RESEARCH REPORT
SWINE HEALTH

Title: A Cell-free Synthesis Approach for the Rapid and Cost-Effective Production of Foot and Mouth Disease Vaccines **NPB# 13-104** **revised**

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Institution: Brigham Young University

Date Submitted: 31 Mar 2015

Industry Summary:

1. **Objectives:**

The objective of this work was to assess a technology's potential to produce the components of a *Foot and Mouth Disease Virus Vaccine* which could be (1) differentiated from infected animals (i.e. DIVA vaccine), (2) inexpensively engineered, produced, and distributed to the producers in a matter of days, and (3) engineered to rapidly target the emerging or pandemic strain. Such a vaccine could reduce/eliminate the threat of a $14+ billion dollar loss if a U.S. FMDV outbreak occurred [1]. The technology capable of potentially meeting these objectives is cell-free protein synthesis. Cell-free protein synthesis is the harvesting of protein-making machinery from bacteria and then turning this machinery on in a test tube to make only the desired proteins (in this case, proteins that would make an FMDV vaccine).

2. **How research was conducted:**

To produce the proteins necessary to make a Foot and Mouth Disease Virus (FMDV) vaccine, we first designed and synthesized only certain genes (DNA) from the FMDV genome. Other genes from FMDV that are necessary to synthesize an infectious virus were not produced. Thus our design enables the production of a DIVA vaccine that would be completely non-infectious. To be certain that the genes necessary for virus infection were not unintentionally produced, the complete FMDV genome was never brought into our lab (only a subset of the genome which was chemically synthesized). Our custom synthetized genes were expressed in our cell-free protein synthesis system to produce the desired proteins. We next assessed for their ability to form a virus-like particle (virus shells that do not contain the virus genome). This virus-like particle is thought to be an effective vaccine. The production system was further optimized by (1) including additional proteins which could help the production of the desired vaccine proteins and (2) varying production conditions.

3. **Research findings:**

Our research demonstrated for the first time that the FMDV structural proteins could be expressed efficiently using our low-cost cell-free system. We also demonstrated the ability of our cell-free production system to be directly manipulated and optimized for improved production.

4. **What these findings mean to the industry:**

Our research findings demonstrating the expression of FMDV proteins with our cell-free system are particularly exciting due to the ability of our cell-free system to rapidly produce large quantities of desired proteins. We have thus provided proof-of-concept data establishing the potential of using

our cell-free technology for the rapid production of vaccine proteins targeted against an emerging or pandemic FMDV strain. To commercialize this technology, future work includes (1) continued optimization of the production of FMDV vaccine proteins, (2) demonstrating the ability of our cell-free system to be stockpiled and rapidly deployed to produce large quantities of strain-specific FMDV vaccine proteins, and (3) verifying the immunogenicity of the FMDV vaccine proteins.

5. **Contact information:**

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6. **Reference**

1. Paarlberg, P.L., J.G. Lee, and A.H. Seitzinger, *Potential revenue impact of an outbreak of foot-and-mouth disease in the United States*. J Am Vet Med Assoc, 2002. **220**:988-92.

Keywords: FMDV, Foot and Mouth Disease, Vaccine, Cell-free, DIVA

Scientific Abstract: Multiple different gene constructs were designed and custom synthesized for the production of the Food and Mouth Disease Virus (FMDV) structural proteins necessary to synthesize a virus-like particle. These genes were based upon the A24 Cruziero FMD strain as this strain remains an outbreak threat, particularly in South America and thus the US. The utility of an *E. coli*-based cell-free system for the production of structural FMDV proteins was demonstrated for the first time. In addition, the cell-free protein synthesis reactions were directly controlled and optimized for improved soluble production of the desired FMDV proteins. The formation of virus-like particles was assessed using sucrose velocity sedimentation assays.

Introduction:

The Foot and Mouth Disease Virus could cost the pork industry a $14+ billion loss with an outbreak [1]. Our research has focused on developing technology to make an inexpensive FMDV vaccine which can be differentiated from infected animals (DIVA) and thus maintain the FMDV-free status of U.S. pork. The technology would also enable rapid vaccine production for better protection and quick response to an outbreak or agroterrorism.

Objectives:

- **Objective #1:** Optimize the sequence of DNA encoding the FMDV protein P1-2A and 3C and synthesize this DNA for expression in *Escherichia coli*-based cell-free protein synthesis reaction.
- **Objective #2:** Express the P1-2A and 3C proteins with *Escherichia coli*-based cell-free protein synthesis reactions and assess for VLP formation.
- **Objective #3:** Engineer the cell-free system to (1) increase the production yields of the FMDV VLP, (2) decrease the cost associated with VLP production, (3) and improve VLP purification.
- **Objective #4:** Disseminate results and prepare proposal for follow-on funding.

