A reporter system for replication-competent gammaretroviruses: the in*Gluc*-MLV-DERSE assay

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Abstract

While novel retroviral vectors for use in gene-therapy products are reducing the potential for formation of replication-competent retrovirus (RCR), it remains crucial to screen products for RCR for both research and clinical purposes. For clinical grade gammaretrovirus-based vectors, RCR screening is achieved by an extended S+L− or marker rescue assay, while standard methods for replication-competent lentivirus detection are still in development. In this report, we describe a rapid and sensitive method for replication-competent gammaretrovirus detection. We used this assay to detect three members of the gammaretrovirus family and compared the sensitivity of our assay with well-established methods for retrovirus detection, including the extended S+L− assay. Results presented here demonstrate that this assay should be useful for gene-therapy product testing.

Keywords

replication-competent gammaretrovirus; gene therapy vectors; infectivity assay

INTRODUCTION

Gammaretroviral vectors were the first viral gene-therapy vectors to enter clinical trials and remain in use today.[1] One potential hazard associated with the use of such vectors is the

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presence of replication-competent retroviruses ("RCR") in the vector preparations, either as a result of recombination events between the plasmids used for vector production or between the plasmids and endogenous retroviral sequences in the packaging cell lines, or as a result of contamination in the laboratory.[2–5] RCR are potentially pathogenic, and have in fact been shown to induce malignancy in mice and non-human primates.[6,7] Therefore, it is critical that vector preparations be rigorously tested to exclude the presence of RCR, and the Food and Drug Administration (FDA) requires that all vector preparations be screened for RCR.[8]

At present, the FDA recommends inoculating an aliquot of each vector preparation into a permissive cell line and passaging the cells for at least three weeks ("amplification phase"). Cell-free media from the amplification phase can then be analyzed by a variety of methods, including S+L– and marker-rescue assays, to demonstrate the presence or absence of RCR (reviewed in Cornetta and Wilson[9]; for a description of the S+L– assay, see Bassin *et. al.*[10]).

We now describe a new method for the detection of RCR in gammaretrovirus-based gene-therapy products and the cell lines used to make them. The assay uses one of a series of DERSE (Detector of Exogenous Retroviral Sequence Elements) plasmids, stably maintained in a cell line permissive for the retrovirus of interest.[11,12] Utilizing the unique reverse-transcription step of the retroviral infection cycle, a functional reporter gene is formed in the DERSE cell culture only after infection with an RCR. The DERSE assay can be constructed, potentially, to detect any RCR and can use any reporter gene.

In this report, we describe the in*Gluc*-MLV-DERSE assay, using a 293T cell line that expresses the receptor for ecotropic MLVs (murine cationic amino acid transporter, mCAT1), and *Gaussia* Luciferase (G*luc*) as the reporter gene.[13–15] We use the in*Gluc*-MLV-DERSE assay to detect three gammaretroviruses commonly used in construction of gene-therapy products: Moloney MLV (MoMLV); 4070A amphotropic MLV (4070A); and Gibbon Ape Leukemia Virus (GALV). Measurement of G*luc* activity in the cell culture supernatant, or the G*luc* gene in MLV-DERSE cell genomic DNA, indicates the presence of RCR in the test sample. The high enzymatic activity of G*luc* and the requirement for RCR infection for G*luc* expression result in an assay with a very high (10^4) signal:background ratio. The fact that G*luc* is secreted from the cell means that a single infected culture can be periodically sampled over time without the need to harvest cells. Most appealingly, the in*Gluc*-MLV-DERSE assay does not require specialized training to perform and compares well to current protocols used for RCR detection. In experiments not shown here, we have also obtained comparable results with other reporters, including GFP.

RESULTS

Generation of the in*Gluc*-MLV-DERSE cell line

The DERSE plasmids use a strategy that was originally devised for detection of retrotransposition by retroelements.[16–18] These plasmids consist of a retroviral genome with an intron-containing indicator sequence replacing the viral coding regions (Figure 1).[12] The viral sequences retained in the plasmid provide all the functions necessary for viral replication and expression (except those functions that are provided by viral proteins). Importantly, the indicator sequence is oriented in a reverse direction relative to the viral

sequences while the intron is oriented in a forward direction. In the absence of an RCR the cell transcribes the indicator sequence, using the viral sequences as a promoter, and removes the intron. The result is a minus-strand (non-coding) copy of the indicator RNA, flanked by viral *cis*-acting sequences that include the viral RNA packaging signal. If this cell is infected with an RCR, the minus-strand indicator sequence can be packaged by viral proteins encoded by the RCR. When this RCR goes on to infect a new cell, reverse transcription of the viral RNA (that now includes the minus-strand indicator sequence) results in a coding indicator gene that is subsequently integrated into, and expressed by, the newly infected cell. Here we describe the in*Gluc*-MLV-DERSE cell line in which the indicator gene is the secreted *Gaussia* luciferase (G*luc*) enzyme, the LTRs and other *cis* acting sequences are from MLV, and the host cell line is 293mCAT1. G*luc* activity is measured by addition of coelenterazine to a small volume (10μL) of cell culture supernatant, followed by measurement of light output.

Detection of virus with the inG*luc* cell line

We initially tested the in*Gluc*-MLV-DERSE cell line in several ways. Firstly, we were interested in the ability of the cell line to detect RCR produced from a chronically infected cell line. To assess this we co-cultured the in*Gluc*-MLV-DERSE cells with NIH-3T3 cells chronically infected with Moloney MLV (MoMLV). We observed a ~3-log increase in G*luc* activity in the cell culture supernatant 3 days after the two cell lines were mixed, indicating that the MoMLV originating from the NIH-3T3 cells had infected and replicated in the in*Gluc*-MLV-DERSE cells, resulting in expression of G*luc* (Figure 2a).

We next investigated the relationship between the amount of virus present at the time of infection and the G*luc* activity measured in the supernatant several days after infection. We infected the in*Gluc*-MLV-DERSE cells with 10-fold serial dilutions of MoMLV and measured G*luc* activity in the supernatant 4, 5 and 6 days after infection (Figure 2b). We found the G*luc* activity level in the culture supernatant increased monotonically with the concentration of MoMLV used for infection. Further, we could follow the spread of MoMLV through the culture as periodic sampling of the culture supernatant demonstrated accumulation of G*luc* activity over time. The dilution end-point for this assay was between 10^{-4} and 10^{-5} (Figure 2b); this result is in excellent agreement with the titer of this virus stock obtained by the direct S+L− focus assay[10] (data not shown; see Figure legend for details.).

Finally, as G*luc* is a secreted protein, we thought it interesting to see whether the G*luc* assay could be conducted directly in the cell culture plate. We tested this by infecting the in*Gluc*-MLV-DERSE cells with MoMLV in a black 96-well plate and then adding G*luc* substrate directly to the cell culture well 3 days after infection (Figure 2c). The MoMLV-infected culture showed G*luc* activity of ~10^7 counts per second (cps), the maximum activity measurable using our instrument, while a mock-infected culture had a background of ~10^3 cps. Thus it is possible to infect and detect RCR in the same plate. The ability to conduct the assay in this way may simplify automation of the assay and could have applications outside of gene-therapy product testing.

