Video Article

Protocol for Recombinant RBD-based SARS Vaccines: Protein Preparation, Animal Vaccination and Neutralization Detection

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Abstract

Based on their safety profile and ability to induce potent immune responses against infections, subunit vaccines have been used as candidates for a wide variety of pathogens [1-3]. Since the mammalian cell system is capable of post-translational modification, thus forming properly folded and glycosylated proteins, recombinant proteins expressed in mammalian cells have shown the greatest potential to maintain high antigenicity and immunogenicity [4-6].

Although no new cases of SARS have been reported since 2004, future outbreaks are a constant threat; therefore, the development of vaccines against SARS-CoV is a prudent preventive step and should be carried out. The RBD of SARS-CoV S protein plays important roles in receptor binding and induction of specific neutralizing antibodies against virus infection [7-9]. Therefore, in this protocol, we describe novel methods for developing a RBD-based subunit vaccine against SARS. Briefly, the recombinant RBD protein (rRBD) was expressed in culture supernatant of mammalian 293T cells to obtain a correctly folded protein with proper conformation and high immunogenicity [6]. The transfection of the recombinant plasmid encoding RBD to the cells was then performed using a calcium phosphate transfection method [6,10] with some modifications. Compared with the lipid transfection method [11,12], this modified calcium phosphate transfection method is cheaper, easier to handle, and has the potential to reach high efficacy once a transfection complex with suitable size and shape is formed [13,14]. Finally, a SARS pseudovirus neutralization assay was introduced in the protocol and used to detect the neutralizing activity of sera of mice vaccinated with rRBD protein. This assay is relatively safe, does not involve an infectious SARS-CoV, and can be performed without the requirement of a biosafety-3 laboratory [15].

The protocol described here can also be used to design and study recombinant subunit vaccines against other viruses with class I fusion proteins, for example, HIV, respiratory syncytial virus (RSV), Ebola virus, influenza virus, as well as Nipah and Handra viruses. In addition, the methods for generating a pseudovirus and subsequently establishing a pseudovirus neutralization assay can be applied to all these viruses.

Video Link

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Protocol

1. Recombinant SARS-CoV RBD Protein Preparation

1. Prepare calcium phosphate transfection reagent
    1. 2X HBS buffer preparation: Mix together 16 g of NaCl, 0.4 g of $Na_2HPO_4*7H_2O$, and 13.0 g of HEPES. Adjust pH to 7.00 and bring up total volume to 1000 mL in distilled water. After filtering the solution for sterilization, aliquot and store it at -20°C.

        Hint: Any variation of the pH value would affect the transfection results. Thus, it is advisable to test several pH values around 7.00 (for example, 6.99, 7.00, or 7.01) and find the best one for the transfection using the method introduced below.
    2. 2.5 M $CaCl_2$ preparation: Add 73.5 g of $CaCl_2*2H_2O$ to distilled water in a final volume of 200 mL. Filter the solution and store at -20°C.
2. Recombinant plasmid transfection and protein purification
    1. Split 293T cells at 50-70% confluency 24 h before transfection. Grow cells in T-175 cm^2 tissue culture flasks in 40 mL DMEM containing 10% heat-inactivated (HI) FBS and 1% Penicillin/Streptomycin (P/S) at 37°C in 5% CO_2.
    2. All transfection reagents should be brought up to room temperature before transfection. These reagents include 2X HBS, 2.5M $CaCl_2$, DiH_2O, and rRBD plasmid [6].

        Hint: The final recombinant plasmid construct used for protein expression should contain a signal peptide to ensure secretion of expressed recombinant proteins to the culture supernatant. A 6x His tag may be added to the C-terminal of the expressed protein for easy purification.

3. Prepare one 50 mL (A) and one 15 mL (B) BD Falcon tube, and add reagents to the tubes as indicated in Table 1. Add 2X HBS buffer to tube A. In tube B, add 2.5M $CaCl_2$ and rRBD plasmid, and bring the volume to the requirement in DiH_2O.

A. One T-175 cm^2 tissue culture flask (4000 µL/flask): prepare for rRBD expression			
Tube A		**Tube B**	
2X HBS	2000 µL	2.5M $CaCl_2$	200 µL
		rRBD plasmid DNA	40 µg
		DiH_2O to	*2000 µL*
B. One 100-mm petri dish (1000 µL/dish): prepare for SARS pseudovirus production			
Tube A		**Tube B**	
2X HBS	500 µL	2.5M $CaCl_2$	50 µL
		SARS-CoV S plasmid DNA	5 µg
		HIV-1 plasmid (pNL4-3.luc.RE)	5 µg
		DiH_2O to	*500 µL*

Table 1. Transfection mixture preparation and volumes

The volumes listed in Table 1 are for one transfection unit. If more flasks or dishes are used for the transfection, adjust volumes accordingly.

