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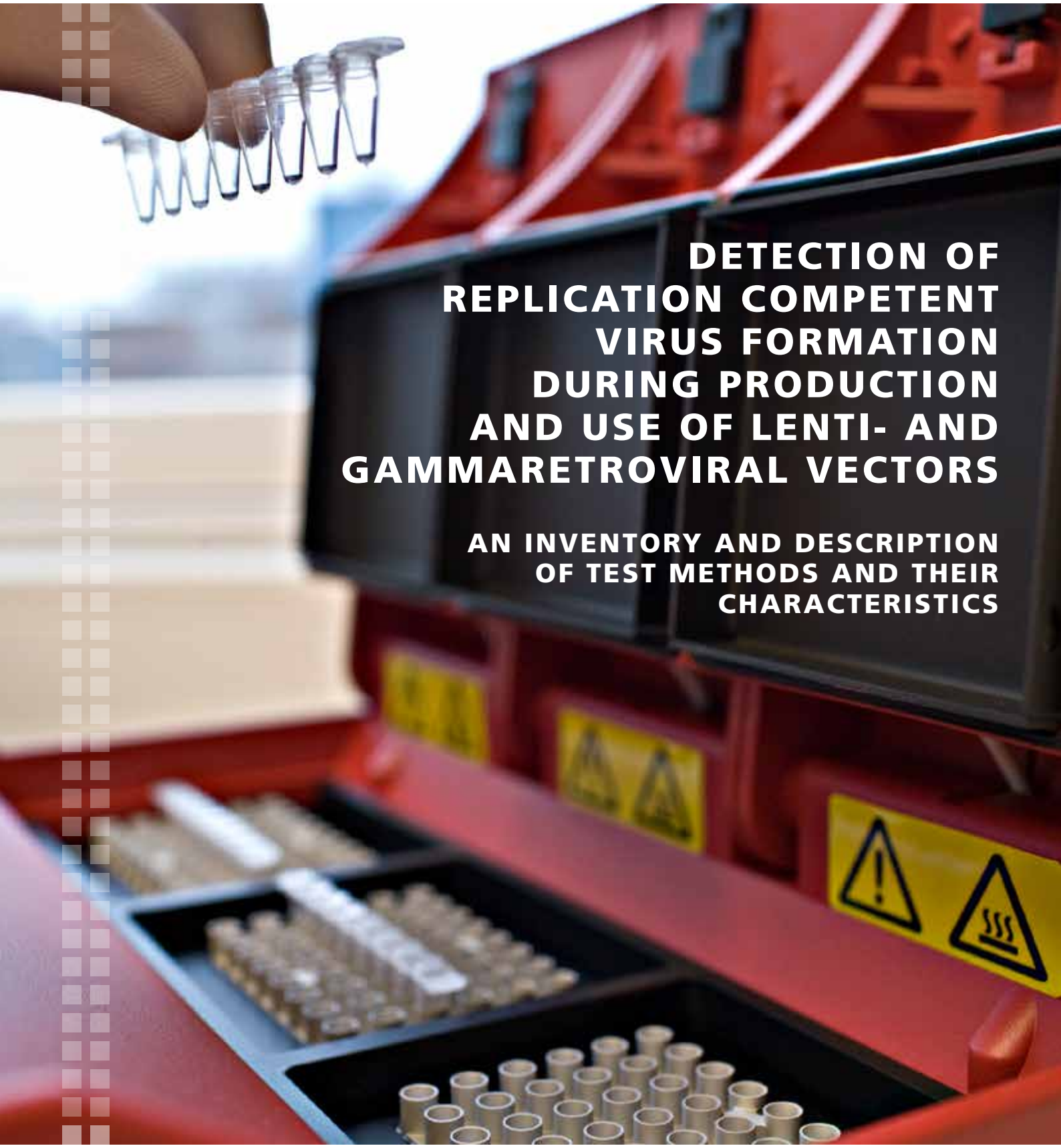

DETECTION OF REPLICATION COMPETENT VIRUS FORMATION DURING PRODUCTION AND USE OF LENTI- AND GAMMARETROVIRAL VECTORS

AN INVENTORY AND DESCRIPTION OF TEST METHODS AND THEIR CHARACTERISTICS

ONAFHANKELIJKE WETENSCHAPPELIJKE ADVISERING EN SIGNALERING VAN DE RIJKSOVERHEID

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Detection of replication competent virus formation during production and use of lenti- and gammaretroviral vectors

An inventory and description of test methods and their characteristics

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Foreword

Gene-transfer vectors derived from retroviruses are widely used as tool in cell biology and in experimental gene therapy. Their capacity to integrate their genetic information into the host cell genome makes the retroviral vectors very suitable for stable genetic modification of mammalian cells. To date vector systems are mainly derived from the murine gammaretroviruses (e.g. murine leukemiavirus, MuLV) and the human lentiviruses (e.g. HIV-1). The parental viruses are pathogenic: the gamma retroviruses are known as slow oncoviruses as they can disturb the expression of genes near their integration site in the genome, leading to cellular transformation and oncogenesis. The lentivirus HIV-1 is the causative agent of AIDS. Hence risk assessment before use of these systems is essential.

One of the risk factors associated with the use of these vectors is the presence of replication-competent retroviruses (RCV) in the replication-deficient retroviral vector preparations. Fortunately, for the production of retroviral vectors safe production systems are available, in which the generation of RCV during production is extremely unlikely. Nevertheless, tests for the presence of RCV in gammaretroviral or lentiviral vector batches are warranted, e.g. when the vectors are intended for use in clinical gene therapy procedures. While RCV tests are in use for several decades, new and in many cases more sensitive tests have become available. It should be clear that the requirements for RCV tests are strict with respect to sensitivity, specificity, and predictive value of the assay.

The COGEM commissioned a research project focused on RCV tests which had two aims. The first was to describe the state of the art of RCV testing based on a literature review. The second was to describe the current practice based on interviews with a number of stakeholders. The results of the work is presented in this report.

The advisory committee of this research project greatly appreciated the pleasant and constructive interaction with the authors and their efficient approach in this project. The committee is pleased with their broad approach and the comprehensive description of the results. This report may facilitate acquiring an up to date perspective on RCV testing for the COGEM and beyond.

Prof. Dr. Rob Hoeben

Chair of the Advisory Committee

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Summary

Taking their capacity for robust and stable integration into the host cell genome into account, vectors based on retroviruses are ideal candidates to deliver genetic information to host cells and to ensure that the inserted transgene is passed to the progeny of the infected cells. Lenti- and gammaretroviral vectors have therefore been used for many years in R&D and clinical applications.

In order to safely use a retrovirus as vector system, hazardous effects of the parental virus have to be eliminated. Viral vectors are therefore designed as such that they lack major virulence factors and are unlikely to recombine into fully replication competent viruses (RCV). An important goal in vector design is therefore to select only the necessary genetic elements and arrange them in such a way that the chance that they can come together and recombine into a RCV is negligible, ideally zero. Omitting specific viral functions, dividing functional genes over multiple plasmids, reducing sequence similarity, including self-inactivating properties in the vector and several other design features have been implemented to increase vector safety.

However, in theory, it is still conceivable that separated or missing viral sequences are reassociated by recombination or complementation. Such could occur during manufacturing of the vector, where there is a stage in which different viral genetic elements come together to assemble the vector (primary RCV), or in host cells during in vitro or in vivo application of the vector whereby related viruses and/or unrelated viruses present in the host provide for the missing viral sequences (secondary RCV).

In early retroviral vector production systems, the formation of RCV has been described. For more recent and safer vector production systems, however, there are no indications in scientific literature that replication competent lentivirus (RCL) or replication competent retrovirus (RCR) were formed either during manufacturing or after clinical application, provided production cells were free of retroviruses that can recombine with / complement the retroviral vector system. Still, as the absence of RCV is a key determining factor for reducing the risk classification of vectors, reliable testing for the absence of RCV is a prominent issue. Since 2000, The Netherlands Commission on Genetic Modification (COGEM) performed risk assessments for activities with lenti- and gammaretroviral vectors and provided recommendations for RCV testing. Hereby, the possibility to downscale the biosafety level of activities with the vectors in contained facilities (laboratories, animal facilities) was an important consideration of the risk assessment. In order to facilitate future assessments and potential downscaling**, this report provides an inventory of available RCV assays that are currently available, and summarizes their characteristics, (dis)advantages and validation.**

An extensive literature review was undertaken compiling scientific information related to RCV testing. The setup was done according to the typical consecutive steps of a systematic review and was based on a pre-specified protocol, including research question, search strategy, inclusion/exclusion criteria for the articles and methods for the analysis. Based on the scientific publications obtained, also an extensive secondary search was performed to retrieve additional relevant publications. Most publications presented applications of a previously set up RCV assay, referring to a common original paper in which that assay was initially described. Apart from the extensive literature review, additional risk considerations were identified in advices and recommendations previously issued by COGEM, as well as on the websites of (inter)national organizations related to (bio)safety, including but not limited to ZKBS, EBSA, ABSA, FDA and EMA.

In addition to researching published information, this study also aimed to include experience with and daily practices of RCV testing from different stakeholders. Such practical experience is important to put the findings from literature in perspective.

Based on the literature review, several RCV assays were identified. They include structural assays, based on the detection of structural components of RCV (e.g. ELISA, RT PCR, PERT), as well as functional assays, whereby the biological activity of an RCV is detected (e.g. marker rescue assay). A major point of attention for the structural assays is that detection of a specific parameter or signal does not necessarily correlates with or predicts the presence of RCV, since the structural components identified (e.g. genetic sequences, proteins) may also be present in replication-

deficient vector particles. As compared to structural assays, functional assays will only provide a positive outcome when RCVs are present since the biological function to be tested is not present in the replication-defective vector particle. Still, care is to be taken in view of interpretation as the biological function may be intrinsically present in the host cell or introduced via wild type retroviruses.

Amplification of the vector batch in cells prior to performing the structural assays is useful to increase the particle number of putative RCVs and decrease the number of replication-defective vector particles and consequently in increasing the sensitivity of an assay. Also, the amplification phase allows sampling at multiple time points, and thus study an increase/decrease of a parameter over time.

Irrespective of the assay used, a clear understanding of the limitations of the assays is important. However, when it comes to validation of the assays, data are limited and hard to compare. This was evident from the literature study as well as from the survey results. Furthermore, it became clear that RCV testing appeared to be more prominent in organizations where vectors are used in view of clinical application than in a more fundamental research environment. In the former environments, RCV testing of vector material (and of transduced medicinal products) is mandatory in view of patient safety. In contrast, in R&D labs vectors are mostly applied under contained used conditions. Still, RCV testing may be of added value in R&D labs to help decide on the downscaling the required containment for handling of transduced host cells or animals.

