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Rapid, seamless generation of recombinant poxviruses using host range and visual selection

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Abstract

Vaccinia virus (VACV) was instrumental in eradicating variola virus (VARV), the causative agent of smallpox, from nature. Since its first use as a vaccine, VACV has been developed as a vector for therapeutic vaccines and as an oncolytic virus. These applications take advantage of VACV's easily manipulated genome and broad host range as an outstanding platform to generate recombinant viruses with a variety of therapeutic applications. Several methods have been developed to generate recombinant VACV, including marker selection methods and transient dominant selection. Here, we present a refinement of a host range selection method coupled with visual identification of recombinant viruses. Our method takes advantage of selective pressure generated by the host antiviral protein kinase R (PKR) coupled with a fluorescent fusion gene expressing mCherry-tagged E3L, one of two VACV PKR antagonists. The cassette, including the gene of interest and the mCherry-E3L fusion is flanked by sequences derived from the VACV genome. Between the gene of interest and mCherry-E3L is a smaller region that is identical to the first ~150 nt of the 3' arm, to promote homologous recombination and loss of the mCherry-E3L gene after selection. We demonstrate that this method permits efficient, seamless generation of rVACV in a variety of cell types without requiring drug selection or extensive screening for mutant viruses.

SUMMARY:

This is a method to generate "scarless" recombinant vaccinia viruses using host-range selection and visual identification of recombinant viruses.

Keywords

Vaccinia virus; Recombination; Poxvirus; Host range; Protein kinase R; E3L-mCherry

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*Authors contributed equally to the work

DISCLOSURES:
The authors declare no competing financial interests.

INTRODUCTION:

Vaccinia virus (VACV) was instrumental for the first successful eradication of a human pathogen, variola virus (VARV), from nature. Ever since the extermination of variola virus, poxviruses including VACV have continued to be useful therapeutic viruses for both human and animal medicine. For example, a VACV-based rabies virus vaccine has been very effective in preventing transmission of sylvatic rabies in Europe[1] and the United States[2]. More recently, recombinant poxviruses expressing a variety of anti-tumor molecules, for example, single-chain antibodies or human erythropoietin, have seen encouraging success as oncolytic agents[3–5]. VACV is particularly attractive as a vector because it is readily amenable to genetic manipulation, possesses a broad host range, and it is stable under a variety of conditions, allowing easy transportation and vaccine viability in the field[6, 7]. While multiple techniques have been developed to generate recombinant VACV for laboratory experiments and vaccine generation, current strategies to generate these viruses have notable limitations.

Because of the utility of VACV, multiple strategies to generate recombinant viruses have been developed. The first strategy employs homologous recombination to introduce a cassette including the transgene and a selectable marker gene such as an antibiotic resistance gene. The cassette is flanked by two ~500nt or larger arms directing the gene to a specific site in the viral genome, which is then stably integrated by double crossover events[8–10]. This strategy is rapid and efficient; however, it results in extra genetic material in the form of the marker gene that may produce unexpected effects. Furthermore, there is a practical upper limit to the number of transgenes that can be introduced limited by the number of unique selectable markers available. Transient dominant selection (TDS) strategies have addressed this issue by facilitating the generation of “scarless” recombinant viruses[11]. Using this strategy, a plasmid containing a mutant VACV gene and a selectable marker gene are integrated into the viral genome, but without additional flanking VACV DNA. This approach results in transient integration of the entire plasmid and duplication of the VACV gene as a result of integration by a single crossover event. This intermediate is stable as long as it is maintained under selection pressure, permitting enrichment of this construct. When selection is removed, the VACV duplication enables a second crossover event that results in the removal of the plasmid and subsequent formation of either the wildtype (wt) or recombinant virus in approximately 50:50 ratio. While TDS generates recombinant viruses without requiring the stable introduction of foreign DNA, multiple virus clones must be screened for the expected mutation by sequencing analysis, a potentially time consuming and costly step.

