

The Williamsburg BioProcessing Foundation

Reference Materials for Retroviruses and Lentiviruses June 5, 2002: The Sheraton Hotel - Boston, MA, USA

Final Report

A growing number of retrovirus -based vectors for gene therapy applications are being developed. Vectors based on animal gammaretroviruses (*formerly oncoretroviruses*) such as the amphotropic strain of murine leukemia virus (MuLV) have been in use for *ex vivo* transductions of hematolymphoid cells for over a decade. Most vectors actually used for clinical gene transfer are hybrids of amphotropic (A) and Moloney (Mo) strains of MuLV– the core proteins, Gag and Pol, are derived from MoMuLV, while the envelope protein, Env, is derived from A -MuLV. While these vectors have demonstrated proof of principle in transfer and expression of transgenes, they have had only limited success in clinical settings. The lack of success has been related to the inability of these vectors to transduce non -dividing cells. Non-dividing cells often represent a large proportion of target cells. In addition, their use for *in vivo* gene therapy applications has been limited due to complement inactivation and poor structural stability. To overcome these limitations, researchers have focused attention on lentiviruses for the development of replication -deficient lentivectors since lentiviruses are potentially capable of transducing both dividing and non -dividing cells and appear to be somewhat more robust for *in vivo* applications. However, several groups have chosen pathogenic human lentiviruses, human immunodeficiency virus-1 (HIV-1) and -2 (HIV-2), or non-human primate lentiviruses, such as simian immunodeficiency virus, as the bases for lentivector development, leading to growing concerns about the relative safety of such vectors. Lentivectors based on non -primate lentiviruses, e.g. feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), are also being developed which appear to pose less of a safety risk, but less is known generally about these viruses than HIV -1 and there could be unpredictable risks with their clinical use. (*General issues about lentivirus vectors were previously discussed by a US FDA advisory committee in October 2001; visit <http://www.fda.gov/ohrms/dockets/ac/cber01.htm#Biological%20Response> for details*).

As with all biologically active medicinal products, gene transfer vectors based on gammaretroviruses and lentiviruses require a good safety profile. This should be derived from product-specific data as well as collective data from products that share common elements in the platform technology. There is a need to characterize each product as fully as possible to ensure that it is fit for its intended purpose. Characterization requires the use of validated analytical tests and assays, which themselves are dependent on the availability of well -characterized reference materials to act as standards or controls. Currently, there is only one reference material for retrovirus -based vectors. This reference material is an infectious preparation derived from a chimeric molecular clone originally constructed by A.D. Miller that carries the LTRs and Gag/Pol coding region from MoMuLV and the Env coding region from A -MuLV. The reference material, provided by the American Type Culture Collection (ATCC), serves to monitor the sensitivity of infectivity assays to detect replication -competent retroviruses (RCR) derived from the manufacture of replication -deficient MuLV vectors or from recombination events in target cells. Since the development of

this reference material, additional gammaretroviral vectors using envelopes from other retroviruses (e.g., Gibbon Ape Leukemia Virus) or other viruses (e.g., VSV -G), as well as lentiviral vectors, is either already in clinical trials, or being explored for their potential utility in clinical gene therapy protocols. Thus, there is a clear need to work towards the development of other reference materials to cover the growing number of novel pseudotyped gammaretroviral vectors and lentivectors currently under development for clinical gene therapy protocols.

To begin to address the requirements for and development of reference materials for gammaretroviruses and lentiviruses, an *Ad hoc* Working Group on the Biological Standardization in Gene Therapy (WGBSGT) had met at The National Institute for Biological Standards and Control (NIBSC), South Mimms, Herts, UK, on September 27-28th, 2001 to review the gammaretroviral/lentiviral field and regulatory concerns for their safe clinical use. The WGBSGT agreed to follow up these discussions at a further meeting in order to try and identify the steps needed to pursue standardization requirements in this area. To this end, The Williamsburg BioProcessing Foundation (WilBio) hosted a 1-day meeting on June 5th, 2002 (Boston, MA). The aim was to bring together representatives from industry, regulatory agencies, contract testing laboratories, academia, and organizations involved in standards development to discuss the testing of vectors based on gammaretroviruses/lentiviruses and whether it was now appropriate to proceed towards the development of suitable reference materials. More than 50 representatives from all sectors attended the meeting. The aims were to discuss:

1. The design and activity of replication -deficient gammaretroviral and lentiviral vectors with regard to safety;
2. How to test such replication-defective vectors for particle enumeration, infectivity, integration capacity and *in vitro* potency;
3. How to test for the presence of replication competent retroviruses (RCRs) and replication competent lentiviruses (RCLs);
4. The stages at which such testing was appropriate;
5. Potential reference materials that would lead to improvements in validating tests and give greater assurance for product safety; and
6. How to maintain continuity for future standardization activities.