Materials & Methods:

Much of the experimental material and methods performed in this work have been reported previously in peer-reviewed publications. The materials and methods for performing our *E. coli*-based cell-free system has been reported in: *Varner CT, Smith MT, Bush DB, Bundy BC. 2012. The incorporation of A2 protein to produce novel Qbeta virus-like particles using cell-free protein synthesis. Biotechnology Progress. 28(2):549-555.* One difference is reactions were templated with T7-promoted expression plasmid pJ411-KanR which contained the synthesized gene cassette (described in Objectives 1 and 3).

The plasmids were added to the reaction at a final concentration of 12nM. The methods for including GroEL/ES in the cell-free system were described previously in: *Smith MT, Hawes AK, Shrestha P, Rainsdon JM, Wu JC, Bundy BC. 2014. Alternative Fermentation Conditions for Improved Escherichia coli-based Cell-free Protein Synthesis Requiring Supplemental Components for Proper Synthesis. Process Biochemistry. 49(2):217-222*. A similar protocol was followed for the co-expression of N-myristoyltransferase from *Saccharomyces cerevisiae* (NMT) during extract preparation such that NMT was present in cell-free reactions that do not contain GroEL/ES. The protein gel electrophoresis, the autoradiograms, the His-tag purification, and the sucrose gradient velocity sedimentation assay materials and methods are reported in the above mentioned papers.

Results:

Objective #1: ***Completed***: Optimize the sequence of DNA encoding the FMDV protein P1-2A and 3C and synthesize this DNA for expression in Escherichia coli-based cell-free protein synthesis reaction.

The genes to produce virus-like particles of A24 Cruziero Foot and Mouth Disease Virus were designed with safety as a priority. The contrast between the native and synthetic gene structure is featured below. Safety measures included:

1. Lack of the 5` and 3` untranslated regions (UTRs) that are required for eukaryotic expression
2. Lack of 3D RNA replicase gene required for genome replication and infection
3. Lack of majority of 2B, 3A and 3B genes that aide in infection cycle and proper replication
4. Inclusion of a T7 RNA polymerase promoter and ribosome binding site (RBS) upstream of the gene to restrict mRNA production to environments with T7 RNA polymerase

The general structure of the native gene was followed to promote the native cleaving of the polyprotein. However, the gene sequence was optimized on a base-pair level for synthesis in *Escherichia coli*, specifically by optimizing the codon selection. After careful consideration of safety and codon selection, the gene was commercially synthesized into a T7 promoted vector and cloned into *E. coli* strain DH5α for preparation of the plasmid DNA. A secondary plasmid for expressing exclusively the gene for 3C was prepared by excising the genes located between the NdeI restriction sites and ligating the NdeI sites together. This allowed for excess 3C protein to be produced. These constructs are illustrated below.

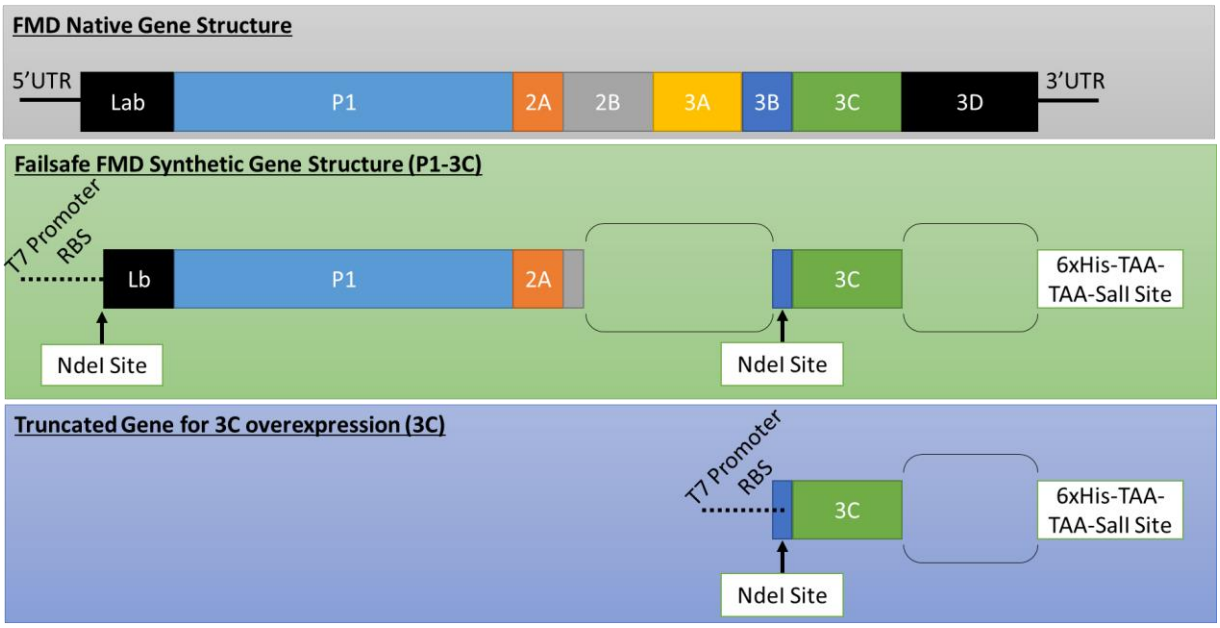

Figure 1: Gene design for cell-free synthesis of FMDV structural proteins

Objective #2: ***Completed***: Express the P1-2A and 3C proteins with Escherichia coli-based cell-free protein synthesis reactions and assess for VLP formation.

