Molecular Therapy
Methods & Clinical Development
Review

Risks of replication-competent retro/lentivirus from associated vector systems: Is it time for a roadmap toward reduced testing?

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The development of retroviral and lentiviral vectors (RV/LVs) has culminated in third-generation vector systems with excellent safety profiles. However, there has been no significant (re) assessment of RCR/RCL formation risk within RV/LV systems regarding the underlying molecular recombination events, despite updated guidance for RCR/RCL assay development/testing issued by the key regulatory agencies. This review reflects on genuine RCRs generated in early vector systems that drove the development of the common safety features of all third-generation systems. We then present a "minimal-path" model for RCL formation within four-component LV systems, which draws on published recombination rates between plasmid DNA in cell lines and retrovirus genomic RNA during reverse transcription. This model indicates that the probability of generating a single RCL genome molecule at 200-L scale is ≤ 1 in 10,000 per bioreactor and is reduced by 100-fold if packaging cell lines containing integrated vector components are used. We propose that the improbability of generating RCR/RCL within contemporary vector systems justifies consideration of a roadmap to reduced testing and provide potential aspects of such a roadmap for the field to contemplate. We also review past and current guidelines for RCL testing and assay development, recommending key aspects of method development toward unification in the field.

INTRODUCTION

To date, viral vectors derived from retroviruses remain the most well-characterized and efficient means for the delivery and long-term expression of large transgene payloads to human cells for therapeutic effect.[1] This is principally due to the remarkable information "compression" encoded by their genomes. This allows them to replicate, assemble, and importantly protect their genetic cargo within the extracellular milieu through cell entry, cytoplasmic and nuclear trafficking (inside the capsid), and to the point of integration into the host cell genome (inside the pre-integration complex).[2] These contemporary viral vector systems share all these fundamental aspects of retroviruses, with the important distinction; they are replication-incompetent, which is a critical safety aspect. Indeed, attaining replication-incompetence was arguably the single most important driver in the evolution of vector design, as summarized in this review. Division of vector components onto separate cassettes, reduction of shared homology between cassettes, and the use of more favorable cell lines have drastically reduced the probability of adverse recombination events that might lead to a replication-competent virus (RCV). This has culminated in third-generation vector systems used to develop products where (as of the time of writing) not a single incidence of RCV has been reported. The assays used to detect the presence of putative RCVs have been borne out of non-binding guidance provided by the regulatory agencies beginning in the 1990s. The non-binding nature of these guidelines has pros and cons. They provide a welcome degree of flexibility in the development of assays that are ultimately attempting to detect theoretical entities at theoretical concentrations within product-specific vector material. Conversely, while there is a substantial amount of overlap between guidance from different regulatory jurisdictions (see later references), the interpretation of certain aspects of the guidance is leading to a degree of drift and misunderstanding by stakeholders within the industry (the authors' professional experience). As retroviral vector (RV) design becomes more complex in the coming age of targeted, *in vivo* gene delivery,[3–6] we believe clarity and unification within RCV testing will benefit the successful development of these potentially transformative medicines, thereby increasing the speed to patient access. Despite the excellent safety data regarding lack of RCV formation over 25 years of use of these third-generation systems, only relatively minor changes have been made to these guidelines. RCV assay conception, development, qualification, and implementation for any given vector product represent a substantial amount of resources within the scope of clinical supply. Our review provides the historical background of RV development, highlighting their common molecular features that form the basis for their observed

https://doi.org/10.1016/j.omtm.2025.101601.

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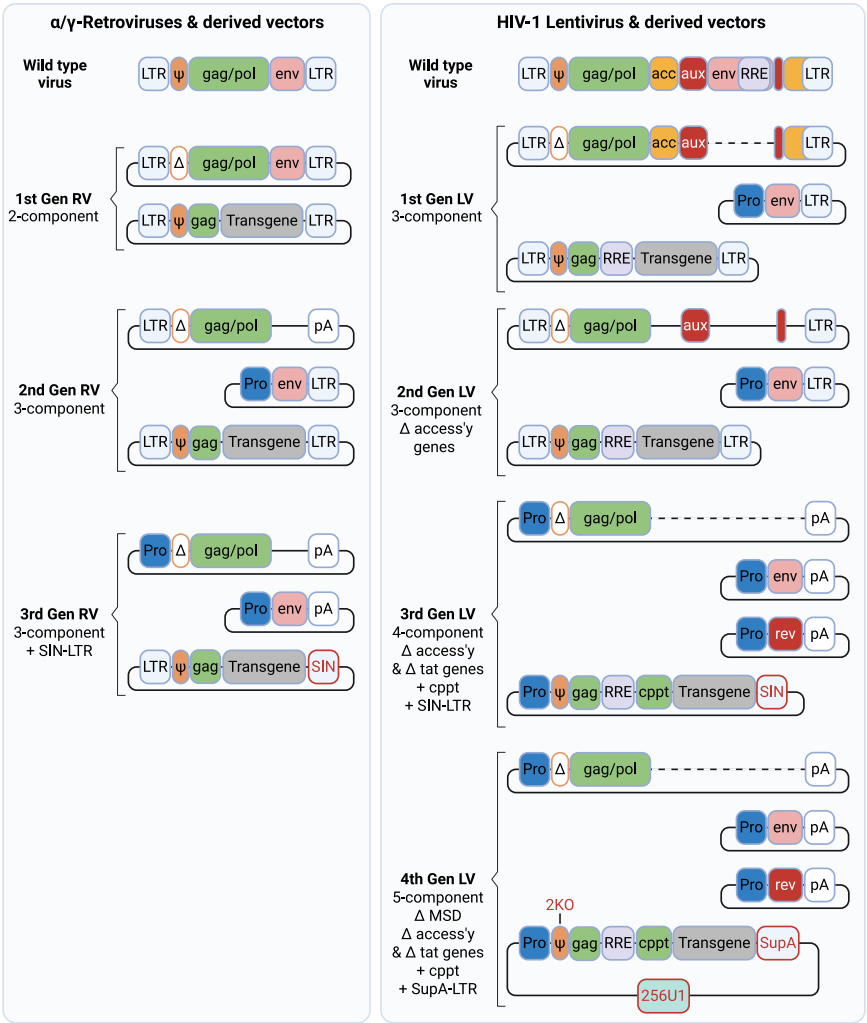

Figure 1. Evolution of components of retroviral and lentiviral systems over multiple generations

Retroviral vectors are typically based on simple alpha-/gamma-retroviruses, whereas lentiviral vectors are mainly derived from the more complex lentiviruses such as HIV-1. HIV-1 replication and infection is dependent on auxiliary genes (tat/rev) in cell lines and additionally auxiliary genes (vif, vpu, vpr, and nef) in primary cells. The general progression of development from early generation vector systems includes: [1] the removal of the packaging signal from packaging component(s); [2] separation of components onto separate constructs; [3] the minimization of homology between constructs; [4] use of heterologous promoters/UTRs/polyAs; [5] removal of tat and accessory genes (LVs); [6] implementation of SIN-LTRs and retention of the central polypurine tract (cppt; LVs). New LV systems using five components include use of two separate cell entry genes (a fusogen and a targeting molecule) or "SupA2KO-LVs" that have been mutated in the major splice donor and are dependent on co-expression of a U1 snRNA-based enhancer (256U1). SupA-LTRs have been engineered to enhance transcriptional insulation of integrated LVs in target cells, thus reducing the likelihood of vgRNA mobilization and potential interaction with hERVs. Created in https://BioRender.com.

safety. Using the known biological pathways that would be needed to generate an RCV, we present a mathematical model to infer that RCV formation during manufacturing is extremely unlikely. We believe this model justifies the collective drafting of a road-map toward reduced testing within the industry, for which we have made a set of tentative suggestions as a starting point. We also provide additional advice from our own and others' experience in RCV assay design and implementation.

A BRIEF HISTORY OF RETROVIRAL AND LENTIVIRAL VECTOR PRODUCTION SYSTEMS

Taming retroviruses

There exists a diversity of vector systems derived from the *Retroviridae* family, but for simplicity we shall focus on the two main systems in use. The development of γ- RVs for the delivery of transgenes to mammalian cells began in the mid-1980s, principally driven by the Verma, Friedmann, Mulligan, Anderson, and Temin labs.[7] Lentiviral vectors (LVs) based on HIV-1 were incorporated into that effort in the mid-1990s mainly by the Verma/Naldini/Trono and Kingsman labs, culminating in the third-generation RV and LV systems by the year 2000.[8,9] The third-generation systems remain in use as part of licensed products since 2017.[10–12]

The minimal functional elements of these replication-incompetent vectors are as follows:

(1) The packaged vector genomic (vg)RNA encoding the transgene and viral *cis*-elements.
(2) The gag/pol gene to provide structural and enzymatic proteins necessary to package, reverse-transcribe, and integrate the vector genome.
(3) An envelope/spike protein to allow entry into target cell.

Figure 1 describes the general transition from first- to third-generation RV/LVs and is overwhelmingly a story of improving safety of these integrating vectors. Early-generation RVs suffered from homologous recombination events between vector components during production, leading to formation of replication-competent retrovirus (RCR), discussed later. The main solution to this was to separate components to different DNA expression plasmids and reduce their sequence homology.

Lentiviral vectors: Slower to tame

The drive to develop LVs was mainly due to the ability of lentiviruses to infect non-dividing cells. Subsequent discovery of the subtle

difference in LV integration site preference also provides reduced risk of insertional mutagenesis. Engineering of LVs was made more difficult since lentivirus genomes like HIV-1 are complicated, harboring multiple accessory factors that modulate productive infection and the fundamental requirement for the auxiliary genes tat and rev. Initially, it was not clear if and to what extent the ability to transduce non-dividing cells was due to the auxiliary and/or accessory genes that are inherent to lentiviruses. The ability to generate a safe LV system in the knowledge that tat and some accessory factors were associated with certain pathogenic maladies (e.g., tat/Kaposi sarcoma) was in tension. The deletion of HIV-1 accessory genes from LV systems was found to have little impact on output titers, although this was primarily due to use of the HEK293(T) cell line for production. In hindsight, it was discovered in the 2000s that the vif, vpu, vpr, and nef accessory genes function to antagonize viral restriction factors.[13–18] The reason why these restriction factors are not substantially expressed in HEK293(T)-based cell was only empirically demonstrated in 2019.[19] A significant step in LV development was the removal of the auxiliary gene tat, which acts to upregulate the native U3 promoter encoded within the LTR by recruiting transcription elongation factors to the emergent genomic RNA transcript. Removal of tat from the third-generation LV platform is an important safety feature since it impacts on many cellular functions.[20,21] This was achieved by replacing the 5′ U3 promoter with powerful constitutive promoters such as the cytomegalovirus (CMV) promoter.

