LABORATORY MANUAL

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Identification of FDMV serotype by Sandwich ELISA

User Manual

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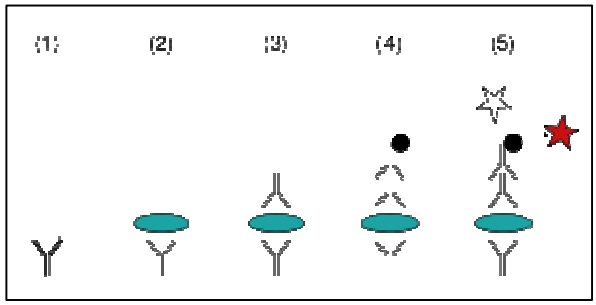
Principle:

Antigen capture sandwich ELISA for virus serotyping is highly sensitive method of virus detection and sero-typing in clinical materials. In this test plates are coated with serotype specific rabbit poly-clonal sera and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype a specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution. The steps in S-ELISA are described step by step:

1. Coating with trapping antibodies
2. Capture of target antigen
3. Tracing of captured antigens
4. Detection of bound antibodies with species specific antibodies conjugated with HRPO
5. Development of color reaction after addition of chromogen and substrate



Kit Components:

1. 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
2. Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV sera raised in rabbits.
3. Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV sera raised in guinea pig.
4. Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
5. FMDV inactivated antigens: sero-type specific inactivated freeze dried antigens
6. Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
7. Hydrogen peroxide (H_2O_2)
8. Phosphate buffer saline vials (Sigma)
9. Substrate buffer capsules

10. Coating buffer tablets

11. Tween-20

Materials needed but not provided:

1. Precision pipettes: multi channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
2. Distilled water
3. H_2SO_4
4. Wash bottle fitted with Immunowasher
5. 1 container: 1 to 2 liters
6. ELISA plate reader, 492 nm filter.
7. Photometer: Multiscan type Micro pate ELISA reader with an interference filter of 492nm and reference filter of 620nm.
8. Refrigerator: range of +2ºC to +6ºC
9. Freezer: range of -15 to -20ºC.
10. Incubator: warm wall incubator maintained at +35ºC to 37ºC.
11. pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
12. Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
13. Weighing balance

Preparation of solutions/ reagents

1. **Coating buffer:** dissolve one capsule in 100ml of distilled water. Check pH if necessary bring pH to 9.2.
2. **Washing buffer:** dissolve one PBS capsule in 1000ml of distilled water check pH and if necessary adjust to pH 7.4. Add tween-20 to final concentration of 0.1% and mix it well.
3. **Blocking Buffer for 1 plate**

   Lactalbumin hydrolysate (LAH) 450mg

Healthy rabbit serum	750µl
Healthy calf serum	750µl
Washing buffer	13.5ml

4. **Substrate Buffer:** dissolve one capsule in 100ml of distilled water. Check pH if necessary adjust pH to 5.2.

5. **Substrate solution for one plate : (prepare fresh)**

Phosphate-Citrate buffer (pH 5.0)	- 7.5 ml
Orthophenylene-diamine dihydrochloride (OPD)	- 5 mg
H_2O_2 (30% w/v)	- 4µl

Procedure

1. **Coating of ELISA wells:** Dilute all the 4 coating sera, as suggested, with coating buffer sufficient for the number of plates to be used (2ml of working dilution per serum per plate). Dispense the diluted coating sera in 50µl volumes per well as indicated later in the plate layout. Gently tap the plates to ensure that the liquid has covered the whole well area and incubate at 37ºC for 1 hour.

2. Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

3. Dispense 50µl of test sample/ antigen per well according to plate layout. In background wells dispense 50µl of blocking buffer in place of antigen and in positive controls dispense 50µl of respective controls provided. Cover the plate with lid and incubate at 37ºC for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

4. Dilute all the 4 tracing sera, as suggested, with blocking buffer sufficient for the number of plates required. Dispense 50µl volumes per well as indicated in plate layout.

5. Incubate the plates at 37ºC for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

6. Prepare working dilution of conjugate, as suggested, in blocking buffer in sufficient volume. Dispense 50µl of diluted conjugate to all the wells of ELISA plate and incubate the plates for 1 hour followed by washing as described in step 2.

7. Prepare substrate solution, as described, and dispense 50µl of substrate solution to each well. Cover the plate with lid and incubate at 37ºC for 15 min in dark. Stop the color reaction by adding 50µl of stopping solution to each well.
8. Measure the optical density of each well at wavelength of 492nm and reference wavelength of 620nm in ELISA reader.

Interpretation of test sample result

Performance of a test can be determined from positive reaction of the known antigens employed in the test and a clear background reaction of all the sera. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).

When a test is conducted properly, the background reaction of all the sera provided will lie between 0.00 to 0.02 OD with substrate. If the reactivity (OD value) of the test antigen with a particular FMDV type serum is $\geq$ 0.10 (with no heterologous reactivity), then antigen in question can be identified as belonging to that type (OD limit 0-2.5).

Precautions:

1. All the glass wares to be used should be clean and sterile.
2. Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH everday before use.
3. Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
4. Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
5. Do not stack plates one over other in incubator.
6. Prepare fresh substrate solution every time.
7. Do not store prepared buffers more than 1 month.
8. Check pH of every buffer every day before starting the test.

Trouble shooting:

1. **No OD in any wells including positive controls:**
    - Improper reconstitution/ preparation of reagents/ buffers
        - Cross check the composition of buffers and reagents
    - Use of conjugate other than anti-guinea pig conjugate.
        - Cross check the conjugate used
    - Lower pH of washing buffer
        - Cross check the pH of washing buffer
2. **Even OD in all the wells**
    - Cross contamination of tips used for conjugate and substrate solution.
    - Coating of wells with tracing antibodies (in spite of coating serum).
3. **Low OD**
    - Improper dilution of reagents
        - Cross check the dilutions recommended for each batch
    - Expiry of reagents of kits
        - Cross check the expiry of reagents
    - High percentage of tween-20 in washing buffer
        - Cross check the tween-20 concentration in washing buffer
    - Lower/ higher temperature in incubator
        - Cross check the temperature of incubator