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Emily Sue Frampton
Utah State University

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by

Emily Sue Frampton

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Approved:

Thesis/Project Advisor
Dr. Sean Johnson

Departmental Honors Advisor
Dr. Alvan Hengge

Director of Honors Program
Dr. Kristine Miller

**UTAH STATE UNIVERSITY
Logan, UT**

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Emily S. Frampton, Jeremy Bakelar, Sean J. Johnson

Department of Biochemistry, Utah State University 0300 Old Main Hill Logan, Utah 84322-300

ABSTRACT: Air2 is a eukaryotic protein involved in multiple biological processes including protein-protein interactions as well as RNA binding. Air2 plays a critical role in RNA quality control and also helps regulate post-translational modification of various proteins. Although previous studies have revealed information regarding Air2's roles within a cell, the molecular and structural basis for Air2 function is unclear. Using a codon-optimized version of the Air2 gene, various constructs were created that improved the expression and solubility of Air2. Additionally a co-expression complex of Air2 with a PRMTI mutant, K13S, was made to obtain the Air2 protein with a native binding partner. It was hypothesized that the co-expression of these two proteins together would lead to the creation of a complex increasing both the stability, expression, and activity of each. These experimental efforts will greatly contribute to the biochemical and structural studies necessary to achieve a better functional understanding of Air2 and thus lead to the improved future characterization of this protein.

INTRODUCTION

Within the cell, many protein-protein interactions are essential for proper cell function and maintenance. For example, protein-protein interactions are necessary for the formation of multiprotein complexes that are needed to carry out specific enzymatic reactions. In addition, many enzymatic processes can be regulated through the inhibition, activation, or modification of

interacting proteins. Inside the nucleus of *Saccharomyces cerevisiae* (*S. cerevisiae*), the Air1 and Air 2 proteins have been implicated in mediating protein-protein interactions in relation to different enzymatic processes (Figure 1). One of the structures within the nucleus of the cell that Air (Arginine methyltransferase Interacting Ring-finger proteins) proteins are compulsory components of is the **Trf4/Air2/Mtr4p Polyadenylation** complex (TRAMP) complex[1-7]. The TRAMP complex's function within the cell involves RNA recognition, degradation, and trimming. Within the TRAMP complex, the Air proteins have been shown to be involved with regulating protein-protein interactions as well as RNA binding[2]. Overall in the TRAMP complex Air proteins are involved in the RNA control processes. In addition to the function of Air1 and Air 2 within TRAMP, the Air proteins are also involved in the regulation of nuclear mRNA transport. Air1 has been shown to modulate the activity of yPRMTI (yeast Arginine methyltransferase)[8] and it is suggested that Air2, being a homologue of Air1, may also modulate PRMTI activity.

(Insert Figure 1)

Although the involvement of the Air proteins in these processes is clear, not much is known regarding the functional and structural basis behind TRAMP or yPRMTI involvement. Detailed biochemical and structural data will significantly improve the understanding of these processes. In order to perform these studies and obtain the necessary empirical data, significant amounts of the purified proteins are required. The Air1 and Air2 proteins have poor solubility and expression, which makes purification and subsequent biochemical studies difficult to conduct. Previously, in the Johnson Lab, a method for improving the solubility and expression of the Air1 protein has been developed. This has been accomplished by designing a codon-optimized version of the Air1 protein.

In a cell, there are multiple codons, three letter sequence portions, which code for particular single amino acids. Certain codons are preferred over others by both Prokaryotic and Eukaryotic cells. The process of codon-optimization of a gene changes its original codons to those codons that are more favored by a particular species, but still code for the same amino acid. The version of Air being studied is from *S. cerevisiae*, but it is expressed in *Escherichia coli* (*E. coli*). Codon optimization of the Air proteins from *S. cerevisiae* replaces the original codons with those codons preferred by *E. coli*. It was found that the codon optimization greatly improved the expression of Air1. This discovery has allowed for the necessary expression, binding, and purification experiments to be conducted. This project aimed to remedy the previous problems encountered with Air2 using the same codon optimization approach as used with Air1.