Another defining feature of third-generation RV/LV systems is the self-inactivating (SIN)-LTR, in which the U3 promoter is deleted from the 3′ LTR. This eliminates the potential of the viral enhancer-promoter sequences within LTRs to transactivate proto-oncogenes laying close to integration sites or distally via chromatin looping.[22] Additionally, mobilization of a putative RCV genome after the first round of infection is made impossible unless the SIN-ΔU3 region somehow "re"-acquires promoter activity from the production system. The initial concerns of wholesale etiological insertional mutagenesis by RVs/LVs—since their presence has the potential to affect every host cell gene into which they integrate—have not been realized at the typical achievable vector copy number per cell required to mediate therapeutic effect. This is partly due to the remarkable redundancy of gene/metabolic pathways and the fact that there are two copies of each gene in the human genome. If this was not the case, gene therapy mediated by RVs/LVs simply would not be operable today. However, the potential mobilization of an RCV might lead to greater genotoxic potential, even if the RCV infection was asymptomatic. The lack of mobilization of SIN vectors in patient cells also means that potential co-packaging/recombination with endogenous retroviruses (ERVs) or with HIV-1 in infected patient cells is greatly minimized.

For HIV-1 LVs, the auxiliary gene rev is required to export/stabilize both viral genomic RNA (vgRNA) and gag/pol mRNA during production, via binding to the rev-response element (RRE) encoded within both cassettes. Therefore, the RRE and partial gag sequence of the vgRNA packaging region (ψ-gag; typically ∼350 bases) remain significant regions of homology between genome and gag/pol cassettes. This can be eliminated by use of codon-optimized gag/pol cassettes, which produce rev-independent gag/pol mRNAs (deleted for RRE) and contain no homology (<10 bases) with the ψ-gag region of vgRNA.[23] It has been shown that "pre-RCL" recombinants are essentially eliminated when using codon-optimized gag/pol compared to wild type.[24]

The early success of RV development, particularly regarding production titers, owes principally to the relative simplicity of retroviral genomes, which unlike lentiviruses, lack dependency on auxiliary or accessory genes in their life cycles. That said, it was also discovered in hindsight that murine leukemia virus (MLV) is sensitive to human restriction factor Serinc5 and expresses a glycosylated form of gag ("glyco-gag") resulting from alternative upstream translation of the gag open reading frame (ORF), which antagonizes this restriction.[25] It is highly likely that putative RCRs from MLV-based RV systems will encode and express glyco-gag, unless researchers have made specific mutations to abolish its expression. It remains to be seen as to whether there are other undiscovered "accessory" functions within retrovirus genomes and retained within RV components.

Third-generation RV and LV systems could therefore only produce putative RCV genomes from at least three or four recombination events between component DNA cassettes, respectively. Recently, a fourth generation of LVs became available (see Figure 1), in which the major splice donor within ψ is inactivated ("2KO" modification) to ablate production of aberrantly spliced (and packaged) vgRNAs.[26] Additionally, the polyadenylation sequences within the SIN-LTRs have been enhanced, which reduces transcriptional read-in to integrated LVs by > 10-fold, thus reducing potential for mobilization of vgRNA in patient cells ("SupA-LTRs"; manuscript under review). Production of these "SupA2KO-LVs" depends on co-expression of a modified U1 snRNA (256U1), which binds to the ψ region and stabilizes the vgRNA. Thus, to generate an RCL from this SupA2KO-LV system, a fifth component must be acquired to enable genomic vgRNA production in subsequent rounds.

Production approaches

Commercial production of RVs/LVs is typically carried out by plasmid transfection of HEK293(T)-based cells in suspension (serum-free) bioreactors, although fed-batch bioreactors can also be used.[27] In principle, packaging cell lines (PaCLs) can also be used, which only require the transfection of the vector genome plasmid, since gag/pol, rev, and envelope genes are stably integrated into the production cell genome.[28] This was relatively commonplace for earlier RV systems, and even RV producer cell lines (PrCLs) additionally stably expressing the vgRNA were used, especially for those encoding non-cytotoxic envelopes, where multiple passages and harvests were possible. Unfortunately, the detection of RCR from some of those early RV production systems (see below) forced the above-described modifications of the third-generation RVs.

The use of RV (and LV) PaCLs/PrCLs has clear advantages over fully transient (all plasmid) production, including simplified and cost-effective scale-up. While output titers from PaCL/PrCLs have not tended to exceed those from fully transient methods, it is anticipated that improved yields will soon be realized. Given that RV/LV component copy number is far lower in PaCL/PrCLs compared to plasmid transfection (and constrained within chromatin), logically the probability of RCV formation from such cell lines is far lower. However, some caution is warranted, first in reviewing those past incidents of RCR formation and second in how best to generate and characterize PaCL/PrCLs of the future.

RCR FORMATION FROM EARLY RV SYSTEMS: LEARNING FROM THE PAST

Horrible homologies and meddlesome mobilization

It is clear that the main driver for RCR formation was the large homology overlap shared between the RV components and generally from earlier generation systems where gag/pol and env genes were encoded on the same helper/packaging construct.[29–31] The risk may have been heightened by possible interaction with endogenous retroviruses (see below), and the ability to prolong production times, extending the time window to form/amplify RCRs.

However, we believe the other contributing aspect to RCR formation is due to the constitutive and high level of expression of the RV U3 promoter, which is not dependent on expression of an auxiliary protein like tat. This likely made the earlier generation of RVs predisposed to greater production of precursor RCR-like vgRNAs, having active 3′ LTRs and allowing subsequent transcription. Therefore, it is conceivable that co-packaging of gag/pol or recombinant gag/pol-env mRNAs within the same virion (all retroviruses specifically package two copies of vgRNAs) provided more templates (and opportunity) to generate an RCR genome via reverse-transcriptase (RT)-driven recombination. This might allow for low-level auto-transduction of production cells and perhaps some further adaptation/refinement of initially unfit RCRs by further rounds of co-packaging and RT-driven recombination events. It has been shown that template switching between the two packaged vgRNAs by RT is as high as 3–4 events per cDNA formation.[32,33] Increasing the length of discrete homology blocks between "different" co-packaged vgRNAs will therefore lead to meaningful recombination rates when considering production of millions of transducing units of RVs.

Improved design of separate vector component cassettes involved the complete removal of LTR/UTR sequences (containing *cis*-elements involved in packaging and RT) in favor of heterologous promoter/UTRs and polyA sequences with no or minimal homology to the vector cassette. As mentioned above, codon optimization of HIV-1 gag/pol, and also rev, eliminates homology to the vector RNA. In contemporary RV/LV systems based in HEK293(T) cells, we suggest that the SIN-LTR is a major contributing feature in reducing the likelihood of generating mobilized RNAs that might otherwise become co-packaged and partake in RT-driven recombination events.

Recombination with endogenous retroviruses: When cousins marry

Another key contributor to the appearance of RCRs from early RV systems is the presence of endogenous retroviruses (ERVs) within mouse-based cell lines such as NIH-3T3. An RCR was reported from a PrCL derived from a PaCL that had been generated from sequential stable transfection of NIH-3T3cells with MLV gag/pol and 4070A (amphotropic) env cassettes.[34] The PrCL was derived by stable transfection of this PaCL with the pBabeNeo series of (wild-type LTR) RV vectors.[35] The RCR was able to mobilize several different RV genomes (all wild-type LTRs) encoding lacZ and puromycin resistance genes, as well as the RV-Neo vgRNA. However, characterization of the RCR revealed that the entire 5′-LTR-gag/pol sequence was derived from an ecotropic ERV sequence, and only the 3′ LTR was acquired from the pBabeNeo vector.[36] These were conjoined together by the 4070A gene sequence, having a ∼600 nucleotide overlap with the upstream pol sequence and harboring a homologous 3′ UTR derived from Friend MLV. It is likely that the 4070A expression cassette employed the entire Friend MLV(C57) LTR to make use of its polyadenylation (polyA) signal, as typical of other RV systems.[37]

Subsequent RV PaCLs further reduced homology between components,[38] and non-murine cell lines lacking homologous endogenous RV are more suited as parental cells.[39] The propensity for cross-packaging of human ERV vgRNAs into RV particles is much less of a concern than for mouse ERVs.[40] The mobilization/cross-packaging of HIV-1 based LVs and human ERVs is highly unlikely, as judged by empirical studies, due to their sequence divergence, and human ERVs are generally far less active than mouse ERVs.[41–43] Where human ERV activation has correlated with HIV-1 infection, this has been shown to be due to the activity of tat and to a lesser extend vif—both of which are absent from third-generation LV systems.[44–46]

Packaging and producer cell lines: Proceed with caution

Finally, it is worth highlighting that it may be possible to accidentally generate recombinant RCV precursor cassettes when carrying out stable transfection of vector components. Co-transfection of all components in one step can lead to shuffling of episomal DNA prior to integration into chromatin (unpublished data). This was a suggested mechanism to partly explain why RCR formation was detected in only one of the two PaCLs produced at the same time by co-transfection (versus serial transfection) with identical RV gag/pol and env components.[47] Recently, methods have been developed to expedite PaCL/PrCL formation by stable transfection using modular plasmids or BACmids to deliver some or all vector components in one step.[48,49] Any subsequent negative RCV assay performed on supernatant derived from clones generated in this way would not reveal the potential for RCL precursor-cassettes generated from pre-shuffled, integrated components. Methods employing transposases to specifically deliver modular components may be less prone to inter-donor DNA recombination prior to integration; regardless, however, these components are delivered, and the final PaCL/PrCL clone

warrants full characterization by modern next-generation sequencing (NGS) methods. In doing so, we propose that such "clean" PaCL/PrCLs form additional basis for reduced RCV testing, as discussed later.

