5-5-2018

Conferring DIVA Capability to a Commercially-Available CSFV ELISA: A Double-Competition Approach

Yuxiang Wang  
*University of Connecticut - Storrs, yuxiang.wang@uconn.edu*

---

**Recommended Citation**

https://opencommons.uconn.edu/gs_theses/1195

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact opencommons@uconn.edu.
Conferring DIVA Capability to a Commercially-Available CSFV ELISA: A Double-Competition Approach

Yuxiang Wang

B.S., Sichuan University, China, 2016

A Thesis
Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science
at the
University of Connecticut
2018
Copyright by

Yuxiang Wang

2018
Conferring DIVA Capability to a Commercially-Available CSFV ELISA: A Double-Competition Approach

Presented by

Yuxiang Wang, B.S.

Major Advisor
Guillermo R. Risatti

Associate Advisor
Paulo H. Verardi

Associate Advisor
Antonio E. Garmendia

University of Connecticut

2018
ACKNOWLEDGEMENTS

I would like to extend my most sincere gratitude to Dr. Guillermo R. Risatti for all that he helped me with. His wealth of knowledge made not only my research work but also other academic activities such as poster presentation run fluently. He is also willing to give advice on my life and career. It has been a valuable and memorable experience for me to do research with him.

I am also very grateful to Dr. Paulo H. Verardi and Dr. Antonio E. Garmendia for being my associate advisors. Their support and suggestions on my research as well as dedication to course teaching made it possible to improve my knowledge and skills throughout the graduate study.

I would like to give thanks to everyone that previously worked in our laboratory: Boris Gavrilov, Kalpanie Bandara and Kara Rogers. It would be hard to imagine how my project could proceed without their explorations on Sf9 cell culture, infection, protein extraction and all analyses. I also want to thank Lu Li in Dr. Xiaohui Zhou laboratory for her assist in western blot image developing with their new system.

I would like to thank all my friends for being around whenever I am in need. I am especially grateful to my parents who have always been supporting and encouraging my pursuit in education and decision in career, without them none of these beings would have been possible.
This Thesis Is Dedicated to My Parents
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
</tr>
<tr>
<td>Approval Page</td>
</tr>
<tr>
<td>Acknowledgements</td>
</tr>
<tr>
<td>Table of Contents</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
</tr>
<tr>
<td>Abstract</td>
</tr>
</tbody>
</table>

## Chapter 1

Introduction ................................................................. 1

1.1 CSF: Introduction .................................................. 2
1.2 Disease ................................................................. 2
1.3 Disease Control ...................................................... 3
1.4 CSFV: Introduction .................................................. 6
1.5 CSFV Pathogenesis .................................................. 7
1.6 Baculovirus/Insect Cell Protein Expression System .......... 8
1.7 Significance and Rationale ....................................... 10

## Chapter 2

Approach, Hypothesis and Specific Aims ........................................ 11

2.1 Approach to fulfilling DIVA criterion ......................... 12
2.2 Hypothesis ............................................................ 12
2.3 Specific Aims

Chapter 3

Specific Aim 1: Biosynthesis of Recombinant Viral Envelope Glycoprotein E2

3.1 Introduction

3.2 Materials and Methods

3.3 Results

3.3.1 Cell culture in Monolayer and Suspension

3.3.2 Infection with Recombinant Baculovirus

3.3.3 Protein Extraction, Purification and Identification

3.4 Discussion

Chapter 4

Specific Aim 2: Modification of A Commercially Available ELISA

4.1 Introduction

4.2 Materials and Methods

4.3 Results

4.4 Discussion

Chapter 5

Conclusion

Supplementary Data

Reference
LIST OF FIGURES AND TABLES

Page

Figures:

Figure 1 ........................................................................................................ 27
Figure 2 ........................................................................................................ 28
Figure 3 ........................................................................................................ 29
Figure 4 ........................................................................................................ 30
Figure 5 ........................................................................................................ 32
Figure 6 ........................................................................................................ 39
Figure 7 ........................................................................................................ 40

Tables:

Table 1 ........................................................................................................ 25
Table 2 ........................................................................................................ 26
ABSTRACT

Classical swine fever (CSF) is a viral infectious disease mainly affecting wild boars and captive pigs. Being highly contagious and lethal, an outbreak causes both tremendous direct economic loss and financial burden before a new round of production. The disease has been long endemic in Asia, Central and South America, Africa and some regions in Europe, while many developed countries including the US, Canada, Australia, New Zealand and several members of the European Union have taken strict measures to successfully eradicate it. Vaccines currently available can protect pigs throughout their economic life, however, countries without endemics choose to not them for the concern of stamping out policy since no sophisticated marker vaccines together with companion tests fulfilling DIVA (differentiation of infected from vaccinated animals) criterion are approved yet.

The causative agent is classical swine fever virus (CSFV), under the family Flaviviridae and genus Pestivirus. Viral particle is 40-60 nm in diameter, containing icosahedral nucleocapsid and its genome is single stranded positive-sense RNA (~12.5 kb). Lipid bilayer envelope is anchored with three viral proteins: E\textsuperscript{NS}, E1 and E2 among which E2 is the most immunogenic and induces strong antibody response in hosts.

Several approaches are currently available for CSFV diagnosis: (i) detection of CSFV, and (ii) detection of CSFV specific antibody. Identifying the existence of virus particle or necropsy is labor-intensive and not suitable for high throughput screening in the event of which ELISA-based test is preferred for detecting virus specific antibodies.
This research project is aimed at modifying a widely used commercial ELISA to serve as a companion test for a previous marker vaccine candidate with the intention of discriminating CSFV naturally infected from vaccinated pigs.

Baculovirus/insect cell protein expression system has been long practiced and proved an effective way to biosynthesize recombinant proteins. Sequences encoding foreign protein can be easily incorporated into recombinant baculoviruses using commercially available kits. In this research, a recombinant baculovirus was constructed and used to infect Sf9 cells to synthesize genetically modified viral envelope glycoprotein E2 which plays a key role in the ELISA-based test.
CHAPTER 1

INTRODUCTION
1.1 CSF: Introduction

The first record of hog cholera which is now known as classical swine fever was presumably in 1833 in Ohio, USA, one of many outbreaks during that time as claimed by Hanson (Hanson, 1957). However, the disease has been suspected to be transmitted from Europe since a similar one was documented in 1822, France (Birch 1992; cited by Cole et al., 1884). Falling into the OIE (World Organization for Animal Health) list, it is required that any infection cases be reported so that elimination of pigs and other measures can be taken to control spread. The disease can almost be considered the most economically significant one affecting pig industry, leading to tremendous loss once an outbreak is confirmed. Despite being widespread in both Europe and the US, till the 1860s, many developed countries including America, Canada, Australia, New Zealand and some members of the European Union have successfully eradicated the endemic of CSF with strict actions such as the stamping out policy. However, the disease is still a big concern in many parts of Asia, Central and South America, Africa and several countries in Europe. Therefore, risk of transmission to and causing outbreaks in nations without such endemic disease does exist since those countries do not adapt routine vaccines against CSFV.

