Construction and expression of modified PRRSV *GP5* encoding GP5 protein targeted to different subcellular compartments

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Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the causative agent of PRRS, the most economically important disease in swine industry worldwide including Thailand. Due to problems associated with Modified Live Vaccine (MLV), recombinant vaccine is being developed and tested as an alternative vaccine against PRRSV. However, the recombinant vaccines developed so far have been relatively ineffective at inducing protective immune responses and improvement is needed. In this study, we aim to improve the immunogenicity of GP5-based PRRSV vaccine by employing subcellular-targeting strategy, by which an antigen is redirected to the compartments involved in stimulation of B cells, CD8 and CD4 T cells. The immunogen GP5, derived from ORF5 of genotype 2 PRRSV, is designed to contain different signal sequences and thus is targeted to cytoplasm, ER, endosome and MHC I pathway. The immunogen GP5 is vectored by DNA plasmid pTH. Using western blot analysis, only the original GP5 and GP5 targeted to cytoplasm and ER could be detected. Localization and immunogenicity will be next studied. This study contributes knowledge and techniques of vaccine design and development to the vaccine research area, which will be beneficial to further development of PRRSV vaccines.

Keywords: Subcellular targeting, PRRSV, GP5, DNA vaccine

Introduction

Porcine Respiratory and reproductive Syndrome Virus (PRRSV, PRRS virus) is a single-stranded positive-sense RNA virus that is classified in the order *Nidovirales*, family *Arteriviridae* and genus *Arterivirus* (Tummaruk, P et al. 2013; Charerntantanakul, W 2012). PRRSV is divided into two genotypes: European (type 1) and North American (type 2) genotype. PRRSV is the causative agent of PRRS in swine. PRRSV infection leads to abortion in the sows and respiratory syndrome in the growing pigs (Nilubol, D., et al. 2012; Tun, H. M et al. 2011). PRRS epidemic continuously has emerged and caused huge economic losses worldwide including Thailand (Nilubol, D., et al. 2012; Tun, H. M et al. 2011).

Modified live attenuated vaccine is widely used to control PRRSV infection. However, MLV can possibly revert to virulent strain (Charerntantanakul, W 2012). Killed viral vaccine is the safe vaccine, but it has poor immunogenicity and poor protective efficacy against either homologous or heterologous PRRSV (Charerntantanakul, W 2012). Recombinant vaccines against PRRSV are now being developed with the ultimate goal of developing a safer and more effective vaccines against PRRSV. However, the recombinant vaccines developed so far have been shown to be relatively inefficient in inducing protective immune responses. Hence, new approaches and new technology are required to help develop a new vaccine.

For development of recombinant vaccine against PRRSV, GP5 protein has been demonstrated to contain major neutralizing epitopes and plays an important role in receptor recognition and viral infectivity, thus making it a leading target for PRRSV vaccine design (Dea, S et al. 2000). Although humoral immune response is considered to play a major role in preventing infection, the importance of cellular immune response in controlling PRRSV infection has also been suggested (Kutzler, M. A. and D. B. Weiner. 2008). For successful induction of CD8 and CD4 T cell immune responses, the antigen-derived peptides should be efficiently delivered to the MHC class I- and class II-restricted presentation pathways, respectively (Starodubova, E. S., et al. 2010). For successful induction of humoral immune responses, exogenous antigen is considered to be crucial (Starodubova, E. S., et al. 2010). One of the promising strategies to improve immunogenicity of a vaccine antigen is subcellular targeting strategy, by which an antigen is modified with signal sequences, resulting in targeting the antigen into distinct subcellular compartments. Targeting an antigen to the subcellular locations involved in MHC class I and MHC class II antigen processing and presentation will consequently be beneficial to the induction of CD8 and CD4 T cell response, respectively (Starodubova, E. S., et al. 2010). On the other hand, targeting the antigen for extracellular secretion will promote stimulation of B cells and CD4 T cells (Starodubova, E. S., et al. 2010).

DNA plasmid is one the vaccine-delivering vehicles that have been most commonly used for vaccine development. DNA vaccines offer several advantages over other vaccines. It is highly stable, relatively temperature insensitive and suitable for mass production and distribution (Kutzler, M. A. and D. B. Weiner. 2008). More importantly, they overcome the safety concerns associated with live vaccines, e.g., reversion of live attenuated vaccines and the risks linked to the manufacture of killed vaccine (Belakova, J et al. 2007; Abdulhaqq, S. A. and D. B. Weiner. 2008).

