Quality Biological™

Neutral Red Solution (0.33%) for Plaque Assay

Introduction

The plaque assay measures virus concentration expressed in infectious units; it is used to determine the viral titer of an infectious sample. A confluent monolayer of host cells is infected with serial dilutions of a lytic virus sample. The infected cells are then immobilized with an overlay material to prevent virus spread and restrict virus growth to the site of initial infection. During incubation, the viral replication and cell death is restricted to the surrounding monolayer leading to plaque formation. Cells are then stained with neutral red to enhance plaque visualization. The virus titer or infectious dose is expressed in plaque-forming units or PFU per milliliter of sample (PFUs/mL). The PFU/mL number represents the concentration of infectious virus particles in the sample based on the assumption that each plaque formed is representative of an initial infection by one infectious virus particle.

Components and Recommended Storage

Component	Catalog No.	Format	Storage *
Neutral red solution 0.33 g/L in ultra-pure water	115-341-061	100 ml	4-8°C

* It is recommended to keep the neutral red solution protected from light as extended exposure to light might lead to degradation.

Protocol

1. Material:

- Host cells: cells susceptible to infection by the virus sample tested.
- Cell maintenance medium and infection medium: Cell maintenance medium and infection medium: the cell maintenance medium is the medium in which the hosts cells are regularly passaged and expanded, generally supplemented with 10% FBS. The infection medium is the same medium supplemented with 2% FBS.
- Trypsin-EDTA solution (**Quality Biological Trypsin (0.25%)-EDTA (0.02%) #118-093-721EA**).
- DPBS without calcium and magnesium (**Quality Biological #114-057-131**).

7581 Lindbergh Drive ● Gaithersburg, MD 20879
(301) 840-9331 ● Toll Free (800) 443-9331 ● Fax (301) 840-0743
Email: customerservice@qualitybiological.com ● Web Site: www.qualitybiological.com

- Overlay diluent:
  500 mL EMEM (2X) without phenol red and L-Glutamine (**Quality Biological #115-073-101**).
  40 mL FBS (**Quality Biological FBS, USDA Approved Origin #110-001-101**).
  10 mL L-Glutamine 200mM (**Quality Biological L-Glutamine 200mM #118-084-721EA**).
  10 mL Non-Essential Amino Acids (**Quality Biological MEM NEAA (100X) #116-078-721EA**).
  7.5 mL sodium bicarbonate (**Quality Biological Sodium Bicarbonate Solution 7.5% #118-085-721EA**).
- 2% Noble agar (see notes).
- Neutral red solution (**Quality Biological #115-341-061**)

2. Samples preparation:

a. Host cells:

- Maintain host cells in cell culture flasks containing cell maintenance medium in an incubator at 37°C with 5% CO_2.
- Trypsinize a confluent flask of host cells and bring the volume to 20 mL with cell maintenance medium. Count cells and prepare a cell suspension at a density of $3x10^5$ cells/mL in cell maintenance medium. Seed four 6-well plates with 2 mL of cell suspension ($6x10^5$ cells/well), each plate will be one replicate. Incubate in an incubator at 37°C with 5% CO_2 overnight to achieve 95-100% confluency.

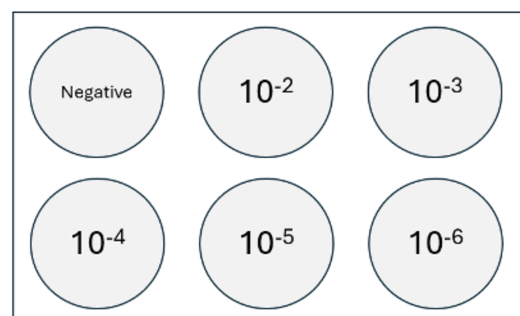
b. Viral sample:

- Serial dilute virus sample six times in infection medium (recommended to prepare 10-fold dilutions such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} including one negative sample containing infection medium only) to obtain 400-500 µL for each dilution (minimum volume required is 100 µL per dilution x 4 replicates).

3. Assay method

a. Infection:

- Label the 6-well plates seeded with the host cells as indicated.
- Remove medium from the wells leaving about 100 µL to prevent host cells from drying.
- Rinse cells with 1 mL of DPBS leaving about 100 µL to prevent host cells from drying.
- Dispense 100 µL of infection medium in well "Negative".
- Dispense 100 µL of diluted viral sample into designated wells.