An *E. coli*-based CFPS reaction, illustrated in Figure 2 below, was performed with the expression plasmid for the P1-3C gene, expression plasmid for the 3C construct and with both expression plasmids at a 1:1 ratio. A schematic of the expected cleavage products from the P1-3C polypeptide from the 3C protease and their resulted hypothesized assembly into a VLP are shown in Figure 3A. Figure 3B reports the cleavage products by gel electrophoresis followed by autoradiography in which the bands shown are the protein products synthesized from the specified cell-free reactions. The coexpression of 3C protease with the P1-3C polypeptide increased cleavage efficiency by over 10%. The top band in the first two lanes for Figure 3B represent the P1-3C polypeptide and the top band of the third lane represents 3C. Additional bands illustrate proteolytic processing of the P1-3C by 3C protease. Sucrose Gradient velocity sedimentation was performed with the cell-free reaction product from the reaction with both plasmids co-expressed and the formation of significant population of virus-like particles was not observed (Figure 3C).

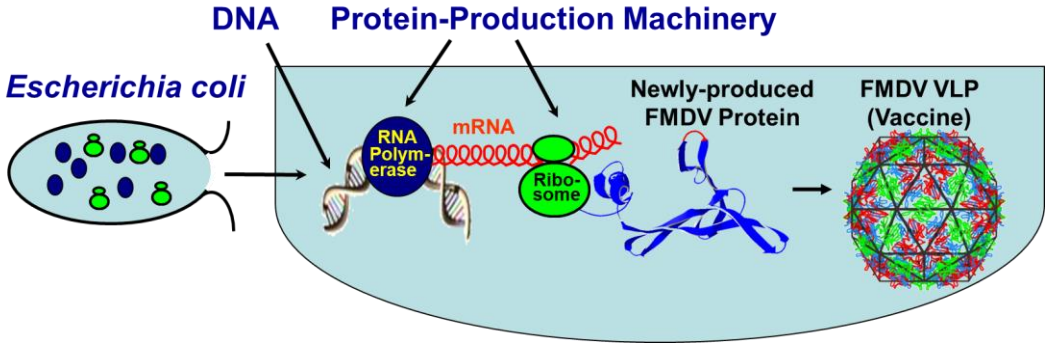

Figure 2: Schematic of cell-free protein synthesis of FMDV structural proteins that could synthesize an FMDV Virus-like particle (VLP).

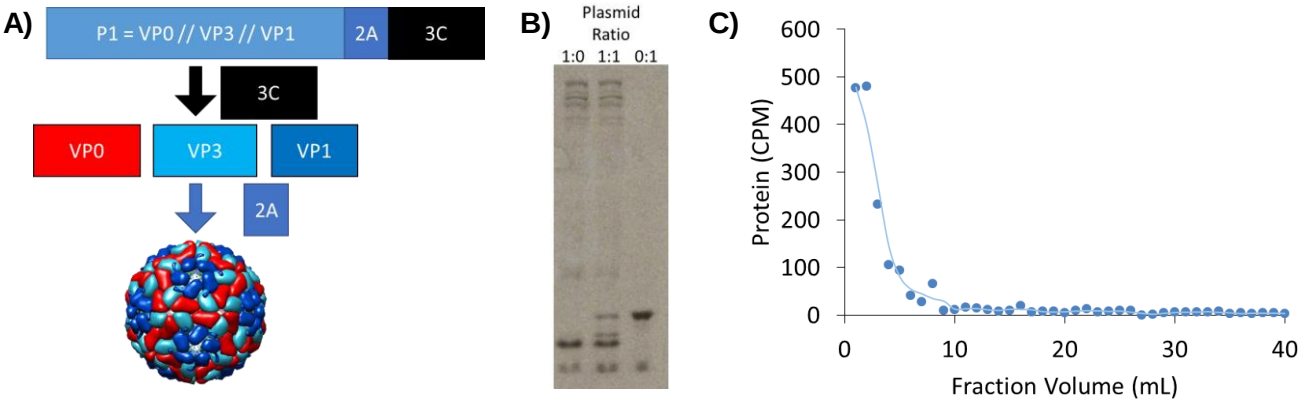

Figure 3: (A) Schematic of the expected cleavage products from the P1-3C polypeptide by the 3C protease and their resulting hypothesized assembly into a VLP. **(B)** Autoradiogram of C^{14}-Leucine-labeled cell-free products following synthesis of the expression plasmid for P1-3C only (left lane), expression plasmid for 3C only (right lane), and both plasmids at equimolar concentrations (center lane). **(C)** Protein content detected from C^{14}-labeled cell-free synthesized protein at different fractions following ultracentrifugation (5hrs at 4°C, 28,000 RPM in SW-32 rotor) of the cell-free product loaded onto 10-40% sucrose gradient. The lack of a peak in 10 to 30 fraction range suggests that VLPs were not significantly formed.

