Chapter 11

Qualitative and Quantitative Determination of MERS-CoV S1-Specific Antibodies Using ELISA

Sawsan S. Al-amri and Anwar M. Hashem

Abstract

Indirect enzyme-linked immunosorbent assay (ELISA) enables detection and quantification of antigen-specific antibodies in biological samples such as human or animal sera. Most current MERS-CoV serological assays such as neutralization, immunofluorescence, or protein microarray rely on handling of live MERS-CoV in high containment laboratories, highly trained personnel as well as the need for expensive and special equipment and reagents representing a hurdle for most laboratories especially when resources are limited. In this chapter, we describe a validated and optimized indirect ELISA protocol based on recombinant S1 subunit (amino acids 1–725) of MERS-CoV for qualitative and quantitative determination of MERS-CoV-binding antibodies.

Key words Antigens, Antibodies, Serology, ELISA, MERS-CoV, Recombinant S1 subunit

1 Introduction

Enzyme-linked immunosorbent assay (ELISA) is an immunological technique used to study the interaction between antigens and antibodies. The use of an enzyme-linked conjugate as well as substrate leads to changes in color which help to determine the presence and the quantity of substances such as peptides, proteins, antibodies, and hormones in a given sample, such as blood or urine samples [1]. The importance of validated ELISA is evident especially when dealing with pathogens requiring high containment facilities such as MERS-CoV as it could provide a rapid, simple, and cheap method for field or clinical use without the need for high containment laboratories.

Different indirect ELISAs based on MERS-CoV nucleocapsid (N) or spike (S) proteins were developed and used in epidemiological and surveillance studies [2–8]. Here, we provide a detailed protocol for indirect ELISA based on recombinant MERS-CoV S1 subunit (amino acids 1–725) for qualitative and quantitative MERS-CoV serological testing. This assay was developed and

Rahul Vijay (ed.), *MERS Coronavirus: Methods and Protocols*, Methods in Molecular Biology, vol. 2099,
https://doi.org/10.1007/978-1-0716-0211-9_11, © Springer Science+Business Media, LLC, part of Springer Nature 2020

validated using a large number of well-characterized human serum samples [8] and could be adapted by any laboratory especially that all required reagents are commercially available.

2 Materials

Prepare all solutions using deionized water and store them at room temperature or 4 °C. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 General Materials

1. 1 L graduated cylinder.
2. 100 mL graduated cylinder.
3. Weighing balance.
4. Magnetic stirrer plate.
5. Magnetic stirrers.
6. PH meter.
7. 1 L glass bottles.
8. 15 and 50 mL falcon tubes.
9. Sterile U-shaped 96-well plate.
10. Multichannel pipette.
11. Sterile disposable tips.
12. Reagent reservoirs (*see* **Note 1**).
13. MaxiSorp flat-bottom 96-well ELISA plates.
14. ELISA plate sealing covers.
15. Automated 96-well plate washer.
16. Automated microplate reader.

2.2 Solution Preparation

1. Phosphate-buffered saline (PBS; 10×): Weigh and transfer 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 to a 1 L graduated cylinder, and add about 800 mL water. Mix for about 15 min and adjust pH to 7.4 with HCl. Make up to 1 L with water. Autoclave the solution and store at room temperature.
2. PBS; 1×: Add 100 mL of 10× PBS to a 1 L graduated cylinder and complete the volume to 1 L by adding 900 mL water. Transfer the buffer to a 1 L glass bottle and store at 4 °C.
3. Coating buffer: PBS; 1× (*see* **Note 2**).
4. Wash buffer: 1× PBS containing 0.1% Tween-20 (TBST). Add 1.0 mL Tween-20 to 1 L 1× PBS (0.1% v/v). Mix and store at 4 °C.
5. Blocking buffer: 5% skim milk in PBST (*see* **Note 3**). Store at 4 °C.

6. Diluent for primary and secondary antibodies: Primary and secondary antibodies should be diluted in blocking buffer (*see* **Note 3**). Store at 4 °C.

