Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up

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Guidance for Industry

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Contains Nonbinding Recommendations

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Guidance for Industry

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I. INTRODUCTION

The potential pathogenicity of replication competent retrovirus (RCR) requires vigilant testing to exclude the presence of RCR in retroviral vector-based human gene therapy products[1] (Ref. 1). We, FDA, are providing you, sponsors of retroviral vector-based human gene therapy products, recommendations regarding the testing for RCR during the manufacture of retroviral vector-based gene therapy products, and during follow-up monitoring of patients who have received retroviral vector-based gene therapy products.[2] Recommendations include the identification and amount of material to be tested as well as general testing methods. In addition, recommendations are provided for monitoring patients for evidence of retroviral infection after administration of retroviral vector-based gene therapy products.

The *Retroviridae* family is composed of two subfamilies: *Orthoretrovirinae*, which consists of six genera of viruses: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, and *Lentivirus*, and *Spumaretrovirinae* (foamy viruses) which has recently been updated to consist of five genera of viruses: *Bovispumavirus*, *Equispumavirus*, *Felispumavirus*, *Prosimiispumavirus*, and *Simiispumavirus* (Refs. 2, 3). RCR may potentially be generated during the manufacture of a retrovirus vector from any of these genera. At this time, the most common retrovirus-based vectors are constructed from gammaretroviruses or lentiviruses, and therefore further details are provided for these genera. Historically, lentivirus RCR is referred to as replication competent lentivirus (RCL).[3]

This guidance supersedes the guidance entitled, "Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy

[1] Retroviral vector-based human gene therapy products include retroviral vector supernatant that can be directly administered and ex vivo genetically modified (transduced) cells.
[2] This guidance does not apply to vaccines for infectious disease indications, bacteriophage products, live biotherapeutic products, fecal microbiota for transplantation (FMT) products and allergenic products.
[3] RCR and RCL are synonymous for the purposes of this guidance.

Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors" dated November 2006 (2006 RCR Guidance) (Ref. 4). This guidance also supplements the following two final guidances: the "Long Term Follow-Up After Administration of Human Gene Therapy Products; Guidance for Industry" dated January 2020 (Long Term Follow-up Guidance) and "Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs); Guidance for Industry" dated January 2020 (CMC Guidance).[4] This guidance finalizes the draft guidance of the same title dated July 2018.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

FDA's Center for Biologics Evaluation and Research (CBER) recommendations for RCR testing during retroviral vector production and patient monitoring were originally developed at a time when clinical experience was limited to a small number of studies using gammaretrovirus vectors (Ref. 5). At that time, the overriding safety concerns associated with the use of retroviral vectors were exemplified by the findings of an animal study involving administration of gammaretroviral vector-transduced bone marrow progenitor cells that had been inadvertently exposed to high-titer RCR, and administered to severely immunosuppressed rhesus monkeys (Ref. 1). In this setting, 3/10 animals developed lymphomas and died within 200 days. The RCR was presumed to be etiologically associated with the disease by virtue of the presence of multiple murine RCR sequences in the lymphomas and an inverse correlation between anti-retroviral antibodies and development of disease (Refs. 6, 7). In contrast, another study in moderately-immunosuppressed cynomolgus monkeys exposed intravenously to RCR showed no signs of disease (Refs. 8, 9).

More than two decades of experience has generated a substantial amount of data on the safety of retroviral vectors in clinical applications for gene therapy, including experience with different vector designs, vector producing cells, RCR detection assays, and lack of positive results from RCR testing of vector lots, ex vivo transduced cells, and patient samples collected during monitoring. These data have provided the basis for public discussions, including Retroviral Breakout Sessions at the 1996 and 1997 FDA/National Institutes of Health (NIH) Gene Therapy Conferences, the 2010 Cellular, Tissue, and Gene Therapies Advisory Committee meeting (Ref. 10), and the 2014 American Society of Gene and Cellular Therapy (ASGCT) Breakout Session on Replication Competent Virus (Ref. 11). In addition, FDA scientists published an evaluation of RCR testing methods associated with the use of retroviral vectors (Ref. 12). During this time, the gene therapy community has improved retroviral vector design to reduce the likelihood of

[4] The Long Term Follow-up Guidance is available at this website: https://www.fda.gov/media/113768/download The CMC Guidance is available at this website: https://www.fda.gov/media/113760/download

generating RCR during the manufacturing process (Refs. 13, 14). For instance, the likelihood that recombination will generate RCR is reduced by manufacturing vectors using a split plasmid design, where the vector genome is on a separate plasmid from the envelope gene and packaging functions. RCR generation can be further reduced by using more than two plasmids for vector production. Lentiviral vectors have been further modified to remove genes encoding viral accessory and regulatory proteins, which would cripple the functionality of an RCR in the event an RCR may be generated (Refs. 15, 16).

