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Project Page Link: https://review.jove.com/files_upload.php?src=21317108

Title: Hepatitis E Virus Fluorescence Reduction Neutralization Test for Measuring Neutralizing Antibody Titers

Authors and Affiliations: Shabnam Ansari¹, Umang Berry¹, Shalimar², Baibaswata Nayak², Milan Surjit¹

¹Virology Laboratory, Centre for Virus Research, Therapeutics and Vaccines, Translational Health Science and Technology Institute, NCR Biotech Science Cluster

²Department of Gastroenterology, All India Institute of Medical Sciences

Corresponding Authors:

Milan Surjit milan@thsti.res.in

Email Addresses for All Authors:

shabnam@thsti.res.in
umangberry@thsti.res.in
drshalimar@gmail.com
baibaswat@gmail.com
milan@thsti.res.in

Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 15

Number of Shots: 47

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Shabnam Ansari:** The main purpose of fluorescence reduction-based neutralization test is to quantify the neutralization potential of vaccine candidates and antibodies.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Umang Berry:** HEV's non-cytopathic nature limits PRNT applicability; this protocol uses immunostaining-based FRNT to visualize infected cells and determine antibody NT50.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Shabnam Ansari:** This protocol can be used to study neutralization potential of anti-HEV and measure their neutralization titer 50%.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Shabnam Ansari:** The most critical steps of this protocol include isolating a high-titer virus for infection, proper cell staining, and acquisition of high-resolution images.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Umang Berry**: Alternative methods such as RT-qPCR and ELISA can be used to evaluate neutralization titer, but they have some limitations.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Ethics Committee (IEC) at the All India Institute of Medical Sciences (AIIMS)

Protocol

NOTE: The videographer has provided a sheet with video file names matched to the shot number and also indicated final takes. Please refer to this document on the project page for logging https://review.jove.com/files/ftp_upload/69892/Post-Shoot-Notes_69892.xlsx

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Hepatitis E Virus (HEV) Infection and Immunofluorescence Analysis

Demonstrator: Umang Berry

- 2.1. To begin, obtain the freshly split Huh7 cells [1] and count the cells [2]. Place a microscopy-grade glass coverslip in a 12-well plate [3]. After adding 1 milliliter complete media containing DMEM (*D-M-E-M*) supplemented with 10 percent FBS (*F-B-S*), seed 0.2×10^6 (*Zero point two million*) cells per well [4]. **NOTE:** VO added for the extra shot
 - 2.1.1. WIDE: Talent bringing freshly split Huh7 cells to the workspace. Added shot(final takeC4649): Cells were counted using Millipore Scepter 3.0 sensor cell counter. *Videographer: Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent placing a glass coverslip into the 12-well plate.
 - 2.1.3. Talent seeding cells into the wells with media.
- 2.2. Add 500 microliters serum-free media containing 1.8×10^4 (*One point eight times ten to the power of four*) genome copies of purified g1-HEV (*G-1-H-E-V*) to the seeded cells [1]. Incubate at 37 degrees Celsius with 5 percent carbon dioxide for 1 hour [2].
 - 2.2.1. Talent adding 500 μ L of virus inoculum to the cells.
 - 2.2.2. Talent placing the plate into the incubator.
- 2.3. Use 500 microliters DPBS (*D-P-B-S*) to wash the cells three times [1-TXT]. Add 2 milliliters of DMEM supplemented with 2 percent FBS to the wells [2]. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 72 hours [3].
 - 2.3.1. Shot of the talent aspirating the media and washing cells with buffer. **TXT: DPBS: Dulbecco's Phosphate-Buffered Saline** **Videographer's NOTE:** This was split into part a and b