Here, we present an approach to generating recombinant poxviruses combining the best aspects of each of these approaches, similar to an approach that has been described for the replication incompetent modified vaccinia Ankara[12–14]. This strategy combines visual and host range selection to rapidly generate recombinant viruses by double crossover events, and subsequently eliminate the selectable marker gene by homologous recombination. This approach permits the rapid generation of mutants mediated by homologous recombination, with the “scarless” nature of TDS approaches, while not requiring a subsequent screening step to distinguish wild type and mutant viruses. Our method also uses host range selection in place of antibiotic selection, eliminating the risk of chemically induced phenotypic

changes in the cell line. For this approach, we have chosen to use the host antiviral protein kinase R (PKR) as the selective agent to generate recombinant VACV. PKR is expressed as an inactive monomer in most cell types[15]. Upon binding double-stranded RNA (dsRNA) at the N-terminal dsRNA-binding domains, PKR dimerizes and is autophosphorylated[16]. This active form of PKR phosphorylates the alpha subunit of the eukaryotic initiation factor 2 (eIF2), ultimately inhibiting delivery of initiator methionyl-tRNA to the ribosome, thereby preventing intracellular translation and broadly inhibiting the replication of many virus families[17, 18].

In response to the broad and potent antiviral activity of PKR, many viruses have evolved at least one strategy to prevent PKR activation. Most poxviruses express two PKR antagonists, encoded by the E3L and K3L genes in VACV, which antagonize PKR through two distinct mechanisms[19]. E3 prevents PKR homodimerization by binding double-stranded RNA[20, 21], while K3 acts as a pseudosubstrate inhibitor by binding directly to activated PKR and thereby inhibiting interaction with its substrate eIF2α[22]. Importantly, these two PKR antagonists do not necessarily inhibit PKR from all species. For example, the K3 homolog from sheeppox virus strongly inhibited PKR from sheep, whereas the sheeppox E3 homolog did not show considerable PKR inhibition[23, 24]. In this study, we present a method to use PKR-mediated selective pressure combined with fluorescence selection to generate a VACV recombinant deleted for E3L and K3L (VC-R4), which cannot replicate in PKR competent cells derived from diverse species. This recombinant virus provides an excellent background for rapid generation of recombinant viruses expressing genes under control of the native E3L promoter.

PROTOCOL:

1. Generating the Recombination Vector

1.1.1 Design primers to generate the selection cassette. We recommend designing each individual amplicon with overlapping sequences with neighboring amplicons and the vector to facilitate isothermal enzymatic assembly of DNA molecules, also called Gibson assembly, using any of several online primer design tools.

NOTE: This protocol can also be completed using traditional restriction endonuclease-based cloning methods. In that case, design primers with the appropriate restriction sites rather than with overlapping sequences.

1.1.2 Using the primers designed in step 1.1.1, PCR amplify the following elements in order from 5’ to 3’ (Figure 1): ~500 nucleotides of the VACV genomic region 5’ of E3L (5’ arm), EGFP or the gene of interest, ~150 nucleotides from the VACV genomic region immediately 3’ of E3L (short 3’ arm), a synthetic early/late poxvirus promoter[25], the mCherry-E3L fusion gene, and ~500 nucleotides from the VACV genomic region 3’ of E3L including the short 3’ arm (long 3’ arm).

1.1.3 In a PCR tube, add the reagents in the following order for each amplicon: DNase free water, primers to a final concentration as recommended by the polymerase manufacturer, PCR buffer to a final concentration of 1X, appropriate template

DNA and a high-fidelity polymerase to a final concentration as recommended by the manufacturer. The concentration of template DNA should be empirically determined, but we generally start with 10 ng/reaction.

NOTE: Adjust the volume of these reagents according to the reaction volume.

1.1.4 Use a three-step (denaturing, annealing, extension) PCR protocol to amplify each fragment from the appropriate template DNA. Determine the melting temperature based on the manufacturer's suggested Tm for each primer set, and determine the appropriate extension time based on the length of each amplicon and the DNA polymerase used. Run this protocol for 25 cycles.

1.1.5 Visualize the amplification products on an agarose gel. Determine the appropriate gel composition based on amplicon size, but in general 1% agarose is acceptable.

1.1.6 Gel purify each amplicon using a commercially available kit. Elute the amplicons in a volume equal to the initial PCR volume in step 1.1.3.

1.1.7 Linearize the cloning vector using either restriction endonuclease digestion, or by PCR. Gel purify the product using a commercially available kit, and elute in a volume sufficient to yield a final concentration of approximately 100 ng/μL.

1.1.8 Ligate all of the individual, gel purified amplicons and the linearized vector using a commercially available isothermal enzymatic assembly kit. Follow the manufacturer's suggested instructions to determine the appropriate concentration of each component, temperature, and time.