Real-time PCR for detection of RCR infection

We found measurement of G*luc* activity in the supernatant to be a rapid and simple method for detecting infection of the inG*luc*-MLV-DERSE cell line. However, since infection of the cell line with a RCR results in the generation of a new gene (the spliced G*luc* gene), it is also possible to use PCR as a read-out for the assay by targeting a primer/probe specifically to the coding G*luc* sequences that flank the intron. We developed a duplex real-time PCR assay that simultaneously amplified the functional G*luc* gene and, as a control for DNA quality and quantity, the single-copy CCR5 gene. Using this PCR, only the spliced, functional G*luc* gene can be amplified (and thus amplification from the G*luc* primer/probe set can only occur following RCR infection of the inG*luc*-MLV-DERSE cell line). Using a plasmid standard, we determined that the real-time PCR could detect 10 – 33 copies of G*luc* in 100ng of inG*luc*-MLV-DERSE cell DNA (data not shown). To test the duplex real time PCR assay, we extracted genomic DNA from inG*luc*-MLV-DERSE cells 3 days after they were infected with serial dilutions of MoMLV. We performed duplex real-time PCR on the DNA and measured G*luc* activity in the supernatant (Figure 3a, b, c and d). The G*luc* probe Ct value was inversely related to the MoMLV concentration used for infection (Figure 3a and c). Further, the G*luc* probe Ct values and the G*luc* activity in the supernatant were in complete concordance - a culture deemed G*luc* positive or negative in the duplex real-time PCR assay gave the same result in the G*luc* activity assay (Figure 3a and d). As expected, CCR5 was amplified in all cases, confirming that the absence of G*luc* probe fluorescence was not due to an absence, or poor quality, of DNA (Figure 3b).

In a separate experiment, we infected the inG*luc*-MLV-DERSE cells with MoMLV and the day after infection performed real-time PCR on the cellular DNA and a G*luc* activity assay on the supernatant (results not shown). In this case, we found that the G*luc* gene was amplified by real-time PCR but G*luc* activity was not detected in the supernatant, presumably because G*luc* protein levels had not accumulated sufficiently. This suggests that the real-time PCR can detect infection of the inG*luc*-MLV-DERSE cell line marginally earlier than measurement of G*luc* activity in the supernatant. However, the extra time and manipulations (which could increase the risk of contamination) required to extract DNA and perform the real-time PCR, as well as the fact that the experiment is terminated once DNA is collected, may make the G*luc* activity more suitable for many purposes.

Comparison of the inG*luc*-MLV-DERSE assay with other methods for gammaretrovirus detection

To support the results from the inG*luc*-MLV-DERSE assay, we compared the assay with two established methods for detecting retrovirus – the reverse transcriptase (RT) assay and immunoblotting against viral proteins. We infected the inG*luc*-MLV-DERSE cells with serial dilutions of 4070A amphotropic MLV, grew the cells, and collected the supernatant for G*luc* activity assay, RT assay or immunoblot (Figure 4a, b and c). Within 16 days of the initial infection, we observed that all cultures infected with the 10^{-5} dilution of 4070A and none of the cultures infected with the 10^{-6} dilution of 4070A had G*luc* activity in the supernatant, making 10^{-5} the dilution end-point for this particular virus stock using this assay (Figure 4a). This result is in reasonable agreement with the results of the standard, extended S^+L^--based assay[19], which gave a titer of 10^6 infectious units/ml for the inoculum

(data not shown). G*luc* activity was first detected in the cultures infected with the 10^{-5} dilution 6 days after infection (Figure 4a).

We next measured RT activity in the supernatant collected during growth of the 4070A-infected inG*luc*-MLV-DERSE cells. Using this particular RT activity assay, we first detected RT activity in supernatant collected 13 days after infection of the inG*luc*-MLV-DERSE cells with a 10^{-5} dilution of 4070A (Figure 4b). RT activity was not detected in supernatant from cells infected with a 10^{-6} dilution of 4070A, even at 16 days after infection. While the G*luc* activity assay proved to be more rapid than the RT assay under these experimental conditions, both assays gave the same dilution endpoint for this 4070A stock in this experiment (Figure 4a and b).

Additionally, we used a broadly reactive antiserum to MLV p30^{CA} in an immunoblot of virus pellets prepared from the supernatants of the 4070A-infected cultures (Figure 4c). Supernatant p30^{CA} levels, as estimated by immunoblot, were proportional to G*luc* activity (Figure 4a and c). Under the conditions used, the G*luc* activity assay was more sensitive than the immunoblot for RCR detection. Thus, while supernatant from the culture infected with a 10^{-5} dilution of 4070A demonstrated G*luc* activity 6 days after infection, p30^{CA} was not observed in the supernatant until 12 days after infection (Figure 4a and c). Again, both assays gave the same endpoint for this 4070A stock, as p30^{CA} was not observed in supernatants collected from cells infected with a 10^{-6} dilution of 4070A.

Comparison of DERSE assay with FDA-compliant protocols for RCR detection

To determine the usefulness of the inG*luc*-MLV-DERSE assay for testing gene-therapy products, we used a single GALV stock and compared G*luc* activity assay results from infection of the inG*luc*-MLV-DERSE line (conducted at NCI) with an extended S+L− assay conducted at the Indiana University Vector Production Facility.[20] Using the S+L− assay, the GALV stock was found to contain 10^5 tissue culture-infectious doses (TCID50) per ml (not shown). We infected the inG*luc*-MLV-DERSE cells with 10-fold dilutions of the virus, in triplicate, and then passaged the cells and monitored G*luc* activity in the cell culture supernatant. Twelve days (and 3 cell passages) after infection, 3/3 of the cultures infected with a 10^{-5} dilution of GALV and 1/3 of the cultures infected with a 10^{-6} dilution of GALV showed maximum G*luc* activity in the supernatant (Figure 5). Using identical infection conditions, we repeated this experiment twice (that is, with 2 different GALV dilution series): in the second and third experiments 3/3 of the cultures infected with a 10^{-5} of GALV were positive and 0/3 of the 10^{-6} infected cultures (results not shown). Thus, the results from the inG*luc*-MLV-DERSE assay are in excellent agreement with those from the S+L− assay.

Finally, using the same GALV stock, we investigated the sensitivity of the inG*luc*-MLV-DERSE assay when infecting with an RCR in the presence of excess replication-incompetent retroviruses, as might be the situation when testing gene-therapy products. High concentrations of vector at the time of infection have been observed to interfere with detection of RCR, likely due to competition for cellular entry receptors.[21,22] To determine the effect of excess vector on our assay, we infected 1.25×10^5 inG*luc*-MLV-DERSE cells with serial dilutions of GALV. The dilutions were made either in media or in 7.6×10^6

infectious units of a GALV-based gene-therapy vector shown to be RCR-negative using an FDA-compliant extended S+L− assay. As expected, in multiple experiments, no RCR were detected in the vector preparation by the inG*luc*-MLV-DERSE assay. However, we did observe somewhat lower G*luc* activity in cell cultures infected with GALV in the presence of vector, compared to cultures infected with GALV alone. This observation was most notable in cultures infected with the 10^{-5} dilution of GALV (Figure 6a and b). Importantly though, the rate at which GALV infection proceeded through the culture was the same regardless of the presence or absence of gene-therapy vector (Figure 6a). As there was no effect of excess gene-therapy vector on the kinetics of RCR spread through the culture, the reduction in G*luc* activity in samples assayed soon after infection was a result of interference by the vector at the time of virus attachment to the cell, and not interference of the vector at a later stage in the retroviral cycle. Most importantly, however, within the resolution of our assay, the dilution end-point for the GALV stock was the same (i.e. 10^{-5}) both in the presence and absence of gene-therapy vector (Figure 6b); as in Figure 5, this result also agreed with the titer of the GALV preparation previously determined by passaging on S+L− cells (data not shown).

