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## Plaquing of Herpes Simplex Viruses

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**TITLE:**

# Plaquing of Herpes Simplex Viruses

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**SUMMARY:**

Plaquing is a routine method used to quantify live viruses in a population. Though plaquing is frequently taught in various microbiology curricula with bacteria and bacteriophages, plaquing of mammalian viruses is more complex and time-consuming. This protocol describes the procedures that function reliably for regular work with herpes simplex viruses.

**ABSTRACT:**

There are numerous published protocols for plaquing viruses, including references within primary literature for methodology. However, plaquing viruses can be difficult to perform, requiring focus on its specifications and refinement. It is an incredibly challenging method for new students to master, mainly because it requires meticulous attention to the most minute details. This demonstration of plaquing herpes simplex viruses should help those who have struggled with visualizing the method, especially its nuances, over the years. While this manuscript is based on the same principles of standard plaquing methodology, it differs in that it contains a detailed description of (1) how best to handle host cells to avoid disruption during the process, (2) a more useful viscous medium than agarose to limit the diffusion of virions; and (3) a simple fixation and staining procedure that produces reliably reproducible results.

Furthermore, the accompanying video helps demonstrate the finer distinctions in the process, which are frequently missed when instructing others on conducting plaque assays.

## INTRODUCTION:

The beginnings of virus plaque assays go back to the first discoveries of viruses in the 1890s[1]. Tobacco mosaic virus was first isolated and passed on tobacco leaves, where individual spots of infection could be recognized and quantified as originating from a single, live virus entity[2], later identified as a virion[2]. Later seminal studies with bacteria and bacteriophages perfected the techniques used to plaque these viruses, including bacteria at the mid-log phase of growth, serial dilution of bacteriophage samples, and top agar with subsequent visualization of literal holes (named plaques) in the bacterial lawn[3].

Plaquing of animal viruses lagged the exciting research being conducted with bacteriophages, mainly because the methods required for growing mammalian cells in culture were not developed until the 1940s[4]. However, the advent of increasing murine cells in the absence of the entire host organism[4] spawned a new era in the ability to culture and count viruses. Such work was extended for the propagation and quantitation of Western Equine Encephalomyelitis virus in chicken cells and poliovirus in human cells[5,6]. As the realm of culturable mammalian cells expanded, the bevy of different host cells for various viral infections gave the world a cornucopia of possibilities to study all manner of viruses[7]. This included the propagation and quantification of human herpesviruses, particularly herpes simplex virus-1 (HSV-1) and -2 (HSV-2), which cause mucocutaneous lesions[8]. Importantly, all plaque assays are dependent on the existence of live virions, which can enter host cells in a receptor-mediated fashion in a sample[9]. Regardless of the ubiquity and multitude of publications on the execution of plaque assays[5,10-16], these methods for HSV-1/-2 are a mixture of both art and science; one cannot conduct the assay without proper attention to every detail in the protocol, nor can one execute a successful assay without a specific eye for subtlety in the process. This manuscript depicts one of the most consistently reproducible methods for HSV-1/-2 plaque assays, with precise details towards the art of the assay that is seldom discussed.

This current protocol obtains live plaque-forming units (PFU) counts for HSV-1 and -2 reliably. Best results are obtained using Vero cells (transformed African green monkey kidney epithelial cells) at low passage (below passage number 155) and routinely grown in alpha-MEM[17] supplemented with 10% fetal calf serum (FCS), L-alanyl-L-glutamine, and an antibiotic/antimycotic mixture[18]. Vero cells are standardly propagated in this medium two to three times per week at a ⅕ dilution each time.

## PROTOCOL:

All procedures with the Vero cells and live herpesviruses have been approved by the Towson University Institutional Biosafety Committee. A generalized scheme of these procedures is represented in **Figure 1**.

### 1. Seeding of the Vero cells

1.1 The day before initiating the plaque assay, trypsinize Vero cells and resuspend them in regular Dulbecco's Modified Eagles Medium (DMEM) and supplement as per standard cell culture methodology[19]. Resuspend the trypsinized cells in 10 mL of DMEM per T-75 flask of the confluent Vero cells (~$10^7$ cells).

NOTE: DMEM is used for plaque assays instead of alpha-MEM because it slightly slows the cell growth rate and favors better plaque assay results.

1.2 Count the cells by one's preferred method (e.g., a standard hemocytometer with Trypan Blue exclusion)[19].

1.3 Seed the cells at 4 x $10^6$ cells/plate; for HSV-2, use 6-well plates with 2.5 mL of DMEM (with supplements) per well; and for HSV-1, use 12-well plates with 1.25 mL of DMEM (with supplements) per well.

NOTE: The number of cells should be constant regardless of the plate used, though the size of the wells matters concerning accuracy in the plaque assay. It is essential to get cells evenly distributed, which can be most effectively accomplished by moving the plate back and forth, not in a circular fashion.