1.1.9 Transform competent *E. coli* with 2 μL of the assembled product from step 1.1.8 as previously described[26, 27]. Plate the transformed cells on LB agarose plates containing the appropriate selective antibiotic for the vector. Incubate the plates overnight at 37°C.

1.1.10 Pick well-isolated colonies, and transfer them to tubes containing Luria broth with the appropriate antibiotic. Incubate the tubes overnight at 37°C while shaking at 225 rpm.

1.1.11 Isolate the plasmids from the overnight culture using a commercially available kit. Check the concentration and purity of the DNA using a spectrophotometer. An A260/A280 ratio between 1.8 and 2.0 is acceptable. Sequence the plasmids to determine whether the desired cloning product is correct. Store the DNA at −20°C.

2. Generating the Recombinant Virus

2.1.1 Infect a confluent monolayer of suitable cells with the virus to be recombined at a multiplicity of infection of 1.0 (MOI = 1.0) in a 6-well plate. Incubate the infected cells at 37°C and 5% CO_2 for one hour, then aspirate the infecting medium and replace it with fresh DMEM.

NOTE: For replication competent viruses such as a vaccinia virus that lacks K3L[22], a cell like such as European rabbit kidney cell line RK13 (ATCC #CCL-37) or BSC-40 is appropriate. However, for replication deficient viruses, such as the virus described in this paper lacking both PKR antagonists E3L and K3L, a complementing cell line expressing these two genes in *trans* or PKR knock-down or knock-out cells are required.

2.1.2 Transfect the infected cells with 500 ng of the vector generated and validated in step 1.1.11 using a commercially available transfection reagent following the manufacturer's protocol. Incubate the cells at 37°C and 5% CO_2 for 48 hours.

NOTE: If using a vaccinia virus lacking both E3L and K3L, PKR-mediated selective pressure will drive selection of recombined viruses and maintain expression of the mCherry-E3L fusion protein in these cells.

If desired, it should also be possible to PCR amplify only the insert to use for transfection instead of the whole plasmid.

2.1.3 48 hours post-infection, harvest the infected monolayer. In some cases, the cells can be harvested by pipetting, but if they are still tightly adhered, harvest them with a cell scraper. Freeze-thaw the cells three times, and then sonicate the lysates for 15 seconds at 50% amplitude. Store this lysate at −80°C until ready to use.

2.1.4 Infect a confluent 6-well plate of a PKR competent cell line such as RK13 cells with serial 10-fold dilutions of the lysate harvested in step 2.1.3. Incubate the infected cells at 37°C and 5% CO_2.

2.1.5 24 to 48 hours post-infection, identify recombinant viruses by fluorescence microscopy. Plaques from recombinant viruses express red fluorescence due to integration the mCherry-E3L fusion gene (Figure 2). If a virus devoid of PKR inhibitors was used initially, all plaques will contain recombinant virus.

2.1.6 Plaque purify recombinant viruses three times on RK13 cells. After the final round of plaque purification, all plaques should express red fluorescence.

2.1.7 Infect a confluent 6-well plate of RK13 cells expressing the VACV PKR inhibitors E3L and K3L (RK13+E3L+K3L cells[28]) with the plaque-purified red fluorescing virus from step 2.1.4. Aim for approximately 50–100 plaques per well.

NOTE: These cells provide the VACV PKR antagonists in *trans* and alleviate the PKR-mediated selective pressure to maintain the mCherry-E3L fusion gene, thus promoting "scarless" generation of the recombinant virus.

2.1.8 Identify collapsed viruses by fluorescence microscopy. Plaques from mutant viruses that have lost the mCherry-E3L fusion gene will be colorless.

NOTE: The frequency at which the mCherry-E3L fusion gene is lost is approximately 2.5% (Table 2).

2.1.9 Plaque purify colorless plaques three times on RK13+E3L+K3L cells. All plaques should be colorless.

2.1.10 Confirm the loss of mCherry-E3L and the presence of the expected mutation by PCR and Sanger sequencing.

NOTE: If the gene or mutation of interest does not have PKR inhibitory activity, recombinant viruses must be grown on RK13+E3L+K3L cells or an equivalent PKR-inhibited or PKR deficient cell line (Figure 3).