Based on the information collected in this study, following conclusions are made:

- The absence of RCV is a key determining factor for reducing the risk level of activities with lenti- or retroviral vectors. In that perspective, different safety features have been combined in the design of the vectors to make formation of RCV extremely unlikely. In addition, a variety of assays has been developed to test for the presence of RCV.
- For applications with (investigational) medicinal products consisting of, or generated with retro- or lentiviral vectors, RCV testing is in many cases mandatory, unless the competent authorities allow alternative approaches (e.g. a risk assessment based on vector design supporting that the likelihood for presence of RCV is negligible).
- In R&D environment, RCV testing is not performed routinely and only when deemed necessary based on a risk assessment.
- RCV assays are either based on structural elements or on functional aspects of RCV. Each assay has different advantages and disadvantages. Including an amplification phase prior to testing increases sensitivity for all assays since the amplification phase increases the number of RCVs and decreases the number of (interfering) vector particles. Amplification may be the only way to discern between RCV and vector particles, although it significantly increases the required time and effort.
- Advanced vectors are designed to reduce the likelihood of RCV formation, and the above-mentioned assays are intended to further substantiate the absence of RCV. As a consequence, in the absence of cases where RCV have been demonstrated, it is very difficult to determine the likelihood of the remaining -so far theoretical- risk.
- There remains uncertainty on the link between the outcome of an RCV assay on the one hand and the biological relevance in view of RCV formation on the other hand. Additional research on the biological relevance of an assay outcome and providing a robust set of validation data would be necessary to complete the framework for risk assessment of the likelihood of RCV formation and to determine the relevance of RCV testing.

Samenvatting

Vanwege hun vermogen tot robuuste en stabiele integratie in het genoom van de gastheercel, zijn lenti- en gammaretrovirale vectoren ideale kandidaten om genetische informatie aan gastheercellen over te dragen. Door de stabiele integratie wordt het ingebrachte transgen bovendien doorgegeven aan de volgende generatie cellen na celdeling. Bijgevolg worden lenti- en gammaretrovirale vectoren reeds vele jaren gebruikt in O&O en klinische toepassingen.

Om een retrovirus veilig als vectorsysteem te kunnen gebruiken, moeten de mogelijk schadelijke eigenschappen van het oudervirus worden geëlimineerd. Virale vectoren worden daarom zo ontworpen dat zij belangrijke virulentiefactoren missen en dat het onwaarschijnlijk is dat zij zullen recombineren tot een volledig replicatiecompetent virus (RCV). Belangrijk hierbij is dat de vectoren alleen de noodzakelijke genetische elementen behouden en dat deze zodanig gerangschikt worden dat de kans dat zij kunnen samenkomen en recombineren tot een RCV verwaarloosbaar klein wordt, idealiter nul. Het weglaten van specifieke virale functies, het verdelen van functionele genen over meerdere plasmiden, het verminderen van de sequentie-overeenkomst, het opnemen van zelf-inactiverende eigenschappen in de vector en verschillende andere ontwerpkenmerken zijn toegepast om de veiligheid van de vector te vergroten.

In theorie is het echter nog steeds denkbaar dat gescheiden of ontbrekende virale sequenties opnieuw worden geassocieerd door recombinatie of complementatie. Dit kan gebeuren tijdens de productie van de vector, waarbij verschillende virale genetische elementen samen worden gebracht om de vector te assembleren (primaire RCV), of in gastheercellen tijdens *in vitro* of *in vivo* toepassing van de vector, waarbij verwante en/of niet-verwante virussen in de gastheer de ontbrekende virale sequenties aanvullen (secundaire RCV).

Voor vroege retrovirale vectorproductiesystemen is de vorming van RCV beschreven. Voor recentere en meer veilige vectorproductiesystemen zijn er in de wetenschappelijke literatuur echter geen aanwijzingen dat replicatiecompetent lentivirussen (RCL) of replicatiecompetent retrovirussen (RCR) werden gevormd, noch tijdens de productie, noch na de klinische toepassing, op voorwaarde dat de productiecellen vrij waren van retrovirussen die kunnen recombineren met het retroviraal vectorsysteem of dit kunnen complementeren. Aangezien de afwezigheid van RCV een belangrijke bepalende factor is voor de risicoclassificatie van vectoren, is betrouwbaar testen op de afwezigheid van RCV nog steeds een belangrijke kwestie. Sinds 2000 heeft de Nederlandse Commissie voor genetische modificatie (COGEM) risicobeoordelingen uitgevoerd voor activiteiten met lenti- en gammaretrovirale vectoren en aanbevelingen gedaan voor RCV-testen. Hierbij was de mogelijkheid om het bioveiligheidsniveau van activiteiten met de vectoren in ingeperkte faciliteiten (laboratoria, dierfaciliteiten) naar beneden bij te stellen een belangrijke overweging van de risicobeoordeling. Om toekomstige beoordelingen en mogelijke omlaagschaling te vergemakkelijken, **geeft dit rapport een inventaris van de momenteel beschikbare tests voor RCV's en geeft het een overzicht van hun kenmerken, voor-/nadelen en validatie.**

Er werd een uitgebreid literatuuronderzoek uitgevoerd om wetenschappelijke informatie in verband met RCV-testen te verzamelen. De opzet verliep volgens de typische opeenvolgende stappen van een systematische review en was gebaseerd op een vooraf gespecificeerd protocol, met inbegrip van de onderzoeksvraag, de zoekstrategie, de in- en exclusiecriteria voor de artikelen en de methoden voor de analyse. Op basis van de verkregen wetenschappelijke publicaties werd ook een uitgebreide secundaire zoekactie uitgevoerd om aanvullende relevante publicaties te vinden. In de meeste publicaties werden toepassingen van een eerder opgezette RCV-test gepresenteerd, waarbij werd verwezen naar een gemeenschappelijk origineel artikel waarin die test aanvankelijk werd beschreven. Naast de uitgebreide literatuurstudie werd naar aanvullende risico-overwegingen gezocht in adviezen en aanbevelingen die eerder door de COGEM zijn uitgebracht, alsmede op de websites van (inter)nationale organisaties op het gebied van (bio)veiligheid, waaronder maar niet beperkt tot ZKBS, EBSA, ABSA, FDA en EMA.

Naast het onderzoek van gepubliceerde informatie werd in deze studie ook getracht de ervaring met en de dagelijkse praktijk van RCV-testen van verschillende belanghebbenden in aanmerking

te nemen. Dergelijke praktijkervaring is belangrijk om de bevindingen uit de literatuur in perspectief te plaatsen.

Op basis van het literatuuronderzoek werden verschillende RCV-testen geïdentificeerd. Deze omvatten zowel structurele testen, gebaseerd op de detectie van structurele componenten van RCV's (bv. ELISA, RT PCR, PERT), als functionele testen, waarbij de biologische activiteit van een RCV wordt gedetecteerd (bv. de “marker rescue assay”). Een belangrijk aandachtspunt voor de structurele testen is dat de detectie van een specifieke parameter of een specifiek signaal niet noodzakelijk correleert met de aanwezigheid van RCV, aangezien de geïdentificeerde structurele componenten (bv. genetische sequenties, eiwitten) ook aanwezig kunnen zijn in replicatie-deficiënte vectordeeltjes. In vergelijking met structurele tests zullen functionele tests alleen een positief resultaat opleveren wanneer er RCV aanwezig zijn, aangezien de te testen biologische functie niet aanwezig is in het replicatie-deficiënte vectordeeltje. Toch is voorzichtigheid geboden bij de interpretatie, aangezien de biologische functie intrinsiek aanwezig kan zijn in de gastheercel of geïntroduceerd kan zijn via retrovirussen van het wilde type.

Amplificatie van de vectorbatch in cellen voorafgaand aan de uitvoering van de structurele testen is nuttig om het aantal vermoedelijke RCV's te verhogen en het aantal replicatie-deficiënte vectordeeltjes te verminderen, en bijgevolg om de gevoeligheid van een test te verhogen. Ook maakt de amplificatiefase bemonstering op meerdere tijdstippen mogelijk, zodat een toename/afname van een parameter in de tijd kan worden bestudeerd.

Ongeacht de aard van de gebruikte test is een duidelijk inzicht in de beperkingen van de testen belangrijk. Wat de validering van de testen betreft, zijn de gegevens echter beperkt en moeilijk te vergelijken. Dit bleek zowel uit de literatuurstudie als uit de enquêteresultaten. Bovendien werd duidelijk dat RCV-testen meer op de voorgrond traden in organisaties waar vectoren worden gebruikt met het oog op klinische toepassing dan in een meer fundamentele onderzoeksomgeving. In de eerste omgeving zijn RCV-tests van vectormateriaal (en van getransduceerde geneesmiddelen) in veel gevallen verplicht met het oog op de veiligheid van de patiënt. In onderzoeks- en ontwikkelingslaboratoria daarentegen worden vectoren meestal toegepast onder ingeperkt gebruiksomstandigheden. Toch kunnen RCV-testen in O&O-laboratoria van toegevoegde waarde zijn om te helpen beslissen over het verlagen van de vereiste inperking voor het hanteren van getransduceerde gastheercellen of -dieren.

Op basis van de in deze studie verzamelde informatie worden de volgende conclusies getrokken:

- De afwezigheid van RCV’s is een doorslaggevende factor om het risiconiveau van activiteiten met lenti- of retrovirale vectoren te verlagen. In dit verband zijn bij het ontwerp van de vectoren verschillende veiligheidskenmerken gecombineerd om de kans op vorming van RCV’s uitermate onwaarschijnlijk te maken. Bovendien is een verscheidenheid van testen ontwikkeld om na te gaan of RCV’s al dan niet gevormd werden.
- Voor onderzoek met of toepassingen van (nieuw ontwikkelde) geneesmiddelen die bestaan uit of worden gegenereerd met retro- of lentivirale vectoren, zijn RCV-testen in veel gevallen verplicht, tenzij de bevoegde autoriteiten alternatieve benaderingen toestaan (bv. een risicobeoordeling op basis van het vectorontwerp waaruit blijkt dat de kans op aanwezigheid van RCV verwaarloosbaar klein is).
- In een O&O-omgeving worden RCV-testen niet routinematig uitgevoerd en alleen wanneer ze op basis van een risicobeoordeling noodzakelijk worden geacht.
- RCV-testen zijn ofwel gebaseerd op structurele elementen of op functionele aspecten van RCV. Elke test heeft zijn specifieke voor- en nadelen. Het uitvoeren van een amplificatiefase vóór het testen verhoogt de gevoeligheid voor alle testen aangezien de amplificatiefase het aantal RCV's verhoogt en het aantal (interfererende) vectordeeltjes verlaagt. Amplificatie kan de enige manier zijn om onderscheid te maken tussen RCV's en vectordeeltjes, hoewel het de vereiste tijd en inspanning aanzienlijk verhoogt.

- Geavanceerde vectoren zijn ontworpen om de kans op de vorming van RCV's te verkleinen. Op basis van de huidig beschikbare testen werden voor deze vectoren nog geen RCV's aangetoond. Bijgevolg is het onmogelijk om de waarschijnlijkheid van het resterende - tot dusver theoretische - risico op RCV's te bepalen.
- Er blijft onzekerheid bestaan over het verband tussen de uitkomst van een RCV-test enerzijds en de biologische relevantie met het oog op de vorming van RCV's anderzijds. Aanvullend onderzoek naar de biologische relevantie van een testuitslag en het verstrekken van een robuuste reeks valideringsgegevens kunnen een belangrijke aanvulling vormen om het kader voor de risicobeoordeling van de waarschijnlijkheid van RCV-vorming te vervolledigen en de relevantie van RCV-testen verder te verfijnen.