1.4 Allow the cells to grow overnight at 37 °C/5% $CO_2$ in a humidified incubator.

NOTE: Humidity is maintained with a pan of distilled water containing an algicide in the bottom of the incubator.

## 2. Sample dilution

2.1 Complete the sample dilution and the addition of virus to cells in one lab session.

CAUTION: HSV-1 and -2 are infectious for humans and must be handled under proper biosafety containment (BSL-2). Keep all materials that come into contact with the virus separate and disinfect with a quaternary agent at 1/256 dilution (see **Table of Materials**), iodophor, bleach, or strong ionic detergent (e.g., SDS) before removing them from the biosafety cabinet.

2.2 The following day, serially dilute each virus sample to be plaqued in 1x PBS; typically use 10-fold dilutions for most precise tracking.

2.3 Make these dilutions through $10^{-4}$ (for low concentrations of viruses) or through $10^{-9}$ (for expectations of higher titer samples) with at least 1 mL of each sample remaining for the plaque assay itself.

2.4 Keep all the viral dilutions on ice (no longer than 1 h) until ready to add these diluted virus samples to the cells.

## 3. Addition of virus to the cells

3.1 Remove the cell culture medium from one or two wells by a pipette.

NOTE: Do not use an aspirator because it removes too much liquid from the cell monolayer and dries the cells out. One crucial consideration is ensuring that the cell monolayer remains hydrated throughout the entire course of the experiment. If the cells dry out, they will wash off the plate in the final step and generate unusable data. Therefore, process only one or two wells at a time to reduce this possibility.

3.2 Carefully add the diluted virus sample (100-400 µL and 50-200 µL of virus sample are used for 6-well and 12-well plates, respectively) dropwise to each monolayer, adding the drops down the side of each well. Repeat the process for every one or two wells until the entire plate is filled with the virus samples being plaqued.

NOTE: Adding the drops to the center of the well may inadvertently slough cells off the substrate.

3.3 Gently rock the entire plate by hand to ensure the PBS that contains the virus covers the entire monolayer of cells in each well, then place the plate in a $CO_2$ incubator at 37 °C to allow the virus to adsorb.

3.4 Every 10 min within 1 h, remove the plate from the incubator, gently rock it again to spread the virus more evenly across each well, and then place it back in the incubator.

NOTE: Each experiment will dictate the number of replicates and which dilutions are used; the reader's preference dictates that decision.

3.5 To diminish the possibility of inadvertently counting unabsorbed inoculum, remove the virus sample from the wells with 1000 µL pipette tips and place it in a waste beaker.

NOTE: Again, this procedure is conducted with only one or two wells at a time to maintain hydration of the cell monolayer.

3.6 Place 2.5 mL of a methylcellulose overlay (dissolve 5% methylcellulose w/w in 100 mL of PBS, autoclave for 15 min at 121 °C and 15 psi on a liquid cycle, then add 375 mL of DMEM plus 25 mL of FBS).

3.7 Once an entire plate contains overlay, place the plate back in the incubator to allow growth for two days.

NOTE: If the virus and cells are allowed to grow for three days, even in DMEM plus supplements, a substantial loss of cells in the monolayer may result, thereby compromising the final data.

3.8 Disinfect the waste beaker, typically with the addition of bleach, an iodophor, or a similar

virucide, before removing it from the biosafety cabinet.

## 4. Staining for plaques

4.1 After the two-day incubation, remove the methylcellulose overlay by a pipette, again one or two wells at a time, and place it in a waste beaker.

4.2 Add ~2 mL of 1% crystal violet (see **Table of Materials**) in 50% ethanol to each well to stain the plaques.

NOTE: It is not essential to remove every last drop of the overlay.

4.3 Incubate the plate with all wells filled with the stain for 30 min at 37 °C. At this point, disinfect the waste beaker as above in step 3.8.

4.4 Wash the plates vigorously with tap water until the runoff is clear.

NOTE: A gentle stream of water is not vigorous enough; full pressure from a lab sink fixture is required.

4.5 At this point, ensure that there are approximately 10-fold differences in the number of plaques across each dilution series.

4.6 Allow the plates to dry overnight upside down, after which the individual plaques may be counted.

## 5. Counting plaques and determining virus titer

5.1 Regardless of the approach (see NOTE below), multiply the number of plaques by the dilution factor (e.g., if the plaques were counted in the $10^{-4}$ well, then the number of plaques would be multiplied by $10^4$).

NOTE: Although counting within a range of 10-100 plaques is useful[5], counting wells with 30-300 plaques are somewhat more reliable and takes into account approximately ½-log dilutions instead of full-log dilutions.

