A reporter system for replication-competent gammaretroviruses: the inGluc-MLV-DERSE assay

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Although 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-1L- marker-rescue assay, whereas 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-1L- assay. Results presented here demonstrate that this assay should be useful for gene-therapy product testing.

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 presence of replication-competent retroviruses (RCRs) 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 vector preparations be rigorously tested to exclude the presence of RCR for both research and clinical purposes. For clinical-

RESULTS

Generation of the inGluc-MLV-DERSE cell line

The DERSE plasmids use a strategy that was originally devised for detection of retrotransposition by retroelements.15-16 These plasmids consist of a retroviral genome with an intraintron-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,
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by, the newly infected cell. Here we describe the indication gene that is subsequently integrated into, and expressed includes the minus-strand indicator sequence) results in a coding infect a new cell, reverse transcription of the viral RNA (that now viral proteins encoded by the RCR. When this RCR goes on to include the viral RNA packaging signal. If this cell is infected with an RCR, the minus-strand indicator sequence can be packaged by the host cell). The plasmid is maintained in 293mCAT1 cells. In the absence of RCR, only minus-strand, spliced Gluc sequences are present in RNA in the cell. An RCR that infects the DERSE cell can package the RNA containing the minus-strand Gluc sequence. In the next round of infection, reverse transcription of the encapsidated RNA produces a double-stranded DNA containing an uninterrupted Gluc gene. This gene is an intact, coding Gluc sequence that is subsequently integrated into the DNA of, and expressed by, the newly infected cell. Expression of Gluc is under the control of the cytomegalovirus (CMV) promoter.

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 inGluc-MLV-DERSE cell line in which the indicator gene is the secreted Gluc enzyme, the long terminal repeats and other cis-acting sequences are from MLV, and the host cell line is 293mCAT1. Gluc activity is measured by the addition of coelenterazine to a small volume (10 μl) of the cell culture supernatant, followed by measurement of light output.

Detection of virus with the inGluc cell line
We initially tested the inGluc-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 inGluc-MLV-DERSE cells with NIH-3T3 cells that were chronically infected with MoMLV. At 3 days after the co-culture was initiated, 10 μl of virus used for infection. The 96-well plates containing 1.25 × 10^3 inGluc-MLV-DERSE cells per well were infected with 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 the supernatant from the mock-infected cultures. Points represent the mean of three infections; (standard error of mean error bars are present, but not visible at this scale). (a) Gluc activity assay can be conducted directly in the cell culture supernatant. (b) Gluc activity in the cell culture supernatant accumulates over time and its level is directly related to the amount of virus used for infection. The 96-well plates containing 1.25 × 10^3 inGluc-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 the supernatant from the mock-infected cultures. Points represent the mean of three infections (standard error of mean error bars are present, but not visible at this scale). (c) Gluc activity assay can be conducted directly in the cell culture plate. inGluc-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 three infections, and error bars represent the standard error of mean.
measured in the supernatant several days after infection. We infected the inGluc-MLV-DERSE cells with 10-fold serial dilutions of MoMLV and measured Gluc activity in the supernatant 4, 5 and 6 days after infection (Figure 2b). We found that the Gluc 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 Gluc activity over time. The dilution end point for this assay was between $10^{-3}$ and $10^{-5}$ (Figure 2b); this result is in excellent agreement with the titer of this virus stock obtained by the direct $5 \times 10^7$ focus assay (data not shown; see figure legend for details).

