 42 °C and placed back on ice. S.O.C. medium (250 µl) was

added to mixture and transfered into a falcon tube then incubated at 37 °C for 1 hour in shaker.

Table 2. PCR Conditions

Step	Temp. (°C)	Time (Min)
1	95	2.0
2	95	0.5
3	(50, 55, 60, 65)*	0.5
4	72	2.5
5	72	30
6	4	120

* Different annealing temperatures were attempted, in order to evaluate which temperature gave the best results, step 3 (50 °C, 55 °C, 60 °C, 65 °C). Steps 2-4 repeated 30 times. Results can be seen in figure 2.

After incubation the mixture (50 µl) was spread onto two selective culture plates (kanamycin), and incubated at 37 °C for 15 hours. After incubation, 10 colonies were selected and grown on a patch plate at 37 °C for 30 minutes. PCR was utilized to confirm the Smad6 MH2 entry clone. Five of the ten samples were positive for the Smad6 MH2 entry clone. From these five sample the QI Aprep Spin Miniprep Kit allowed the preparation of the plasmid as stated above and an agarose gel confirmed the entry of the plasmid (gel can be seen in figure 2 (right gel)). The gel confirmed that all five samples

were positive and each were sequenced to ensure no mutation have occurred in the Smad6 MH2 gene.

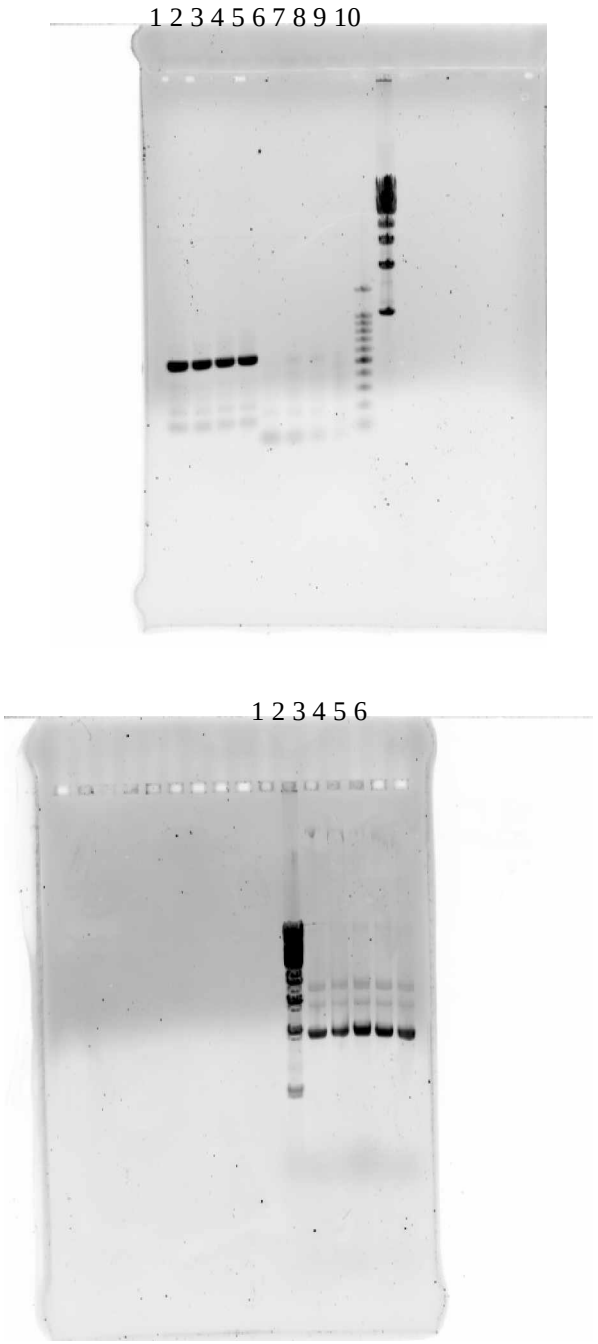

Figure 2. Agarose gel of PCR product (lanes 1-4, from left are positive bands of PCR product desired, last two lanes far right are markers)(left gel). Agarose gel after miniprep was preformed to confirm the gene was inserted into plasmid (lane 1

is weight marker, and lanes 2-6 are positive bands of plasmid with gene, reading form left to right)(right gel).