5.2 Divide this number by the volume of inoculum (as above in step 3.2), and average all wells that have plaques within the stated range to get the most accurate titer of HSV in the original sample.

5.3 Perform the calculations in steps 5.3.1 to 5.3.5, considering the use of mock data in **Table 1**.

5.3.1 Consider only wells with 30-300 plaques (step 5.1). Hence, in **Table 1**, use only the $10^{-5}$

column for replicates 1, 2, and 3.

5.3.2 Take the number of plaques at the given dilution and multiply by the reciprocal of the dilution factor. Hence, the $10^{-5}$ column of sample 1 goes from 81 plaques at the $10^{-5}$ dilution to 81 plaques times $10^{5}$.

5.3.3 Then divide that number by the volume (in mL) of the virus dilution added to the cells in that well. Assuming the data on HSV-1 in **Table 1** are from a 12-well plate, 0.2 mL would be the most appropriate measurement. Hence the calculation from step 5.3.2 becomes 81 x $10^{5}$ divided by 0.2 mL, or 4.0 x $10^{7}$ PFU/mL of HSV-1.

5.3.4 Repeat this process for all useful data and average. Hence conduct the calculation in 5.3.2-5.3.3 on all samples in the $10^{-5}$ column, assuming they originated from the same virus sample and the biological replicates were conducted to obtain the most accurate titer measurement. In the case of **Table 1**, average 4.0 x $10^{7}$, 2.1 x $10^{7}$, and 2.6 x $10^{7}$ PFU/mL to obtain a final average titer of 2.9 x $10^{7}$ ± 0.98 x $10^{7}$ PFU/mL for this sample.

**REPRESENTATIVE RESULTS:**

**Table 1** shows an experiment that has optimal results. All 10-fold dilutions follow an approximately 10-fold decrease in plaque counts. These kinds of data can also be seen in **Figure 2,** an actual plaque assay where the countable number of plaques fell in the $10^{-4}$ range for all three replicates. The same can be seen in **Figure 3,** the top row, where the countable number of plaques was in the $10^{-3}$ dilution.

However, **Figure 3,** bottom row, shows an entirely different result when a plaque assay is not conducted well. First, there are very few plaques visible from any dilution; hence, the data are unusable. Second, there are visible areas around the top where the Vero cells have entirely lifted from the substrate, evidence either of allowing the cells to dry out in the process or of pipetting too vigorously in that region of the well.

**Table 2** also shows a set of potentially aberrant outcomes in plaque counts and requires cautions along the way. Of note, neither the top row nor the bottom row of replicates follows a regular 10-fold difference in plaque counts as one moves from one dilution to the next; these data indicate user error during the dilution of the virus addition process. Furthermore, it is not likely that a user would accurately count over 15,000 plaques, so one must call those data into question. Finally, one may note that in replicate 1, $10^{-6}$ dilution, there is a plaque count in the 30-300 range; nonetheless, given the poor nature of the rest of the data in this replicate, it is unlikely that the 42 plaque count is an accurate number. Hence, the only believable data in **Table 2** are in replicate 2.

**Figure 4** shows the same problems with cells drying out or being mishandled during the plaque assay. However, **Figure 4** critically shows poor Vero cell seeding; every well shows darker purple spots, indicative of cells not spread well across the well and piled on top of one another in clusters.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Overall plaque assay scheme**. Adapted from Biorender template, "Viral Plaque Assay Protocol," by BioRender.com.

**Figure 2: Plaque assay for HSV-1 in twelve-well plates**. Biological replicates were conducted as outlined in **Figure 1** on one sample of HSV-1. Serial 10-fold dilutions of an HSV-1 sample (starting at the $10^{-2}$ dilution) were added to the Vero cells, incubated, then stained with crystal violet.

**Figure 3: Plaque assay for HSV-2 in six-well plates**. Biological replicates were conducted as outlined in **Figure 1** on one sample of HSV-2. Serial 10-fold dilutions of an HSV-2 sample (starting at the $10^{-2}$ dilution) were added to Vero cells, incubated, then stained with crystal violet.

**Figure 4: Plaque assay for HSV-1 in twelve-well plates**. Biological replicates were conducted as outlined in **Figure 1** on one sample of HSV-1. Serial 10-fold dilutions of an HSV-1 sample (starting at the $10^{-2}$ dilution) were added to Vero cells, incubated, then stained with crystal violet.

**Table 1: Representative plaque counts from one successful assay**. Serial dilutions from each biological replicate were conducted; representative plaque counts (starting at the $10^{-3}$ dilution) are shown for each dilution.

**Table 2: Representative plaque counts from one unsuccessful assay**. Serial dilutions from each biological replicate were conducted; representative plaque counts (starting at the $10^{-3}$ dilution) are shown for each dilution.

