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EPPIC (Efficient Purification by Parental Inducer Constraint) Platform for Rapid Generation of Recombinant Vaccinia Viruses

Brittany Jasperse,[1,2] Caitlin M. O'Connell,[1] Yuxiang Wang,[1] and Paulo H. Verardi[1]

[1]Department of Pathobiology and Veterinary Science and Center of Excellence for Vaccine Research, College of Agriculture, Health and Natural Resources, University of Connecticut, Storrs, CT 06269, USA

Vaccinia virus (VACV) was successfully used as a vaccine in the smallpox eradication campaign. Since then, it has been widely used in the development of vaccine and therapeutic vectors. However, methods of generating and purifying recombinant VACVs (rVACVs) are often time-consuming, cumbersome, and in some cases require specialized cell lines or equipment. Here, we describe a novel EPPIC (Efficient Purification by Parental Inducer Constraint) platform for the rapid generation of rVACVs using a replication-inducible VACV (vIND) as a parental virus for homologous recombination. Purification of the rVACV from the parental vIND is achieved by two serial passages in the absence of inducer (i.e., parental inducer "constraint") in standard laboratory cell lines, without the need for specialized equipment, within 1 week. We determined the optimal conditions for homologous recombination and serial purification and generated a suite of vIND parental viruses to facilitate customization of the platform. Importantly, the EPPIC platform can be adapted to rapidly generate replication-deficient and replication-competent rVACVs expressing vaccine or therapeutic antigens, with or without screening markers, by simple modifications to a DNA shuttle vector, thus allowing the rapid development, updating, and refinement of personalized or custom vaccines and therapeutic vectors in a matter of days.

INTRODUCTION

Vaccinia virus (VACV) is a large, double-stranded DNA virus in the *Poxviridae* family, along with variola virus and monkeypox virus. VACV is most notable for its use as the vaccine that led to the eradication of smallpox by the World Health Organization in 1979.[1] Since the eradication of smallpox, there has been renewed interest in the development of next-generation smallpox vaccines due to the threat of bioterrorism and the possible emergence of other orthopoxviruses (such as monkeypox virus) as significant human pathogens. VACV has also been successfully used as a viral vector for the development of effective human and animal vaccines as it elicits strong and long-lasting humoral and cell-mediated immune responses to heterologous antigens expressed in its genome, is thermally stable, can accommodate up to 25 kilobases of heterologous DNA, is easy to propagate, and is not oncogenic.[2]

In an effort to increase the safety of VACV, we recently generated VACV vectors with a built-in safety mechanism that replicate only in the presence of inducer.[3,17] These replication-inducible VACVs (vINDs) contain elements of the *tetracycline* (*tet*) or *lactose* (*lac*) operons such that transcription of a gene essential to VACV replication occurs only in the presence of inducers such as tetracycline antibiotics or the synthetic inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), respectively. The *tet*-inducible VACV (viTet) or *lac*-inducible VACV (viLac) vectors replicate freely (like traditional replication-competent VACVs) in the presence of inducer but are replication-defective in the absence of inducer. Unlike modified vaccinia Ankara (MVA), our replication-inducible VACVs can be propagated in standard cell lines at high titers in the presence of inducer,[3,17] making large-scale production of vIND vectors more time- and cost-effective.
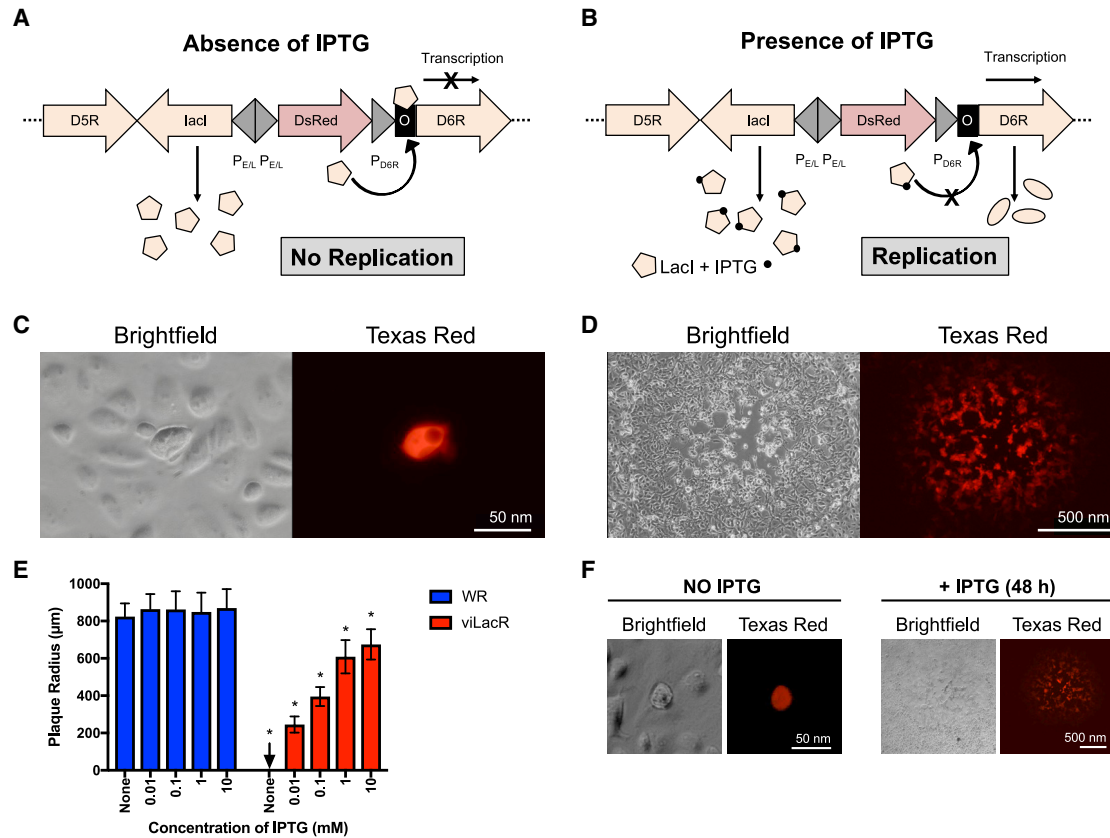
Traditional methods of generating and purifying rVACVs (replication-inducible or otherwise) are time-consuming (requiring many rounds of purification) and laborious (typically utilizing selection media, agarose overlay, special equipment, or specialized cell lines), and often require inclusion of screening and/or selection markers in the final vector. Transient dominant selection[4] has been used to generate marker-free rVACVs but requires repeated passage in the presence of selection media followed by passage in the absence of selection media and extensive screening,[5] and can take months. A more recent method utilizes the antibiotic coumermycin to selectively remove coumermycin-sensitive parental VACVs from rVACVs using minimal rounds of plaque purification.[6] Other recent methods of rVACV purification utilize fluorescence-activated cell sorting (FACS) to sort rVACVs by either differential fluorescent marker expression[7,8] or a selectable and excisable marker.[9–11] While these methods enable the generation of marker-free rVACVs in as little as 10 days,[9] they require special cell lines (to excise the marker) and FACS equipment, which