Table 3. TOPO Cloning Reaction Mixture

Solution	**Volume (μL)**
PCR Product (~5 ng/μl)	1
SaH Solution (from kit)	1
Sterile Water	3
TOPO Vector (from kit)	1

The samples from the miniprep were prepared for sequencing through PCR by adding ~0.5 μl of the template (miniprep product), 3.5 μl water, 3.0 μl of 5 mM $MgCl_2$, 2.0 μl primer (M-13 forward), and 1.0 μl of ABI Big Dye, for each of the five different samples, the program SEQUENCE was used in PCR reaction. The PCR products were taken to the Indiana Molecular Biology Institute for sequencing. The results from sequencing established that four out of the five samples were correct, and the incorrect sample was discarded.

LR recombination reaction was preformed for only one of the entry clones with the proper sequence, the sample from lane 2, figure 2 (right gel). Sample from lane 2 (0.5 μl) entry clone (Smad6 MH2 (1)) (150 ng) was mixed with 1 ul of destination vector (150 ng/μl) and both PDEST-15 (GST_{10}-tag), and PDEST-17 (His-tag) with 6.5 μl TE buffer pH 8.0, followed by the LR Clonase II enzyme (2 μl). A total of four tubes were made,

two for PDEST-15, and two for PDEST-17, with each one having a control. The product from the above mixture was transformed into One Shot® TOP 10 competent cells and plated onto ampicillin plates, incubated at 37 °C for 16 hours, and stored at 4 °C. From the plates cultures, single colonies were grown, also Smad6 Full length colonies were grown and miniprep was preformed from the LR reaction products (preparation of expression plasmid). After the miniprep was preformed both the Smad6 MH2 PDEST-15 and Smad6 MH2 PDEST-17 were transformed into BL21-AT™ competent cell, while the Smad6 full length transformed into BL21-CDE plys S. competent cells.

Once the cells were cultured, a small-scale expression test was preformed (5 ml cultures grown) from three different single colonies from each PDEST-15, PDEST-17, and full length (9 tubes total). Once the cultures (incubated in shaker at 37 °C for 1.5 hours) of the expression samples were grown (5 ml), a 0.5 ml aliquot was taken from and analyzed by SDS-PAGE (non-induced sample). An additional 0.75 ml was taken from each tube and combined with sterile glycerol for a glycerol (0.2 ml) stock solution, which was stored at -70 °C for future expression of the Smad6 MH2 PDEST-15/PDEST-17 and full length gene. To the remaining samples (~4 ml) the cells were induced with 40 µl L-arabinose (final concentration 0.2%) for PDEST-15/PDEST-17 and added 3.5 µl of IPTG (final conc. 1 mmol) to full length sample and incubated in shaker at 37 °C for 3.5 hours. After incubation a 0.5 ml aliquot was taken from each sample culture for SDS-PAGE (induced sample), the samples for SDS-PAGE both non-induced and induced were centrifuged with a tabletop centrifuge at 14,000 rpms for 1 minute. After centrifugation the supernatant was discarded and the pellets were resuspended in milli pore water (50 µl). To this 4x loading buffer (17 µl) was added and the samples were heated at 95 °C.

Samples were ran on a 12% SDS-PAGE gel at 200 volts for 50 minutes. The gel was stained/destained and analyzed, the results indicated that Smad6 MH2 PDEST-17 did not express, but there was good expression for both Smad6 MH2 PDEST-15 and the full length genes.

The previous procedure was repeated for a large scale expression producing a larger amount of protein to be purified.

Based on the results from the small-scale expression test, a small-scale solubility analysis was preformed for Smad6 MH2 PDEST-15. Two cultures (5 ml) were inoculated with the Smad6 MH2 PDEST-15 in BL21-AI cells from glycerol stock, and were allowed to grow for 15 hours at 37 °C in the shaker. After 15 hours the cultures were distributed (2.5 ml) into four different 250 ml flasks with 100 ml of LB medium and 100 µl ampicillin (100 mg/ml) added. This mixture was incubated in the shaker at 37 °C for 2 hours. After the incubation 1 ml of L-arobinose was added and the mixtures were submitted to different induction periods and temperatures. Two flasks were induced at room temperature, one for 6 hours and the other for 15 hours each. The other two samples were induced at 4 °C in the cold room for 6 hours and 15 hours each respectively. After induction (either 6 or 15 hours) cultures were collected and centrifuged at 8,000 rpms for 5 minutes and the supernatants were discarded. The two with 6 hours induction samples (pelleted cells) were placed at -70 °C, so all samples (6 hours and 15 hours) could be resuspended in lysis buffer at the same time. The lysis buffer was prepared as stated in table 4, the pellets were resuspended with lysis buffer and the cells were lysised with the

sonicator. Lysis buffer (20 ml) was added to each of the four pelleted cells, and the cells were resuspended and sonicated on power setting 5 for 10 minutes (pulse time) with a 1 second pulse, 3 second pause cycle. When sonicating the tubes the cells in the lysis buffer were placed in ice to prevent denaturation of the proteins.