**DISCUSSION:**

While plaque assays are almost as old as mammalian cell culture itself, it seems that each lab has its own set of protocols to execute this basic assay[5,6,10-16,20]. Although the instructions in each published version of a plaque assay differ ever so slightly, few of these manuscripts elucidate the critical nuances involved in obtaining consistent results.

Hence, there are some parts of this protocol that are more art than science. When Vero cells are seeded in wells for the assay, they must be spread evenly throughout the well (**Figure 4**). All steps involving a liquid transfer (e.g., steps 3.1, 3.2, 3.5, 3.6, 4.1, and 4.2) must be done carefully to keep the Vero cell monolayer hydrated (**Figure 3** and **Figure 4**). It is also crucial not to touch the bottom of the well with any pipette, or the cells may scrape off, further compromising the final data (**Figure 4**).

While methylcellulose is advantageous as an overlay material to prevent diffusion of virions across the entire well, alternatives may be used. For example, one may use microcrystalline cellulose[21,22] or carboxymethyl cellulose[21]. Regardless, methylcellulose is one of the easiest and least expensive liquid overlay media to use, though. One may also use a different dye[21] or fixing

solution (e.g., paraformaldehyde)[21] for the staining part of the protocol (step 4), but crystal violet in 50% ethanol is the easiest and least expensive among the options.

While it may be overly straightforward to conduct these assays, they do require training and practice. It is well worth using samples that are not irreplaceable for learning this method so that one has mastered the technique when it matters most.

Plaque assays themselves should only be used, though, when one needs to count bona fide live virions. Plaquing is a much older methodology to measure virus particles[5,10] compared to newer quantitative methods like fluorescence microscopy[23], ELISA[24], enzymatic activity[25,26], or qPCR[27]. However, these latter methods generate surrogate data that suggest the presence of whole virions, yet may lead to a gross overestimate in viable particles because the user would be quantifying an indicator of infection, not real virions. Fluorescence microscopy measures infectious virions by how many cells exhibit the presence of a protein expressed by the virus but does not necessarily detect complete, replication-competent virions. ELISA similarly detects the presence of virus proteins that may exist without an utterly infectious virion. Assaying enzymatic activity, while suggestive of an intact virion, only shows that a single active enzyme is present and does not reflect the true contagious nature of a whole virion. And qPCR merely quantifies copies of a virus' genome, whether that genome is defective or not. The only method comparable to a plaque assay to quantify live viruses is an endpoint dilution assay[28], which still requires a statistical estimation via the Reed-Muench or Spearman-Kärber methods[29-31] to determine virion concentration.

While plaque assays are a routine method used throughout virology, there are particular cases where they are not the quantitative assay of choice. For example, plaque assays require host cells to attach to a substrate; if the best host cell for infection is non-adherent, plaques will never form[32]. Some viruses grow more slowly than the typical cell cycle of their hosts, and therefore any burgeoning plaques would be overgrown by the host cells themselves[21]. Plaques may sometimes not form well, just by the nature of the cytopathic effect, making the determination of bona fide plaques a limitation[21].

The approach to plaquing herpesviruses presented here is not necessarily unique. However, this in-depth description of the minor caveats associated with a successful HSV plaque assay may diminish the difficulties experienced by many users, especially novices. Regardless, the methodology described is also broadly applicable to plaque assays with many other viruses, including but not limited to picornaviruses[33], orthomyxoviruses[34], flaviviruses[35], coronaviruses[36], and many other systems in which a quantifiable amount of infectious animal viruses are required.

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**DISCLOSURES:**

The authors declare no conflict of interest.