DISCUSSION

While advances in gene-therapy vector preparation have reduced the risks of RCR formation during gene-therapy vector production, screening of these products remains essential. Screening for RCR in both gammaretrovirus- and lentivirus-based vectors is a time-consuming and meticulous process. In particular, the S+L− assay used for screening gammaretrovirus based vectors requires counting of RCR-induced foci in a cell monolayer. This is a tedious and somewhat subjective measurement. Furthermore, the FDA-compliant biologic assays are generally a two-stage process, with vector product (or co-culture when testing cell lines and patient cells) being first passaged on a permissive cell line for 3 weeks (to amplify any RCR) prior to conducting a 4–6 day S+L− assay or a marker rescue assay (RCR detection).

The inG*luc*-MLV-DERSE assay described here does not require the identification of foci, a substantial improvement on the S+L− assay. Further, the DERSE assay combines the amplification and detection stages for relatively rapid, real-time monitoring of RCR infection of the inG*luc*-MLV-DERSE cell culture. We show here the assay sensitively detects RCR in supernatant and produces results that are comparable to other RCR detection techniques (Figure 4 and 5). Moreover, the inG*luc*-MLV-DERSE assay is capable of directly detecting RCR by simple cocultivation with a producer cell line (Figure 2a). Thus it should be useful for screening both gene-therapy products and the packaging cell lines used for vector production (though the latter were not investigated here).

What makes the DERSE assay unique, however, is that the indicator gene (or protein) is only formed if the inG*luc*-MLV-DERSE cells are infected with RCR. This should result in a high signal: background ratio, with resulting enhancement of detection sensitivity, regardless of the indicator gene used. Furthermore, as far as we know, any replication-competent gammaretrovirus would register in this assay. Thus, in addition to applications in

gene-therapy product testing, the generic DERSE assay will undoubtedly have multiple applications for basic RCR research.

In this work, we chose *Gaussia* luciferase as our indicator gene as it has two advantages over other reporter proteins. Firstly, G*luc* is a predominantly secreted protein and thus there is no need for cell lysis, as is required for assays of firefly luciferase. In fact, since G*luc* is secreted from the cell, the G*luc* activity assay can be conducted directly in the cell culture plate (Figure 2c). Secondly, this highly active enzyme can produce luminescent signal intensities of at least 10^7 counts per second, a signal ~10^4 times greater than the background of our assay. The combination of these two properties of the G*luc* enzyme means that, potentially, this particular DERSE assay could become fully automated rather simply (again, with possible applications outside of gene-therapy product testing).

For gamma-RCR detection, we chose to maintain the inG*luc*-MLV-DERSE vector in mCAT1-expressing 293T cells (293mCAT1 cells). We show here that these cells are sufficient for infection and rapid amplification of gamma-RCR that are commonly used with vectors for gene delivery, namely GALV, MoMLV and 4070A. Additionally, we have used the inG*luc*-MLV-DERSE 293mCAT1 cell line to detect several other gammaretroviruses including members of the xenotropic MLV family. [5] While we did not compare the infectability of these cells against other cells used with GALV, MoMLV or 4070A, previous work has demonstrated that 293 cells are most suitable for GALV amplification.[20] We would expect the 293mCAT1 cells used here to be equally suitable for GALV amplification when compared to 293 cells. While *Mus Dunni* cells are currently recommended for amplification of MLV-based vectors, we did not investigate an inG*luc*-MLV-DERSE assay in these cells.[22] It may well be that *Mus Dunni* cells offer enhanced virus amplification when assessing MLV-based vectors; it is important to note that the indicator plasmid can be maintained in any cell line deemed optimal for growth and detection of a particular retrovirus.

We used several cell densities and flask/plate sizes while developing this assay, eventually settling on the use of 6-well plates as these provided us with ample cells for DNA extraction and supernatant for RT assay or immunoblot, without using large amounts of space or reagents. While such a small plate size is likely not compatible with the requirement to test 5% of the final gene-therapy product volume, it should be sufficient for research purposes. In setting these parameters, it is crucial to remember that infection by gammaretroviruses requires that the host cell be dividing, while relatively high cell densities are optimal for virus spread.[20,22] In general, we found that passaging the cells such that they were confluent in 3–4 days provided a good environment for rapid RCR amplification. Using the standardized 6-well plate assay, we observed that inG*luc*-MLV-DERSE cells initially infected with a few virus particles had detectable G*luc* activity in the culture supernatant 6 – 7 days after infection and became completely infected 10 – 14 days after infection (Figure 4 and 5). While vigilant RCR detection may still benefit from a 3-week amplification stage (to ensure detection of low numbers or slowly replicating RCR), the inG*luc*-MLV-DERSE assay remains a more rapid method for detecting small quantities of virus than the currently used two-stage protocols as it combines the amplification and detection steps.

Similar to previous reports, we found that the presence of excess gene-therapy vector interferes somewhat with the initial amplification of RCR (Figure 6).[21,22] As expected, this was most clearly observed at the limiting dilution (10^{-5}) of GALV where the ratio of RCR: vector is lowest. However, at least for this combination of RCR and vector, the vector did not detectably affect the titer of GALV obtained in the inG*luc* assay. Thus, the data indicate that the effect of vector is minimal, and is presumably similar to the decrease in sensitivity observed in the extended S+L− assay.

It can easily be imagined that an RCR would be partially replication-defective, and would replicate too slowly to be detected by the new assay. However, the results indicate that the inG*luc* signal appears very rapidly (see Fig. 4 and 6A), so that efficiently replicating viruses are detected well before the 3 weeks required by the FDA. It seems likely that inefficiently replicating viruses will be detected sooner by the new assay than by standard assays. In fact, we have shown that the inG*Luc* assay could detect MLVs produced by the VCaP and EKVX tumor cell lines[5], although these MLVs had very poor infectivity.[5,23] It is also conceivable that the efficient packaging of the inG*luc* genome might competitively inhibit the spread of an RCR in the indicator cells; in this case, modulation of its expression level and/or its packaging signal might further enhance the sensitivity of the assay.

Yet another advantage of the DERSE assay is that a generic PCR, targeted to the indicator gene rather than the RCR itself, can be used to detect RCR (Fig. 3). Thus, unlike traditional PCR-based assays for RCR detection, PCR directed towards the G*luc* gene is a functional assay, as it will only be positive in the presence of an RCR. Further, the PCR overcomes the requirement for virus-specific primer and probe design: any RCR capable of rescuing the inG*luc* vector, regardless of its viral origin or the recombination event(s) that generated it, will result in the production of a functional G*luc* gene.

Importantly, the assay concept presented here is highly versatile and can be extended for detection of, potentially, any RCR simply by replacing the MLV non-coding regions with those of the retrovirus of interest. We have previously reported on DERSE plasmids comprising inG*luc* or in*luc* in the context of HTLV non-coding sequence.[11,12] Of particular interest to the gene-therapy community, HIV-DERSE plasmids containing HIV genomic sequences in place of the MLV sequences described here, should allow detection of RCR (sometimes called "RCL") in lentivirus-based gene-therapy vectors.[12]

Furthermore, the indicator gene can be replaced with any gene of interest allowing a researcher to customize the assay read-out to suit available equipment. We have successfully detected gamma-RCR infection of an inGFP-MLV-DERSE/LnCaP cell culture by using either FACS analysis or fluorescence microscopy to measure the appearance of GFP-positive cells following RCR infection (not shown). Here again, the fact that GFP is expressed only after RCR infection of the inGFP-MLV-DERSE cell line offers the possibility of a large signal: background ratio, particularly in comparison to other fluorescence-based RCR detection techniques that rely on enhancement, rather than initiation, of GFP expression.[24] Observation of GFP-positive cells using fluorescence microscopy may represent a very simple method for RCR detection.