Table 4. Lysis Buffer Mixture

Reagents	**Volume (ml)**
PBS	100.0
DTT (1 M)	0.2
Lysozyme (10 mg/ml)	1.0
Protease Inhibitor Cocktail	0.1

Once the cells were sonicated the solutions were centrifuged at 15,000 rpms for 30 minutes, the supernatant was removed, and a 10 µl aliquot was taken from each supernatant and combined 10 µl of water with pellet (very small amount) in a micocentrifuge tube. To this mixture was added 4x loading buffer (5 µl) for all 8 samples. The mixture was heated at 95 °C for 15 minutes and then ran SDS-PAGE gel (Figure 3).

The previous procedure was repeated for a large-scale expression, but two 1 L cultures were made from Smad6 MH2 PDEST-15 glycerol stock, cultures contained 1ml ampicillin (100 mg/ml) were incubated in shaker at 37 °C for 3 hours, the optical density of the cells should be between 0.6-0.8 measured with spectrophotometer. The cultures were induced with L-arobinose and placed in a shaker in the cold room at 4 °C for 15 hours. The cultures were centrifuged at 8,000 rpms for 5 minutes, the supernatant was discarded, and the pellets resuspented with lysis buffer (mixture found in table 5). Once

the pellets were suspended in the lysis buffer the samples were sonicated for 3 minutes (total pulse time) with 1 second pulse, 3 second pause cycles. The tubes were placed in ice to keep solutions from over heating while sonicating. After sonication the samples were centrifuged at 15,000 rpms for 30 minutes, the supernatant was used in the rest of the purification steps.

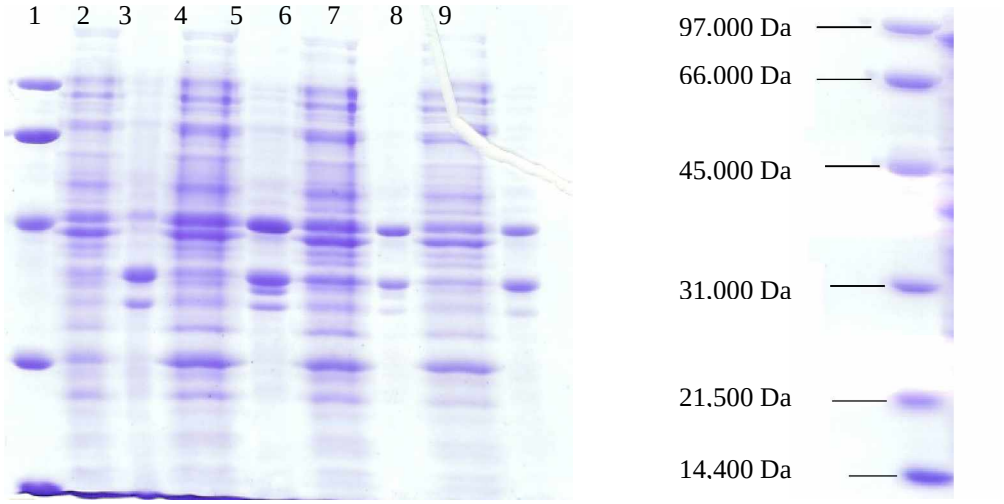

Figure 3. From left to right: lane (**1**) low range molecular marker, (**2**) supernatant (16 °C, 6 hrs), (**3**) pellet (16 °C, 6 hrs), (**4**) supernatant (16 °C, 15 hrs), (**5**) pellet (16 °C, 15 hrs), (**6**) supernatant (RT, 6 hrs), (**7**) pellet (RT, 6 hrs), (**8**) supernatant (RT, 15 hrs), (**9**) pellet (RT, 15 hrs). Figure on the right shows the low range molecular weight marker (Daltons) used for all figure.

A GST-column (Bio-rad Econo-Pac column was used) was set up for purification of the supernatant with a bed volume of 4 ml GST beads. The protein was put on the column, but no protein passed through the column; the protein seemed to precipitate while in the column, resulting in the loss of all the protein. Next was the large-scale expression

and purification of the Smad6 Full Length protein, which the procedures are the same as for the large-scale expression and purification of the Smad6 MH2 PDEST-15 above, except that instead of using LB medium for the culture 2xYT medium was used with ampicillin and Chloramphenicol. After the cells were cultured and centrifuged (same as above) the pellets were resuspended in lysis buffer (mixture from table 5), and sonicated, and centrifuged. The supernatant (10 μl) and pellet (in 10 μl of water) were saved for SDS-PAGE, the rest of the pellet was discarded and the supernatant was purified with the GST-column.

