Enhanced Stability of Inactivated Foot-And-Mouth Disease Virus Vaccine With A Novel Solution Buffer.

Jing Li  
Gansu Agricultural University  https://orcid.org/0000-0002-8119-5430

Yanming Wei (weiym@gsau.edu.cn)  
Gansu Agricultural University

Rong Zhang  
Science and Technology Co, Ltd

Huiqing Yang  
Science and Technology Co, Ltd

Xuerong Liu  
Science and Technology Co, Ltd

Research Article

Keywords: Foot-and-mouth disease, Inactivated vaccine, Novel solution buffer, Stability, HPSEC

Posted Date: December 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1035449/v1

License: ©️ This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Objectives: Stability is vital for potency of food-and-mouth disease virus vaccine preparation. However, the assembly of inactivated foot-and-mouth disease virus is poor stable and prone to dissociate into 12s under mild acidic or heating conditions, especially emulsified with oil-adjuvant. Thus, it is crucial to explore a suitable medium and condition to improve the stability and efficiency of inactivated FMDV vaccine.

Results: In this study, the basic solution buffer and a serious of potential stabilizers, such as carbohydrate, amino acid, antioxidant, salt and antioxidant were screened for evaluating stable effect on FMDV antigen with aid of high performance size exclusion chromatography (HPSEC). On this basis, orthogonal experiment was performed to optimize and finally confirm the formulation. Anti-aging test were carried out to asses the efficiency of formulation on vaccine stability and the results showed that the vaccine was more stabler either stored at 37°C or 4°C. Moreover, physicochemical monitoring revealed that formulation had no influence on the properties of vaccine. The combined results suggested that the novel solution buffer would lower degradation and prolong shelf life of vaccine. In a word, the novel buffer is beneficial to make FMD vaccine more stable and effective, reducing the dependence on cold delivery and storage. This study also provides insight into the processes of optimization and inactivated vaccine development.

Introduction

Foot-and-mouth disease (FMD) is a severe economically and socially animal disease that hinder the development of stock raising (Kitching 2005; Porphyre et al. 2018). FMD virus is the cause of disease which contains a single-stranded positive-sense RNA and capsid protein composed of 60 copies of VP1, VP2, VP3 and VP4, each of which arranged in 12 pentamer (Fry et al. 2005). As an antigenically variable virus, FMDV consists of seven serotypes and a large number of subtypes, which is a big challenge to prevent and control FMD effectively (Paton et al. 2009). Although slaughtering animal was used as control measures in disease-free-areas, vaccination against FMDV is still an effective measure to block the spread of epidemic in developing countries. Vaccine potency is largely determined by the content of intact viral capsid (referred to as 146s) (Rodriguez et al. 2011; Spitteler et al. 2011). However, 146s is both sensitive to mild acidic or elevated temperature, dissociating into 12s easily (Kotecha et al. 2017; Kotecha et al. 2015; Harmsen et al. 2010). This disadvantage results in reduced shelf life and insufficient efficacy. Therefore, there is a need for developing effective and safe strategies to improve the stability of FMDV (Kartoglu et al. 2014).

Excipient, a supplement, formulated alongside the active ingredient of a medication has been used for the purpose of long-term stabilization. Wide variety of excipients including carbohydrate, bovine serum albumin, amino acid, metal ion were functioned as filler, buffer agent or establishing salt bridge to stabilize the antigen. It has been demonstrated that these excipients could protect FMDV from dissociation, especially 20% sucrose (w/v). However, the high concentration could make vaccine thicker, hardly absorbed by body. Therefore, a combination of excipients with reasonable content could stabilize the antigen with no influence on vaccine viscosity. It is known that formulation had been applied to several virus vaccines, such as Newcastle Disease (Wambura 2011), Marek (Colwell et al. 1975), and Classical swine fever (Pachauri et al. 2020), but no investigation on FMD. Hence, in this study, a novel solution buffer was confirmed and evaluated the stable effect on inactivated vaccines.

