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Report from the Lentivirus Vector Working Group: *Issues for Developing Assays and Reference Materials for Detecting Replication-Competent Lentivirus in Production Lots of Lentivirus Vectors*

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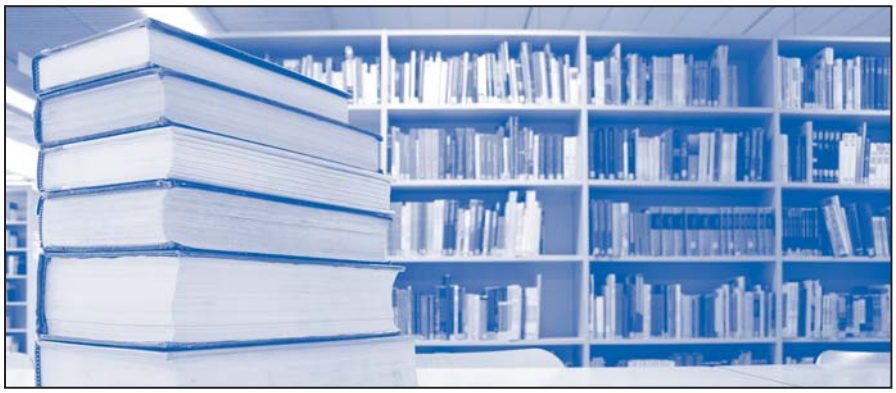

The Lentiviral Vector Reference Working Group (LVRWG) was created at the conclusion of a meeting organized by The Williamsburg BioProcessing Foundation in June 2002, in conjunction with the American Society of Gene Therapy (ASGT) annual conference. The meeting participants were gathered to evaluate the need for developing reference material to ensure comparability of lentiviral and retroviral vectors, in a similar spirit to the Adenovirus Reference Material program that had just been completed.[1] The consensus at the conclusion of this meeting was that the diversity in the lentiviral vector field, which includes vectors derived from different parental viruses and with various designs, does not allow for identification of a single reference material that would benefit more than a single or very few investigators. However, the participants agreed on the need to develop recommendations for assays used to characterize lentiviral vectors. Hence, during the June 2003 annual meeting of ASGT in Washington, D.C., the LVRWG met again to discuss the general principles that should guide the development of vector characterization assays. The major topics discussed by the LVRWG participants were detection of replication-competent lentiviruses (RCL) and vector dose definition. This report presents the suggestions that the working group agreed upon in their effort to develop a common approach to the characterization of different vector systems and provide some degree of comparability between different lentiviral vectors.

RCL DETECTION

RCL Definition

A critical safety issue associated with the lentiviral vector technology is the possibility that components of the vector system, and potentially other genetic elements from packaging cells, would recombine to generate a virus that has reacquired some replication capability and is related to the parental potentially pathogenic virus. These recombinants

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are defined as replication-competent lentiviruses or RCL. In lentiviral vector systems, the helper functions may be provided by a single plasmid or may be split among two or more plasmids. Therefore, recombinations may also be expected between subsets of the system components, generating new genetic entities that cannot replicate, such as recombinants between the vector genome and the gag-pol sequence in the helper system. Such so-called partial recombinants may be detected by molecular biology methods or biological rescue assays.[2] While partial recombinants lack the capacity to replicate autonomously, they are likely precursors to replicating recombinants. However, FDA's Biologics Response Modifiers Advisory Committee [now called the Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC)] and the LVRWG recommended that initial emphasis on assay development should be placed on the detection of biologically active recombinants capable of autonomous replication (RCL).