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Figure 1



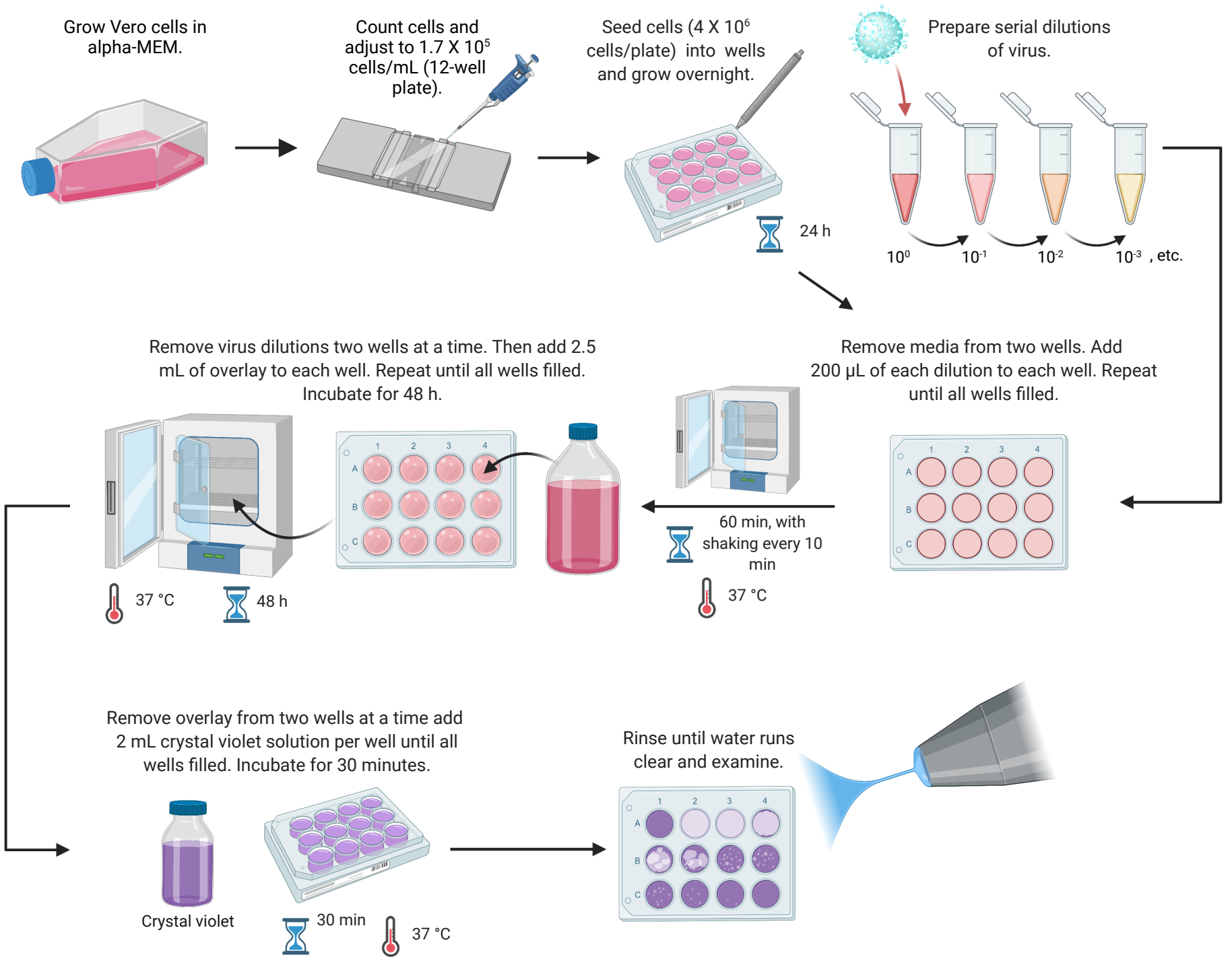


Figure 2