To start the meeting, K. Carson, chairman of WilBio, introduced the company and its goals to the group. Presentations by T. Meager (NIBSC), D. Takefman (CBER/FDA), E. Aguilar-Cordova (Harvard Gene Therapy Initiative), and M. Wisher (BioReliance Corporation) covered perspectives and issues surrounding the testing of gene transfer products in general, and the more specific retroviral/lentiviral vectors. It was evident that some methods to estimate particle numbers, infectivity, and transducing potency had been, or were being, developed. However, it was more difficult to enumerate retrovirions than adenovirus particles because of their greater plasticity and instability. For example, electron microscopic determinations are insensitive and not very quantitative. No single alternative method could distinguish between full and empty particles. By utilizing immunofluorescent tagging of viral envelope or capsid proteins, and then visualizing these by confocal microscopy in relation to fluorescent microspheres of known concentration, or by interpolating capsid (Gag) concentration, or reverse transcriptase (Pol) activity to particle numbers based on estimated correlation of these parameters with particle number (e.g. 1pg Gag

= 12000 particles) we can successfully estimate total particle numbers. An HIV p24 Gag reference reagent (NIBSC 90/636) was available and probably could have been used to calibrate ELISA for HIV p24 Gag determinations, providing a method for determination of particle number of HIV -lentiviral vector preparations. Regarding infectivity of retroviruses, it was well known that the particle to infectious unit ratio was high, perhaps as high as 1000:1, and that infectivity assays suffered low reproducibility. A hybrid amphotropic -MoMuLV preparation (VR-1450), available from the ATCC, could be used to monitor the sensitivity of infectivity assays for detection of RCR in gammaretroviral vectors arising from the manufacture of replication-deficient MuLV-based vectors. However, this preparation was not suitable for assessing sensitivity of assays to determine infectivity of RCRs arising from production of Gibbon Ape Leukemia Virus (GALV) envelope pseudotyped gammaretroviral-vectors or RCLs arising from production of lentiviral vectors or rescue by infectious lentiviruses, e.g. HIV -1. Besides standard infectivity measurements, some regulatory authorities (e.g. France) were asking for further evidence of freedom from RCRs by asking sponsors to use "marker -rescue assays." The current reference material VR -1450 could be used in a marker -rescue assay, so long as it was for a vector with amphotropic Env, not GALV.

Regarding transducing capacity, PCR methods are being developed to determine the number of proviral DNA integrations per cell, but these tend to be transgene -specific and generally difficult to perform due to the need for combining optimized cell culture and DNA extraction procedures. Reference materials for this purpose are not available. The number of integrations per cell is an important safety consideration given the risk of insertional mutagenesis with the use of gammaretroviral or lentiviral vectors. Functional activity of the integrated transgene is usually assessed by measurement of its translation product, either in an assay to determine amount, e.g. by ELISA, or in an assay to measure enzymatic or biological activity. These assays are almost always transgene -specific, so it is difficult to have other than transgene product-specific reference materials.

While it was apparent there was a lack of reference materials, the need to develop any reference material should be placed in the context of whether any "added value" would accrue relating to safety of gammaretroviral/lentiviral vector products if tests were subsequently validated with the reference material. There is a clear requirement for particle number determination for certain viral vectors for *in vivo* clinical applications, as particle numbers will define doses. Adenoviral vectors exemplify this where the necessity for an adenovirus reference material was widely recognized as crucial for the characterization and safety of these vectors. A wild -type adenovirus serotype 5 (Ad5) reference material has now been prepared and characterized with respect to particle number and infectivity, and is available from the ATCC (B. Hutchins, see later mention). However, the need for a retrovirus preparation to act as a reference material for particle counts may not be so strong. There is a lack of observed toxicity from most gammaretroviral particle proteins, and the majority of retroviral vectors are used for *ex vivo* transductions of hematolymphoid cells, reducing the need for particle counts. Efficacy and transduction efficiency will probably be of more importance than particle numbers, which may have only limited impact on the overall safety of such systems (excess particles will wash away). Nevertheless, particle enumeration should strengthen the characterization profile of

retroviral vector products, which also becomes important for lentivectors (and modified gammaretroviral vectors) developed for *in vivo* applications. The availability of reference materials provides "po ints in the sand" and enables cross talk among laboratories/manufacturers using identical or similar QA strategies. However, there exists a need to not only identify suitable reference materials (not easy when the field of retroviral vectorology presents a moving target with development of variously pseudotyped vectors, e.g. GALV, VSV, RD114 Envs, and increasingly focusing on lentiviral vectors) but also to define their purpose(s). For example, what assays should be standardized? Should assays be standardized for sensitivity or for end-point determinations?