1.2 Disease

Classical swine fever is mostly seen transmitted oronasally, while transmission via fomite or skin wound is possible. Transplacental infection was also reported (Oirschot &
Terpstra, 1977). Many factors including viral virulence, age and breed of infected swine, etc., (Depner et al., 1997; Moennig & Plagemann, 1992; Van Oirschot, 1999) contribute to severity and disease related signs which are characteristically exhibited by pyrexia, hemorrhage of the outer skin and mucosae and central nervous system disorders (Moennig, 2000). Mortality rate among young pigs is especially high, being around 90%-100% and death can occur two to three weeks after initiation of acute infection. Prenatal infection of sows may lead to abortion, stillbirth, malformations and persistently viremic piglets (Meyer, Liess, Hermanns, & Trautwein, 1980).

Density of pig population is largely related to the risk of disease transmission and a potential outbreak. Some regions adapt the intensification pattern of producing pigs to a large-scale while others prefer to raise them in small-sized farms. Despite the high concentration in mass pig production, it is relatively easier to regulate and perform quality control. Pigs in small private farms may be confronted with less risk of cross infection, but the general management and surveillance of these farms are more difficult to conduct. Infected wild boars or feral pigs can, if having contact with, transmit virus to domestic pigs both in a direct and indirect (fomite transmission) way.

1.3 Disease Control

Effective control of CSFV requires establishment of epidemiological work, e.g. investigation and tracking of each outbreak and performing virus isolate typing (Greiser-Wilke, Depner, Fritzemeier, Haas, & Moennig, 1998; Greiser-Wilke, Zimmermann,
Apart from a well-developed understanding of epidemiological situation, an accurate and efficient laboratory diagnosis is indispensable. Modern molecular approaches have been utilized for screening from two perspectives: (i) detection of virus, and (ii) detection of virus specific antibodies.

To detect the presence of viral particles, isolation of virus in cell culture is still the ‘gold standard’. Ongoing viremia is required for the isolation of CSFV from animal spleen, tonsils, lymph nodes, parotid glands and kidneys (Anonymous, 1980, 1996). Isolated virus is then grown in susceptible cell lines of porcine to be tested with CSFV specific monoclonal antibodies (Anonymous, 1980, 1996; Cay et al., 1989). Two less sensitive tests are adapted instead for the purpose of reducing labor work: in situ antigen demonstration with fluorescent antibody and ELISA targeting specific CSFV antigens. Furthermore, detection of viral RNA by RT-PCR and following sequencing have become an alternative (Greiser-Wilke et al., 1998; Hofmann, Brechtbühl, & Stäuber, 1994; Lowings, Ibata, Needham, & Paton, 1996; Paton, McGoldrick, Belak, et al., 2000).

Serological test is also favored given its rapidness and sensitivity. Virus neutralization tests are designed for CSF antibody detection in which case serum is mixed with CSF reference virus so that neutralizing reaction occurs in the presence of antibody. However, antibodies generated against ruminant pestivirus may cross-react with CSF reference virus thus a second ruminant pestivirus neutralizing test is required for differentiation. In large-scale screening, ELISA tests targeting CSFV specific antibody in serum are more accepted.
owing to its convenience. Samples tested positive or ambiguous should be processed with neutralization test again to confirm that they are not fake positive.

There have been many trails to develop CSF vaccines since 1940s. The first attempt to attenuate CSFV (Baker, 1946; Koprowski, James, & Cox, 1946) was not initially successful but eventually led to safe and efficacious vaccines. China-strain (C-strain) based attenuated vaccine is the most widely accepted and used to protect domestic pigs as well as wild boars from CSF. It is attenuated by adapting in rabbit and can still promote potent antibody response which protects pigs from virulent CSFV infection as early as five-day post vaccination. This vaccine is also highly safe and can be given to pregnant animals. However, such vaccine does not fulfill DIVA (differentiation of infected from vaccinated animals) criterion since both types of animals exhibit the same pattern of antibody response.

Therefore, a marker vaccine is needed. E2 subunit vaccine was designed aiming at only inducing antibody response against viral glycosylated envelope protein E2 in vaccinated animals, while naturally infected animals generate many more classes of antibodies including those against viral envelope protein E\textsuperscript{ms}. Therefore, detection of antibody with high affinity for E\textsuperscript{ms} indicates infection. However, subunit vaccines are less immunogenic and protection of E2 subunit vaccine is not substantial until 21 dpv. Additionally, sensitivity of ELISA test designed for detection of E\textsuperscript{ms} specific antibodies is low. Live attenuated marker vaccine is a more promising option given higher immunogenicity and potent protection. A successful practice FlagT4G vaccine (Holinka et
al., 2014) proven to be capable of inducing effective protection against CSFV challenge as early as 7 dpv. Two markers are involved in the vaccine: (i) a positive marker, Flag® epitope, inserted in E1 glycoprotein, and (ii) a mutation introduced into the WH303 epitope in E2 glycoprotein (wild type: TAVSPTTLR to mutant: TSFGMDTLR). Positive detection of antibody against Flag® epitope and simultaneous negative detection of antibody against TAVSPTTLR epitope indicates vaccination, while presence of TAVSPTTLR epitope specific antibodies is interpreted as infection.

Countries with and without endemics of CSF take rather different control policies. The former adapts a routine of vaccination which protects pigs throughout their economic lives and only pigs showing clinical signs will be eliminated, but the possibility of virus being hidden from screening cannot be ruled out. As a result, their meat product is not accepted for importing by those countries without CSF occurring. In regions where no CSF endemic exists, governments conduct a stamp-out policy for both herds with infected pigs and neighboring herds, including epidemiological investigations, clinical diagnosis, tracking and restriction for movement of live pigs, meat product and vectors with potential capability of transmitting virus, etc. (Anonymous, 1980; Edwards et al., 2000) Death caused by CSFV related symptoms actually only accounted for a small proportion, the majority of pigs were killed out of disease control purpose and such eradication led to severe economic loss.