Table 5. Large-Scale Lysis Buffer

Reagents	**Volume (ml)**
PBS buffer	100.0
Lysozyome (10 mg/ml)	1.0
DTT (1 M)	0.5
Protease Inhibitor Cocktail	0.2
Dnase (10 mg/ml)	0.1
Rnase (27 mg/ml)	0.04
$MgCl_2$ (1 M)	0.5

The results from the GST-column can be seen in figure 4, from lane 2 there is some soluble protein in the supernatant, and some purification from the GST-column. The results from the GST-column show there was protein in the elution sample so a test was preformed to see if time was a factor when cleaving the tags off of the protein. To do this the elution sample (1ml) was removed from the sample and placed in a 1.5 ml centrifuge

tube, and 1 unit of the thrombin enzyme was added and after 1, 2, 3, 4, 18.5 hours a 10 µl aliquot was taken from the sample and 4x loading buffer (5 µl) was added to the samples. The samples were heated at 95 °C to stop the cleavage and all the sample were ran on a 12 % SDS-PAGE gel to analyze the results (figure 5).

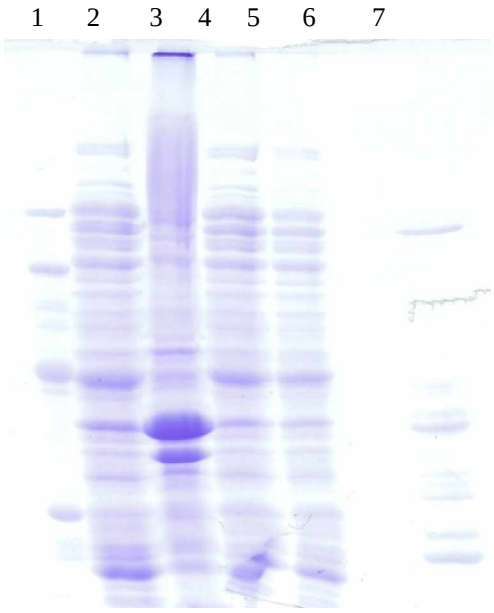

Figure 4. From left to right: lane (**1**) low range marker, (**2**) Smad6 Full length supernatant, (**3**) Smad6 Full length pellet, (**4**) Smad6 Full length flow through, (**5**) Smad6 Full length first wash, (**6**) Smad6 Full length second wash, (**7**) Smad6 Full length elution sample.

The remainder of the elution sample was treated with 1 unit of thrombin enzyme for 2 hours. Before Resource S-column purification the buffer was exchanged. The buffer was prepared according to table 6. The buffer exchange was preformed by added the cleaved elution sample (~50 ml) to a Amicon Ultra centrifuge filter device (Millipore-15, 30,000 MW), added in (4 x 15 ml) increments over a period of 2 hour centrifugation

(5,000 rpms) until there was only 1 ml remaining in the filter. The filter was than washed with 20 mM Mes (from table 6), 1 ml at time, and then placed in new 25 ml tube, a buffer exchange total volume was 15 ml. The sample was run through the Resource S-column (FPLC).

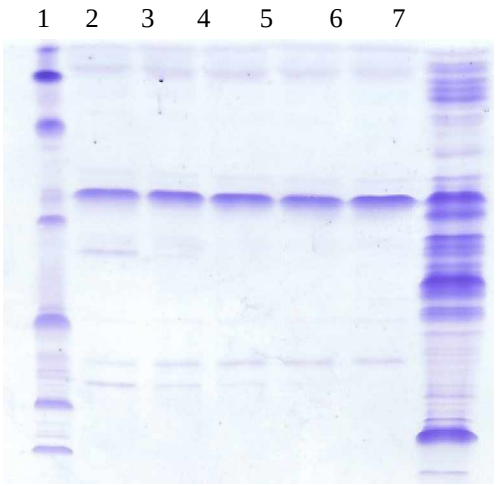

Figure 5. From left to right: lane (**1**) low range marker, (**2**) Smad6 Full length cleaved for 1 hour, (**3**) Smad6 Full length cleaved for 2 hours, (**4**) Smad6 Full length cleaved for 3 hours, (**5**) Smad6 Full length cleaved for 4 hours, (**6**) Smad6 Full length cleaved for 18.5 hours, (**7**) Smad6 Full length pellet from precipitation after buffer exchange.