Objective #3: ***Completed***: Engineer the cell-free system to (1) increase the production yields of the FMDV VLP, (2) decrease the cost associated with VLP production, (3) and improve VLP purification.

The lack of detectible VLP formation as reported in Objective #2 led to an optimization effort to improve the synthesis of soluble FMDV protein. We first sought to enable myristoylation as myristoylation of VP0 is thought to be important for VLP formation. N-myristoyltransferase from *Saccharomyces cerevisiae* (NMT) was overexpressed during cell-free extract preparation such that it was abundant in the cell-free reaction and myristic acid was added to the reaction in excess (20 μg/mL). The resulting cell-free reaction in which the P1-3C and 3C expression plasmids template the reaction in equimolar concentrations did not result in detectible assembly of VLP (Figure 4).

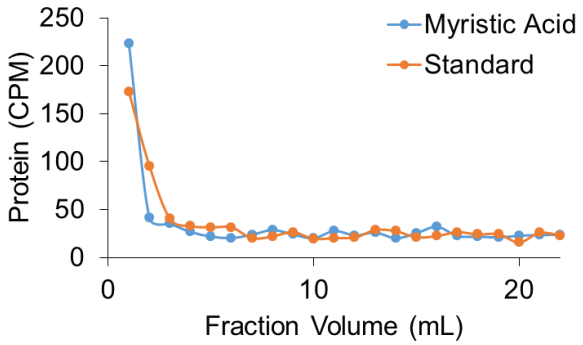

Figure 4: Assessment of Myristoylation on VLP assembly. The protein content in each fraction was detected by the radition from the C^{14}-labeled cell-free synthesized protein. Fractions were collected following ultracentrifugation (5hrs at 4°C, 28,000 RPM in SW-32 rotor) of a 10-40% sucrose gradient loaded with the cell-free product (separately for the standard reaction and the reaction containing NMT and myristic acid). The lack of a peak in 10 to 30 fraction range suggests that VLPs were not significantly formed.

We next sought to improve solubility by cloning mutants of 3C protease that have been reported to improve the solubility of 3C protease. This resulted in a 4 fold improvement in 3C protease solubility (Figure 5).

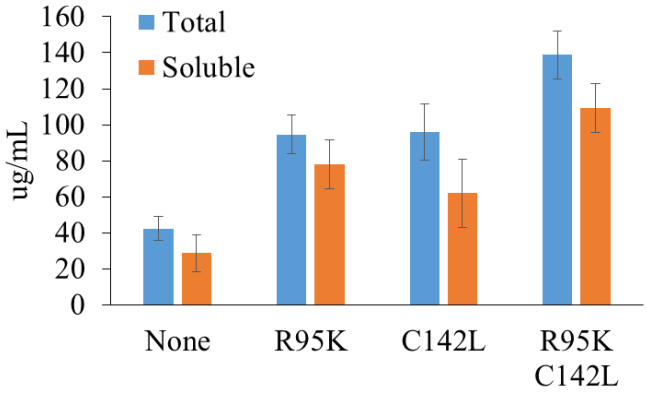

Figure 5: 3C protease mutant total and soluble expression yields (μg/mL) after synthesis in cell-free reactions. Substitution mutations in 3C protease are specified on the x-axis. (n = 3, error bars = standard deviation)

The use of these 3C protease mutants improved the overall solubility of P1-3C and 3C co-expression reactions by 35% (Figure 6) and increased the concentration of soluble cleaved products (such as the VP0, VP1, and VP3 capsid proteins that are necessary for the formation of VLPs) as shown in Figure 7.

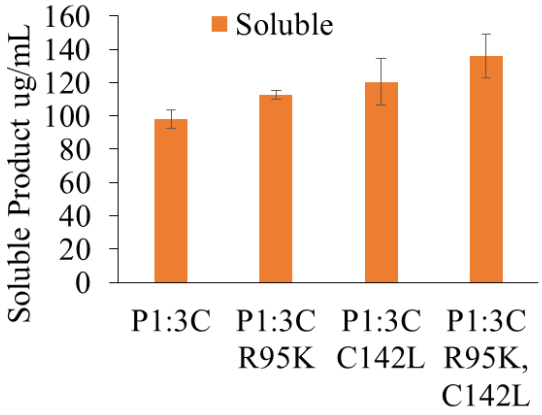

Figure 6: Soluble cell-free synthesis product yield (µg/mL) in reactions containing the native and mutant 3C proteases coexpressed equamolarly with the P1-3C gene. (n = 3, error bars = standard deviation)