Development of Guidelines and Standards: Dr. C. Wilson (Division of Cellular and Gene Therapies, CBER, FDA, USA) presented a review of FDA guidance on testing for RCR. She pointed out a major safety concern regarding vector sequences with helper or endogenous retroviral elements for generating RCR: it could potentially cause neoplastic transformation following multiple cycles of infection and integration. A study of immunosuppressed monkeys whose bone marrow cells were exposed and transduced with a preparation of MuLV RCR +ve vector found that 3/10 monkeys developed lymphoma and died in 200 days (Donahue et al, 1992). RCR with recombinant sequences from helper and endogenous retroelements were present in the monkeys. Since by analogy there was a perceived risk that RCR could cause similar tumors in humans, a rationale was provided for testing gammaretroviral vectors to show the absence of RCR, which led to CBER's initial recommendations on RCR testing in 1993. These recommendations required RCR testing of the master cell bank, working cell bank, production lot, and *ex vivo* transduced cells for each lot of retroviral vector and *ex vivo* transduced cells. The amounts of materials required for RCR testing were 5% of retroviral vector -containing supernatant and 1% or 10^8 cells.

A CBER expert group was set up to evaluate the 1993 recommendations and evaluations were considered at a Forum for Gene Therapy in 1996, co-sponsored by CBER, FDA and NCI, NIH. The outcome of this Forum was published in 1997 (Wilson et al, *Hum. Gene Ther.* 8:7). Further proposals regarding RCR testing were made at a second Gene Therapy Forum in 1997, with final guidance published in October 2000. This guidance provided information on when and what to test based on cumulative experience from RCR testing of both cells and supernatant. For example, unpublished data suggested that infection of human cells with a low -level RCR preparation required at least 4 days of culture for detection by current methods.

It was clear that infectivity assays to detect and quantify RCR would benefit by being standardized for sensitivity by the use of a suitable MoMuLV preparation with an assigned infectivity titer. To this end, A.D. Miller's group supplied ATCC with a molecular clone and producer cells for the development and characterization of a hybrid amphotropic-MoMuLV standard. ATCC then produced a crude virus -containing supernatant from the producer cells, which, with the collaboration of industry, had been characterized in 2-3 laboratories for infectivity titer and particle count. They prepared two lots, the more recent of which was undergoing stability testing one year after laying down stock ampoules at -20°C . The first lot of this preparation, VR-1450, had since been used as a virus standard for calibrating the sensitivity of infectivity assays. Its use allowed statistical analysis of the sample size of retroviral vector supernatants required for testing. The amount of retroviral vector

supernatant required for RCR testing remained 5% by default, but if the total volume of supernatant exceeded 6 liters an alternative approach was possible. Using VR - 1450, it had been estimated that the volume of retroviral vector supernatant required to detect RCR with a probability of 95% if the RCR concentration is >1 RCR/100mL is 300mL. In order to apply the "alternative approach," the MoMLV standard VR - 1450 had to be used to determine the sensitivity of RCR detection assays.

The main questions for discussion were:

1. How had we arrived at the requirement for a 4-day culture of transduced cells before RCR testing?
2. What was the stability of VR-1450 when put through repeated freeze-thaw cycles?
3. Would VR-1450 be suitable for standardizing other analytical procedures?

At the FDA/NIH Gene Therapy Conference in 1997, G. McGarrity (GTI) verbally presented the data demonstrating that infection of human cells with low -level RCR preparations required at least 4 days of culture before progeny RCR could be detected by current assay methods. Dr. Wilson clarified that the data had not been published, and exhaustive searches by GTI suggested that the data was no longer available in the company archives. Dr. Cornetta of the National Gene Vector Laboratory (NVGL) Program suggested that the NGVL facility at Indiana University could perform the experiments required to confirm that a minimum four days of culture would be required before low level RCR are amplified to a detectable level. As an alternative approach, M. Kenter (Central Committee involving Human Subjects [CCMO], The Netherlands) proposed the requirement by CCMO of 5 dry runs on each vector batch to indicate absence of RCR generation in *ex vivo* transduced cells, irrespective of the time in culture of transduced cells. Each dry run should consist of a transduction of human cells from different donors at the proposed clinical scale, subsequent co -cultivations with RCR -permissive cell lines, and a read-out in a validated RCR assay. If no RCR can be detected from any of the 5 dry runs, the investigator can then use the vector batch for *ex vivo* transduction of cells and it requires no further RCR testing prior to administering the transduced cells to the patient. However, archiving a quantity of the transduced cells is recommended in case a need for retrospective RCR testing arose.