Materials And Methods

Materials

FMDV O/MYA98/BY/2010 strain supernatant disposed with cultivation, inactivation and pretreatment (Li et al. 2015) was provided by Zhongnongweite biotechnology Co., Ltd. sucrose, trehalose, bovine serum albumin (BSA), amino acids including glycine (Gly), cysteine (Cys) and lysine (Lys) were purchased from sigma (MO, USA). Polyethylene glycol with molecular weight of 6000 were from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). All other chemicals were analytical grade reagents purchased from Beijing Chemical works (Beijing, China) and all solutions were prepared using Milli-Qgrade water (Millipore, USA).

Thermal stability study
The antigen and vaccine prepared with normal solution buffer (PBS) were stored at 4°C, 25°C and 37°C for a period of time to determine the thermal stability, respectively. The vaccine collected at 5 d, 15 d and 30 d was mixed with 1-pentanol in a 10 ml centrifuge tube at a ratio of 9:1. The mixture was shaken fully to break the emulsion. After placed at 4°C for 1 h, the upper oil phase was removed and the aqueous phase at the bottom was absorbed slowly for 146s determination. The antigen sample were collected at same time point for detection directly. The method of HPSEC was performed to measure the remaining 146s content and studied the influence of temperature on stability (Yang et al. 2015). Briefly, samples of 100ul were injected and eluted at 0.6 ml/min with 50 mM phosphate buffer at pH 7.2 containing 100 mM Na₂SO₄. The peak area at 259 nm was linearly proportional to 146s concentration with established standard curve. Therefore, 146s content could be calculated according to the peak area.

**Screening of basic solution buffer**

Three types of buffer were used for antigen preparation, including Tris·Cl(pH 8.0), PBS (pH 8.0) and the combination of 50% Tris·Cl with 50% PBS. The stable effect was evaluated by measure the residual 146s in antigen after stored at 37°C for 5 d. The initial concentration of antigen was 20 µg/ml, and the HPSEC was performed to measure the residual 146s.

**Stability study of excipients**

Several substances were analyzed as stabilizer for the inactivated vaccine. These excipients included sucrose, trehalose, Tween-20, arginine, cysteine, BSA, calcium chloride and ascorbic acid. The stock solution of each excipients was prepared in PBS and then diluted further to obtain the final working concentration. We test two levels of all excipients as supplements for stabilizer. The details were showed in Table 1. Antigen stability was examined by incubating at 37°C for remaining 146s detection, using HPSEC described previously. The determined composition were used for subsequent study.

**Optimization the formulation of solution buffer**

After determination of basic solution buffer and stabilizers, the optimal formulation was analyzed by Adopt L₈ (2⁷) orthogonal mode and the stability effect was evaluated by vaccine anti-aging test performed at 37°C. There were totally eight groups included in this study. All solution buffers were adjusted to pH 8.0 with 1N NaOH, sterilized by 0.2 µm needle filter. Antigen prepared with different solution buffer was at an initial 146s concentration of 20 µg/mL and then emulsified with 206 adjuvant to produce the inactivated vaccine. The control one is the sample with no excipient. When treated at 37°C, residual 146s content in vaccine was measured by HPSEC. The one with more residual 146s was regarded to be more effective for vaccine stabilization and used for subsequent experiments.

**Vaccine preparation**

Vaccines were prepared by emulsification of 75 ml FMDV antigen (20 µg/ml 146s) in various solution buffer with 50% (w/w; 88ml) ISA 206 VG adjuvant. The mechanical impeller with 4 blades was used for stirring emulsion. The antigenic media was added into the adjuvant beaker slowly after warmed up at 32°C for 30 min, maintained the agitation at 350 rpm for 5 min and then placed the beaker in a cooling bath at 20°C for 1 h.

**Statistical analysis**

The means and standard deviations(n=3) of all values were analyzed with descriptive statistics. The statistical analysis of excipients was performed with a t-test, and differences between samples were considered statistically significant at p<0.05.