Finally, as Gluc is a secreted protein, we thought it interesting to see whether the Gluc assay could be conducted directly in the cell culture plate. We tested this by infecting the inGluc-MLV-DERSE cells with MoMLV in a black 96-well plate and then adding Gluc substrate directly to the cell culture well 3 days after infection (Figure 2c). The MoMLV-infected culture showed Gluc activity of $\sim 10^7$ counts per second (c.p.s.), the maximum activity measurable using our instrument, whereas a mock-infected culture had a background of $\sim 10^6$ c.p.s. 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 polymerase chain reaction for detection of RCR infection
We found measurement of Gluc activity in the supernatant to be a rapid and simple method for detecting infection of the inGluc-MLV-DERSE cell line. However, as infection of the cell line with an RCR results in the generation of a new gene (the spliced Gluc gene), it is also possible to use polymerase chain reaction (PCR) as a read-out for the assay by targeting a primer/probe specifically to the coding Gluc sequences that flank the intron. We developed a duplex real-time PCR assay that simultaneously amplified the functional Gluc gene and, as a control for DNA quality and quantity, the single-copy CCR5 gene. Using this PCR, only the spliced, functional Gluc gene can be amplified (and thus amplification from the Gluc primer/probe set can only occur following RCR infection of the inGluc-MLV-DERSE cell line). Using a plasmid standard, we determined that the real-time PCR could detect 10-33 copies of Gluc in 100 ng of inGluc-MLV-DERSE cell DNA (data not shown). To test the duplex real-time PCR assay, we extracted genomic DNA from inGluc-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 Gluc activity in the supernatant (Figures 3a–d). The Gluc probe Ct value was inversely related to the MoMLV concentration used for infection (Figures 3a and c). Further, the Gluc probe Ct values and the Gluc activity in the supernatant were in complete concordance—a culture deemed Gluc positive or negative in the duplex real-time PCR assay gave the same result in the Gluc activity assay (Figures 3a and d). As expected, CCR5 was amplified in all cases, confirming that the absence of Gluc probe fluorescence was not due to an absence, or poor quality, of DNA (Figure 3b).

In a separate experiment, we infected the inGluc-MLV-DERSE cells with MoMLV and the day after infection performed real-time PCR on the cellular DNA and a Gluc activity assay on the supernatant (results not shown). In this case, we found that the Gluc gene was amplified by real-time PCR, but Gluc activity

![Figure 3](image-url)
was not detected in the supernatant, presumably because Gluc protein levels had not accumulated sufficiently. This suggests that real-time PCR can detect infection of the inGlu-c-MLV-DERSE cell line marginally earlier than the measurement of Gluc 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 Gluc activity more suitable for many purposes.

Comparison of the inGlu-c-MLV-DERSE assay with other methods for gammaretrovirus detection
To support the results from the inGlu-c-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 inGlu-c-MLV-DERSE cells with serial dilutions of 4070A amphotropic MLV, grew the cells and collected the supernatant for Gluc activity assay, RT assay or immunoblot (Figures 4a–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 Gluc 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⁻¹ L⁻¹-based assay, which gave a titer of 10⁶ infectious units per ml for the inoculum (data not shown). Gluc 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 inGlu-c-MLV-DERSE cells. Using this particular RT activity assay, we first detected RT activity in the supernatant collected 13 days after infection of the inGlu-c-MLV-DERSE cells with a 10⁻⁵ dilution of 4070A (Figure 4b). RT activity was not detected in the supernatant from cells infected with a 10⁻⁶ dilution of 4070A, even at 16 days after infection. Although the Gluc activity assay proved to be more rapid than the RT assay under these experimental conditions, both assays gave the same dilution end point for this 4070A stock in this experiment (Figures 4a and b).

In addition, we used a broadly reactive antiserum to MLV p3₀⁶⁶⁶⁶ in an immunoblot of virus pellets prepared from the supernatants of the 4070A-infected cultures (Figure 4c). Supernatant p3₀⁶⁶⁶⁶ levels, as estimated by immunoblot, were proportional to Gluc activity (Figures 4a and c). Under the conditions used, the Gluc 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 Gluc activity 6 days after infection, p3₀⁶⁶⁶ was not observed in the supernatant until 13 days after infection (Figures 4a and c). Again, both assays gave the same end point for this 4070A stock, as p3₀⁶⁶⁶ 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 inGlu-c-MLV-DERSE assay for testing gene-therapy products, we used a single GALV stock and compared Gluc activity assay results from infection of the inGlu-c-MLV-DERSE line (conducted at National Cancer Institute, Frederick, MD, USA), with an extended S⁻¹ L⁻¹ assay conducted at the Indiana University Vector Production Facility. Using the S⁻¹ L⁻¹ assay, the GALV stock was found to contain 10⁶ tissue culture-infectious doses (TCID50) per ml (data not shown). We infected the inGlu-c-MLV-DERSE cells with 10-fold dilutions of the virus, in triplicate, and then passaged the cells and monitored Gluc activity in the cell culture supernatant. At 12 days (and three cell passages) after infection, 3/3 of the cultures infected with a 10⁻⁵ dilution of GALV activity assay results from infection of the inGlu-c-MLV-DERSE assay proved to be more rapid than the RT assay under these experimental conditions, both assays gave the same dilution end point for this 4070A stock, as p3₀⁶⁶⁶ was not observed in the supernatant until 13 days after infection (Figures 4a and c). Again, both assays gave the same end point for this 4070A stock, as p3₀⁶⁶⁶ was not observed in supernatants collected from cells infected with a 10⁻⁶ dilution of 4070A.