Approaches to the development of any future reference materials for gammaretroviruses and lentiviruses, and vectors based on these viruses, could be modeled on those used for the development of an adenovirus reference material, which is complete (B. Hutchins, Canji). In this case, an action group, the Adenovirus Reference Material Working Group (chaired by B. Hutchins and E. Aguilar-Cordova) has worked for over two years to specifically undertake the development of a wild type Ad5 reference material. Several companies donated activities in the preparation of this Ad5 reference material, directed by the ARMWG, along with the collaboration of CBER/FDA. Following vialing and storage at ATCC, an international collaborative study involving many expert testing laboratories characterized the Ad5 reference material in assays to determine physical particle counts, infectivity titer, and stability. The ARMWG also recommended that study participants use SOPs for particle counting and infectivity assays. However, the standardization of specific methods or the endorsement of specific cell culture, viral culture, purification, formulation, or

analytical methods was seen to be outside of the ARMWG's mission. The raw data received from participants had been collated and sent for statistical analysis to enable calculation and assignment of particle and infectivity titers assigned to the Ad5 reference material. The outcome of that analysis was discussed imminently at the next ARMWG meeting (6th June, 2002, in conjunction with the 5th ASGT Annual Meeting, Boston, MA). These assignments were made, and the Ad5 reference material is now available on a request basis from the ATCC, allowing comparability of results from different laboratories in both pre-clinical and clinical studies, thus aiding the production of more consistent and safer lots of adenoviral products for gene therapy applications.

In discussion following this presentation, it was mentioned that T. Flotte of the National Gene Vector Laboratory had developed a reference material for adeno-associated virus type 2 (AAV -2). However, details of its development and characterization were not available to attendees and there was no consensus concerning its utility.

Technical Review: Expert practitioners of gammaretrovirus/lentivirus research gave a series of presentations on analytical methods used for characterization. These presentations highlighted issues of design, production, analytical procedures, and safety of gammaretroviral and lentiviral vectors. The overall goal was to build a platform of information upon which to base considerations for development of future reference materials.

The production of gammaretroviral vectors and the attendant risks of generation of RCR were reviewed by K. Cornetta (National Gene Vector Laboratory, Indiana). Detection and quantification of RCR contamination of gammaretroviral vector lots — an FDA requirement — was the focus of a presentation by N. Sajjadi (Sajjadi Associates, Encinitas, CA). The FDA specification is set for complete absence of RCR. However, absence of RCR has been equated to "none detected," which is subject to many factors that define the capacity of the test system (assay) to detect RCR. These factors include the sensitivity and efficiency of the assay, the inherent variability of the assay, the amount of sample assayed, and the concentration of RCR in the sample.

Assay validation requires careful experimental design. Since RCR amplification tests may yield negative or positive results, validation testing must be performed using an input RCR concentration where both negative and positive results can be obtained. The outcome of testing is subject to a number of factors that influence RCR detection. These include sampling errors, number of replicates, sensitivity and variability (reproducibility) of the RCR detection method. In order to study these variables, a general approach is advocated where, assuming that the assigned label titer for the amphotropic-MoMuLV standard VR-1450 is the "true value," VR -1450 is diluted to 0.1-1.0 infectious unit (iu) per assay volume input and, using multiple replicates, the proportion of non-detection (negative) results is determined. It can be shown that the probability of non-detection (p) is a function of RCR input amount (C). Based on a Poisson distribution, it has been shown that an average input of 0.7 IU per replicate will yield 50% positive and 50% negative results. The 95% confidence limits for detection/non-detection are a function of N , the number of replicates tested. For example, 95% confidence limits using an RCR input amount of 0.7 iu were 0.30 -

1.35 for $N = 18$. However, "true" confidence limits are also a function of assay variability, e.g. at 50% variability the 95% confidence limits for RCR input of 0.7 iu, $N = 18$, increase to 0.27 - 1.37; at 100% variability, to 0.19 - 1.42. Although defining assay validation parameters and acceptance criteria should be done by consensus, it was suggested that the proposed approach using 0.7 iu of the VR -1450 MLV reference material with 18 replicates would provide a useful model for detection assay validation procedures. There could, however, be problems for validation of detection assays in general if the VR -1450 standard exhibited instability or if its label titer is an underestimate of its "true value," e.g. if the detection assay being validated were more sensitive than the assays used to assign titer to VR -1450.