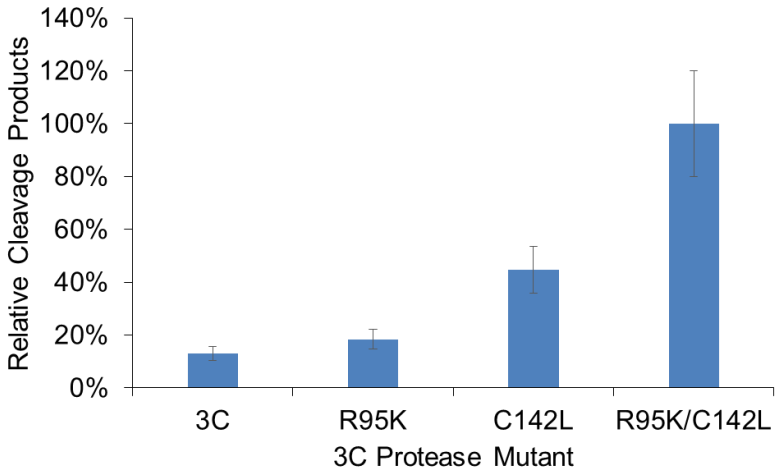

Figure 7: Using electrophoresis, autoradiography, and densitometry, the relative concentration of soluble cleavage products from the P1-3C construct were determined when P1-3C was co-expressed with the native 3C or the 3C mutants as listed on the x-axis of the figure. (n = 1, error bars = 20% based on accuracy of densitometry)

Although the use of the 3C mutants significantly improved the soluble yield of the P1-3C proteolized products, we had yet to observe the formation of a VLP with our improved system. This negative result is not entirely surprising considering FMDV assembles in mammalian cells and we are using the an *E. coli*-based system. However, at this point in our experimentation we were alerted to the first reported synthesis of FMDV VLP using an *E. coli*-based *in vivo* system (*Guo HC, Sun SQ, Jin Y, Yang SL, Wei YQ, Sun DH, Yin SH, Ma JW, Liu ZX, Guo JH, Luo JX, Yin H, Liu XT, Liu DX. 2013. Foot-and-mouth disease virus-like particles produced by a SUMO fusion protein system in Escherichia coli induce potent protective immune responses in guinea pigs, swine and cattle. Veterinary Research 44:48*). We therefore custom synthesized Small-ubiquitin-like modifier proteins (SUMOs) SMT3 fusions onto VP0, VP1, and VP3 as illustrated in Figure 8.

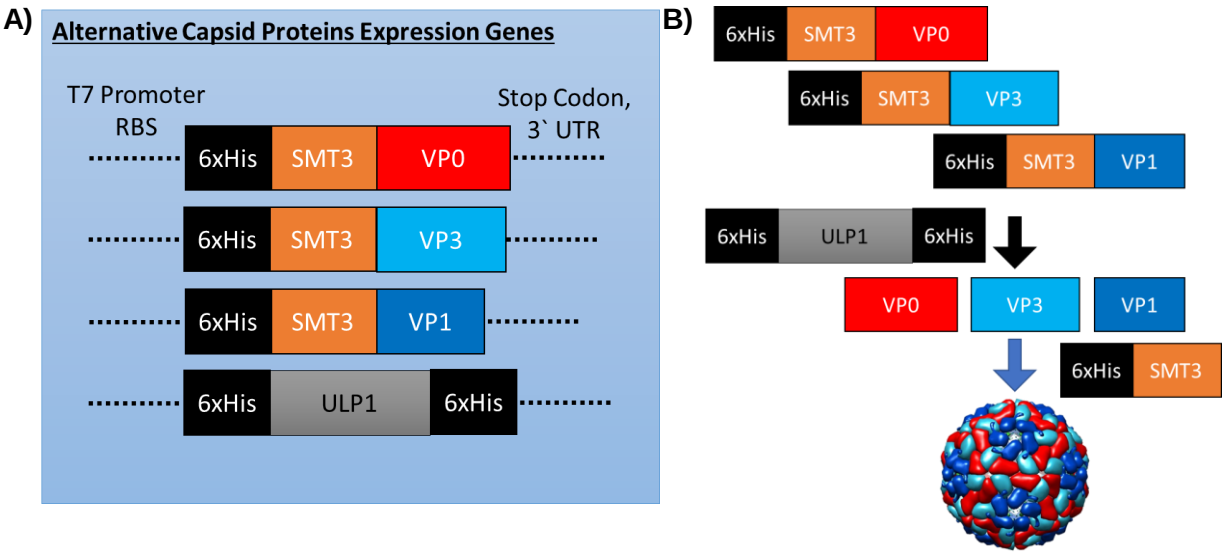

Figure 8: (A) Schematic of the custom synthesized genes each inserted into its own expression plasmid. The ULP1 is the protease that cleaves away the 6xHis-tag and SMT3 fusion from the VP0, VP3, and VP1 constructs. **(B)** Schematic of the ULP1 processing of the fusion proteins liberating VP0, VP3, and VP1 to form VLPs after purification from *E. coli*-based expression.

Although the SMT3 fusion was reported to significantly increase solubility, we found that while our constructs were expressed at very high yields in our cell-free system, the solubility was very low (ranging from 2-15%). Coexpression of the VP constructs did improve the overall solubility, but the solubility remained low (10-20%) as shown in Figure 9. The corresponding autoradiograms are found in Figure 10.