Figure 4. Comparison of inGlu-c-MLV-DERSE assay with RT assay and immunoblot. In total, 1.25 × 10⁵ inGlu-c-MLV-DERSE cells in a six-well (9.6 cm²) plate were infected with 1 ml of dilutions of a 4070A MLV preparation that had a titer of 10⁵ infectious units per ml in an S⁻¹ L⁻¹-based assay. The supernatant was collected and cells split at 3, 6, 9, 13 and 16 days post-infection (d.p.i.). (a) Using the Gluc activity assay, the virus dilution end point (10⁻⁵) is first detected between 3 (circles) and 6 (squares) d.p.i. The dashed line represents average counts per second in the supernatant from mock-infected cultures. Error bars represent standard error of mean for three infections. (b) RT assays of the culture supernatants give the same dilution end point as the Gluc activity assay. A measure of 1 μl of the supernatant from 9 (circles), 13 (squares) and 16 (triangles) d.p.i. were assayed for RT activity. The dashed line represents average counts per minute (c.p.m.) in the supernatant from mock-infected cultures. Error bars represent standard error of mean for three RT assays. (c) Gluc activity and p3₀⁶⁶⁶ levels, as estimated by immunoblot with MLV30 antiserum, gave the same dilution end point. Virus pellets from 1 ml of the supernatant from cultures infected with 10⁻⁵, 10⁻⁶, 10⁻⁵ (9 and 13 d.p.i.) and 10⁻⁶ dilutions of 4070A (13 d.p.i.) were used in the immunoblot.
and 1/3 of the cultures infected with a $10^{-6}$ dilution of GALV showed maximum Gluc activity in the supernatant (Figure 5). Using identical infection conditions, we repeated this experiment twice (that is, with two different GALV dilution series); in the second and third experiments 3/3 of the cultures infected with a $10^{-5}$ of GALV and 0/3 of the $10^{-6}$ infected cultures were positive (results not shown). Thus, the results from the inGluc-MLV-DERSE assay are in excellent agreement with those from the S/L-DERSE assay.

Finally, using the same GALV stock, we investigated the sensitivity of the inGluc-MLV-DERSE assay when infecting with an RCR in the presence of excess RCRs, 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. To determine the effect of excess vector on our assay, we infected $1.25 \times 10^3$ inGluc-MLV-DERSE cells with serial dilutions of GALV. The dilutions were made either in media or in $7.6 \times 10^6$ 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 inGluc-MLV-DERSE assay. However, we did observe somewhat lower Gluc activity in cell cultures infected with GALV in the presence of vector, compared with cultures infected with GALV alone. This observation was most notable in cultures infected with the $10^{-5}$ dilution of GALV (Figures 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 Gluc 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 (that is, $10^{-5}$) 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**

Although 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 biological 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) before conducting a 4- to 6-day S/L assay or a marker-rescue assay (RCR detection).

The inGluc-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 inGluC-MLV-DERSE cell culture. We show here that the assay sensitively detects RCR in the supernatant and produces results that are comparable to other RCR detection techniques (Figures 4 and 5). Moreover, the inGluC-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 (although 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 inGluC-MLV-DERSE cells are infected with RCR. This should result in a high signal to 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 Gluc as our indicator gene as it has two advantages over other reporter proteins. Firstly, Gluc is a predominantly secreted protein and thus there is no need for cell lysis, as is required for assays of firefly luciferase. In fact, as Gluc is secreted from the cell, the Gluc 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 $10^4$ c.p.s., a signal $\sim 10^7$ times greater than the background of our assay. The combination of these two properties of the Gluc 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 inGluC-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. In addition, we have used the inGluC-MLV-DERSE 293mCAT1 cell line to detect several other gammaretroviruses, including members of the xenotropic MLV family.5 Although 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 with 293 cells. While Mus Dunni cells are currently recommended for amplification of MLV-based vectors, we did not investigate an inGluC-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 six-well plates as these provided us with ample cells for DNA extraction and PCR assay or immunoblotting, without using large amounts of space or reagents. Whereas 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 six-well plate assay, we observed that inGluC-MLV-DERSE cells initially infected with a few virus particles had detectable Gluc activity in the culture supernatant 6-7 days after infection and became completely infected 10-14 days after infection (Figures 4 and 5).

