Briefing Document — Testing for Replication Competent Retrovirus (RCR)/Lentivirus (RCL) in Retroviral and Lentiviral Vector Based Gene Therapy Products — Revisiting Current FDA Recommendations

INTRODUCTION

Vectors derived from retroviruses, such as MLV-based gammaretroviral vectors, have been used to introduce therapeutic genes into target cells in various clinical gene transfer applications (Hu and Pathak 2000). The stable integration of the vector provides the theoretical potential for long term therapeutic gene expression and may offer persistent beneficial clinical effects in treated patients. Lentiviral based vectors, such as HIV-1 based vectors, have also been used in clinical trials over the last few years. Lentiviral vectors are able to transduce non-dividing cells more efficiently and therefore may also be used for transduction of more quiescent or more differentiated cells or tissue types (Fassati 2006).

Retroviral vectors are engineered to be replication defective; however replication competent retroviruses (RCR) may be generated during manufacturing through homologous or non-homologous recombination between the transfer vector, packaging components and endogenous retroviral elements in producer cells (Chong *et al*. 1998; Garrett *et al*. 2000). RCR were in fact detected from early generation vector production systems (Miller 1990). As will be discussed later in this document, vector production systems have been improved to reduce the risk of RCR generation. Similarly, generation of replication competent lentivirus (RCL) is a possibility although there have been no reports of RCL occurrences to date.

Many gammaretroviruses are known to cause tumors in animals (Rosenberg and Jolicoeur 1997). After the reported observation that three of ten Rhesus monkeys developed lymphomas after transplantation with cells transduced with a gammaretroviral vector lot that was inadvertently contaminated with RCR (Donahue *et al*. 1992), FDA began requesting manufacturers and investigators to test the retroviral vector products at multiple stages in manufacturing to ensure absence of contaminating RCR and to monitor the treated patients for evidence of RCR.

FDA has made specific recommendations for the testing of replication competent retrovirus (RCR) used in retroviral vector based gene therapy products in the "Guidance for Industry—Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors". As described in the Guidance, RCR testing is performed throughout the manufacturing process and during follow-up

monitoring of patients in clinical trials using retroviral vectors. To ensure the final product is free of RCR, the RCR testing is performed at the following steps: master cell bank for vector producer cells, vector supernatants, end-of-production cells (EOPC), and final vector transduced cells that underwent cell expansion in culture media *ex vivo* for more than 4 days. FDA has additional recommendations for the testing of patient samples for possible RCRs after infusion of the vector or vector transduced cell product during clinical monitoring.

The RCR testing recommendations were established before lentiviral vectors were developed for clinical use, therefore do not directly address the issues of testing for replication competent lentivirus (RCL) in lentiviral vector products. Based on similar approaches of vector construction (due to similar viral genomic organization and biology of the viruses) and safety concerns, these recommendations are equally applicable to lentiviral based vectors and their transduced cell products (Pauwels *et al*. 2009). Therefore lentiviral vectors and transduced cell products must also be tested for and shown to be free of replication competent lentivirus (RCL) prior to be administered into clinical research subjects. As in the case of retroviral vectors, FDA also recommends testing for evidence of RCL occurrence during patient monitoring after infusion of the vector or the vector transduced cell products.

FDA recommends that the most sensitive available methods for RCR or RCL testing be utilized. The current method of choice for RCR/RCL testing is the cell culture based method in which test articles are co-cultured with a highly permissive cell line for amplification followed by endpoint detection of RCR/RCL specific components.

In the past ten years, there have been no reports of RCR or RCL positive results in samples of vector lots that were used for clinical studies. This may be due to the improvements in vector production systems designed to reduce the chance of RCR or RCL generation. Furthermore, FDA has not received any reports of positive results for RCR or RCL from samples of the cell products transduced with vector lots that passed RCR or RCL testing criteria. Finally, no positive RCR or RCL results from samples obtained from patients as part of monitoring after product infusion have been reported to FDA in clinical trials that involve the use of retroviral or lentiviral vectors.