Summary of Revisions from the 2006 RCR Guidance:

With consideration of the accrued scientific evidence of safety associated with retroviral vector design and testing, we are revising our current recommendations for RCR testing during retroviral vector-based gene therapy product manufacture and patient monitoring. More specifically, we are no longer recommending RCR testing on working cell banks for retroviral producer cells. We have also revised our recommendations regarding the amount of vector that should be tested (section III.B and Appendix 1-1 of this document). Briefly, we are recommending that you test a sufficient amount of vector to demonstrate that your vector contains <1 RCR per patient dose, when applicable to your clinical manufacturing practice. Additionally, we are recommending that all retroviral vector transduced cell products be tested for RCR, including those cultured for 4 days or less. We have found no convincing evidence that the length of culture time influences the likelihood of RCR development in transduced cells. However, if you have accumulated manufacturing and clinical experience that demonstrates that your transduced cell product is consistently RCR-negative (section III.A.4 of this document), we recommend that you provide this data to support reduction or elimination of testing ex vivo genetically modified cells for RCR. We have also revised our recommendations regarding assays for testing (section III.C of this document). Specifically, alternative methods may be appropriate for lot release testing of ex vivo transduced cells in lieu of culture based methods, particularly when there are time constraints present. Finally, we have revised our advice for active monitoring of patients following administration of retroviral vector-based products (section IV of this document), and added post-licensure considerations for RCR testing and risk assessment (section VI of this document). Because considerations for RCR assay development and testing schedule during retroviral vector-based gene therapy product manufacture and patient monitoring are generally product-specific, FDA recommends communication with the Office of Tissues and Advanced Therapies (OTAT) at CBER, preferably early, i.e., during your pre-IND meeting, as well as throughout your product development.

III. RECOMMENDATIONS FOR PRODUCT TESTING

A. Material for Testing

Generally, retroviral vectors are manufactured by collection of supernatant following transient or stable production from cultured cells. RCR may develop at any step during manufacturing, from the initial transfection or transduction steps through production of the retroviral vector supernatant. In addition, if the retroviral vector is used for ex vivo genetic modification of cells, the expansion of ex vivo transduced cells in culture

provides the potential for amplification of an RCR contaminant that may be below the level of detection in the retroviral vector supernatant. Therefore, current recommendations include testing of material from multiple stages of product manufacture (see Table of this document).

When the vector is produced by transient transfection, all cell banks should be qualified according to section V.A.2.c.iii.a of the CMC Guidance. Retroviral vector RCR-specific testing requirements are outlined below for the vector supernatant (section III.A.3 of this document), end of production cells (section III.A.3 of this document), and ex vivo transduced cells (section III.A.4 of this document), if applicable.

We recommend use of a stably-transfected Vector Producer Cell (VPC) bank system, when possible, in order to ensure an adequate and consistent supply of retroviral vector. The generation of a Master Cell Bank (MCB) for the VPC allows for the collection of cells of uniform composition derived from a single cell clone. The Working Cell Bank (WCB) is derived from the MCB, following expansion by serial subculture to a specified passage number (refer to "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" dated May 1993)[5]. When the vector is produced using VPC banks, RCR-specific testing of the VPC MCB (section III.A.1 of this document) is recommended in addition to vector supernatant (section III.A.3 of this document), end of production cells (section III.A.3 of this document), and ex vivo transduced cells (section III.A.4 of this document), if applicable.

1. Vector Producer Cell Master Cell Bank

Both cells and supernatant from the VPC MCB should be tested for RCR using a cell line selected *based on the tropism of the parental virus used to generate the vector*. For example, VPC containing envelopes such as gibbon ape leukemia virus (GALV) envelope or vesicular stomatitis virus glycoprotein (VSV-G) are typically tested on a human cell line. Other retroviral envelopes should be tested on a cell line permissive for infection by the relevant RCR.

If the VPC MCB was produced using a retroviral vector pseudotyped with an envelope distinct from the clinical vector product, for example, an ecotropic Murine Leukemia Virus (MLV), the potential exists for introduction of an RCR with that distinct envelope. Even though an ecotropic MLV RCR may present a minimal direct safety risk to humans, the presence of any replication-competent genome in the VPC MCB is problematic because of the increased probability of generating an RCR with a human host range through recombination with elements within the VPC.