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Abbreviations

ABSA	American Biological Safety Association
AIDS	Acquired Immunodeficiency Syndrome
BSD	Blasticidin
COGEM	Commissie Genetische Modificatie Netherlands Commission on Genetic Modification
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBSA	European Biosafety Association
ELISA	Enzyme-Linked Immuno Sorbent Assay
EM	Electron microscopy
env	Envelope protein gene
EOP	End-of-production
FDA	U.S. Food and Drug Administration
FeLV	Feline Leukemia Virus
gag	Polyprotein gene (acronym for Group Antigens) encoding matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins
GaLV	Gibbon ape Leukemia Virus
GFP	Green fluorescent protein
HIV	Human Immunodeficiency Virus
HTLV	Human T-lymphotropic virus
ICH	International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IN	Integrase
IND	Investigational New Drug
LoD	Limit of detection
LTR	Long Terminal Repeat
MCB	Master Cell Bank
MOI	Multiplicity of Infection
MoMLV	Moloney murine leukemia virus
MLV	Murine Leukemia Virus
MSV	Murine Sarcoma Virus
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
NLM	U.S. National Library of Medicine
PCR	Polymerase chain reaction
PERT	Product Enhanced Reverse Transcriptase
pol	Polyprotein gene encoding protease (PR), reverse transcriptase (RT) and integrase (IN)
PR	Protease
qPCR	Quantitative PCR
qRT- PCR	Quantitative reverse transcriptase PCR
RCL	Replication Competent Lentivirus
RCR	Replication Competent Retrovirus
RCV	Replication Competent Virus
rev	Regulator of viral expression
RNA	Ribonucleic acid
RRE	Rev response element

RT	Reverse transcriptase
SG-PERT	SYBR Green I-based PERT assay
SIN	Self-inactivating
tat	Transcriptional transactivator
TMB	Tetramethyl Benzidine
VSV-G	Spike glycoprotein of the Vesicular Stomatitis Virus
WHO	World Health Organization
wPRE	Woodchuck posttranscriptional regulatory element
ZKBS	Zentrale Kommission für die Biologische Sicherheit Central Committee on Biological Safety

Introduction

Taking their capacity for robust and stable integration into the host cell genome into account, vectors based on retroviruses are ideal candidates to deliver genetic information to host cells and to ensure that the inserted transgene is passed to the progeny of the infected cells. In order to safely use a retrovirus as vector system, hazardous effects of the parental virus had to be eliminated. Viral vectors are therefore designed as such that they lack major virulence factors and are unlikely to recombine into fully replication competent viruses (RCV). An important goal in vector design is to remove and arrange only the necessary genetic elements in such a way that the chance that they can come together and recombine into a RCV is reduced, ideally to zero.

However, in theory, it is still conceivable that separated or missing viral sequences are reassociated by recombination or complementation. Such could occur during manufacturing of the vector, where there is a stage in which different viral genetic elements come together to assemble the vector (primary RCV), or in host cells during in vitro or in vivo application of the vector whereby related viruses and/or unrelated viruses present in the host provide for the missing viral sequences (secondary RCV). To document the efficiency of a design strategy and to substantiate the absence of RCVs, reliable testing is of utmost importance.

Since 2000, The Netherlands Commission on Genetic Modification (COGEM) performed risk assessments for activities with lenti- and gammaretroviral vectors and provided recommendations for RCV testing. Hereby, the possibility to downscale the biosafety level of activities with the vectors in contained facilities (laboratories, animal facilities) was an important consideration of the risk assessment. In order to facilitate future assessments, the potential downscaling, in particular during production of vector, and to evaluate the relevance of RCV testing, this study gathered information on available RCV assays, both in literature and in practice, and reviewed their characteristics, (dis)advantages and validation.

1 Methods

1.1 Literature study

A literature review was undertaken compiling information related to the test methods that are currently being used to detect replication competent viruses in lenti- and gammaretroviral vector preparations.

In order to formulate **search strings**, the following keywords were selected:

- Vector / Lentivirus / Gammaretrovirus
- Replication competent / RCV
- Detection / Test / Assay

After selecting keywords, a typical search string was composed using Boolean operators: vector* AND ("replication competent" OR RCV) AND (lentivir* OR gamma*retrovir*) AND (detect* OR assay* OR test* OR assess* OR evaluat*) NOT ("secondary replication competent" OR "secondary RCV").

Three electronic bibliographic multi-disciplinary databases were chosen to **search for relevant publications**:

- Web of Science™ core collection[1]
- Web of Science™ core collection consists of six online databases indexing scholarly books, peer reviewed journals, original research articles, reviews, editorials, chronologies, abstracts, as well as other items. Disciplines included in this index are agriculture, biological sciences, engineering, medical and life sciences, physical and chemical sciences, and many others. The database contains 1.4 billion cited references going back to 1900.
- Scopus®[2]
- Scopus® by Elsevier is an abstract and citation database of peer-reviewed literature, including scientific journals, books and conference proceedings, covering research topics across all scientific and technical disciplines, ranging from medicine and social sciences to arts and humanities. Scopus® is updated daily and includes over 71 million records and over 1.4 billion cited references after 1970.
- PubMed®[3].
- PubMed® is a free resource supporting the search and retrieval of biomedical and life sciences literature with the aim of improving health, both globally and personally. The PubMed database contains more than 33 million citations and abstracts of biomedical literature. Available to the public online since 1996, PubMed was developed and is maintained by the National Center for Biotechnology Information (NCBI), at the U.S. National Library of Medicine (NLM), located at the National Institutes of Health (NIH).

Each search was expected to result in the identification of publications in English or, if in another language, having a title, abstract and/ or keywords in English. The searches were not limited in time. Initially, only original articles were searched for. In case the number of articles on a specific topic was large, relevant review articles were consulted.

In the first stage of **selection**, the title, keywords and abstract of the retrieved references were screened. This resulted in a reduction of the number of potentially relevant publications. Of the selected references an attempt was made to retrieve a full text document, after which the full content was examined. The references of the included studies were manually screened to search for further papers. No language or publication restrictions were applied, and studies were not selected based on quality.

The full text documents were further screened for relevant data and the final set of publications was selected. The **key findings** of the selected, full text papers were then summarized including, but not limited to, information on the vector system, its application, potential assays used to detect RCV,

[1] https://clarivate.com/products/web-of-science/databases/
[2] https://www.scopus.com
[3] https://pubmed.ncbi.nlm.nih.gov

available data to support the validity of the RCV assay, as well as the outcome of the RCV assay used.

The literature search was concluded on February 16, 2022. Additional publications were retrieved until end of May 2022, and were based on publications referenced in the primary literature study, on references in COGEM advices, and on internet searches using terms relevant for the current study. In total, approximately 100 publications were consulted. Most publications presented applications of a previously set up RCV assay, referring to a common original paper in which that assay was initially described.

1.2 Survey of advisory documents and recommendations

Further insight in RCV test methods and their application was obtained through an analysis of **advices and recommendations previously issued by COGEM**. For that, the COGEM website [4] was consulted. In total, 34 documents were identified since 2000 (see Section 5.1.1).

Additionally, the website of the US Food & Drug Administration [5], the European Pharmacopoeia [6], and European Medicines Agency (EMA) [7] were consulted. Following sources were identified as specifically referring to RCV test methods:

- EMA 'Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products' (CPMP/BWP/3088/99) (EMA, 2001)
- EMA 'Guideline on development and manufacture of lentiviral vectors' provides examples of suitable RCL testing (EMEA, 2005)
- EMA 'Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products' (EMA, 2018)
- EMA 'Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells' (EMA, 2020).
- European Pharmacopoeia Chapter 5.14, Gene transfer medicinal products for human use (Ph. Eur., 2019)
- FDA Guidance document for Industry 'Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up' (Version January 2020) (FDA, 2020)

Finally, advices and recommendations were searched on the websites of **(inter)national organizations related to (bio)safety** using the search terms as described in Chapter 1.1. Organizations included but were not limited to the European Biosafety Association (EBSA), ABSA, National Institutes of Health (NIH), ZKBS, and WHO. Only one document was identified as specifically referring to RCV testing ("General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability: Gene transfers using retroviral vectors" (ZKBS, 2011)).

1.3 Survey amongst stakeholders

A survey was made to gain information about:

- vectors used;
- activities performed with the vectors;
- RCV testing and the validation of the assays used.

Survey participants included scientist using retroviral vectors in the context of R&D, pharmaceutical companies applying vectors to generate medicinal products containing genetically modified cells, as well as service providers that either produce vectors or perform RCV testing as a service. Participants were selected based on contacts within the network of Perseus as well as contacts suggested by the

[4] https://cogem.net/
[5] https://www.fda.gov
[6] https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition-
[7] https://www.ema.europa.eu/en

Advisory Committee. The questionnaire was distributed to participants by email in April - May 2022. If no response was obtained within 1 month, a reminder was sent. The survey was closed on June 21, 2022.

Survey data were collected and analyzed. Where additional information was required, participants were contacted either by phone or email. Data were grouped based on the inquiries as mentioned above.

While the data collected from various stakeholders did not include personal or sensitive information, the collection and processing took place in accordance with the indications of Regulation (EU) 2016/679 of the European Parliament and of the Council on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (General Data Protection Regulation).

1.4 Expert consultations

The study was further supported by scientific guidance and critical review by Prof. Dr. R. Gijsbers (Molecular Virology and Gene Therapy - Laboratory for Vectorology and Gene Therapy, KU Leuven) and Dr. C. Van den Haute (Molecular Virology and Gene Therapy - Research Group for Neurobiology and Gene Therapy, KU Leuven). In addition, the Advisory Committee provided useful suggestions on studies and publications.

2 Retroviruses

2.1 Virus structure

Lenti- and gammaretroviruses belong to the family *Retroviridae*, genus lentivirus and gammaretrovirus respectively. Below, the structure of HIV, a representative member of the genus lentivirus, is described. It consists of a lipid bilayer membrane or envelope that surrounds the nucleocapsid (Figure 1). Within the capsid, two copies of the genomic RNA are present. During reverse transcription, catalyzed by the retroviral polymerase, a double-stranded DNA intermediate is formed, which then stably integrates into the genome of the infected cell (provirus).

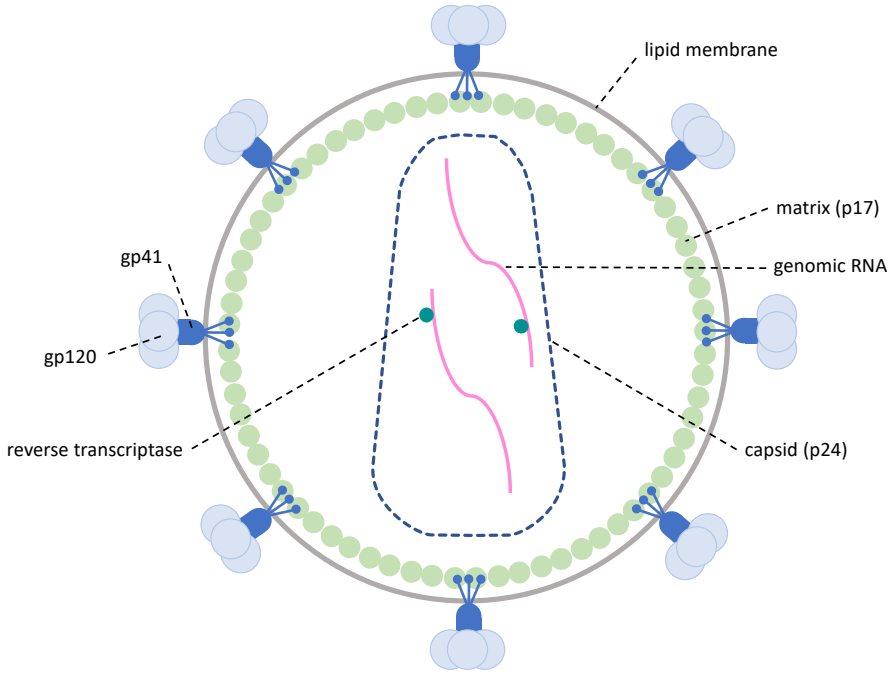

Figure 1. Schematic representation of an HIV particle, a representative member of the *Retroviridae*

Based on: http://microbialcell.com/figure-2-hiv-aids-pandemic/

The **HIV** genome is about 10 kb long and can be divided into three major open reading frames: *gag*, *pol* and *env* (Figure 2a). The *gag* gene encodes the group-specific antigen, which is proteolytically processed into four structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The *pol* gene encodes for a polyprotein which is processed during virion maturation into three enzymes, i.e. protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene is translated into the envelope protein(s) which in lentiviruses includes the surface (gp120) and transmembrane (gp41) proteins. The genome also contains long terminal repeats (LTR) being repetitive sequences that occur at both termini of the integrated DNA provirus and harbor the enhancer, promoter and poly-adenylation signals.