Recently FDA has seen a large increase in the number of clinical trials using retroviral and lentiviral vectors. The majority of these trials utilize autologous patient cells that have been transduced and cultured for more than 4 days. Given the improvement in vector systems being used clinically and the supported by the fact that (1) RCR or RCL has not been reported in vector lots for several years, (2) no RCR or RCL has been reported to date in transduced patient cells or during patient monitoring, FDA is seeking the advisory committee's advice on (1) the need for a cell culture based RCR/RCL assay as a lot release criterion for retroviral or lentiviral vector transduced cell products while keeping the RCR/RCL testing at all other prior steps in the manufacturing process, and (2) additional modifications to other aspects of the current FDA recommendations for RCR/RCL testing and patient monitoring.

To facilitate the advisory committee discussion, analyses and deliberation of the recommendations, this briefing document provides the following overviews:

1. Manufacturing of retroviral or lentiviral vectors and vector transduced cell products
2. Factors that affect probability of RCR generation
3. Evolution of retroviral and lentiviral vector production systems
4. Possible pathogenic effects of RCR/RCL in humans
5. Current recommendations for RCR/RCL testing
6. Draft questions for advisory committee

MANUFACTURING OF RETROVIRAL OR LENTIVIRAL VECTORS AND VECTOR TRANSDUCED CELL PRODUCTS

Retroviral vectors typically are manufactured by use of mammalian cell lines (known as vector producer cell lines, VPCs) engineered to express retroviral helper sequences and retroviral vector sequences. The retroviral helper sequences encode retroviral structural and enzymatic proteins required to make vector particles. The retroviral vector sequences have deletions in retroviral protein coding sequences, while retaining the *cis*-acting elements that are required for packaging the vector RNA into the vector, reverse transcription of RNA into DNA, integration of retroviral DNA into the host cell genome, and transcription of viral mRNA. VPCs produce vector particles that are structurally similar to a retrovirus, but lack the genetic sequences required to make progeny virions, therefore the vectors are replication-defective.

The precursor to the VPC are packaging cell lines designed to synthesize all retroviral proteins required for producing functional retroviral vector particles, but do not include the vector genome. A master cell bank (MCB) is usually established and certified for a packaging cell line. To prepare a vector producer cell line, a transfer vector is introduced into the packaging cell line by either transfection of a plasmid encoding the vector genome or transduction of another retroviral vector containing the desired vector genome. High vector titer clones are selected and characterized. The VPC master cell banks for these high titer clones are then established and certified. The certification of these master cell banks includes an extensive battery of safety testing. Relevant to this discussion, FDA recommends that the MCB be screened using a sensitive cell culture-based RCR detection assay. A working cell bank (WCB) may be established and tested before the vector production step. Less extensive testing is required for a WCB. To manufacture a retroviral vector lot, seed vials from either a working cell bank or a master cell bank are expanded in culture media under appropriate conditions. The batches of supernatant that contain the vector particles are harvested over several days, as determined based on preliminary experiments that demonstrate the period of greatest vector yield. In some cases, vector containing supernatant may be subjected to limited purification steps to remove cell debris. The bulk harvest supernatant is tested for RCR as part of lot release testing.

Alternatively, vector may be produced by transient production methods that require co-transfection of plasmids that encode the vector genome and packaging constructs into a

host cell. Transient production has been primarily used to produce lentiviral vectors since packaging cell lines for lentiviral vectors are not widely available in part due to the cytotoxic effects of certain packaging components (HIV gag and VSV-G); although the transient production method has also been applied to gammaretroviral vectors. This method bypasses the need for VPC. However, the cells used for the transfection of the plasmids are typically expanded and characterized in a manner similar to those tests used for a master cell bank and a working cell bank of a VPC. The resultant vector preparations have high levels of contaminating plasmid DNAs used during the transfection. Therefore, vectors produced transiently often undergo additional steps to remove the plasmid DNAs, such as DNase digestion followed by subsequent purification steps.

While retroviral or lentiviral vectors may be directly administered into patients as a final product, most clinical applications involve *ex vivo* transduction of patient (autologous) or allogeneic target cells that are subsequently infused into a patient. Transduction is typically accomplished by incubation of the target cells with vector supernatant under appropriate conditions. In these applications the transduced patient cells are the final product and subjected to additional testing including RCR/RCL testing.