Click here to access/download;Figure;Fig-2.-HSV-1.pdf

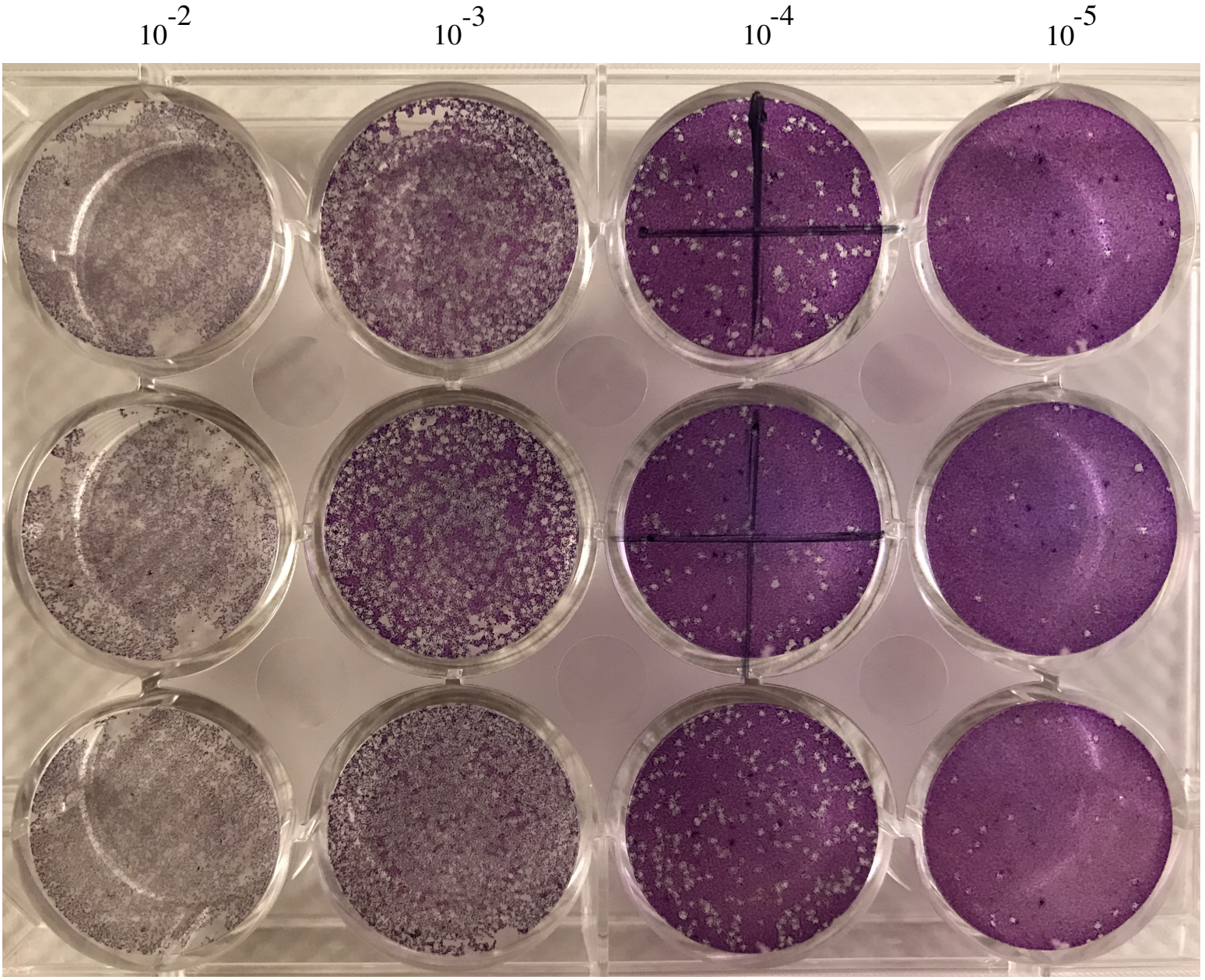


Figure 3