Table 6. 20 mM Mes buffer pH 6.0, 10 mM β-Mercaptoethanol

Reagents	**Volume (ml)**
Mes pH 6.0 (1 M)	10.0
β-Mercaptoethanol	0.35

Milli pore water	487.65

After the sample was passed through the Resource S-column there were 8 fractions that showed peaks, each of these fractions developed on a 12% SDS-PAGE gel (figure 6). After purification with the Resource S-column there was not enough sample purified protein to continue, so a new cloning system was tested to analyze if the protein would be more stable in a different cloning system.

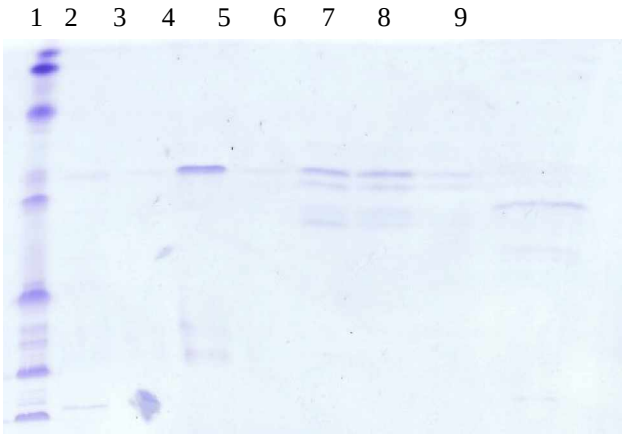

Figure 6. From left to right: lane (**1**) low range marker, Lanes 2-9 are taken from fraction off the Resource S-column where peaks were present; (**2**) fraction 9, (**3**) fraction 25, (**4**) fraction 26, (**5**) fraction 27, (**6**) fraction 36, (**7**) fraction 38, (**8**) fraction 25, (**9**) fraction 63.

Primers were purchased for the LIC system and PCR was preformed with the PCR mixture that is shown in table 7, and the PCR conditions are stated in table 8. Once the PCR product was obtained the PCR products were ran on a 0.8 % agarose gel, with positive results for all the PCR products tested. The PCR products were stored at -20 °C

until vector digestion was complete and both the vectors and the PCR product were ready for T4 polymerase treatment.

To continue, the vectors pMCSG-7 and pMCSG-9 needed to be digested and purified, pMCSG-10 will also be used, but was already digested earlier. The vector digestion mixtures are given in table 9, and the digestion mixtures were incubated at 37 °C for 2.5 hours. A small sample was removed to run an agarose gel, only one band was present indicating sample was digested correctly, so incubation was extended for an additional 1.5 hours at 37 °C. After incubation was complete another gel was ran.

Table 7. PCR mixture for Smad6 LIC system

Reagents	**Volume (μl)**
Sterile water	71
DMSO	5
10x Herculase reaction buffer	10
dNTP (2.5 mM)	8
Template (PGEXSmad6) (263μg/ml)	1
Sense- Primer*	2
Anti-sense- Primer (Smad6LIC-antisense) (39.2 nmol)	2
Herculase Hotstart DNA Polymerase	1

* Two sense-primers were used one fro the full length gene and the other for the MH2 domain, (Smad6LIC-sense, Smad6MH2LIC-sense) respectfully.

Table 8. PCR Conditions (LIC system)*

Step	Temperature (°C)	Time (sec)
1	98	180
2	98	60
3	60	40
4	72	120
5	72	30 (min)
6	4	5 (hours)

*Steps 2-4 were repeated 30 times (cycles).

Table 9. Vector Digestion Mixtures

Reagents	**Volume (μl)**
Vector pMCSG-7*	35.6 (10 μl total vector)
10x SspI buffer E	6.0
SsPI	2.0
Water	16.4
BSA	0.6
Vector pMCSG-9*	34.4 (10 μl total vector)
10x SspI buffer E	6.0
SsPI	2.0
Water	17.6
BSA	0.6

* Vector concentrations are: pMCSG-7 281 μg/ml, pMCSG-9 291 μg/ml

A gel extraction kit was used in this process (QIAquick Gel Extration Kit). Once extraction was complete the concentration for each of the two vectors was determined by spectrophotometry, pMCSG-7 concentration was 86.33 μg/ml, and pMCSG-9 concentration was 100.03 μg/ml. The PCR product and the purified digested vector were each treated with T4 polymerases, the mixture is shown in table 10.

Table 10. T4 Polymerase Treatment Mixture

Reagents	**Final Concentration**	**Volume (μl)**
DNA in H_2O*	200 ng (total= 5 ng/μl)	7.0
10x Reaction Buffer	1x	4.0
dGTP (100 mM)**	2.5 mM	10.0
DTT (100 mM)	5 mM	2.0
Water	-	16.2
T4 DNA Polymerase	2units (total= 0.05/μl)	0.8

* Three different DNA vectors were used (pMCSG-7, pMCSG-9, pMCSG-10),

** dGTP is used with the vectors, dCTP is used in place of dGTP for the treatment of the PCR product, and in place of the vector is the PCR product (concentration of 20 ng/ml).