**Results**

**Thermal stability study**

In order to carry out quantitative analysis of 146s antigen, we firstly established a standard curve by plotting peak area against FMDV 146s antigen standard concentration. It was found that the standard curve had strong linear correlation (R²=0.9994). Then we generated corresponding linear regression equation with x slope and y intercept (Fig. 1)
It has been reported that the suitable pH for FMDV stabilization was 8.0, therefore, thermal stability investigation was based on this condition. Thermal stability of antigens and vaccines stored at 4°C, 25°C and 37°C for 30 d were evaluated respectively by monitoring the changes of 146s concentration. The stability of antigen was negatively correlated with temperature (Fig. 1c). The residual 146s was 19.3 µg/ml at 4°C; however, it was dissociated significantly at 25°C and 37°C with 7.1 µg/ml and 2.5 µg/ml, respectively. Similarly, 146s content in vaccine were also influenced by temperature, moreover, it dissociated rapid than antigen, with residual 146s of 7.3 µg/ml, 4.1 µg/ml and 0.8 µg/ml at 4°C, 25°C and 37°C respectively.

The typical changes of 146s content was illustrated (Fig. 1b). As shown, 146s content was calculated according to peak area appearing at 12.5 min. During storage, along with the reduced peak area, another peak appeared at 20 min, which indicated the dissociation of 146s and formation of small degradation.

Screening basic solution buffer

PBS and Tris-Cl are in common use for protein treatment, thus, in this study, we assessed the effect of PBS, Tris-Cl and a combination of PBS and Tris-Cl (1:1) on FMDV antigen stability. To quickly find the potential basic solution buffer, the antigen prepared with three types of solution buffer were incubated at 37°C for 5 d (Fig. 2). Our results showed that the concentration of 146s was higher in PBS with 10.2 µg/ml than in Tris-Cl and a combination with 2.8 µg/ml and 4.7 µg/ml, respectively. Therefore, we concluded that the PBS was suitable for stabilizing the antigen and used for subsequent experiments.

Effect of excipient on thermal stability

In order to quantitatively evaluate the effect of excipients on thermal stability, we defined a percentage of dissociation (PD) as follows: \(PD=1-(\frac{PA_{\text{excipient}}}{PA_{\text{control}}})/(\frac{PA_{\text{initial}}}{PA_{\text{control}}})\times100\%\). \(PA_{\text{initial}}\) and \(PA_{\text{excipient}}\) represent the 146s concentration in antigen before and after heating, respectively. Our results showed that all the excipients were available for inhibiting dissociation at different levels, and the PD value was negatively correlated with concentration (Table. 1). Among the excipients tested, 5% trehalose exhibited most effective, and 146s was almost completely remained. While only 40% was maintained when the concentration decreased to 3%. The PD value for tween was nearly 90%, indicating it had no obvious protection against dissociation. Therefore, we determined sucrose, arginine, calcium chloride, BSA, cysteine and ascorbic acid as the composition for novel solution buffer.

Formulation determination

The composition of novel solution buffer for thermal stability of capsid was successfully determined. The optimized buffer concentration was analyzed by orthogonal experiments. The optimized combination was A1, B1, C1, D2, E2, F1 and G2 (Table. 2). The effects of excipients on thermal stability was trehalose > calcium chloride > cysteine > BSA > sucrose > arginine > ascorbic acid.

Therefore, the optimal concentration of supplemented excipients were 5% sucrose, 0.01 M arginine, 0.01 M calcium chloride, 1% BSA, 0.05 M cysteine, 0.001 M ascorbic acid and 5% trehalose.

Evaluation of vaccine stability on storage

Increased shelf life and reduced necessity of cold storage are crucial destination when deriving vaccine formulation. To investigate the stability of vaccine on storage, we monitored the 146s content in vaccines during preserved at 37°C and 4°C respectively. As indicated in Fig. 3a, the vaccine prepared with novel solution buffer were more stabler, with the date inducing 50% dissociation prolonged to 20 d at 37°C. Furthermore, reduced dissociation at 4°C was also observed, with shelf life prolonged to more than 300 d, while the normal one was dissociated absolutely (Fig. 3b). These results demonstrated that the novel solution buffer could stabilize the vaccine efficiently.

Assessment of vaccine physical properties

The analysis of particle size distribution revealed that the vaccine formulated with novel solution buffer is similar to the control one (formulated with normal solution buffer) in particle size about 220 nm (Fig. 3c), thus, it has no influence on vaccine physical
properties.