A MATHEMATICAL MODEL FOR RCL FORMATION FROM THIRD-GENERATION LV SYSTEMS

Definition of a minimal RCL

The consensus is that RCV formation from third-generation systems is "extremely unlikely." Yet the field lacks a model that attempts to quantify this risk, which would be of great value for informing on appropriate RCV testing. Consequently, we have derived probabilistic values for hypothetical RCL formation within a fully transient, four-component HIV-1 derived LV production at 200-L bioreactor scale. This is based on our "minimal-path" model presented in Figure 2, where the fewest inter-dependent recombination steps are required to produce a minimal RCL genome. We consider this to be a rev-dependent, packageable vgRNA expression cassette, encoding gag/pol, rev, and env and flanked by fully functional LTRs. A more detailed description of the model is within the supplemental file, and the RCL probability calculator is available as an Excel file (Table S2).

Note that while we might expect such a minimal RCL to replicate within the HEK293(T) manufacturing cell line (having low/negligible expression of restriction factors[19]), it would be reasonable to expect it to be extremely unfit *in vivo*. Due to the absence of accessory genes during production, the RCL would not likely be able to acquire countermeasures for the known primary-cell restriction factors. Moreover, any initial replication within the HEK293(T) cells would have no selective pressure to acquire such functions. HIV-1 lacking just nef replicates poorly within primary blood monocytes and not at all when all four accessory genes are deleted.[50] We also highlight the fact that our model assumes that the post-recombined RCL genome is fully functional in every necessary life-cycle step. Just because a recombination event might be able to occur, this does not mean that optimum configuration of sequences is realized. It should also be noted that even when heterologous envelopes have been purposely and rationally engineered into lentivirus genomes, the resultant virus is typically not fit.[51,52]

The minimal RCL genome may or may not acquire internal heterologous promoter(s) to drive gag/pol and/or rev/env ORFs, but this would deviate from the classic retrovirus genome structure, where these components are generally dependent on 5′ LTR promoter activity and alternative splicing. Moreover, transcription of packageable vgRNA would likely be greatly inhibited by promoter competition, as demonstrated in RV design.[53] Encoded promoter sequences would also make the RCL vgRNA longer than wild type and, potentially encoding unstable sequences, might have reduced abundance in the cell. Irrespective of its viral replication fitness, how might this minimal RCL arrive in the first infected cell?

Summary of the "minimal-path" model to a minimal RCL

Working back from the delivery of this single RCL genome, we envisage the following previous steps being necessary (in reverse order):

(1) A final, RT-driven recombination event between two co-packaged "pre-RCL" vgRNAs.
   a. An RCL formation event that is purely driven by RT-based recombination can only occur between the two co-packaged vgRNAs. There is no evidence that recombination can occur between vgRNA and passively packaged mRNAs from the production cell. Consequently, our model assumes the separation of the minimal features across two co-packaged vgRNAs, therefore requiring prior recombination of vector component plasmid DNAs (pDNAs) (see point 3).
   b. We use a conservative 1% ($p = 0.01$; see Equation 4) recombination rate from a study that assessed recombination between HIV-1-based genomes encoding dissimilar gag/pol clade genes (as low as 70%) and a broken GFP gene.[54]

(2) Competition of co-packaging of the two pre-RCL vgRNAs by the LV product vgRNA.
   a. The LV product vgRNA will be in large excess over both pre-RCL vgRNAs within the cell.
   b. There is a very good correlation between the mass ratio of input LV-pDNA and the ratio of packaged vgRNA within LV virions (unpublished data).
   c. We consider the probability of packaging either one of the two pre-RCL vgRNAs being hetero-dimeric with LV vector vgRNA as not greater than 1 in 50.
   d. Hence, the probability of co-packaging both pre-RCL vgRNAs (i.e., excluding LV vector vgRNA) is both individual probabilities multiplied (i.e., $p = 4 \times 10^{-4}$; see Equation 3).
   e. We assume an optimistic similar steady-state level of pre-RCL vgRNAs in the cell, as per the LV vector vgRNA.

(3) Generation of transcriptionally active DNA cassettes encoding the two pre-RCL vgRNAs.
   a. Our model considers two different recombination pathways (1ab and 2ab) necessarily occurring within the same cell but independently of each other.
   b. The 1a cassette encoding for LTR-ψ-gag/pol-RRE is derived by homologous recombination between either gag (wild-type) or RRE sequences on the LV genome and gag/pol plasmids. The insertion of the rev ORF (step 1b) to generate 1ab is dependent on the pre-existence of 1a, although these two steps could occur in inverse order (the overall probability is the same).
   c. Similarly, the 2ab cassette, encoding LTR-ψ-env-ProLTR, must result from interdependent recombination events that could in theory occur in either order.
   d. Inter-plasmid recombination probabilities are based on rates in COS cells by Ayares et al., who assessed a range of overlapping homology blocks from 25 to 501 nucleotides.[55]
   e. The Ayares study was performed using linearized plasmids, which are known to be substantially more efficient templates for recombination (at least an order of magnitude).[56]

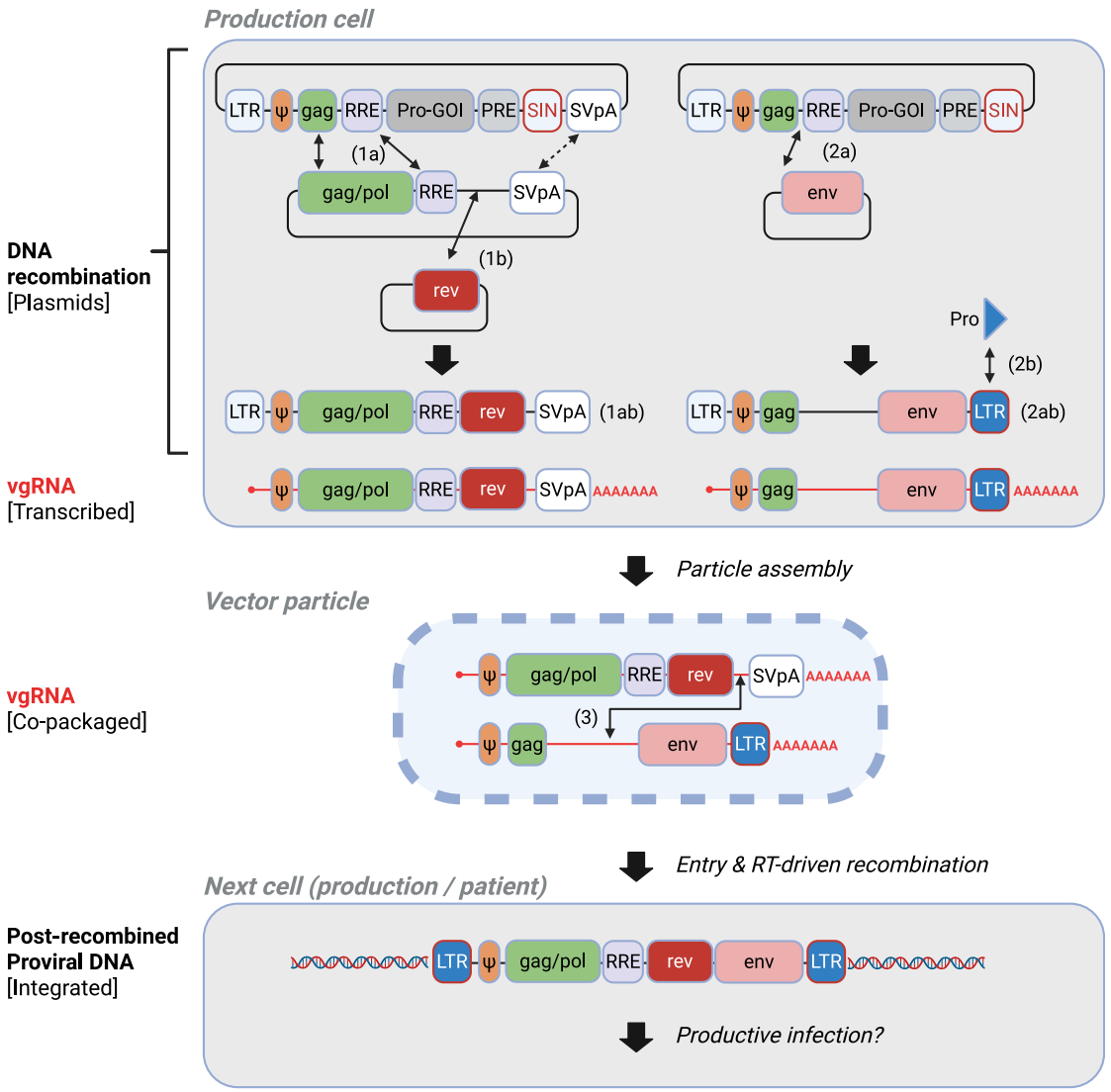

Figure 2. Minimal-path model for RCL formation from a four-component LV system

Our model assumes contribution of both pDNA and vgRNA recombination steps based on observed recombination rates and constrained by retrovirus biology. The minimal RCL is an LTR-flanked cassette encoding gag/pol, rev, and env, with packaging signal (ψ) and RRE. Working backward, only the two co-packaged "pre-RCL" vgRNAs can partake in RT-driven template switching (event 3), indicating that all features of the final RCL must be shared across the two vgRNAs. The simplest template switch is between one vgRNA encoding ψ-gag/pol-RRE-Rev and one vgRNA encoding ψ-gag'-env-LTR; our model assumes a 1% rate. Co-packaging of these two vgRNAs would be in substantial competition with the LV product vgRNA; our model sets co-packaging at no more frequently than 1 in 250 virions per cell. The two DNA cassettes (1ab and 2ab) that encode the two pre-RCL vgRNAs must form in the same cell, and each is based on serially dependent recombination events (1a>1b and 2a>2b). Requirements for acquisition of additional vector components would add onto either of these pathways. Our model uses reported inter-plasmid recombination rates in COS cells performed on linearized plasmids, specifically recombination rates for 330 nt (1a), 110 nt (2a), and 25 nt (1b, 2b) blocks of homology. Since the vast majority of pDNA in RV/LV transfection will be circular, we applied a five-fold reduction factor to the reported recombination rates. We assume a total of 500 transcriptionally active plasmids per nucleus and use typical pDNA input ratios. See main text for references. Created in https://BioRender.com.