FACTORS THAT IMPACT PROBABILITY OF RCR GENERATION

Despite the fact that retroviral vectors are designed to be replication defective, replication competent retrovirus (RCR) may result if recombination occurs between related retroviral sequences present in the vector producer cells in a manner that generates a single viral genome fully constituted with all sequences necessary for viral replication. Several key factors influence the frequency of recombination events, and have been taken into considerations in design of safer vector production systems. These factors are described below.

1. Reduction of homology between vector and helper sequences.

   Homologous recombination of retroviruses occurs at a frequency at least 1000-fold greater than non-homologous recombination (Zhang and Temin 1993). Therefore, vector producer systems have been designed with reduced lengths of homologous sequences between different elements present in the cells. Helper sequences may use heterologous promoters or poly-adenylation sequences in lieu of corresponding retroviral elements (long terminal repeats, or LTRs) to reduce sequence homology.

2. Reduction in homology between vector/helper sequences and cellular DNA.

   Sequences endogenous to the cells being used may also contribute to the extent of homologous sequences. Murine cells carry endogenous retroviral sequences with homology to the MLV-based gammaretroviral vectors. Recombination between the introduced exogenous retroviral vector sequences and the endogenous retroviral sequences may give rise to RCR. For example, such recombinant retroviruses were reported in monkeys that developed lymphomas and died after exposure to RCR-

contaminated retroviral vector (Purcell *et al*. 1996). In more recently developed VPCs, cells of a different species (such as human) have been used to avoid the potential for homologous endogenous retroviral sequences that could serve as a substrate for recombination (Cosset *et al*.1995; Forestell *et al*. 1997; Sheridan *et al*. 2000; Ghani *et al*. 2007).

3. Division of helper sequences to more than one expression cassette to increase the number of recombination events required to generate RCR.

   Early vector producer cell systems used a single expression cassette for all the retroviral helper functions. In this case, RCR generation may result after a single recombination event. Later vector producer cell systems divided the helper functions into at least two expression cassettes. Therefore, a minimum of two recombination events would be required to generate RCR, reducing the likelihood of generating RCR.

4. Other changes in the vector sequences.

   Additional design elements include changes in the vector sequences, such as introduction of stop codons into an open reading frame. If recombination events occur with this type of vector, retroviral proteins can not be expressed, preventing generation of RCR.

EVOLUTION OF RETROVIRAL AND LENTIVIRAL VECTOR PRODUCTION SYSTEMS

An early version of retroviral vector production system was created by only deleting the viral packaging sequences (Mann *et al*. 1983). The viral structural genes that provide packaging functions were on a single expression unit making it relatively easy to recombine with the transfer vector backbone and thus were prone to RCR generations.

Improvements were made to reduce the chance of generating RCR over the years. The PA317 packaging cell line was derived from a virus genome from which the packaging signal was deleted and the 3' LTR was replaced with a polyadenylation site from SV40 and the 5' end of the 5' LTR was deleted (Miller and Buttimore 1986). In doing so, many signals required for reverse transcription and integration were missing, thus preventing RCR generation even if the viral RNA genome was co-packaged. This vector production system was used in the first human gene transfer experiments (Rosenberg *et al*. 1990; Blaese *et al*. 1995)

Because of its propensity to generate RCR (Donahue *et al*. 1992), the PA317 cell line was superseded by the murine GP + envAm12 system (Markowitz *et al*. 1988a). By segregating the *Gag-Pol* and *Env* coding regions onto separate plasmids and by minimizing the homology between vector and packaging sequences, the RCR incidence was reduced substantially, but not eliminated (Danos and Mulligan 1988; Markowitz *et al*. 1988a; 1988b).

PG13 (Miller *et al*. 1991) split packaging cell lines were generated with Gibbon Ape Leukemia Virus (GALV) envelope-pseudotyped gammaretroviral vector particles. GALV envelope pseudotyping may prevent re-infection of vector particles and thus are expected to reduce the chance of recombination.

More advanced packaging cell lines for clinical gammaretroviral vectors were developed with human cells (Cosset *et al*.1995; Forestell *et al*. 1997; Sheridan *et al*. 2000; Ghani *et al*. 2007). The human genome does not contain DNA sequences that are homologous to retroviral sequences and therefore reduces the chance of RCR generation.