Discussion

The safety and effectiveness of conventional inactivated FMD vaccine has been widely recognized, while, the structural instability of vaccine antigens is one of the biggest challenges affecting the quality of vaccine. Thus, it is urgent to find an suitable solution for stabilizing the antigen and then prolonging the shelf life of vaccine.

Several studies on acid stability of FMDV had shown that a cluster of His residual located on the petameric interface were more likely protonation on low pH, inducing the dissociation of 146s (Ellard et al. 1999; Hong et al. 2017; Vazquez-Calvo et al. 2014), therefore, in this study, all experiments were carried out at pH 8.0 (Caridi et al. 2015). By quantitative analysis of 146s with HPSEC method, the thermal stability was performed, and the results exhibited that FMDV antigen was sensitive to temperature, with residual 146s of 2.5 µg/ml at 37°C for one month, lower than that at 4°C (19.3 µg/ml). Moreover, the vaccine was prone to dissociation easily than antigen stored at same temperature. This result was consistent with previous studies demonstrated that emulsification with Freund adjuvant can induce the denaturation of β-lactamase and then decrease the stability (Shimizu et al. 1993; Van de Weert et al. 2000). Hence, the interaction between solvent and water interface may be a factor influencing the stability of 146s, but the mechanism was not sure.

Many strategies have been used to improve the stability of vaccine. Targeted mutations of viral structural proteins have been proven effectively, but would possibly reduce viral infection, hinder viral propagation and even alter the antigenicity of the virus, leading vaccination less effective (Biswal et al. 2016; Lopez-Arguello et al. 2019). Considering the safe, reliable and application for large-scale production, a novel solution buffer might be employed. Initially, different solution buffers were screened as the basic buffer, and the results showed that PBS could improve the stability effectively, with residual 146s of 10.2 µg/ml, higher than the Tris·Cl (2.8 µg/ml) and the combination (4.7 µg/ml), thus, PBS was determined to be the basal buffer.

Excipients such as sucrose, arginine, calcium chloride, BSA, cysteine, ascorbic acid, trehalose and tween has been reported to be potential stabilizers for FMDV (Harmsen et al. 2015). In this study, we firstly evaluated the effect of excipients respectively, and the results showed that 5% trehalose exhibited effectively, while, tween was indistinctively with a PD value of nearly 90%, others showed moderate effect. Based on these results, orthogonal experiment was preformed to further optimize the combination of excipients for novel solution buffer. Although sucrose has been reported to increase the transition temperature related to 146s, our findings revealed that the novel solution buffer with only 5% sucrose could also improved the stability. Besides, as supplement in vaccine, the excipients with low concentration could maintain vaccine viscosity, easily absorbed by animal body. These results indicated that the novel solution buffer could be applied to vaccine preparation and application. Therefore, we achieved a novel solution buffer against FMDV dissociation.

To further confirm the stable effect on vaccine, the changes of 146s content in vaccine was monitored, the results revealed that the half shelf life of vaccine could prolonged from 10 d to 20 d at 37°C and the shelf life at 4°C was also prolonged to 300 d, suggesting the enhanced stability on inactivated vaccine. Meanwhile, the analysis of particle size distribution showed that supplement of excipients could not change the particle size, the diameter of most vaccine droplet were about 220 nm, consistent with normal one, thus, the novel solution buffer has no impact on vaccine physical properties, available for large-scale production.

Adjuvant, a key component of vaccine, has a very important effect on the stability of vaccine. In this study, we found that emulsification with ISA206 would stimulate the dissociation of 146s, while, the situation about other adjuvant such as ISA201 was not clear, thus, future investigation should be required to assess the stability in vaccine prepared with ISA201. In addition, antibody titer is an important parameter to evaluate the vaccine quantity. The problems whether the excipients could influence the immunogenicity and could stabilized vaccine contribute to antibody titer are the two significant aspects we should fully considered, therefore, further study should also take immune response in animals into account. Overall, these studies would provide a new insight into improvement the stability of inactivated vaccine.
In conclusion, our results demonstrated that the novel solution could increase the stability of vaccine antigen as well as prolong the shelf life of vaccine, suggesting the potential application of this solution in vaccine production regimens for FMD prevention.