REPRESENTATIVE RESULTS:

We used the procedure diagrammed in Figure 1 to generate a VACV lacking both PKR antagonists E3L and K3L, by replacing E3L with EGFP in a virus already deleted for K3L (vP872). Figure 2 shows red fluorescent plaques in PKR competent RK13 cells indicative of viral expression of mCherry-E3L, as well as EGFP expressed in RK13+E3L+K3L cells confirming the loss of E3L and collapse of the mCherry-E3L selection marker. Figure 3 confirms that this recombinant virus, VC-R4, lacking both PKR antagonists cannot replicate in PKR competent RK13 cells, while the parent virus, vP872 expressing E3L, is replication competent. To confirm that this inability to replicate in RK13 cells was only due to the loss of E3L, we replaced EGFP in VC-R4 with E3L, to generate a revertant virus using the same selection protocol. Figure 3 also validates that this revertant virus replicates as efficiently as vP872 in RK13 cells. Interestingly, colorless plaques consistent with collapse of the mCherry-E3L selection marker were identified prior to selection in RK13+E3+K3 cells that are generally required to select “scarless” recombinants, likely because of the extended sequence identity between the mCherry-E3L recombination cassette and the E3L gene being inserted into VC-R4. Therefore, to determine the efficiency of recombination and the rate of collapse we elected to produce viruses expressing the poxvirus PKR antagonist K3L to avoid the problem of early collapse[23]. Figure 4 indicates the appearance of colorless plaques (arrowheads) after infection of RK13+E3L+K3L cells. Table 1 shows the results of three independent experiments, where on average 12.6% of progeny virions had undergone recombination with the transfected plasmid, similar to previously reported frequencies[29–31]. Table 2 details the frequency of colorless plaques relative to total plaques in RK13+E3L +K3L cells, demonstrating the rate of collapse and loss of the mCherry-E3L selection marker occurred at a frequency of approximately 1.8%.

DISCUSSION:

Here we present a variation of a transient marker selection strategy[32] to generate recombinant vaccinia viruses without retaining foreign DNA in the final recombinant virus. Our strategy uses selective pressure mediated by the host antiviral protein PKR rather than other forms of selective pressure such as antibiotics. The use of host antiviral genes eliminates the possibility of chemically induced phenotypic changes in the cells, or increased risk of mutation due to selection drugs. Furthermore, unlike with drug selection, there is no lag phase for our approach, because PKR is expressed constitutively in all cells. Secondary visual selection based on mCherry expression also improves the specificity of this method by ensuring that only plaques expressing the transgene are picked during the

first phase, and is equally efficient as a negative selective marker while selecting mature recombinant viruses that have lost the mCherry-E3L gene.

The most critical steps for this recombination strategy are the generation of the appropriate recombination vector, and appropriate plaque purification to ensure that the selected virus is clonal. In this paper we suggest “Gibson assembly” to generate the recombination vector. This strategy is extremely efficient and allows assembly of all the fragments comprising the recombination vector in a single day. However, because the short 3’ arm and the long 3’ arm share identical sequences, these fragments have the potential to be joined together during the cloning reaction, and some vectors may not contain the mCherry-E3L cassette. In our experience this is rare, but confirming the structure of the vector after cloning is necessary. We have also generated recombination vectors for this strategy using traditional endonuclease and ligase methods. This strategy avoids the problem described above, but can be more labor intensive. Plaque purification is generally straightforward, and is primarily reliant on using appropriate permissive cells for the initial recombination, PKR-competent cells for initial plaque purification to ensure that only recombinant viruses can replicate, and then permissive cells again to facilitate intramolecular recombination and loss of the selectable marker. Close attention to cell lines is therefore critical for the successful and efficient application of this strategy.

In this study, we demonstrate the use of this method to generate a VACV recombinant deleted for both PKR antagonists E3L and K3L and expressing EGFP under control of the E3L promoter. Going forward, this virus will serve as an efficient background for future recombinant viruses, as it is incapable of replicating in PKR competent cells. Therefore, there will be strong PKR-mediated selective pressure to drive the mCherry-E3L recombination cassette into progeny virions while at the same time essentially preventing replication of non-recombinant virus. Furthermore, the loss of EGFP by uptake of the recombination cassette is a useful secondary selection marker to ensure picked plaques are not co-infected with a non-recombinant virus. We observed rates of recombination consistent with previously reported rates for VACV, but the visual fluorescent markers increase the efficiency of generating recombinant viruses by ensuring that increasing the likelihood that the appropriate recombinant viruses are selected. Our observation of colorless plaques after two rounds of selection on PKR-competent cells, presumably due to the increased length of identical sequence between E3L and the mCherry-E3L marker gene, suggests that the rate of mCherry-E3L loss may be “tuned” by increasing or decreasing the length of the 3’ short arm. The primary limitation of this technique is the use of PKR as the selective pressure for recombinants. The most efficient use of this recombination strategy is generating these viruses in a background lacking PKR antagonists. However, the colorimetric selection marker allows this recombination strategy to be used even without the selection mediated by PKR, simply by plaque purifying mCherry-expressing plaques. While the lack of PKR-mediated selective pressure will reduce the efficiency of the first screening step, the percentage of mCherry expressing plaques is still high enough that color-based selection is viable. Thus, this method can be used to insert nearly any gene into the poxvirus genome.