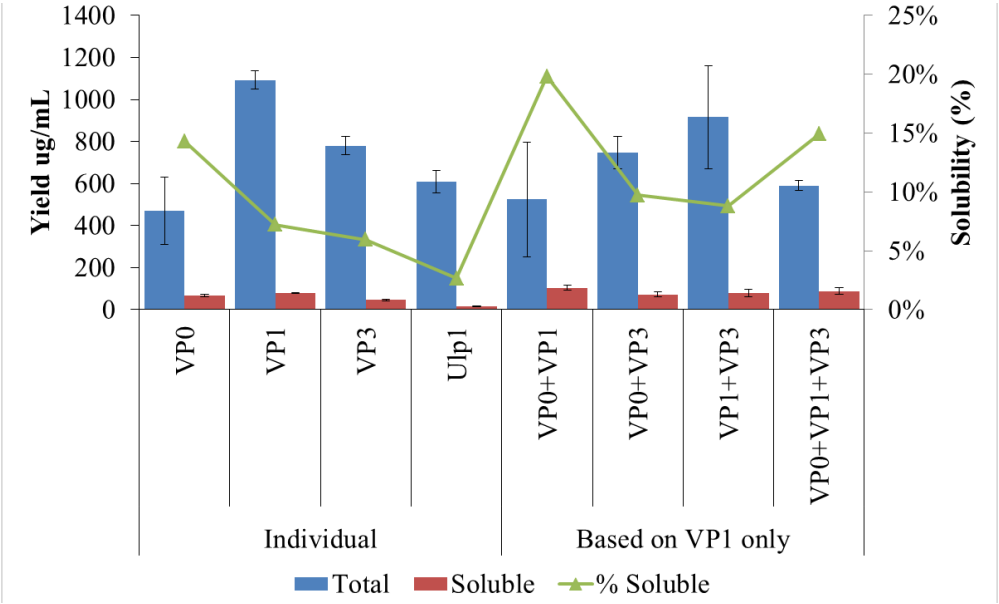

Figure 9: Total and soluble cell-free expression yields and solubilities of the constructs in Figure 8 individually expressed and coexpressed. Note that the VP0, VP1, VP3 constructs are fusions with the His-tag and SMT3 and that Ulp1 contains n- and c-terminal His-tags. (n = 3, error bars = standard deviation)

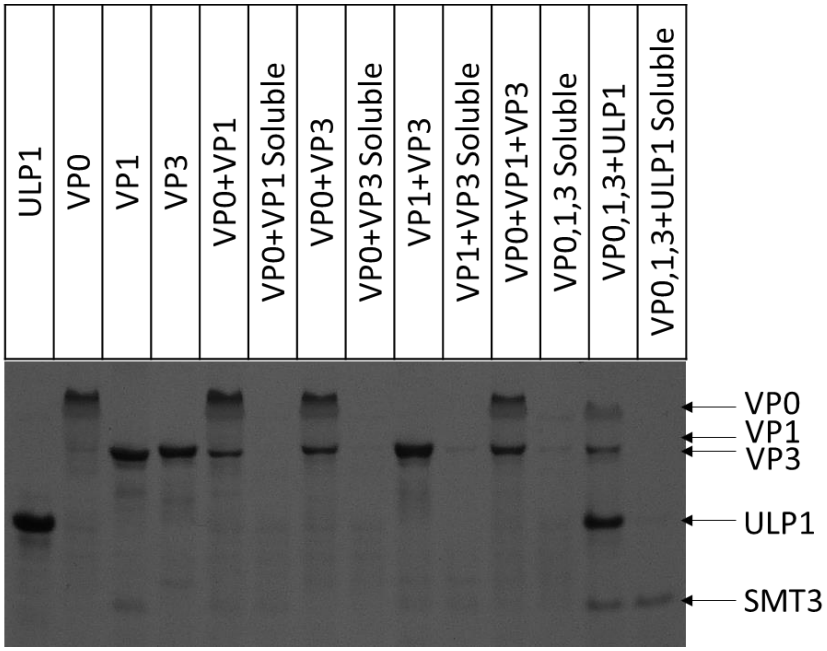

Figure 10: Autoradiogram of the electrophoresis gel containing cell-free reactions producing the constructs specified above the gel lanes. Note that the VP0, VP1, and VP3 constructs are fusions with the His-tag and SMT3 and that Ulp1 contains n- and c-terminal His-tags.

To improve solubility, cell-free reactions were performed with the *E. coli* extract containing overexpressed GroEL/ES or NMT. This resulted in some improvement in the percent of soluble product, but the overall soluble yield remained significantly lower than the total production yield (Figure 11).