Another experimental approach that aids in stabilizing and improving the solubility and expression of a protein is obtaining the protein in complex with a native binding partner. In this more natural conformation, proteins tend to behave more optimally and are more easily purified. This second experimental approach was additionally hypothesized and utilized to improve the expression of Air2, by coupling it with a mutated version of PRMTI, K13S, to create a coexpression complex.

Combining both of the above experimental approaches, this research sought to improve the expression and solubility of the Air2 protein to aid in future purification and characterization studies.

EXPERIMENTAL METHODS

Molecular Cloning

In order to improve expression and subsequent purification of the Air2 protein, a codon optimized version of the gene Air2 was ordered from a commercial vendor (Genscript). The

codon optimized Air2 gene was derived from the first half of the gene sequence, which is (Insert Figure 2 and 3) comprised of the N terminus and includes all five zinc knuckles (Figure 2). Several restriction enzyme sites were also inserted into the codon-optimized gene to aid cloning into different expression vectors. The codon optimized Air2 gene came in a puc57 vector. A total of two constructs have been successfully completed thus far, a His-tagged only N terminus through zinc knuckle five version as well as a N terminus through zinc knuckle five Flag tagged version both in pET Duet vectors (Figure 3). Each of these constructs was obtained through similar molecular cloning techniques and strategies.

The cloning strategies for each are outlined in Figure 4. The His-tagged only version was obtained by cutting the codon optimized gene out of the puc57 vector using the BamHI and EcoRI restriction sites and cutting with these same sites and ligating into the His tag containing pET Duet vector. The other Flag tagged Air2 construct was obtained in a similar manner by ligating into the same vector, but splicing with different restriction sites, namely NcoI and EcoRI. The attached Flag tag will allow us to assess binding of Air2 to other proteins through pull down studies, and the proteins without a Flag tag will be used for crystallization trials in order to study structure. Both of these constructs were successfully cloned into the corresponding pET Duet vector as confirmed by sequencing. Each plasmid was transformed into competent DH5α and βL2I cells.

Different cloning methods were also utilized to obtain Air2 with a PRMTI mutant, namely K13S. Multiple approaches were attempted, but the arrangement that was finally successful was accomplished by splicing the K13S construct out of the pET Duet vector and pasting it into another pET Duet vector containing His-tagged Air2 N-Zk5 (Figure 4). K13S was ligated into the second cloning site of the Air2 containing pET Duet vector utilizing the NdeI and

XhoI restriction enzymes as above. The identity of this completed coexpression complex was (Insert Figure 4) verified via restriction analysis as well as DNA sequencing. The completed coexpression vector was then transformed into competent DH5α and βL2I cells.

Expression Tests of Codon Optimized Air2 Constructs

A variety of small-scale *E. coli* growth cultures were used to conduct expression tests on each of the two different Air2 constructs. A standard protocol (based on the current Air1 expression protocol) included inoculation of a 500 ml SuperBroth growth with a 30 ml lysogeny (LB) broth overnight that has been transformed with a βL21 *E. coli* colony; incubation in a shaker a 37 °C; Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction at and optical density (OD) of 0.6; and the cells are harvested after six hours at room temperature in a shaker. After the cells are harvested from the growth media via centrifugation, using the SLC 4000 FIBERLite® (Piramoon Tech-nologies Inc.) rotor and placed into the Sorvall® RC 6 Plus (Thermo Elctron Corporation) centrifuge at 8,000 rpm for 12 minutes.

The appropriate buffers were prepared before the expression tests were to be conducted. Buffers included a lysis buffer, a buffer B, and a 500 mMol imidazole elution buffer. The frozen cell pellet was resuspended using a 1:3 cell pellet weight to lysis buffer dilution. The cells were then lysed via a Sonifier 450 (Branson). The lysed protein mix was then centrifuged at rpm for 30 minutes to obtain the lysate. Samples of both the lysate and pellet were kept for SDS-PAGE analysis. The protein lysate was then added to a half of a milliliter nickel resin and incubated on the resin for an hour. The resin was then washed with lysis buffer 3 times. A sample was kept after the third wash. Two elutions were then performed using 500 mMol imidazole. Samples

were kept of each of the two elutions. The collected samples were than analyzed for expression, solubility, and purity via SDS page analysis on 12% acrylamide gels stained with Coomasie blue.