**Declarations**

The authors declare that there are no conflicts of interest.

**Acknowledgments**

The authors would like to thank Rong Zhang, Huiqing Yang for vaccine preparation and performing HPSEC tests.

**Funding** This study was supported by grants from National Modern Agricultural Industrial Technology System (CARS-37) and Industry Support Project of colleges in Gansu province (2020C-14).

**Availability of data and materials** The whole raw dataset used may be available from the corresponding author on reasonable request.

**References**

1. Kitching RP (2005) Global epidemiology and prospects for control of foot-and-mouth disease. Curr Top Microbiol Immunol 288:133–148. https://doi.org/10.1007/3-540-27109-0_6
2. Porphyre T, Rich KM, Auty HK (2018) Assessing the Economic Impact of Vaccine Availability When Controlling Foot and Mouth Disease Outbreaks. Front Vet Sci. 5:47. https://doi.org/10.3389/fvets.2018.00047. eCollection 2018
3. Fry EE, Stuart DI, Rowlands DJ (2005) The structure of foot-and-mouth disease virus. Curr Top Microbiol Immunol 288:71–101. https://doi.org/10.1007/3-540-27109-0_4
4. Paton DJ, Sumption KJ, Charleston B (2009) Options for control of foot-and-mouth disease: knowledge, capability and policy. Philos Trans R Soc Lond B Biol Sci 364:2657–2667. doi.org/10.1098/rstb.2009.0100
5. Rodriguez LL, Gay CG (2011) Development of vaccines toward the global control and eradication of foot-and-mouth disease. Expert Rev Vaccines 10:377–387. doi: 10.1586/erv.11.4
6. Spitteler MA, Fernandez I, Schabes E, Krimer A, Regulier EG, Guinzburg M (2011) Foot mouth disease (FMD) virus: quantification of whole virus particles during the vaccine manufacturing process by size exclusion chromatography. Vaccine. 29: 7182–7. https://doi.org/10.1016/j.vaccine.2011.05.078
7. Kotecha A, Wang Q, Dong X, Ilca SL, Ondiviela M, Zihe R, Seago J, Charleston B, Fry EE, Abrescia NGA, Springer TA, Huiskonen JT, Stuart DI (2017) Rules of engagement between avb6 integrin and foot-and-mouth disease virus. Nat Commun 8:15408. https://doi.org/10.1038/ncomms15408
8. Kotecha A, Seago J, Scott K, Burman A, Loureiro S, Ren JS, Porta C, Ginn HM, Jackson T, Perez-Martin E, Siebert CA, Paul G, Huiskonen JT, Jones IM, Esnouf RM, Fry EE, Maree FF, Charleston B, Stuart DI (2015) Structure-based energetics of protein interfaces guides foot-and-mouth disease virus vaccine design. Nat Struct Mol Biol 22:788–794. doi.org/10.1038/nsmb.3096
9. Harmsen MM, Jansen J, Westra DF, Coco-Martijn JM (2010) Characterization of foot-and-mouth disease virus antigen by surface-enhanced laser desorption ionization-time of flight–mass spectrometry in aqueous and oil-emulsion formulation. Vaccine 28:3363–3370. https://doi.org/10.1016/j.vaccine.2010.02.084
10. Kartoglu U, Milstien J (2014) Tools and approaches to ensure quality of vaccines through the cold chain. Expert Rev Vaccines 13:843–854. https://doi.org/10.1586/14760584.2014.923761
11. Wambura PN (2011) Formulation of novel nano-encapsulated Newcastle disease vaccine tablets for vaccination of village chickens. Trop Anim Health Prod 43:165–169. https://doi.org/10.1007/s11250-010-9669-0
12. Colwell WM, Simmons DG, Harris JR, Fulg TG, Carrozza BH, Maag TA (1975) Influence of some physical factors on survival of Marek’s disease vaccine virus. Avian Dis 19:781–790
13. Pachauri R, Manu M, Vishnoi P (2020) Stability of live attenuated classical swine fever cell culture vaccine virus in liquid form for developing an oral vaccine. Biologicals 68:108–111. https://doi.org/10.1016/j.biologicals.2020.07.004