Lentiviruses have a more complex genomic structure that contains regulatory and accessory genes in addition to *gag-pol* and *env*. HIV-1 possesses regulatory functions encoded by the *tat* and *rev* genes as well as accessory genes that include *vif*, *vpr*, *vpu*, and *nef*. The accessory genes of HIV-1 are not required for virus replication in appropriate cell culture systems *in vitro*. Therefore they are typically not included in vector production systems. Because the accessory genes play a role in viral pathogenesis *in vivo*, the lack of these genes in HIV-1 based vector production systems may increase the margin of safety in terms of generation of pathogenic RCL. Lentiviral vector particles are typically generated by co-expressing the vector sequences and the viral *Gag-Pol* and *Env* coding region using separate plasmids by transient transfection.

Stable packaging cell lines for lentiviral vectors are not readily available. For lentiviral vectors, transient transfection systems with three or four plasmids are usually employed for vector production (Dull *et al*. 1998; Naldini *et al*. 1996). Vesicular stomatitis virus glycoprotein (VSV-G) is frequently used as the choice of envelope for HIV-1 based lentiviral vector due to its broad tropism and sturdiness during purification process. So far replication-competent lentivirus (RCL) has not been reported with the commonly used lentiviral production systems that lack accessory protein functions (Sastry and Cornetta 2009).

Several vector systems have been used to produce retroviral and lentiviral vectors for clinical studies and are listed in Table 1 below. The list is not exhaustive and only contains the vector systems widely used in clinical studies. RCR have been observed in early generations of gammaretroviral vector production systems (PA317, AM-12, Psi-CRIP). The findings of RCR in certain vector production systems were discussed in an FDA advisory committee meeting (BRMAC, 2001). FDA asked the committee if the FDA should not allow certain vector production systems to be used clinically. The committee recommended that FDA should evaluate each vector production system on a case-by-case basis.

Table 1. Summary of Vector Production System Characteristics and RCR/RCL Generation

Vector Production System	Cell Line Origin	Design of Helper Sequences	Pseudotyping Envelope	RCR/RCL Observation
PA317	Murine	One Expression Unit, 5' LTR, heterologous polyA	Amphotropic murine leukemia virus	Yes*

AM-12	Murine	Two Expression Units, 5' LTR, heterologous polyA	Amphotropic murine leukemia virus	Yes*
Psi-CRIP	Murine	Two Expression Units, 5' LTR, heterologous polyA	Amphotropic murine leukemia virus	Yes*
PG13	Murine	Two Expression Units, 5' LTR, heterologous polyA	Gibbon ape leukemia virus	No
Other gammaretroviral vectors	Human	Two Expression Units	Amphotropic murine leukemia virus	No
HIV-1 based lentiviral vectors	Human	3 or 4 plasmid transient transfection	VSV-G	No

*It is worth noting that to date no RCR or RCL incidences have been reported for patient cell lots transduced with retroviral or lentiviral vectors in clinical studies. In each of the cases reported, the RCR was only observed in the production lot, not in the Master Cell Bank.

POSSIBLE PATHOGENIC EFFECTS OF RCR OR RCL IN HUMANS

There are no established known diseases in humans caused by natural infection of gammaretroviruses. Early studies showed that four Cynomolgus monkeys showed no evidence of pathology or retroviremia during a 2- to 3-year follow-up after infusion of bone marrow cells transduced with a retroviral vector preparation that contained amphotropic replication-competent retrovirus that arose through recombination between vector and packaging components in VPC cells (Kantoff *et al*. 1987; Cornetta *et al*. 1991). However, another study showed that 3 out of 10 Rhesus monkeys died of lymphomas at around 6 months after transplantation of vector transduced bone marrow cells contaminated with replication-competent virus (Donahue *et al*. 1992). The difference in results between these two studies may be explained by the fact that the three animals that developed lymphomas were conditioned with lethal irradiation and a T-cell depleted marrow graft. This demonstrates that recombinant amphotropic retroviruses that arise in packaging cells can cause disease in primates under conditions of severe immune deficiency.