In conclusion, it was noted that negative RCR results were only meaningful to the extent that the statistical confidence in detecting "one RCR" is understood. The statistical confidence for +/- assays is a function of the binomial distribution of the RCR result outcomes, the assay sensitivity, and the variability of the assay. Therefore, validation approaches should be standardized to ensure comparability of data.

Design of assays to detect a single RCR was also the subject of the presentation of S.K. Dusing (BioReliance, Rockville, MD). Studies carried out at BioReliance compared the use of 4070A amphotropic MuLV (A-MuLV) to that of a hybrid Moloney/amphotropic MLV (Mo/A-MuLV) as a positive control for assays in which cells and culture supernatant are tested for the presence of RCR. (*This is a stock produced and titered at BioReliance by infecting mouse 3T3 cells with the VR-1450 virus*). *Mus Dunningi* cells seeded in flasks or roller bottles were inoculated with 1 and 10 Focus Forming Units (FFU) of A-MuLV or Mo/A-MuLV in 10 and 100 mL of culture medium in amplification assays designed to detect RCR in cell banks or transduced cell culture supernatant. Based upon focus formation in feline PG -4 cells, the 1 FFU input of A-MuLV was detected in culture supernatant harvested at the final passage in 15/15 tests using this amplification protocol. However, the 1 FFU input of Mo/A-MuLV was detected in approximately two-thirds of cultures inoculated with either the 10 mL volume (6/9 positive) or the 100 mL volume (4/6 positive). In testing for RCR by co-cultivation of target with test cells, the 1 and 10 FFU quantities of A-MuLV and Mo/A-MuLV were added as positive control spikes to suspensions of 10^7 *Mus Dunningi* cells seeded in flasks. Under these conditions, the 10 FFU inputs of both A-MuLV and Mo/A-MuLV yielded, after 5 passages, RCR detectable by the PG-4 S⁺L⁻ assay. At the 1 FFU input level, 8/10 (80%) of A-MuLV infected *Mus Dunningi* test cultures yielded detectable RCR, whereas 7/12 (approx. 60%) of Mo/A-MuLV infected test cultures were positive for RCR. The variability in assay sensitivity with regard to the type of MuLV used as a positive control did not appear to be related to inoculum volume, or number of cell passages in culture, or the virus lot used. Because virus stock was diluted to 1 to 10 FFU based upon titer in the PG -4 assay, these results may relate to the extent that the virus titer established in feline S⁺L⁻ cells corresponds to the level of infectivity for *Mus Dunningi* cells for the two viruses.

Although regulations require researchers to show absence of RCR in gammaretroviral vector lots to prevent accidental exposure of patients' cells to RCR, there is little data on the capacity of T lymphocytes, often a prime target for transduction, to amplify RCR. S. Ebeling (University Medical Centre, Utrecht, The Netherlands) outlined her work aimed at making a risk assessment of exposure of T lymphocytes to RCR. In a clinical trial by the Utrecht group, human primary T lymphocytes were transduced

with an amphotropic MLV-vector. A feasibility study to validate RCR-assay of *in vitro* transduced T lymphocytes, in which various cell types (NIH 3T3, HEK 293, Jurkat, and T lymphocytes) were spiked with the reference Mo/A-MuLV RCR-producing cells (VR-1448) or supernatant (VR-1450), was described. The presence of RCRs, without prior amplification in *Mus Dunni* cells, was determined by real-time RT-PCR using specific primers of the amphotropic MuLV 4070A strain Env gene. The NIH 3T3 cell line produced the highest number (ca. 10^9) of RCR, but there was also efficient propagation of infectious MuLV in Jurkat and HEK 293. In contrast, the capacity of freshly isolated T lymphocytes, stimulated with anti-CD3 and interleukin-2 (IL-2), to produce infectious MuLV was less than 1% that of murine NIH 3T3 cells. RCR was also quantified in PG-4 S⁺L⁻ infectivity bioassays. For NIH 3T3 and HEK 293 cells, there was a close correlation between PCR and infectivity titers, but in Jurkat cells the infectivity titer was about 20-fold lower than the PCR titer. This difference was more striking in T lymphocytes where only 1 particle in 250, as measured by PCR, was infectious. Together, the data obtained from PCR and infectivity assays permitted calculation of the ratio of input and output RCR for the different cell types. For T lymphocytes, this ratio varied between 68:1 and 3125:1, indicating that T lymphocytes do not amplify RCR during 1-2 weeks in culture. This observation may have relevance to the FDA when guidelines develop for testing for RCR in gammaretroviral vector transduced cells.