The T4 polymerase mixtures were incubated at room temperature for 30 minutes, and the T4 polymerase was inactivated by heating the sample at 75 °C for 20 minutes, at which time the vector is ready for LIC cloning. The same procedure was preformed with the PCR product, and when both were treated with T4 polymerase the vector and the PCR products were transformed into XL-Blue cells according to table 11.

Once the mixture in table 11 was made the mixture was incubated for 30 minutes at 0 °C, then heat shocked at 42 °C for 45 seconds and placed back on ice. Then S.O.C. medium (0.45 ml) was added to the mixture, and then incubated at 37 °C in shaker for one hour.

Table 11. Transformation Mixture

Reagents	**Volume (μl) pMCSG-10**	**Volume (μl) pMCSG-9**	**Volume (μl) pMCSG-7**
Vector DNA (15 ng total)	3.0	10.0	10.0
PCR product (30-45 ng)	3.0	6.0	6.0
Incubated for 30 minutes on ice			
Add XL-Blue cells	65.0	167.5	167.5

After incubation the culture was centrifuged at 4,000 rpms for 5 minutes, discarding all but 100 μl of the supernatant. The pellet is resuspended with the remaining medium and was plated on a ampicillin plate and incubated at 37 °C for 15 hours. From the plates only the transformants with pMCSG-7 and pMCSG-10 grew and miniprep was preformed to prepare the vector DNA on these samples and was given pMCSG-9 for miniprep (same as miniprep was preformed above). Miniprep was completed for all samples and then transformed into BL21 DE3 cells and plated on ampicillin plates. All plates successfully grew single colonies, which were used for small-scale expression test. The expression was preformed the same as stated above and glycerol stocks were made, (figure 7). The cells with pMCSG-9 were not induced correctly and discarded.

Large-scale cultures were prepared for both the Smad6 MH2-7 and Smad6 MH2-10 using the same method as above inducing the cells with IPTG and placing in 4 °C for induction. The cells were collected, and added to lysis buffer (table 12).

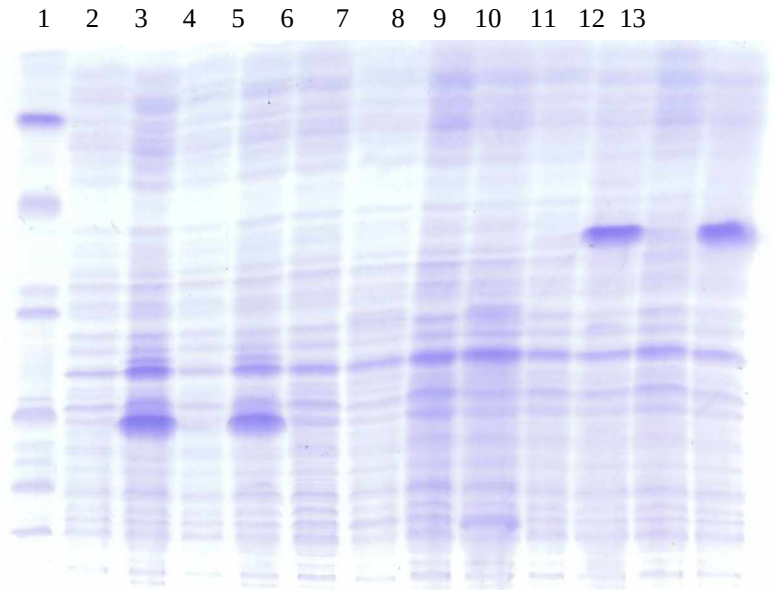

Figure 7. From left to right: lane (**1**) low range marker, (**2**) vector 10 (NI), (**3**) vector 10 (I), (**4**) vector 10 (NI), (**5**) vector 10 (I), (**6**) vector 9 (NI), (**7**) vector 9 (I), (**8**) vector 9 (NI), (**9**) vector 9 (I), (**10**) vector 7 (NI), (**11**) vector 7 (I), (**12**) vector 7 (NI), (**13**) vector 7 (I). Vector 10 is pMCSG-10, vector 9 is pMCSG-9, and vector 7 is pMCSG-7, (NI-non-induced), (I-induced).

The pellets from both Smad6 HM2-7 and Smad6 MH2-10 were resuspended in the MEGA lysis buffer and sonicated for a total of 15 minutes (pulse time) in a 5 second pulse, 10 second pause cycle. Sample were prepared for SDS-PAGE (figure 8), with both proteins being found in the supernatant (proteins are soluble).