Expression Tests of the Coexpression Construct

Two different growth protocols were evaluated to test the expression and complex formation of the Air2-K13S coexpression construct. The first small scale growths were conducted using a similar protocol as was used with the codon optimized constructs. A 30 ml SuperBroth overnight using a βL21 colony containing the His Air2 N-Zk5 + K13S plasmid was used to inoculate two 500ml growth flasks. Each flask was inoculated with 10 ml of the overnight and allowed to grow in a 37 °C shaker till it reached an optical density (OD) of 0.6. At this optic density the growths were then induced with 50 mM IPTG and allowed to grow at room temperature for 4 hours. The cells were then harvested in the same method previously utilized. The second growth protocol, adapted from the Hevel Biochemistry lab, used LB media instead of SuperBroth. The overnight was thus conducted in 30 ml of LB media and two growth flasks each containing 500 ml of LB media were inoculated with 10 ml of the overnight. After inoculation, the flasks were placed in a 37 °C shaker till an OD of 0.3 was reached. The growths were then induced with 0.05 mM IPTG and allowed to grow for 20 hours until they were harvested via centrifugation as above.

A specific lysis buffer was prepared prior to each expression test. The frozen cells pellets were resuspended in lysis buffer and lysed in a manner similar to the protocol above. The lysed cellular material was the centrifuged and prepared in the same using the same methodology as was employed previously. The resulting lysate was then applied to nickel resin and the

expression test proceeded was outlined above. Samples were kept and analyzed via SDS-PAGE as previously detailed.

RESULTS

Codon Optimized Constructs

The proper fragments for the cloning of the constructs were visualized via ethidium bromide staining and agarose gel electrophoresis. Both the vector and the inserts were seen to be running at the proper base pair amounts in comparison with the ladder. After ligation and transformation of the fragments, the identity of each of the plasmids was confirmed via DNA sequencing and basic local alignment search tool (BLAST) analysis. Once each of the constructs were completed, expression tests were performed. The Flag tagged Air2 N-Zk5 showed slightly increased expression when compared to previous Air2 expression tests, but the solubility was relatively the same (Figures 5 and 6). In contrast, the His-tagged only Air2 N-Zk5 construct showed greatly improved expression and solubility (Figure 7).

(Insert Figures 5, 6, and 7)

Coexpression Complex

The proper insert and vector fragments were identified via agarose gel electrophoresis and ethidium bromide staining. Restriction analysis of the coexpression complex revealed an Air2 fragment of the appropriate molecular weight. Given this result the vector was sent to sequencing. Sequencing and BLAST revealed that the molecular cloning techniques had produced the desired vector. Analysis of the SDS-PAGE gels revealed the expression of both the Air2 and K13S proteins, but no there was no evidence of a complex being formed (Figure 8).

(Insert Figure 8)

DISCUSSION

Codon Optimized Constructs

The expression and solubility seen in the first expression test of this construct, when compared to previous expression tests, is the best achieved for an Air2 construct thus far. The codon optimization thus achieved what it was hypothesized to accomplish. It greatly improved the solubility as well as the expression of this protein within *E. coli.*

Coexpression Complex

Good expression of both proteins is observable from the SDS-PAGE gels. Although expression of each of the proteins is evident from the gels, it appears that a complex between the two proteins is not being formed. Each of the four expression tests that have been conducted have had parameters that have been adjusted in attempts to favor the formation of a complex, but none of the tests have been successful thus far.

CONCLUSION

Codon Optimized Constructs

Codon optimization of Air2 greatly improved the solubility and expression in the His-tagged version. Expression and solubility were also seen to be improved to a smaller degree in the Flag tagged Air2 N-zk5 construct. These constructs will help tremendously in future *in vitro* growth, expression, and purification experiments necessary to obtain purified Air2 protein for crystallization trials. Larger scale growths are underway with the His-tagged Air2 N-Zk5 construct to optimize growth conditions in order obtain more protein for *in vitro* experiments. The His-tagged version was also used to obtain the coexpression vector outlined in this paper

Coexpression Complex

The coexpression vector achieved expression of both Air2 and K13S in synchrony. Despite the success of their dual expression, there is an unknown factor preventing the formation of a complex or a complex that is stable enough to be detected. Many factors contribute to the formation and isolation of a complex including growth parameters, buffer constituents, lysing temperature, and the amount of resin used.