In the present study, the immunogen GP5 derived from ORF5 of type 2 PRRSV was designed to be redirected to (i) cytoplasm, (ii) endoplasmic reticulum (ER) and secretory pathway, (iii) endosome, and (iv) MHC-I pathway, by conjugating with different signal sequences. The GP5 is delivered by DNA vector pTH. The expression of GP5 and its variants by vaccine vector pTH were investigated using western blot analysis and the result showed that different GP5 variants had different patterns of expression. However, localization and immunogenicity of GP5-based vaccines remain to be studied.

Methodology

1. Construction of different *GP5* variants

The immunogen *GP5* used in this study is derived from ORF5 of type 2 PRRSV (North American genotype), collected from a swine farm in Ratchaburi. The native sequence of *GP5* was replaced with pig's most frequently used codons and the codon-optimized *GP5*, designated *GP5opt*, is synthetically made (GeneArt, Germany). DNA fragments of all five *GP5opt* constructs were generated by PCR amplification. All PCR amplifications were performed using KAPA^{TM} Hot Start High-Fidelity DNA Polymerase (NEB), following manufacturer's instruction. The signal sequences were added to the GP5opt by overlapping primers. Five different variants were amplified using the following set of primers: (i) GP5opt; 5'Flanking-F, GP5opt-F1, GP5opt-F2, GP5opt-F3, Gp5opt-F4 and V5-R2, (ii) GP5opt_Cy; 5'Flanking-F, GP5opt(-SS)-F1 and V5-R2, (ii) GP5opt_ER; 5'Flanking-F, tPA-F1, tPA-F2, GP5opt(-SS)-tPA-F1 and V5-R2, (iv) GP5opt_En, 5'Flanking-F, LAMP1-(SS)-F1, LAMP1-(SS)-F2, GP5opt-LAMP1(SS)-F1 and V5-R2, (v) GP5opt_MHCI; 5'Flanking-F and MHCI(TM+CY)-R1. Sequence and Tm profiles of each primer are shown in **Table 1**.

Following PCR amplification, gel extraction was performed using Gel DNA Fragments Extraction Kit (Geneaid).

Table 1 Sequence and Tm profile of the primers used in PCR amplification.

Primer name	Sequence (5'→3')	Tm (°C)
5'Flanking-F	ATTCCTGCAGGAAGCTTTTCCCCGGGGCCACCATG	74
GP5opt-F1	ATTCGAAGCTTTTCCCCGGGGCCACCATGCTGGGCAA GTGCCTG	75
GP5opt-F2	ATGCTGGGCAAGTGCCTGACCGCCGGCTGCTACTCCC GCCTGCTGTCCCTG	77.4
GP5opt-F3	TCCCGCCTGCTGTCCCTGTGGTGCATCGTGCTGTCCTC GTTCGCCGTGCTG	76.5
GP5opt-F4	TCCTGGTTCGCCGTGCTGGTGAACGCCAACAGCACCA GCAGCAGC	75.3
V5-R2	TAAGCCCGGGGCCGCTTATCAGGTGCTGTCCAGGCCC AGCAG	78
GP5opt-(-SS)-F1	ATTCGAAGCTTTTCCCCGGGGCCACCATGAACAGCAC CAGCAGCAGC	86
tPA-F1	TTCCCCGGGGCCACCATGTACGCCCTGAAGCGCGAGC TGTGGTGCGTGCTG	80
tPA-F2	GAGCTGTGGTGCGTGCTGCTGTGCGGCGCCATCTGCA CCTCCCCCTCC	77
GP5opt(-SS)-tPA-F1	ATCTGCACCTCCCCCTCCAACAGCACCAGCAGCTCC	72
LAMP1(SS)-F1	TTCCCCGGGGCCACCATGGCCGCCCCCGGCGGCGCCT GGCGCCGCCCCCTGCTGCTG	88
LAMP1(SS)-F2	CGCCGCCCCCTGCTGCTGCTGCTGCTGCTGGGCC GGCCCGCGGCGCCAGCGCC	87
GP5opt-LAMP1(-SS)-F1	GCCCGCGGCGCCAGCGCCAACAGCACCAGCAGCAGC	78
MHCI(TM+Cy)-R1	TAAGCCCGGGGCGGCCGCTTATCACACGCGGGGGTC TTGGTCAG	89