- 2.3.2. Talent adding fresh media to the cells.
- 2.3.3. Talent placing the plate into the incubator.
- 2.4. Wash the cells with 500 microliters DPBS [1]. Incubate it in a shaker for 5 minutes at room temperature [2]. Repeat the wash and incubation cycle three times [3].
 - 2.4.1. Talent washing the cells with 500 μ L of DPBS. *Videographer: Please capture multiple reusable takes of this shot.*
 - 2.4.2. Talent placing the plate on shaker at room temperature.
 - 2.4.3. *Reuse 2.4.1*
- 2.5. Add 500 microliters 4 percent paraformaldehyde to the cells and incubate it at room temperature for 20 minutes [1]. Then, perform the wash-incubate cycle as demonstrated earlier [2]. Incubate the cells in buffer for 30 minutes at room temperature [3].
 - 2.5.1. Talent adding 4% paraformaldehyde to the cells and leaving it undisturbed.
 - 2.5.2. *Reuse 2.4.1*
 - 2.5.3. Talent leaving cells in buffer.
- 2.6. Add 250 microliters blocking solution containing 5 percent normal goat serum, 0.5 percent bovine serum albumin, and 0.3 percent Triton X-100 in buffer [1]. Incubate in a shaker for 1 hour at room temperature [2].
 - 2.6.1. Talent adding 250 μ L blocking solution to the wells.
 - 2.6.2. Talent placing the plate on shaker at room temperature.
- 2.7. Remove the blocking solution [1] and wash once with 500 microliters DPBS buffer [2]. Add 250 microliters of primary antibody solution at 1 to 500 dilution [3]. Incubate the cells overnight at 4 degrees Celsius [4]. Perform the wash-incubate cycle as demonstrated earlier [5].
 - 2.7.1. Talent aspirating blocking solution.
 - 2.7.2. Talent washing cells once with 500 μ L buffer.
 - 2.7.3. Talent adding 250 μ L primary antibody
 - 2.7.4. Talent placing the plate for cold incubation.
 - 2.7.5. *Reuse 2.4.1*
- 2.8. Add 100 microlitres of secondary antibody at 1 to 1000 dilution and incubate for 1 hour at room temperature in a humified chamber [1]. Perform the wash-incubate cycle as demonstrated earlier [2]. Add 10 microliters antifade reagent with DAPI (*dapi*) onto a glass slide [3]. Mount the coverslip with cells facing the reagent while avoiding air

bubbles [4]. Seal the coverslip with nail polish to preserve it for confocal microscopy visualization [5].

- 2.8.1. Talent adding secondary antibody to the cells and placing it on shaker for 1 h at room temperature. **Videographer's NOTE: This was split into part a and b**
- 2.8.2. *Reuse 2.4.1*
- 2.8.3. Talent placing antifade reagent with DAPI on a glass slide.
- 2.8.4. Talent mounting the coverslip onto the slide with the cells facing the reagent.
- 2.8.5. Talent sealing the edges of the coverslip with nail paint.

3. Fluorescence Reduction Neutralization Test (FRNT)

Demonstrator: Shabnam Ansari

- 3.1. Obtain the freshly split Huh7 cells [1]. Place a microscopy-grade glass coverslip in a 12-well plate [2]. Seed 0.2×10^6 cells per well in 1 milliliter complete media [3].
 - 3.1.1. *Reuse 2.1.1*
 - 3.1.2. *Reuse 2.1.2*
 - 3.1.3. *Reuse 2.1.3*
- 3.2. A day later, obtain anti-ORF2 and rabbit immunoglobulin G antibodies in serum-free medium at a starting concentration of 25 micrograms per milliliter [1]. Perform successive one-to-ten dilutions up to a final dilution of ten to the power of minus five [2]. Add 1.8×10^4 genome copies of purified g1-HEV to each antibody dilution [3].
 - 3.2.1. Talent keeping the antibodies at a starting concentration of 25 micrograms per milliliter ready **Videographer's NOTE: This was split into part a and b**
 - 3.2.2. Shot of the talent preparing serial dilutions in tubes.
 - 3.2.3. Talent adding the virus to each of the dilution tubes.
- 3.3. Incubate the antibody-virus mixture at 37 degrees Celsius with 5 percent carbon dioxide for 1 hour [1]. Wash the cells with 500 microliters of DPBS [2].
 - 3.3.1. Talent placing tubes into the incubator.
 - 3.3.2. Talent washing cells with 500 μ L of DPBS.
- 3.4. Add the antibody-virus mixture to the cells [1]. Incubate at 37 degrees Celsius with 5 percent carbon dioxide for 1 hour [2]. Wash the cells with 500 microliters buffer [3].

After adding DMEM with 2 percent FBS, incubate for 72 hours at 37 degrees Celsius with 5 percent carbon dioxide [4].

