The 3 Rs Of Replication-Competent Lentivirus (RCL) Formation Risk: Real, Rare or Artificial?

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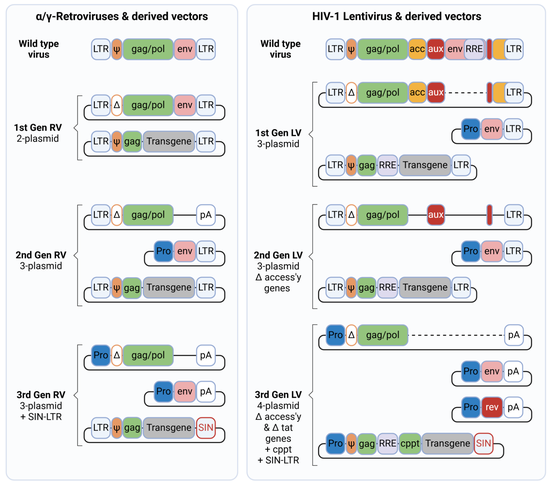
1 Generations of γ-Retro/Lentiviral (RV/LV) vectors and their safety features

- Early vector components shared sequence homology.
- RCRs were generated by recombination between just two components, and/or with endogenous retroviruses (ERVs).
- All four accessory genes & tat were deleted from HIV-1 based LVs.
- Self-inactivating (SIN) LTR lacks native promoter & stops mobilisation of pre-cursor RCR/RCL vgRNA.
- RV production methods stopped using mouse cell lines.

1st to 3rd Gen RV/LV vector component engineering



α/γ-Retroviruses are simpler than Lentiviruses and were easier to engineer. RVs and LVs followed similar development paths through three generations, where functional components were progressively separated to different expression constructs.

Early RV production systems used fewer component constructs and were based on mouse cell lines; cases of genuine RCRs were reported due to homologous recombination between constructs and mouse endogenous retroviruses.

Engineering of LVs (based on HIV-1) involved removal of accessory genes and tat. Rev is retained so that full length vgRNA is exported for packaging, via binding to the RRE. Contemporary LV systems use at least 4 component constructs.

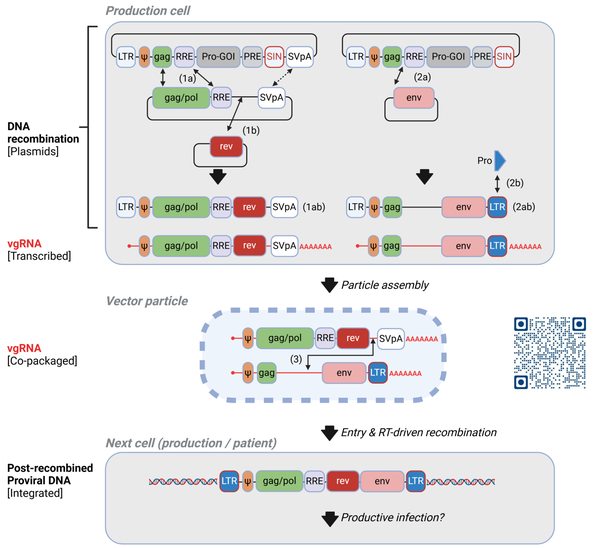
Use of human production cell lines (HEK293[T]) that do not highly express ERVs, as well as the employment of the self-inactivating (SIN) LTR, means that recombination and mobilisation of pre-cursor RCR/RCL-like entities during production is made extremely unlikely.

Conclusion: the reduction in sequence homology, the use of separate constructs and the SIN-LTR, and the implementation of HEK293[T] cells makes it extremely unlikely that RCR/RCLs will be generated from 3rd Gen RV/LV systems.

2 Modelling formation of a hypothetical 'minimal RCL' from a 3rd Gen LV system

- A minimal RCL must encode: active LTRs; a packaging signal; the RRE, and viral genes gagpol, rev and envelope.
- We propose a final step being RT-driven recombination between two co-packaged vgRNAs that provide these sequences.
- Therefore, two precursor vgRNA must be produced from two independent DNA cassettes derived from in-cell recombined pDNA.
- Our model indicates p = <1-in-10,000 per 200L bioreactor.

Modelling inter-pDNA and vector RNA recombination during LV production



Our minimal-path model suggests the formation of two independent DNA cassettes derived from serial inter-plasmid recombination in the same cell. The rates of recombination are based on published inter-plasmid recombination, where homology overlap is considered[1], as well as plasmid copy-number per nucleus[2]. The two precursor vgRNAs must compete with packaging of the LV product vgRNA into virions. The RT-driven recombination step rate is based on HIV-1 studies[3]. This model assumes nothing about the fitness of the single, integrated RCL genome molecule produced.

Using this model, an RCL Probability Calculator tool (at the QR code above) predicts that RCL formation will not be more likely than 1 in 10,000 LV production runs at 200L scale. Inputting numbers to reflect lower LV component copies, as for a packaging cell line, further reduces RCL probability by >100-fold. Use of 5-component LV systems (e.g. targeting and fusion proteins, instead of a single env) further reduces probability by >1000-fold.