As demonstrated by the insertion of EGFP, with this approach, any gene can be rapidly inserted into the E3L locus under control of the native promoter, provided that PKR null cells or complimenting cell lines are used for downstream experiments if the transgene is not a PKR antagonist. This strategy, combined with the VC-R4 virus that we report here, adds a new and potent method to rapidly and reliably generate recombinant vaccinia viruses using host-mediated selective pressure and visual identification of recombinants early in the process.

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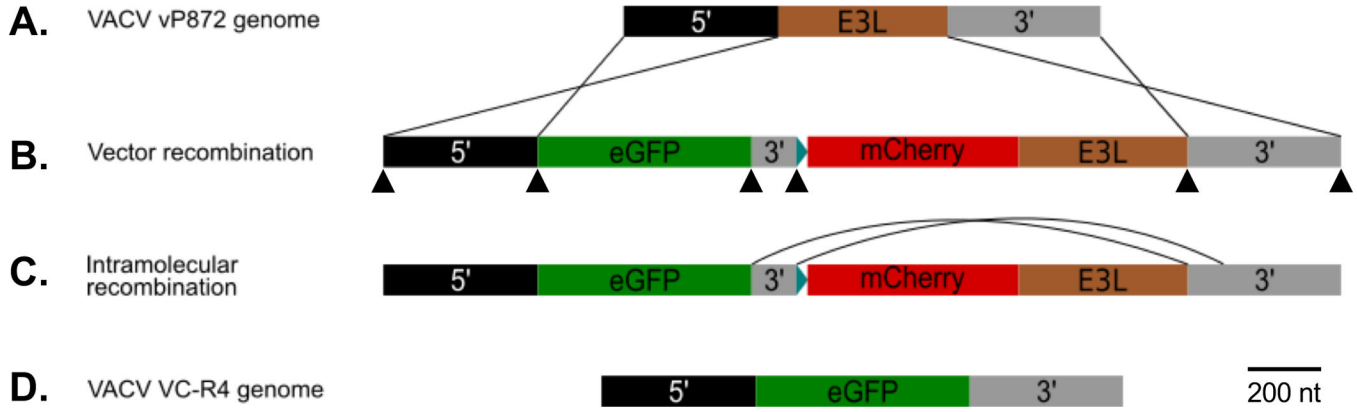

Figure 1.

Diagram of p837-GOI-mCherry-E3L as well as the host-range and visual recombination strategy. **(A)** 5' arm (black) and 3' arm (grey) flank the E3L locus (brown) in VACV. **(B)** In p837-GOI-mCherry-E3L, these arms flank a cassette containing the gene of interest (GOI), in this case EGFP, (green) separated from an mCherry-E3L (red) fusion gene under control of the synthetic early/late poxvirus promoter[25] blue) by a short 3' arm (grey). These external arms drive homologous recombination between VACV and the p837-GOI-mCherry-E3L. Black arrowheads indicate the sites of the overlapping primers used to generate this plasmid by Gibson cloning. **(C)** When PKR selective pressure is removed, viruses that have undergone intramolecular recombination between the short and long 3' arms can be selected. **(D)** Resulting in a virus (VC-R4) containing only the gene of interest in the E3L locus.