It has also been shown that insertions of replication defective retroviral vectors into the host genome in the absence of RCR caused clonal outgrowth that led to leukemogenesis in X-linked SCID clinical studies (Hacein-Bey-Abina *et al.* 2008 and Howe *et al.* 2008). In an X-linked chronic granulomatous disease (X-CGD) trial, retroviral vector insertions caused over expression of an oncogene, *EVI1*, which eventually led to genomic instability and clonal progression to myelodysplasia (Stein *et al.* 2010).

More recent detection of novel MLV-like viruses or MXRVs in patient samples with chronic fatigue syndrome (CFS) and human prostate cancer raised concerns that RCR may have similar effects on humans (Urisman *et al*. 2006; Fischer *et al*. 2008; Lo *et al*. 2010). It should be noted that the causality has not been firmly established.

As is well known, lentiviruses cause diseases in their respective hosts. HIV-1/2 causes AIDS in humans by infecting and destroying the critical CD4+ T lymphocytes. Equine Infectious Anemia Virus (EIAV) causes recurring febrile episodes with associating viremia, fever, thrombocytopenia, and wasting symptoms in horses. Feline Immunodeficiency Virus (FIV) is the etiology of the feline immunodeficiency syndrome. Field isolates of Bovine Immunodeficiency Virus (BIV) can cause transient viremia and leukocytosis with no apparent clinical persistence possibly due to effective host immune response. Natural lentivirus infections are limited to its respective species due to viral binding receptor and other host restrictive factors. However, lentiviral vectors are engineered to specifically infect human cells by changing the envelope (pseudotyping). Therefore potential RCL risk derived from pseudotyping remains and lentiviral vector products must be demonstrated to be free of RCL for clinical applications.

CURRENT REGULATORY RECOMENDATIONS FOR RCR/RCL TESTING

Introduction and Background

Despite the fact that MLVs can induce neoplastic transformation in mice under certain conditions (Chatis *et al*. 1983; Gardner 1978; Nazarov *et al*. 1994), early studies in nonhuman primates suggested that murine RCRs present in retroviral vector lots did not present serious safety concerns in non-rodent species (Cornetta *et al*. 1990; Cornetta *et al*. 1991).

However, as mentioned previously in this document, in an experiment using severely immunosuppressed Rhesus monkeys, administration of *ex vivo*-transduced bone marrow progenitor cells that had been exposed to a retroviral vector preparation contaminated with RCR, 3 of 10 animals developed lymphomas and died within 200 days (Donahue *et al*. 1992). The lymphomas were attributed to the RCR because the RCR, but not the vector, was later found in the tumor cells and RCR mediated retroviremia was detected in the affected animals (Purcell *et al*. 1996; Vanin *et al*. 1994).

This precedent-setting event prompted extensive public discussions which led to FDA recommendations for RCR testing for retroviral vector products in 1993 (Gunter *et al.* 1993). These recommendations, endorsed by the Vaccines and Related Biological Products Advisory Committee (October 25, 1993), called for extensive RCR testing at multiple time points during retroviral vector production, in addition to testing of *ex vivo* transduced cells. To mitigate the risk to patients in clinical studies and increase our knowledge base regarding long lasting safety concerns in the event of patient exposure to RCR, FDA also asked sponsors to monitor patients for the presence of RCR (see "Letter to Sponsors of INDs using Retroviral Vectors"—9/20/93).

Over the next few years following the 1993 discussions, experience with different vector production systems, RCR detection assays and results from patient monitoring, resulted in an accumulated experience and information on the safe use of retroviral vectors in clinical applications of gene therapy. This information base formed a framework for

further discussion of the RCR recommendations by the FDA and the gene therapy community. At the 1996 and 1997 FDA/NIH Gene Therapy Conferences, public discussion and development of these supplemental recommendations took place during the Retroviral Breakout Sessions with representatives of the gene therapy community. The results of the discussions were published (Wilson *et al*. 1997) and provided the framework for the current FDA recommendations on RCR testing (Guidance for Industry—Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors).