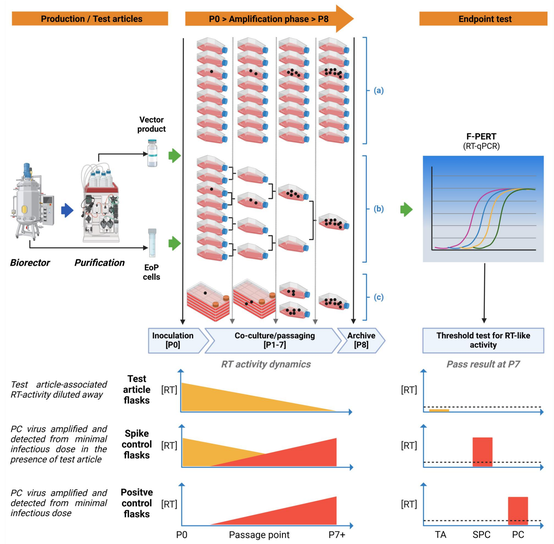
This model supports the existing excellent safety record of 3rd Gen RV/LVs, where no RCR/RCL has been reported to date. Use of well-characterized packaging/producer cell lines would further reduce risks, although we believe this model justifies reduced testing of clinical LV product generated by widespread transient production methods.

Conclusion: the probability of RCL formation from typical 4-component LV systems is extremely low, and we believe justifies the development of a roadmap to reduced RCL testing for clinical batch release.

3 RCL assay format for testing vector product and end-of-production cells (EoPCs)

- Current regulatory guidance requires testing of vector (3x patient doses) and $1x10^8$ EoPCs in separate assays.
- Standard format is to incubate the test article with permissive (amplification) cells across multiple flasks, plus controls.
- Assay handling challenges can be addressed by pooling or using larger vessels; RT signal from test article must be diluted away. A highly sensitive RT-qPCR endpoint is best.

Amplification in cell culture and RT-based endpoint test (F-PERT)



The above schematic shows a typical RCL assay amplification phase and endpoint assay tests for RT-activity, a necessary property of any retrovirus/RCL. The F-PERT (RT-qPCR) assay is extremely sensitive, requiring the RT activity associated with the LV product and EoPCs to be diluted away during the amplification phase to avoid false-positive results.

Challenges of assay scale (due to high amount of LV product to test) can be addressed by alternative culturing formats, including pooling (b and c). We recommend taking whole culture (viable cells and supernatant) archive samples at P0 and at endpoint, and small sampling throughout for potential troubleshooting.

To theoretically obtain RCL-infected cells within the $1x10^8$ EoPCs sampled from a typical 200L scale batch (with 95% confidence), the RCL must infect at least 30,000 EoPCs. (assumes $1x10^{12}$ total cells). At this level of infection, the RCL would readily be detected in the LV product RCL test, questioning the added value of testing EoPCs.

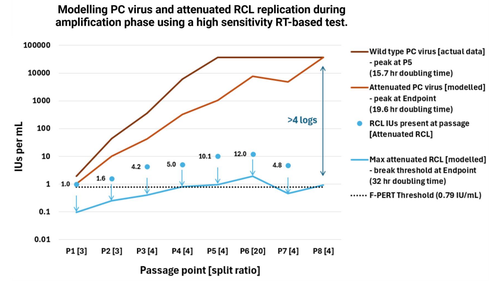
Testing of 3x patient doses will be challenging for high concentration, low-dose-per-batch *in vivo* therapies, and also ignores the underlying safety features shared by all 3rd Gen LV products. Our RCL formation model indicates that the same type and number of recombination events will need to occur, irrespective of the GOI and final application.

Conclusion: we propose a roadmap to reduced RCL testing including the removal of EoPC testing and testing of a fixed amount (vgRNAs) of LV product per clinical batch (similarly to Adenovirus RCA testing).

4 Assay sensitivity versus robustness: what's the right balance?

- Demonstrating assay sensitivity during qualification is important, but the final GMP assay also needs to be robust.
- An RT-based endpoint test (F-PERT) provides a large dynamic range (> 4 logs), meaning that a 'slow' replicating RCL will be detected above the threshold.
- A positive control virus (typically wild-type) that initiates infection from fewer particles better controls for potential inhibition.

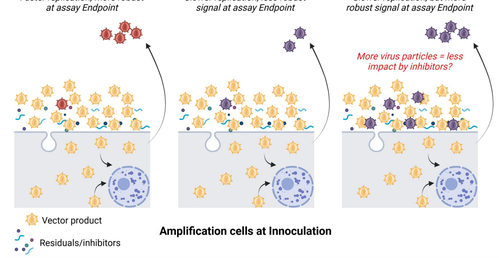
Modelling extremely attenuated RCL replication based on PC virus



Modelling replication of a wild-type (~16hr doubling; real data) or weakly attenuated (~20hr doubling; theoretical) PC virus indicates that after 8 passages the RT activity at endpoint is >4 logs greater than Threshold. An extremely attenuated RCL with 32hr doubling would still be detected in this assay format.

Conclusion: the assay can detect a retrovirus/RCL with any (realistic) replication kinetics i.e. genetic attenuation of PC virus is not important for the final GMP assay.

Wild-type vs attenuated PC virus: which is better at modelling inhibition?



An attenuated PC virus may not consistently yield sufficiently high RT signal above Threshold to enable a robust GMP assay. Simply increasing the dose at inoculation to avoid this risks masking inhibitors of PC virus and putative RCL at entry, since more attenuated PC virus particles will be present in the Spike control (co-inoculated with test article) compared to wild-type PC virus

Conclusion: having demonstrated during assay development/qualification that the assay can detect attenuated PC virus from minimal doses, the wild-type PC virus may be more appropriate to control for potential inhibitors of infection at inoculation in the final GMP assay.

QR code takes you to the RCL Probability calculator here: doi.org/10.5281/zenodo.15081753

[1]doi: 10.1073/pnas.83.14.5199 [2]10.1016/j.jconrel.2008.12.016 [3]10.1016/j.jmb.2011.01.052