Therefore, in cases where VPC are derived, at any step, by transduction with an ecotropic retroviral vector, testing of the MCB for the presence of ecotropic RCR is recommended, in addition to amphotropic RCR testing. For example, VPC possibly containing ecotropic MLV envelope should be tested for RCR on an

[5] https://www.fda.gov/media/76255/download

appropriate cell line which is permissive to infection by ecotropic MLV-like RCR (Ref. 17).

2. Vector Producer Cell Working Cell Bank

The risk of generating RCR during vector production with a VPC WCB that is derived from an appropriately qualified VPC MCB is relatively low based on our accumulated experience. Therefore, we do not recommend RCR testing of the VPC WCB except when RCR testing of the VPC MCB is insufficient based on recommendations described herein.

3. Retroviral Vector Supernatant Product and End of Production Cells

Both retroviral vector supernatant lots and end of production (EOP) cells should be tested for RCR. EOP cells are defined as cells from which a single bulk harvest of retrovirus-containing supernatant is taken or cells from which the last of a serial set of supernatant harvests is taken. This recommendation is based on data and experience reported at the 1997 FDA/NIH Gene Therapy Conference, where it was reported that RCR in vector production lots was not always consistently detected in both vector supernatant and EOP cells. These data support the position that dual testing provides a complementary approach to assuring RCR-free retroviral vector.

4. Ex Vivo Transduced Cells

If the retroviral vector is used for ex vivo genetic modification of cells, it is possible that RCR may be present in your vector at undetectable levels, which could be amplified during the manufacture of ex vivo transduced cells. Therefore, we recommend that each lot of ex vivo retroviral transduced cells be tested for RCR. This recommendation applies regardless of the length of time that the cells are cultured after transduction, because the length of culture time (e.g., greater than 4 days) has not been shown to strongly influence the likelihood of RCR development.

However, experience with vectors that have been deliberately designed to minimize the likelihood of recombination suggests that amplification of RCR in transduced cells is unlikely for many vectors. If you have accumulated manufacturing and clinical experience that demonstrates that your transduced cell product is consistently RCR-negative, we recommend that you provide this data to support reduction or elimination of testing ex vivo genetically modified cells for RCR. We recommend you include a discussion of safety features in the vector design that reduces the likelihood of generating RCR, a description of vector testing in accordance with current guidance, and your experience manufacturing RCR-free cell products. You may provide information supporting removal of RCR testing for lot release of ex vivo transduced cells in your IND (i.e., in the section titled: Manufacturing Process Development Section 3.2.S.2.6 or 3.2.P.2.3

of the electronic Common Technical Document (eCTD)) or discuss with the FDA during your pre-IND meeting.

If the cumulative information sufficiently supports discontinuation of RCR testing as part of your ex vivo retroviral transduced cell product release testing, we recommend archiving a sample for at least 6 months after the product expiration date. We recommend that you retain a sufficient amount (section III.B.2 and Appendix of this document) of the cell product to perform RCR testing in the future if necessary (section IV of this document). Samples should be archived with appropriate safeguards to ensure long-term storage (e.g., a monitored freezer alarm storage system) and an efficient system for the prompt linkage and retrieval of the stored samples with the medical records of the patient and the production lot records. We do not recommend archiving if the ex vivo transduced cells are tested for RCR at product release.

Table. Recommendations for Product Testing

Material to be Tested	**Frequency of Testing**	**Testing for Expected RCR –** *Cells and Supernatant* [1]	**Testing for Ecotropic RCR –** *Cells and Supernatant*
MCB -Derived by transduction with ecotropic vector -Derived by transfection of retroviral vector plasmid	One-time	Yes Yes	Yes NA[2]
Vector Harvest Material -EOP cells -Vector supernatant	Product release	Yes Yes	NA
Ex vivo Transduced Cells	Product release	Yes, cells only OR archive[3]	NA

[1] RCR testing should be based on the type of vector envelopes used. Consult text in section III.A.1 of this document for details.
[2] NA, not applicable.
[3] If an agreement reached with FDA to discontinue testing; consult text in section III.A.4 of this document.