Lentiviral vector systems currently under development already incorporate many features aimed at reducing the risk of using lentiviral vectors, many of which prevent the generation of RCL, such as: (1) reduced homology between the vector and helper (by deletion or codon optimization of viral sequences), (2) deletion of "accessory" genes, (3) use of multiple plasmids to provide the helper functions, hence increasing the number of recombination events required to generate an RCL, (4) introduction of transcriptional separation elements in single-helper plasmids to block read through transcripts, (5) conservation of the Rev-dependency for gag–pol and vector genome expression, (6) deletions of 3' enhancer elements in self-inactivating LTR (SIN), (7) use of a heterologous envelope, or (8) use of "suicide genes" as a transgene.[3–12]

Even with the safety features listed, a critical component of qualifying lentiviral vectors for clinical use will be to develop assays to detect RCL. Developing sensitive and specific RCL assays presents unique challenges. First, the features that decrease the likelihood of generating RCL may make it difficult to accurately predict the structure of a viral genome that would be replication competent. Second, although major efforts across the field are directed toward the derivation of stable lentiviral vector producer cell lines, these efforts have so far remained largely unsatisfactory and many production systems employ transient transfection procedures. Vector manufacturing that relies on transient transfection tends to result in detectable levels of residual input plasmid DNA, and its derivative RNA and proteins. These contaminants complicate interpretation of RCL assays that rely on molecular methods applied directly to the lentiviral vector product, necessitating an efficacious vector purification process. Therefore, biological culture-based infectivity assays that include amplification of RCL by culture on a permissive cell line currently provide the best system for achieving sensitive and specific detection of RCL over partial recombinants and residual contaminants.

RCL Positive Control

The development and qualification of biological RCL detection assays requires a positive control that adequately represents the replication characteristics of the RCL predicted to arise from any particular lentiviral vector production system. The working group suggested that an adequate positive control should be derived from a molecular clone encoding a minimally replicating virus based upon the viral genome most closely related to the lentiviral vector. For instance, a positive control for an HIV-based vector might be an HIV-1 strain attenuated by deletion of the accessory genes that are also absent from the vector system; for example, *vif, nef, vpr,* and *vpu.* Ideally, the positive control virus should carry the same envelope as that used in the vector particles, in order to reflect the same *in vitro* cell entry properties. However, in those cases for which the introduction of the envelope into the positive control viral genome will expand the host range beyond that of the parental virus (for example, the use of VSV-G envelope for HIV), the creation of a replication competent virus with expanded tropism may pose a biosafety risk. An alternative approach was reported in which the positive-control virus was generated as a phenotypic pseudotype (i.e., a phenotypically mixed viral particle bearing both envelope proteins) rather than as a single genome capable of encoding the resulting pseudotype.[13] The use of phenotypic pseudotyping would allow for a single round of infection utilizing the vector envelope glycoprotein, with subsequent amplification depending on the native envelope of the lentivirus. This approach is considered preferable to the creation of a genetic pseudotype with expanded tropism, due to the biosafety risks. A recently derived molecular clone that included the VSV-G envelope coding region in *cis* allowed direct examination of a genetic pseudotype's behavior and determination of the sensitivity of its detection in indicator cells.[14] Thus investigators were able to detect a true VSV-G-expressing RCL.

In accordance with these suggestions, the choice of an appropriate positive control will be specific to each lentiviral vector system. To provide some level of comparability between the sensitivity of different RCL assays, the working group suggested that doses of positive control used to establish the assay sensitivity should be reported both as a particle count and infectious dose. The particle count, or physical titer, can be measured by one of several methods, such as quantification of a viral structural protein (with an extrapolation of particle number using a conversion factor derived from the virions' average composition), viral RNA copy number (the nuclease-resistant fraction), and other methods for quantifying encapsidated genomes.[15] The infectious titer of a positive control should be measured in the cell line used for the RCL assay and under similar assay conditions.