7581 Lindbergh Drive • Gaithersburg, MD 20879
(301) 840-9331 • Toll Free (800) 443-9331 • Fax (301) 840-0743
Email: customerservice@qualitybiological.com • Web Site: www.qualitybiological.com

- Incubate in an incubator at 37°C with 5% CO_2 for 1 hour gently rocking every 15 minutes. Do not swirl the plate but rather rock front-to-back and side-to-side to distribute the viral sample across the host cells monolayer.

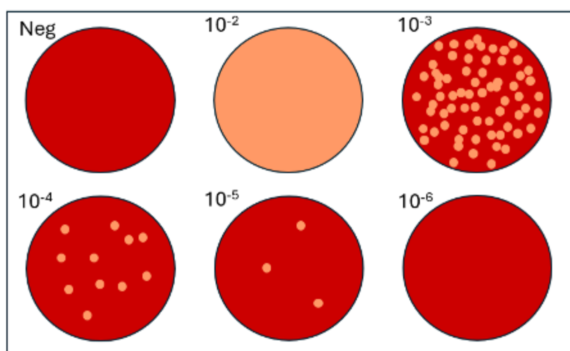
b. Primary overlay:

- Prepare the primary overlay by dissolving 2% Noble agar in warm overlay diluent; this step can be achieved by using a microwave for better results. Make sure to let the agar solution cool down before adding it to the plates to prevent cell damage.
- Add 3 ml of primary overlay to each well of the plate. Allow primary overlay to solidify and incubate the plate in an incubator at 37°C with 5% CO_2 for 48 hours.

c. Secondary overlay and staining:

- Prepare the secondary overlay by dissolving 2% Noble agar in warm overlay diluent; this step can be achieved by using a microwave for better results.
- Add neutral red solution to a final concentration of 0.01%. The volume of neutral red solution = total volume of secondary overlay x 0.03. Mix the solution to ensure even distribution of the neutral red.
- Add 2 mL of secondary overlay to each well of the plate. Allow secondary overlay to solidify and incubate the plate in an incubator at 37°C with 5% CO_2 overnight.

4. Enumeration of plaques and titer calculation:



- The plaques will appear as light orange-pink circles on a brownish red background of cells. The negative control will be a uniform monochrome layer used as color reference.
- Record the number of plaques identified per well at each dilution

- Calculate the average number of plaques = sum of plaques from 4 replicates of the same dilution/4
- Calculate the virus titer = average number of plaques calculated above/(dilution factor x 0.1 mL)

7581 Lindbergh Drive ● Gaithersburg, MD 20879
(301) 840-9331 ● Toll Free (800) 443-9331 ● Fax (301) 840-0743
Email: customerservice@qualitybiological.com ● Web Site: www.qualitybiological.com

Notes

- Always wear appropriate personal protective equipment including lab coat, gloves, and safety glasses when handling the neutral red solution and infectious material.
- This protocol consists of general guidelines to perform a plaque assay; it may be necessary to optimize conditions, such as culture medium, overlay diluent, number of days post-infection to enumerate plaques.
- It is recommended to use Noble agar instead of regular agar; Noble agar is a bleached and washed derivative of agar resulting in a whiter overlay allowing for a better visualization.
- It is recommended to perform preliminary testing to determine the optimal conditions for generating and enumerating plaques.
- After extended storage of the neutral red solution, a precipitate might occur in the form of small particles. If the neutral red solution contains precipitates, use the supernatant only, or filter the solution to remove the precipitates.
- The Quality Biological neutral red solution is for research or further manufacturing use only, not for diagnostics or therapeutic use.

References

- Martin S.J. (1978). The Biochemistry of Viruses. Cambridge University press. ISBN 0-12-402033-X
- Baer A. & Kehn-Hall K. (2014). Viral concentration determination through plaque assays: using traditional and novel overlay systems. Journal of Visualized Experiments, 93, e52065. doi:10.3791/52065.
- Burnett L.C. et al. (2009) Biosafety: guidelines for working with pathogenic and infectious microorganisms. Current Protocols in Microbiology. doi:10.1002/9780471729259.mc01a01s13