2. Generation of recombinant DNA plasmids

DNA fragments of all GP5opt variants were digested with restriction enzymes *Pst*I and *Not*I (NEB). Following double-digestion, DNA fragments were purified using PCR DNA Fragments Extraction Kit (Geneaid). The GP5opt constructs were ligated into pTH vector between *Pst*I and *Not*I sites. To generate recombinant clones, *E. coli* DH5α was transformed with ligation mixtures by heat shock method. Transformed *E. coli* cells were spread on LB agar containing Ampicillin and incubated at 37°C overnight (12-16 h). Recombinant clones were screened and selected using either colony PCR or rapid size screening. Selected clones were subsequently cultured in LB medium and subjected to plasmid purification using PureLink® HiPure Plasmid Miniprep Kit (Thermo Fisher Scientific Inc.). After the nucleotide sequences had been confirmed by automated DNA sequencing, plasmid DNA stock was prepared using GenElute HP Endotoxin-Free Plasmid Megaprep Kit (Sigma). The DNA concentration was subsequently determined using Nanodrop (Thermo Fisher Scientific) and the DNA is stored at -20°C until use.

3. Transfection of different pTH.GP5opt DNA vaccines

Human Embryonic Kidney 293A cells (HEK293A) were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. When cells reached 80% confluency, transfection was performed with 1 µg of a plasmid using polyethylenimine (PEI). Three days later, culture medium and transfected HEK293A cells were harvested and analyzed using western blotting.

4. SDS-PAGE and Western blot analysis

After culture medium was collected, transfected HEK293 cells were washed with PBS. Cold lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% Glycerol, 1% NP40) was added into the cells that had been placed on ice. Cells were scraped, transferred to 1.5 mL eppendorf tube, vortexed, incubated on ice for 1 h, and spun at 13,000 rpm for 10 minutes. Soluble proteins were separated on a 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membrane was blocked with PBS containing 2% w/v skimmed milk for 1 hour, prior to incubation with anti-V5 tag mAb conjugated with Alkaline Phosphatase in PBS containing 1% w/v skimmed milk for 2 hours at room temperature. The signal was developed using AP Conjugate Substrate Kit (Bio-Rad Laboratories, Inc.).

Results

1. Design and Construction of GP5opt variants

The immunnogen gene that encodes GP5 is derived from ORF5 of type 2 PRRSV (North American genotype), collected from the swine farm in Ratchaburi. The immunogen gene, designated *GP5opt,* was codon-optimized with pig's codon usage and synthetically made. Kozak sequence (GCCACCATGG) is added to the 5' end of the GP5opt constructs to enhance translation of the protein. V5-tag (GKPIPNPLGLDST) is added to the C-terminus of the GP5 in order to facilitate antigen detection.

To redirect the GP5 protein to different subcellular compartments, GP5 was modified using the following strategies: (i) removing the existing signal sequence to redirect the GP5 to cytoplasm, (ii) conjugating with signal sequence derived from swine's tissue plasminogen activator (tPA) gene to redirect the GP5 to the ER and secretory pathway, (iii) conjugating with signal sequence coupled with transmembrane and cytoplasmic tail sequences of swine's LAMP-1 to redirect the GP5 to endosome, (iv) conjugating with signal sequence coupled with transmembrane and cytoplasmic tail sequences of swine's MHC-I to redirect the GP5 to the MHC-I trafficking pathway (**Fig. 1**). Signal sequences were added to each construct by a set of primers that contain specific signal sequence. The products with expected size of 704, 611, 875, 680, and 800 bp were obtained from the PCR amplification of GP5opt, GP5opt_Cy, GP5opt_MHCI, GP5opt_ER and GP5opt_En, respectively **(Fig. 2)**.