RCL Detection Assay

An effective way to achieve high sensitivity and specificity of RCL detection is to amplify the RCL by *in vitro* culture before detection by a relevant endpoint assay. To increase the sensitivity, it is important to identify a cell line that permits efficient replication of the relevant positive control, for each vector-specific positive control. The guiding principle

is to choose the cell line (which may not always be a human line) that will identify the relevant positive-control RCL most reliably and with greatest sensitivity. For example, when detecting replication-competent retrovirus (RCR) in a production lot of retroviral vector that uses an amphotropic murine leukemia virus envelope, two non-human cell lines are most commonly used: amplification of the virus is performed on *Mus dunni* tail fibroblasts (derived from Indian wild mice), and the detection assay is performed on PG-4 cells, a feline glioblastoma line.[16]

In a recent survey of human cell lines, C8166-45 (a human T-cell line) was identified as the most sensitive of a panel of seven candidates for amplification of an attenuated molecular clone of HIV-1 as the positive control RCL.[13] An independent report describing the advantages of C8166-45 cells in RCL detection has also been published.[17] If future vectors depart significantly from those tested in these two studies, screening of additional cell lines may become necessary.

After amplification in a qualified cell line, the assay should then include an endpoint analysis. Given the elusive nature of RCL, there is a risk in relying on a single endpoint analysis for their detection. Multiple endpoints should be considered that target structural features (e.g., viral RNA, proviral DNA, or proteins), as well as enzymatic activities (e.g., reverse transcriptase (RT) activity). The analyses based on the detection of structural features of an RCL should focus on the most likely identity and structure of the predicted RCL. For example, the choice of p24 as an indicator of the presence of a replicating virus in a preparation of HIV-1-derived vector is based on the assumption that relevant recombination events involve the gag-pol proteins of this vector system. Additional components may be useful to target in an endpoint assay, such as detection of VSV-G protein or nucleic acid coding sequences (if the vector is pseudotyped with this envelope). When an assay relies on detection of a specific sequence or protein, it relies on the accuracy of the predicted RCL. Therefore, it is also important to consider using a less specific method that does not rely on a prior knowledge of the genetic structure of the RCL, such as an assay for RT activity. When using RT activity as an endpoint, the sensitivity should be determined with an appropriate RT standard and assessment of potential cross reactivity of cellular enzymes.

An alternative approach to RCL detection is a marker-rescue assay. Marker rescue has been developed for detection of gag-pol recombinant intermediates, as well as HIV-based RCL, and has been widely used for the detection of RCR in gammaretrovirus-based vector systems.[2,14,17–20]

Regardless of the specific methods chosen for RCL detection, care must be taken in all cases to choose a time for analysis using the endpoint assay that is sufficiently beyond the latest time point when the vector components from the input production material would still be detectable. For example, with gammaretrovirus it has been shown that residual DNA from the vector producer cell line can interfere with the interpretation of PCR-based assays for RCR detection in *ex vivo* transduced cells.[21] Likewise, input HIV-1-based vector capsid protein can be detected in a p24 ELISA for at least 20 days, although its concentration decreases while the RCL-positive control concentration increases.[13,17] In such cases, careful assessment of the detection assay is required to determine how best to differentiate between a true RCL and the replication-defective vector (e.g., by length of the amplification culture). PCR and RT-PCR methods may suffer similar background problems due to the contamination of the vector preparations with vector RNA and DNA, which can persist in culture. Partial subgenomic recombinants that are replication-defective may also contribute background to PCR-based detection assays.[17] If the sensitivity of the endpoint analysis is limited by the presence of background in the amplification culture, this limitation may be alleviated by introducing an additional step that consists of transferring the amplification culture supernatant to a separate culture of indicator cells.[17] A true RCL — one that is not a result of partial recombination or contamination with residual nucleic acid — should have the ability to be transferred via culture supernatant to naïve indicator cells, in which any RCL would replicate and result in a detectable level that increases over time. For this purpose, the indicator cells may or may not be the same as the amplification cells. In this case, the sensitivity of the indicator cells to infection by the positive control should be at least comparable to the amplification cell line.