Figure 1. Replication of viLacR, a VACV Inducibly Expressing the Essential D6R Gene, Occurs Only in the Presence of Inducer, IPTG

A cassette containing the putative D6R promoter (P_{D6R}) followed by the *lac* operator (O) sequence was inserted upstream of the D6R gene. The cassette also contained the *lacI* repressor gene and *dsRed* screening gene under back-to-back synthetic early/late VACV promoters ($P_{E/L}$). (A) In the absence of IPTG, LacI binds *lacO*, preventing transcription of the D6R gene and virus replication. (B) In the presence of IPTG, LacI binds IPTG, undergoes a conformational change and is unable to bind *lacO*, thus allowing transcription from the *lacO*-controlled P_{D6R} promoter and viLacR replication. (C) viLacR forms abortive infections in the absence of IPTG that can be distinguished by expression of dsRed. (D) viLacR forms dsRed+ plaques in the presence of IPTG. Images taken with a fluorescence microscope 2 DPI of wells infected with approximately 20 PFU. (E) BS-C-1 cell monolayers were infected with the VACVs at approximately 30 PFU/well in the absence or presence of multiple concentrations of IPTG. After 2 days, cells were stained and fixed in 0.5% crystal violet/20% ethanol and the size (radius) of approximately 30 representative plaques was measured. Asterisks represent statistical significance ($p < 0.05$) by one-way ANOVA with Dunnett's multiple comparisons test compared to wild-type WR at 10 mM IPTG. Bars represent geometric mean and error bars represent standard deviation. Arrow indicates absence of plaques. (F) In the absence of IPTG, only single dsRed+ cells were observed 2 DPI, and under high magnification, dsRed expression was contained to single cells and was the only indication of infection, suggesting abortive infections. When IPTG was added 48 h after infection, plaques were visible 2 days later (4 DPI). Schematics not drawn to scale.

may not be feasible for all researchers due to biosafety restrictions on sorting risk group 2 infectious viruses.

We developed an EPPIC (Efficient Purification by Parental Inducer Constraint) platform to generate and rapidly purify rVACVs by utilizing a vIND as the parental virus. This platform uses standard cell lines, standard cell culture media (no agarose overlay), and minimal equipment (fluorescence and/or light microscope). The main objectives of this study were to determine optimal conditions for homologous recombination and serial purification and to generate a suite of vIND parental viruses to facilitate customization of the EPPIC platform. This platform allows for the generation and purification of replication-competent or replication-inducible VACV vectors, with

or without screening markers, by simple modifications to the DNA shuttle vector, thus facilitating the development, updating, and refinement of personalized or custom vaccines in a matter of days. Most importantly, rVACVs can be purified from the vIND parental virus within 1 week by simple withdrawal of inducer (i.e., inducer constraint) during serial purification. Here, we demonstrate the EPPIC platform through the generation of viTetG, a vIND expressing enhanced green fluorescence protein (EGFP).