Although vigilant RCR detection may still benefit from a 3-week amplification stage (to ensure detection of low numbers or slowly replicating RCR), the inGluC-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 to 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 inGluC assay. Thus, the data indicate that the efficiency of vector is minimal, and is presumably similar to the decrease in sensitivity observed in the extended time 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 inGluC signal appears very rapidly (Figures 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 inGluC assay could detect MLVs produced by the VCaP and EKVX tumor cell lines,6 although these MLVs had very poor infectivity.23 It is also conceivable that the efficient packaging of the inGluC 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 (Figure 3). Thus, unlike traditional PCR-based assays for RCR detection, PCR directed towards the Gluc 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 inGluC vector, regardless of its viral origin or the recombination event(s) that generated it, will result in the production of a functional Gluc 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 inGluC or inLuc 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 fluorescence microscopy, without the need for reverse transcription or fluorescence microscopy to measure the appearance of GFP-positive cells following RCR infection (data not shown). Here again, the fact that GFP is expressed only after RCR infection of the 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 inGluC-MLV-DERSE assay is a rapid and sensitive method for detection of RCR. End-point dilution experiments using the inGluC-MLV-DERSE assay gave results that were consistent with established retroviral detection methods (RT assay, immunoblotting, etc.)...
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. Although 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 also 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 an 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 Gluc coding sequences interrupted by the second intron of the rabbit \( \beta \)-globin gene at position 144, and the SV40 polyA signal.14

Preparation of inG Luc-MLV-DERSE cells

The 293mCAT1 cells were seeded at \( 1 \times 10^6 \) cells/10 cm plate in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 U ml⁻¹ penicillin, 100 \( \mu \)g ml⁻¹ streptomycin and 0.292 mg ml⁻¹ glutamine (DM). The following day cells were transfected with 10 \( \mu \)g of the inG Luc-MLV-DERSE plasmid using Transit 293 from Mirus Bio LLC (Madison, WI, USA), according to the manufacturer’s instructions. The inG Luc-MLV-DERSE plasmid contains the pac gene conferring puromycin resistance. Stable transfectants were selected in \( 5 \mu \)g ml⁻¹ puromycin and were subsequently maintained in 1 \( \mu \)g ml⁻¹ puromycin (DM-Puro).

Gluc activity assay

The BioLux Gaussia Luciferase Flex Assay Kit from New England Biolabs (Ipswich, MA, USA) was used to assay for Gluc activity. Coelenterazine was prepared according to the manufacturer’s instructions. The stabilizer reagent was not used. As Gluc is a secreted protein, 10 \( \mu \)l of the cell culture supernatant was assayed (with 50 \( \mu \)l of coelenterazine reagent). Light output was measured in a microtiter Trilux luminescence and liquid scintillation counter for 1 s per sample.

Viruses

MoMLV was obtained from 0.45 \( \mu \)m 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 The 4070A MLV was purchased from the American Type Culture Collection (no. 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⁻ focus assay.27 With the probe and primer set-forward: \( 5’\) CGTCTCCTACCAAGACCA CATATCC-3’; reverse: \( 5’\) CGTCTCCTACCAGAACCA CATATCC-3’; probe: \( 5’\) FAM-CCGTGTTTTTTGGCCCGCACTGAGG-TAMRA-3’. Infection and co-culture of inG Luc-MLV-DERSE cells

The inG Luc-MLV-DERSE cells were seeded one day before infection. On the day of infection, the cells were treated with 20 \( \mu \)g ml⁻¹ DEAE-dextran for 30 min. DEAE-dextran was removed and cells were rinsed twice with growth medium. Virus and POLYBRENE (8 \( \mu \)g ml⁻¹ final concentration) were added to cells and the cultures incubated for 4 h. The virus was replaced with DM-Puro. Experiment-specific cell quantities and volumes are given below.