Therefore, we use more conservative inter-plasmid recombination rates (5-fold lower) than those reported.

f. While the average total number of plasmid molecules per cell may be in the thousands, we argue that only transcriptionally active plasmids should be counted; ultimately, cassettes 1ab and 2ab need to transcribe vgRNA-like products for co-packaging. We assume 500 transcriptionally active pDNAs per nucleus (available for recombination), based on the work published by Cohen et al., who showed that transgene expression plateaus at ~200 plasmids/nucleus.[57]

Using the rates above, and typical pDNA mass input ratios (we assume similar plasmid sizes), our model derives the total number of

gag/pol and env pDNA molecules available per cell for recombination into an LV genome plasmid to initiate the pathway to 1ab or 2ab vgRNA cassettes, respectively. The probabilities of producing cassette 1ab ($p = 1.43 \times 10^{-5}$) and cassette 2ab ($p = 1.4 \times 10^{-6}$) in the same cell—now capable of producing both pre-RCL vgRNAs—are multiplied to give $p = 1.9 \times 10^{-11}$ (see Equation 2). Using the other parameters from pre-RCL vgRNA co-packaging and recombination events, an overall probability at 200-L scale (containing 1×10^{12} cells; see Equation 1) can be estimated.

The final analysis

Altogether, the probability of generating a single copy of the minimal RCL within a single 200-L bioreactor is taken as the following:

$$p(RCL\ Formation) = Cells_{Total} \times p(pre-RCL\ DNA\ recombination) \times p(vgRNA\ co-packaging) \times p(RT-driven\ recombination)$$

$$p(RCL\ Formation) = (1 \times 10^{12}) \times (1.9 \times 10^{-11}) \times (4 \times 10^{-4}) \times (1 \times 10^{-2})$$

$$p(RCL\ Formation) = 7.7 \times 10^{-5}$$

where

$$Cells_{Total} - Total\ number\ of\ production\ cells\ in\ bioreactor = 1 \times 10^{12} \quad \text{(Equation 1)}$$

$$p(pre\text{-}RCL\ DNA\ recombination) - Probability\ per\ cell\ generating\ both\ pre\text{-}RCL\ DNA\ casssettes = 1.9 \times 10^{-11} \quad \text{(Equation 2)}$$

$$p(vgRNA\ co\text{-}packaging) - Probability\ of\ virions\ co\text{-}packaging\ both\ pre\text{-}RCL\ vgRNAs = 4 \times 10^{-4} \quad \text{(Equation 3)}$$

$$p(RT\text{-}driven\ recombination) - Probability\ of\ a\ single\ RT\text{-}driven\ recombination\ event = 1 \times 10^{-2} \quad \text{(Equation 4)}$$

Thus, the model estimates that a four-component system would not likely generate a single RCL genome molecule in 10,000 bioreactor batches at 200-L scale. We estimate that current LV supply is in the order of 500× 200-L bioreactor equivalents per year, and while this number is set to double over the next decade, our model suggests that a single RCL genome molecule is not likely to form in any batch within that decade.

In a previous publication, measurable recombination rates per transducing unit (TU) for a one-step event were obtained by selection of transduced cells.[58] This produced ∼64 events for every 1 million GFP TUs, modeling a homology of ∼10% (i.e., gag and RRE) between LV vgRNA and gag/pol mRNA, with the latter additionally encoding a selection marker. Our model returns similar values when inputting for a 1-step recombination step such as this.

When we input values into our model to reflect lower, integrated copy numbers of gag/pol (10), rev (5), and env (5), representative of a PaCL (i.e., transfecting only pLV-Genome), the probability of RCL formation at the same scale drops by at least 100-fold. In other words, moving to PaCLs would push the likelihood further out into the next millennium.

We also note that the probability of RCL formation from systems employing two-component envelopes such as the Sendai F/HN genes[59] or a fusogen and targeting molecule (i.e., five-component systems) is reduced by at least 1,000-fold according to our model. In fact, the likelihood of producing an RCL that has acquired both envelope components may actually be no greater than acquiring one of the 94 single human ERV envelope genes from the host cell genome.[60] Utilization of SupA2KO-LVs, which rely on a 5th component (256U1) to produce full-length vgRNA, would also be > 1,000-fold less likely to generate an RCL compared to a four-component system.

PAST AND CURRENT GUIDELINES FOR RCV TESTING: RATIONALE AND LIMITATIONS

General principles

The regulatory authorities of the USA (Food and Drug Administration [FDA]),[61] the European Union (European Medicines Agency [EMA]),[62] and United Kingdom (Medicines and Healthcare Products Regulatory Agency [MHRA]; at the time of writing, the British Pharmacopeia had released new draft guidance for comment) all provide non-binding guidelines for RCV testing. The FDA guidance has tended to set precedence, since the recommendations were published first (from 2006) and provide greater levels of detail. Consequently, our review of testing requirements is focused primarily on the current[61] and previous FDA guidance.[63] We will not generally comment on guidance regarding patient follow-up but regard current recommendations as appropriate.

We consider testing based on RT activity, as the most meaningful criterion to determine the presence/absence of RCV; by definition, a retrovirus is characterized by such activity. Highly sensitive RT-qPCR assays such as fluorescent product-enhanced reverse transcriptase (F-PERT) are able to detect extremely low levels of RT activity in a wide range of matrices and importantly do so in an RT-sequence-independent manner.[64] A hypothetical RCV containing a mutated vector Pol-RT ORF or an ERV Pol-RT will still be detected within the PERT assay. This is not necessarily the case for endpoint assays reliant on antibodies to capsid[65] or sequence-specific qPCR assays for the detection of recombinants.[66] The PERT assay has been shown to outperform the p24 ELISA for

HIV-1 titer determination by lower inter-run variation, lower cost, and higher linear range.[67] While NGS can provide deep sequence analysis, in our opinion this approach would not be likely to provide practical or technical advantage over those methods mentioned above. First, the incidence of RCV formation (as predicted by the model) is extremely low, and consequently direct analysis of the relatively small sample of RV/LV product would unlikely be able to reveal an RCV without an initial viral amplification phase, which also enables separation of the signal from the background vector material. Second, to justify any potential truncation of the amplification phase (to streamline a future testing approach), the detection limit for RCV vgRNA would need to be substantially lower (i.e., more sensitive) than is currently achieved, even with the most sensitive detection methods. Third, the presence of the full-length vgRNA of the RCV would only be unambiguously determined using long-read sequencing, and therefore short-read NGS would need to be complemented by confirmatory long-read NGS, resulting in a complex workflow not well suited for routine implementation in a QC environment. Finally, any NGS method would require significant further development and support in terms of bioinformatics pipeline, in order to handle large amounts of data in a QC context. This would lead to increased complexity and costs, which is not required for established highly sensitive methods such as F-PERT. A summary of typical endpoint assays is given in Table S1 and elsewhere.[68]

Testing of *ex vivo* transduced cell products

It is now recommended that all *ex vivo* transduced cell products are tested for RCV, irrespective of *in vitro* expansion time. This is with the caveat that simplified or no testing at all may be justified on condition that sufficient supporting data/rationale is submitted within the IND document. The basis of testing is essentially the same as for EoPCs/vector material, whereby transduced cells are co-cultured with amplification cells, followed by a suitable endpoint test (as per Table S1—see references therein). However, as has been discussed above, it is highly unlikely an attenuated RCL lacking accessary gene functions will replicate more efficiently (or only) within primary (target) cells, compared to an RCL amplification cell line.[50] While *in vitro* infection of human cell lines by wild-type MLV (γ-retrovirus) is well known, there are no established diseases in humans caused by natural infection of γ-retroviruses, implying a broad level of restriction. The administration of RV contaminated with RCR to Rhesus monkeys, leading to 3 out of 10 deaths, likely only occurred because they were severely immunosuppressed.[31] Moreover, given that direct *in vivo* transduction of T cells by LVs is emerging as an alternative mode for CAR-T therapy, testing of representative transduced cells would be a technical challenge (requiring post-treatment sampling and analysis) and regardless would be "post-hoc" in nature. We believe the most appropriate and meaningful test is done on the vector product. Indeed, the EMA no longer requires testing of *ex vivo* transduced cell products so long as RCV testing for vector material provides the necessary confidence of using RCV-free vector under a risk assessment.[62]

One-off testing of master cell banks

The characterization of MCBs for GMP manufacturing includes a raft of wider tests but includes the one-off testing for endogenous retrovirus, and a straight-forward test for RT activity within parental (e.g., HEK293(T))-cell-derived material is appropriate. The current FDA guidelines also recommend typical RCV testing (i.e., with an appropriate amplification/indicator cell line phase) for any derivative cell line that has been generated by transduction of a "component-delivery" RV (CD-RV) encoding at least one of the vector system components. If the CD-RV was pseudotyped with an envelope different from the envelope of the end-product, a suitable one-off test with a permissive cell line for the presence of the envelope of the CD-RV is advised. However, it is very unlikely that stable transmission of the "delivery" envelope to a PaCL/PrCL would occur, assuming the CD-RV itself is also a third-generation vector. Moreover, we propose that MCBs of clonal PaCLs/PrCLs need only be fully characterized by one-off NGS to demonstrate (1) the absence of detection of pre-RCV cassettes resulting from inadvertent recombination between vector components (as discussed above) and (2) the absence of detection of other unwanted sequences, including heterologous envelopes and/or transposase genes that may have been used to generate these cell lines.

Routine testing of end-of-production cells: Does it make sense?