Apart from the proteins mentioned above, the lentiviral genome also encodes six additional or accessory proteins. These include Vif, Vpr, and Nef that are found in the viral particle, the transcriptional transactivator Tat and the regulator of viral expression Rev, the latter two having essential gene regulatory functions, and the Vpu protein involved in the assembly of the viral particle.

The genome of the **murine gammaretrovirus**, a representative member of the genus gammaretrovirus, is simpler than that of HIV. It contains the LTRs and the major open reading frames *gag*, *pol* and *env* (Figure 2b). However, it lacks the genetic sequences coding for the accessory proteins.

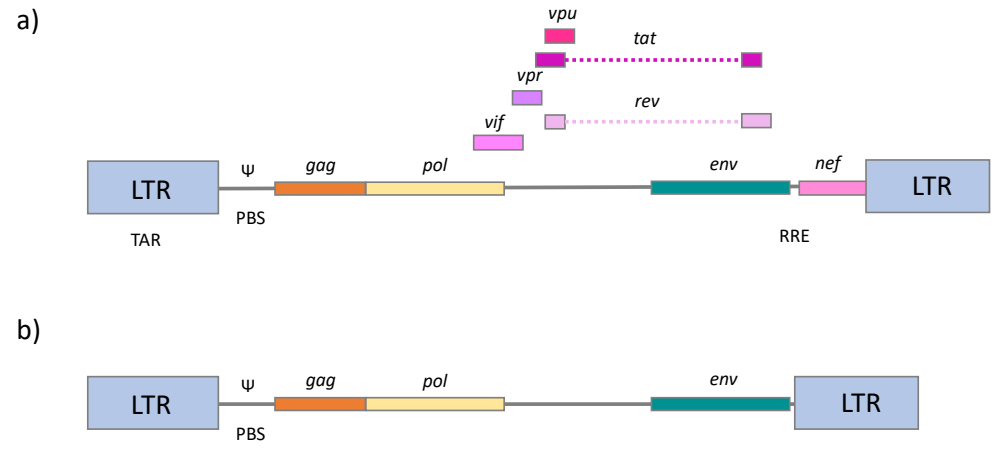

Figure 2. Schematic representation of the integrated retroviral genome

PBS (primer binding site) denotes the binding site for the tRNA primer; Ψ marks the packaging signal; gag (group specific antigens, matrix, capsid and nucleocapsid proteins), pol (protease, reverse transcriptase and integrase) and env (envelope proteins) are coding regions; LTR (long terminal repeats) contain repetitive sequences that occur at both termini of the integrated DNA provirus

(a) Complex retrovirus using the example of human immunodeficiency virus (HIV); In addition to the regulatory elements such as LTR, packaging signal Ψ, PBS, the trans-activation response element (TAR) and the rev response element (RRE) as well as the *gag*, *pol* and *env* genes, HIV has further reading frames encoding regulatory proteins

(b) Murine leukemia virus (MLV)

Based on: General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability: Stable and transient gene expression using γ-retroviral and lentiviral vectors. (ZKBS, 2011)

2.2 Virus replication

A schematic picture of the retroviral replication cycle is shown in Figure 3.

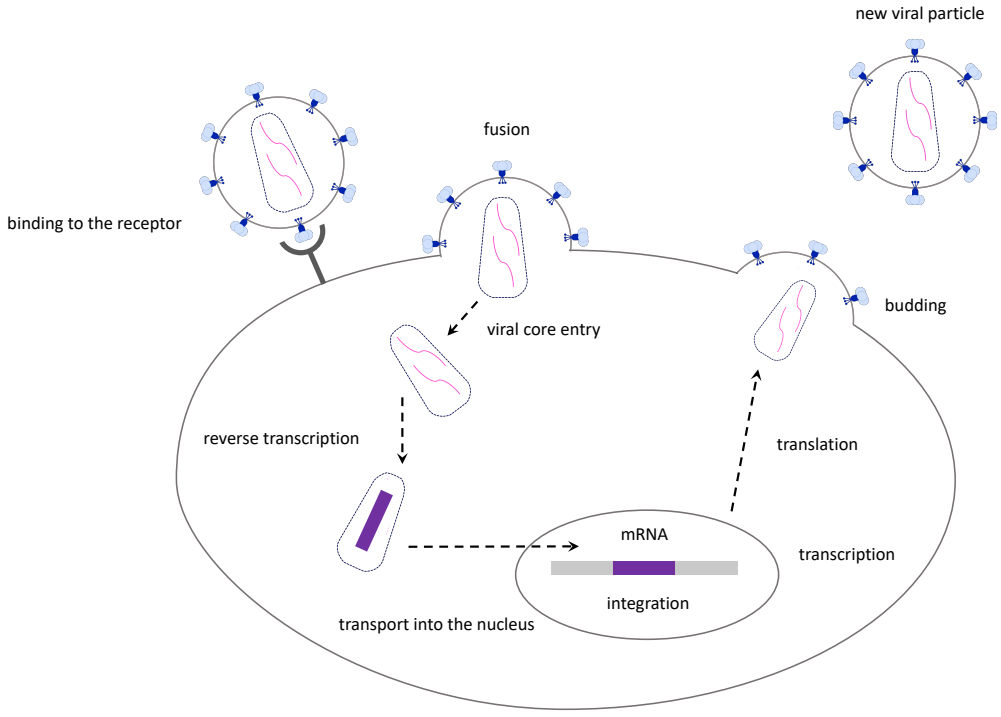

Figure 3. Schematic representation of the retroviral replication cycle

Based on: Shalev et al. (2009)

The retroviral replication cycle starts with binding of the virus to the host cell surface. This binding is receptor mediated and involves the envelop proteins of the retroviral particle on the one hand and host cell receptors on the other hand. The capsid then enters the cell, either by fusion of the viral and cellular lipid membranes or by transition through the endosome. Subsequently, the capsid partially disintegrates and the retroviral protein called reverse transcriptase transcribes the viral RNA into a double stranded DNA intermediate. The viral DNA is transported across the nucleus, where the retroviral protein integrase integrates the viral DNA into the host's DNA. The host's normal transcription machinery transcribes viral DNA into multiple copies of new viral RNA. Part of this RNA forms the genome of new viral particles, whereas other copies are used by the cell to make new retroviral proteins. The new viral RNA and proteins assemble to form an immature viral particle that leaves the cell by budding. The retroviral protease finally cleaves newly synthesized polyproteins to form a mature infectious virus particle.

Whereas lentiviruses are capable of productively infecting both dividing and non-dividing cells, productive gammaretrovirus infection is limited to dividing cells.

2.3 Risk group classification

Most applications of lentiviral vector systems are derived from HIV, the causative agent of acquired immunodeficiency syndrome (AIDS), a severe disease in humans. Taking into account the pathogenicity of the virus, the bloodborne route of transmission, a variety of treatments, and the lack of prophylaxis, COGEM classified the virus as a risk group 3 agent in accordance with EU legislation [8].

For retroviral vector development, vectors based on murine leukemia virus (MLV) are the most frequently used. The host range of MLV is dependent on the specificity of the viral envelope. Ecotropic MLV infect only rodent cells, whereas xenotropic and amphotropic MLV can infect rodent and non-rodent cells, including human cells. MLVs are generally benign at the cellular level, but they can induce lymphomas and neurological diseases in mice. The virus is classified as risk group 2 for animals (COGEM, 2021). Although at some point, a correlation was reported between xenotropic MLV and chronic fatigue syndrome as well as prostate cancer in humans (Lombardi et al., 2009; Urisman et al., 2006), later studies clearly indicated that the detection of the xenotropic MLV was due to contaminated samples and was not a marker of or a causal factor in prostate cancer or CFS (e.g., Johnson and Cohn, 2016; Panelli et al., 2017).

As other gammaretroviruses such as feline leukemia virus (FeLV) and gibbon ape leukemia virus (GaLV) are uncommon in vector development, they are not further discussed.

[8] Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (https://eur-lex.europa.eu/eli/dir/2000/54/2020-06-24)

3 Lenti- and retroviral vectors

3.1 Introduction

Taking their capacity for robust and stable integration into the host cell genome into account, vectors based on retroviruses are ideal candidates to deliver genetic information to host cells and to ensure that the inserted transgene is passed to the progeny of the infected cells. However, in order to safely use a retrovirus as vector system, hazardous effects of the parental virus have to be eliminated. Viral vectors are therefore designed as such that they lack major virulence factors and are unlikely to recombine into fully replication competent viruses (RCV). The sections below will focus on the design elements that aim to reduce the likelihood of RCV formation. Other design features are only touched upon briefly.

3.2 Basic components of lenti- and retroviral vectors

Vectors based on lenti- or gammaretroviruses require three components: (1) a transfer plasmid similar to a retroviral provirus with the gene of interest but lacking the sequences coding for the viral proteins, (2) (retro)viral structural proteins to form virus-like particles, and (3) retroviral nonstructural proteins for reverse transcription and integration of the gene of interest into the genome of a target cell. The latter two can either be delivered via trans-acting helper plasmids or via stably transfected cells. Bringing together the three elements in a vector producing cell will result in the production of replication-deficient vector particles, that in turn can be used to transduce [9] a host cell of interest. However, since a packaging signal is only included in the transfer plasmid, the replication deficient vector particles will only contain the genetic information of this plasmid, but lack genetic information encoding for other viral structures. Consequently, the vector particles are not able to generate infectious progeny. A schematic overview of vector components and production is summarized in Figure 4.

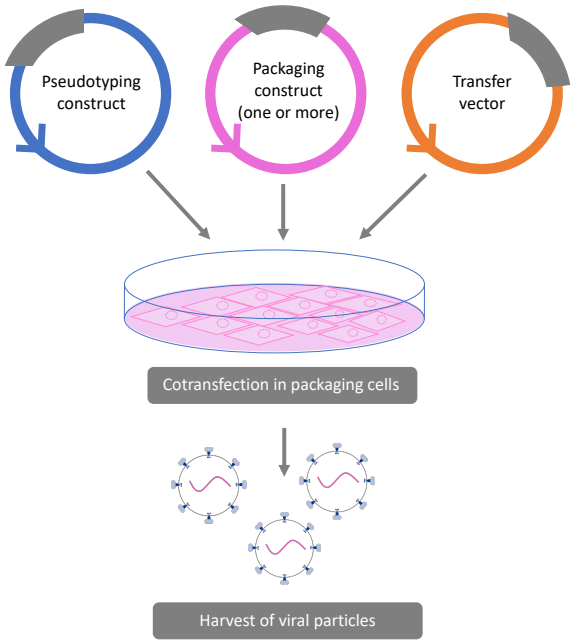

Figure 4. Vector components and production of vector particles

[9] Transduction meaning the transfer of genetic material from one (micro)organism to another by a viral agent

3.3 Replication competent viruses

The main principle in the above-mentioned vector design is to avoid that the different genetic elements can recombine into a replication competent virus. In theory, it is however conceivable that separated or missing viral sequences are reassociated by recombination.