$10^{-2}$ $10^{-3}$ $10^{-4}$

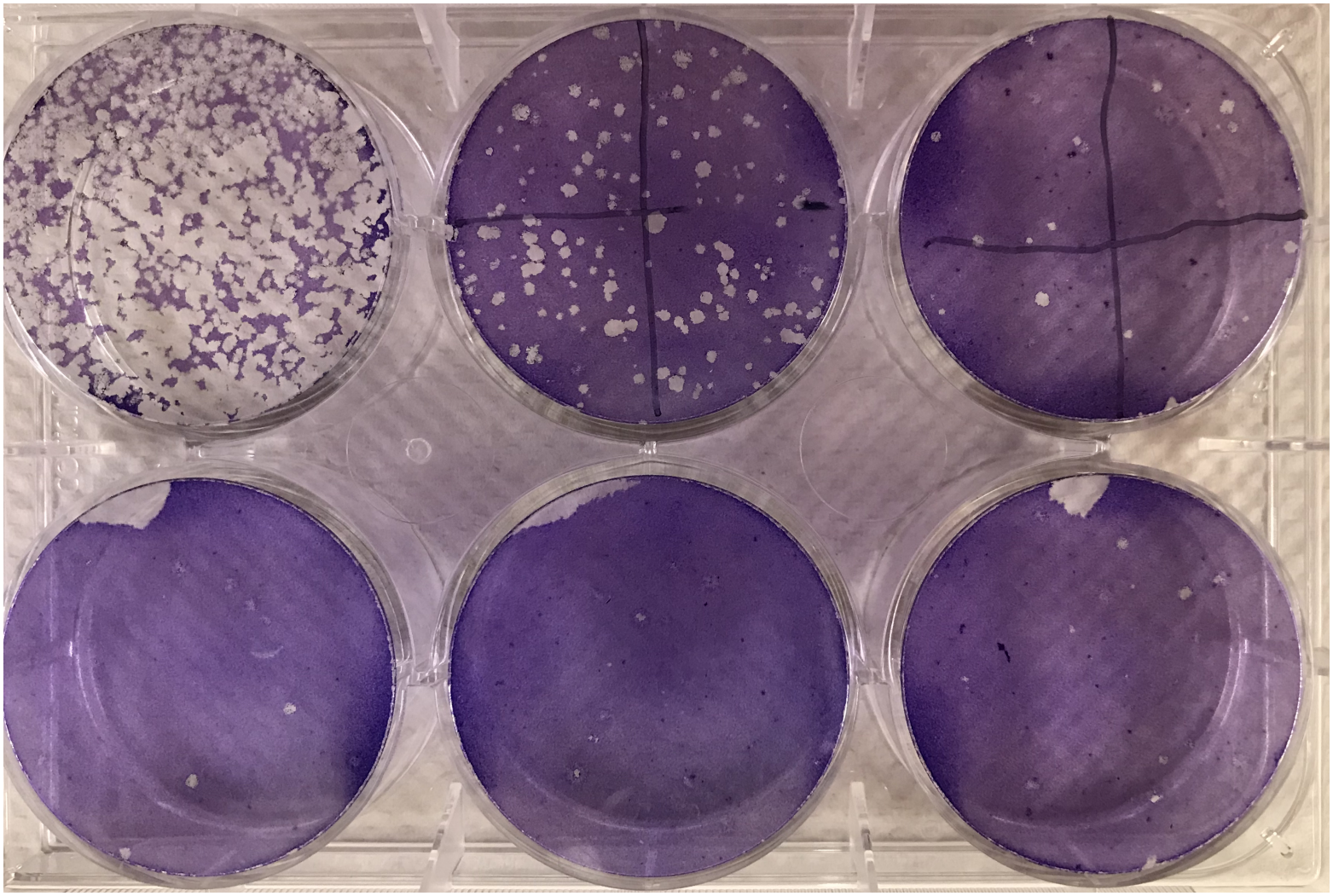
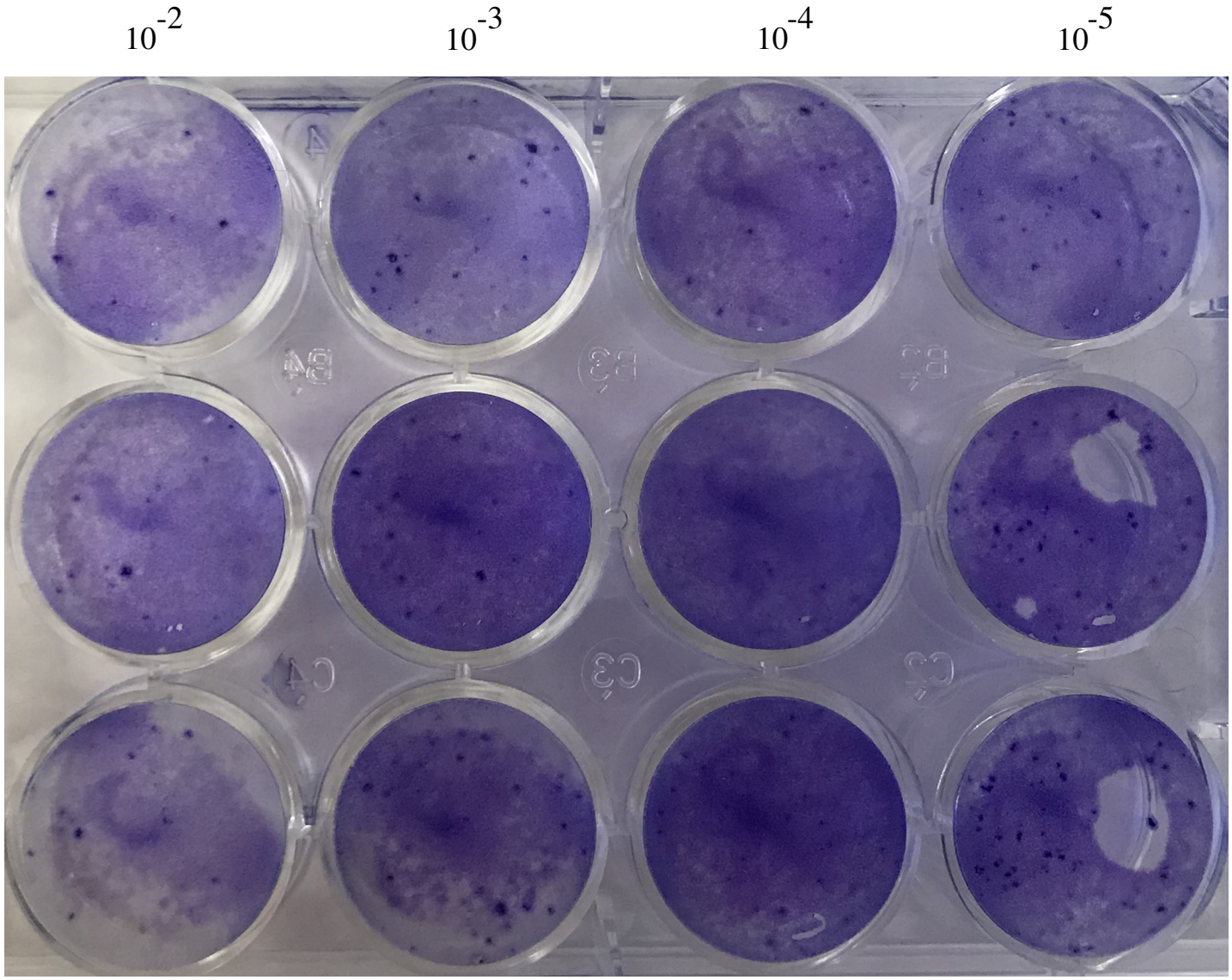