The inG*luc*-MLV-DERSE assay is a rapid and sensitive method for detection of RCR. End-point dilution experiments using the inG*luc*-MLV-DERSE assay gave results that were consistent with established retroviral detection methods (RT assay, immunoblot, extended S +L− assay). The presence of a great excess of gene-therapy vector did not change the limit of RCR detection at the resolution used (10-fold RCR dilutions). Further, the assay gave reproducible results over multiple experiments. While further assay development would be required to implement this method into the FDA guidelines, the work presented here demonstrates the potential of this assay for RCR detection in gene-therapy products. Future work may involve development of similar assays for lentiviral vectors. In conclusion, the DERSE assay is a versatile method for detecting RCR which should be useful not only for the gene-therapy community but for any researcher studying or working with RCR.

MATERIALS AND METHODS

Preparation of the inG*luc*-MLV-DERSE plasmid

The inG*luc* cassette was inserted as a SmaI to Acc65 fragment into the pBabe-puro retroviral vector between the BsrGI and the blunt-ended SalI sites.[25] The inG*luc* cassette consists of the CMV immediately early promoter, the human codon-optimized *Gaussia* luciferase coding sequences interrupted by the second intron of the rabbit β-*globin* gene at position 144, and the SV40 polyA signal.[14]

Preparation of inG*luc*-MLV-DERSE cells

293mCAT1 cells were seeded at 1×10^6 cells/10cm plate in DMEM containing 10% fetal calf serum, 100 Units/mL penicillin, 100μg/mL streptomycin and 0.292 mg/mL glutamine (DM). The following day cells were transfected with 10μg of the inG*luc*-MLV-DERSE plasmid using Transit 293 from Mirus Bio LLC (Madison, WI), according to the manufacturer's instructions. The inG*luc*-MLV-DERSE plasmid contains the pac gene conferring puromycin resistance. Stable transfectants were selected in 5μg/mL puromycin and were subsequently maintained in 1μg/mL puromycin (DM-Puro).

Gaussia Luciferase Activity Assay

The BioLux™ Gaussia Luciferase Flex Assay Kit from New England Biolabs (Ipswich, MA) was used to assay for G*luc* activity. Coelenterazine was prepared according to the manufacturer's instructions. The stabilizer reagent was not used. As *Gaussia* luciferase is a secreted protein, 10μL of cell culture supernatant was assayed (with 50μL of coelenterazine reagent). Light output was measured in a microbeta Trilux luminescence and liquid scintillation counter for 1 second per sample.

Viruses

MoMLV was obtained from 0.45μm filtered culture supernatant from a chronically infected NIH 3T3 cell line,[26] and the titer of this virus preparation was determined by direct S+L− focus assay.[10] 4070A MLV was purchased from the American Type Culture Collection (#VR-1445) and was titered as described.[19] GALV SEATO virus was a kind gift from Mary Beth Eiden, NIH, and its titer was measured by the S+L− assay as described;[20] this assay was initiated by addition of 1 ml of the virus preparation to 1×10^6 293 cells. Clinical grade

retroviral vector expressing the Fanconi A gene and pseudotyped with the GALV *Env* was produced by the Indiana University Vector Production Facility. The vector stock had previously screened negative for RCR using the extended S+L− assay according to current FDA requirements for clinical products. The vector titer was determined by quantitative PCR using the methods previously described[27] with the following probe and primer set: Forward: 5'-CGC AAC CCT GGG AGA CGT CCC-3', Reverse: 5'-CGT CTC CTA CCA GAA CCA CAT ATC C-3'; Probe: 5'-FAM-CCG TTT TTG TGG CCC GAC CTG AGG-TAMRA-3'.

Infection and co-culture of in*Gluc*-MLV-DERSE cells

in*Gluc*-MLV-DERSE cells were seeded one day prior to infection. On the day of infection the cells were treated with 20μg/mL DEAE-Dextran for 30 minutes. DEAE-Dextran was removed and cells were rinsed twice with growth medium. Virus and POLYBRENE® (8μg/mL final concentration) were added to cells and the cultures incubated for 4 hours. Virus was replaced with DM-Puro. Experiment-specific cell quantities and volumes are given below.

In-well Gluc activity assay—in*Gluc*-MLV-DERSE cells were seeded at 1250 cells/well/ 100μL in DM-Puro, in a black μclear, Advanced TC, 96-well plate from Greiner Bio-one (Frickenhausen, Germany). 30μL of DEAE-Dextran and MoMLV were used and virus was replaced with 50μL DM-Puro. 3 days after infection, 100μL of coelenterazine reagent was added to each well.

Co-culture assay—in*Gluc*-MLV-DERSE cells and NIH-3T3 cells chronically infected with MoMLV were seeded at 1250 cells (each) per well of a 96-well plate. No DEAE-dextran treatment, POLYBRENE®, or puromycin were used.

4070A and GALV end-point experiments—in*Gluc*-MLV-DERSE cells were seeded at 1.25×10^5 cells/well/2mL in a 6-well plate. 1mL of DEAE-Dextran and virus were used. Cells were passaged such that they were confluent every 3 – 4 days.

Immunoblot

Supernatants from infected in*Gluc*-MLV-DERSE cells were first filtered through a 0.45μm filter. Supernatants were then centrifuged through a 20% sucrose cushion at 110,000×g for 1 hour. The virus pellet was resuspended in 2xNuPAGE sample buffer (Invitrogen, Carlsbad, CA). Antiserum prepared against purified MLV p30^{CA} (MLV30) was used.[28] Detection used Western Lightning Plus-ECL from Perkin Elmer (Waltham, MA).

Reverse Transcriptase (RT) Assay

5μL of in*Gluc*-MLV-DERSE cell culture supernatant (0.45μm filtered) was mixed with 25μL of RT assay buffer (62.5mM Tris pH 8.0, 25mM KCl, 0.6mM $MnCl_2$ (added immediately before use), 10mM DTT (added immediately before use), 5μg/mL oligo(dT), 50μg/mL poly(rA), 0.25% v/v Nonidet P40 and 3.75 Ci/mmol [α-^{32}P]-TTP (added immediately before use)). Oligo(dT) 12-18mer was purchased from Gene Link (Hawthorne, NY), poly(rA) from GE Healthcare (Piscataway, NJ) and [α-^{32}P]-TTP from Perkin Elmer

(Waltham, MA). Assays were incubated for 4 hours at 32°C and then 5μL of the mixture was spotted onto a DEAE filter mat (Perkin Elmer, Waltham, MA). The filter mat was dried (85°C for 10 minutes) and then washed 4 times for 10 minutes each in 2xSSC then twice for 5 minutes each in ethanol. The filter mat was dried again (85°C for 10 minutes) and then placed in a ^{32}P cassette (Perkin Elmer, Waltham, MA). ^{32}P activity was measured in a microbeta Trilux luminescence and liquid scintillation counter for 1 minute per sample.