14. Li H, Yang Y, Zhang Y, Zhang S, Zhao Q, Zhu Y (2015) A hydrophobic interaction chromatography strategy for purification of inactivated foot-and-mouth disease virus. Protein Expr Purif 113:23–29. https://doi.org/10.1016/j.pep.2015.04.011

15. Yang YL, Li H, Li ZJ, Zhang Y, Zhang SP, Chen Y (2015) Size-exclusion HPLC provides a simple, rapid, and versatile alternative method for quality control of vaccines by characterizing the assembly of antigens. Vaccine. 33:1143–50. https://doi.org/10.1016/j.vaccine.2015.01.031

16. Ellard FM, Drew J, Blakemore WE, Stuart DI, King AMQ (1999) Evidence for the role of His-142 of protein 1C in the acid-induced disassembly of foot-and-mouth disease virus capsids. J Gen Virol 80:1911–1918. https://doi.org/10.1099/0022-1317-80-8-1911

17. Yuan H, Li P, Ma X (2017) The pH stability of foot-and-mouth disease virus. Virol J 14:233. https://doi.org/10.1186/s12985-017-0897-z

18. Vazquez-Calvo A, Caridi F, Sobrino F, Martin-Acebes MA (2014) An increase in acid resistance of foot-and-mouth disease virus capsid is mediated by a tyrosine replacement of the VP2 histidine previously associated with VP0 cleavage. J Virol. 5:3039–3042. https://doi.org/10.1128/JVI.03222-13

19. Caridi F, Vázquez-Calvo A, Sobrino F, Martin-Acebes MA (2015) The pH stability of foot-and-mouth disease virus particles is modulated by residues located at the pentameric interface and in the N terminus of VP1. J Virol. 89:5633–5642. https://doi.org/10.1128/JVI.03358-14

20. Shimizu M, Miwa Y, Hashimoto K, Goto A (1993) Encapsulation of chicken egg yolk immunoglobulin G (IgY) by liposomes. Biosci Biotechnol Biochem 57:1445–1449. doi.org/10.1271/bbb.57.1445

21. Van de Weert M, Hoechstetter J, Hennink WE, Crommelin DJ (2000) The effect of a water/organic solvent interface on the structural stability of lysozyme. J Control Release 68:351–359. https://doi.org/10.1016/s0168-3659(00)00277-7

22. Biswal JK, Das B, Sharma GK, Khulape SA, Pattnaik B (2016) Role of a single amino acid substitution of VP3 H142D for increased acid resistance of foot-and-mouth disease virus serotype A. Virus Genes 52:235–243. https://doi.org/10.1007/s11262-016-1294-1

23. Lopez-Arguello S, Rincon V, Rodriguez-Huete A, Martinez-Salas E, Belsham GJ, Valbuena A, Mateu MG (2019) Thermostability of the foot-and-mouth disease virus capsid is modulated by lethal and viability-restoring compensatory amino acid substitution. J Virol 93:e02293–e02218. https://doi.org/10.1128/JVI.02293-18

24. Harmsen MM, Fijten HP, Westra DF, Dekker A (2015) Stabilizing effects of excipients on dissociation of intact(146S) foot-and-mouth disease virions into 12S particles during storage as oil-emulsion vaccine. Vaccine. 33:2477–2484. https://doi.org/10.1016/j.vaccine.2015.03.066

Tables

**Table 1** Efficacy of excipients in stabilizing FMDV at 37°C
Excipients | Concentration | PD(%)  
--- | --- | ---  
| | Molarity or % w/v |  
Trehalose | (1) 3% | 61.2±2.1  
| | (2) 5% | 42.8±1.7  
Sucrose | (1) 3% | 41.5±2.4  
| | (2) 5% | 28.4±3.1  
Arginine | (1) 0.01M | 55.1±0.1  
| | (2) 0.05M | 35.1±0.1  
Cysteine | (1) 0.01M | 60.2±0.3  
| | (2) 0.05M | 48.7±0.3  
BSA | (1) 0.5% | 75.4±0.1  
| | (2) 1% | 80.3±0.1  
Tween-20 | (1) 0.1% | 98.2±0.1  
| | (2) 0.5% | 93.4±0.1  
Calcium chloride | (1) 0.01M | 21.2±2.5  
| | (2) 0.05M | 10.7±1.8  
Ascorbic acid | (1) 0.001M | 59.1±2.7  
| | (2) 0.005M | 34.2±3.1  

