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MVA titration by plaque assay using crystal violet staining in DF-1 cells

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Abstract

Modified Vaccinia Ankara (MVA) is a highly promising vector for generating safe vaccine candidates against many pathogens, such as HIV-1, SARS-CoV-2, and influenza viruses. The gold standard method to titrate MVA involves visualizing MVA plaques in chicken embryo fibroblasts after immunostaining. However, this method is time-consuming and costly. In this study, we evaluated the visualization of MVA plaques formed in continuous chicken embryo fibroblasts DF-1 cells using crystal violet staining. We found that MVA titration by plaque assay using crystal violet staining in DF-1 cells yielded similar results to immunostaining, with substantially reduced time and costs. The MVA plaque assay by crystal violet staining in DF-1 cells is a reliable method with accurate results and low time and financial costs.

Keywords MVA, Plaque assay, Crystal violet staining, DF-1 cell

To the editor

While many members of poxviruses cause severe diseases in humans and animals [1, 2], some are promising candidates for vaccine vectors due to their broad host tropism, accommodating up to 25 kbp of foreign DNA and eliciting strong immune responses [3]. Modified Vaccinia virus Ankara (MVA) is a highly attenuated virus that is unable to replicate in most mammalian cells due to the accumulation of mutations/deletions from the original genome after 570 passages of the vaccinia Ankara strain in chicken embryo fibroblasts (CEFs), leading to approximately 10% genome lost [4]. The inability to replicate in mammals makes MVA a highly safe and attractive vaccine vector for human and animal vaccine development [5, 6].

Due to its limited replication ability, it is very challenging to titrate MVA conveniently using immortal cell lines. The current gold standard MVA titration method is through immunostaining the plaques formed in MVA-infected CEFs [7]. The procedure includes the culture of fresh CEFs made from chicken embryos, infection of the cultured CEFs by a stock of MVA with a series of MVA dilutions to form well-separated plaques, incubated from two–three days in Dulbecco's Modified Eagle Medium (DMEM) containing 0.5% methylcellulose (to prevent secondary plaque formation), followed by fixation with methanol/acetone 1:1 and staining with primary rabbit anti-vaccinia virus serum and secondary anti-rabbit IgG conjugated with Horseradish peroxidase (HRP). Subsequently, the immune-stained plaques are visualized using a diaminobenzidine (DAB) substrate containing the substrate peroxidase (approximately half to one day) [7]. The method is time and cost consuming due to the CEFs culture, anti-vaccinia virus serum usage, and immunostaining. In addition to the lengthy fresh CEFs culture, the whole procedure requires three to four days starting from the MVA infection.

Some previous studies used continuous chicken embryo fibroblasts DF-1 cells, which are cultured and passaged similarly to immortalized or transformed mammalian cells, to replace the CEFs, in MVA titration [8]. The use of DF-1 cells simplifies the cell culture part of the MVA titration. We established the use of DF-1 cells

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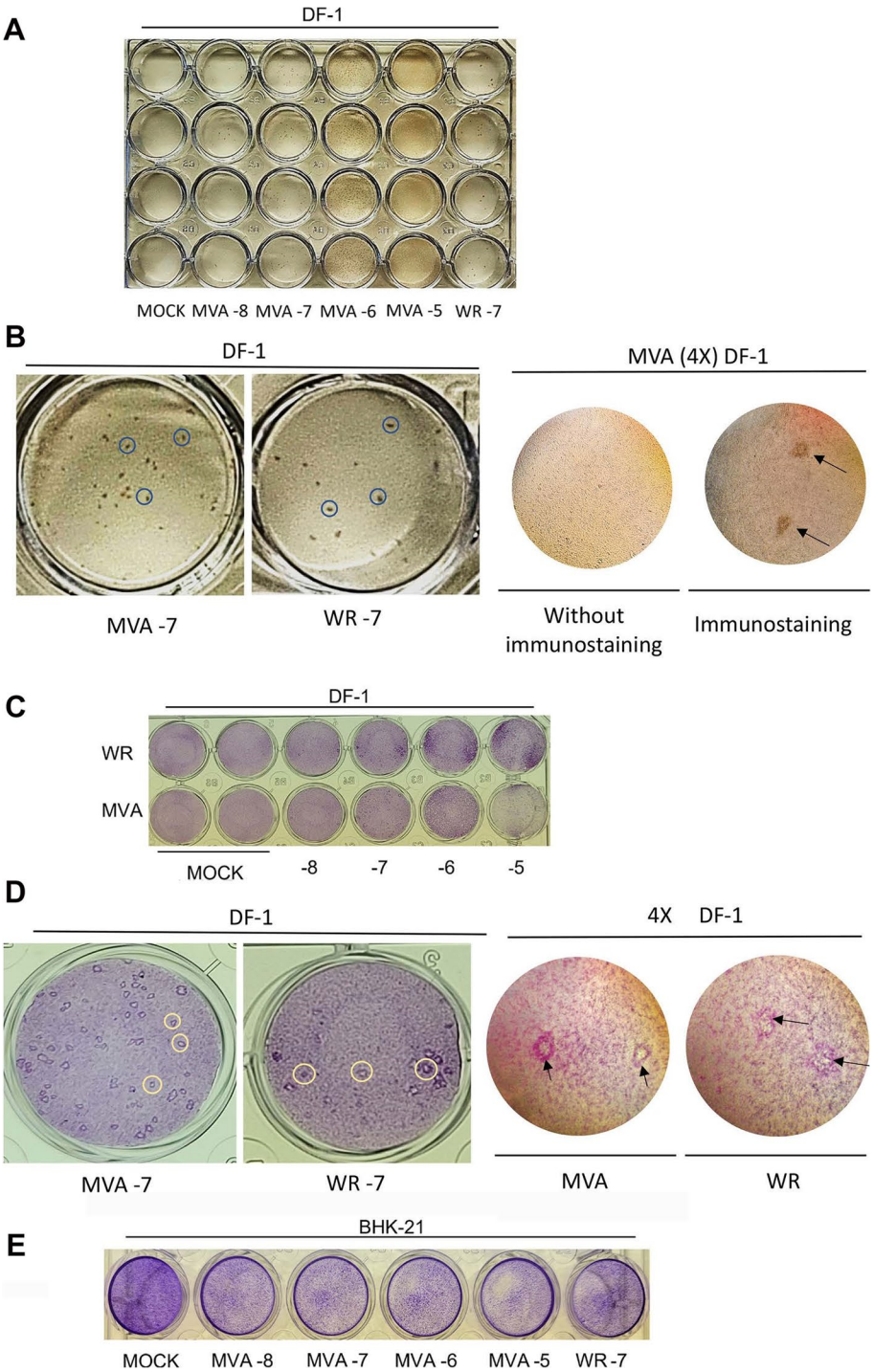