First, in the manufacturing of lenti- and retroviral vectors there is a stage in which different viral genetic elements come together to assemble the vector. In that stage, there is a potential risk for recombination with the formation of **'primary' RCV**. Primary RCV formation could occur through homologous or nonhomologous recombination between the transfer vector, packaging components and/or 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 (Chong et al., 1998; FDA, 2020; Garrett et al., 2000). Retroviral elements could be provided by e.g. wild type HIV, wild type gammaretrovirus, or, in theory, another similar virus such as human T-lymphotropic virus (HTLV) present in the genome of the production cell.

It is worth noticing that murine cells used for production of gammaretroviral vectors frequently contain endogenous retroviral sequences that show similarity to the murine-based gammaretroviral vectors (summarized in CGM/210218-01 [10]). Recombination between the introduced exogenous retroviral vector sequences and the endogenous retroviral sequences may give rise to primary 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 line with this, regulators require that the Master Cell Bank (MCB) and/or cells cultured up to or beyond the limit of *in vitro* cell age [11, 12], which are used for production of retroviral vectors is tested for endogenous retroviral sequences. For gammaretroviral vectors, using vector producing cells of a non-murine species will help to reduce the likelihood of this type of recombination (Cosset et al., 1995; Forestell et al., 1997; Ghani et al., 2007). For example, in human cell lines, presence of (nearly) intact endogenous gammaretroviruses can be excluded and RCR have never been reported in cells, even when they contained endogenous gammaretroviral sequences (summarized in CGM/210218-01)

Second, recombination can occur in host cells during *in vitro* or *in vivo* application of the vector whereby related viruses and/or unrelated viruses present in the host provide for the missing viral sequences. The formation of such **secondary RCV** is outside the scope of the current study.

3.4 Vector design as a means to reduce the likelihood of recombination

The likelihood for recombination can be reduced by several design approaches. Main approaches include separation of genetic elements, reducing sequence similarity, introduction of a self-inactivating (SIN) property, and regulation at translational and posttranslational level. Additionally, genetic modification can be used to affect cell tropism. Each of the approaches are discussed in more detail below. Design approaches other that those focused on limiting the likelihood of RCV are outside the scope of the current study.

Additionally, it is highlighted that apart from the design approaches, also the means of production may affect the likelihood of RVC formation. In brief, for lentiviral vectors, mostly batch-wise production systems based on transient expression achieved by plasmid transfection are used. In contrast, production systems for gammaretroviral vectors are frequently based on stably transfected cell lines. The latter type of production system is likely to provide a much longer window of opportunity for RCV generation and expansion.

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[10] CGM/210218-01: Heroverweging inschaling werkzaamheden met replicatiedeficiënte lenti- en gammaretrovirale vectordeeltjes onder Ingeperkt Gebruik (https://cogem.net/app/uploads/2021/02/210218-01-Advies-Inschaling-werkzaamheden-RD-lenti-en-retrovirale-vectordeelties-onder-IG.pdf)

[11] A collection of cells of uniform composition derived from a single source prepared under defined culture conditions (FDA Guidance for Industry - Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications)

[12] ICH Topic Q 5 A (R1) Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (CPMP/ICH/295/95)

3.4.1 Separation of genetic elements

As a rule of thumb, when more recombinations are required to obtain a complete viral genome, the likelihood of RCV formation is lower (unless the transgene would affect the recombination ability) and thus the probability that a fully replication competent virus can emerge will be lower. Therefore, vector design aims at separating the trans-acting sequences onto more than one expression cassette.

Over time, different production systems have been described for **lentiviral vectors** in order to obtain a separation of genetic elements and thus a reduced probability of recombination (Table 1).

For lentiviral vectors generated with a production system of the first generation, the following plasmids are typically used:

- a packaging construct containing the genes encoding for the structural, accessory and regulatory genes, except *env*,
- a transfer vector similar to a retroviral provirus with the foreign gene to be transferred as well as the LTRs and a packaging signal, and
- a packaging construct containing a gene encoding a heterologous envelop such as VSV-G.

Provided that the gene encoding HIV Env is no longer present in the vector particle after assembly, no mature HIV virus particle can be formed. In the packaging construct, psi is deleted to avoid integration of the structural, accessory and regulatory genes in the assembled virus particle. The 5' and 3' LTR sequences have been replaced with a CMV promoter and a polyadenylation signal, respectively.

When using a production system of the second generation, not only *env*, but also the genes encoding the accessory factors of Nef, Vif, Vpr and Vpu have been deleted from the packaging construct (Zufferey et al., 1998). It is known that wild type HIV loses it pathogenicity if devoid of one of those proteins. On the other hand, for most cell types, removing the accessory genes only has a minor impact on vector performance.

For vectors generated with a production system of the third generation, the regulatory gene *tat* is additionally removed and, moreover, the remaining regulatory gene encoding Rev is separated from the structural genes *gag* and *pol* by placing it on a separate non-overlapping expression plasmid (Dull et al., 1998; Kim et al., 1998).

Further efforts to separate genes were undertaken and included supplying *gag* separate from *pol* (Wu et al., 2000), as well as independently supplying the gene coding for protease (PR) (Westerman et al., 2007). Separating the overlapping *gag-pol* structure on two or more plasmids prevents the formation of functional *gag-pol* structures, which are essential for vector mobilization (Kappes and Wu, 2001), but lowers the viral titer that can be obtained with the production system.

Similar as for HIV-derived vectors, also **gammaretroviral vector** design is based on separation of genetic elements in different expression plasmids (reviewed by Maetzig et al., 2011). This can be achieved by the so-called split packaging design. In brief, the helper plasmid provides the genetic sequences coding for the viral Gag/Pol and Env proteins but lacks all other retroviral components including the retroviral packaging signal psi. A separate plasmid harbors the gene of interest, flanked by the LTRs, and psi for efficient packaging of the gene of interest into the viral particle. The result is a retroviral vector, which harbors the transgene but does not contain the genes encoding for structural and enzymatic retroviral proteins. Consequently, the generation of replication competent retroviral vector progeny is prevented.

Alternatively, separation of genetic elements is pursued by providing the gammaretroviral Gag/Pol and Env proteins via stably transfected packaging cells (Miller et al., 1991). This approach proved difficult for lentiviral vectors, since not all components are well tolerated within the packaging cell line over extended periods of time, but stable packaging cell lines for conventional LTR-driven gammaretroviral vectors have been described.

Table 1. Comparison of different lentiviral vector production systems (based on Pauwels et al. (2009))

	Production system				
	1st generation	2nd generation	3rd generation	Translentiviral ™	Super-split system
Number of plasmids	3	3	4	6	7
Number of packaging plasmids containing HIV genes	1	1	2	3	6
Accessory genes *vif, vpr, vpu, nef*	All present	All absent	All absent	All absent, except for non-functional *vpr* that is fused to coding sequence of Pol and is packed into the particles formed as a fusion protein with RT and IN	*Vpu* and *nef* are absent, *vpr* is fused to PR and RT/IN - *Vif* functions which are delivered on separate plasmids
Sequences encoding Tat and Rev protein	Tat and Rev are present on a single packaging construct	Tat and Rev are present on a single packaging construct	Tat is absent, Rev is expressed from a separate, nonoverlapping construct	Tat and Rev are present on a single separate construct	Tat and Rev are present on a single separate construct
Overlapping Gag and Pol polyprotein structures	On the same plasmid	On the same plasmid	On the same plasmid	Split over 2 plasmids	Split over 3 plasmids

RT: reverse transcriptase; IN: integrase; PR: protease

3.4.2 Reducing sequence similarity

The likelihood for recombination can also be reduced by limiting the sequence similarity between the transfer vector and the trans-acting helper sequences. In view of this, it is noted that homologous recombination of retroviruses occurs at a frequency at least 1000-fold greater than non-homologous recombination (Zhang and Temin, 1993).

Strategies such as using a non-overlapping expression construct for Rev expression as well as a Rev-independent codon-optimized gag-pol expression plasmid in vectors produced by a system of the third generation will be useful to reduce the likelihood of recombination (Molina et al., 2004; Wagner et al., 2000). Also, hybrid vectors have been designed to reduce the probability of homologous recombination. Hybrid vectors are generated by cross-packaging of transfer vectors from one virus with the packaging machinery from a second virus. The rationale behind this approach is that the difference in sequence between the viruses could be sufficient to inhibit recombination, but on the other hand, their similarity would still ensure the formation of a functional particle (Kaye and Lever, 1998; Sachdeva et al., 2007; Tareen et al., 2013; White et al., 1999); Tareen et al. (2013). When using lentiviral vector preparations in a drug selection assay involving a lentiviral packaging construct containing a drug-resistance gene encoding blasticidin (BSD) resistance, Kuate et al. (2014) could demonstrate an up to sevenfold reduction of the frequency of BSD-resistant colonies when substituting the Rev response element (RRE) present in the vector construct with a heterologous RRE derived from simian immunodeficiency virus, indicating that the capacity to form partial recombinants was diminished.

Nevertheless, it must be highlighted that for lentiviral vectors, minimizing or avoiding sequence similarity may conflict with the attempt to generate high vector titers. Indeed, distinct retroviral cis-acting sequences that overlap with coding regions regulate packaging of proviral RNA, reverse transcription, genomic insertion, and transgene expression, and are thus important for production of high-titer vector stocks (Baum et al., 2006; Tareen et al., 2013).

For gammaretroviral vectors, there is no overlap with the coding regions except for the splice acceptor site located downstream of the packaging and dimerization (psi) motif (Baum et al., 2006). To eradicate this sequence overlap and thus minimize the risk of homologous recombination, the splice acceptor site can be condensed to a partially degenerate oligonucleotide (Hildinger et al., 1999), completely wobbled, or replaced by alternative cellular sequences (Lee et al., 2004). Also, the translation initiation codon of Gag can be mutated (Bender et al., 1987).

3.4.3 Self-inactivating design

An additional approach to reduce the risk of recombination and, consequently RCV, is the introduction of the so-called self-inactivating (SIN) property which has been described for lentiviral as well as for gammaretroviral vectors. The first gammaretroviral SIN vector was already described in 1986 by Yu and colleagues (Yu et al., 1986). In brief, the promotor and enhancer elements located in the U3 region of the 3'LTR are removed. This modified U3 sequence is copied to the 5'LTR during reverse transcription. In a wild-type virus, the 5'LTR functions as promoter and drives viral transcription. The SIN modifications significantly reduce the promotor activity to 10% of the original activity (reviewed by Pauwels et al., 2009). The SIN modifications in the transfer vector thus reduce the consequences of a potential recombination, because even if multiple recombination events would occur with reuptake of the missing accessory and regulatory genes into the vector genome, the recombinant would still contain a crippled LTR promoter and not regain original replication competence. Furthermore, also the risk of tumor formation, as well as the likelihood of mobilization of the vector (upon infection with a wild-type virus) are reduced for a SIN vector.