RESULTS

Parental vIND, viLacR, Replicates Only in the Presence of IPTG
We developed a novel platform, based on our vIND vectors, to generate and purify rVACVs in as little as 6 days without any
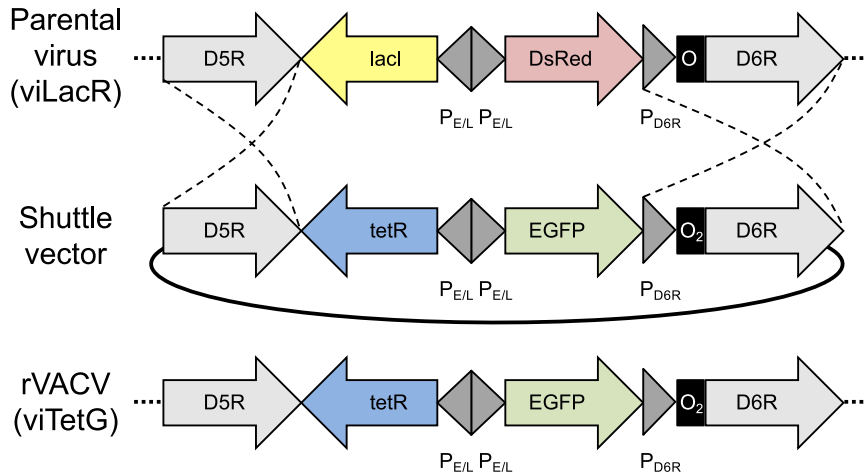
Figure 2. rVACV viTetG Was Generated by Homologous Recombination between Parental viLacR and a DNA Shuttle Vector

viLacR (top panel) contained the D6R promoter (P_{D6R}) followed by the *lacO* sequence (O) and the *lacI* and *dsRed* genes under back-to-back synthetic $P_{E/L}$ promoters. The DNA shuttle vector (middle panel) contained the D6R gene under a *tet* operator (O_2)-controlled P_{D6R} promoter and *tetR* and *EGFP* genes under back-to-back $P_{E/L}$ promoters, flanked by regions homologous to viLacR. Homologous recombination (dashed lines) between the shuttle vector (middle panel) and viLacR (top panel) generated viTetG, a *tet*-inducible rVACV expressing EGFP (bottom panel). Schematics not drawn to scale.

specialized equipment or special cell lines. Our EPPIC platform utilizes a vIND[3,17] as the parental virus for homologous recombination with a DNA shuttle vector to generate a rVACV. By incorporating elements from either the *tet* or *lac* operon, we have generated viTet (e.g., viD6R[3]) or viLac viruses, respectively, that tightly regulate transcription of a gene essential to VACV replication and thereby replicate exclusively in the presence of inducer (e.g., tetracyclines or IPTG, respectively). We have generated vINDs based on several essential VACV genes.[3,17]

To generate the rVACV viTetG (a vIND expressing EGFP), we selected a *lac*-inducible parental virus expressing a dsRed reporter protein (viLacR). Replication of viLacR is based on the conditional expression of the essential D6R gene (encoding the small subunit of the VACV early transcription factor) (Figure 1). In the absence of inducer (IPTG), the LacI repressor is expressed, binds the *lacO* operator sequence, and blocks transcription of the downstream D6R gene (Figure 1A), thus preventing virus replication and resulting in an abortive infection (Figure 1C), where the infected cell expresses viral-encoded genes such as the reporter dsRed gene but does not produce progeny virus.[3,17] However, in the presence of IPTG, LacI is expressed but binds IPTG and undergoes a conformational change and is therefore unable to bind the *lacO* operator sequence. Thus, transcription of the essential gene D6R occurs (Figure 1B), and viLacR replicates freely, forming a plaque (Figure 1D). Replication of viLacR is dependent on IPTG (Figure 1E), with no plaques observed in the absence of IPTG, and nearly wild-type size plaques observed in the presence of at least 1 mM IPTG. Furthermore, while only single infected cells (dsRed+) can be detected 2 days post-infection (DPI) in the absence of IPTG, addition of IPTG 2 DPI results in resumption of viral replication and formation of a plaque by 4 DPI (Figure 1F).

Homologous Recombination between Shuttle Vector and Parental viLacR Generates rVACV viTetG

The first step of the EPPIC platform is to design a DNA shuttle vector that contains any promoter(s) and gene(s) of interest (GOI) flanked by regions homologous to the VACV genome. The homologous regions are determined by the essential gene controlled by the inducible mechanism (right border) and its upstream gene (left border). For example, to generate viTetG (a vIND expressing EGFP), a shuttle vector was designed such that elements of the *lac* operon were replaced with those from the *tet* operon (*lacI* replaced with *tetR* and *lacO* replaced with *tetO₂*), the GOI (EGFP) was placed under the control of a synthetic $P_{E/L}$ VACV promoter, and the entire cassette was flanked by the upstream D5R gene and the D6R essential gene (Figure 2, middle panel). Homologous recombination between the DNA shuttle vector and parental virus viLacR generates viTetG, a *tet*-inducible rVACV expressing EGFP (Figure 2).