PD represented the percentage of 146s dissociation that was calculated as $\frac{1}{\text{PA}_{\text{excipient}}/\text{PA}_{\text{control}}} \times 100\%$. \(\text{PA}_{\text{initial}}, \text{PA}_{\text{excipient}}, \text{PA}_{\text{control}}\), represent the 146s remainings before and after inoculated at 37°C with and without excipient respectively.

**Table 2** Orthogonal test and visual analysis of efficiency of novel solution buffer
| Groups | A  | B  | C  | D  | E  | F  | G  | remaining 146s(μg/ml) | Mean value |
|--------|----|----|----|----|----|----|----|-----------------------|------------|
| 1      | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 14.203                | 13.973     | 13.982 | 14.053 |
| 2      | 1  | 1  | 1  | 2  | 2  | 2  | 2  | 14.566                | 14.312     | 14.265 | 14.381 |
| 3      | 1  | 2  | 2  | 1  | 2  | 2  | 2  | 14.152                | 14.061     | 13.892 | 14.035 |
| 4      | 1  | 2  | 2  | 2  | 2  | 1  | 1  | 14.210                | 14.062     | 14.001 | 14.091 |
| 5      | 2  | 1  | 2  | 1  | 2  | 2  | 1  | 14.222                | 14.243     | 13.991 | 14.152 |
| 6      | 2  | 1  | 2  | 2  | 2  | 1  | 2  | 14.077                | 13.991     | 13.804 | 13.957 |
| 7      | 2  | 2  | 1  | 1  | 2  | 2  | 1  | 14.157                | 14.163     | 13.994 | 14.105 |
| 8      | 2  | 2  | 1  | 2  | 1  | 1  | 2  | 14.407                | 14.221     | 14.021 | 14.216 |
| 9(control) | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 11.406                | 11.491     | 11.502 | 11.466 |
| K1     | 14.140 | 14.136 | 14.189 | 14.086 | 14.065 | 14.128 | 14.052 |
| K2     | 14.108 | 14.112 | 14.059 | 14.161 | 14.182 | 14.12 | 14.196 |
| R      | 0.032 | 0.024 | 0.130 | 0.075 | 0.117 | 0.008 | 0.114 |

Annotation: column A represent sucrose. B is Arginine. C is Calcium chloride. D is BSA. E is Cysteine. F is ascorbic acid. G is Trehalose.

**Figures**

**Figure 1**

**HPSEC for determination of 146s remaining.** A Standard curve of qualification of inactivated FMDV 146s antigen; B Chromatogram of FMDV during incubated at 37°C. 5 d, blue. 15 d, green. 30 d, black. C Dissociation of 146s in antigen and vaccine at 4°C(C-1), 25°C(C-2) and 37°C(C-3) respectively. Antigen and vaccine were prepared with normal PBS buffer at 146s concentration of 20μg/ml. During storage, the sample were collected at 5th, 15th and 30th day respectively. After demulsification and extraction, antigen in vaccine were obtained and dected with HPSEC, the antigen were measured directly with no treatment.

**Figure 2**

**Comparison the stability of three types basic solution buffer.** Antigens prepared with different solution buffer were sampled in regular time point and analyzed by HPSEC for residual 146s content.
Figure 3

Shelf life and particle size of vaccine. A Comparison the half shelf life of vaccine at 37°C. Vaccines prepared with normal and novel solution buffer with 146s 20μg/ml were inocubated at 37°C and the concentration of 146s was analyzed by HPSEC every 10 day. The length of time that induced 50% dissociation of 146s was defined as half shelf life. B Monitor the changes of 146s content of vaccine during stored at 4°C. C Measure and comparison the particle sizing of vaccine droplets with and without excipient. The red curve indicated the normal vaccine (without excipient), green one was vaccine formulated with excipient. The particle size were about 200 nm.