3.4.4 Other design features

3.4.4.1 Pseudotyping

Modifications may also be used to affect the tropism and stability of the parental virus. This is called pseudotyping. Pseudotyping can be performed irrespective of the production system used to create the vector and of the SIN properties.

For **lentiviral vectors based on HIV**, pseudotyping is mostly used to expand the range of cells susceptible to vector transduction. Indeed, the HIV-1 envelope glycoprotein restricts tropism of the vector to CD4+ cells. Most commonly, the HIV envelop protein is replaced with the vesicular

stomatitis virus G glycoprotein (VSV-G) which results in a very broad tropism (Burns et al., 1993). However, also other envelop proteins have been used to circumvent the shortcomings of VSV-G pseudotyping (e.g. serum inactivation, toxicity) or to obtain a targeted cell tropism, including hard-to-transduce cells (reviewed by Joglekar and Sandoval, 2017). Even though bringing in genes encoding for heterologous envelop proteins allows great customization of lentiviral vectors for many purposes, it also triggers biosafety concerns, especially for vectors produced with a system of the first generation. Indeed, recombination with a wild type virus could potentially result in a RCV with a broader host tropism than HIV itself (Escarpe et al., 2003; Farson et al., 2001).

In view of tropism and pseudotyping, it is highlighted that naturally occurring murine leukemia viruses, the most commonly used backbone for **gammaretroviral vector** design, already have different host tropisms depending on the envelop protein present. The envelop protein of ecotropic MLV binds to a murine receptor only, and consequently, derived vectors only mediate transduction of murine and rat cells. Amphotropic and xenotropic MLVs, in contrast, have envelop proteins that interact with receptors that occur in a broader species spectrum, including humans (Albritton et al., 1989; Miller et al., 1994; Tailor et al., 1999). Thus, by switching the MLV envelop proteins, the tropism of the MLV vector can be changed. Apart from the MLV envelop proteins, also non-MLV envelop proteins can be used to affect the cell tropism of the MLV vector and its stability, such as VSV-G, simian endogenous retrovirus envelop protein RD114, gibbon ape leukemia virus envelop protein, or hepatitis B virus envelop protein. For an overview of the most important pseudotypes, their receptors and potential envelope modifications required for pseudotyping of MLV it is referred to the review by Maetzig et al. (2011).

3.4.4.2 Regulation on transcriptional and posttranscriptional level

The 5′ end of the retroviral LTR serves as a promoter that plays an essential role in driving viral gene transcription. By modifying the 5’ LTR, viral gene expression can thus be affected. For example, by replacing the internal viral promoter sequence(s) with strong ubiquitously active viral promoters or cellular promoters, expression of the gene of interest can be made tissue or cell type specific.

Furthermore, transgene expression of the vector can also be regulated on the posttranscriptional level. For example, incorporation of repeats of microRNA target sites into the transgene harboring gammaretroviral RNA cells which express the corresponding microRNA can downregulate transgene expression, thereby enabling more targeted transgene expression (Brown et al., 2007; Brown et al., 2006). On the other hand, retroviral transcripts can also be stabilized and mRNA export and translatability can be increased e.g. by incorporation of the woodchuck posttranscriptional regulatory element (wPRE), by codon optimization, or by improving their polyadenylation (reviewed by Maetzig et al., 2011; Pauwels et al., 2009).

Finally, stop codons can be introduced into an open reading frame. If recombination events would occur with this type of vector, retroviral proteins cannot be expressed, preventing generation of RCV (FDA, 2020).

3.5 Occurrence of RCV in practice

RCR were detected in the early retroviral vector production systems where vector packaging cell lines were used in which all viral genes were expressed from a single plasmid (Mann et al., 1983). These original packaging cell lines developed RCV after relatively few passages (Bosselman et al., 1987; Cone and Mulligan, 1984; Danos and Mulligan, 1988; Miller and Buttimore, 1986; Miller et al., 1984; Sorge and Hughes, 1982). The replicating virus was generated by linking the helper genes to the LTR and psi sequences of the vector by recombination. Donahue et al. (1992) additionally showed that presence of RCR in the vector batch could induce T cell lymphoma in nonhuman primates after retroviral medicated gene transfer.

However, since those early days, vector production systems have been significantly improved to reduce the risk of RCV generation (see above). For the improved vector systems, there are no indications in scientific literature that RCL or RCR were formed either during manufacturing or after clinical application (Bear et al., 2012; Cornetta et al., 2018; Holzinger et al., 2016; Lyon et al., 2018; Marcucci et al., 2018; Naldini et al., 2016; Sastry et al., 2003).

4 RCV assays, a critical assessment based on literature

4.1 Introduction

As illustrated in the previous sections, different safety features are combined to ensure the safety of viral vector systems, thereby making the formation of RCV extremely unlikely. In consequence, the risk level of and the required containment level for activities with such vectors have been lowered in some instances. The absence of RCV is a key determining factor for reducing the risk level, and therefore the level of certainty on this absence has been a prominent issue. This section provides an overview of the assays most commonly used to substantiate the absence of RCV as well as available data on their validation.

4.2 Amplification

Irrespective of the assay used to detect RCV, an amplification phase in cells can be used prior to the actual testing.

4.2.1 Methodology

First, a preset volume of vector batch is inoculated on a sensitive cell line such as C8166-45 [13] or HEK293 cells for lentiviral vectors, and CRL2017 or PG4 cells for gammaretroviral vectors (see also 4.2.4). After this initial inoculation, regular passages are made whereby the supernatant is transferred to multiple fresh cell cultures. Passaging is continued for up to three weeks in the presence of a virus-transduction stimulating agent such as protamine sulphate or polybrene, during which potentially present RCV can replicate.

Figure 5 presents the RCV assay principle including an amplification phase.

During and/or after the amplification phase, the presence of RCV is determined by one of the assays described in Section 4.3, 4.4 and 4.5.

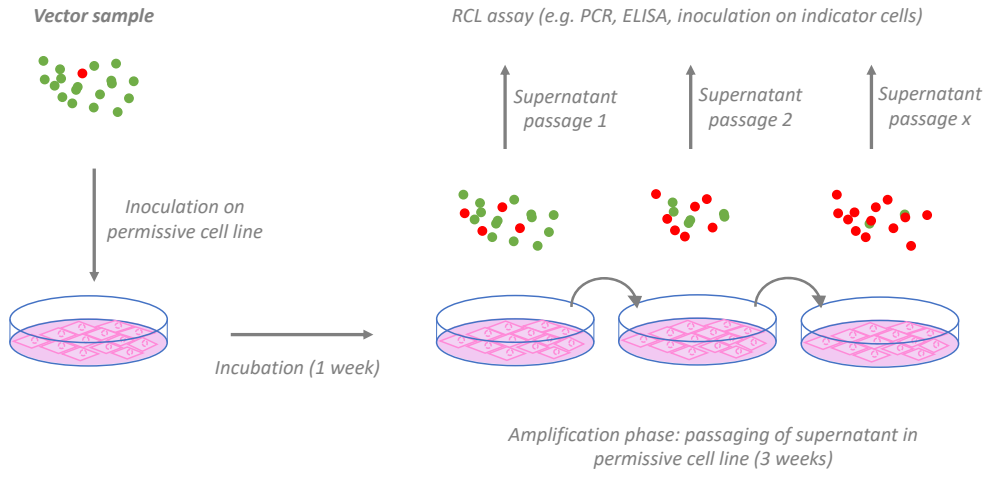

Figure 5. RCV assay principle including an amplification phase in cells

Green dots represent vector particles, red dots represent RCV

[13] In several cell lines used for amplification (e.g. C8166-45, MT-2, MT-4) human T-lymphotropic virus (HTLV) sequences may be present

4.2.2 Advantages

First, the main advantage to include an amplification phase in RCV testing is that it allows small amounts of putative RCV to be multiplied. Where the initial amounts in the vector batch may not be detectable, subsequent amplification of RCV during the 3-week culture period can increase the sensitivity of the assay.

Second, when an assay is performed on a production batch, there will be concentrated vector material present in which the (potentially) low levels of RCV have to be detected. If such assay is then based on a parameter that is present in both RCV and vector particle (e.g. p24 ELISA), the high amounts of vector particles may overshadow the low level of RCV, resulting in a false positive result (Cornetta et al., 2011; Escarpe et al., 2003). By including an amplification phase, vector particles will gradually be removed during passaging, allowing a more accurate detection of RCV during the indicator phase. This was clearly illustrated by a study of Escarpe et al. (2003). They showed that the p24 concentration of cell cultures exposed to purified vector gradually decreased with p24 becoming undetectable after 10 passages. When spiking cells and vector with a single 50% tissue culture infectious dose (TCID50) of a positive control virus, p24 originating from the control virus became detectable after the 4^{th} passage with a p24 concentration 1000-fold higher than in the vector preparation.

Third, high vector particle concentrations may compete for receptors also to be used by potentially present RCV. This was illustrated by introducing a small amount of the GFP-expressing vector (sufficient to transduce 10 to 20% of C8166 cells) with varying concentrations of a vector that lacks an expressed transgene (Cornetta et al., 2011). Infectivity was monitored by assessing the number of GFP-expressing cells after transfection. There was a dose-dependent decrease in the number of GFP-expressing cells indicating that excess of vector particles that lacked an expressed transgene limited the ability of the GFP-expressing vector to transduce the target cells. To avoid false negative results, vector supernatant may need to be diluted to a concentration whereby no interference occurs.

4.2.3 Disadvantages

The main disadvantage of including an amplification phase is that the whole process may take up to 6 weeks to complete, whereas a determination directly in vector batch material can be completed within a much shorter time frame.

Also, the large amounts of consumables and volumes when testing a bulk production of vector product brings challenges to the amplification phase. Corre et al. (2016) therefore developed a pooling assay for lentiviral vectors (Figure 6). In brief, sensitive cells are transduced with the vector at day 0 in several flasks in order to analyze 5% of the bulk volume with a given Multiplicity of Infection (MOI), as required by the regulators. An amplification period of 8 days allows any potential RCL to be amplified. Each flask is then diluted 1/4 and maintained until day 11 when pairwise pooling is started (5 ml from two flasks in 30 ml medium) until day 29 for final harvest. The p24 concentration was measured throughout the process and it was found that pooling did not cause a loss of sensitivity. The authors do recommend that pooling is not performed prior to day 11, to avoid false-negative results and allowing amplification of any potential RCL during the early phase of the infection in a high-cell-density environment.