Table 12. MEGA Lysis Buffer Mixture

Reagents	**Final Concentration**	**Volume (ml)**
NPI-10*	-	100.0
Glycerol	5 %	5.000
Lysozyme (10 mg/ml)	0.1 mg/ml	1.000
DNase (10 mg/ml)	10 mg/ml	0.100
RNase (27 mg/ml)	10 mg/ml	0.038
$MgCl_2$ (1 M)	4 mM	0.400
β-Mercaptoethanol	5 mM	0.035
Protease Inhibitor Cocktail	-	0.100

* NPI-10 was made from mixing 1 ml of NPI-1000 to 99 ml of NPI-0, solution consists for 50 mM sodium phosphate buffer (pH 8.0), 300 mM sodium chloride, and 10 mM imidazole.

Two Ni-columns were set up and the supernatants passed through a Ni-column, and analyzed to see what concentration of imidazole would elute the protein from the Ni-column (the solutions were prepared according to table 13). It was shown that for Smad6 MH2-7 protein eluted slightly with NPI-60, but mostly with NPI-250, and for Smad6 MH2-10 NPI-250 eluted the protein. These two fractions were collected for each of the two (Smad6 MH2-7 fractions NPI-60/250 (20 ml), and for Smad6 MH2-10 fraction NPI-250 (10 ml)) and added TEV protease to both samples to cleave off the tags.

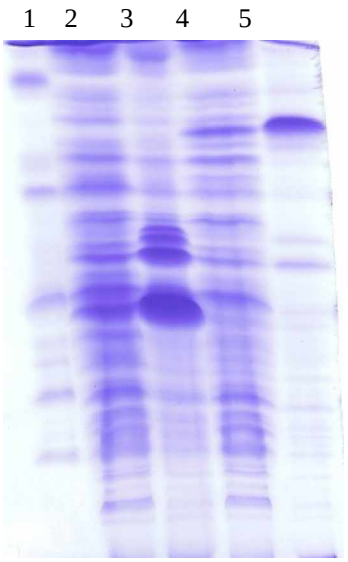

Figure 8. From left to right: lane (**1**) low range marker, (**2**) supernatant from Smad6 MH2-10, (**3**) pellet from Smad6 MH2-10, (**4**) supernatant from Smad6 MH2-7, (**5**) pellet from Smad6 MH2-7.

Table 13. NPI Solution Mixtures*

NPI Solution	NPI-0 Volume (ml)	NPI-1000 Volume (ml)	Glycerol (5 % total conc.) Volume (ml)	β-Mercaptoethanol (5 mM final conc.) Volume (μl)
NPI-10 (50 ml)	49.5	0.5	2.5	17.5
NPI-20 (30 ml)	29.4	0.6	1.5	10.5
NPI-40 (30 ml)	28.8	1.2	1.5	10.5
NPI-60 (30 ml)	28.2	1.8	1.5	10.5
NPI-250 (30 ml)	22.5	7.5	1.5	10.5
NPI-1000 (30 ml)	-	30.0	1.5	10.5

*All solutions were kept on ice.

After cleavage (cleavage time 12 hours) both solutions had precipitated the solutions were placed (Smad6 MH2-10) at 4 °C and the rest of the purification was preformed with Smad6 MH2-7. The sample was centrifuged at 14,000 rpms for 5 minutes with table top centrifuge, and ran a SDS-PAGE gel to determine if there was any residual protein in the supernatant. From the gel it was determined that all the protein had precipitated of out of the solution.

A large-scale expression and purification was preformed again with Smad6 MH2-7 using the same methods as before, except the lysis buffer used this time was NPI-10 from Table 13, made fresh on the day to be used. The sample was shown to be soluble in the supernatant as before after sonication 20 total minutes pulse time, with a 3 second pulse, 3 second pause cycle. The supernatant was purified through a Ni-column with Chaps added to a final concentration of 2 mmol to stabilize the elution fraction. After 24 hours, the solution that was at 4 °C had precipitated, and as a result the sample was centrifuged at 14,000 rpms for 5 minutes and both the supernatant and pellet were tested along with the multiple fractions from the previous Ni-column (figure 9). Before the sample could be cleaved the buffer needed to be changed from NPI-250 to NPI-10 (10% glycerol, 5 mM β-Mercaptoethanol, and 2 mM Chaps).