B. Amounts for Testing

1. Supernatant Testing

In all cases, we recommend testing at least 5% of the total supernatant by amplification on a permissive cell line. Historically, we have recommended an alternative testing volume of 300 mL for vector lots over 6 liters. This volume was set based on our experience at the time with gammaretrovirus vector production lot size, reference material, and patient dosing. However, this recommendation may not be applicable to all current clinical manufacturing practices. Current manufacturing experience indicates that <1 RCR/dose equivalent is an achievable level for retroviral vector preparations intended for clinical use. Therefore, we now recommend that sufficient supernatant be tested to ensure a 95% probability of detection of RCR if present at a concentration of 1 RCR/dose equivalent. A more detailed explanation of the rationale and the mathematical formulas applied to achieve this level of testing confidence is found in the example in Appendix 1-1 of this document. You should detail the amount to be tested, and provide a justification for the proposed testing volume, in the description of RCR testing procedures included in your IND (in the eCTD section: Analytical Procedures 3.2.P.5.2).

To support the underlying assumption that a single retrovirus will be detected, one should determine a volume in which a single RCR can be detected by an individual RCR assay. Based on the determination of this volume, the total test volume should then be divided into replicate samples, each containing the volume demonstrated to detect a single RCR. When large volumes or high titer retroviral vector preparations are used, interference in RCR detection may occur. Sponsors are encouraged to develop more sensitive detection methods that overcome the interference effect of high titer retroviral vector preparations in order to use the alternative approach.

2. Cell Testing

We recommend that you test 1% or 10^8 (whichever is less) pooled vector-producing cells or ex vivo transduced cells by co-culture with a permissive cell line. This recommendation is unchanged from previous recommendations and is consistent with public consensus expressed at the 1996 and 1997 FDA/NIH Gene Therapy Conferences.

C. Assays for Testing

Vector supernatant assays should include culture of supernatant on a permissive cell line in order to amplify any potential RCR present. Similarly, cell testing should be accomplished by co-culture with a permissive cell line in order to amplify any potential RCR present. Typically, RCR culture assays have included a minimum of five passages. Assay optimization may allow for fewer passages if demonstrated to achieve similar

assay sensitivity. Sponsors are encouraged to develop RCR assays that support virus entry, amplification, and particle production specific to vector design (e.g., *Mus dunni* for ecotropic MLV (Ref. 17), C8166 cells for VSV-G pseudotyped HIV-1 (Ref. 18), or 293F-DCSIGN cells for E1001 enveloped HIV-1 (Ref. 19), noting that certain production systems may require more than one amplification cell line. The amplified material may then be detected in an appropriate indicator cell assay (e.g., PG-4 S+L- (Ref. 20), XC (Ref. 21), or by PERT (Ref. 22), or by psi-gag or VSV-G polymerase chain reaction (PCR) (Ref. 23), or by a commercially available p24 ELISA. All assays should include relevant positive and negative controls to assess specificity, sensitivity, and reproducibility of the detection method employed. Each lot of retroviral vector supernatant should be tested for inhibitory effects on detection of RCR by using positive control samples that are added to vector supernatant.

Alternative methods, such as PCR, may be appropriate for lot release testing of ex vivo transduced cells in lieu of culture based methods; particularly, when time constraints are present.

Any alternative methods should be developed in consultation with CBER. Data on sensitivity, specificity and reproducibility should be provided to support the use of alternative methods.

For assay development, you should develop a standard virus stock for use as a positive control and for method validation. The standard virus stock can be used for determination of the volume in which a single RCR can be determined. Gammaretrovirus RCR standard virus stocks have been developed, their infectious titers ($TCID_{50}$) have been determined, and are available through the American Type Culture Collection (ATCC) (4070A virus) or from the National Gene Vector Biorepository (NGVB) (GALV SEATO virus). Refer to Appendices 1-2 and 1-3 of this document for detailed information about the gammaretrovirus RCR standard and how it can be used to determine the replicate size and number for RCR detection. Standard virus stocks have not yet been developed for other retrovirus vectors. We recommend that you develop an in-house standard virus stock that represents your clinical vector attributes, including, the genetic background, envelope protein, and deletion of accessory proteins. The standard virus stock should be characterized for growth kinetics in the cells used for the RCR assay and tested for stability. The standard virus stock should be used to validate the RCR assay prior to licensure. For more information on assay validation, please refer to FDA's "Analytical Procedures and Methods Validation for Drugs and Biologics; Guidance for Industry," dated July 2015.[6]

IV. RECOMMENDATIONS FOR PATIENT MONITORING

Previous FDA guidance for active patient monitoring recommended RCR testing and/or archiving of patient samples at regular intervals for fifteen (15) years (Refs. 4, 24). To date,

[6] https://www.fda.gov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics

RCR or delayed adverse events related to RCR have not been reported in patients who have received retrovirus-based gene therapies (Refs. 5, 25, 26, 27, 28).