Historically, the testing of EoPCs has continued to be recommended,[61,69] since it was shown that an RCR generated in an early RV system was more robustly detected within a co-culture EoPC assay compared to the vector substance assay.[47] However, in that report it seems the inhibition of the RCR was directly related to the co-presence of vector substance, indicating that such inhibition could in principle have been avoided by diluting-out the vector substance. Indeed, this aspect of RCV assay design is already recommended within FDA guidance. The maximum concentration of vector substance that does not have an impact on detection rate of the positive control (PC) virus from minimal infectious dose should be empirically determined during assay qualification. This forms the basis for Spike controls, i.e., monitoring of general inhibition. In the case above, if the observed inhibition was due to both the RCR and RV product sharing the same receptor (i.e., they both had the amphotropic envelope), then within current state-of-the-art RCL assay development (EoPC co-culture or vector substance) it is not possible to assess potential specific receptor competition in an analogous manner. For example, direct inhibition of a putative vesicular stomatitis virus G protein (VSVG)-encoding RCL by high concentrations of VSVG-pseudotyped LV product cannot be modeled using an HIV-1-based PC virus (i.e., gp120 envelope), which uses a different receptor. Conversely, it should also be noted that LV-VSVG-product-based inhibition of a putative VSVG-encoding RCL would also be manifest at the point of target cell entry, decreasing risk to the patient. Spike controls are valuable and informative in terms of monitoring general aspects of assay inhibition, such as non-specific receptor blocking (crowding) and competition of post-entry steps.

Even if engineering of an HIV-1 PC virus encoding the relevant heterologous envelope was successful,[70] deliberately altering/expanding

the tropism of HIV-1 PC virus is considered an unacceptable risk to assay operatives and wider community. Indeed, the greatest risk to humans by replication-competent virus across the entire scope of LV manufacturing, testing, and clinical/commercial use is likely that experienced by operators of RCL assays, who must handle the HIV-1 PC virus, albeit in a highly controlled biological safety level (BSL) of 2+ (US) or 3 (UK). It is important to remember that large quantities of concentrated RVs/LVs are handled at BSL-1/2 in manufacturing facilities by many operators under the reasonable assumption that the material is RCV-free prior to RCV testing.

Another argument for testing EoPCs is that local (higher) concentration of an RCV budding from an infected EoPC might better contribute to initiation of cell-mediated infection of adjacent co-cultured amplification cells. The current guidelines state that 1% or 1×10^8 EoPCs (which ever the least) are tested per batch. If we consider a typical 200-L bioreactor containing 1×10^{12} EoPCs, then the probability of selecting a single infected EoPC (as per our model) is just 1-in-10,000 ($1 \times 10^8/1 \times 10^{12}$). Therefore, in this context, for the EoPC test to provide any meaningful assessment, we must assume that the RCV will have already infected at least 30,000 EoPCs. Only at this level of infection will we have 95% confidence interval of obtaining an RCV-infected cell within this cell sample according to Poisson calculations (see later), i.e., 3 in 1×10^8 EoPCs.

As our model indicates, spontaneous RCV formation in more than one cell per bioreactor is highly implausible, as is the idea that 30,000 infectious units (IUs) can be generated from a single cell. Logically, we are therefore forced to assume a degree of RCV replication between the transfection/induction step and harvest, to yield at least 30,000 IUs of RCV within the bioreactor supernatant, resulting in the 30,000 infected EoPCs. This would be 15 IUs per 100 mL or 1,500 IUs per 5% crude supernatant volume. To put this in context, previous FDA guidance indicated that RCL assays needed to be sufficiently sensitive to detect $\geq$1 RCV per 100 mL. In other words, to expect to be able to detect RCL from 1×10^8 EoPCs, it is inconceivable that the RCL will not also be present at readily detectable levels within the vector substance. In the extremely unlikely scenario that an RCV is strictly only transmitted via cell-mediated infection (and sheds no virus), then is there an actual risk of transmitting such an RCV using the vector product on target cells? We reiterate that the most meaningful material for RCV testing is the vector product, since this is the material that contacts and modifies the patient cells *ex vivo* or *in vivo*.

Vector substance testing: Past and present

As mentioned above, previous FDA guidelines proposed that an attenuated RCV might only be present within the crude vector supernatant at 1 IU per 100 mL (i.e., 2,000 IUs in a 200-L bioreactor). According to the Poisson distribution, the probability of infection/detection is p = 1-EXP(-c x Vt), where c is the concentration of the RCV (or PC virus), and Vt is the test volume. To obtain 95% confidence interval of a pass/fail result, under the assumption that c is 0.01 IU/mL, a maximum of 300 mL of crude supernatant must be tested. Initial guidance prescribed 5% volume or 300 mL crude supernatant to test, whichever is the lower amount; thus, production scales of $\geq$ 6 L need only test 300 mL. This approach has been developed to include testing of final vector product at 300 mL "equivalent" amount; the test volume of final product is determined as containing the same number of RV/LV TUs as present within the 300 mL crude supernatant prior to processing. This plausibly assumes that any RCV particles would be similarly and proportionately recovered in parallel to RV/LV particles across the entire process. This approach to vector substance testing was the mainstay of RCV testing until 2020, when the FDA brought out new guidance to simplify these considerations.

To short-circuit all the assumptions regarding likely RCV concentration within crude supernatant, as well as potential differentials in RCV versus RV/LV process recoveries, the testing of three patient doses (3×PD) was introduced. Accordingly, the Poisson calculation applies to the testing of 3×PDs, providing 95% confidence of the absence of RCV within a single dose. Superficially, we can see why this was an attractive development in terms of clarifying guidance and in our experience has generally led to a modest but welcome reduction in burden of testing for typical *ex vivo* (CAR-T) vector batches. However, both theoretical and practical problems with this approach are hiding under the surface, particularly for high-concentration-per-dose, low-dose-number-per-batch LV products for *in vivo* indications.

Problems with three-patient dose testing

The testing of 3×PDs completely disconnects the fundamental considerations of RCV formation within standard third-generation RV/LV systems from probabilistic risk to the patient. For an *ex vivo* product, for example (e.g., 1×10^8 TU per dose for CAR-T), the implication is that the incidence of RCV formation is less than 1 in 3×10^8 TUs produced. If 3×PD testing is applied to a high-concentration-per-dose LV product (e.g., 1×10^{10} TU per dose of a lung/liver treatment), the implication is that the incidence of RCV formation is less than 1 in 3×10^{10} TUs produced, even though both products share all the same safety features. Implicitly, the high-dose product is being differentially assumed to be 100 times less likely to generate an RCV (i.e., is safer) such that 100-fold more vector substance is prescribed for testing to be able to potentially detect one. While we recognize that 100 times more product is being administered to the patient, we are arguing that the probability of detecting an RCV in the dose would also increase by 100 times. Therefore, this justifies testing less, and indeed the same (standardized), amount for any RV/LV product.

From a practical perspective, the testing of so much vector product becomes extremely challenging, with potentially many 10s to potentially >100 test article flasks required to service the assay. In our experience, the handling of 15× T225 flasks (plus 5× control flasks) within GMP assays performed under (UK) BSL-3 facilities/codes of practice pushes the limit of what can be achieved at each passage

point, which includes taking various samples. Going beyond this scale substantially increases risk of handling errors or other factors leading to a failed or invalid assay. Additionally, for low-dose-per-batch products, each assay will require 4–5×PDs (depending on number of Spike controls), which may be a substantial proportion of the batch and, together with retains for other QC tests, could render an otherwise commercially viable and highly important (for patients) product non-viable.

Toward unification: Fixed vector product testing based on vgRNA copies?

Unlike replication-competent adenovirus (RCA) testing, which fixes testing at 3×10^{10} viral particles for any product,[71] no such standardized testing for RVs/LVs exists. This has been due to the lack of a standard assay that would define a "transducing unit." However, as we have already established, the transducing capacity of any given RV/LV product in no way predicts the infectivity of a putative RCV generated in parallel.

We propose that unification of RV/LV vector substance testing could be achieved by testing a fixed number of vector product vgRNA copies, as quantified by RT-qPCR to ψ-gag, a common target sequence of the vector that is already widely used to determine integration titers. This would bring formal physical quantification of RV/LV vector stocks in line with rAAV titration methods and is also a good measure of the "mass output" of an entire process. The minimal physical characteristic of an RCV core is two vgRNAs packaged into a capsid; a measure of vgRNA copies avoids having to consider empty capsids that would be quantified in capsid ELISA assays. Detection of vgRNA signal is dependent on the vgRNA being inside a capsid, where it is protected from nucleases.

For example, testing 1.5×10^{11} vgRNA per batch would approximate to 3×10^{8} TUs of a typical LV product, assuming a particle-to-infectivity (P:I) ratio of 500:1. Note that the P:I ratio is a currently non-standardized calculation, because the cell-based method employed for functional titer determination is influenced by a wide range of assay variables, which are not yet standardized—volume, cell type, cell growth mode etc., all of which can have a profound effect on the reported value in "TU"/mL. The actual number of fixed vgRNAs could be derived from the large body of data from previous RCV testing that currently sits with regulatory authorities and could be reviewed and agreed upon by several stakeholders in the field prior to implementation. Adoption of a physical measure of titer to determine vector product test volume also provides the regulatory bodies the opportunity to generate vgRNA standards so that the industry is further aligned. This would also build a dataset to understand how many vector particles are being delivered to patients in different contexts.

Fundamentally, this proposal makes the reasonable assumption that the probability of RCV formation within contemporary multi-component RV/LV systems is similar, irrespective of the transgene sequence, given that the same number/type of recombination events are required within each platform. Products employing a greater number of components (and therefore substantially lower risk of RCV formation) could potentially justify reduced (or no) testing on a case-by-case basis. Considerations regarding how this could be implemented across different RV/LV products is discussed later.

RCV ASSAY DEVELOPMENT: BEST PRACTICE AND RECOMMENDATIONS

First things first: Essentials versus desirables

OXB has experience in the development of RCV assays over several decades, encompassing vector platforms derived from MLV, EIAV, HIV-1, and SIV-1. While official guidelines are nonbinding, there are aspects to assay design that make for a final format that regulatory bodies are more likely to accept. The primary factors in setting out plans for RCV assay development are choice of amplification cell line and identity of the PC virus.