4. Add the DNA-calcium solution in tube B into tube A in a dropwise manner, while maintaining constant and gentle mixing in a vortex. Let the mixture sit at room temperature for 20-30 min. The key for successful calcium phosphate transfection depends on the size and shape of the precipitate formed. Thus, the mixture should be vortexed constantly and slowly to meet this requirement and, as a result, improve transfection efficacy.
5. Add mixture in a dropwise and even manner into 293T cells (4000 µL/flask). Culture cells in incubator at 37°C in 5% CO_2.
6. Replace the culture medium with fresh serum-free OPTI-MEM I Reduced-Serum Medium (50 mL/flask) 8-10 h post-transfection. Continue to culture cells for two more days in the same condition.
7. Collect supernatant containing expressed rRBD protein 72 h post-transfection. Centrifuge at 6000 rpm for 15 min to remove cell debris. Add protease inhibitor cocktail to the collected supernatant and store at 4°C overnight.
8. On the next day, purify rRBD recombinant protein from the culture supernatant using Ni-NTA Superflow following manufacturer's instructions.
9. Concentrate purified protein using Amicon Ultra -15 concentration tubes. After protein concentration, add PBS to the concentration tubes and centrifuge again to remove imidazole in elution buffer. Calculate protein concentration and store purified protein at -80°C until use.

2. Mouse Immunization and Sample Collection

1. Pre-warm Sigma adjuvant system (SAS) to 40-45°C according to manufacturer's instructions. Add 1 mL PBS per vial and mix thoroughly.
2. Prepare protein-adjuvant emulsion according to the protocol in Table 2. Pipette calculated rRBD protein into a 1.5 mL tube. Add equal volume of SAS adjuvant to the tube and vortex vigorously for 2-3 min to form emulsion.

Hint: The volumes listed in Table 2 are for one mouse. Adjust volumes according to the actual mouse numbers used. For each group, mix together the proteins and adjuvant required for each vaccine. Always prepare one extra sample for the vaccination to ensure accuracy.

Group	**1st vaccine** (200 µL/mouse)	**2nd vaccine** (200 µL/mouse)	**3rd vaccine** (200 µL/mouse)
rRBD protein	20 µg protein in PBS (100 µL) + 100 µL SAS	10 µg protein in PBS (100 µL) + 100 µL SAS	10 µg protein in PBS (100 µL) + 100 µL SAS
PBS control	100 µL PBS + 100 µL SAS	100 µL PBS + 100 µL SAS	100 µL PBS + 100 µL SAS

Table 2. Mouse immunization protocol

3. Subcutaneously prime-immunize female BALB/c mice (4-6 weeks old, 5 mice/group), and boost twice with rRBD and SAS as indicated in Table 2. Use PBS group as the control. The site for subcutaneous injections usually chosen is the loose skin between the shoulder blades. Alternatively, the ventral abdomen is commonly used, because it is easier to inject, and may observe any leakage from the injection sites. When repeated doses of vaccines are used, it is easy to select various injection sites, preventing potential local skin reactions.

4. Bleed mice via retro-orbital with anesthetic before immunization and 10 days after each vaccination, and heat sera at 56°C for 30 min to inactivate complement. Store mouse sera at -20°C until use.

3. Neutralization Detection using Pseudovirus Neutralization Assay

1. Prepare SARS pseudovirus
    1. Split 293T cells in 100 mm tissue culture petri dishes ($2x10^6$ cells/dish) 16 h before transfection, and grow cells as above.
    2. Prepare transfection reagents as indicated in Table 1. Co-transfect a plasmid encoding SARS-CoV S protein and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) using calcium phosphate transfection reagent.
    3. Replace medium with 10 mL fresh DMEM containing 10% FBS and 1% P/S 8-10 h post-transfection. Collect supernatant containing SARS pseudovirus 72 h post-transfection.
    4. Filter pseudovirus through a 0.45 μm filter. Aliquot and store at -80°C until use.

2. SARS pseudovirus neutralization assay
    1. Split 293T cells expressing SARS-CoV receptor ACE2 (ACE2/293T) at 10^4 cells/100 μL/well in 96-well tissue culture plates 16 h before infection.
    2. Dilute SARS pseudovirus by 2-fold to detect the virus titer in ACE2/293T cells.
    3. Serially dilute mouse sera in 96-well tissue culture plates, and add equal volume of titrated SARS pseudovirus. Preincubate the plates at 37°C for 1 h.
    4. After incubation, add 100 μL of the sera-pseudovirus mixture to ACE2/293T cells, and continue to grow cells at 37°C in 5% CO_2. Add fresh DMEM 24 h later.
    5. Completely remove culture supernatants from the plates 72 h post-infection. Add 1X luciferase cell culture lysis reagent (60 μL/well), and promote cell lysis with constant shaking of plates for 1-2 h at room temperature.
    6. Transfer cell lysates (50 μL/well) into luminometer plates (Microfluor 96-well plates). Add luciferase substrate (50 μL/well) included in luciferase assay system, and detect relative luciferase activity in Ultra 384 luminometer.
    7. Calculate SARS pseudovirus neutralization titer and present as 50% neutralizing antibody titer (NT_{50}) [6].

Discussion

Cell density is an important factor affecting the efficacy of calcium phosphate-based transfection. In our experience, less than 70% confluency of the cells brings the best results. Thus, in order to improve transfection efficiency, it is recommended that cell density be kept at around 50-70% of confluency. The calcium phosphate transfection method is generally thought to be less efficient compared with other transfection methods, such as lipid transfection [16]. However, in this protocol, we used a modified calcium phosphate transfection method whereby constant and slow mixing of the transfection solution in a vortex ensures the formation of a precipitate with the appropriate size and shape, thus improving transfection efficiency.

Disclosures

No conflicts of interest declared.

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