A. RCR Testing Schedule

We recommend the monitoring schedule to include analysis of patient samples at the following time points: pre-treatment, followed by testing at three, six, and twelve months after treatment, and yearly for up to fifteen (15) years. If any post-treatment samples are positive, further analysis of the RCR, and more extensive patient follow-up should be undertaken, in consultation with CBER. However, if all post-treatment assays for an individual patient are negative during the first year, collection of the yearly follow-up samples may be discontinued for that individual, and yearly review of medical history will generally be sufficient for the patient.

After you have accumulated patient monitoring data with your product, you may provide a rationale to discontinue all active testing of patient samples for RCR in the safety monitoring section of your clinical protocol. The rationale may include a discussion of safety features in the vector design that reduce the likelihood of generating RCR, as well as results of your previous clinical testing experience.

If the data sufficiently support discontinuation of active testing of patient samples, we recommend a yearly review of medical history be collected in lieu of patient samples, as a part of long-term follow-up. This history should be targeted towards determination of clinical outcomes suggestive of retroviral disease, such as cancer, neurologic disorders, or hematologic disorders. As part of the long-term follow-up protocol, a yearly long-term follow-up clinical report[7] should be submitted to the IND.

Relevant clinical samples should be collected and tested for RCR upon development of an adverse event suggestive of a retrovirus-associated disease. If patients die of a suspected retrovirus-associated disease or develop neoplasms within 15 years during a gene therapy trial, you should assay for RCR in a biopsy sample of the neoplastic tissue or the pertinent autopsy tissue. Sample collection and storage should be compatible with the expected testing strategy. Additional recommendations for long-term follow-up of patients in clinical trials using retroviral vectors are discussed in the Long Term Follow-up Guidance.

B. Recommended Assays

We recommend two methods that are currently in use for detecting evidence of RCR infection in patients: 1) serologic detection of RCR-specific antibodies; and 2) analysis of patient peripheral blood mononuclear cells by PCR for RCR-specific DNA sequences. The choice of assay may depend on the vector, mode of vector administration, and the clinical indication. For example, it has been shown that direct administration of VPC or repeat direct injection of a vector can result in vector-specific antibodies that do not

[7] For more information, refer to section V.D.3 and Appendices of the Long Term Follow-up Guidance ("Recommendations for Protocols for Long Term Follow-Up Observations: Clinical Considerations").

correlate with the presence of RCR (Refs. 29, 30). Therefore, in cases where vector or VPCs are directly administered, a PCR assay may be preferable over serologic monitoring. Additionally, monitoring of patient samples by PCR may be preferable over serologic monitoring if the patients are immunocompromised to an extent that antibody production may be minimal or not at all. In either situation, all confirmed positive results should be pursued by direct culture assay to obtain and characterize the infectious viral isolate.

V. DOCUMENTATION OF RCR TESTING RESULTS

RCR testing results from production lots and patient monitoring should be documented in amendments to the IND file. Positive results from patient monitoring should be reported immediately as an adverse experience in the form of an IND safety report (21 CFR 312.32). Negative results should be reported by way of the IND annual report (21 CFR 312.33) or Development Safety Update Report (DSUR), if used in lieu of reporting in annual reports. In addition, to enhance the accumulation of data on RCR testing assays, CBER encourages members of the gene therapy community to publish data and/or discuss data publicly regarding their experience with different vector producer cell lines, patient monitoring, and safety.

VI. POST-LICENSURE CONSIDERATIONS

We recommend that labeling for retroviral vector-based gene therapy products incorporate relevant data and information to clearly present the immediate and long-term risks associated with RCR. As a critical safety test for retroviral vectors, testing for RCR during vector manufacture and release should continue after licensure.

At the time of submission of your Biologics License Application (BLA),[8] you should have accumulated sufficient manufacturing and clinical safety data to determine whether there is a significant risk of RCR developing with your product. This risk assessment may be used to propose that periodic patient monitoring for RCR would not be warranted for your product post-licensure. However, you should include a provision in the BLA to collect relevant clinical samples from patients for RCR testing upon development of an adverse event suggestive of a retrovirus-associated disease. In the event patients die from a disease potentially associated with a retrovirus or develop neoplasms within 15 years following product administration, every effort should be made to assay for RCR in a biopsy sample of the neoplastic tissue or the pertinent autopsy tissue.