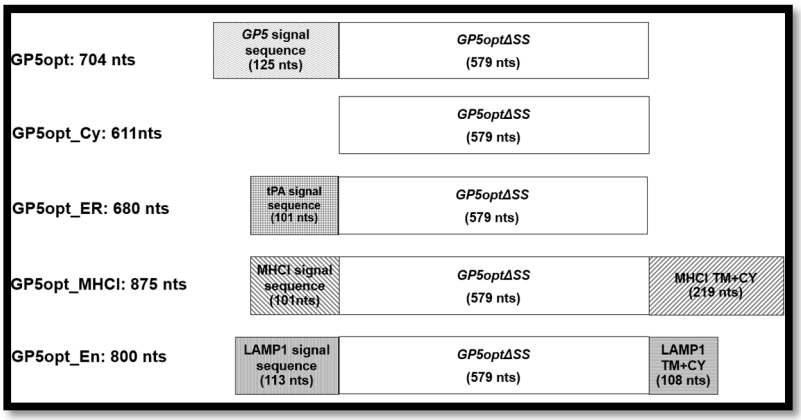

Figure1: Schematic diagram of different GP5opt variants. Components of GP5opt, GP5opt_Cy, GP5opt_MHCI, GP5opt_ER and GP5opt_En, are shown.

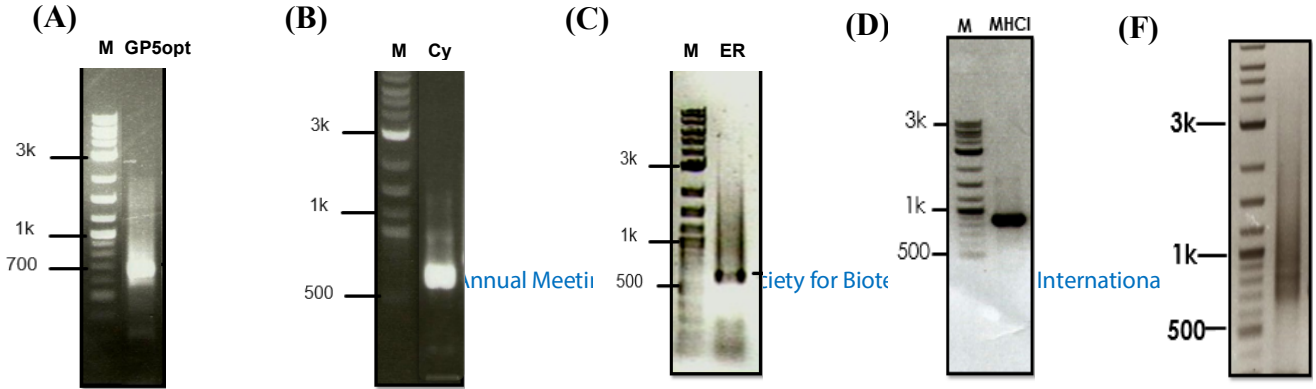

Figure 2: PCR amplification of different GP5opt constructs. PCR products of GP5opt **(A)**, GP5opt_Cy **(B)**, GP5opt_ER **(C)**, GP5opt_MHCI **(D),** and GP5opt_En, **(E),** are shown M represents 2-Log DNA ladder**.**

2. Generation of recombinant pTH plasmid

All modified GP5opt constructs were designed to be vectored by plasmid pTH. Following transformation of *E.coli* DH5α with ligation mixture, *E.coli* clones that carry recombinant pTH plasmid were screened using colony PCR and rapid size screening. Colony PCR was performed to determine recombinant clones of pTH.GP5opt, pTH.GP5opt_Cy, and pTH.GP5opt_Cy **(Fig. 3A-C).** Whereas, rapid size screening was carried out to determine recombinant clones of pTH.GP5opt_En and pTH.GP5opt_MHCI **(Fig. 3D-E)**. The DNA sequencing results demonstrated that nucleotide sequences of clone no. 13 of pTH_GP5opt, clone no. 8 and 18 of pTH_GP5opt_Cy, clone no. 1 and 2 of pTH_GP5opt_ER, clone no. 15 of pTH_GP5opt_En and clone no. 7 of pTH_GP5opt_MHCI are 100% correct.