The preferred PC virus is derived from the wild-type retrovirus on which the vector system is derived. However, we argue that an RCV derived from human HEK293(T) cells is most likely to be human cell tropic, given that some low-level replication within the bioreactor is likely to be needed for it to be both detectable and a risk to patients. This represents a challenge if the vector system is derived from a retrovirus that does not replicate in human cell lines, for example, EIAV.[72,73]

Additionally, the aspects of vector envelope tropism need to be considered, since for single-envelope component systems at least, this is the envelope most likely to be acquired by the RCV. If the only available candidate cell line that is permissive for entry by the specific envelope does not support replication of a PC virus parental to the vector system, a surrogate PC virus from a different class of retrovirus must be used.[52,72] Other complicating factors include whether a particular vector envelope component requires some form of conditional activation that is absent from the production cell culturing phase, for example, post-harvest trypsin activation of Sendai F.[59] In-bioreactor replication of an RCV with such an envelope would not be possible. The only way to include effective trypsin treatment within the amplification phase would be to remove serum, and this would require serum-free adaptation of several candidate cell lines prior to screening. In our experience, this is not a successful route to development of a credible assay system; for example, serum-free adapted C8166 cells are at least 10-fold less permissive to HIV-1- and SIV-1-based viruses, as well as having lower routine viability, rendering them unsuitable (unpublished data).

Consequently, we recommend the following selection criteria for a limited number of candidate amplification cell lines, unless the "off-the-shelf" RCL assays (see later) available for biosimilar vectors are appropriate.

Essential properties

(1) Human cell line; absent/negligible endogenous RT-like activity.
(2) Robust growth and viability (enabling effective cell banking).

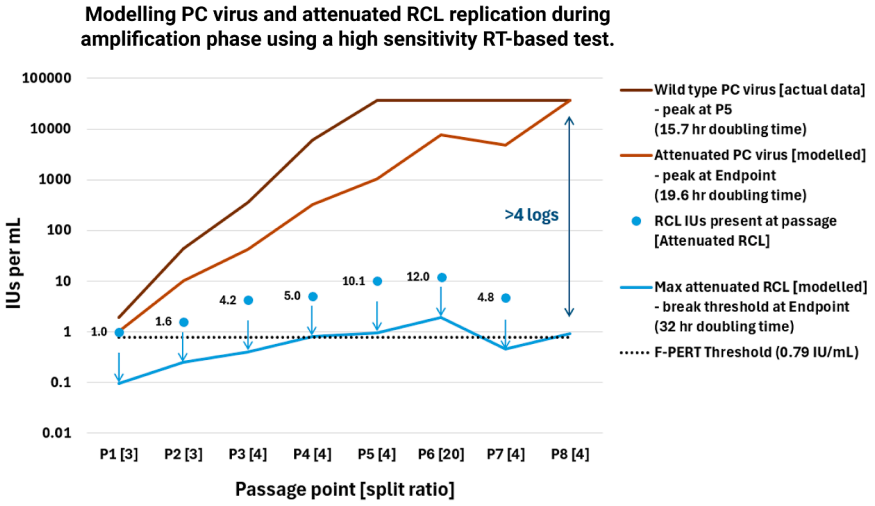

Figure 3. High sensitivity and dynamic range of F-PERT assay at endpoint allows detection of replication competent viruses of widely different replication kinetics

Actual RCL assay amplification phase data plotted for a wild-type PC virus inoculated at a minimal infectious dose peaks at passage 5 (dark maroon line; unpublished data); infectious units (IUs) of wild type PC virus per mL (log-10 scale). This is a virus doubling time of 15.7 h. An attenuated PC virus (red line) models doubling every 19.6 h and would still reach maximal infection by endpoint at P8. The stated passaging regime (1:3–4) is a cell-only split, including a 1:20 supernatant-only split at P6. Under this passaging regime (where necessarily, the bulk of infected cultures are discarded), the most attenuated RCL (blue line) is modeled such that it still breaks the endpoint F-PERT threshold in P8 samples. This is modeled from a single IU of RCL present at P0–P1 and displays the minimum number of IUs (blue dots) that would present in each inoculum at each passage point in order to break threshold at P8. This severely attenuated RCL would have a doubling time of 32 h. The dynamic range of signal of the F-PERT assay means that fully infected cell cultures at endpoint contain 4–5 orders of magnitude more RT activity than the threshold level. Therefore, the assay is capable of detecting an RCL irrespective of whether it is replicating with fast or slow kinetics. Created in https://BioRender.com.

(3) Permissive for vector product envelope.
(4) Efficient at shedding particles of the vector gag/pol gene employed.

Desirable properties

(1) Can support pseudotyping of particles with vector product envelope.
(2) Culturing format mirrors other envelope-specific dependencies.
(3) Can support robust replication of the parental virus as the PC control (preference: parental > retrovirus class > other retrovirus).

Pre-qualification stages of RCV assay development

Given the relative maturity of RV/LV systems, there are already well-characterized cell lines being used in RCV assays. For RV-based products, this includes the use of HEK293 cells and MLV-4070A as the PC virus.[74] For most LV-based products, the C8166 T cell line is used with attenuated HIV-1 as the PC virus.[75,76] We have also evaluated wild-type SIVagm for the PC virus in C8166 cells for SIV-1-based LVs (unpublished data). These two cell lines are highly permissive for their respective PC viruses, likely due to their high metabolic states resulting from endogenous expression of other viral factors (HTLV-1 Tax in C8166 cells and Adenovirus E1 in HEK293 cells), as well as negligible/low expression of restriction factors.

We recommend for new products that employ alternative envelopes to those used broadly (e.g., VSV-G); developers focus on these two cell lines (plus an appropriate third option) as candidate amplification cell lines. This would likely involve stably introducing the receptor for the vector envelope, assuming that the cells are not already permissive. A phased program of work would then follow, taking the 2–3 cell lines through assessments for the essential and then desirable properties, arriving at a data-driven decision to identify the best PC virus. This type of program to develop an assay that was considered suitable by the regulators for one specific application and unique vector system type is described in Farley et al.[52]

PC virus controls: What are they controlling?

As discussed above, often the PC virus cannot fully model all aspects of a putative RCV that might conceivably be derived from the vector production system, particularly the specific route of entry. However, successful amplification and detection of the PC virus from limiting quantities will report on the general permissiveness/health of the amplification cell line (PC control). The Spike control reports on potential inhibition of infection (cell surface crowding, post-entry competition) by the test article at inoculation. Here, we need to comment on the properties of the PC virus in terms of the importance of maintaining sensitivity at inoculation versus whether it should directly model the potentially slower kinetics of an attenuated RCL. In our experience, the use of the F-PERT assay provides an extremely wide range of sensitivity within endpoint samples after multiple passages (e.g., ∼8), such that RT activity of fully infected cultures are 4–5 orders of magnitude greater than the threshold (pass/fail) point. This means RCVs replicating with extremely low (and possibly unrealistic) kinetics will be detected at endpoint (Figure 3). In other words, the replication kinetics *per se* are not as important as the sensitivity to infection at inoculation. This raises the valid point that a non-attenuated PC virus, when initiating infection from fewer particles—and therefore is likely to be more sensitive to inhibition—may actually be a more appropriate virus than an attenuated variant, which may need more particles to initiate infection to achieve robust detection levels at endpoint (Figure 4). It is therefore sensible to monitor the relative detection level over the endpoint threshold value during comparisons of wild-type versus attenuated PC viruses and set an additional selection criteria (e.g.,

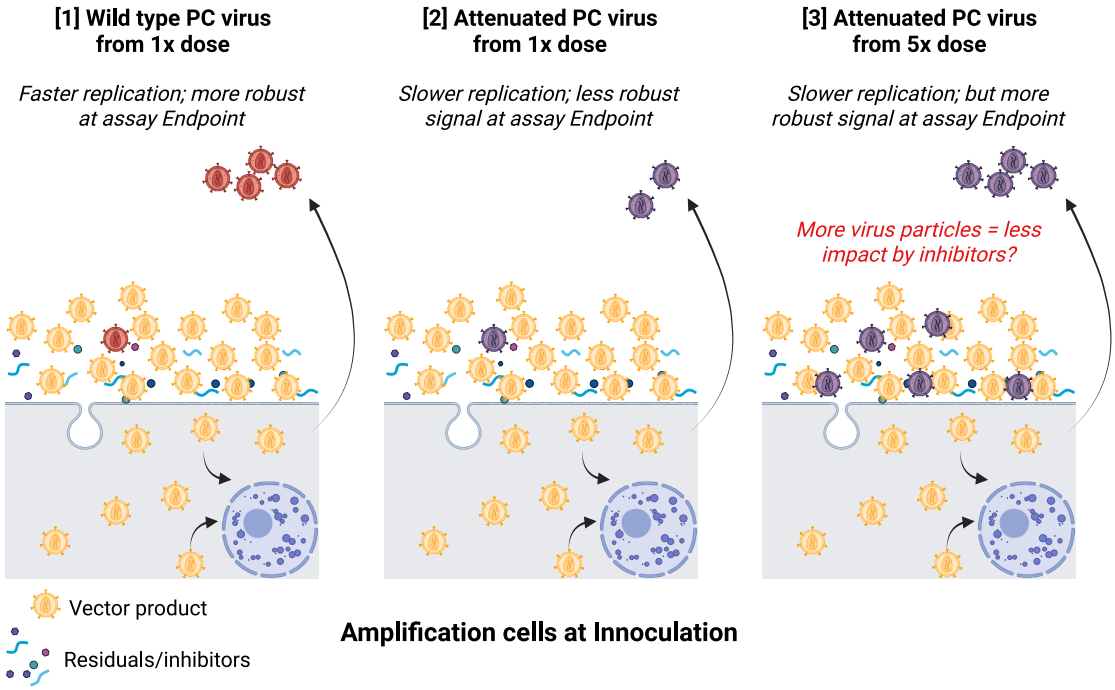

Figure 4. PC virus type and dosing at inoculation in Spike controls—pros and cons

Historical guidance has tended to recommend the use of the most attenuated PC virus, to attempt to model an RCL. While it is important to demonstrate that the final assay format is sensitive, and capable of detecting an attenuated PC virus (see Figure 3), its routine use in the assay can lead to false-negative rates and often necessary (but arbitrary) increases in dose.[76] Increasing dose of attenuated PC virus particles at inoculation not only contrasts with a general RCL formation model that predicts low amounts of RCL particles will be formed but may also be less impacted by general inhibitors in the test article, compared to a wild-type PC virus that is capable of initiating infection (that will be more robustly detected at endpoint) from lower numbers of particles. Therefore, after empirical testing of wild-type versus attenuated PC viruses, the wild-type PC virus may be considered more appropriate in controlling/monitoring inhibition at the inoculation step. Created in https://BioRender.com.