The current RCR testing recommendations were developed primarily based on the experience with gammaretroviral vectors (MLV based vectors) which have been used in most retroviral vector based clinical trials. However, the principle may be applied to other retroviral vectors including lentiviral vectors with careful considerations for special issues with each vector class. Lentivirus is a genus of Retroviridae family and has similar general characteristics with that of gammaretrovirus' in terms of genomic structure and viral life cycle. Since 1996, lentiviral vectors have been developed and used for clinical applications. Examples include HIV-1 and EIAV based lentiviral vectors which offer some unique properties such as the ability to transduce non-dividing cells and may have more prolonged expression of the therapeutic transgene than MLV based vectors. Special safety issues associated with lentiviral vectors, HIV-1 based lentiviral vectors in particular, were discussed extensively at an advisory committee meeting (BRMAC) in 2001. Although FDA does not yet have a specific guidance for lentiviral vectors *per se*, the existing guidance documents governing the retroviral vectors and other gene therapy products also apply to lentiviral vectors. FDA collaborated with industry and academia and addressed the specific issues related to RCL testing during the 6th annual American Society for Gene Therapy (ASGT) meeting in 2003. This effort resulted in a publication outlining the recommendations for developing assays and reference materials for detecting replication-competent lentivirus in production lots of lentiviral vectors. (Kiermer *et al*. 2005)

RCR Assay Method

A cell culture based assay is recommended for RCR testing performed throughout the retroviral product manufacturing process. This assay allows for *in vitro* amplification followed by a sensitive endpoint detection assay.

The two types of cell culture based assays most commonly used to detect RCR are the S^+/L^- assay and the marker rescue assay (Sastry and Cornetta 2009). In these assays, test samples are co-cultured with a permissive cell line for at least 3 weeks (amplification phase).

In the S^+/L^- assay, RCR is detected using indicator cell lines, such as the cat cell line PG-4 (Haapala *et al*. 1985). The PG-4 cell line is referred to as a S^+/L^- cell line, as it contains the murine sarcoma virus genome (S^+) but lacks the murine leukemia virus genome (L^-). Cells that express the murine sarcoma virus induce a transformed phenotype but only in

cells co-expressing a murine leukemia virus. In this assay, cell culture supernatant aliquots collected during the amplification phase are placed on PG-4 cells and transformation of cells indicates the presence of RCR. While *Mus dunni* cells are suitable for amplification of RCR with a murine amphotropic envelope, they are not permissible for GALV envelope. To address this, 293 cells are substituted for *Mus dunni* during the amplification phase.

In the marker rescue assay, the permissive cell line contains a retroviral vector with an easily identifiable marker transgene (Danos and Mulligan 1988). After three-week incubation to amplify any potential RCR, the cell supernatant is collected. If RCR is present, it will package both the RCR genome and "rescue" the vector containing the marker transgene. Upon transduction of a naïve cell line a positive RCR will result in the marker gene being expressed.

Appropriate positive and negative controls are included in the assay system. The known concentration of the positive control virus is used to calibrate the assay sensitivity which can detect one viable RCR in a certain volume of test sample with a 95% confidence interval.

Although highly sensitive, the cell culture based RCR or RCL assays are time consuming and labor intensive. It takes up to six weeks to complete the assay; and is challenging for vector transduced cells that cannot be cryopreserved. For this reason, a PCR based rapid alternative RCR or RCL assay may be used as a lot release test for the transduced cell product (Martineau *et al*. 1997); but a cell culture based RCR or RCL assay is initiated at the same time for the purpose of retrospective confirmation. Particular care must be taken when performing a PCR shortly after vector exposure because contaminating plasmids carried over from transient production methods or DNA from producer cell lines could yield a false positive result suggesting the presence of RCR (Chen *et al*. 2001).

