

Measuring Genetic Robustness in Vesicular Stomatitis Virus

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[Abstract] Genetic robustness is the ability of a genome to incorporate mutations with the result of no fitness changes. Thus, more robust viruses have an increased neutral mutation rate. This property is particularly important in RNA viruses due to their high mutation rates. The most direct way of measuring robustness in vesicular stomatitis virus (VSV) is to carry out clonal analysis of populations: randomly isolating individual VSV strains (plaques), measuring the fitness of each one and generating fitness distributions (Novella *et al.*, 2010). A second possibility is to carry out multiple replicates of repeated plaque-to-plaque passages, determining fitness in progeny populations and generating fitness distributions (Novella *et al.*, 2010). Depending on the expected differences, the former may require hundreds of determinations, while the latter may require tens of determinations. A third approach consists of increasing the mutation rate of populations under analysis to magnify any differences that may exist and, instead of measuring fitness, measuring survival (Novella *et al.*, 2013). One caveat of this method is that changes in survival can also be explained by changes in polymerase fidelity. For that reason, it is important to perform complementary experiments, in this case quantifying mutant frequency.

Materials and Reagents

1. Test and reference VSV strains

Note : The former is the strain under investigation, the latter is the control (typically the progenitor).

2. 10x Trypsin/EDTA (Life Technologies, Gibco®, catalog number: 15400)
3. Baby hamster kidney cells (BHK-21)
4. I1 Monoclonal antibody (I1Mab) hybridoma (Holland *et al.*, 1991) (ATCC, catalog number: CRL-2700)

Note: This antibody recognizes the G glycoprotein of VSV.

5. I14 Mab (Holland *et al.*, 1991) (<https://www.kerast.com/p-172-hybridoma-ie9f9-i14.aspx>)

Note: This antibody recognizes the G glycoprotein of VSV.

6. Proteose peptone No. 3 (PP3) (BD Difco™, catalog number: 212230) (12 g/L in dH₂O, autoclaved)
7. Bovine Calf Serum (BCS) (Life Technologies, Gibco®, catalog number: 16170-078)

Note: BCS is a good choice to carry out plaque assays and it is a lot cheaper than FBS.

8. Fetal Bovine Serum (FBS) (Life Technologies, Gibco®, catalog number: 10437)
9. 10% CO₂
10. 5-Fluorouracyl (5-FU) (Sigma-Aldrich, catalog number F-6627) (10 mg/ml in ethanol, filtered)
11. Agarose (Lonza, Seqplaque™ GTG™, catalog number: 50111) (40 g/L in dH₂O, autoclaved)
12. 4.2% bicarbonate
13. Penicillin/streptomycin mixture (Mediatech, Cellgro®, catalog number: 30-004-CI)
14. Saline solution (see Recipes)
15. Minimal Essential Medium with Hank's salts (MEM-H) (Mediatech, Cellgro®, catalog number: 50-019-PB) (see Recipes)
16. Crystal violet solution (see Recipes)

Equipment

1. T25 plug-seal flasks (CytoOne®, catalog number: CC7682-4325)
2. 5 ml and 25 ml pipetes
3. Plugged Pasteur pipetes
4. 1.5 ml tubes
5. Type II biosafety hood
6. 37 °C, CO₂ cell culture incubator
7. Transilluminator (optional)

Procedure

1. Make I1 Mab stock and titrate I1 and I14 antibodies; use at enough concentration to produce full inhibition of wild type. Alternatively, I1 Mab can be purchased from Kerfast (<https://www.kerfast.com/p-171-hybridoma-8g5f11-i1.aspx>).

Notes:

- a. *Because of high mutation rates leading to high frequency of antibody-resistance mutants, viral stocks can never be fully neutralized. The goal of Mab titration is to produce plaque assays with less than 10⁻⁴ (if viral stocks are neutralized) or less than 10⁻³ (if the Mab is added to the overlay medium) mutants/wt.*
- b. *Antibodies other than I14 (<https://www.kerfast.com>) can be used to calculate mutant frequencies.*

2. BHK-21 cells are washed twice with saline solution, trypsinized and $0.8-1.0 \times 10^5$ are seeded in T25 flasks with MEM-H supplemented with 7% BCS and 0.06% PP3. The flasks are gassed for 2 sec with 10% CO₂ using a plugged Pasteur pipette. Caps are locked.
3. The cells are incubated for 24 h to produce monolayers 90% confluent.
4. On day 2 cell monolayers are treated with no mutagen (mock) or with the mutagen 5-FU at a range of concentrations between 1 and 100 µg/ml for 6 h at 37 °C (5, 10, 25, 50 and 100 µg/ml).
5. Dilute viral stocks in MEM-H+FBS as needed to produce a 10^6 plaque forming units (PFU)/ml solution.
 - a. Use 200 µl to infect a BHK-21 monolayer for each 5-FU concentration. Incubate 10 min at room temperature (RT) and 40 min at 37 °C. Add 5 ml of MEM-H+FBS+5-FU (Figure 1, right).
 - b. From the 10^6 PFU/ml solution carry out additional, 10-fold, 100-fold and 1000-fold dilutions. Use each of the three dilutions (10^5 , 10^4 and 10^3 PFU/ml solutions) to infect at least 3 BHK-21 monolayers with 200 µl of the dilution to carry out plaque assay in the presence of I1Mab. Do not neutralize the mixture with I14Mab prior to infection, as it will result in incorrect data due to phenotypic mixing and hiding (Valcarcel and Ortin, 1989; Holland *et al.*, 1989). Incubate 10 min at room temperature (RT) and 40 min at 37 °C. Prewarm a mixture of MEM-H+FBS and I14Mab, add agarose to a final concentration of 0.2%, and add 5 ml of mixture to flasks (Figure 1, left).