In-well Gluc activity assay. inG Luc-MLV-DERSE cells were seeded at 1250 cells per well in 100 \( \mu \)l in DM-Puro, in a black \( \mu \)clear, Advanced TC, 96-well plate from Greiner Bio-one (Frickenhausen, Germany). A measure of 30 \( \mu \)l of DEAE-dextran and MoMLV were used and virus was replaced with 50 \( \mu \)l DM-Puro. At 3 days after infection, 100 \( \mu \)l of coelenterazone reagent was added to each well.

Co-culture assay. The inG Luc-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 was used.

4070A and GALV end-point experiments. The inG Luc-MLV-DERSE cells were seeded at 1.25 \( \times 10^5 \) cells per well in 2 ml in a six-well plate. A measure of 1 ml of DEAE-dextran and virus were used. Cells were passaged such that they were confluent every 3–4 days.

Immunoblot

 Supernatants from infected inG Luc-MLV-DERSE cells were first filtered through a 0.45 \( \mu \)m filter. Supernatants were then centrifuged through a 20% sucrose cushion at 110 000 g for 1 h. The virus pellet was resuspended in 2 \( \mu \)l NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA). Antiserum prepared against purified MLV p30-46 (MLV30) was used.28 Detection used Western Lightning Plus-ECL from Perkin Elmer (Waltham, MA, USA).

RT assay

A measure of 5 \( \mu \)l of inG Luc-MLV-DERSE cell culture supernatant (0.45 \( \mu \)m filtered) was mixed with 25 \( \mu \)l of RT assay buffer (62.5 \( \mu \)M Tris (pH 8.0), 25 \( \mu \)M KCl, 0.6 \( \mu \)M MnCl₂ (added immediately before use), 10 \( \mu \)M DTT (added immediately before use), 5 \( \mu \)g ml⁻¹ oligo-(dT), 50 \( \mu \)g ml⁻¹ poly-(rA), 0.25\% (v/v) Nonidet P40 and 7.5 \( \mu \)g/ml (\( \times 32\)P)TTP (added immediately before use). Oligo (dT) 12-18mer was purchased from Gene Link (Hawthorne, NY, USA), poly-(rA) from GE Healthcare (Piscataway, NJ, USA) and (\( \times 32\)P)TTP from Perkin Elmer. Assays were incubated for 4 h at 32°C and then 5 \( \mu \)l of the mixture was spotted onto a DEAE filter mat (Perkin Elmer). The filter mat was dried (85°C for 10 min) and then washed four times for 10 min each in 2 \( \times \) SSC and then twice for 5 min each in ethanol. The filter mat was dried again (85°C for 10 min) and then placed in a 32P cassette (Perkin Elmer). 32P activity was measured in a microtiter Trilux luminescence and liquid scintillation counter for 1 min 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 (\( 5’\)-CCAGGAAATCTCAGATAAGTC-3’); inG Luc-F (\( 5’\)-FAM-TGGGACAGGCA GTGAGACAG-3’); and SpG Luc-R (\( 5’\)-GGAACTGGCGGCCAA-3’). The five nucleotides on the 5’ end of the SpG Luc-R primer target Gluc sequence 5’ of the intron, whereas 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 (\( 5’\)-TCCAGAAAGCTGACATCGG-3’); CCR5-PR (\( 5’\)-HEX-TCCCTTCAAGAGAAAATCT CCACCC-GHG-3’); and CCR5-R (\( 5’\)-GCCAAAGCTGAGGTATG-3’). AmpliTaq Gold polymerase and associated buffer were purchased from Applied Biosystems/Life Technologies (Foster City, CA, USA). A master mix of PCR reagents was prepared such that the final concentrations in the PCRs were: 1 \( \times \) PCR buffer; 300 nM dNTPs; 2.5 mM MgCl₂; 50 nM CCR5-F; 50 nM CCR5-R; 50 nM CCR5-PR; 500 nM 3’-Gluc-F; 500 nM SpG Luc-R; 100 nM inG Luc and 0.06 \( \mu \)M \( \mu \)l⁻¹ polymerase. Reactions were heated to 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 60°C for 90 s. Reactions were performed using a DNA Engine Opticon 2 instrument (MJ Instruments, now BioRad, Hercules, CA, USA). Fluorescence profiles are presented on a log scale and are global minimum background subtracted as described by the Opticon Monitor 2 software.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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