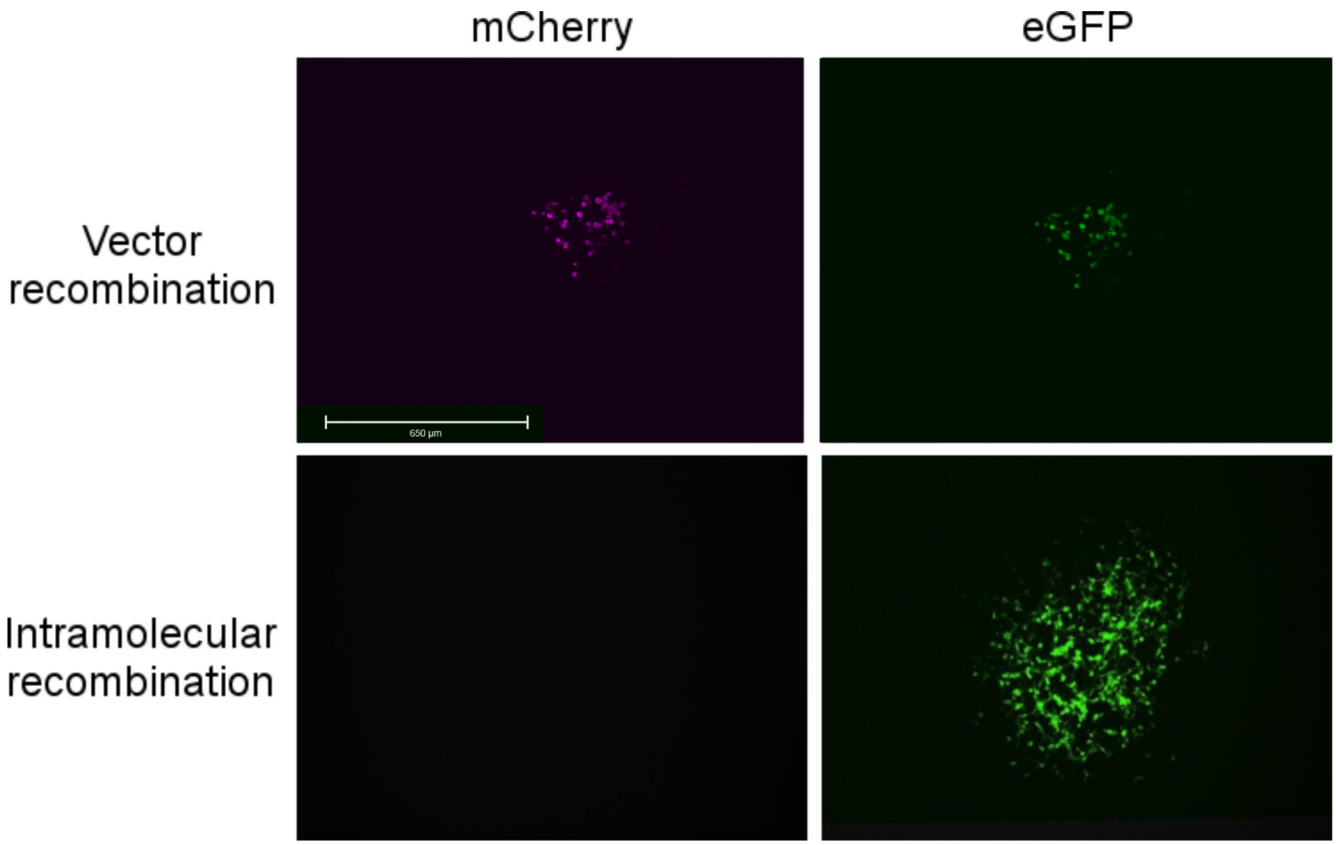

Figure 2.

Fluorescent micrographs of (top) a recombinant virus plaque 24 hours after recombination with p837-GOI-mCherry-E3L expressing both mCherry (left) and EGFP (right) in RK13 cells. (Bottom) Micrograph of a recombinant virus plaque 48 hours after PKR-mediated selective pressure has been removed in RK13++ cells, expressing EGFP (right) but not mCherry (left). The scale bar indicates 650 µm for all panels.