There has been increasing interest in developing pseudotyped MuLV vectors as these may better facilitate the transduction of human hematopoietic stem cells compared to non-pseudotyped MuLV vectors. One of the retroviral Env proteins for pseudotyping MuLV is that derived from Gibbon Ape Leukemia Virus (GALV). Facts about GALV were summarized by MV Eiden (NIMH, NIH, MD). The GALV family comprises 5 strains of exogenous gammaretroviridae isolated from non-human primates in various disease states. One of these isolates, GALV strain SEATO, has been used to construct the PG13 packaging cell line. PG13 cells are derived from NIH 3T3 and express GALV SEATO Env together with MLV Gag/Pol components. In addition, a plasmid, pGALV386, containing a full-length infectious clone of GALV SEATO has been developed and used to make vectors containing not only GALV Env, but also GALV core components and GALV-based packageable genomes as well. Since GALV does not, due to the absence of a GALV receptor, infect murine cells, assays reliant on amplification of RCR in murine cell lines are unsuitable for GALV-derived RCR.

The pGALV386 plasmid should prove useful as a reference reagent in GALV-RCR assays because it contains the gene encoding the GALV-SEATO Env – the same Env incorporated in vectors produced in PG13 cells. Additionally, the pGALV386 clone is infectious, making it possible to obtain low-passage GALV SEATO producer cells that would generate fewer defective forms of the virus. The pGALV386 plasmid and early passage pGALV386 producer cells, as reference materials for assessing GALV-based retroviral vectors for contamination with GALV RCRs, will be deposited with the ATCC shortly.

The theme of detection of GALV RCR was continued in the presentation of M Audit (Genethon, France). Two methods for detecting RCR in amphotropic and GALV-pseudotyped MuLV vector preparations were developed at Genethon. The first was based on RCR detection by PCR, and the second was based on the "marker-rescue" principle. In comparison to these methods, the more traditional RCR infectivity assays

using PG4 S⁺L⁻ cells were found to perform poorly due to poor focus formation. The PCR detection method also had drawbacks:

1. The need for a large number of amplification cycles, potentially leading to false positives; and
2. RCR whose sequences differ from those of the PCR primers cannot be detected.

Therefore, the marker -rescue assay has become the method of choice for detecting Amphotropic or GALV RCRs.

A chimeric infectious clone of the MuLV genome with a SU and TM coding sequences from the Env gene of the GALV SEATO was constructed. Since this infectious virus replicates normally and maintains the GALV tropism, it was used as positive control for RCR detection in GALV-pseudotyped retroviral vector preparations. The amphotropic positive control (ATCC, VR-1450) and the GALV positive control were used in marker -rescue assays to compare the sensitivity of RCR detection, either in amphotropic or in GALV vector preparations. Cells containing a packageable Hygromycin resistance gene (HT1080-Hygro[®]) are infected with amphotropic or GALV-based retroviral vector preparations, which, if they contain RCR, will rescue and package the Hygro[®] gene construct into progeny virions. The latter can then be quantified by infection of indicator cells (HCT1 16) and the scoring of Hygromycin resistant colonies. The GALV RCR marker -rescue assay is as sensitive as the currently validated amphotropic assay, and it will be a useful reagent for testing clinical-grade vector preparations.

While there was no equivalent lentivirus standard to the amphotropic -MoMuLV standard VR-1450, there was perceived to be a growing need to develop lentivirus standards to monitor the sensitivity of infectivity assays and other analytical tests. The F. Borellini (Cell Genesys, Foster City, CA) outlined the approaches taken by the Cell Genesys Lentivirus Group to developing the appropriate assays and reference for HIV-1-based lentivectors. The critical parameters for characterization of lentivectors were physical particle titer, infectious titer and the detection and quantification of RCLs. Physical particle number could be estimated from interpolation of measurements of HIV -1 p24 Gag (by ELISA, for which a WHO HIV -1 p24 Gag reference reagent, 90/636 is available from NIBSC) or vector genomes (by PCR methods). However, measurement of filled (genome -containing) particles as opposed to measurement of filled and empty particles together was only possible by application of an RT PCR procedure to estimate the amount of vector RNA. The read-outs from any of these assays could be enhanced by contaminating side products such as unincorporated Gag (ELISA) or plasmid DNAs (PCR). The issue of whether the determination of empty versus filled particles would be important for the safe use of lentivectors was raised. It was recognized that, in terms of safety, total particle concentration is more representative of the clinical dose, even though only 0.1 -1.0 % of particles is apparently infectious.