1.4 CSFV: Introduction
Classical swine fever virus is a spherical enveloped particle of 40 to 60 nm in diameter, the icosahedral nucleocapsid of which contains 12.3 kb single stranded RNA genome with positive polarity. The virus belongs to the pestivirus genus of the Flaviviridae family (Wengler, 1991) and is closely related to other two viruses in the same genus: bovine viral diarrhea virus (BVDV) and border disease virus (BDV) of sheep. Viral infectious cDNAs have been successfully produced by some laboratories (Meyers, Thiel, & Rümenapf, 1996; Moormann, Van Gennip, Miedema, Hulst, & Van Rijn, 1996; Ruggli, Tratschin, Mittelholzer, & Hofmann, 1996). Four structure proteins: core protein C, envelope glycoproteins E\textsuperscript{envelope}, E1, E2 and seven non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B are encoded in the viral genome (Beer, Reimann, Hoffmann, & Depner, 2007; Elbers et al., 1996; Meyers & Thiel, 1996). Viral glycoprotein E2 is the main virulence determinant and is responsible for attachment and invasion to host cells. Being highly immunogenic, E2 is the main target of neutralizing antibody and cytotoxic T cell response. Stable existence of virus usually occurs in humid animal excretion and fresh meat product (Savi, Torlone, & Titoli, 1965), but inactivation of virus can be easily achieved by damaging viral envelope and nucleocapsid with heat, detergent, lipid solvent, protease and disinfectants (Edwards et al., 2000).

1.5 CSFV Pathogenesis

Classical swine fever virus preferentially infects endothelial cells, some epithelial cells, lymphoreticular cells and macrophages (Moennig, 2000). Gestation period infection
causes abnormal tissue and organ differentiation and maturation in fetus. Postnatally infected pigs may develop thrombosis, endothelial damage due to the invasion of virus into such host cell types. These lesions are usually accompanied with hemorrhage or petechial bleeding. Pulmonary as well as bronchial epithelial cells infected by CSFV may lose normal physiological function to a degree where bronchopneumonia occurs. Central nervous system disorders are presumably resulted by infection of cerebrovascular endothelial cells and thrombosis given severe vasculitis concomitantly observed with encephalitis. Depletion of B cells in peripheral blood and lymphoid tissues was reported in the very late phase of acute infection (Susa, König, Saalmüller, Reddehase, & Thiel, 1992). Antibody response against CSFV may protect pigs up to years or even throughout entire life (Van Oirschot, 1999). Neutralizing antibody can be detected as early as 11-day post infection and remain high level up to 100 dpi with data support. In the case of chronic infection, neutralizing antibody may only be detectable in the few days after 30 dpi (Cheville & Mengeling, 1969; Depner, Rodriguez, Pohlenz, & Liess, 1996). Prenatally infected pigs which develop persistent viremia hardly generate CSFV specific antibodies (Van Oirschot, 1999). Postnatal piglets are protected by passive immunity including maternal antibodies of which the half-life is about two weeks, however, virus is still capable of replicating and shedding under such circumstance. T cell immunity in defense of CSFV is comparatively less elucidated.

We realized that the closely related virus BVDV is less virulent than CSFV and when their E2 protein coding sequences were aligned, significant difference was only observed
within the WH303 epitope. Because our previous few attempts to attenuate the CSFV by arbitrarily introducing mutations into the WH303 epitope ended up being lethal to the virus, we then only replaced the amino acid within the WH303 epitope of CSFV E2 protein with that of BVDV correspondingly. Starting from the first amino acid alanine, when all five were mutated (AVSPT to SFGMD), the virus was fully attenuated but it could still elicit strong and protective immune response in pigs. This phenomenon indicates the WH303 epitope plays an important role in viral reproduction as well as pathogenesis.

1.6 Baculovirus/Insect Cell Protein Expression System

Many insect cell lines have been exploited for expression of heterologous proteins including *Bombyx mori*, *Mamestra brassicae*, *Spodoptera frugiperda*, *Trichoplusia ni*, and *Drosophila melanogaster* among which *Spodoptera frugiperda* SF9 cell might be the most commonly chosen one. The ability to undergo posttranslational modification e.g., glycosylation, grants insect cells superiority to prokaryotic cell in many recombinant protein expressions especially those required to be similar to mammalian proteins. Additionally, the convenience, effectiveness and safety of this approach and the fact that numerous protein products on market are manufactured in this way may strongly support the advantages of insect cell protein expression system.

Recombinant baculovirus serves as the vector carrying the foreign genes and *Autographa californica* multiple nuclear polyhedrosis virus particularly infects SF9 cells
The genome of baculovirus is double-stranded, circular, supercoiled DNA. The life cycle of wild-type baculovirus includes two forms, the ODV (occlusion derived virus) and BD (budded virus), however, the recombinant baculovirus loses the polyhedrin gene which is replaced by a foreign gene of interest. Three typical phases: early, late and very late phase are involved in wild-type virus infection cycle. In early phase (0-6 hours), virus attaches to, invades host cell, initiates early viral gene expression and interferes with host gene expression. During late phase (6-24 hours), viral DNA is replicated and genes responsible for assembly are expressed to form and release budded viral particles. Very late phase (24-96 hours) is marked by formation of ODVs and cell lysis. Recombinant baculovirus does synthesize polyhedrin coat of ODV, but foreign gene is expressed instead. Cytopathic effects are less noticeable which are merely reflected by, if grown in T-75 flask, increased cell diameter and enlarged nuclei in the early phase, cessation of cell growth and dislodgment in late phase as well as cell lysis in very late phase.

1.7 Significance and Rationale

ELISA targeting CSF specific antibody has been widely used as an approach for CSF disease screening due to its convenience and high through-put. In particular, countries without endemics of CSF including the US adapt a non-vaccine policy in the event of which detection of CSFV specific antibody in serum means elimination of these animals has to be implemented, leading to tremendous economic loss. However, a marker vaccine
and its companion test may exempt healthy vaccinated animals from elimination. In order to promote the potential wide acceptance and application of a previous CSF live attenuated marker vaccine candidate, a companion serological test fulfilling DIVA criterion is urgently needed.