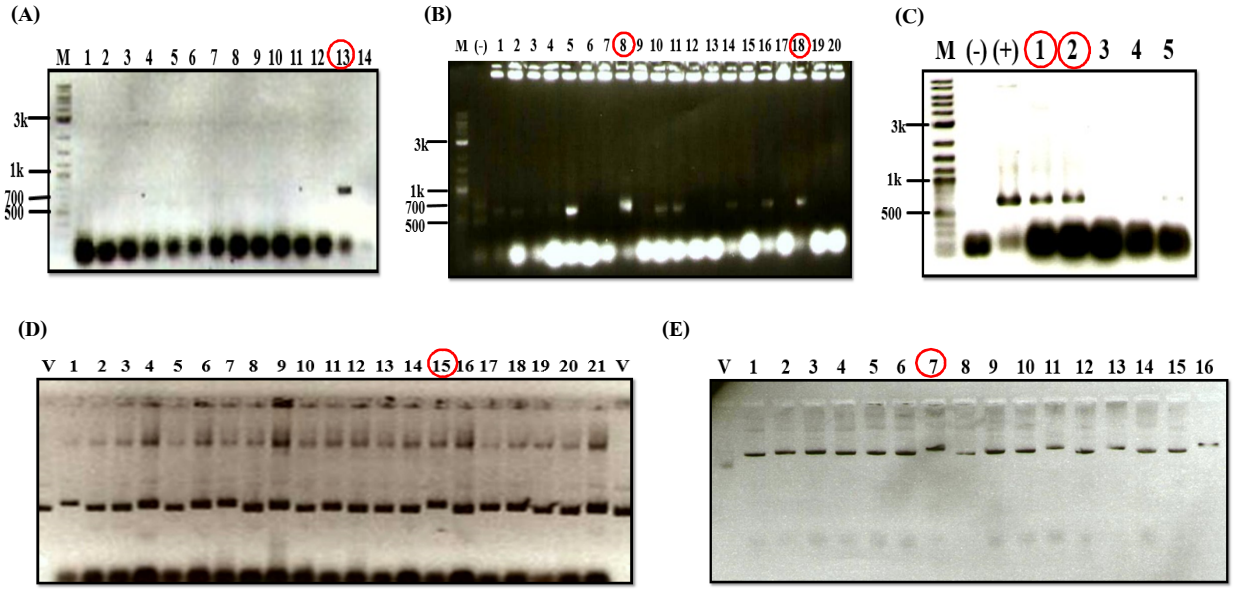

Figure 3: Screening of recombinant clones. Recombinant clones of pTH.GP5opt **(A),** pTH.GP5opt_Cy **(B)** and pTH.GP5opt_ER**(C)** were identified using colony PCR method. Rapid size screening was performed to identify the recombinant clones of pTH.GP5opt_En **(D)** and pTH.GP5opt_MHCI **(E)**. M represents 2-Log DNA ladder**.** V represents plasmid pTH.

3. Expression of the modified GP5 proteins

To study the expression of GP5 protein and its variants, SDS-PAGE and western blot analysis were performed. HEK293 cells were transfected with 1 ug of pTH.GP5opt, pTH.GP5opt_Cy, pTH.GP5opt_ER, pTH.GP5opt_En and pTH.GP5opt_MHCI. By using anti-V5 tag mAb, V5-tagged GP5 protein with the molecular mass of 25 kDa was detected in the cell lysates obtained from HEK293 cells transfected with pTH.GP5opt, pTH.GP5opt_Cy and pTH.GP5opt_ER. No expression of the GP5 protein by pTH.GP5opt_En and pTH.GP5opt_MHCI was observed **(Fig. 3)**.