Since lentivectors are replication defective, infectious titers can only be estimated from indirect measurements of vector activity. RT -PCR on RNA extracted from vector preparations does not accurately reflect the infectious titer due to the presence of defective interfering particles, plasmid DNA contamination and inhibitors of

transduction. Therefore, infectious titer is related to either the number of proviral integration events or to transgene expression (protein) in transduced cells. Integration is measured by QPCR of, for example, the packaging signal ψ following transduction, but the experimental procedures are complex. Measurement of transgene expression suffers several drawbacks, including the fact that any assay will have to be transgene-specific. If assays are relatively insensitive, low-level transgene expression may be missed, and may not distinguish cells with multiple copies. Detection and quantification of ψ DNA by PCR in transduced cells is therefore preferred for assessment of infectious titer. However, such an assay requires standardized procedures, including specification of the cell line used for transduction, cell culture conditions for transduction, timing of PCR testing after transduction and a pseudotype-specific lentivirus reference reagent.

The generation of RCLs is the major safety concern associated with the clinical use of lentivectors. However, current design of lentivectors has virtually eliminated the possibility of RCL formation. Nevertheless, assays to detect and quantify RCLs are required to prove absence of RCLs in lentivector products. Any RCL generated will contain p24 Gag and thus it is possible to measure p24 Gag to quantify RCL. In the RCL assay developed by Cell Genesys, human C8166 cells are exposed to a lentivector preparation and infectious lentivirus amplified by at least 4 passages (21 days) in C8166 before p24 Gag quantification. In spike and recovery studies with a positive control (RCL-like lentivirus), this assay has been shown to be very sensitive, possibly because the HTLV-1 sequences contained in C8166 cells help the amplification of infectious lentivirus. However, the choice of a suitable, representative, positive control is critical for demonstrating the sensitivity of the RCL assay. For HIV-1-based vectors there is the need to create a pseudotype-specific, accessory gene minus (but *tat* and *rev* plus), attenuated strain of HIV-1. Cell Genesys has developed such a strain, which is co-pseudotyped with VSV-G and HIV-1 Env. The positive control is produced in a VSV-G expressing cell line, so that the first infection of C8166 is mediated by VSV-G, but subsequent infections are mediated by HIV-1 Env since neither C8166 nor the positive control carry the VSV-G gene. This RCL-like positive control may overcome the safety concerns about using an HIV-1 derivative that contains the VSV-G pseudotyping gene in its genome.

Conclusion:

The conclusions are that p24 Gag ELISA is suitable for particle determinations, that ψ DNA PCR quantification in transduced cells is suitable for infectious titer estimations, and that RCL could be quantified in appropriate cultured cell lines followed by end-point analysis. For ψ DNA PCR and RCL assays, suitable standards/positive controls need to be identified.

While much recent research had focused on the development of lentivectors based on HIV-1, and for which specific HIV-1 RCL testing methods were available, there was a perceived need to look for ways to test for the presence of less well-defined RCL that might arise from a product based on non-primate lentiviruses, e.g. equine infectious anemia virus (EIAV), as currently being developed by Oxford-BioMedica. D. Jolly (BioMedica Inc, CA) outlined the RCL testing strategy in development at his company. This strategy involved designing a meaningful assay system able to detect a wide range of retrovirus-like elements, and then control it with a relevant robust spike control agent. It was proposed to use a test system that detects reverse transcriptase

(RT) activity. While earlier RT assays were relatively insensitive, the combination of RT activity with PCR amplification led to the development of ultrasensitive "product-enhanced reverse transcriptase" (PERT) assays. This assay was based on reverse-transcribing a defined RNA template into cDNA with RT released from the putative retroviral particles, followed by PCR amplification. As the usual test samples included a vector that carries RT, it was necessary to amplify the replicating entity on a cell line while the vector RT decayed to zero. The amplifying cell line should be human, and the spike control could be any well-characterized retroviral vector that amplifies in the human cell line. The existing ATCC standard for gammaretroviruses (VR-1450) is one possibility. The assay appeared to easily detect one biologically active vector particle.

Rapid progress in designing and producing lentivectors has inevitably led to their use in clinical trials. One such HIV-1-based lentivector aimed at transducing T lymphocytes from HIV-1 infected patients with an anti-HIV anti-sense sequence was described by B. Dropulic (VIRxSYS Corporation, MD). The lentivector was generated by transfection of 293 cells with two plasmids, one (VIRPAC) containing all the necessary helper genes and the other the HIV-1 vector containing the anti-sense transgene, and optionally, a GFP marker gene. Very efficient transduction of CD4+ T lymphocytes was demonstrated using the lentivector with GFP gene VRX494 (VRX496 is the lentivector without the GFP gene for clinical application). A quantitative relationship between GFP expression and vector copy number per cell was demonstrated. VRX494 transduction led to strong inhibition of the X4 and R5 HIV-1 strains. It was found that VRX496 was selectively packaged into virions from CD4+ T cells infected with wild type HIV-1, but did not efficiently mobilize in uninfected CD4+ cells. RCL testing was carried out for the lentivector product and the "end of production" 293 cells. This test involved infection/cocultivation with MT4/C8166 cells and Taqman RT PCR analysis on supernatant using HIV-1 Gag and VSV-G primers after 6-12 passages of the indicator cells; attenuated HIV-1 (del Vif, Vpr, Vpu, and Nef) was used as positive control. RCL testing of VRX494/496-transduced HIV-1 infected cells was done similarly, except HT1080 cells were used as indicator/amplifier cells and the positive control was a VSV-G pseudotyped HIV vector. In all cases, lentivector and transduced cells were only released if RCL tests were negative; if positive, they would not have been released and the RCL would become isolated and characterized. No RCL has been detected to-date.