The double-antigenic live attenuated CSF FlagT4G virus (FlagT4Gv) (Holinka et al., 2014) contains two modifications critical for DIVA purpose one of which is the mutation in WH303 (mAbWH303) epitope (TAVSPTTLR to TSFGMDTLR) in E2 glycoprotein. Due to the absence of antibody binding WH303 epitope in vaccinated pigs, CSFV naturally infected pigs can be discriminated serologically by detecting the presence of such antibody. Thus, we hypothesize to modify a commercially available ELISA (IDEXX CSFV Ab Test) to produce a double-competition assay to fulfill this mechanism and differentiate infection from vaccination.
CHAPTER 2

APPROACH, HYPOTHESIS AND SPECIFIC AIMS
2.1 Approach to fulfilling DIVA criterion

Here we propose to discriminate CSFV naturally infected from vaccinated pigs using a double-competition ELISA by detecting a subclass of antibodies which is only present in sera of infected pigs. Wild type CSFV envelope glycoprotein E2 contains a typical epitope TAVSPTTLR (amino acid sequence), the immunogenicity of which is rather high that majority of CSFV specific antibodies generated by infected pigs is against this epitope. However, a previously developed marker vaccine candidate has a mutation that epitope TAVSPTTLR is replaced by TSFGMDTLR, leading to vaccinated animals lacking of antibodies against TAVSPTTLR epitope. By incubating sera with a recombinant peptide termed E2T4G that is identical to the wild type E2 protein except for the absence of WH303 epitope, antibodies generated by infected pigs remain dissociative thus being detected by the IDEXX ELISA.

2.2 Hypothesis

The failure to distinguish between naturally infected and FT4G vaccinated pigs using the commercially-available IDEXX ELISA is due to the inability of conjugates to bind in the presence of irrelevant antibodies against non-WH303 epitopes in E2 protein.

Recombinant protein E2T4G can sequester irrelevant antibodies and enrich anti-303 antibodies, thus making the difference distinguishable.

2.3 Specific aims

1. Biosynthesize genetically modified viral envelope glycoprotein E2 (E2T4G) with
baculovirus/insect cell protein expression system.

2. Interfere commercial ELISA with E2T4G to enrich antibodies that are merely present in sera from naturally infected pigs.
CHAPTER 3

SPECIFIC AIM 1:

BIOSYNTHESIS OF RECOMBINANT

VIRAL ENVELOPE GLYCOPROTEIN E2
3.1 Introduction

Baculovirus/insect cell protein expression system has been long practiced to successfully produce recombinant proteins. In this research, recombinant baculovirus encoding genetically modified viral envelope glycoprotein E2 (E2T4G) was constructed, amplified, titrated and stocked at 4 °C. Sf9 cells were grown firstly in a monolayer then suspension culture was adapted for the purpose of reducing consumption, labor work, contamination risk and increasing cell viability. The recombinant protein E2T4G is not secretory but loses the transmembrane domain so that extraction is simply by adding lysis buffer and high-speed centrifugation. The loss of such transmembrane domain will also lead to a decreased molecular weight compared to the approximate 55 kDa of the wild type viral E2 protein. Anti-his-tag Western blot was performed to identify the presence of E2T4G in clarified cell lysate followed by cobalt column purification, desalting, BCA assay and on-chip electrophoresis. Protocols and conditions had been explored to achieve high protein expression and to reduce unnecessary loss during extraction and purification.

3.2 Materials and methods

Cell culture Sf9 cells were purchased from Invitrogen® and preserved at -80°C at a concentration of 1*10^6 cell/ml. Gibco Grace’s Medium (1X) was supplemented with 20% heat inactivated FBS (RMBI). Gentamicin (SIGMA) was added to a final concentration of 0.05 mg/ml. To initiate an adherent culture, Sf9 cells were thawed at room temperature and mixed with 10 ml of growth media before centrifugation for 5 min at 1000 rpm.
Cellular pellet was suspended with 6 ml of media and transferred to a T-25 flask, growing at 27°C. 24 hours after inoculation, media was replaced with fresh ones to remove any remaining DMSO. Cells were scraped down and transferred to a T-75 flask when confluency reached 100% and volume of medium used in a T-75 was 15 ml. To start a culture in suspension, Sf9 cells were taken from a T-75 flask with 100% confluency and added to a 250-ml conical glass flask to reach a concentration of 1*10^6 cell/ml in a total volume of 30 ml. 300 ul pluronic® (GIBCO, 10%) was added to protect cells from shear force at the shake speed of 125 rpm. Growth state was monitored daily by counting using hemocytometer and trypan blue staining. Culture was expanded to a 500-ml conical flask under the circumstance that viability was no lower than 90% and concentration reached 2*10^6 cell/ml.

**Baculovirus construction**  
Recombinant baculovirus was constructed on the basis of site-specific recombination with the Gateway® Technology of Invitrogen. DNA sequence of recombinant viral envelope glycoprotein E2 (E2T4G) was synthesized into a bacmid containing two att recombinant sites by GENEWIZ. Recombination was performed following manufacturer’s protocol (BaculoDirect™ Baculovirus Expression System, Invitrogen). Virus passage III was titrated and stocked at 4 and -80°C.

**Infection and harvest**  
Sf9 cells in monolayer were infected with a MOI of 0.1~5 when confluency reached 90% and were scraped down at 7 dpi when cell viability
decreased to approximately 80%. Infection in suspension was initiated when cell concentration was over $2 \times 10^6$ cell/ml and was terminated when cell viability dropped below 80%. Cell suspension was centrifuged at 3200 rpm for 5 min. Supernatant containing virus was collected for future infection and cellular pellet was washed twice with PBS (1X, GIBCO) before 10 ul of RIPA cell lysis buffer (Thermo) was added per $1 \times 10^6$ cells. After 10 min on-ice incubation, 10 min centrifugation at 13200 rpm was performed to collect clarified cell lysate, stored at -20°C.

**Qualitative and quantitative test**  Western blot was performed to detect the presence of recombinant protein in both whole cell lysate and clarified cell lysate. Standard molecular weight marker, Bis-Tris gel, MES SDS running buffer, transfer buffer, PVDF membrane, anti-his primary antibody (C-term) as well as chemiluminescent immunodetection kit were purchased from Invitrogen. Image developing was done with BIO-RAD ChemiDoc™ Imaging System with exposure time starting at 30s to as long as 300s. Clarified cell lysate samples showing high expression were purified and desalted following manufacture’s protocol with HisPur™ Cobalt Purification Kit (Thermo) and Zeba™ Spin Desalting Column (Thermo). Western blot was also performed with purified and desalted samples. Concentration of total proteins was measured by Pierce BCA Protein Assay (Thermo) and the percentage of E2T4G was determined by Agilent High Sensitivity Protein 250 Kit (Agilent Technologies) thus the exact concentration of E2T4G could be calculated.
3.3 Results

3.3.1 Cell culture in Monolayer and Suspension

Unlike most mammalian cells, Sf9 cells adhere weakly to T-25/75 flasks and get dislodged easily. It is normal that some floating cells could be observed under inverted microscope especially when flask was moved, rocked or the confluency of monolayer increased above 100%. However, too many floating cells in the event of overnight incubation after thawed and transferred to T-25 flask or confluency lower than 100%, still indicate unhealthy cell state. Cells of low passage number (< p.20) replicated faster, usually taking three days to reach 100% in a T-25 flask after seeding at a number of $1\times10^6$ and two to three days to fully grow in a T-75 flask after splitting at a ratio of 1/3. Cells of high passage number (> p.26) took longer to multiply, nearly twice of what was needed by those of low passage number. Based on practice, at 100% confluency the total number of cells in a T-75 flask fluctuated around $4.5\times10^7$ and corresponding concentration was around $3\times10^6$ cell/ml, in a volume of 15 ml of media. Counting with hemocytometer for culture in monolayer required cells to be scraped down, therefore shear force applied to cells decreased viability to approximately 93% and clumps of cells led to inaccuracy in calculation.