Fig. 1 Visualization of MVA Plaques Using Immunostaining and Crystal Violet Staining in DF-1 Cells. MVA plaques were visualized in DF-1 cells using the gold standard immunostaining with anti-VACV serum and secondary antibody anti-rabbit IgG conjugated with HRP (**A**, **B**) and crystal violet staining (**C**, **D**). Following infection with MVA, the DF-1 cells were incubated in medium containing 0.5% methylcellulose for two to three days to facilitate plaque formation. Immunostaining revealed distinct brown plaques (**A**), while crystal violet staining provided distinguishable plaques (**C**). VACV-Western Reserve (WR) strain was used as a comparison. Each circle in the left of (**B**) and (**D**) indicates a single plaque view in B & D. Zoomed-in views (4x) of the plaques are shown in (**B**) and (**D**) in the right. The numbers following virus strains (MVA or WR) indicate the dilution fold of infection (logarithm). **E** MVA plaque assay using crystal violet staining in BHK-21 cells does not present visible and countable plaques compared to DF-1 cells

Table 1 Procedure of MVA titration by plaque assay using crystal violet staining in DF-1 cells

1) Seed DF-1 cells in 24-well plate until confluence in DMEM containing 10% FBS
2) Infect the cells with MVA using a series of dilutions using DMEM containing 2.5% FBS
3) One hour after infection, replace the media with DMEM containing 2.5% FBS and 0.5% methylcellulose
4) After 48 h of incubation, fix and stain DF-1 cells with a solution of 20% methanol and 0.1% crystal violet for 20 min
5) Wash the wells, visualize and count the plaques

Table 2 Comparison of MVA plaque assay using Immunostaining and crystal violet staining using DF-1 cells

	Time	Cost	Accuracy
Crystal violet staining	2–3 days	Low	Comparable to immunostaining
Immuno- staining	3–4 days	High due to anti-VACV serum and immune detection reagents	Gold standard

for MVA titration without the need to prepare CEFs from fresh chicken embryos. After immunostaining, we observed well-distinguishable, countable brown, irregular MVA plaques in DF-1 cells (Fig. 1A and B). Using this method, we determined the titer of an MVA stock as 2.0×10^9 PFU/mL.

We then sought to examine whether we could further simplify the MVA titration procedure by replacing the immunostaining with crystal violet staining a cost-efficient method of plaque visualization. Crystal violet staining that take only 15–20 min for the staining procedure compared to about 4–8 h required when using immunostaining [7]. Crystal violet has been widely used to stain plaques formed by many replicating-competent poxviruses and non-poxviruses. However, it was thought that MVA plaques are challenging to distinguish using crystal violet staining due to their limited spreading ability and irregular plaque shapes. Interestingly, after incubating DF-1 cells infected with MVA for two-three days, we observed well-distinguishable plaques with clear boundaries after fixation and staining with 0.1% crystal violet (Fig. 1C and D). The plaques were also readily countable. Using the same MVA stock in Fig. 1A and B, the titer was determined as 2.3×10^9 PFU/mL using the crystal violet staining, which highly agreed with the titer determined by the immunostaining (2.0×10^9 PFU/mL). After performing this procedure (summarized in Table 1) several times with slight variations, we found that highly confluent DF-1 cells at the time of MVA infection are essential for forming well-recognizable plaques using crystal violet staining. We also noticed that the use of methylcellulose during the two–three days of incubation time of viral spreading is optional, likely due to the limited spreading ability of MVA. Despite losing the ability to replicate in most mammalian cells, MVA can replicate in Baby Hamster Kidney 21 (BHK-21) cell line [6]. However, after examining if BHK-21 cells could be used for MVA plaque assay using crystal violet staining, we found that the plaques were not readily visualized and counted (Fig. 1E).

In conclusion, our findings indicate that the MVA plaque assay using crystal violet staining in DF-1 cells is reliable and comparable to quantifying MVA titers by the gold standard method of immunostaining. Furthermore, compared to immunostaining, crystal violet staining is substantially less expensive, less time- and labor-consuming (Table 2).

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Authors' contributions
S.N.F & L.D. performed the experiments; S.N.F. & Z.Y. drafted and edited the manuscript; Z.Y. supervised the project. All authors read and approved the final manuscript.

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