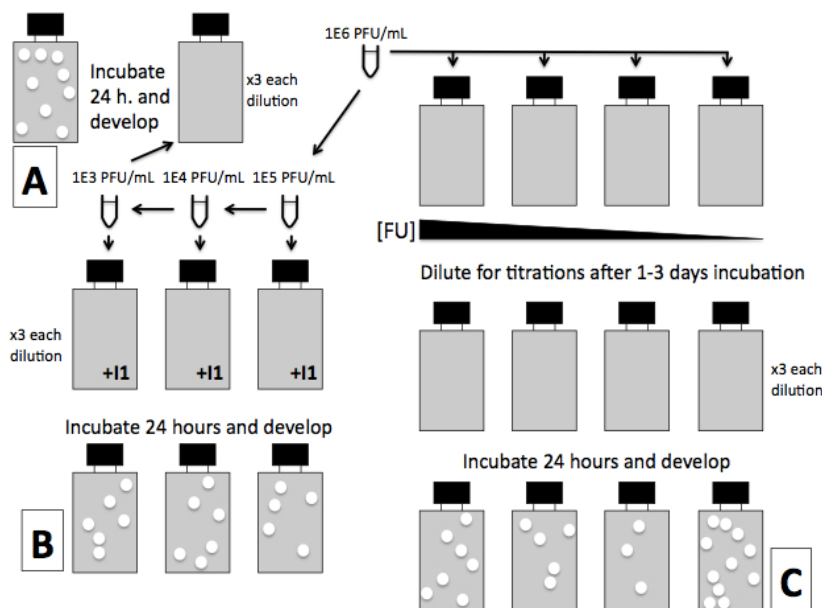


Figure 1. Flowchart showing the infections needed to calculate the frequency of I1 MARM and the sensitivity to 5-FU. Details are given in the text.

Note: Even if the virus stock has a known concentration it is recommended that a titration is done in parallel by triplicate plaque assay Figure 1, top left).

6. Incubate mutagenized infections and plaque assays for 24 h at 37 °C (Figure 1).
7. Develop all plaque assays (Figure 1). Because of the low agarose concentration there is no need to fix the cells. Just let the medium overlay slide out and add 2-3 ml of crystal violet solution. Wait for 5 min at RT, discard crystal violet and rinse with tap water.
8. Count plaques and calculate mutant frequency by dividing Monoclonal antibody resistant mutant (MARM) titers (Figure 1B) by wt titers (Figure 1A).
9. Check virus replication by examining monolayers under the microscope. When cytopathic effect is > 90%, recover mutagenized populations.

Notes:

- a. *For lower FU concentrations 24 h are usually sufficient, but for FU concentrations over 30 µg/ml it may take up to three days.*
 - b. *Samples may be frozen so titrations can be carried out for the complete set of mutagenized samples in a single assay.*
10. Carry out titrations of control and mutagenized viral yields. To improve statistical significance it is recommended to do duplicate or triplicate plaque assays for each dilution. Allow 24-36 h of incubation.
 11. Develop plaque assays with crystal violet and count plaque (Figure 1C).
 12. Robustness can be calculated as the slope of the regression of log transformed normalized viral titers vs. square-root transformed 5-FU concentration (Figure 2). The more robust the strain is the closer to a slope of 1.

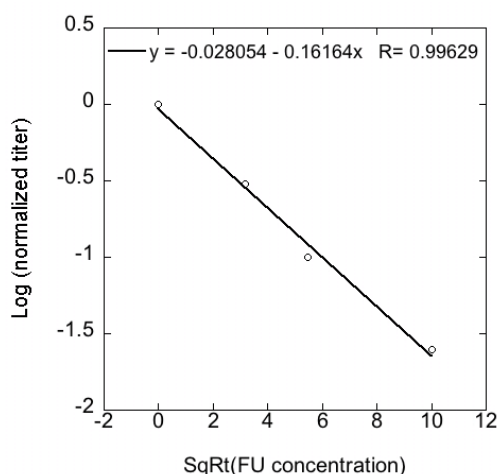


Figure 2. To calculate robustness the log-transformed viral titer is represented against the square root of mutagen concentration. Robustness is the slope of the regression (shown at the top of the graph).

13. To test whether changes in survival are the result of changes in the overall mutation rate, and not in the neutral mutation rate, mutant frequencies must be compared. When all other environmental parameters are the same, mutant frequencies correlate with mutation rates. If mutant frequency and survival to mutagenesis correlate inversely, the latter may not represent robustness. In such case, a clonal analysis is indicated.

Recipes

1. Saline solution

Add 7 g of NaCl to dH₂O to 1,000 ml

Autoclave 20 min

Stored at RT

2. MEM-H

Mix powder with 9.65 L of dH₂O

Add:

250 ml of 4.2% bicarbonate

100 ml of penicillin/streptomycin mixture

Filter and aliquote in 1 L bottles. At this time the medium can be stored in the fridge.

When ready for use add:

70 ml of serum (FEB or BCS)

5 ml of 12% PP3 (if needed)

Notes:

- a. *Making medium is worth it if there is a large volume of cell culture performed in the laboratory. Ready-to-use MEM-E can also be purchased from several companies.*
- b. *MEM with Earls salts (MEM-E) is also a good choice, but requires a CO₂ incubator.*

3. Crystal violet solution

Mix 750 ml H₂O, 250 ml Ethanol and 5 g crystal violet

Stir until dissolved and stored at RT

Acknowledgments

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