2.3 Antigen and Conjugates

1. Recombinant MERS-CoV S1 subunit protein.
2. Primary antibody (human or animal serum samples).
3. Secondary antibody (horseradish peroxidase (HRP)-conjugated anti-human IgG antibody could be used if testing human sera).
4. KPL SureBlue tetramethylbenzidine (TMB) substrate.
5. KPL TMB BlueSTOP solution.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Qualitative Detection of MERS-CoV S1-Specific Antibodies

1. Dilute recombinant MERS-CoV S1 subunit protein (antigen) to a final concentration of 1 μg/mL using 1× PBS buffer. You need 10 mL per 96-well ELISA plate.
2. Transfer the diluted recombinant protein to a clean reagent reservoir and coat the 96-well ELISA plate with 100 μL/well using multichannel pipette.
3. Seal the plate using ELISA plate sealing cover (adhesive film) and incubate at 4 °C overnight to allow the antigen to adsorb to the well surface (*see* **Note 4**).
4. Wash the 96-well ELISA plate three times using automated 96-well plate washer with PBST. Use 350 μL wash buffer per well (*see* **Note 5**).
5. After washing, invert the plate and tap it firmly on an absorbent paper to remove any residual liquid.
6. Block the plate with blocking buffer (300 μL/well), seal the plate and incubate for 1 h at room temperature (*see* **Note 6**).
7. During incubation, dilute serum samples in blocking buffer in duplicate at a final dilution of 1:200 or 1:400 (*see* **Notes 7** and **8**).
8. Wash the plate as indicated in **steps 4** and **5**.
9. Add 100 μL of diluted serum samples or controls to each well, seal the plate, and incubate at 37 °C for 1 h (*see* **Note 9**).
10. During incubation, dilute appropriate HRP-conjugated secondary antibody in blocking solution according to manufacturer's instructions.
11. Wash the plate as indicated in **steps 4** and **5** (*see* **Note 10**).

12. Add 100 μL of diluted secondary antibody to each well and incubate at 37 °C for 1 h.
13. Wash the plate as indicated in **steps 4** and **5** (*see* **Note 11**).
14. Add 100 μL of TMB substrate to each well and incubate at room temperature for 30 min in the dark for colorimetric development (*see* **Notes 12** and **13**).
15. Stop reaction with equal volume of TMB BlueSTOP solution.
16. Read absorbance in the plate on automated microplate reader at 630 nm (*see* **Note 14**).
17. Samples with absorbance above the cutoff value of 0.34 are considered positive (assay sensitivity and specificity are 94.9% and 95.2%, respectively [8]) (*see* **Note 15**).

3.2 End-Point Titration of MERS-CoV S1-Specific Antibodies

1. Coat and block the plate as indicated in the **steps 1–6** in Subheading 3.1.
2. In a new sterile U-shaped 96-well plate, add 297 μL blocking buffer to all wells in column 1 (*see* **Note 16**).
3. Add 150 μL blocking buffer to all remaining wells in the plate (Fig. 1).
4. Add 3 μL from each serum per well in all wells in column 1 to have 1:100 dilution (Fig. 1). Test each serum sample in duplicates (*see* **Notes 7** and **8**).