Figure 4





| | Dilution factor | | | |
|---|---|---|---|---|
| **Replicate** | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| 1 | 5721 | 635 | 81 | 5 |
| 2 | 4592 | 365 | 42 | 0 |
| 3 | 5,519 | 521 | 53 | 1 |

Table 2



| | Dilution factor | | | |
|---|---|---|---|---|
| **Replicate** | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| 1 | 15225 | 635 | 450 | 42 |
| 2 | 4592 | 365 | 42 | 0 |
| 3 | 5519 | 5400 | 53 | 1 |

To the editors:

All revisions are commented in the manuscript file. Please note that the upload of Figs. 2-4 did not permit them to be named “Figure” as the Item; they needed a designation in the editorial management system to proceed, and the closest Item type available was “Video or Animated Figure;” regardless, these are simple figures, like Fig 1, and should be treated as such.

We would also prefer to discuss whether steps 4.1-4.5 will be filmed because they routinely occur two days after the previous steps. It will be better to talk about what to do by phone instead of written comments going back and forth.

Thank you for your consideration.

We thank the reviewers and editors for their useful comments. Given the plethora of editorial comments and the need for extensive detailed revisions of working, we have opted to address those changes by making them in green text throughout the manuscript via "Track Changes." Similarly, the reviewer's point for revision are also marked in red and green (dependent on how MS-Word formatted things). Please also note that because of the need to add two references, the entire set of citations and the bibliography were automatically marked as "changed" and are green.

The following items, though, are addressed here (with their corresponding number) so that the changes are clearer:

2. One new author was added, and all email addresses are provided below that list of authors.
4. Glutamax is the only available form of the relevant reagent. However, its generic type of additive is now in the text and it is listed by both the generic and trade names in the Table of Materials.
8. An appropriate reference to a cell culture manual has been added.
12. The quaternary agent is now listed in the Table of Materials.
17. We believe the original Discussion, consisting of 6 paragraphs, contains all the necessary items as listed. Please advise more specifically if something is missing.
18. We used the officially available Endnote JoVE format for references (as supplied on the JoVE website). However, we have complied and edited the formatting directly in the text to meet the guidelines listed. Note that we retained the original JoVE Endnote style format for listing more than 5 authors as *et al.;* please advise if every author's name is required.
19. Please find the BioRender license attached with the revised submission. The citation of BioRender has already been recorded according to their specifications (https://help.biorender.com/en/articles/3619405-how-do-i-cite-biorender); we believe it needs no further modification beyond what has already been supplied in the original text.
20. The suggested change has been made.

As for the one reviewer comment, a sentence has been added after the phrase "which cause mucocutaneous lesions," with the suggested citation. We hope this addresses the reviewer's concern.

Field Code Changed

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To whom this may concern,

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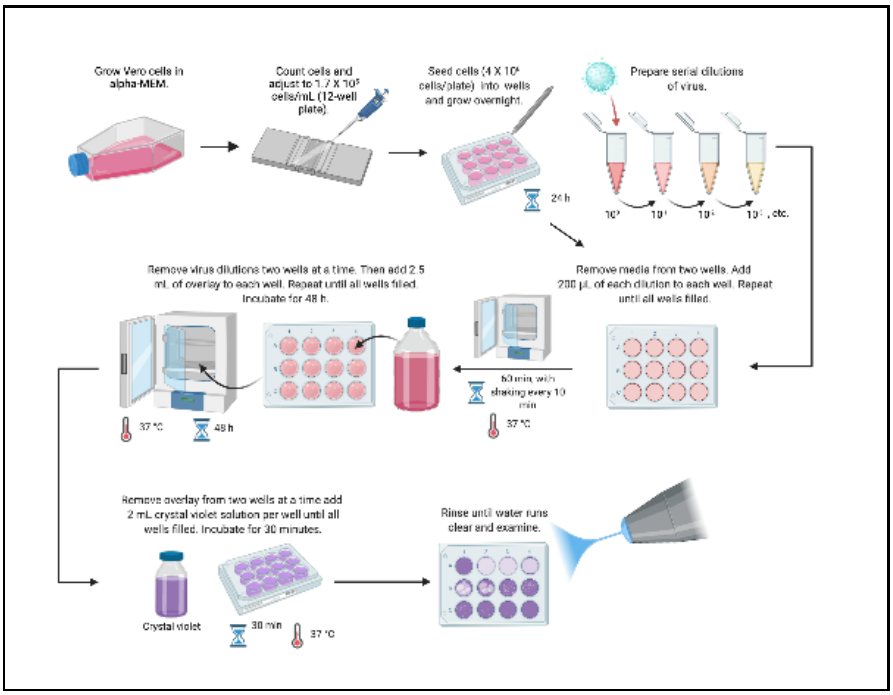


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