RCL Assay Sensitivity

Because RCL generation is expected to be a very rare event, the detection assays should be as sensitive as technically feasible. Effective RCL amplification is crucial and it is important to empirically determine, using the relevant positive control RCL, the minimal duration of the culture period that is required to ensure the detection of limiting amounts of RCL. The assay sensitivity must be evaluated in the presence of vector particles at concentrations likely to be present in the final product preparations of the lentiviral vector. It has been shown that gammaretroviral vector particles reduce the sensitivity of the detection of RCR.[20] However, in a similar study of an HIV-1-based vector, RCL detection was not inhibited by the presence of vector particles.[13] Residual viral proteins from the production process may also affect RCL infectivity. Therefore, the sensitivity of the detection assay should be qualified by performing spike and recovery experiments of the positive control in a background of test article to determine the detection limit attained in the presence of vector particles.

Qualification of the detection assay should include a determination of the largest volume of vector supernatant that allows detection of a single RCL. For release testing, if the appropriate test volume of vector is larger than the predetermined amount that can be tested without interference, the test article should be divided into several duplicate samples of the predetermined volume and be assayed concurrently. Similarly, the appropriate multiplicity of infection (MOI) will need to be determined due to potential vector toxicity to the indicator cells. The appropriate volume of vector to be tested for release must also

be determined carefully. A statistical approach is suggested to determine the test dose. Ideally, a release assay should demonstrate that there is no RCL in a patient dose. Considering that the RCL distribution in a vector sample obeys a Poisson distribution, it would be necessary to test the equivalent of three full patient doses with an assay capable of detecting a single RCL, in order to determine with 95% confidence that there is less than one RCL in a patient dose.[16,22] However, at the current scale of lentiviral vector production, this ideal approach may not be feasible. An alternative statistical approach is to determine the test dose based on an estimate of what would constitute an infectious dose of RCL. This rationalization should be based on the infectivity of the parental virus compared to the estimated infectivity of the RCL positive control, taking into consideration the effects on replication that would reduce infectivity (such as deletions of accessory genes) as well as those that may increase infectivity or cell tropism (such as the use of VSV-G envelope). Although these estimates may be extremely difficult to make accurately, they may provide a rough approximation of the minimal RCL infectious dose. Using this dose of concern, a statistical rationale based on the Poisson distribution, with a high confidence interval, can be applied to determine the amount of vector that should be tested to detect an RCL infectious dose.

In the CBER Guidance for Industry for RCR testing, the end-of-production (EOP) cells are tested directly for the presence of an RCR.[16,22] Testing of the EOP cells was originally intended for use on a packaging cell line, in which the cells making the vector would be well represented at the end of production. Currently, the majority of lentiviral vectors are made using a transient transfection process in which considerable cell toxicity may result from overexpression of helper functions. In these cases, testing of the EOP may be problematic. The decision to test EOP cells should be based on sound data from the specific manufacturing procedure that indicates whether the testing of EOP will be useful.

Standardization of Vector Titer and Dose Definitions

As more lentiviral vectors move toward clinical development, it becomes crucial that the experience gained with specific pre-clinical and clinical protocols be used to drive regulatory decisions regarding the safety of new investigational products. A critical point in comparability of different clinical protocols is the vector dose. Although doses of various vectors with different designs cannot be directly matched, the following suggestions on dose definition should improve the ability to compare across different protocols.

The dose-defining titer should be based on an infectivity or transduction titer. However, there is considerable variability in a transduction titer determination depending on the target cell line and the details of the transduction procedures. Therefore, the working group advised that reports of transduction titers be accompanied by a detailed description of the methodology that includes the target cell line used. In addition, when possible, a physical titer or particle count should also be reported for both *ex vivo* and *in vivo* applications to aid in comparisons between different vector systems.

Given that the field of lentiviral vectors is relatively new and in development, it is important to place the issues presented here in the context of the current understanding of lentiviral vectors. As clinical development of these vectors proceeds, new considerations will need to be addressed. It is hoped that this document will provide a basis for further refinement of critical safety testing. Evaluation of vector safety will be critical to the clinical progress of lentiviral vectors. The issues herein, it is hoped, will facilitate the development of well-qualified, sensitive, and specific assays that can be used in lentiviral vector safety assessment to support clinical use.

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