Duplex Real Time PCR

Genomic DNA was extracted using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany). Amplification of the *Gluc* gene was achieved using 3'*Gluc*-F (5'-CAG GAA TCT CAG GAA TGT C-3'); In*Gluc* (5'-FAM-TGG GAC AGG CAG ATC AGA CAG-BHQ1-3') and SpG*luc*-R (5'-GGG AAG TTG CCC GGG AA-3'). The 5 nucleotides on the 5'end of the SpG*luc*-R primer target *Gluc* sequence 5' of the intron, while the 12 nucleotides on the 3'end of the SpG*luc*-R primer target *Gluc* sequence 3' of the intron. In this way, SpG*luc*-R targets only the functional *Gluc* gene. CCR5 was amplified using CCR5-F (5-CCAGAAGAGCTGAGACATCCG-3); CCR5-PR (5'-HEXTCCCCTACAAGAAACTCTCCCCGG-BHQ1-3'); and CCR5-R (5'-GCCAAGCAGCTGAGAGGTTACT-3').[28] AmpliTaq Gold polymerase and associated buffer were purchased from Applied Biosystems/Life Technologies (Foster City, CA). A master mix of PCR reagents was prepared such that the final concentrations in the PCRs were: 1xPCR buffer; 300nM dNTPs; 2.5mM MgCl$_2$; 50nM CCR5-F; 50nM CCR-R; 50nM CCR-PR; 500nM G*luc*-F; 500nM SpG*luc*-R; 100nM InG*luc* and 0.06 Units/μL polymerase. Reactions were heated to 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds and 60°C for 90 seconds. Reactions were performed using a DNA engine Opticon 2 instrument (MJ Instruments, now BioRad, Hercules, CA). Fluorescence profiles are presented on a log scale and are global minimum background subtracted as described by the Opticon Monitor 2 software.

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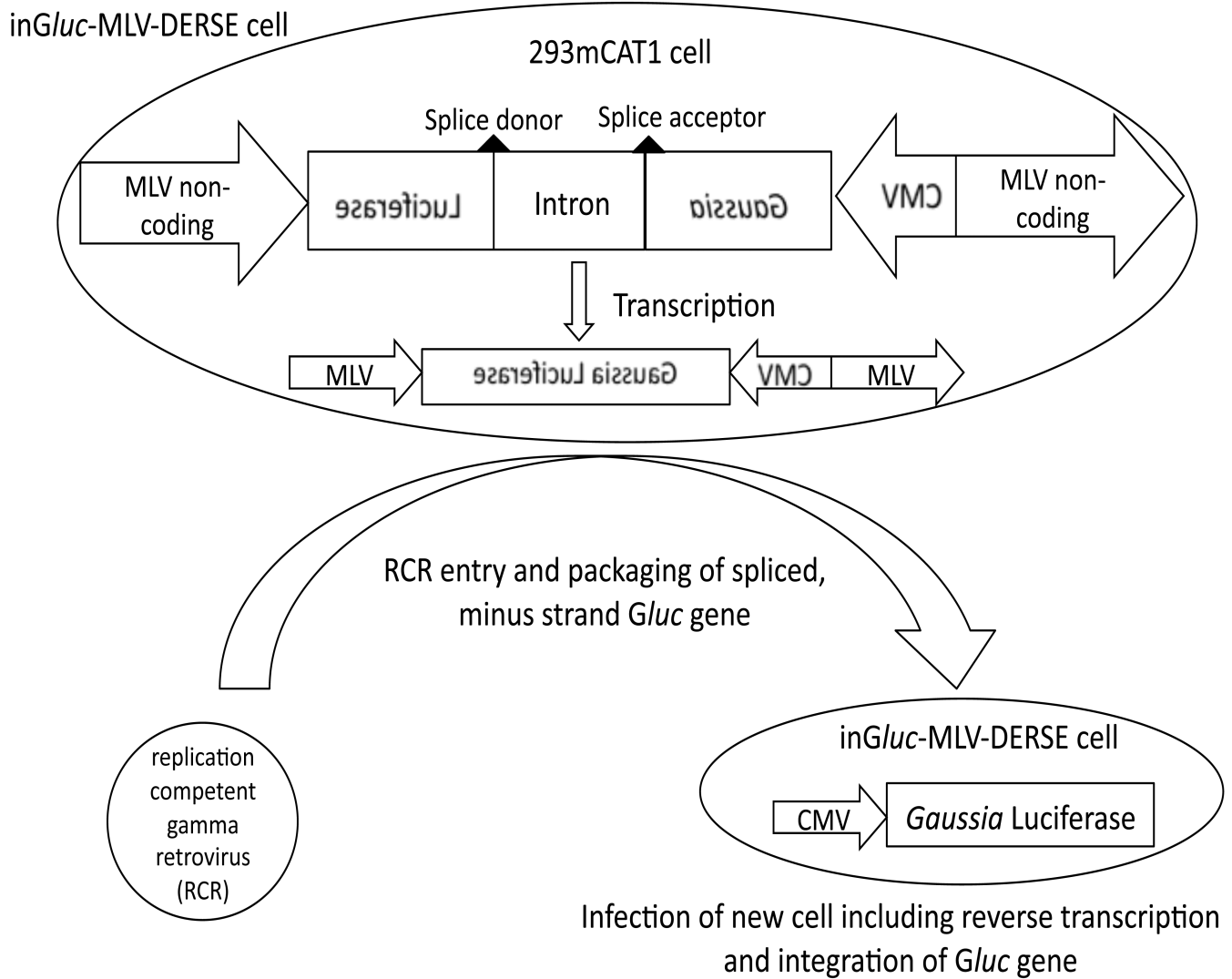
Figure 1. Schematic of inG*luc*-MLV-DERSE assay

The inG*luc*-MLV-DERSE plasmid consists of a *Gaussia* Luciferase (G*luc*) sequence oriented in a reverse direction with respect to flanking MLV non-coding sequences. Within the non-coding G*luc* sequence is an intron that is oriented in a forward direction relative to the viral non-coding regions (and can be spliced by the host cell). The plasmid is maintained in 293mCAT1 cells. In the absence of RCR, only minus-strand, spliced G*luc* sequences are present in the cell. An RCR that infects the DERSE cell can package the RNA containing the minus-strand G*luc* sequence. In the next round of infection reverse transcription of the encapsidated RNA produces a double-stranded DNA containing an uninterrupted G*luc* gene. This gene is an intact, coding G*luc* sequence that is subsequently integrated into the DNA of, and expressed by, the newly infected cell. Expression of G*luc* is under the control of the cytomegalovirus (CMV) promoter.
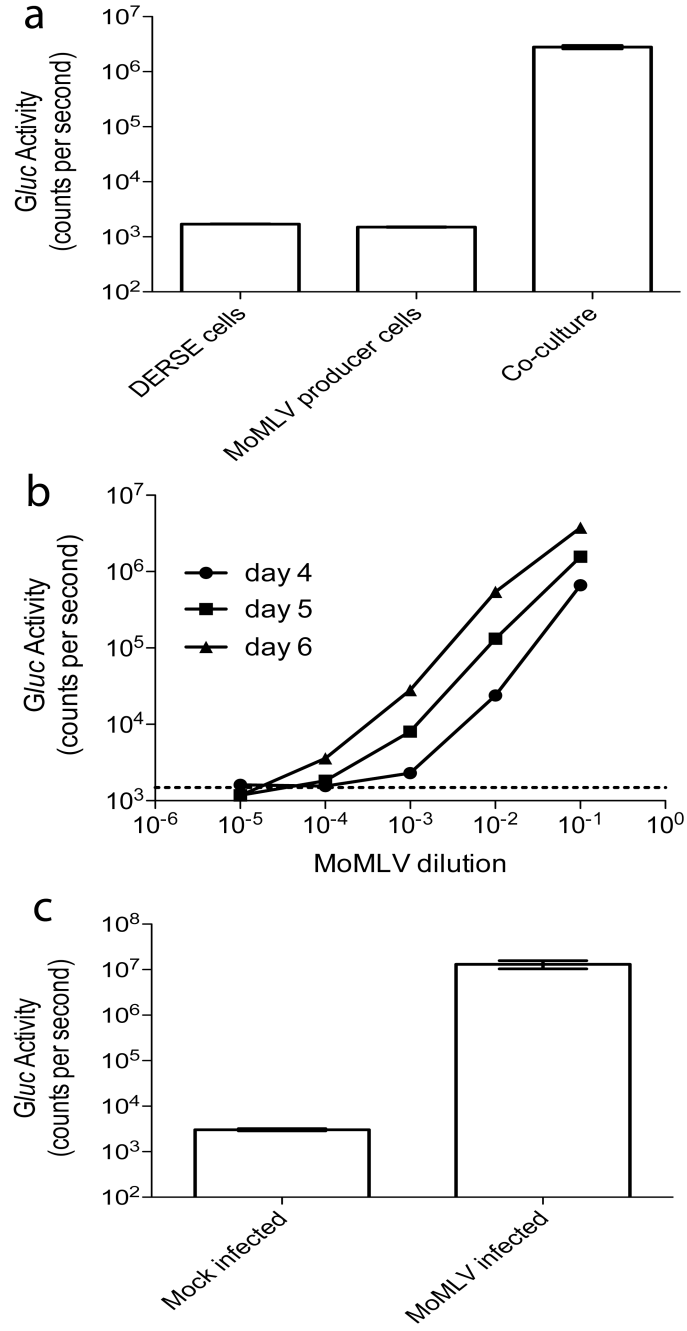
Figure 2. MoMLV infection of in*Gluc*-MLV-DERSE cells produces G*luc* activity in the culture supernatant