RT levels must be routinely 100-fold greater than threshold) to identify the most appropriate virus to use in the final assay.

Sensitivity versus robustness

Once the most appropriate PC virus has been selected and a virus bank generated, it must be empirically titrated under the appropriately modeled and representative conditions intended at the inoculation step of the RCV assay. This essentially means titration of the virus stock at the same cell concentration intended for the final assay, which itself reflects a sensible density that allows for co-cultivation with EoPCs/vector substance, and an appropriate incubation time before the first passage is required. This is usually a density where the cells are in vast excess over the PC virus, and it is most sensible to view the PC virus (and RCV) inoculated as a discrete entity, rather than a concentration. In doing so, we can see that infection rate of the minimal infectious dose will be the same irrespective of vessel size, but only if the cell concentration at inoculation is maintained.[73] The larger the vessel size, the longer it takes to amplify and reach full infection. This means that titration can be carried out at multi-well format (48-/24-well), enabling sufficient replicates to appropriately power the subsequent back-calculations required to derive an accurate titer value, using the proportion of positively infected vs. uninfected wells. A similar process is carried out for the Spike control, but where multi-well plates are co-inoculated with a single IU of PC virus and increasing concentrations of the vector substance, or the number of EoPCs. This will identify the maximum concentration of test article at inoculation that does not impact PC virus infection rate.

During qualification, the detection of the PC virus from a single IU should be demonstrated at final assay scale. This can be done by inoculating 3x independent cell culture vessels, each receiving a single IU at P0, and passaging until endpoint. The outcome (i.e. the endpoint threshold pass/fail result) will yield one or more positively infected flasks, with 95% confidence. Having demonstrated that the assay format can detect the PC virus from a single IU under these conditions, the final qualified assay typically utilizes positive and Spike control vessels where the PC virus is inoculated at 3 IUs ("1×PC" and "1×SPC") so that only one (rather than three) vessel can be used, while ensuring assay robustness (i.e., 95% pass rate). Additionally, "10×PC" and "10×SPC" flasks can be used as part of assay acceptance criteria, since it can be challenging to practically dose vessels with such low amounts of PC virus within the requisite precision/accuracy, and pass/failure across these additional PC and SPC flasks can be useful for troubleshooting unexpected outcomes.

In summary, PC virus dosing across development and qualification phases need to balance the demonstration of assay sensitivity with final assay robustness, to minimize failures due to false-negative results in these controls.

Other recommendations

We recommend developing standard procedures for generation of PC virus stocks, to minimize potential differences between successive banks. We typically transfect HEK293T cells with proviral DNA, rather than regenerate virus stocks from the amplification cell line, to avoid potential genetic drift. The use of retains from the older stock allows cross-referencing during titration of subsequent stocks.

If PC virus replication leads to cell lysis during mid-to-latter stages of amplification (as does HIV-1 in C8166 cells), this might lead to loss of infection by the end of the assay and by implication, possibly loss of RCL infection. We therefore recommend adding fresh cells during passaging (e.g., from passage 3) to avoid this.

It is prudent to retain representative culture archive samples (viable cell banks and supernatant) at early (P0–P1) and endpoint times, and smaller supernatant samples at every passage point, to facilitate potential troubleshooting. If a positive result unexpectedly occurs in a test article flask at endpoint, this would require investigation and root cause analysis. Further troubleshooting and characterization of the RCV (if it exists) or determining if this was a false-positive result is facilitated by these samples.

Finally, we recommend assessing alternative/novel approaches to handling scale issues. Progressive pooling of test article flasks as reported by Corre et al.[77] is one approach, or alternatively the vector substance test vessel could be a single multilayer cell factory that can handle much larger numbers of vector TUs to expedite tissue culture handling times (Figure 5). Note that PC virus controls could still be handled at smaller scale, being mindful that these controls would likely take less time to become fully infected. We have automated the EoPC co-cultivation assay (and certain vector substance assays) at multi-well scale, showing that this aspect can also be streamlined (unpublished data).

Final thoughts and a proposed roadmap for reduced testing

Batch testing for RCV remains a substantial burden for RV/LV product development and is often a rate-determining step within the batch release process; the assay is lengthy and can only be initiated with the availability of certain critical QC data. Historical issues with RCR formation was limited to early first- and second-generation RVs, and the advances representative of third-generation systems appears to have solved these issues.[68,78,79] Our proposal for simplification and potential reduced testing (see below) is based on the central argument that, irrespective of the genetic payload, if an RV/LV product is a third-generation (or greater), replication-incompetent vector, there are a minimal number and type of recombination events that would need to occur to "reverse" the common safety features employed. There may be additional safety features employed in product-specific vectors, such as further division of components to separate cassettes (≥five-component), and/or use of fully sequenced PaCLs/PrCLs, that could be used to justify a further reduction in testing; such arguments would need to be made on a case-by-case basis. Conversely, for example, an approach to deliver HIV-1 gag or an envelope-like protein as an antigen via an LV will necessarily produce a vector that may already constitute a pre-cursor RCL vgRNA molecule (e.g., LTR-Gag-SINLTR or LTR-Env-SINLTR). Consequently, fewer recombination events would be needed to arrive at the minimal RCL genome; under these specific circumstances it would be reasonable to expect RCL testing for every batch, as is currently required. Vector engineers should be careful to assess "unplanned" regions of homology between transgene sequences and the vector components and seek to minimize these as much as possible. We also note that there are conditionally replicating RVs (crRVs) being used for cancer therapy,[80] where generation of RCV risk must be considered in a different light. Here, presumably the risks (and detection assays) must be directed to potential for the crRV to escape the target tumor environment and be capable of replication in somatic cells. The basis of safety to mitigate such risk appears to be a combination of the fact that the crRV requires the relatively greater cell division rate of tumor cells (although many somatic cell types do divide) and general innate restriction of retroviruses in somatic tissues. It seems to us that the risk of escape by a crRV used for cancer therapy is much greater than RCV formation from RV/LV systems, and yet the ability to develop comprehensive *in vitro* primary cell assays to measure the potential for "escape" RCVs is clearly limited (which and how many primary cell assays are to be performed?).

The risk of RCV from contemporary vector systems remains purely theoretical, and we have generated a mathematical model for the assessment of RCL formation from four-component, third-generation LV systems. Given the extremely low probability of RCV formation and that the same number/type of recombination events are required irrespective of the product, we propose that a roadmap for reduced testing should now be considered; these are also presented graphically in Figure 6.

Vector product testing

Standardized to a fixed number of vgRNAs per batch, in the range of $1–5 \times 10^{11}$ vgRNA or 3× patient doses, whichever is lower. The final number should be determined by regulatory bodies and agreed in consultation with industry.

EoPC and ex vivo *cell product testing*

Both to be reconsidered, as it can be reasonably argued (as we have here) that this testing is no longer warranted/necessary.

Reduction in testing regimes

All clinical trial supply would still require testing, and this could be used as a period to acquire relevant and informative data. We propose finite batch testing for products that enter commercial/clinical