RCL Assay Method

To maximize the assay sensitivity, a cell culture based RCL assay with an appropriate permissive cell line is used to allow viral amplification and end point detection. In a typical assay for detecting RCL associated with HIV-1-based vectors, vector aliquots were used to inoculate a human T-cell line (C8166) that is permissive for infection and growth of HIV-1 (Escarpe *et al*. 2003; Sastry *et al*. 2003). The cells were cultured for 21 days to amplify any potential RCL. An attenuated HIV-1 virus lacking the accessory protein-encoding genes was used as a positive control (Escarpe *et al*. 2003). The medium from amplified cells was then used to infect naïve C8166 indicator cells, which were cultured for an additional seven days and analyzed for the presence of viral proteins or nucleic acids. To detect RCL, sensitive end-point assays have been developed including an ELISA-based p24 Gag antigen assay (Mochizuki *et al*. 1998), a product enhanced reverse transcriptase (PERT) assay that involves the vector's reverse transcriptase (Miskin *et al*. 2006; Sastry *et al*. 2005), a PCR-based assay that detects Psi-Gag sequences from a recombination event between vector and packaging constructs, and PCR-based assays to detect the VSV-G Env used for pseudotyping (Sastry *et al*. 2003).

Current Recommendations for RCR Testing

FDA currently recommends testing for RCR at multiple stages throughout the product manufacturing process. Figure 1 depicts the recommendations for RCR testing and patient monitoring. Although not mentioned in these recommendations, FDA is currently applying the same principles to RCL testing and monitoring for lentiviral vector products where applicable.

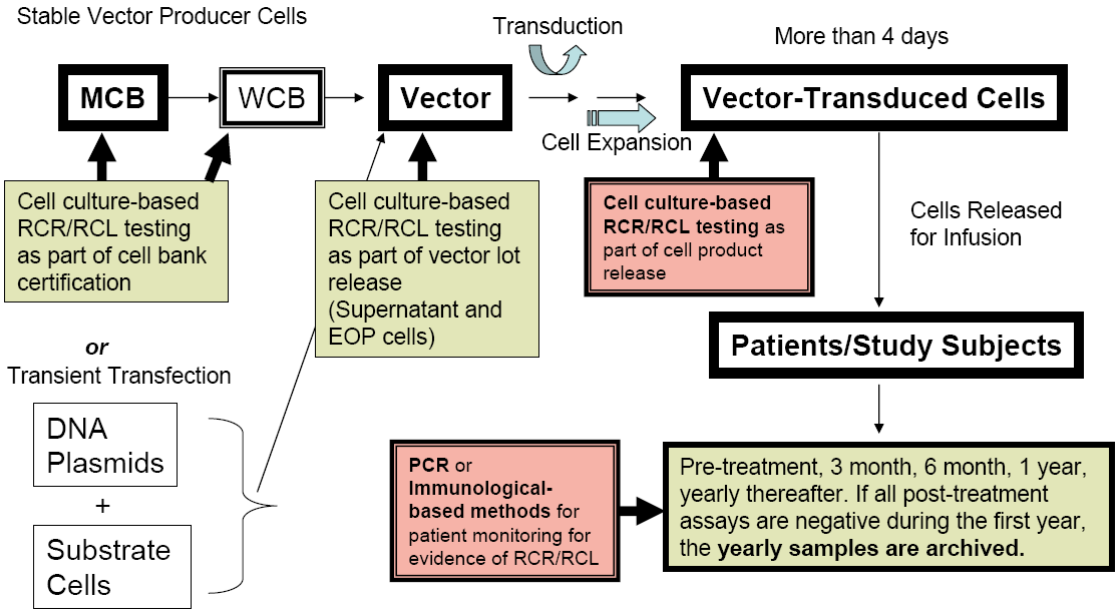

Figure 1. RCR/RCL Testing Schedule for Retroviral or Lentiviral Vector Transduced Cell Products

RCRs are tested for in the Master Cell Bank (MCB) (1 time), Working Cell Bank (WCB) (1 time), vector supernatant, end-of-production cells (EOPs) (1 time), and vector transduced cells (if expanded *ex vivo* for more than 4 days post transduction). A cell based co-culture RCR test method is used with a known positive control. The assay sensitivity and specificity must be demonstrated.

In addition, an immunological or PCR based method with known sensitivity and specificity is used to monitor the presence of RCR in samples obtained from treated patients after infusion of the vector products.

The specific recommendations are outlined in the following sections:

RCR Testing during MCB Certification of Vector producer Cells

Both VPC and supernatant from production of a MCB are tested for RCR using a cell line permissive for the RCR most likely to be generated in a given producer cell line.