At the present stage of lentivector development, when all safety concerns, especially for *in vivo* applications, had not been fully resolved, it is pertinent to ask what sorts of studies should be done with lentivectors. This question, and others relating to the safe use of lentivectors, was addressed by A. Lever (University of Cambridge, UK). In his opinion, lentivectors should only be used where other known safe vectors did not, or would not be expected to work, and where transduction of a small number of cells may be effective, e.g. stem cells, direct *in vivo* neurological or liver gene transduction. Further, it was important to characterize lentivector lots with regards to particle numbers and infectious titer. Direct quantification of vector particles could be achieved using immunofluorescent tagging methods, although these did not distinguish between full and empty particles (p24 Gag alone can make particles). If possible, for quantification of infectious titer, vectors should be titrated in cell lines comparable to the target cells. The integration (copy) number of vector proviral DNA per cell should be estimated and, since integration may cause insertional mutagenesis,

an acceptable maximum copy number per cell established. This number could be based on figures derived from cells cultured *in vitro*. There is verbal evidence from a Cold Spring Harbor Symposium that 35% of 500 mapped HIV proviral DNA integrations *in vitro* fell into transcriptionally-active sites of nuclear DNA; this frequency was even higher for both HIV and HIV-based vectors *in vivo*, where preferential integration into gene-rich chromosomes could also be observed.

It is also debatable on which type of lentivirus one ought to base lentivector development and use. There are arguments for and against using lentivectors based on lentiviruses that infect humans. Since there is no clear answer on which of these vectors based on human, non-human primate, or non-primate lentiviruses are the safer, the issue of which is the most efficient lentivector becomes more prominent.

Review of group discussions and wrap-up

Following discussions among meeting participants, consensus was reached on the following points:

1. There is currently much diversity in vector type and design. Therefore, any specific reference materials only serve a few specific vectors.
2. Generic reference materials may be possible for determining physical particle numbers, infectivity, and replication-competent retroviruses. A reverse transcriptase (RT) reference standard is considered to be potentially useful since all retroviruses contain RT. However, an RT standard must be appropriate to the needs of vector producers; the nature and characterization of such a standard requires further consideration. PERT assays may be appropriate for both gammaretroviruses and lentiviruses.
3. Regarding specifically gammaretroviruses, the current amphotropic MuLV reference preparation is useful for monitoring the sensitivity of assays to detect RCR. With further characterization, it may also be useful for other purposes, e.g. RT assays. GALV-pseudotyped gammaretroviral vectors are increasingly used for transductions and a need for an appropriate reference material has been perceived. However, no conclusion has been reached on which type of GALV-pseudotyped virus or vector would be the most appropriate. The Question on how to proceed on GALV- and other viral envelope-pseudotyped gammaretroviruses has been left open for further discussion.
4. Specifically regarding lentiviruses, the consensus is that it is too early to identify and develop reference materials, since at this point in time many distinct primate and non-primate lentiviral vectors are in development. Manufacturers should, however, develop their own "in-house" reference materials according to their own specific needs. In the case of HIV-based lentiviral vectors, concern has been expressed about the safety of attenuated and/or pseudotyped HIV as a positive control for RCL tests. If possible, surrogate positive controls should be identified and developed.
5. Further discussions on reference materials leading to prioritization of the development of the most valuable reference materials for the purposes of characterization, standardization and safety assessment are important for the advancement of this field.
6. It was further agreed that two Action Groups, one for gammaretroviral vectors and one for lentivectors, should be formed to oversee future activities for

standardization and reference material development in these respective vectorology fields. Participants were encouraged to enlist in either or both of these Action Groups. F. Borellini and K. Cornetta will co-chair the lentivector Action Group. Chairpersons for the gammaretroviral group have yet to be identified.

The names and contact details of the members of the two Action Groups are listed at: <http://www.isbiotech.org/ReferenceMaterials/retlentupdate.html>.