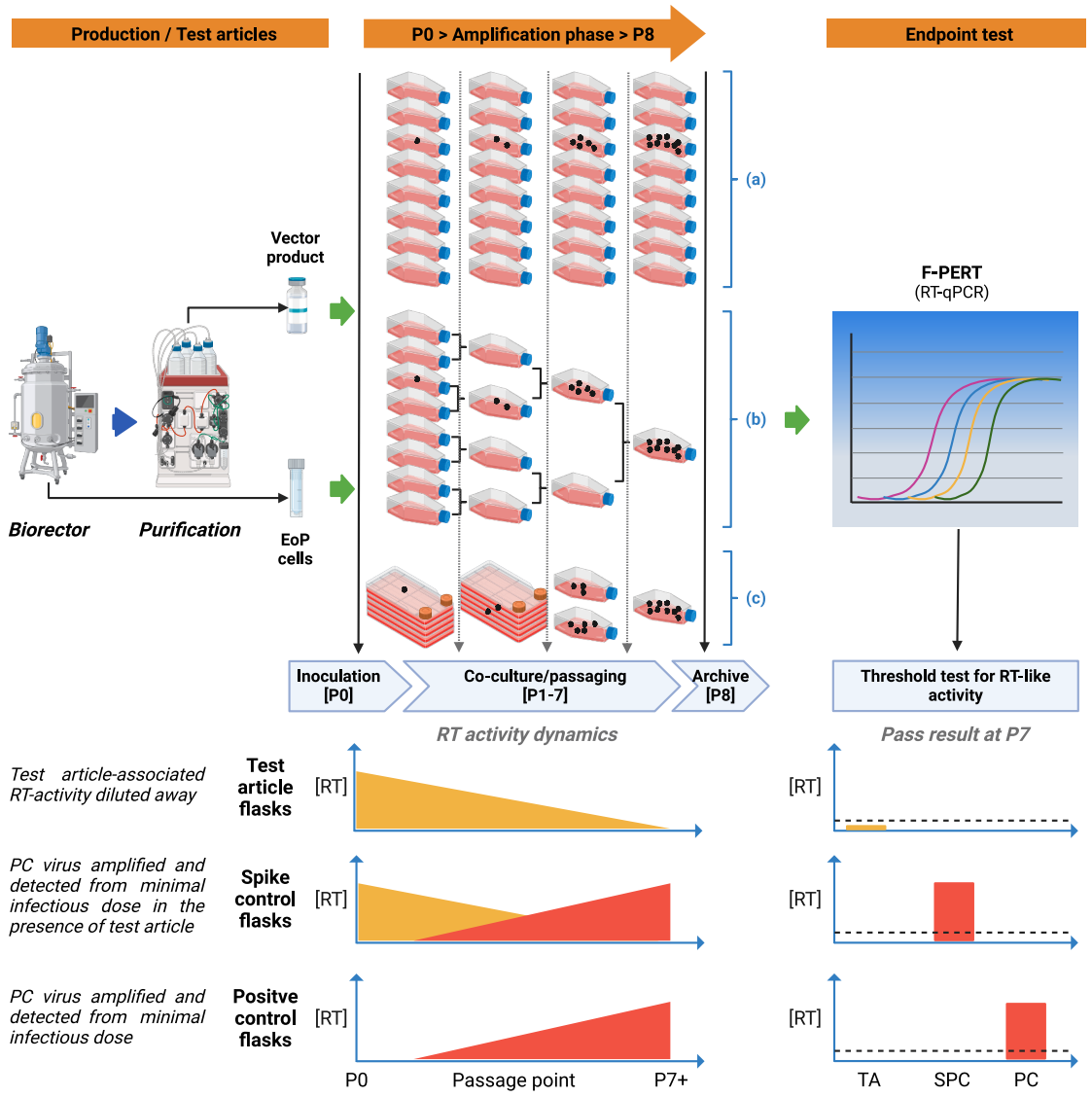

Figure 5. RCV assay format based on endpoint RT activity

Vector substance (product) and end-of-production cells are routine assay test articles, tested in separate but similar assays. Inoculation of test article equally across flasks containing amplification cells begins at P0, followed by passaging until P7/8. Co-culturing of EoPCs typically ceases within P1-3, and a supernatant-only passaging is taken to amplification cell cultures only thereafter. The standard test article flask format is shown in (A), whereas a flask pooling strategy (B) or scale-down from a multilayer cell factory (C) can help with handling initially high amounts of vector substance at inoculation (black dots represent putative RCL particles). Since RT detection methods such as F-PERT are highly sensitive, the RT activity associated with test article material (orange) must be sufficiently diluted out during amplification phase to avoid false-positive results at endpoint. The final result is a threshold pass/fail, according to validated testing of background levels within non-infected amplification-cell-conditioned media. Assays typically take 3–4 weeks to complete. Created in https://BioRender.com.

supply. For fully transient production methods, perhaps only the first 15 commercial/clinical batches should need to be tested. For fully characterized PaCLs/PrCLs where NGS has demonstrated the absence of pre-RCL cassettes, perhaps only the first commercial/clinical batch should be tested. For ≥five-component RV/LV systems (or integration-defective vectors), we suggest that no testing may be justified, or the number of limited/intermittent batch testing is discussed during pre-/IND submission.

Patient follow-up

To remain in place under current guidelines.

Industry standards

Central provision of stocks of vgRNAs for reference standards for RV/RCR and LV/RCL RT-qPCR assays and potentially primer/probe sequences. There are compelling reasons why the HEK293 cell line and MLV-4070A (as the PC virus) could be justified and

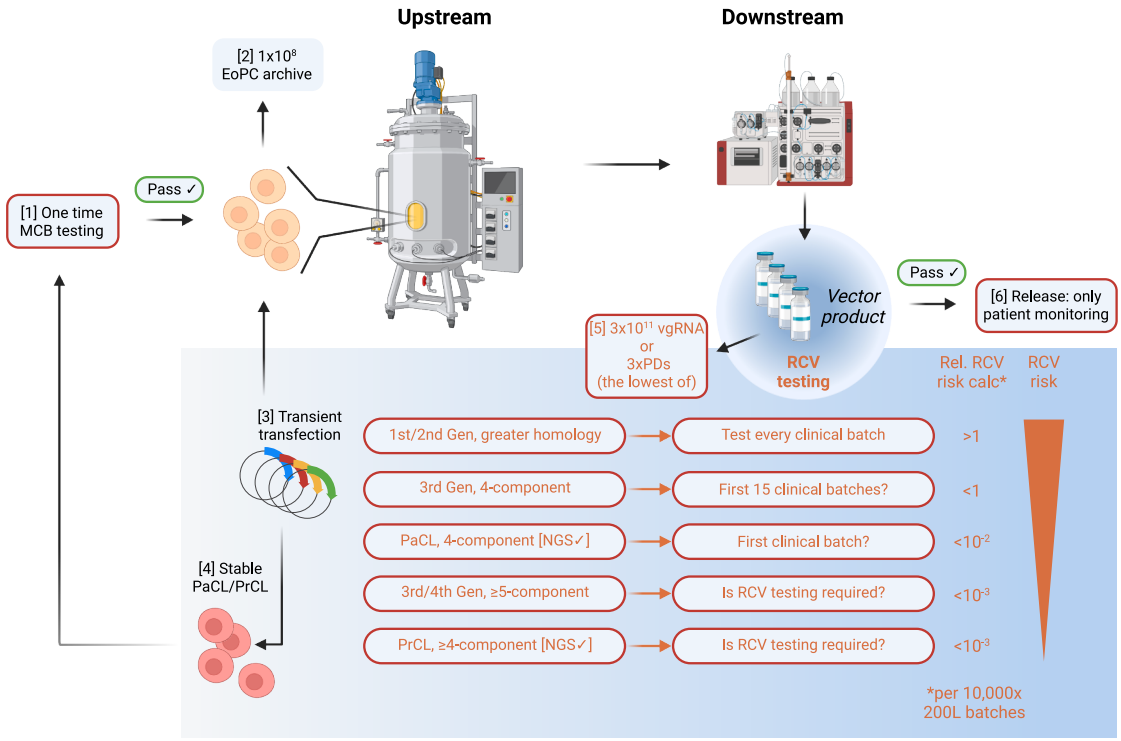

Figure 6. Overview of the model-driven basis for reduced RCV testing

[1] One-time testing of master cell banks (MCBs) is typically done by an RT-activity test such as F-PERT; note this ideally should be done on parental HEK293/T cells prior to PaCL/PrCL development. We have made a reasonable argument that EoPCs no longer need to be tested for RCV but recommend such samples are still taken and archived [2]. Vector production is currently mediated by transient transfection of four components as plasmids, assuming third-generation LVs are being used (three-component for RVs) [3]. Similar expression cassettes are stably integrated into PaCL/PrCLs [4], which we suggest are "one-time" sequenced using NGS to demonstrate lack of detection of unwanted (recombined) sequences that constitute pre-cursor RCV vgRNA cassettes. We propose that RCV testing is performed on vector product and is set to a fixed amount of 1–5 $\times$ 10^{11} vgRNAs per batch (to be agreed by stakeholders), or 3$\times$PDs, whichever is the lower volume, to integrate with current guidance [5]. The extent and regularity of RCV testing (of this fixed amount) would depend on the generation of RV/LV system employed and the relative (fewer) copies of vector components per cell (i.e., PaCL/PrCL). The greater number of recombination events needed, the fewer and less frequent batch testing would be required. Note that we have not calculated the probability RCV formation of first-/second-generation vectors (or third-generation vectors encoding gag or env) in our model. Neither have we done this for PrCLs and have simply indicated that it is no more probable than $\geq$five-component transient transfection approaches. Additionally, the use of codon-optimized GagPol rather than wild-type GagPol would also contribute to lowering overall probability of RCL formation in any of these settings (see Tareen et al., 2013). We recommend endpoint testing of RT activity by F-PERT, but other sensitive methods are available (and acceptable to the regulatory agencies) and described in Table S1. Once a vector product has passed RCV testing, no further testing would be required (i.e., on *ex vivo* transduced cells), and only patient monitoring is warranted. Created in https://BioRender.com.

used as an industrial standard format for testing RCV for new products going forward. This human cell line is amenable to engineering for introduction of specific RV/LV product receptors, lacks restriction factors, is parental to HEK293(T) production cells, and hence scores highly for most/all essential/desirable properties. The genome of the RCV would look more like MLV-4070A, lacking key accessory genes.

CONCLUSION

The conception, development (of new controls and cell lines), and qualification of RCV assays requires a substantial allotment of time and resources during product development. A significant amount of vector product (and retains) is required to service RCV and other safety/QC tests, which contributes to batch "losses" and therefore overall cost per dose. Consequently, for the successful development of such potentially transformative medicines, it is vital to ensure manufacturing costs, and CMC costs more generally are carefully managed. Due to the non-binding nature of guidelines—and hence a level of (product-specific) interpretation by product developers—the submission of proposed RCV testing methods to regulatory agencies during early interactions is typically accompanied with a degree of trepidation. Any requirement to return to RCV assay development will significantly impact product development timelines and increase costs further. The minimal RCL model/calculator we present is the first of its kind, and we believe both the assumptions and values used are reasonable (even conservative) ones. The model generates RCL probabilities that are in line with the current safety profile of third-generation RV/LVs. This forms the basis for our proposition for a roadmap toward unified and reduced testing within the field, so long as the defining safety features of third-generation (and beyond) are included as standard. We welcome feedback from the wider community and the regulatory agencies in terms of

how the model may be improved and the recommendations herein. We also consider that the topic merits a comprehensive re-assessment by a wide range of stakeholders (perhaps as a workshop series) with the overall intent to avoid unwarranted testing and make products more rapidly available to patients at a reasonable cost of goods.

ACKNOWLEDGMENTS

We express our thanks to all the OXB research and lab support staff, as well as clients/collaborators, who have contributed to RCV assay development and the wealth of knowledge that we have been able to draw on to write this review article.

AUTHOR CONTRIBUTIONS

D.F. wrote the bulk of the article, with contributions from J.R., J.M., and K.M. D.F. and S. S. derived the minimal-path model and associated mathematics. C.M.-K. produced the code to generate the RCL Probability Calculator tool.

DECLARATION OF INTERESTS

All authors except J.R. hold stock or stock options in OXB and are currently employed by OXB; J.M. is a recent employee of OXB. J.M. is a non-executive director of eXmoor Pharma and special advisor at ViroCell Biologics. J.M. was not a paid consultant relating to this manuscript. J.R. is Senior Director, Regulatory Affairs at Vector BioMed Inc. He was not a paid consultant relating to this manuscript. D.F. is an inventor on the following patent applications relating to "SupA2KO-LVs" and the 256U1 enhancer molecule referenced within the manuscript: WO2023062365A2, WO2021160993A1, and WO2021014157A1. Funding was internal to OXB.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2025.101601.

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