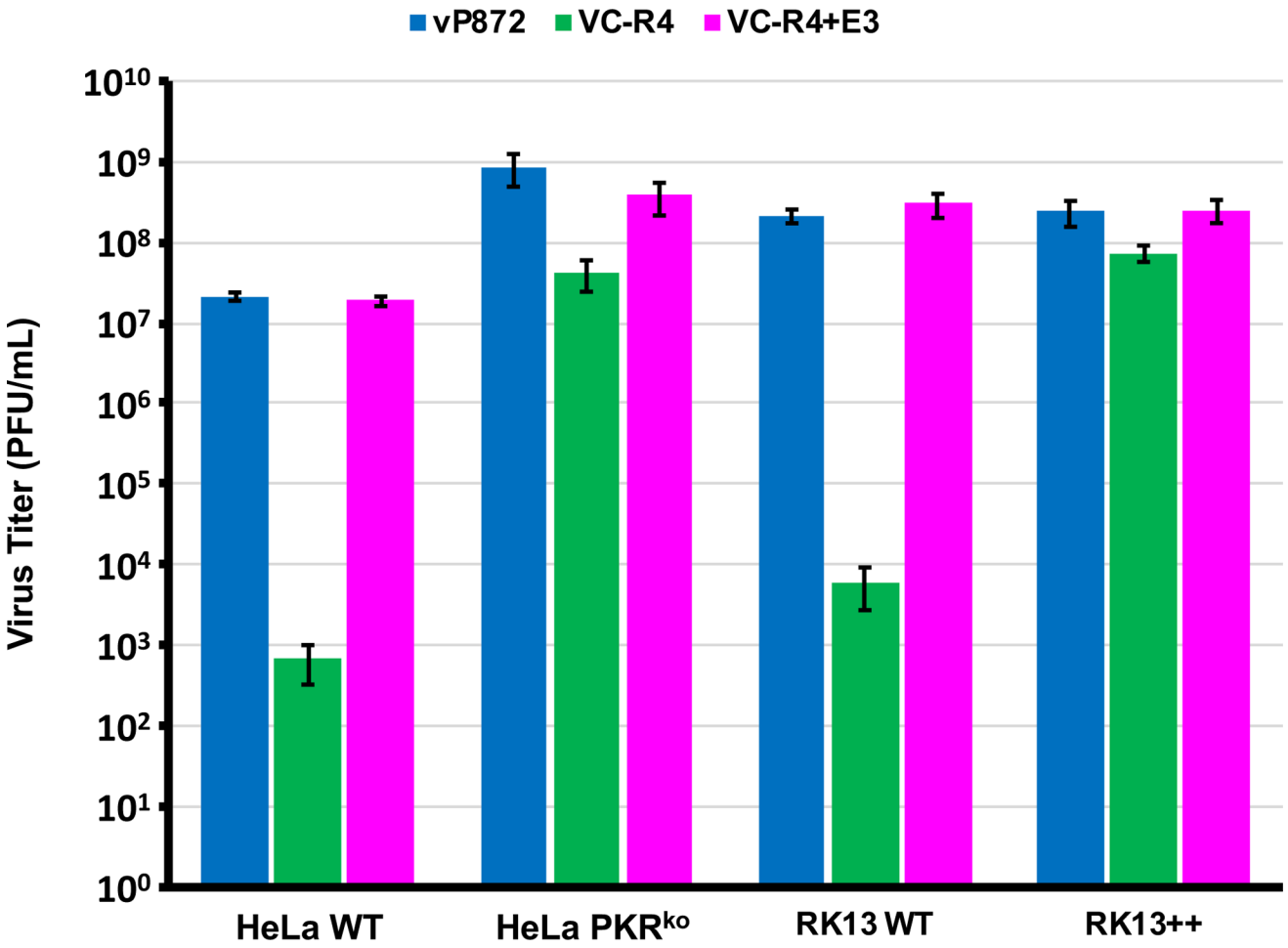

Figure 3.

VC-R4 cannot replicate in PKR competent cells. The indicated cell lines were infected with vP872 (blue), VC-R4 (green), or VC-R4+E3L (magenta) at MOI = 0.1. 48 hours post-infection the infected cells were harvested and titered by serial dilution on RK13+E3L+K3L cells. Titers are reported in PFU/mL, errors bars represent the standard deviation of three replicate experiments.