Culture in suspension was then adapted to replace growing with T-75 flasks due to many disadvantages of the latter. Cells grew to 100% confluency in a T-75 flask was still needed to initiate culture in a 250-ml conical glass flask at a concentration of $1\times10^6$
cell/ml in a total volume of 30 ml of grow media. Cells with low passage number multiplied quickly and took two days to reach $2 \times 10^6$ cell/ml when they need to be transferred to a 500-ml flask where an additional two days were usually necessary for growing back to $2 \times 10^6$ cell/ml. Cell viability observed was higher than that in monolayer, being around 99%-100%. A typical growth curve is provided in figure 1.

### 3.3.2 Infection with Recombinant Baculovirus

Cells growing in monolayer were infected when confluency reached 90% by directly adding virus stock or medium containing virus particle which was collected from a previous protein production with a MOI ranging from 0.1 to 5. No conspicuous cytopathic effect could be observed in the first two to three-day post infection except that nuclei might appear to be enlarged due to virus replication. The most obvious cytopathic effect is elevated dislodging of cells from monolayer compared to non-infected group, occurring as early as 3 dpi and culminating at 7 dpi when more than 80% cells floated in the medium. Harvest was done at 3/4/5/6/7/8 dpi and only cell pellets were kept. Grow media were discarded after centrifugation. Proteins were extracted by adding lysis buffer to cell pellets and both cell lysate and clarified cell lysate were tested by Western blot (WB) for the expression of recombinant. The highest expression of E2T4G was achieved at 7 dpi with cell viability around 85% and a total concentration around $2 \times 10^6$ cell/ml.

Infection in suspension was done with the same MOI by directly adding virus stock or medium from a previous infection. After infection, cell concentration still increased
from $2 \times 10^6$ cell/ml to approximately $4 \times 10^6$ cell/ml during the first three days. Significant decrease in viability was not observed until 3 dpi when 96% were alive following by 90% at 4 dpi and 80% at 5 dpi. A large decrease in viability from 80% to 40%-60% would be observed if not harvested which would cause a tremendous loss of target protein. A typical growth curve is shown in figure 2.

For both of the two growths, non-typical growth state/curve inconsistent with the description above was also observed, reflected by higher cell density ($> 2 \times 10^6$ cell/ml) at expansion or infection time point or higher viability ($> 80\%$) at harvest day, which might be attributed to different cell passages, inaccurate cell counting and temperature fluctuation.

Comparison of different parameters between culture in monolayer and suspension is shown in Table 1. Advantages of production with growth in suspension include reduced consumption of medium, flasks, labor work and risk of contamination as well as increased cell viability.

### 3.3.3 Protein Extraction, Purification and Identification

Western blot was performed according to manufacturer’s protocol. Bands of molecular weight marker and target protein were recognizable as early as 30-second exposure with ChemiDoc™ Imaging System (BIO-RAD). Band of target protein E2T4G lies around 50 kDa instead of 55 kDa as the case of wild type E2 protein. Such reduction in size might be attributed to the deletion of transmembrane domain. Different from
clarified cell lysate, whole cell lysate exhibited an additional band around 40 kDa. These peptides also had his-tag but were of different molecular weight, indicating more than one version of E2T4G was synthesized which could be variously glycosylated proteins or other non-specific proteins. Some E2T4G appeared to lost during centrifugation too, leading to a slightly weaker band corresponding to that of whole cell lysate (figure 3A).

It was observed that whole cell lysate was much more viscous than clarified cell lysate, as a result of which the former was extremely difficult to load into the gel well for WB and band shown after image developing, though intensity being higher, was not as sharp compared to clarified lysate. An explanation is that high concentration of protein contributes to the viscosity of sample.

Purified samples lost considerable proportion of target protein, reflected by much weaker band in WB image. Proteins were eluted from purification column three times with elution 2 usually having the strongest band. Desalting was performed to further increase purity but a conspicuous loss of protein was unavoidable (data not shown).

BCA assay was done to measure the concentration of total proteins in purified and desalted products. Elution 1 of purified sample shown the highest concentration, with elution 2, 3 and desalted product showing a gradual decrease. Data are provided in table 2. Given the fact that non-specific proteins were synthesized and they might all contain a ‘his-tag’, in order to determine the comparatively accurate percentage of E2T4G, protein on-chip electrophoresis was performed. Proportion of each protein component was readable with bio-analyzer. By comparing to a highly purified positive control which was
a different recombinant viral E2 produced using the same expression system is our lab previously. Considering both the concentration of total proteins in the solution and the percent of E2T4G, its actual concentration could be calculated (table 2). Schematic picture of E2T4G is shown in figure 5.

A typical result of on-chip protein electrophoresis is shown in figure 4. Molecular weight of a band cannot be simply determined based on ladder because the ladder is computer generated and only represents the migration speed. Instead, by comparing to the positive control, if similar pattern was observed, the sample would be considered of good quality. Each peak represents one protein component and height of the peak indicates the relative amount, which means samples exhibiting few and high peaks and were purer. Elution 1, 2 and 3 all contained many different kinds of non-specific proteins and the proportion believed to be E2T4G is respectively 15%, 21.6% and 20% (table 2, figure 4). Desalting process further excluded non-specific proteins but recovery was low, therefore desalted elution 3 had higher purity (figure 4) but almost 60% of protein was lost (table 2).

3.4 Discussion

Previous research and manufacturer’s protocol validate the feasibility of adapting suspension culture for Sf9 cell growth. The exploration on growth conditions took months and ultimately exhibited conspicuous superiority to monolayer culture (table 1). A typical round of production using adherent culture required thirty T-75 flasks but actually
another fourteen flasks were needed to finally generate thirty. Considering 15 ml growth media was added to each flask, an entire volume of 660 ml media was needed. Therefore, culture in suspension is much more efficient and largely reduces labor work, consumptions and risk of contamination. In addition, without shear force applied to cells when they were scraped down, those growing in suspension exhibited much higher viability, nearly 100%, which was desired for protein expression. Some disadvantages of suspension culture pointed out by previous research include forming of clumps and potential risk of contamination due to growth in conical flask. However, clumps of cell were hardly observed and contamination never occurred in my practice.