The type of media, amount of IPTG, OD, and the length of growth were all adjusted in an attempt to promote the formation of a complex, but were unsuccessful. Buffers constituents were also adjusted. Altering the amount of salt, the type of buffer utilized HEPES and TRIS, and the amount of imidazole were all adjusted in attempts to form a complex. Better regulation of the lysis temperature has not been tested. The amount of resin used has been adjusted and tested for to ensure that an overloading of the resin was not the cause of a possible complex not adhering to the resin. Additionally, it is hypothesized that since zinc was not added into the growth media nor the buffers that Air2 may not have had the amount of zinc necessary for it to fold properly. If Air2 is not folding properly, it will not form a complex. Thus the next growths and expressions conducted will contain zinc to evaluate if that change will contribute to the formation of a complex between the two proteins.

FUTURE DIRECTIONS

More codon optimized versions of Air2 may be created such as knuckle specific truncations to conduct experiments identifying which regions of Air2 are necessary for different cellular interactions and processes. Additional coexpression constructs are being hypothesized and designed, such as a coexpression complex with Air2 and different yPRMTI versions that may more favorably form a complex. One of particular interest contains an yPRMTI construct

that is a truncation of the amino or N terminus off of the protein. This yPRMTI construct has previously been crystallized and had its structure determined via x-ray crystallography. A complex containing Air1 may also be pursued depending upon the success of the Air2 constructs. Different growth conditions, buffer constituents, and expression test designs will be attempted to identify the conditions that will produce a complex.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

S. cerevisiae, Saccharomyces cerevisiae; Air (1 and 2), Arginine methyltransferase Interacting Ring-finger proteins; TRAMP, **Tr**f4/**A**ir2/**M**tr4p **P**olyadenylation complex; yPRMTI, yeast Arginine methyltransferase; *E. coli, Escherichia coli*; OD, optical density; LB, lysogeny broth; IPTG, Isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BLAST, basic local alignment search tool; HEPES, *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*; TRIS, tris(hydroxymethyl)aminomethane.

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FIGURES

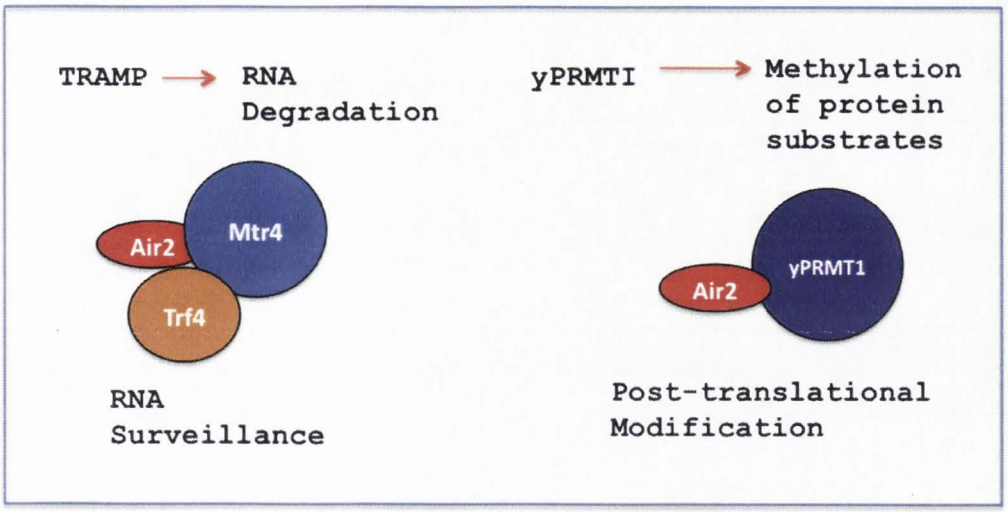

Figure 1: Roles of Air2 within the cell.