- 3.4.1. Talent adding mixture to wells.
- 3.4.2. Talent placing plate into incubator.
- 3.4.3. Talent washing cells with 500 µL of DPBS. **Videographer's NOTE:** This was same as 3.3.2
- 3.4.4. Talent placing plate into incubator. **Videographer's NOTE:** This was same as 3.4.2

3.5. Process the cells for immunofluorescence staining as demonstrated previously [1]. After acquiring the fluorescence images, open the Image-J software [2]. Select **File** and **open** the image [3]. Then, select **Image, Type** and click **8-bit** to convert the RGB image to 8-bit format [4].

- 3.5.1. Representative shot of the talent washing the cells with 500 µL DPBS.
- 3.5.2. SCREEN: Screen-3.5.2and 3.5.3. 00:00-00;03
- 3.5.3. SCREEN: Screen-3.5.2and 3.5.3. 00:04-00;08.
- 3.5.4. SCREEN: Screen-3.5.2and 3.5.3. 00:09-00;13.

3.6. Adjust the threshold by selecting **Image** and choosing **Adjust**, then **Threshold**, setting the lower level to 40 and upper level to 255 [1]. Select **Analyze**, choose **Analyze Particles** and enable display results, clear results, summarize, exclude on edges, and composite regions of interest [2]. Then click **Ok** to obtain fluorescent spot counts [3].

- 3.6.1. SCREEN: Screen-3.6.1.
- 3.6.2. SCREEN: Screen-3.6.2 and 3.6.3. 00:00-00:17
- 3.6.3. SCREEN: Screen-3.6.2 and 3.6.3. 00:21-00:25.

3.7. Calculate percent neutralization, and NT₅₀ (*N-T-50*) as the antilog of Log₁₀ NT₅₀ (*log ten N-T fifty*) [1] using the formula provided [2]. Plot the graph showing mean and standard deviation of all fields [3].

- 3.7.1. SCREEN: Screen-3.7.1
- 3.7.2. TEXT ON PLAIN BACKGROUND:

% Neutralization:

% Neutralization= 100-[((ORF2-Positive cells in dilution)/(ORF2-positive cells in virus control)) X 100]

NT₅₀:

$\log_{10}(\text{NT}_{50}) = \log_{10}D1 + (50 - N1 / N2 - N1) \times (\log_{10}D2 - \log_{10}D1)$

Where, D1 = Dilution at which % Neutralization is below 50

D2 = Dilution at which % Neutralization is above 50

N1= % Neutralization below 50

N2= % Neutralization above 50

3.7.3. SCREEN: 00:02-00:15 and 00:25-00:27.

Results

4. Results

4.1. A significant level of g1-HEV ribonucleic acid was detected in infected cells compared to mock-infected cells [1].

4.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the bar under "G1-HEV Infection: +", which is much taller than the bar under "-"*

4.2. Immunofluorescence assay using anti-ORF2 antibody confirmed ORF2 expression in the infected Huh7 cells [1].

4.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the bright red signal in the panel labelled "G1-HEV infected"*

4.3. The 50% neutralization titer of the anti-ORF2 antibody was estimated to be 2.1×10^3 [1].

4.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar labelled "Anti-ORF2," which is positioned around 10^3 on the vertical axis*

4.4. Representative images showed the appearance of mock-infected [1] and g1-HEV-infected Huh7 cells in the fluorescence reduction neutralization test [2].

4.4.1. LAB MEDIA: Figure 2D. *Video editor: Highlight the panel labelled "Mock Infected"*

4.4.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the panel labelled "G1-HEV infected"*