To generate viTetG, we infected BS-C-1 cells with the parental virus viLacR in the presence of parental inducer (IPTG) and rVACV inducer (tetracyclines such as doxycycline [DOX]) and subsequently transfected them with the shuttle vector (Figure 3). Infected/transfected cells were incubated for 2 days and subsequently examined with light and fluorescence microscopy to check for cytopathic effect (CPE) and expression of fluorescence proteins (viLacR-based expression of dsRed and viTetG-based expression of EGFP). Next, the cells were collected and processed (series of three freeze/thaw cycles, sonication, and trypsinization) to obtain the intracellular virus fraction for further purification. During optimization of the EPPIC platform, we determined that addition of parental inducer during the infection/transfection step more reliably resulted in rVACV generation. However, the addition of 0.1 mM IPTG (10-fold less parental inducer than that typically used for propagation of parental viLacR) was sufficient for rVACV generation and conveniently, should decrease the concentration of residual IPTG carried over into subsequent purifications.

rVACV viTetG Was Purified from Parental viLacR in the Absence of IPTG and Presence of DOX

Since viTetG should replicate only in the presence of DOX and viLacR should replicate only in the presence of IPTG, purification was performed in the presence of DOX (viTetG inducer) and absence of IPTG (viLacR inducer). During the first purification step, the processed lysate from the infection/transfection was used to infect fresh
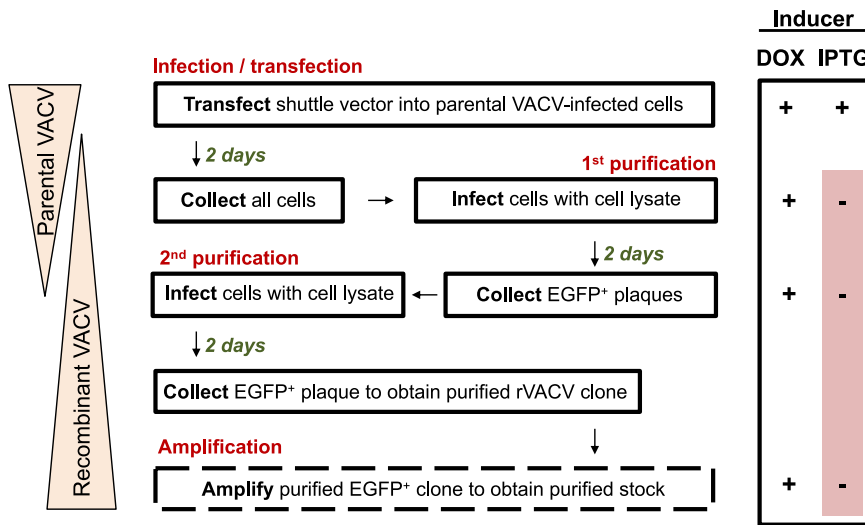
Figure 3. rVACV viTetG Was Generated Using the Optimized Steps of the EPPIC Platform

Infection/transfection was performed at an MOI of 0.1 in the presence of viTetG inducer (DOX) and parental viLacR inducer (IPTG) and resulted in generation of viTetG by homologous recombination between a parental VACV (viLacR) and DNA shuttle vector. Purification of viTetG from viLacR was achieved by serial purification in the presence of DOX and absence of IPTG (parental inducer constraint). A purified viTetG clone was obtained within 6 days, which was then amplified to generate a working stock.

cells under "parental inducer constraint" (absence of IPTG and presence of DOX) in the absence of any special overlay (e.g., agarose) (Figure 3). Two days later, cells were scanned under a fluorescence microscope to identify EGFP+ plaques (evidence of viTetG replication). While the vast majority of cells were infected by viLacR (dsRed+ cells) due to the high proportion of parental virus remaining in the transfection/infection lysate, EGFP+ plaques were identified. During our optimization, we determined that it was prudent to infect with several dilutions (e.g., 10^{-1}, 10^{-2}) and in a cell culture plate with a large surface area (e.g., 100 mm dish or 6-well plate) to more easily detect the rare rVACV among the abundant parental virus. Once viTetG (EGFP+) plaques were identified, the plaques were simply collected with a pipet tip from the monolayer (see Materials and Methods) and processed (series of three freeze/thaw cycles, sonication, and trypsinization).