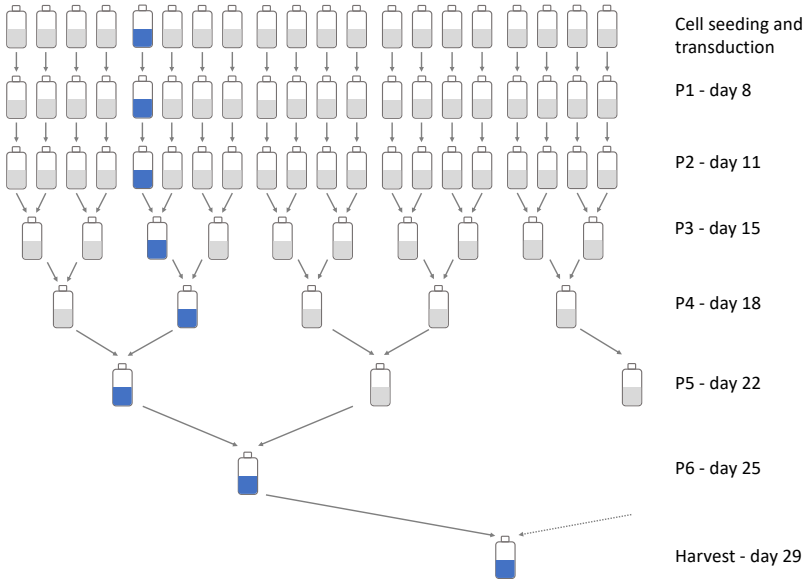

Figure 6. Schematic overview of the RCL pooling assay strategy

Based on: Corre et al. (2016)

4.2.4 Points of attention

When selecting a cell line for the amplification phase, it is important to select a line that is sensitive for the potential RCV that may be formed. Escarpe et al. (2003) compared six cell lines for their capacity to support replication of a putative RCL as represented by a VSV-G pseudotyped HIV devoid of its accessory genes (R8.71-VSV G). They determined the amount of virus required to infect 50% of the cells (TCID50) in C8166-45, CEM-SS, MT4, PM1, H9, SupT1 and primary PBMC. The TCID50 was the lowest in C8166-45, indicating that C8166-45 was the most permissive cell line. Other cell types had at least a 10-fold higher TCID50 and thus seemed less sensitive to support putative RCL replication.

When highly specific envelop proteins are used for pseudotyping, standard cell lines as described by Escarpe et al. (2003) or as recommended by the regulators, may not be suitable for the amplification phase. For example, Farley and colleagues (2015) designed an HIV-based vector produced by a third generation production system and pseudotyped with a modified Sindbis virus gP E1001. This pseudotyping allowed the vector to specifically target dendritic cells. For their amplification phase, modified 293F cells expressing the E1001 receptor DC-SIGN had to be used because the specific pseudotyping no longer allowed the use of e.g. C8166-45 cells.

A final point of attention for the amplification phase (and by default, any cellular assay) is the potential inhibitory effect of high vector particle concentrations on cell growth. Cornetta and colleagues (2011) noted a dose-dependent inhibition of cell growth after 48h post exposure to various vector dilutions, with the greatest effect noted in MRC-5 cells. Growth inhibition was noted with ~60% of C8166 cells present after exposure to vector at the 10.000 ng/ml concentration. At a vector concentration of 1.000 ng/ml the growth inhibition of C8166 cells was modest with ~90% of cells surviving. Also for clinical grade vector, growth inhibition of cells was observed. Initial vector batch concentrations to be used when starting the amplification phase are thus to be chosen carefully to avoid that inhibited cell growth negatively affects amplification of potentially present RCVs (see section 4.6).

4.3 Cellular assays

4.3.1 Main principle and examples

Cellular assays are based on inducing a specific phenotypic change in a suitable indicator cell line that can only be caused by an RCV, not by the vector particle. Various approaches have been described.

A marker rescue assay is based on the rescue of a specific marker gene by RCV. For example, Chang and Yee (2012) described an assay for retroviral vectors using the HT1080-based cell line, HT/*hyg*, with an integrated retroviral vector carrying the *hygR* gene. Potential RCV in the harvested vector preparation was first amplified in HT/*hyg* cells for a minimum of 3 weeks. During this time, potentially amplified RCV could rescue (i.e. mobilize) the *hygR* vector and when subsequently introduced on naïve cells, hygromycin resistance can be conferred in the cells.

Sakaguchi et al. (2008) described a *lacZ* marker rescue assay to detect infectious gammaretrovirus in a supernatant. In this approach, initially a cell line is stably transfected with the *lacZ* gene (in provirus or retroviral construct), rather than using the pseudotype virus. When the *lacZ* transfected cells are exposed to the supernatant to be tested and infectious gammaretrovirus is present in the supernatant, virus particles containing the *lacZ* gene will be generated. A subsequent inoculation on a fresh and non-*lacZ* containing cell culture and staining with beta-galactosidase will result in blue foci (Figure 7).

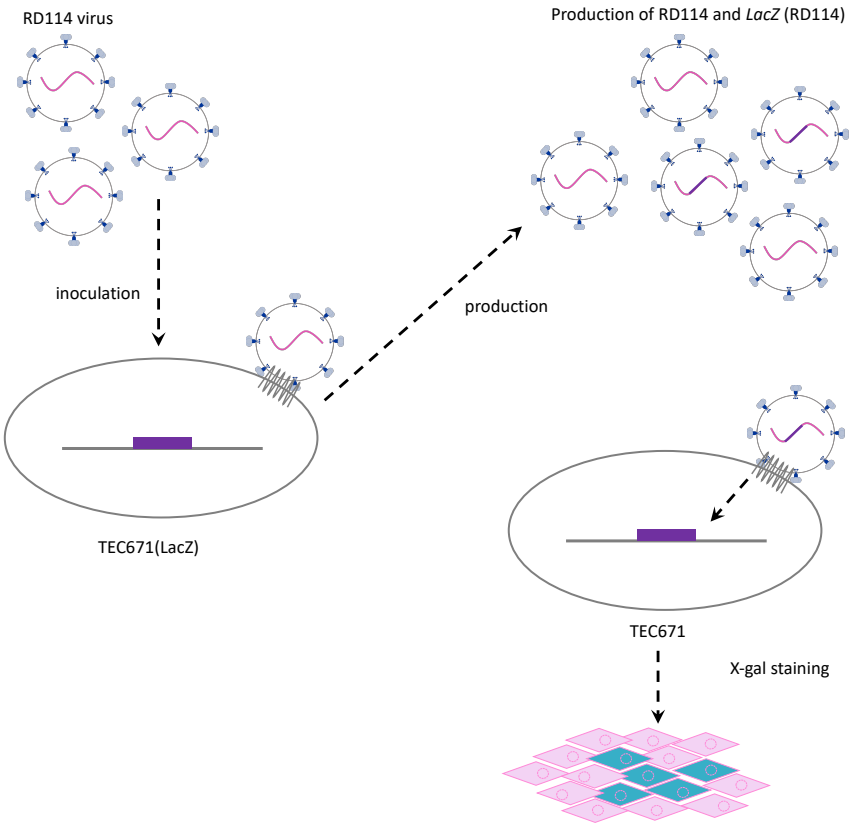

Figure 7. Principle of the lacZ marker rescue assay

Based on: Sakaguchi et al. (2008)

Hashimoto-Gotoh et al. (2015) described an assay that can be used to detect contaminating adventitious gammaretroviruses in producer cells. The cells potentially containing the infectious gammaretrovirus are exposed to a pseudotype virus containing an easily distinguishable marker transgene, the *lacZ* gene. The *lacZ* gene will subsequently be packaged into the newly formed viral particles of the gammaretrovirus, if present. *lacZ* containing infectious virus is then detected as blue

foci in nuclei upon beta-galactosidase staining. If no infectious gammaretroviruses are present, the *lacZ* gene will not be incorporated in the cells and no foci are formed.

Yet another assay, the sarcoma-positive leukemia-negative (S+/L-) assay, was first documented by Bassin et al. (1971) describing that a group of murine 3T3 cells containing murine sarcoma virus (MSV) genome induced foci when they were infected with replication-competent MLV. For the S+/L- assay, an indicator cell line such as PG-4 or MiCl1 is used. This cell line, also referred to as a S+/L- cell line, contains the MSV genome (S+), but lacks the MLV genome (L-). Cells that express the MSV induce a transformed phenotype, represented by foci formation, but only when there is co-expression of a MLV, e.g. when RCR are present. The number of foci that form in a monolayer after inoculation with a sample is proportional to the number of infectious units in the inoculum.

4.3.2 Advantages and disadvantages

The main advantage of the cellular assays is that they detect infective viruses, in contrast to the molecular assays as described below. Consequently, there is no concern for interference by non-infective vector particles.

However, for the *lacZ* marker rescue assay, interference is described with retroviruses present in the cell line used to detect the foci (Sakaguchi et al., 2008). Presence of these retrovirus in the fresh cell culture completely interfered with infection by the *lacZ* containing virus, resulting in the absence of foci and thus a false negative result of the assay. On the other hand, when apoptotic cells are present (e.g. due to suboptimal buffer conditions), they may color blue and thus provide a false positive result of the assay.

An additional disadvantage to be considered is that the indicator cell lines may only support replication of amphotropic retroviruses, which is the case for the S+/L- cell lines. Therefore, that assay cannot be used when ecotropic pseudotyped viral vectors are used.

4.3.3 Validation

Annex 8.2 provides a non-exhaustive overview of representative parameters that can be used for validation of an assay.

Sakaguchi et al. (2008) studied the sensitivity of the *lacZ* marker rescue assay. They serially diluted a stock of RD114 viruses and inoculated on TE671(*lacZ*) cells. The inoculated cells were then passaged at multiple time points. The viruses produced in the culture supernatant were subsequently titrated on TE671 cells by the *lacZ* marker rescue assay. Simultaneously, virus-inoculated cells were subjected to DNA isolation at the indicated times, and proviral DNA was detected by PCR. Using the *lacZ* marker rescue assay, *lacZ* -positive signals were detected in a 10^4-fold-diluted sample at 4 days after inoculation and the endpoint dilution (10^5-fold dilution) could be determined at 12 days after inoculation. By the PCR test using env primers, proviral DNA was detected to be as sensitive as the *lacZ* marker rescue assay; however, the sensitivity was lower when the PCR test was conducted using pol primers.

No other validation data were found for the cellular assays.

4.4 Immunoassays

4.4.1 Main principle and examples

The main principle of the immunoassays is to detect viral proteins by means of an ELISA. In brief, a plate is coated with antibodies against the viral protein of interest, after which the sample to be studied is added to the plate. If the viral proteins of interest are present, they will bind with the antibodies. Subsequently, enzyme labeled antibodies are added to the plate followed by an incubation period. Finally, substrate is added that will be processed into a product by the enzyme and which can be quantified (Figure 8).

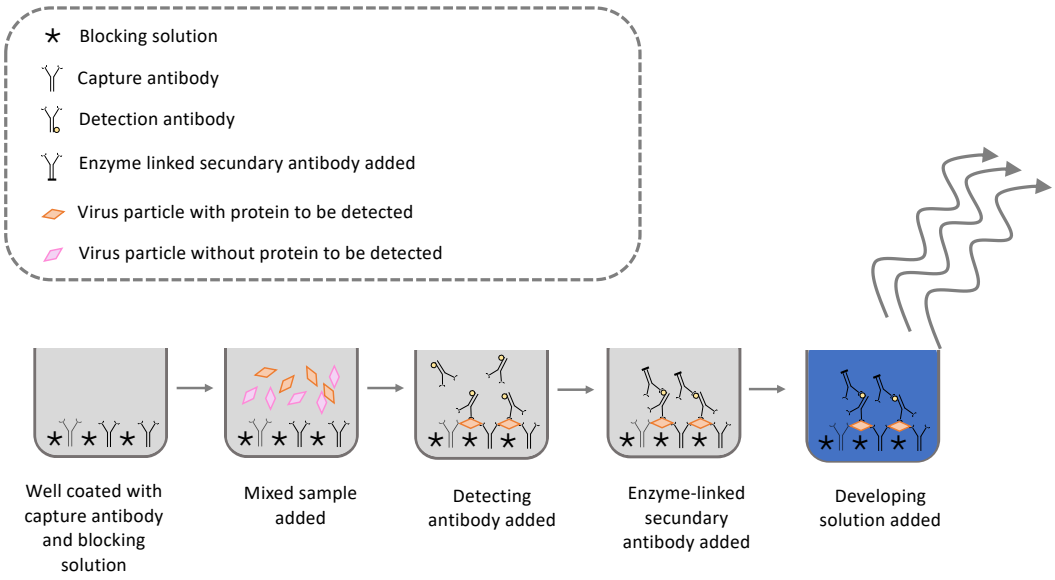

Figure 8. General principle of an immunoassay to detect viral proteins

Based on: Burguillos, 2003

The most frequently described immunoassay is the p24 ELISA for lentiviral vectors. The p24 protein is a component of the capsid of HIV. The p24 ELISA quantitatively measures p24 in the sample. To this end, the ELISA plate is coated with anti-p24 antibodies, incubated with the sample potentially containing RCL and subsequently with HRP-labelled anti-p24 antibodies. After incubation and washing, a tetramethyl benzidine (TMB) substrate is added. If p24 is present in the sample, HRP will be present and able to transform TMB in a water-soluble blue reaction product that can be measured spectrophotometrically.