1. Hepatitis E Virus (HEV)

Pronunciation link: <https://www.merriam-webster.com/dictionary/hepatitis>

IPA: /ˌhep.əˈtaɪ.təs/

Phonetic Spelling: hep·uh·tai·tuhs

2. Immunofluorescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>

IPA: /ɪˌmjuː.nouˈflʊˈres.əns/

Phonetic Spelling: ih·myoo·noh·fluo·res·uhns

3. Microscopy

Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>

- IPA: /maɪˈkraː.skə.pi/
Phonetic Spelling: my·kraa·skuh·pee
4. Coverslip
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /ˈkʌv.ər.slɪp/
Phonetic Spelling: kuv·er·slip
 5. DMEM
Pronunciation link: No confirmed link found
IPA: /ˌdiː.ɛm.iːˈɛm/
Phonetic Spelling: dee·em·ee·em
 6. Genome
Pronunciation link: <https://www.merriam-webster.com/dictionary/genome>
IPA: /ˈdʒiː.noʊm/
Phonetic Spelling: jee·nohm
 7. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪn.kjəˌbeɪt/
Phonetic Spelling: in·kyuh·bayt
 8. Carbon Dioxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/carbon%20dioxide>
IPA: /ˌkɑːr.bən daɪˈɑːk.saɪd/
Phonetic Spelling: kar·bun dye·aak·side
 9. DPBS
Pronunciation link: No confirmed link found
IPA: /ˌdiː.piː.biːˈɛs/
Phonetic Spelling: dee·pee·bee·ess
 10. Paraformaldehyde
Pronunciation link: <https://www.merriam-webster.com/medical/paraformaldehyde>
IPA: /ˌpɛr.əˈfɔːrˈmæl.dəˌhaɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
 11. Bovine
Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>
IPA: /ˈboʊ.vɪn/
Phonetic Spelling: boh·vine
 12. Triton X-100
Pronunciation link: No confirmed link found
IPA: /ˈtraɪ.təːn ɛks wʌn ˈhʌn.drəd/
Phonetic Spelling: try·ton ex one hundred
 13. Antibody
Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>
IPA: /ˈæn.tɪˌbɑː.di/
Phonetic Spelling: an·tee·baa·dee
 14. Antifade
Pronunciation link: No confirmed link found

- IPA: /ˌæn.tiˈfeɪd/
Phonetic Spelling: an·tee·fayd
15. DAPI
Pronunciation link: <https://www.howtopronounce.com/dapi>
IPA: /ˈdæpi/
Phonetic Spelling: dap·ee
16. Confocal
Pronunciation link: <https://www.merriam-webster.com/medical/confocal>
IPA: /kɑːnˈfoʊ.kəl/
Phonetic Spelling: kon·foh·kuhl
17. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈres.əns/
Phonetic Spelling: fluo·res·uhns
18. Immunoglobulin
Pronunciation link: <https://www.merriam-webster.com/medical/immunoglobulin>
IPA: /ɪˌmjuː.noʊˈɡləːbjə.lɪn/
Phonetic Spelling: ih·myoo·noh·glaa·byuh·lin
19. Dilution
Pronunciation link: <https://www.merriam-webster.com/dictionary/dilution>
IPA: /daɪˈluːʃən/
Phonetic Spelling: dye·loo·shuhn
20. Ribonucleic Acid
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleic%20acid>
IPA: /ˌraɪ.boʊ.nuːˈkliː.ɪk ˈæs.ɪd/
Phonetic Spelling: rye·boh·noo·klee·ik as·id
21. ImageJ
Pronunciation link: No confirmed link found
IPA: /ˈɪm.ɪdʒ dʒeɪ/
Phonetic Spelling: im·ij·jay
22. Threshold
Pronunciation link: <https://www.merriam-webster.com/dictionary/threshold>
IPA: /ˈθreʃ.hoʊld/
Phonetic Spelling: thresh·hohld
23. Neutralization
Pronunciation link: <https://www.merriam-webster.com/dictionary/neutralization>
IPA: /ˌnuː.trə.ləˈzeɪ.ʃən/
Phonetic Spelling: noo·truh·luh·zay·shuhn
24. Antilog
Pronunciation link: <https://www.merriam-webster.com/dictionary/antilogarithm>
IPA: /ˌæn.tɪ.ləːɡ/
Phonetic Spelling: an·tee·log
25. Standard Deviation
Pronunciation link: <https://www.merriam-webster.com/dictionary/standard%20deviation>

IPA: /'stæn.dərd ,di:.vi'eɪ.fən/

Phonetic Spelling: stan·derd dee·vee·ay·shuhn

26. ORF2

Pronunciation link: No confirmed link found

IPA: /oo ,ɑ:r ,ɛf 'tu:/

Phonetic Spelling: oh·ar·ef two

27. Fluorescent

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>

IPA: /flʊ'res.ənt/

Phonetic Spelling: fluo·res·uhnt

28. Mock-infected

Pronunciation link: No confirmed link found

IPA: /mɑ:k ɪn'fek.tɪd/

Phonetic Spelling: maok in·fek·tid