During the second purification step of the EPPIC platform, the cell lysate collected during the first purification step was transferred to fresh cell monolayers (several wells of a 24-well plate using dilutions of 10^{-1} and 10^{-2}) under parental inducer constraint (presence of DOX and absence of IPTG) (Figure 3). Any plaques that develop should be due to viTetG replication, since any residual parental viLacR should only undergo abortive infections in the absence of IPTG (Figure 1C). Two days later, cells were examined under fluorescence microscopy. At this stage, the proportion of rVACV in the virus pool was substantially increased, such that only occasional abortive infections (due to viLacR) were detected. Individual (well-separated) viTetG (EGFP+) plaques were identified, collected, and processed. In this manner, a purified clone of viTetG was obtained in only 6 days.

Following isolation of a purified viTetG clone, the virus was amplified further to generate a working stock (Figure 3). To accomplish this, we infected cell monolayers with several dilutions (e.g., 10^{-2}, 10^{-3}) of the purified viTetG clone (i.e., cell lysate collected after the second purification step). Two days later, wells were observed under fluores-

cence microscopy to check for development of viTetG (EGFP+) plaques. Wells containing a single plaque (in this case, EGFP+) were examined carefully to ensure they were free of residual viLacR (i.e., dsRed−) and were then incubated for several days, until the entire well exhibited CPE. At that time, the cells were collected and processed to obtain a working stock of viTetG.

viTetG Abortively Infects Cells in the Absence of DOX and Forms Plaques in the Presence of DOX

Once a working stock of viTetG was obtained, the virus was characterized to ensure the correct replication phenotype (i.e., replication-inducible by DOX). Cells in 24-well culture dishes were infected with either the parental virus viLacR or rVACV viTetG at ~20 plaque-forming unit (PFU)/well. After 2 days, cells were imaged using a fluorescence microscope and subsequently stained and fixed with crystal violet (Figure 4). As expected, parental virus viLacR formed dsRed+ plaques only in the presence of IPTG and abortively infected cells (detected by dsRed expression) in the absence of IPTG. Furthermore, rVACV viTetG formed EGFP+ plaques only in the presence of DOX and abortively infected cells (detected by EGFP expression) in the absence of DOX.

Marker-free rVACVs Can Be Purified Using the EPPIC Platform

While the addition of a screening marker (e.g., dsRed or EGFP) within the rVACV makes this platform extremely straightforward, to be of maximal utility, the EPPIC platform should be customizable to purify marker-free rVACVs as well. As a proof of concept, we used viTetG (tet-inducible VACV expressing EGFP) as the parental virus to generate a marker-free replication-constitutive rVACV (vFREE) expressing a GOI (in this case, dsRed). During purification, cells were infected in the absence of DOX and were examined using light microscopy only (no fluorescence) to demonstrate the ability to purify a marker-free rVACV (i.e., relying solely on cytopathic effect). During the first purification step (Figure 5), plaques were identified under bright field (black circles) and were secondarily confirmed to be dsRed+ (expressed by vFREE and not by parental virus viTetG). vFREE plaques were readily identified by cytopathic effect and purification was continued (as in Figure 3) to obtain a purified vFREE clone.
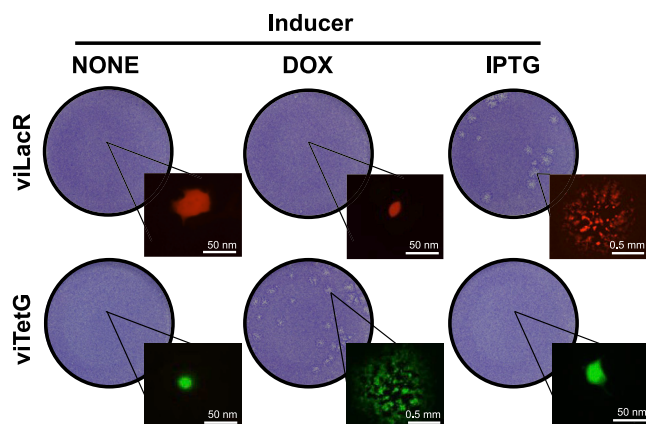
Figure 4. Parental Virus viLacR and rVACV viTetG Form Plaques Only in the Presence of the Appropriate Inducer
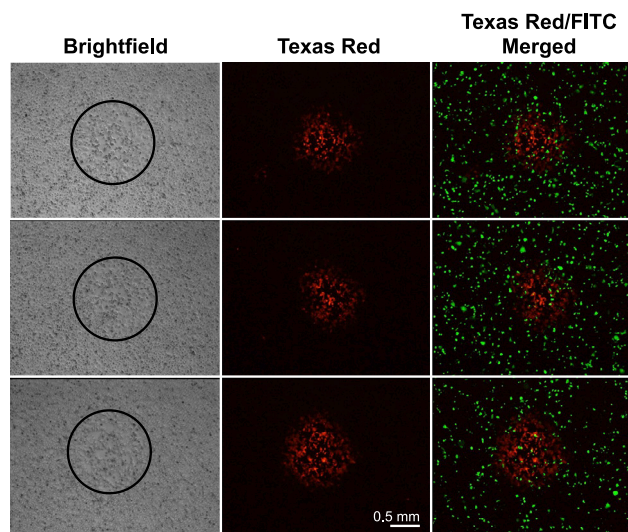
Cells were infected with viTetG or viLacR at ~20 PFU/well and incubated in the absence or presence of inducer (1 μg/mL DOX or 1 mM IPTG). After 2 days, representative images were taken using an inverted fluorescence microscope. Cells were then stained and fixed in 0.5% crystal violet/20% ethanol and the entire well was imaged.