To perform the buffer exchange the same Amicon Ultra centrifuge filter device (Millipore-15, 30,000 MW) used before with the same procedures. After the buffer exchange and before cleavage occurred the OD was measured. The OD was 0.57 at 280 nm, and 0.64 at 260 nm. Cleavage with the TEV protease was allowed to react for 13 hours, with little precipitation occurring. The sample was centrifuged and the pellet and supernatant will be tested with SDS-PAGE with other samples.

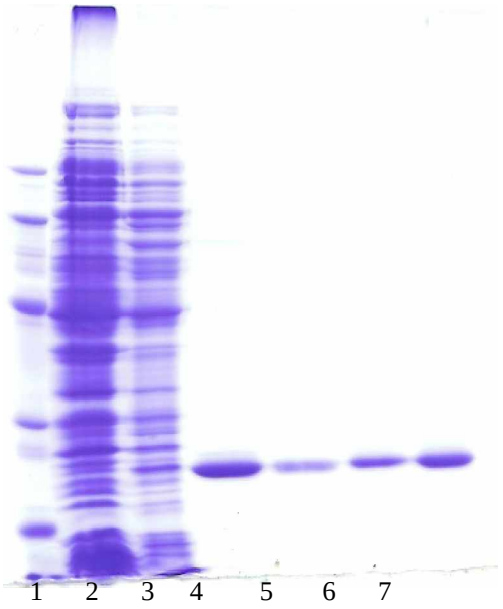

Figure 9. All samples are of Smad6 MH2-7; From left to right: lane (**1**) low range marker, (**2**) first flow through, (**3**) NPI-40 wash, (**4**) NPI-250 wash, (**5**) NPI-1000 wash, (**6**) supernatant, (**7**) pellet.

The OD was determined after cleavage with TEV protease, the OD was 0.59 at 280 nm, and 0.59 at 260 nm. The sample was passed through a Ni-column once again to further purify the protein, with the tag cleaved off the tag should stay in the column with the protein passes through the column. Once the sample was passed through the column the OD was taken again, the OD 0.32 at 280 nm, and 0.46 at 260 nm.

The results from buffer exchange to the final purification through the Ni-column are shown in figure 10, the sample still was not pure enough for crystallization screening to be performed. A buffer exchange was needed from NPI-10 to a Tris buffer (pH 8.0). After the buffer exchange there was not a sufficient amount of the protein to further purify the protein.

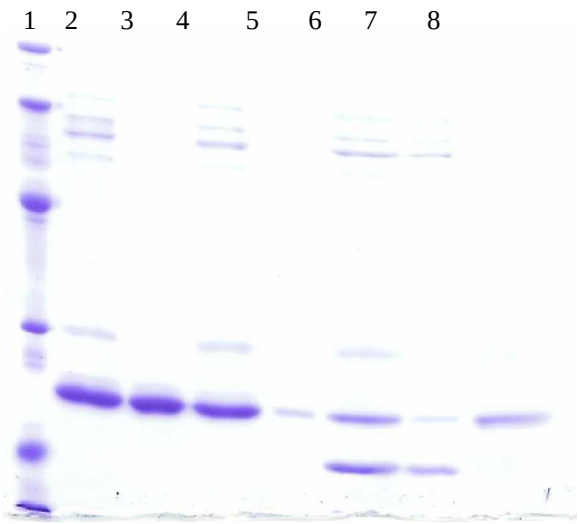

Figure 10. All samples are of Smad6 MH2-7; From left to right: lane (**1**) low range marker, (**2**) NPI-250 fraction before buffer exchange and centrifugation, (**3**) NPI-10 fraction after buffer exchange and centrifugation (pellet), (**4**) NPI-10 fraction after buffer exchange and centrifugation (supernatant), (**5**) NPI-10 fraction after cleavage and centrifugation (pellet), (**6**) NPI-10 fraction after cleavage and centrifugation (supernatant), (**7**) NPI-10 fraction purified protein after cleavage and Ni-column, (**8**) NPI-1000 fraction (wash to see what was left on Ni-column).

Section 6. Conclusion

It was found that it was difficult to purify the Smad6 Full length gene as well as the MH2 domain using both the Gateway® system as well as in the LIC system. In each stage of purification the protein precipitated. It was also shown that these proteins were soluble, but were not stable in solution at 4 °C for more than 12 hours. The LIC system was found to be slightly easier to work with and the use of the Ni-column was much more efficient than the GST-column, which was used in the Gateway® system as a means to purify this

Smad6 protein/domain. It was also found to be extremely difficult to perform a solvent exchange with this protein/domain without considerable protein precipitation.

In the future I will utilize the LIC cloning system with the pMCSG-9 vector, which is more stable, for purification of the Smad6 Full length and MH2 domain. Also I would like to test varied conditions to optimize the stability of this protein in solution.

Section 7.

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