If derivation of VPC includes use of a retroviral vector containing an envelope distinct from the packaging vector, for example, an ecotropic MLV, testing of the MCB for the

presence of ecotropic RCR is recommended. Both cells and supernatants are tested using a method validated to detect the appropriate positive control.

Working Cell Bank Testing (one time testing)

Either supernatant testing or cocultivation of cells for RCR is recommended.

Vector Supernatant Lot Release Testing

Both retroviral vector supernatant lots and end of production cells (EOPC) (one time) are tested for RCR.

Testing of *Ex Vivo* Transduced Cells

a. For Cells Cultured < 4 days after transduction

For *ex vivo* transduced cells cultured for a period less than four days, active RCR testing is not required, but archiving of the quantity of product needed to perform RCR testing is recommended.

b. For Cells Cultured > 4 days

When *ex vivo* transduced cells are in culture for a period of time greater than or equal to 4 days from the start of transduction, cells and the appropriate volume of culture supernatant are tested for RCR. In situations where *ex vivo* transduced cells cannot be cryopreserved during testing, and must be administered to patients prior to the availability of testing results, the culture assays should be initiated at the time of patient administration. An alternative rapid method such as PCR may be appropriate to provide an initial analysis.

Clinical Monitoring for Evidence of RCR/RCL

a. Testing Schedule

The patient monitoring schedule includes analysis of patient samples at the following time points: pre-treatment, 3 months, 6 months, 1 year after treatment and yearly thereafter. If all post-treatment assays are negative during the first year, the subsequent annual samples are archived. If any post-treatment samples are positive, further analysis of the RCR and more extensive patient follow-up should be undertaken.

b. Recommended Assays

Two methods are currently in use and recommended for detecting evidence of RCR infection in patients: 1) detection of RCR-specific antibodies; and 2) analysis of patient peripheral blood mononuclear cells by polymerase chain reaction (PCR) for RCR-specific DNA sequences. The choice of assay may depend on the mode of vector

administration and the clinical indication. All positive results are further pursued by direct culture assay to obtain and characterize the infectious viral isolate.

Guidance for Long-Term Follow-UP

It is worth noting that FDA has a separate guidance document for observation of delayed adverse events in gene therapy clinical trials (Guidance for Industry Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events). It specifically addresses the issues of delayed adverse events that may be caused by gene transfer vectors because of its persistence in treated patients. The guidance calls for documentation of observations of delayed clinical adverse events (malignancies, hematological, neurological, and rheumatologic or autoimmune disorders) at appropriate intervals for 15 years post treatment. Adverse experiences associated with the use of the product are reported as IND Safety Reports.

In particular for integrating vectors (e.g. retroviral and lentiviral vectors), vector persistence is monitored at intervals of no greater than six months for the first five years and then no greater than yearly for the next ten years, or until such time that no vector sequences are detectable. If more than 1% of cells in the test samples are tested positive for vector sequences, the pattern of vector integration sites are analyzed. Furthermore, if the integration pattern analysis suggests a predominant clone, the specific integration locations on the host chromosome are determined. Any positive results of predominant clones are reported to FDA in the form of information amendment within 30 days.

DRAFT QUESTIONS FOR ADVISORY COMMITTEE

1. Based on the current vector systems being used for clinical production and supported by the data presented at this meeting regarding RCR/RCL testing results, please discuss the need for a cell culture based RCR/RCL assay as a lot release criterion for retroviral or lentiviral vector transduced cell products while keeping the RCR/RCL testing at all other prior steps in the manufacturing process.

   Please consider each of the following in your discussions:

   a. Different vector designs and production systems may have different risk profiles with respect to RCR/RCL generation;
   b. Accumulated experience with gammaretroviral vector vs. less extensive experience with lentiviral vectors;
   c. Alternatives to the cell culture based RCR/RCL assay for the transduced cell products:
      i. PCR based RCR/RCL as a lot release test when feasible;
      ii. Qualify the cell manufacturing process by testing the first few patient cell lots manufactured;
      iii. Qualify the cell manufacturing process and demonstrate the process will not amplify potential RCR/RCL with spiking experiments;
      iv. Other possibilities…

2. Do you recommend additional modifications to other aspects of the current FDA recommendations for RCR/RCL testing or patient monitoring?

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