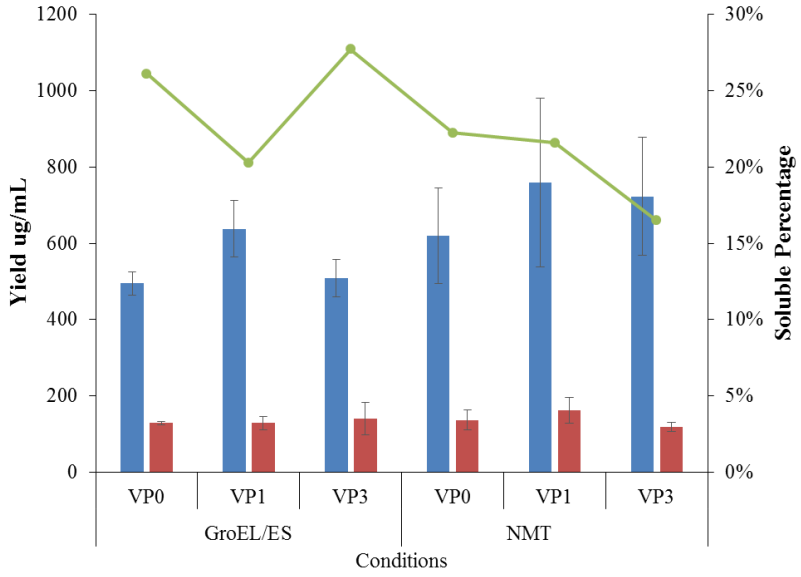

Figure 11: Total and soluble cell-free expression yields and solubilities of the constructs in Figure 8 individually expressed in reactions with GroEL/ES (left) or NMT (right) overexpressed. Note that the VP0, VP1, and VP3 constructs are fusions with the His-tag and SMT3. (n = 3, error bars = standard deviation)

We next assessed the effect of lowering the temperature of the cell-free reaction as lowering the temperature slows the translation rate giving the protein product more time to fold correctly. Lowering the temperature had a marked improvement in solubility with room temperature (25°C) resulting in significantly higher soluble product yields (Figure 12).

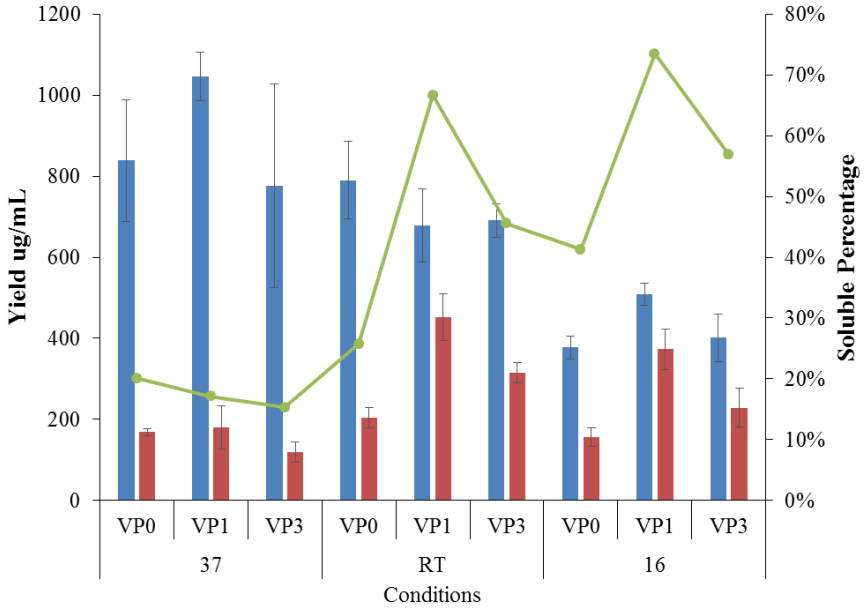

Figure 12: Total and soluble cell-free expression yields and solubilities of the constructs in Figure 8 individually expressed in reactions at 37°C, 25°C (RT), and 16°C. Note that the VP0, VP1, and VP3 constructs are fusions with the His-tag and SMT3. (n = 3, error bars = standard deviation)

Once soluble yields were optimized to respectable levels, we next assessed for VLP formation. To enable VLP assembly, we first produced SMT3-VP0, SMT3-VP1, and SMT3-VP3 fusion in separate cell-free reactions at room temperature (25°C). The equivalent volumes of each reaction were then combined and incubated for 1 hour at room temperature in the presence of Ni-affinity column purified ULP1. The combined incubated sample was then split into two equivalent aliquots, with one aliquot dialized against PBS in 6kD cut-off dialysis tubing for 24 hours and the other aliquot combined with 1 volume equivalent of 10 M urea and incubated for 1 hour at room temperature before similarly dializing against PBS for 24 hours. The soluble fraction of each of the two samples were then loaded onto sucrose gradient 10-40%, spun at 28,000 RPM in SW-32 rotor at 4°C, and fractionated with the results shown in Figure 13. The presence of a radioactive peak caused by cell-free synthesized protein in fractions 20 to 30 suggest the possibility of VLP formation. We are currently preparing the collected fractions for TEM imaging to verify VLP formation. We are also currently further manipulating the expression and incubation conditions to further improve soluble protein production and the conditions to facilitate VLP assembly. We are also currently using Ni-affinity chromatography to purify the subunits of the VLP from the cell-free reaction environment.

