TRAINING SCHOOL

ONWARD ratHEV

LABORATORY PROTOCOL SERIES

PROTOCOL 2

Enzymatic immunoassay (EIA) for ratHEV

Serology

COST Action CA24140

Hosted by the Clinical Virology and Zoonoses group, IMIBIC

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Enzymatic immunoassay (EIA) for ratHEV

An enzymatic immunoassay was developed to assess exposure to ratHEV (Situ et al., *JHEP Reports*, 2023). The assay requires each serum sample to be tested in parallel against HEV (*P. balayani*) and ratHEV (*R. ratti*) antigens to differentiate antibody responses to each hepevirus.

- **Reagents/Equipment**

1. **Sample dilution buffer**: 1% casein (Ref C5890, Sigma) in PBS with 0.1% tween20:
    a. 10g casein in 1L PBS by autoclave
    b. After cooling, add 1 ml Tween 20
2. **Wash buffer**: 0.1 % tween 20 in PBS
    a. 1 ml Tween20 + 1L PBS
3. **HRP conjugate buffer**: 20% Goat serum (Ref: 16210072 Thermofisher) in PBS with 0.5% tween 20.
    a. 20 ml goat serum + 79.5 ml PBS + 0.5ml tween 20
4. **Secondary Antibody**: 1: 8000 dilution goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody HRP (Ref A18811, Invitrogen)
    a. 1.25ul Secondary Antibody add in ~10ml HRP conjugate buffer
5. **TMB Chromogen Solution (for ELISA)** (Ref 002023, Thermofisher)
6. **Stop buffer:** 0.3 M H2SO4
    a. 16ml H_2SO_4 + 984ml H_2O
7. **ELISA plates** (Ref 504201, NEST)
8. **ELISA cover plates** (XXXXXXXXX)
8. **Microplate reader**: Absorbance detection wavelengths: 450nm and 620nm

- **Protocol**

1. **Preparation**: Allow the reagents to reach room temperature 18-30°C
2. **Add diluent**: Add 200µl of Sample dilution buffer into 1.5ml tube
3. **Add specimens:** Add 1 µL of the positive control or specimen to the corresponding 1.5 mL tube containing 200 µL of sample dilution buffer. Mix thoroughly and transfer 100 µL of the diluted sample to the corresponding well. Each specimen must be tested in parallel on both the HEV and ratHEV plates.
4. **Incubating**: Cover the plate with the plate cover and incubate at 37°C for 1 hour
5. **Washing**: At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with 300µl Wash Buffer. After the final washing cycle, turn down the plate onto blotting paper or clean towel, and tap it to remove any remainders
6. **Adding HRP-Conjugate**: Add 100µl of HRP-Conjugate buffer with Secondary Antibody (1:8000) into each well
7. **Incubating**: Cover the plate with the plate cover and incubate at 37°C for 30 minutes
8. **Washing**: At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with 300µl Wash Buffer. After the final washing cycle, turn down the plate onto blotting paper or clean towel, and tap it to remove any remainders
9. **Coloring**: Add 100µl TMB into each well, mix gently. Incubate the plate at room temperature for 5 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and HEV IgG positive specimen wells
10. **Stopping Reaction**: Add 100µl of Stop buffer into each well and mix gently. Intensive yellow color develops in Positive control and HEV IgG positive specimen wells

11. **Measuring the Absorbance**: Calibrate the plate reader and read the absorbance at 450nm. If a dual filter instrument is used, set the reference wavelength at 620nm. (Note: read the absorbance within 10 minutes after stopping the reaction)

- ## Interpretation

HEV EIA result positive IF OD ≥ 0.437	**ratHEV EIA result positive IF OD ≥ 0.864**	**Interpretation**
Negative	Negative	Seronegative for hepeviruses
Positive	Negative	Past exposure to HEV
Negative	Positive	Past exposure to ratHEV
Positive	Positive	Assess HEV/ratHEV OD ratio: If ≥ 2.947, consistent with exposure to HEV, but additional ratHEV exposure is possible If < 2.947, consistent with exposure to ratHEV, but additional HEV exposure is possible