(**a**) in*Gluc*-MLV-DERSE cells can detect RCR produced by a chronically infected cell-line. in*Gluc*-MLV-DERSE cells were co-cultured with NIH-3T3 cells that were chronically infected with MoMLV. Three days after the co-culture was initiated, 10μL of culture supernatant was assayed for G*luc* activity. Cultures and co-cultures were conducted in triplicate. Columns represent the mean of 3 cultures (standard error of mean error bars are present, but not visible at this scale). (**b**) G*luc* activity in the cell culture supernatant

accumulates over time and its level is directly related to the amount of virus used for infection. 96-well plates containing 1.25×10^3 in$Gluc$-MLV-DERSE cells per well were infected with 0.03 ml of serial dilutions of MoMLV; $Gluc$ activity in the supernatant was measured at 4 (circles), 5 (squares) and 6 (triangles) days post infection. The dashed line represents the average counts per second in supernatant from the mock-infected cultures. Points represent the mean of 3 infections (standard error of mean error bars are present, but not visible at this scale). The 10^{-1} dilution of the inoculum contained 1.5×10^3 focus-inducing units per 0.03 ml.[10] (**c**) Gluc activity assay can be conducted directly in the cell-culture plate. in$Gluc$-MLV-DERSE cells were infected with MoMLV and 3 days after infection Gluc substrate was added directly to the culture wells and Gluc activity measured. Columns represent the mean of 3 infections, error bars represent the standard error of mean.
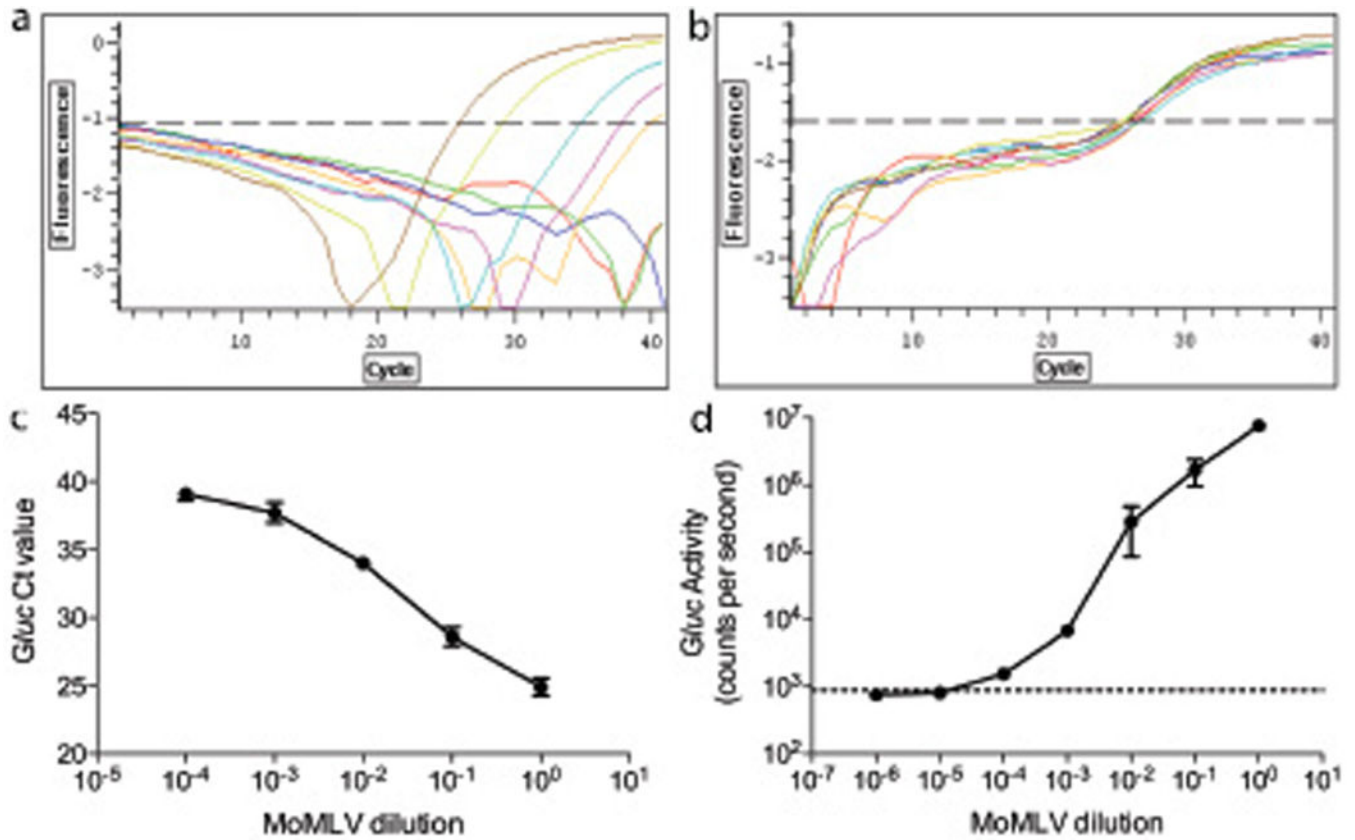
Figure 3. inG*luc*-MLV-DERSE cell infection can be detected using PCR directed to the G*luc* gene