Figure 5. Marker-free rVACVs can be purified using the EPPIC platform

vFREE, a replication-constitutive rVACV expressing a GOI (*dsRed*), was generated by homologous recombination with viTetG, a *tet*-inducible virus expressing EGFP. vFREE was purified under light microscopy to demonstrate purification of a marker-free rVACV. Cell monolayers in 100 mm dishes were infected with infection/transfection lysate at a dilution of 10^{-2}, incubated for 2 days, then imaged using an inverted fluorescence microscope. Plaques were readily identified under brightfield and then confirmed to be a result of vFREE replication (dsRed⁺). Parental viTetG was detected by EGFP expression (abortive infections).

Following purification, vFREE was characterized *in vitro* by comparing the size of plaques formed by vFREE, wild-type VACV (Western Reserve [WR]), and the parental VACV (viTetG) in the absence or presence of 1 μg/mL DOX (Figure 6). As expected, viTetG formed plaques only in the presence of DOX, while only abortive infections were detected in the absence of DOX. Importantly, plaques formed by vFREE were comparable to or near wild-type size in both the absence and presence of DOX. Thus, marker-free rVACVs can be readily purified using the EPPIC platform by simply using light microscopy to identify regions of cytopathic effect.

DISCUSSION

Here, we describe an EPPIC platform for rapid and simple purification of rVACVs in as little as 6 days. This platform utilizes previously generated replication-inducible VACVs[3,17] as parental viruses that can be rapidly and efficiently removed from the parental/rVACV pool following homologous recombination by simple serial passage in the absence of parental inducer. Notably, this platform uses standard cell lines and does not require selection media, agarose overlay, or specialized equipment such as FACS. Importantly, the resulting rVACV is identical to parental with the sole exception of the precise introduction of the sequences (in the case of vFREE, the promoter P_{11} and GOI dsRed) between the homologous regions.

The EPPIC platform is entirely customizable, allowing for the generation and purification of replication-competent, *tet*-inducible, or *lac*-inducible rVACVs, with or without screening markers using the purification workflow described above. To facilitate customization, we have developed a suite of viLac and viTet parental VACVs that contain the inducible mechanism at different loci within the VACV genome (e.g., D6R, A6L, F17R) with various markers (e.g., dsRed,

EGFP, *gusA*,[12] or lacZ). For example, to generate a *lac*-inducible rVACV expressing EGFP, a *tet*-inducible parental VACV expressing LacZ could be used and purification performed in the absence of DOX and presence of IPTG. Conversely, to generate a *tet*-inducible rVACV expressing LacZ, a *lac*-inducible parental VACV expressing dsRed could be used and purification performed in the absence of IPTG and presence of DOX. In this manner, one can simply and rapidly shuffle back and forth between viLac and viTet vectors by strategic selection of the parental virus and swapping inducer constraint during purification.

The EPPIC platform utilizes reagents and equipment found in most standard virology laboratories. Nevertheless, if fluorescence microscopy is unavailable, we have investigated alternative strategies. First, we have used this platform to successfully purify rVACVs through "blind" passaging by simply transferring the supernatant of infected cells, rather than the cell lysate. In the absence of inducer, the vIND parental virus only abortively infects cells and therefore, few infectious parental VACV particles are released into the supernatant. Therefore, the vast majority of virions released into the supernatant are the rVACV. While the technique of serial passage of supernatant is technically simpler, the concentration of rVACV in the supernatant is typically low. Thus, in our hands serial passage of cell lysate results in a more reliable and therefore faster purification. Second, we have successfully purified a marker-free rVACV by relying solely on light microscopy to identify regions of cytopathic effect (a result of rVACV
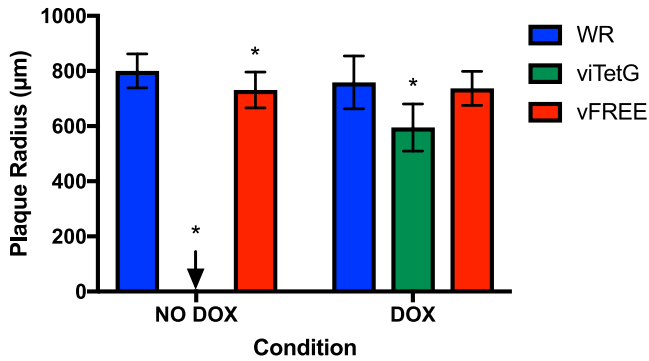
Figure 6. Marker-free rVACV, vFREE, Forms Plaques Comparable to Wild-Type (WR) in the Presence and Absence of DOX, while Parental VACV, viTetG, Forms Plaques only in the Presence of DOX