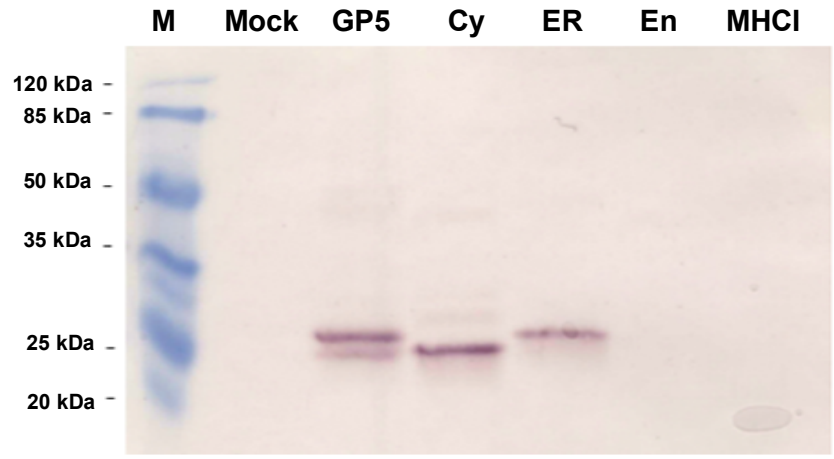

Figure 4: Detection of GP5 protein expression using western blot analysis. HEK293 cells were transfected with pTH.GP5opt (GP5), pTH.GP5opt_Cy (Cy), pTH.GP5opt_ER (ER), pTH.GP5opt_En (En) and pTH.GP5opt_MHCI (MHCI). Transfection without plasmid (Mock) was also performed in parallel. Transfected cells were collected 72 h after transfection and cell lysates were subjected to Western blot analysis. V5-tagged GP5 was detected by anti-V5 mAb. M represents protein standard marker.

Discussion

Antigen processing and presentation are important steps for activation of T cells. One of the most promising approaches to improve immunogenicity of a vaccine antigen is subcellular targeting strategy. In this approach, a vaccine antigen is modified by conjugating with signal sequences, resulting in targeting the antigen into different subcellular compartments and altering antigen processing and presentation.

In the present study, the immunogen GP5 was modified to be redirected to different subcellular compartments and localizations involved in antigen processing and presentation for T and B cell stimulation. These include cytoplasm, endoplasmic reticulum, endosome and MHC I pathway. The original full-length GP5 produced by DNA vaccine pTH.GP5opt would mimic the GP5 produced by the PRRS virus, in terms of expression, localization and its ability to induce immune responses in the host cell system. pTH.GP5opt_Cy produces GP5 that is translocated to cytoplasm and this would increase the chance of antigen degradation by the proteasome, thus producing more antigenic peptides that can be presented in a complex with MHC-I to stimulate a cytotoxic immune response. To enhance cytotoxic T cell stimulation, GP5 was also designed to be redirected to ER and MHC class I pathway, the locations involved in antigen presentation of MHC-I pathway. On the other hand, targeting the GP5 to the ER could also result in extracellular secretion of the protein and this secreted protein could be recognized by B cell receptor (BCR), leading to B cell stimulation and antibody production. Additionally, modification of the GP5 with LAMP1 sequences will result in redirection of the GP5 to the endosome, a compartment where MHC class II binds an antigenic peptide prior to presentation to CD4 T cells, thus improving a T helper cell response.

A number of studies have reported that subcellular targeting strategy could enhance immunogenicity of the vaccine. Compared to the unmodified E7, E7 modified with ER signal sequence showed more effective formation of E7 antigen/MHC I complex, leading to a considerably increased capacity of E7 to activate specific CD8+ T-cells (Hung C.F. et al. 2001). Targeting the PCV2 Cap protein to cytoplasm, secretory pathway, cell membrane and

nucleus, has demonstrated to enhance the immunogenicity of PCV2 Cap2-based vaccine, but to different extents (Fan, H., et al. 2008).

According to the western blot result, the expression of GP5 protein could be seen in the lysate samples of pTH.GP5opt, pTH.GP5opt_Cy and pTH.GP5opt.ER, but not pTH.GP5opt_MHCI and pTH.GP5opt_En. The negative result of GP5 expression by pTH.GP5opt_MHCI could be due to a degradation of the GP5 during migration from the ER to cell surface and/or extracellular secretion after the protein reaches cell membrane. For the GP5 fused with endosome-targeted sequence, it is possible that GP5 trafficking to the endosome were rapidly cleaved by endosomal proteases, thus, no intact full-length protein remains.

Conclusion

The immunogen GP5, derived from ORF5 of the PRRSV type 2, was designed to be redirected to cytoplasm, ER, endosome and MHC class I pathway, by conjugating with particular signal sequences. DNA vaccines (pTH.GP5opt, pTH.GP5opt_Cy, pTH.GP5opt_ER, pTH.GP5opt_En and pTH.GP5opt_MHCI) were successfully generated. Transfection and western blot results shows that GP5 protein could be detected only in the cells transfected with pTH.GP5opt, pTH.GP5opt_Cy and pTH.GP5opt_ER. However, localization and immunogenicity of different GP5 variants will be further studied.

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