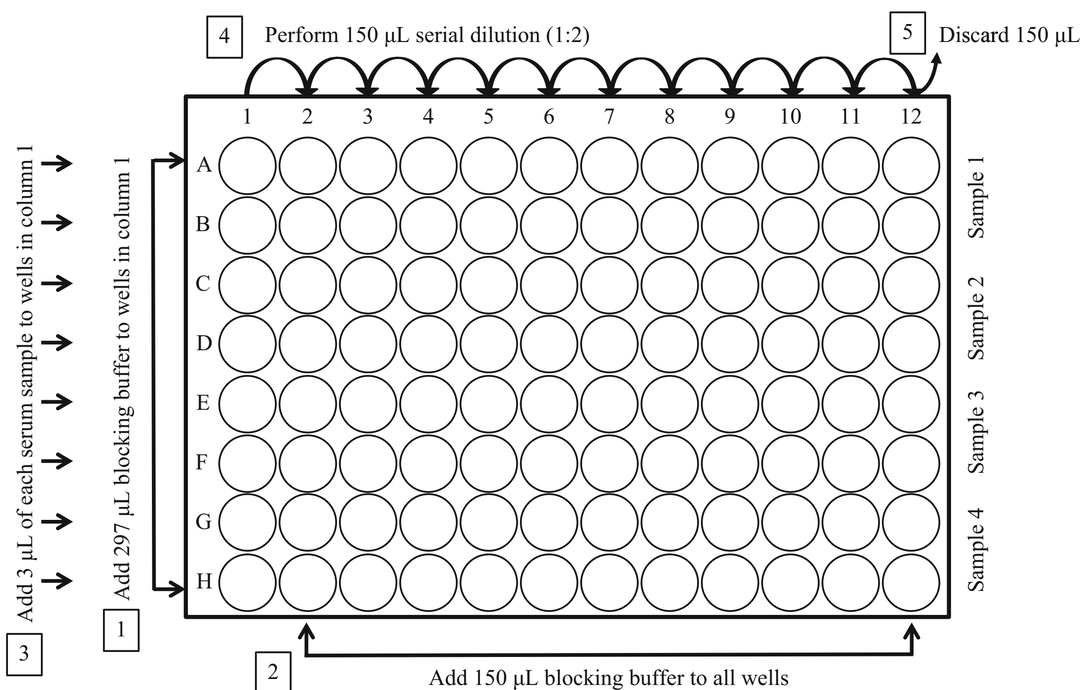

Fig. 1 Plate layout for serum sample serial dilution

5. Perform twofold serial dilutions by transferring 150 μL progressively from column to column using a multichannel pipette (Fig. 1).
6. During each dilution step mix well by pipetting eight times up and down (*see* **Note 17**).
7. Discard the final 150 μL after column 12.
8. Wash the incubated plate as indicated in **steps 4** and **5** in Subheading 3.1 to remove blocking buffer.
9. Add 100 μL from each dilution to each well using a multichannel pipette, seal the plate, and incubate at 37 °C for 1 h (*see* **Note 17**).
10. Continue using **steps 10–16** from Subheading 3.1 and save the results.
11. The last dilution needs to reach a signal equivalent to the background reading from blocking buffer without serum (*see* **Note 18**).
12. The every last dilution that gives twice the signal of the background indicates the end-point titer of the sample; otherwise, antibody titer can be determined using four-parameter logistic (4PL) regression curve in SigmaPlot or GraphPad Prism software.

4 Notes

1. Use different reagent reservoir for each buffer.
2. Alternatively, carbonate-bicarbonate buffer (50 mM), pH 9.6, could be used as coating buffer: Prepare buffer by adding 2.88 g of sodium bicarbonate ($NaHCO_3$) and 1.67 g of sodium carbonate (Na2CO3) to ~980 mL water. Adjust the pH to 9.6 with HCl if needed and complete the volume to 1 L with water.
3. Measure 100 mL of 1× PBST to a 100 mL graduated cylinder and transfer the volume to a glass bottle. Transfer 5 g skim milk powder into the bottle and stir until dissolved.
4. Coating buffer helps to bind antigen to the wells. During coating, sealing the plates will help prevent any reagents from evaporating overnight when leaving them in the refrigerator.
5. Washing the 96-well ELISA plate with PBST will help remove any unbound antigens from the wells.
6. Blocking helps in preventing nonspecific binding of detection antibodies to the microplate surface, reducing signal background and improving the signal-to-noise ratio. Blocking could be done at 4 °C for overnight.

7. Use heat-inactivated serum samples at 56 °C for 30 min.
8. Mix thawed serum samples before and after dilution with a vortex for about 10 s.
9. No primary antibody control could be included by adding 100 μL of blocking buffer per well.
10. This washing step will help removing nonspecific or unbound antibodies.
11. This washing step is critical to reduce background signal.
12. Warm TMB substrate and stop solution to room temperature before use. Never pipette directly from the bottle. Pour out needed amount into a plastic reservoir. Do not return excess to the primary container.
13. Avoid shaking.
14. Stopped reactions should be read within 30 min. TMB BlueSTOP allows the chromophore to remain blue, instead of turning yellow. If using a stop solution resulting in a yellow reaction, read the plate at 450 nm.
15. Samples with absorbance values that fall 0.26 and 0.34 should be considered "indeterminate" and should be validated with other methods if possible.
16. Do **steps 2–7** during incubation with blocking buffer.
17. Change pipette tips between wells.
18. Higher dilutions of the samples may be required in case the last dilution did not reach a signal equivalent to the background.

Acknowledgment

This work was supported by King Abdulaziz City for Science and Technology (KACST) through the MERS-CoV research grant program (number 09-1 to AMH), which is a part of the Targeted Research Program.

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