BS-C-1 cell monolayers were infected with the VACVs at approximately 30 PFU/well in the absence or presence of 1 µg/mL DOX. After 2 days, cells were stained and fixed in 0.5% crystal violet/20% ethanol and the size (radius) of approximately 30 representative plaques was measured. Asterisks represent statistical significance ($p < 0.05$) by one-way ANOVA with Dunnett's multiple comparisons test compared to WR at a given DOX condition. Bars represent geometric mean and error bars represent standard deviation. Arrow indicates absence of plaques.

replication since purification is performed in the absence of parental VACV inducer; Figure 5). Thus, even if a fluorescence microscope is unavailable, the EPPIC platform can be utilized for the rapid purification of rVACVs.

Since we have generated vIND parental viruses at multiple loci in VACV, the EPPIC platform can be used to rapidly generate rVACVs that contain two (or more) heterologous DNA constructs at distinct (and distant) genetic loci to express multiple GOIs (e.g., for multi-pathogen vaccines). Separating the genetic constructs into distant VACV loci would allow repetition of VACV promoters or other genetic elements, or incorporation of multiple similar antigens (e.g., glycoproteins of related viruses) while minimizing the risk of homologous recombination and genetic instability.

This novel platform enables the rapid generation and characterization of VACV vectors for a variety of applications, including standard rVACV generation for research use, rapid generation of marker-free VACV vectors for clinical use, as well as rapid characterization of vaccine antigen strategies for emerging pathogens. For example, we recently generated a number of VACV-vectored vaccine candidates against Zika virus by performing serial purifications for each of the candidates in parallel, to rapidly determine the best antigen strategy. Furthermore, the EPPIC platform could enable researchers to rapidly and simply generate, update, and refine personalized or custom VACV-vectored vaccines or therapeutic vectors in a matter of days.

MATERIALS AND METHODS
Cells, Viruses, and Reagents
African green monkey BS-C-1 (CCL-26) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and

were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% tetracycline-tested fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA). All cells were grown at 37°C in 5% CO_2. The L-variant of VACV strain WR was obtained from ATCC (VR-2035) and a clone (9.2.4.8) derived by sequential plaque purification[12] was used to generate the recombinant viruses in this study. DOX (Sigma-Aldrich, cat. # D9891, ≥98% TLC) and IPTG (Gold Biotechnology, cat. #12481C) were added to DMEM where indicated.

Generation and Characterization of Parental VACV viLacR
The *lac*-inducible parental virus expressing dsRed (viLacR) was generated exactly as previously described for *tet*-inducible VACVs,[3,17] with two modifications. During construction of the shuttle vector to generate viLacR, the *tetR* gene and *tet* operator (O_2) sequence were replaced with a modified *lacI* gene[13] (encoding the repressor protein of the *lac* operon and based on GenBank: U00096), and a synthetic 22 bp *lacO* sequence (5′-GAATTGT GAGCGCTCACAATTC-3′),[14] respectively. The dsRed-express gene (dsRed) encoding a red fluorescent protein and the *lacI* gene were placed under the control of the back-to-back $P_{E/L}$ synthetic promoters,[15] as previously described.[12] Propagation of viLacR was performed in the presence of 1 mM IPTG. To characterize viLacR, we infected BS-C-1 cell monolayers with viLacR or wild-type WR at approximately 30 PFU/well in the absence or presence of multiple concentrations of IPTG. After 2 days, cells were stained and fixed in 0.5% crystal violet/20% ethanol and the size (radius) of approximately 30 representative plaques was measured under an inverted microscope with measurement-capable software (AxioVision 4.8.1, Carl Zeiss).

viTetG Shuttle Vector Design
The viTetG shuttle vector was generated by a combination of DNA synthesis (Atum, Newark, CA) and standard subcloning, using engineered restriction endonuclease sites to facilitate assembly. The *tetR* gene (based on GenBank: X00694) was synthesized with an internal VACV early transcriptional termination sequence (TTTTTNT) removed from the middle of the gene (Leu codon at position 358 changed from TTA to CTT) to ensure early gene expression.[3,12,17] The *tetR* and *EGFP* genes were placed under back-to-back $P_{E/L}$ synthetic promoters.[15] The shuttle vector also contained the putative VACV D6R promoter region with a $tetO_2$[16] placed immediately after the late transcriptional initiator element sequences. The cassette was surrounded by 600 bp of VACV genomic sequences (based on Gen-Bank: NC_006998) to the left (upstream D5R gene) and to the right (D6R gene) to direct homologous recombination and insertion of the cassette into the appropriate genomic location. The plasmid was verified after construction by restriction enzyme digestion and PCR analysis.