2.5×10^5 inG*luc*-MLV-DERSE cells in 20 cm$_2$ dishes were infected, in triplicate, with 1 ml of serial dilutions of an MoMLV preparation that had a titer of 1.5×10^6 focus-inducing units per ml in the direct S+L− focus assay. Three days after infection, cell culture supernatant and cellular genomic DNA were collected. Duplex real-time PCR, directed to the G*luc* and CCR5 genes, was conducted on 100ng of genomic DNA from inG*luc*-MLV-DERSE cells infected with undiluted (brown); 10^{-1} (mustard); 10^{-2} (aqua); 10^{-3} (pink); 10^{-4} (orange); 10^{-5} (green) or 10^{-6} (blue) dilutions of MoMLV; or on 100ng of genomic DNA from mock infected inG*luc*-MLV-DERSE cells (red). (**a**) Fluorescence from G*luc* probe. (**b**) Fluorescence from CCR5 probe. (**c**) G*luc* probe Ct values from the duplex real time PCR, plotted against the dilution of MoMLV used for infection of the inG*luc*-MLV-DERSE cells. G*luc* copy numbers were calculated as 9749, 1923, 179, 35 and 19 for the undiluted through 10^{-4} infections, respectively. Error bars represent standard error of mean for 5 PCRs. (**d**) G*luc* activity in 10μL of cell culture supernatant from MoMLV-infected inG*luc*-MLV-DERSE cells. The dashed line represents average counts per second in supernatant from the mock-infected cultures. Error bars represent standard error of mean for 3 infections.
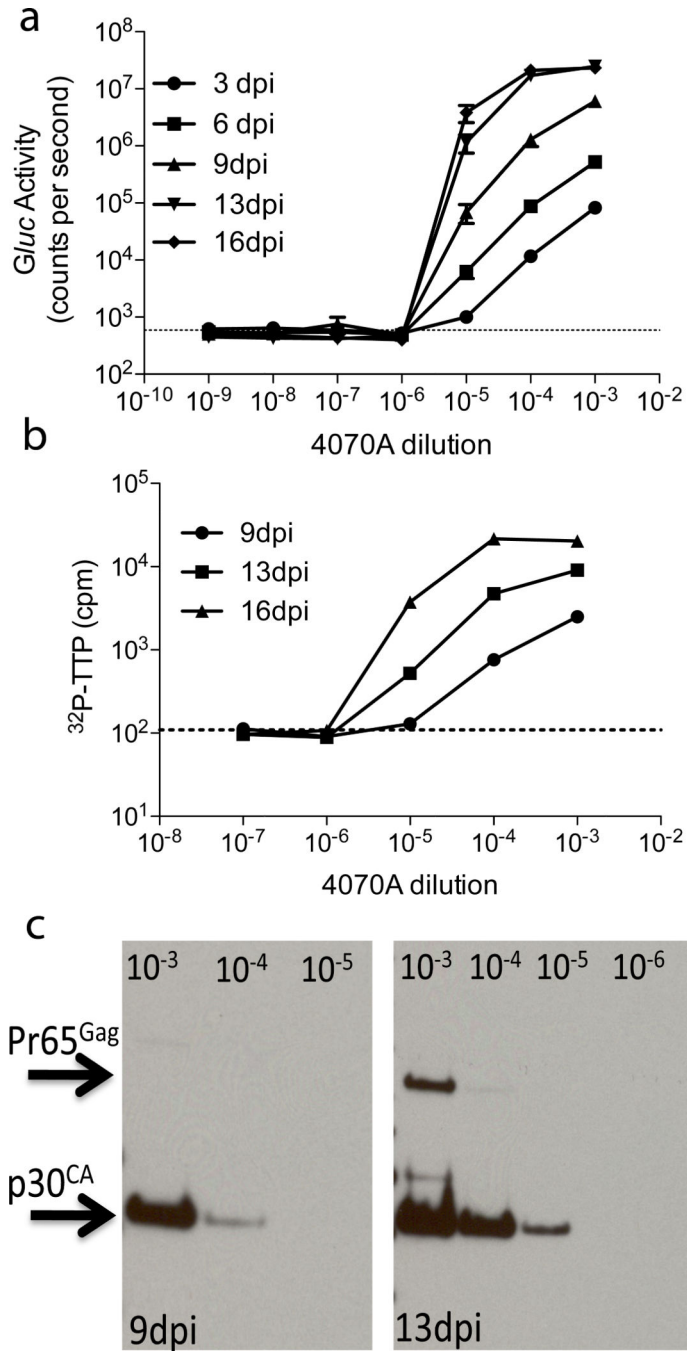
Figure 4. Comparison of in*Gluc*-MLV-DERSE assay with RT assay and immunoblot

1.25×10^5 in*Gluc*-MLV-DERSE cells cells in a 6-well (9.6 cm^2) plate were infected with 1 ml of dilutions of a 4070A MLV preparation that had a titer of 10^6 infectious units/ml in an S $^+$L$^-$-based assay.[19] The supernatant was collected and cells split at 3, 6, 9, 13 and 16 days post infection (dpi). (**a**) Using the G*luc* activity assay, the virus dilution end-point (10^{-5}) is first detected between 3 (circles) and 6 (squares) days post infection. The dashed line represents average counts per second in supernatant from mock-infected cultures. Error bars represent standard error of mean for 3 infections. (**b**) RT assays of the culture supernatants