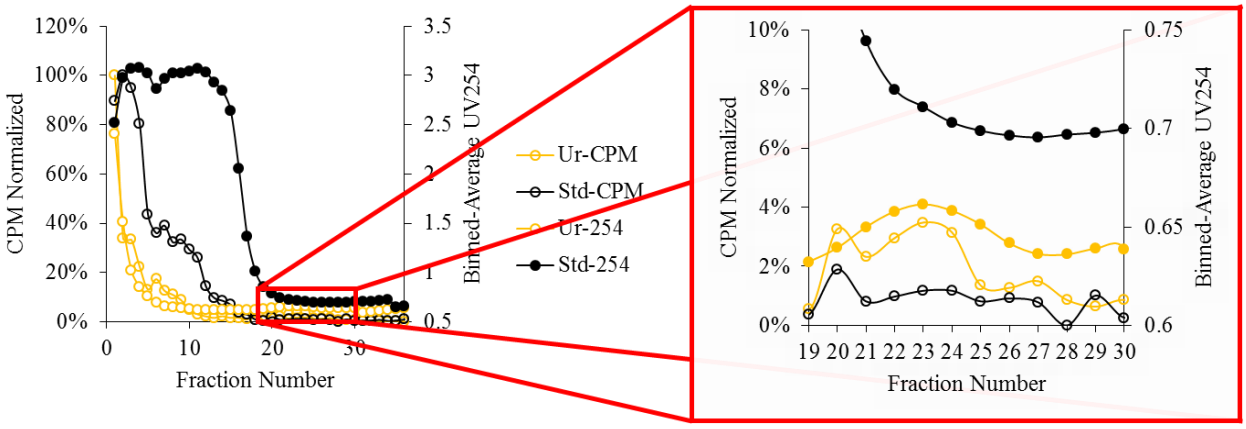

Figure 13: Assessing for VLP formation. Protein content detected from radiation (left axis in CPM) and UV 254 nm abosorbance (right axis) of C^{14}-labeled cell-free synthesized protein following ultracentrifugation and fractionation. Ultrafiltration was performed for 5hrs at 28,000 RPM in SW-32 rotor kept at 4°C using a 10-40% sucrose gradient. Prior to ultracentfugation individual cell-free reactions expressing VP0, VP1, and VP3 were combined in a 1 hour incubation with ULP1 and then subjected to or not subjected to a 1 hour incubation with an volume equivalent of 10 M urea incubation. Both samples were dialized for 24 hours against PBS with the soluble component loaded onto the sucrose gradient. The presence of a peak following in the 20 to 30 fraction range (especially in the urea treated sample) suggests the presence of VLPs.

Objective #4: *Completed*: Disseminate results and prepare proposal for follow-on funding.

We have published an archival peer-reviewed article in Vaccine in which we acknowledged the National Pork Board as requested with the following statement: "The authors would like to acknowledge that funding, wholly or in part, was provided by the National Pork Board13-104". The article reference is: *Smith MT, Bennett AM, Grubman MJ, Bundy BC. 2014. Foot and Mouth Disease: Technical and Political Challenges to Eradication. Vaccine. 32(31):3902-3908.* The preproof version of the article is available online free of charge from the author's personal website at:
http://bundy.byu.edu/publications/Bundy%20-%202014%20-%20Foot-and-mouth%20disease%20technical%20and%20political%20challenges%20to%20eradication%20-%20Preproof.pdf.

We are currently drafting an additional publication for the work performed by this research and are currently drafting a proposal for continued funding.
The following presentations have been made to the community: *Bennet AM, Smith MT, Bundy BC. Engineering Novel Vaccines for Foot-and-Mouth Disease. Emerging Ideas in Biomedical Research. Provo, UT. Oct 2014.* and *Bennett AM, Smith MT, Bundy BC. Foot-and-Mouth Disease Vaccine: Technical and Political Challenges to Vaccine-based Eradication. Utah Conference on Undergraduate Education. Brigham Young University. Provo, UT. Feb 2014*.

Discussion:
We have custom designed and synthesized non-infectious gene constructs for the production of FMDV structural proteins with cell-free protein synthesis. Our research has focused on assessing our cell-free technology's capability to make an inexpensive FMDV vaccine which can be differentiated from infected animals (DIVA) and thus maintain the FMDV-free status of U.S. pork. We have for the first time demonstrated that ability of a cell-free system to produce FMDV structural proteins and also the ability to directly modify, control, and optimize the system to increase solubility of these proteins. We have gathered evidence of VLP assembly using our cell-free system and are currently further verifying the presence of such VLP as well as further optimizing our system for improved structural protein expression and VLP assembly. The transportability, low-cost, and scalability of the cell-free system are highly advantageous for rapid production of targeted FMDV vaccines for use in the U.S. and in neighboring locations. Overall we are excited about the technology's potential and are grateful to the National Pork Board for initiating our continued research efforts on FMDV vaccine design and production.

Reference:
1. Paarlberg, P.L., J.G. Lee, and A.H. Seitzinger, *Potential revenue impact of an outbreak of foot-and-mouth disease in the United States*. J Am Vet Med Assoc, 2002. **220**:988-92.