These post-licensure considerations should also include continued long-term patient follow-up, up to fifteen (15) years after dosing, in accordance with the Long Term Follow-up Guidance. For more information, refer to section VI of the Long Term Follow-up Guidance ("General Considerations for Post-Marketing Monitoring Plans for Gene Therapy Products").

[8] 21 CFR 601.2.

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APPENDIX

1-1. Derivation of Recommendation for Test Volume for RCR Detection

Assuming the RCR are present in the production lot at a concentration (**c**) and that an assay will detect a single retrovirus in the sample, the probability (**p**) of detecting retrovirus in a volume (**Vt**) is given by the formula: **p = 1-exp(-cVt)**, because the number of RCR in **Vt** follows a Poisson distribution with a parameter **cVt**. Solving for **Vt**, one gets the following equation:

$$\mathbf{Vt = - (1/c) \ln (1-p)}$$

where **ln** denotes the natural logarithm.

Value for p
For the use of this formula, it is recommended that the value for **p** be set at 0.95. With the recommended replicate size and number defined in Appendix 1-3 of this document, **p** becomes the probability of detecting an RCR in the production lot.

Value for c
We recommend that the value for **c** be set no higher than 1 RCR/dose equivalent. If the concentration of RCR in the production lot is 1 RCR/dose equivalent or greater, then the probability of detection is at least 0.95. If the production lot contains RCR at a concentration of <1 RCR/dose equivalent, the RCR may not be detected and would be administered to the patient. We also recommend that a dose equivalent be defined as the maximum amount of vector expected to be administered at one time. For ex vivo genetically modified cells, a dose equivalent is the amount of vector used to transduce the maximum number of target cells for each production lot.

Value for Vt

With the recommended value for **p** and **c**, the total volume of retroviral supernatant to be tested, independent of lot size, is calculated as follows:

Vt = - (1 / (1 RCR/dose equivalent)) ln (1 -0.95)

Direct administration example:
If your product is administered at $1x10^{10}$ TU (transducing unit)
Vt = - (1 / ($1/1x10^{10}$ TU)) ln (1 -0.95) = $3x10^{10}$ TU

Ex vivo genetic modification example:
If you aim to transduce up to $1x10^8$ cells at an MOI (multiplicity of infection) of 0.5 with a titer of $1x10^7$ TU/mL:
Dose equivalent = ($1x10^8$ cells) (0.5 TU/cell) / ($1x10^7$ TU/mL) = 5 mL
Vt = - (1 / (1/5 mL)) ln (1 -0.95) = 15 mL

Proposals to use smaller volumes should be developed and reviewed in consultation with CBER.

1-2. Empirical Determination of Assay Sensitivity

In collaboration with the ATCC, a standard gammaretroviral stock (ATCC # VR-1450) has been established for use in determination of sensitivity and validation of assays used to detect the presence of RCR which would be produced from VPC containing an amphotropic envelope. This stock can be used to determine the relative assay sensitivity for detecting RCR. This information can subsequently be used to determine the size of replicates of retroviral supernatant to be tested that will ensure detection of a single retrovirus and thus, the number of replicates to ensure an adequate total volume, **Vt,** as specified in this guidance (Appendix 1-3 of this document). The virus stock is derived from a cell line which has been transfected with a molecular clone encoding MoMLV with a substitution of the envelope coding region from the 4070A strain of amphotropic MLV (Ref. 31). Therefore, this virus stock represents a typical recombinant virus that could be generated in a retroviral packaging cell line containing coding sequences for a MLV envelope.

The standard virus stock and its infectious titer can be used as a positive control to empirically determine the relative sensitivity of assay methods used for detection of RCR in retroviral vectors. In particular, this stock will allow investigators to determine the largest test volume in which a single RCR can be detected. The determination should be performed in the presence of a retroviral vector supernatant typical of a production lot in order to control for inhibitory effects of the retroviral vector particles on detection of RCR. Availability of this standard should allow individual investigators to establish this methodology in their own laboratories, as well as allow exploration of alternative methods for detection of RCR.

1-3. Formula to Determine Replicate Size and Number

Depending on the volume in which a single RCR can be detected by an individual RCR assay (as determined by use of the RCR standard, Appendix 1-2 of this document), it may be necessary to divide the total test volume into several replicate samples to ensure the detection of RCR in the sample. The number of replicates (**r**), can be determined using the formula,

$$\mathbf{r = Vt / Vs}$$

where **Vs** is the volume in which one RCR can be consistently detected (Appendix 1-1 of this document for determination of **Vt**).