give the same dilution end-point as the G*luc* activity assay. 1μL of supernatant from 9 (circles), 13 (squares) and 16 (triangles) dpi were assayed for RT activity. The dashed line represents average counts per minute (cpm) in supernatant from mock-infected cultures. Error bars represent standard error of mean for 3 RT assays. (**c**) G*luc* activity and p30^{CA} levels, as estimated by immunoblot with MLV30 antiserum, gave the same dilution end-point. Virus pellets from 1mL of supernatant from cultures infected with 10^{-3}, 10^{-4}, 10^{-5} (9 and 13dpi) and 10^{-6} dilutions of 4070A (13 dpi) were used in the immunoblot.
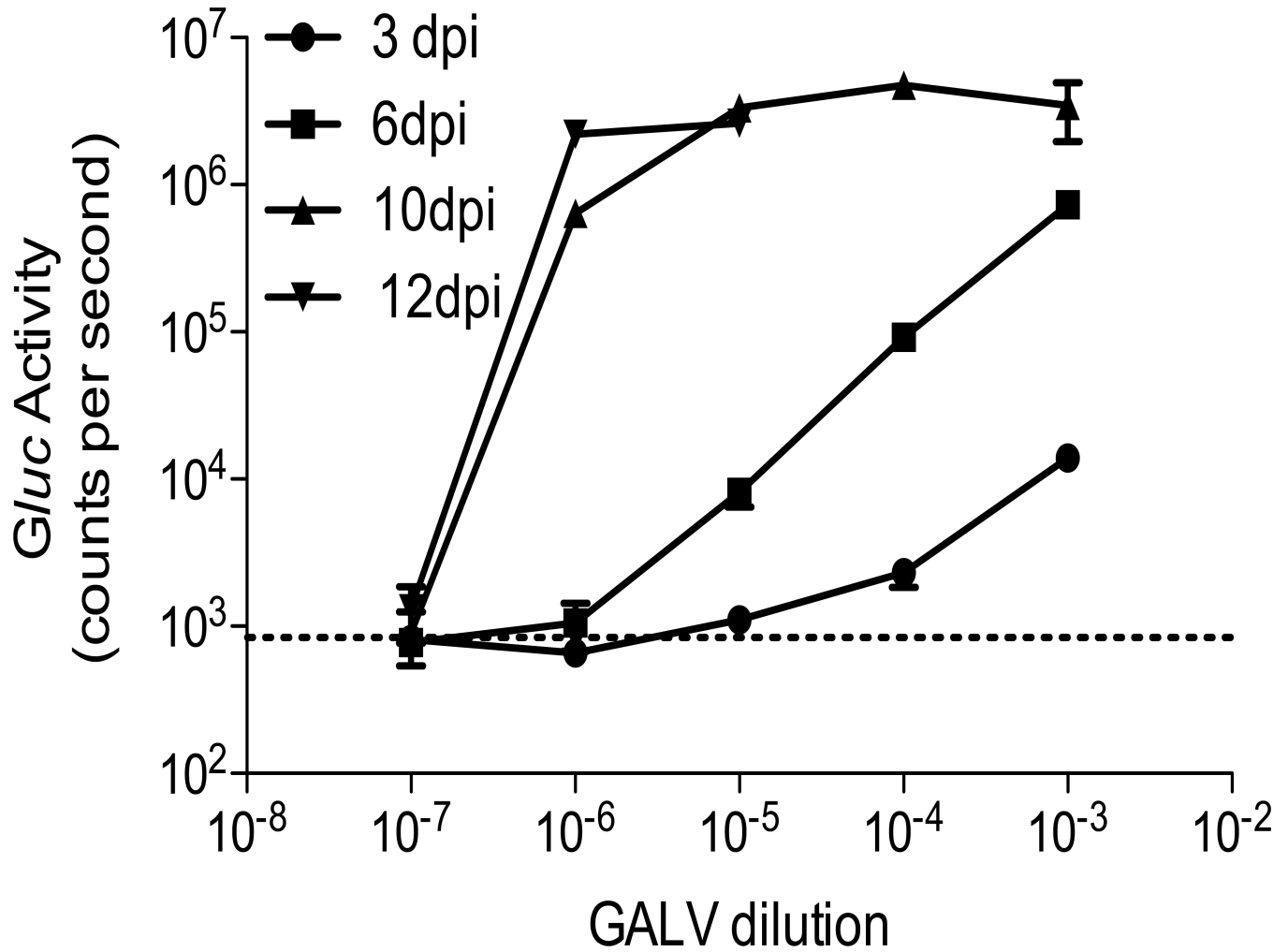
Figure 5.
Comparison of GALV titer using the inG*luc*-MLV-DERSE assay with that from the S+L−
assay. 1.25×10^5 inG*luc*-MLV-DERSE cells in 9.6 cm^2 wells were infected in triplicate
with 1 ml of serial dilutions of a GALV stock that had an S+L− determined end-point of
between 10^{-5} and 10^{-6} infectious units/ml.[20] Cells were passaged and G*luc* activity in the
supernatant was monitored at 3 (circles), 6 (squares), 10 (triangles) and 12 (inverted
triangles) days post infection (dpi). A representative experiment of three is shown. The
dashed line represents average counts per second in supernatant from mock-infected
cultures. Error bars represent standard error of mean for the triplicate infections of one
experiment. For clarity, G*luc* activity is only shown for the positive culture infected with the
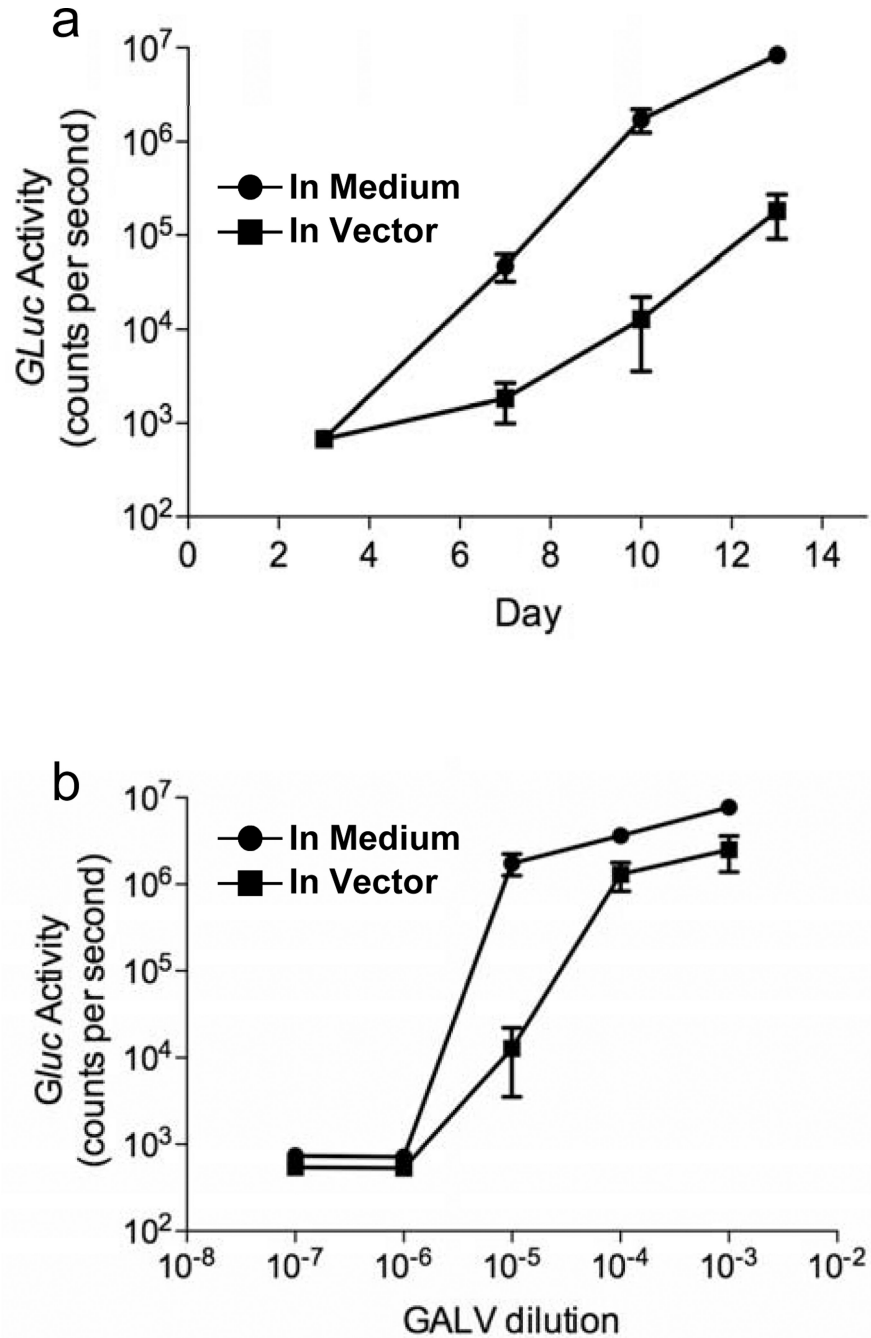10^{-6} dilution of GALV, and not for the negative cultures.

Figure 6. Effect of clinical-grade GALV vector on the infectivity of in*Gluc*-MLV-DERSE cells
in*Gluc*-MLV-DERSE were infected as in Fig. 5 with dilutions of GALV prepared either in media (circles) or in the presence of 7.6×10^6 infectious units of clinical-grade GALV vector (squares), in triplicate. Cells were passaged and G*luc* activity in the supernatant was monitored at 3, 7, 10 and 13 days post infection. (**a**) Kinetics of virus spread in cultures infected with the 10^{-5} dilution of GALV. (**b**) The dilution endpoint of the GALV RCR is determined to be 10^{-5}, regardless of the presence or absence of GALV vector. G*luc* activity was measured in the supernatant of cultures from 10 days after infection. The dashed line

represents average counts per second in supernatant from the mock-infected cultures. Error bars represent standard error of mean for 3 infections.