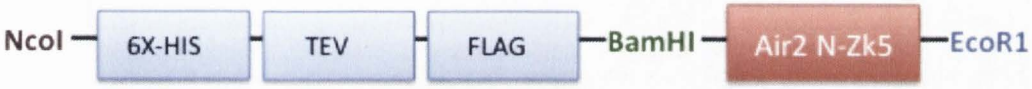

Figure 2: Design of the Air2 codon optimized gene.

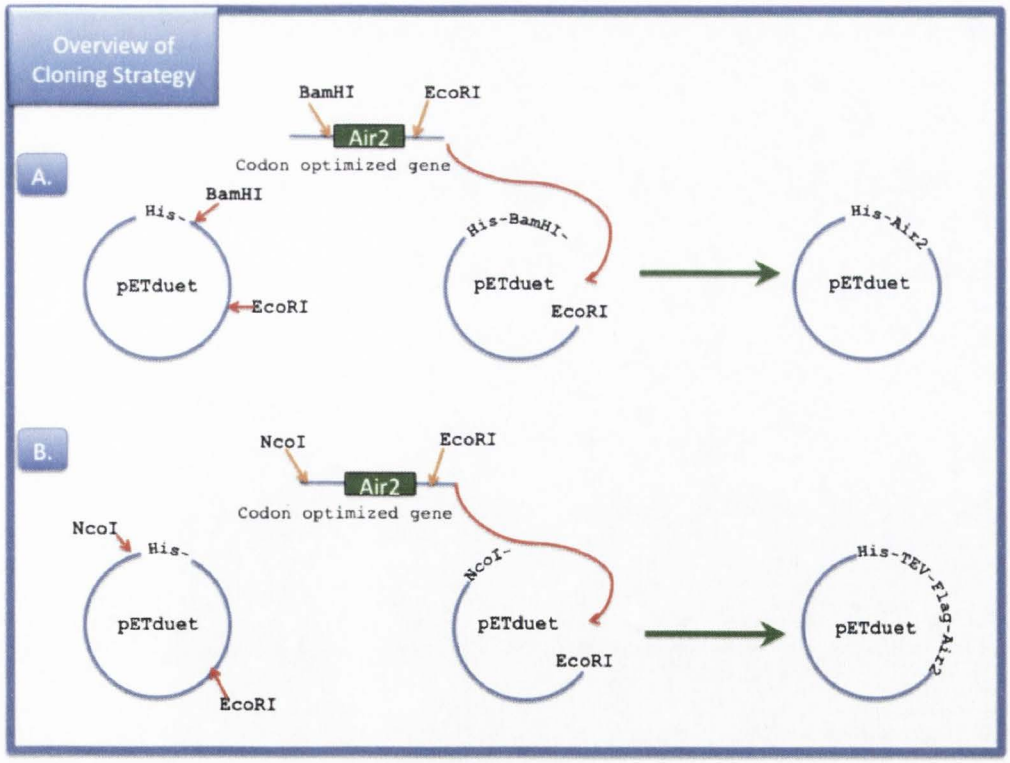

Figure 3: Overview of cloning strategy for (A) the His-tagged only Air2 N-Zk5 and the (B) Flag tagged Air2 N-Zk5.