Generation of rVACV viTetG
To generate viTetG, we seeded BS-C-1 cells in 12-well culture plates to approximately 85%–95% confluency. Cells were washed once with DMEM and infected with parental virus (viLacR) at a multiplicity of

infection (MOI) of 0.1 for 1 h. Cells were then washed once with DMEM and overlaid with DMEM containing 2.5% FBS and both 0.1 mM IPTG and 1 μg/mL DOX. The infected cells were then transfected with a shuttle vector using FuGENE HD transfection reagent (Promega, Madison, WI, USA) in duplicate wells. Two days later, cells were observed under light and fluorescence microscopy for evidence of viLacR replication (dsRed expression and CPE) and transfection efficiency. Cells were collected by scraping the entire surface of the well with the blunt end of a micropipette tip, centrifugation to pellet the cells, and resuspension in 0.5 mL fresh media. Cell lysates were processed by a series of three freeze/thaw cycles, sonication, trypsinization, and finally centrifugation to clarify the virus suspension.

Purification of viTetG from Parental viLacR

To purify viTetG from parental viLacR, we used the cell lysate collected after the initial infection/transfection to infect 100 mm cell culture dishes at various dilutions (e.g., 10^{-1}, 10^{-2}) in the presence of 1 μg/mL DOX (viTetG inducer) and absence of IPTG (viLacR inducer). Two days later, cells were examined with light and fluorescence microscopy for the presence of rVACV (EGFP+ plaques). EGFP+ plaques were collected using a standard micropipette, using an EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA) placed inside a biosafety cabinet to facilitate simultaneous visualization and collection. The cells were collected by aspiration of ~50 μL of overlay media while scraping the well surface to lift off EGFP+ cells. The collected cells were then resuspended in 0.5 mL of fresh media, processed (series of three freeze/thaw cycles, sonication, and trypsinization), and used to infect 24-well culture plates at several dilutions (e.g., 10^{-1}, 10^{-2}) in the presence of 1 μg/mL DOX and absence of IPTG. Two days later, cells were observed under light and fluorescence microscopy for the presence of EGFP+ plaques (viTetG replication), which were then collected and processed in the same manner as above.

Amplification of viTetG was achieved by infecting several wells of a 24-well culture plate with several dilutions (e.g., 10^{-1}, 10^{-2}, 10^{-3}) of the lysate collected during the second purification step. Two days later, cells were observed under light and fluorescence microscopy for the presence of EGFP+ plaques. Wells containing a single EGFP+ plaque were incubated for several days until the entire well exhibited CPE. At that time, the cells were collected by scraping the entire surface of the well with the blunt end of a micropipette tip, centrifugation to pellet the cells, and resuspension in 0.5 mL fresh media. Cell lysates were processed by a series of three freeze/thaw cycles, sonication, trypsinization, and finally centrifugation to clarify the virus suspension.

Characterization of viTetG

DNA was isolated from 2 μL of viTetG stock according to the manufacturer's protocol (NucleoSpin Blood, Macharey Nagel, Germany) and characterized by PCR using in-house primers targeting multiple regions of the genetic cassette, to confirm purity (absence of viLacR DNA) and identity (correct components and proper orientation within the genetic construct), using primers previously described.[3] To confirm the correct replication phenotype (i.e., replication-induc-

ible), we used amplified viTetG (rVACV) and viLacR (parental) stocks to infect fresh cell monolayers (24-well plates) at ~20 PFU/well and incubated in the absence and presence of inducers (1 μg/mL DOX or 1 mM IPTG) for 2 days. After 2 days, cells were observed under light and fluorescence microscopy, and representative images were taken using a Carl Zeiss Axio Observer D1 microscope (Oberkochen, Germany). Cells were then stained and fixed in 0.5% crystal violet/20% ethanol and the entire well was imaged.

Purification and Characterization of vFREE

To generate vFREE, we designed a DNA shuttle vector that contained a GOI (dsRed) under the control of the promoter for the VACV F17R gene (P_{11}) between the homologous flanking regions, and a tet-inducible VACV expressing EGFP (viTetG) was selected as the parental virus. The infection/transfection was performed as described above, in the presence of 0.1 μg/mL DOX. Purification was performed in the absence of DOX, during which plaques were identified using light microscopy and secondarily confirmed by expression of the GOI (dsRed). To characterize vFREE, we infected BS-C-1 cell monolayers with vFREE, viTetG, or wild-type WR at approximately 30 PFU/well in the absence or presence of 1 μg/mL DOX. After 2 days, cells were stained and fixed in 0.5% crystal violet/20% ethanol and the size (radius) of approximately 30 representative plaques was measured under an inverted microscope with measurement-capable software (AxioVision 4.8.1, Carl Zeiss).

Data Availability

Sequences of plasmids used in the current study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

B.J., C.M.O., and P.H.V. designed the research. B.J. performed the experiments with assistance from C.M.O. and Y.W. B.J. and P.H.V. wrote the manuscript. All authors revised and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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