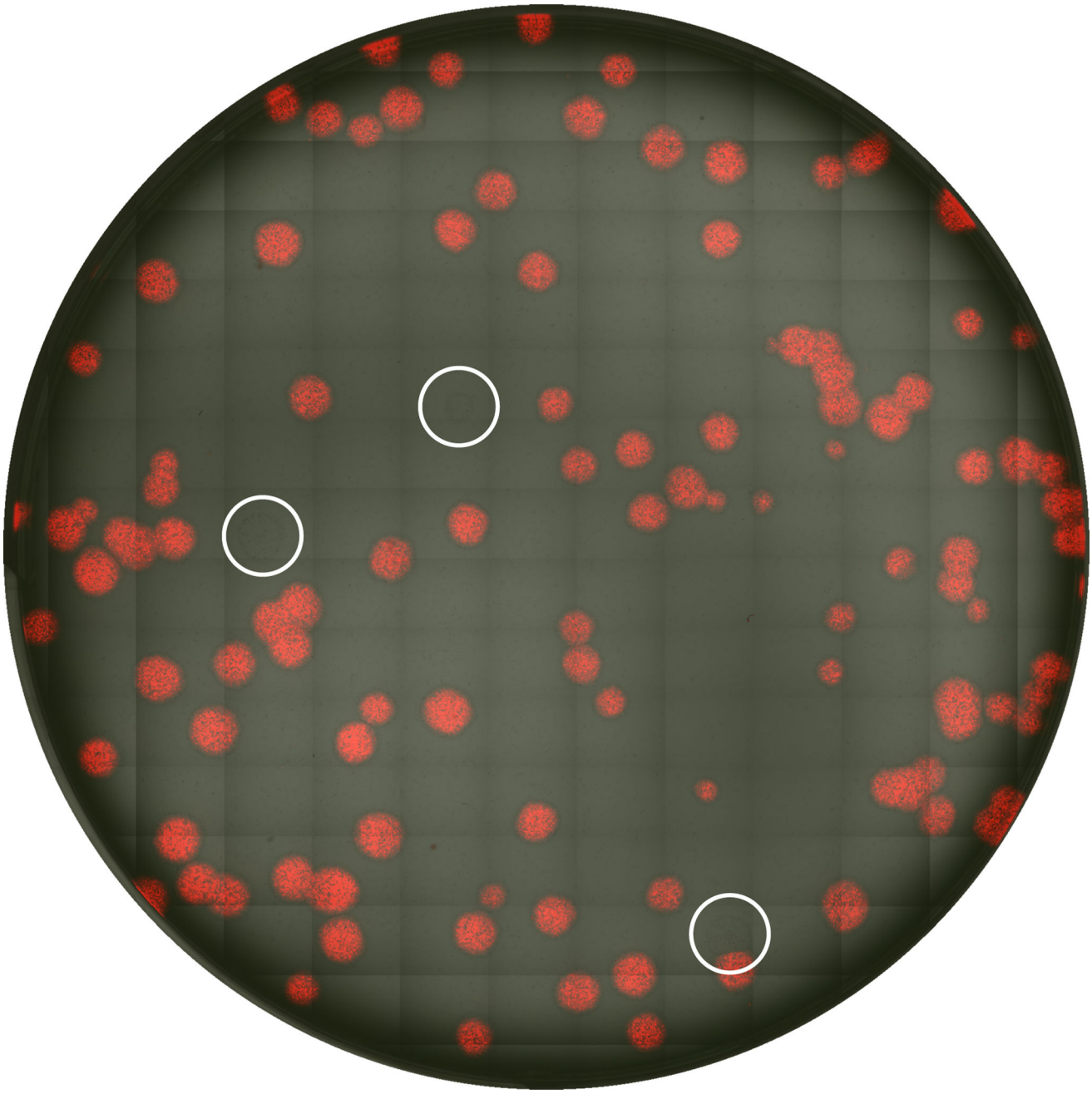

Figure 4.

Loss of mCherry-E3L expression in RK13+E3L+K3L cells. Overlay of fluorescent and phase contrast micrographs of VC-R4+K3L-mCherry-E3L infected RK13+E3L+K3L cells. Three plaques no longer express mCherry (circles) due to collapse of the selection cassette yielding VC-R4+K3L.

Table 1.

Recombination frequency of VACV with the p837-K3L-mCherry-E3L plasmid.

	Experiment 1	Experiment 2	Experiment 3
Red plaques (RK13)	30	11	18
Total plaques (RK13+E3L+K3L)	225	64	249
Recombination Rate	13.3%	17.2%	7.2%

Table 2.

Frequency of mCherry-E3L loss from VC-R4+K3L-mCherry-E3L in RK13+E3+K3 cells.

	Experiment 1	Experiment 2	Experiment 3
Total plaques (RK13+E3L+K3L)	115	44	210
Colorless plaques (RK13+E3L+K3L)	3	1	1
Recombination Rate	2.6%	2.3%	0.5%