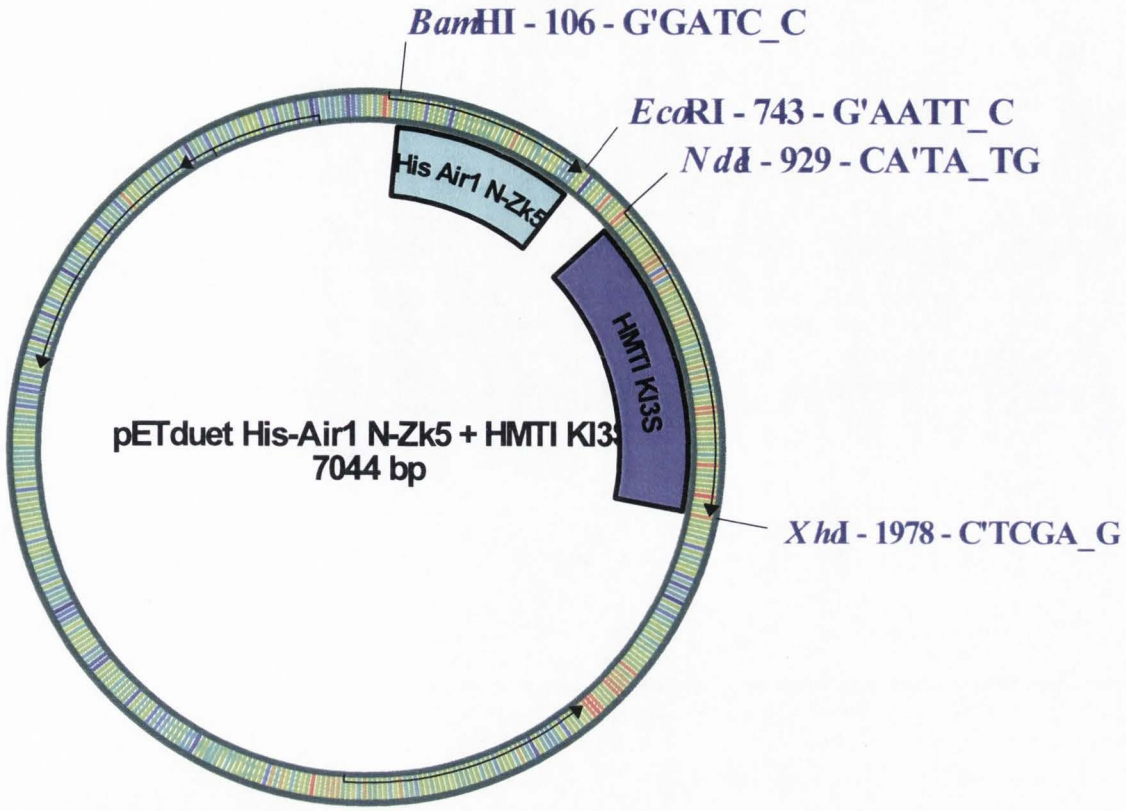

Figure 4: Coexpression complex design. The first cloning site containing His-tagged Air2 N-Zk5 and the second containing K13S. This is also the final completed format that was obtained through molecular cloning techniques and

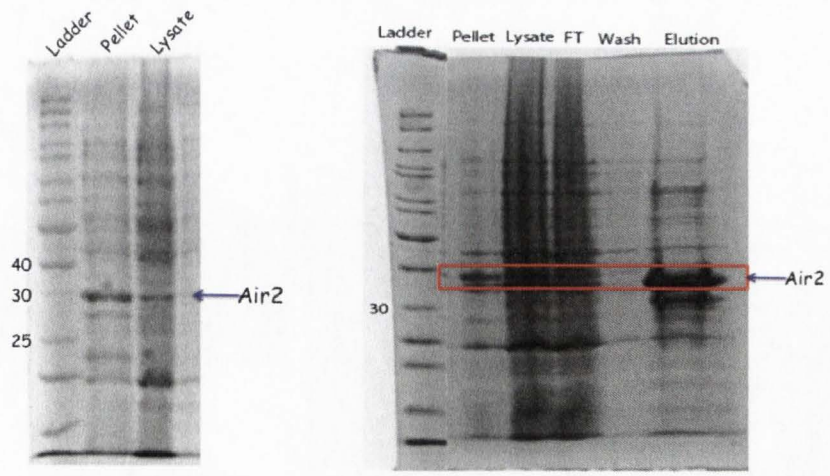

Figure 5 and 6: The SDS-PAGE gel on the left shows non-codon optimized Air 2 expression. The SDS-PAGE gel on the left is expression of codon optimized Flag tagged Air2 N-Zk5 expression and initial purification over a nickel resin.