Infection was done at a low MOI, 0.1-5 as recommended by manufacture. In this research, media containing viral particles was collected and saved for next round of infection. If good protein expression was gained from such infection with this virus stock, it would also be used for following several rounds of production. Viral titration was not performed for these virus stocks due to unsuccessful attempts, mostly unsuccessful staining using neutral red. However, manufacturer’s protocol mentions that such virus stock typically has a pfu of 5*10⁷ to 1*10⁸ per milliliter. Given the number of cells (2*10⁸) and volume of virus (500 ul) added in suspension when infection was initiated, the MOI I actually ended up using should be around 0.125-0.25 which fell into the recommended range. Moreover, the growth curve was consistent with this low MOI, reflected by a slight increasing in density and prolonged high viability within three to four days after infection.
Though designed to lose the transmembrane domain, the recombinant E2T4G was not easily extracted and recovered from cells. Cell lysis buffer was used in our research to break Sf9 cells and release protein of interest in which case it should be dissociative. However, shown by Western blot, samples lost large proportion of E2T4G after centrifugation, indicating much was associated with cell debris. Incubation time with lysis buffer was increased as well as repetitive freezing and thawing was adapted for the purpose of breaking potential linkage between E2T4G with cell debris, but no substantial improvement was observed. Cellular pellet was washed with ice cold PBS and centrifugation after adding lysis buffer was performed at 4 °C laboratory with the intention of protecting E2T4G from degradation. Still, no apparent increase in protein expression was achieved.

Given that recombinant E2T4G undergoes glycosylation, more than one version of proteins were probably synthesized. Based on western blot, whole cell lysate shown an additional band of slightly lower molecular weight which disappeared in clarified cell lysate. Bio-analyzer shown that purified product elution 3 lost many non-specific proteins after desalting. Both the band disappeared in clarified cell lysate and peaks lost in desalted elution 3 might be E2T4G with different degrees of glycosylation.
Table 1. Comparison of Culture in Monolayer and in Suspension.

<table>
<thead>
<tr>
<th>CATEGORIES</th>
<th>MONOLAYER</th>
<th>SUSPENSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of T-25 flask</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of T-75 flask</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>Volume of media</td>
<td>660</td>
<td>300</td>
</tr>
<tr>
<td>Number of cells (harvested)</td>
<td>$8 \times 10^8$</td>
<td>$10 \times 10^8$</td>
</tr>
<tr>
<td>Cell viability (prior to infection)</td>
<td>95%</td>
<td>100%</td>
</tr>
</tbody>
</table>

References: (1) The number of cells that can be produced in each batch of production in suspension is approximately equivalent to 1/3 of that in T-75 flasks. (2) Numbers were selected from samples with typical growth state/curve. Exceptions were not included (data not shown).
Table 2. Quantitation of Purified and Desalted Sample.

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>ELUTION 1</th>
<th>ELUTION 2</th>
<th>ELUTION 3</th>
<th>DESALTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of total protein</td>
<td>270</td>
<td>244</td>
<td>97</td>
<td>37</td>
</tr>
<tr>
<td>(ng/ul)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Percentage of E2T4G</td>
<td>15</td>
<td>21.6</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>Concentration of E2T4G</td>
<td>40.5</td>
<td>52.7</td>
<td>19.4</td>
<td>10</td>
</tr>
<tr>
<td>(ng/ul)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References: (1) Elution 3 was desalted and recovery rate was significantly lower than expected, though purity increased. No desalting treatment was done for elution 1 or 2.
Figure 1. Growth curve of Sf9 cell in suspension (250-ml conical flask). (A)

Figure 2. Growth curve of Sf9 cell in suspension after transferred to a 500-ml conical flask and infection. Infection was initiated at 72-hour after transferred by directly adding virus stock or medium containing virus collected from a previous round of production.

Figure 3. Western blot of whole, clarified cell lysate and purification product. Exposure time was set to be 300 seconds. (A) Protein was extracted from cells growing in suspension. Lane 2 shows whole cell lysate, the larger band being approximately 50 kDa and the other being slightly below 40 kDa. Lane 1 shows the corresponding clarified cell lysate, with the same molecular weight as the larger band in lane 2. (B) Clarified cell lysate from another round of production with adherent culture was purified. Lane 1 to lane 3 represents elution 1 to elution 3 of purified product respectively, with the same weight around 50 kDa.
Figure 4. On-chip protein electrophoresis revealed a variety of proteins in purified and desalted products. (A) Gel-like picture. Ladder does not show molecular weight but exhibits the migration speed. Duplicates were made for each sample. E1: elution 1, E2: elution 2, E3: elution 3, P: positive control and D: desalted elution 3. (B) Purified product elution 1 still contained many non-specific proteins reflected as multiple peaks. The peak considered to be E2T4G is pointed out with an arrow. (C) Elution 2 contained a slightly higher percentage of E2T4G reflected by a higher peak. (D) In elution 3, relatively fewer non-specific proteins were present. The proportion of E2T4G was similar to elution 2. (E) Desalted elution 3 contained conspicuously fewer non-specific proteins as well as a much
higher percentage of E2T4G. (F) Positive control used here is a different kind of recombinant viral E2 protein produced in our lab previously using the same expression system. The purity was apparently higher than any of other E2T4G samples. (G) A typical running pattern of ladder with seven peaks, indicating successful on-chip electrophoresis.
Figure 5. Comparison of E2 and E2T4G. The only difference in recombinant viral glycosylated envelope protein E2 from wild type one is the mutation in WH303 epitope, TAVSPTTTLR to TSFGMDTLR (amino acid sequence). Polyclonal antibodies generated by infected pigs contain those with high affinity to TAVSPTTTLR epitope but in vaccinated pigs such antibody is missing. Antibodies against other epitopes should be the same in both naturally infected and vaccinated pigs.
CHAPTER 4

SPECIFIC AIM 2:

MODIFICATION OF A COMMERCIALy AVAILABLE ELISA
4.1 Introduction

ELISA detecting the presence of antibody in serum against CSFV envelope glycosylated protein E2 has been widely used for high-throughput screenings within herds due to its convenience, high sensitivity and specificity. IDEXX CSFV Ab Test is a commercially available ELISA kit designed for accurate detection of CSFV specific antibodies in serum. It is widely accepted and more importantly it is used by the USDA for screening and surveillance. CSFV glycosylated envelope protein E2 is coated on the plate by manufacture and serum will be incubated in the well either for two hours or overnight. Positive serum control comes with the kit and will be treated in the same way. Enzyme linked monoclonal antibody (anti-E2) can be added to the well after washing away sera. Polyclonal antibody in pig sera against E2 binding to E2 in the plate will block secondary monoclonal antibody from binding, thus exhibiting an inhibition rate. Samples giving inhibition rate higher than 40% will be considered as infection. In this research, recombinant E2T4G was co-incubated with pig sera in the plate with the intention of sequestering irrelevant antibodies (anti-E2 but not anti-303) and enriching anti-303 antibodies. In this setting, inhibition rates higher than 40% would still be considered as infection but lower ones would be regarded as vaccination. Schematic pictures are shown in figure 6. However, we had another expectation that in the presence of E2T4G, the inhibition rate of serum samples from a FT4G vaccinated animal would still be higher than 40% but significantly lower than in the absence of E2T4G.
4.2 Materials and Methods

_Vaccination and challenge_ Sera tested in this research were stocked at -80°C freezer. Animals were previously allocated into three groups with 4 pigs in each. Group 1 and 2 were inoculated with $10^5$ TCID50 FlagT4v (Holinka et al., 2009) intranasally (IN), and group 3 was made a mock of infection. Pigs in group 1 and group 2 were challenged IN with $10^5$ TCID50 CSFV Brescia strain (BICv) (Risatti, Borca, et al., 2005) at 3 DPI and 28 DPI respectively, along with animals in group 3. Daily clinical signs including body temperature were monitored throughout the experiment as previously described (Risatti, Holinka, et al., 2005). Peripheral blood was collected at different time points post-vaccination and post-challenge from the anterior vena cava in EDTA-containing tubes (Vacutainer). Samples were preserved at -80°C freezer for subsequent use. All experiments were performed in Plum Island Animal Disease Center (NY).

_ELISA_ IDEXX CSFV Ab Test Kit was used for the detection of CSFV E2 specific antibody in animal sera. Test was done as recommended by manufacturer’s instructions. 25 ul of the sample diluent and 25 ul of E2T4G were added to all wells first, then 50 ul positive or negative control or serum samples were added respectively. Plate was incubated for 2 hours. Each well was added with 100 ul conjugated after rinse using 300 ul of wash solution. Incubation of 30 min was required before addition of substrate. Reaction was allowed to develop for 10 min then absorbance of the samples and controls was measured at 450 nm with Bio Tek ELx 808 ELISA Reader. Negative control mean =
(NC \( A_{450} \) + NC2 \( A_{450} \))/2, positive control mean = (PC \( A_{450} \) + PC \( A_{450} \))/2, blocking percentage (rate of inhibition) = (NCmean \( A_{450} \) – Sample \( A_{450} \))/NCmean \( A_{450} \)*100. Sample with a calculation of blocking percentage \( \geq 40\% \) was considered positive while \( \leq 30\% \) was considered negative. All tests were performed in Plum Island Animal Disease Center (NY).

4.3 Results

4.3.1 \textit{E2T4G decreased inhibition in the case of vaccination instead of infection.}

Desalted elution 3 containing E2T4G was co-incubated with sera in ELISA plate. Wash steps and incubation of enzyme-linked secondary antibody were performed according to manufacturer’s protocol. Readings of ELISA reader was calculated for percentage of inhibition (figure 6) and column chart is shown in figure 7. Sera from four vaccinated (FlagT4v) pigs at 28 DPI revealed an inhibition rate ranging from 45% to 62%, while in the addition of E2T4G a 10% decrease was observed and one pig (No. 10) exhibited inhibition lower than 40%. Sera collected at 42 DPI from six vaccinated pigs had an obviously higher inhibition ranging from 68% to 78% mostly attributed to further developed antibody response. Co-incubation of E2T4G led to an average decrease in inhibition of 15% but one pig (No. 16) shown no more than 5% decrease. The lowest inhibition rate was 52% (pig No. 13). After challenging with CSFV Brescia strain, pigs generated antibodies against WH303 epitope which was enriched in the presence of E2T4G and led to a high inhibition rate. Consistent to this, sera taken from four pigs at
17-day post-challenge all exhibited high inhibition ranging from 72% to 88% regardless of addition of E2T4G. Our observation is actually consistent with our second expectation which is that in the presence of E2T4G, serum samples would have a lower inhibition rate than in the absence of E2T4G but such rate would still be higher than 40%. Because the serum samples taken from infected animals exhibited a very similar inhibition rate irrespective of the addition of E2T4G, while serum samples form vaccinated pigs showed clearly different inhibition rates depending on E2T4G, we conclude that adding the recombinant viral E2 protein E2T4G can confer DIVA capability to the commercial IDEXX ELISA Kit.

4.4 Discussion

The modification we made to the commercially available IDEXX ELISA exhibited capability of differentiating CSFV naturally infected pigs from vaccinated ones. However, the inhibition rate of infected and vaccinated cohorts did not significantly differ and compared to manufacture’s cutoff line 40%, the 10%-15% decrease in inhibition did not substantially distinguish between positive and negative samples. We attribute the unexpected low percentage of inhibition to suboptimal ELISA protocol as well as insufficient amount and purity of recombinant glycoprotein E2T4G.

ELISA is now a widely used tool for the purpose of sensitive, specific and convenient detection which, however, requires much experience and well controlled experiment conditions. We attempted to increase inhibition rate by pre-incubating serum
with recombinant protein or using different combinations of diluted serum and E2T4G, but no significant improvement was observed (data not shown). Nevertheless, there are more conditions remaining to be adjusted including temperature, incubation time, volume of each reagent and serum or E2T4G of higher or lower dilution rate. Additionally, given the relative low concentration of recombinant E2T4G in the reaction, it will not be surprising if the anti-WH303 antibodies cannot be thoroughly adsorbed and precipitated.