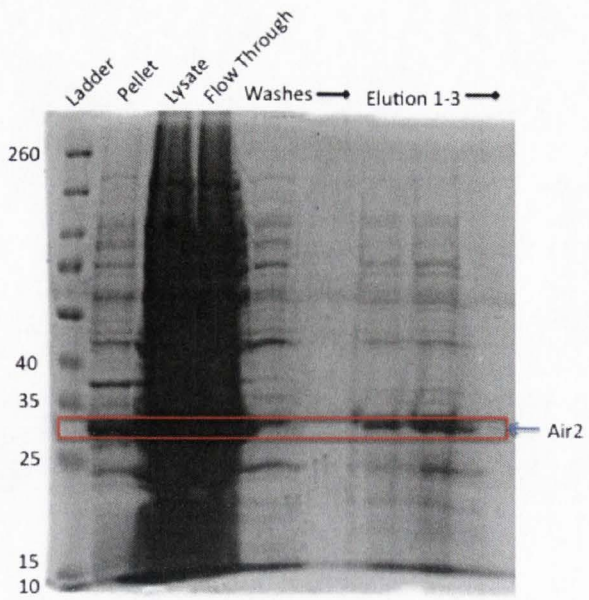

Figure 7: First expression test and initial purification of His-tagged only Air2 N-Zk5. Over 80% solubility is seen in the lysate, which is a significant improvement as compared to the solubility of previous expression tests (see Figures 4 and 5).

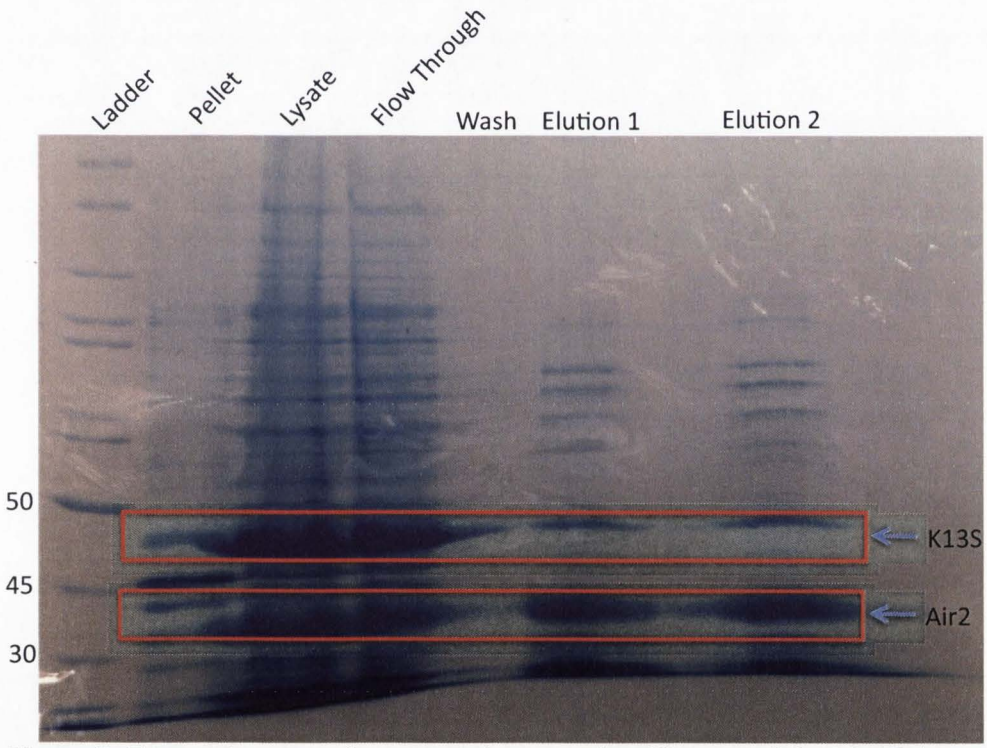

Figure 8: Expression test of coexpression complex. His tagged Air2 N-Zk5 is seen to run around ~31 kDa, which allows us to properly identify it. K13S typically runs around ~45 kDa allowing for proper identification from the gel. Expression of both proteins is observed on this gel, but there is no evidence of a complex being formed.