Despite the insignificant difference in inhibition percentage, our exploration on making the commercial ELISA fulfill DIVA criterion is successful and our strategy is promising for the development of such DIVA ELISA. With this being said, more work will have to focus on both optimizing ELISA protocol and increasing concentration and purity of recombinant E2T4G. In order to obtain high amount of purified E2T4G, efforts can be made on redesigning protein coding sequence, improving cell culture methodology, adjusting protein extraction and purification process and utilizing other protein biosynthesis systems.
Figure 6. Schematic picture of traditional and modified ELISA. (A) Polyclonal antibodies in serum samples from an infected pig, including those with high affinity for TAVSPTTLR epitope, bind to E2 at the bottom, exhibiting a high inhibition rate. (B) Polyclonal antibodies in serum samples from a vaccinated pig, those against TAVSPTTLR epitope not included, can also bind to E2, exhibiting high inhibition. (C) In the addition of E2T4G, in serum samples from an infected pig, only antibodies against TAVSPTTLR epitope can block E2. (D) Addition of E2T4G sequesters antibodies in serum samples from a vaccinated pig, thus showing a low/no inhibition rate.
Figure 7. Addition of E2T4G decreased percent of inhibition in the case of vaccination instead of infection in preliminary trails. Control group: ELISA run without addition of E2T4G, test group: ELISA run in the presence of E2T4G. (A) Sera were taken from four
vaccinated pigs at 28 DPI. Addition of E2T4G decreased inhibition by 12%, 7%, 13% and 8% respectively. (B) Sera were collected from another six vaccinated pigs at 42 DPI. Addition of E2T4G decreased inhibition by 17%, 17%, 15%, 14%, 15% and 6% respectively. (C) Sera were taken from pigs at 17-day post-challenge with CSFV Brescia strain. Addition of E2T4G did not result in significant difference in inhibition, only 2%, 4%, 2% and 2% respectively.
CHAPTER 5

CONCLUSION
5.1 Conclusion

The production of recombinant CSFV glycoprotein E2 through baculovirus/insect cell protein expression system is successful. Growing and infecting Sf9 insect cells in suspension is proved in my trial to be more effective, material-saving and significantly less labor-intensive compared to adherent culture. The recombinant protein E2T4G was obtained and quantitated after protein extraction and purification.

The modification on commercially available ELISA, IDEXX CSFV Ab Test, meet our expectation as the difference in inhibition rate between infected and vaccinated pigs was clear and distinct.

In conclusion, the specific aim 1 of this research is successfully achieved and the result of specific aim 2 potently supports the described strategy for the future development of a CSFV DIVA ELISA for the FT4G vaccine candidate.

More work remains to be done including achieving higher concentration and purity of the recombinant protein E2T4G as well as improving the ELISA protocol. In order to produce E2T4G of better quality, codon optimization, different culture conditions, different extraction and purification procedures can be tried. In terms of improving ELISA protocol, more conditions can be explored: the order of reagents added to the ELISA plate, pre-incubation of E2T4G with serum samples, different incubation time of E2T4G with serum samples, different concentrations of E2T4G, different dilutions of serum samples, serum samples collected at different dpi.
SUPPLEMENTARY DATA
Supplement 1. Nucleotide sequence of recombinant CSFV glycoprotein E2.

Mellitin-E2-T4G-deltaTMR-6XHis:

```
gagatcc\1ATGAAATTCATTAGTCAACGTTGCCCCTTTTTATGGCTGATACATTTCTCATTGACCATGCGCG
C\2CTAGCCTGCAAGGAAGATCACAGGTACGCTATATCAACAACCAATTTGCAACTGGATAGGGCTACTTTGGGGCCGAAG
GTCTCACTACACCTGGAAAGAATAACAACCAATTGCAACTGGATAGGGCTACTTTGGGGCCGAAG
ATGGCAGGTCTCCTTTTAAAGTCACTACATCTAATGTGGGTAGGATTAGTCTATCTGACATATTGACATAAGGAA
CGCTTTTACACCACCGCCGTACATTCGAGCTCTCCTGTTGACCGGGACACGCACCATTGACCGAGGAAATGGGAG
ATGACTTCCGGTGACCTGTCCGATATCTCAGAGCCTGGCTAAGGTGGAAGGTGATCTGGCATCATTACATAAGGAG
TTGAATGCTGACATTCTACCTAGTCTTTGCCCATTAGGGTGGACCGGCTTATAGAGGATGCACCGATATGAGGAG
aatggatACTCTGAGA\3ACAGAATGTTGAAGAGACCTCTAGAAGAGGAAAACCCCTTCGCTAGACAGAAGGATT
GTGACCACTACAGTGGAAAATGAAGATCTATTCTACTGAAATGGGGGAATGGACATATGCTGAAA
GGTGAAACCAGTGCCTACTACGCGGGGGGCAATTCCAAATGCAGATGGTGTGGCTTCGACTTCAATGAGCC
TGACGGACTCCCACACTACCCCACATGCTGTGGAAGTTAGAGCAGTTAACAGAATAGTGGATT
CAACGGACTGTAACAGAGATGGCGGTGTTAGAAAGACTTAGGCGCCCTATGCGCATAGGCCCTAAGGAAGATCGTCTCTAG
ACTGTCAGGGTGATGACATTAGTAAAGAGACTTAGGCGCCCTATGCGCATAGGCCCTAAGGAAGATCGTCTCTAG
TGCGGGACCTGTAAGGAAAACCTCTCCTGACATTACACTGCACAAAACCTCTGAGGAACAGGTATATTATGAGC
CCAGGGACAGCTATTTCCAACATATATGCTCAAGGCGGAGTATCGATCTGTTGATCTGGATGTGAC
GACCGCCACTCAGATTACTTGCAGAAGACACACATCACCACATCAAA\4gaatc\gagatcc
```

Reference: 1. Two restriction enzyme recognition sites (BamH1: GGATCC, EcoR1: GAATTC). 2. Coding sequence of signal peptide for secretion. 3. Mutagenized WH303 epitope coding sequence. 4. (6x) His-tag coding sequence.
Supplement 2. Amino acid (one letter) sequence of recombinant CSFV glycoprotein E2.

MKFLVNLVPMVVYISYIYAR{LACKEDHRYAISTTNEIGLLGAEGLTTTWKEYNHNLQLDDGTVAICMAGSFKVTAALNVSRRLHLKDALPSTVFELLFDGPSLTEREMGDDFGFGLCPYDTSPVVKGKYNTTLLNGSalyLVCPIGWTGVIECTSGMDTLR{TEVVKTPRREEPVYRRDCVTHTTVENEDLFYCKWGGNWTVCVKGEPVYTGGPKQCRWCGDFNEPDGLPHYPIKGCIANETGRIVDSTDNCRDGVVISTEGSHECLIGNTTVKHALDERLGPMPCRPKEIVSSAGFVRKTSCTFNYAKTLRNRYEPRDSYFQQYMLKGEYQYWFDDVTDRHSDYFAEHHHHHH

Reference: 1. Additional hydrophobic signal peptide for secretion. However, the recombinant E2 lacks terminal hydrophobic amino acid sequence (about 32 aa, not shown) which is believed to be the transmembrane domain. 2. Mutagenized WH303 epitope. Five altered amino acids are S, F, G, M and D which in wild type E2 correspond to A, V, S, P and T respectively. 3. Six-histidine tag for detection with Western blot.
REFERENCE