The VSV-G ELISA which can be used for VSV-G pseudotyped vectors, is less frequently described. The principle of the VSV-G immunoassay is highly similar to that of the p24 ELISA, except that VSV-G is quantitatively monitored. The VSV-G ELISA is only applicable for vectors pseudotyped with VSV-G. In case vectors pseudotyped with other envelop proteins are used, the immunoassay should target those proteins.

4.4.2 Advantages and disadvantages

The main advantage of the p24 and VSV-G ELISA is that they are easy to perform and commercially available.

However, as mentioned above, since also vector particles (may) contain the antigen to be detected in the assay, the outcome of the ELISA may be influenced by the vector particles (see Section 4.2). Therefore, detection of RCL in a vector production batch by means of the p24 or VSV-G ELISA is to be performed in combination with an amplification phase to clearly monitor a decay in p24 concentrations as proof of absence of RCL, since passaging on the permissive cells allows for a wash out of interfering vector particles (Escarpe et al., 2003; Ni et al., 2005).

4.4.3 Validation

For the p24 ELISA, different authors report the limit of detection (LoD) (Dull et al., 1998; Escarpe et al., 2003; Farson et al., 2001; Sastry et al., 2005; Sastry et al., 2003) (Table 2). Reported LoDs are highly similar between the authors and range from 2.5 pg/ml up to 5.5 pg/ml. Interestingly, Sastry and colleagues (2005) highlight that the LoD as provided by the supplier of the commercial ELISA is lower (3 pg/ml) than that found during their experimental procedures (5.5 pg/ml).

Table 2. Limit of detection for RCV immunoassays described in literature

Vector	Assay	Sample tested	Limit of detection	Reference
Lentiviral	p24 ELISA	Vector batch	3.9 pg / ml	Dull et al. (1998)
		Attenuated VSV-G pseudotyped HIV	2.5 pg / ml ≈ 12 virions	Farson et al. (2001)
		Supernatant collected during amplification in C8166 cells	3 pg / ml*	Escarpe et al. (2003)
		R7 HIV-GFP (virus)	+/- 1 IU / ml	Sastry et al. (2003)
		R7 HIV-GFP (virus)	5.5 pg / ml	Sastry et al. (2005)

* Reported as indicated by the provider of the commercial kit.

No other validation data were found for the p24, the VSV-G ELISA or any other immunoassay used to detect RCV.

4.5 Molecular assays

4.5.1 Main principle and examples of representative assays

Molecular assays include the quantitative reverse transcriptase (qRT) PCR as well as the Product Enhanced Reverse Transcriptase (PERT) assays. Both assays are based on two main steps, i.e. transcribing a viral RNA of interest into a DNA and subsequently multiplying the DNA of interest by means of a quantitative PCR. The PCR product is then measured e.g. by means of measuring pre-included reporters such as SYBR green, a labeled oligo or ethidium bromide.

Below, various qRT-PCR approaches and the PERT assay are described in more detail. Although both qRT-PCR and PERT are indicated here as molecular assays, it must be noted that there are fundamental differences. The standard qRT-PCR approach measures viral RNA and reverse transcriptase is externally added to the sample to be tested, whereas the PERT assay measures viral reverse transcriptase and an RNA template is added.

qRT-PCR

A standard qRT-PCR approach (Figure 9) has been applied for sequences coding for a variety of viral proteins, including VSV-G, *psi-gag*, *gag-pol* and *tat*. Most frequently described is the qRT-PCR to detect part of the gene encoding for the VSV-G protein (Escarpe et al., 2003; Sastry et al., 2003; Skrdlant et al., 2017; Wiltshire et al., 2021). Within the VSV-G qRT-PCR approach, different methods have been described to improve PCR product detection, or to reduce manual steps. As mentioned for the immunoassays, an approach based on detecting VSV-G is only applicable for VSV-G pseudotyped vectors. In case other envelop proteins are used, the primers used in the qRT-PCR should be directed to sequences encoding those proteins.

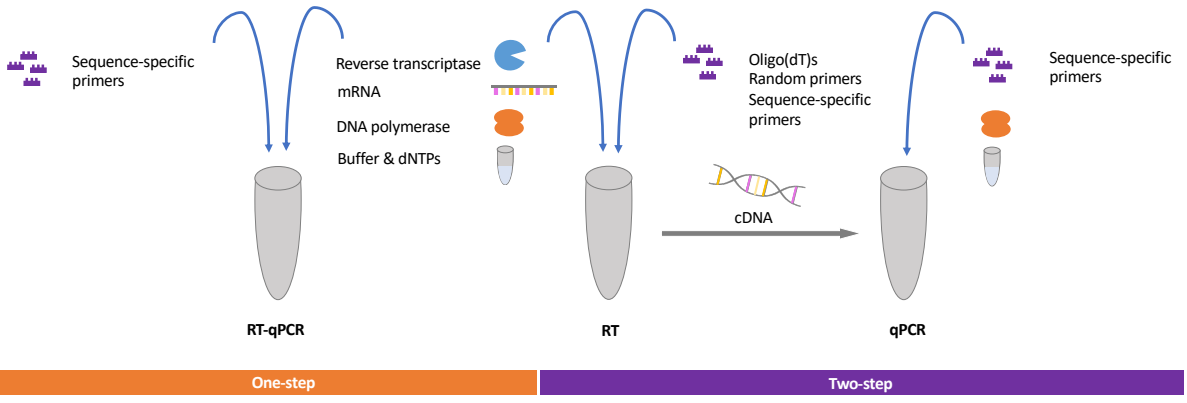

Figure 9. Basic principle of the qRT-PCR

Based on: https://www.thermofisher.com/be/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/spotlight-articles/basic-principles-rt-qpcr.html

The *psi-gag* qRT-PCR has been described by Sastry et al. (2003), mainly to study the relevance of partial recombinants. The authors showed that *psi-gag* recombinants were routinely detected by means of the PCR assay (20 of 21 analyses) upon transduction of C8166 and 293 cells. However, when using a 21-day cell-free medium obtained from these cells to transduce naïve cells, no evidence of *psi-gag* transfer to the naïve cells was detected, indicating that these recombination events alone were insufficient to reconstitute a true RCL.

The *tat* qRT-PCR aims to detect *tat* sequences from a packaging construct. The assay was used to detect RCV in cultures infected with bovine Jembrana disease virus (JDV)-based vectors (Metharom et al., 2000). However, as *tat* is not present in lentiviral packaging constructs used for later production systems, this assay has limited applicability for RCL testing. Moreover, as *tat* is only present in lentiviruses, this assay is not suitable for gammaretroviruses.

PERT assays

Also PERT assays (Figure 10) have been described by multiple authors (Farley et al., 2015; Miskin et al., 2006; Sastry et al., 2003; Vermeire et al., 2012). The assays included different methodologies in view of PCR product detection, or reduction of manual steps such as the SYBR Green I-based PERT assay (SG-PERT) (Vermeire et al., 2012).

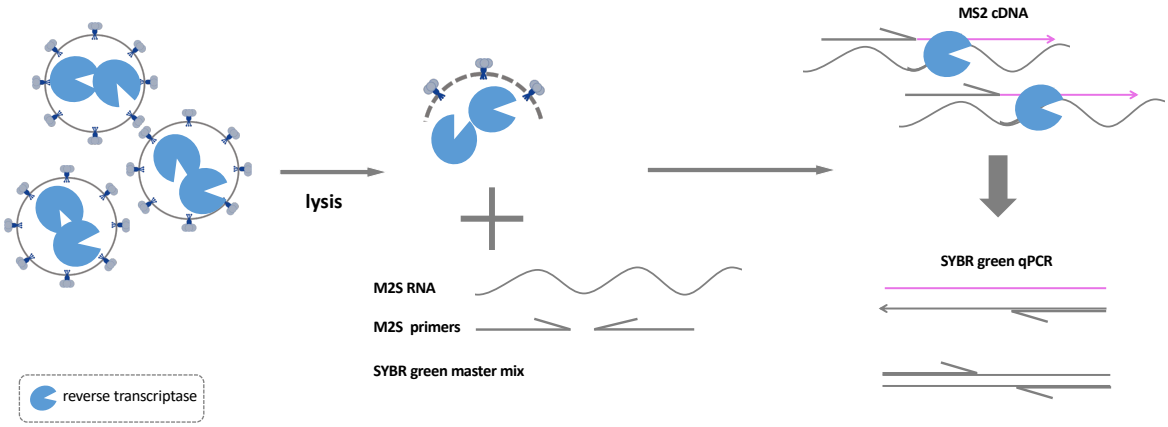

Figure 10. Basic principle of the PERT assay

Based on: Vermeire et al. (2012)

4.5.2 Advantages and disadvantages

The qPCR assays are reliable along a broad range of concentrations.

A disadvantage of any PCR is its low tolerability towards suboptimal PCR conditions and interferences. In that perspective, Wiltshire et al. (2021) developed a digital droplet PCR to increase sensitivity and obtain a greater tolerance of suboptimal PCR conditions and interferences. In brief, in this quantitative PCR (qPCR) individual samples and PCR reagents are partitioned into a large number of individual reaction compartments. At the end of regular endpoint PCR amplification, all generated emulsion droplets are run through an automated droplet reader, which counts every acceptable droplet and measures fluorescence emission from each droplet (positive or negative). Since the read-out is binary for each droplet, occasional false priming in one droplet or another with increased cycle numbers will have a negligible effect on the final result. By contrast, in standard qRT PCR approaches, false priming affects the whole reaction. Additionally, there is no need for external calibration curves, and the analytical performance of the assay is highly predictable.

Additionally, a qRT-PCR detects the presence of a sequence only, irrespective of whether it is functionally active (Sastry et al., 2003). Similarly, the PERT assay detects RT activity, irrespective of whether it is originating from an RCV or from any other source such as cellular DNA polymerases or endogenous reverse transcriptases (Arnold et al., 1998; Pyra et al., 1994).

4.5.3 Validation

Mainly, authors report the limit of detection LoD for molecular assays, as outlined in Table 3. Importantly, the samples used to determine the LoD were quite variable between the different studies, making it difficult to compare values obtained.

Escarpe et al. (2003) spiked VSV-G plasmid into human genomic DNA. They were able to detect as little as 5 copies of VSV-G per 1.4 x 10^5 cells. Second, they mixed C8166 cells, which are frequently used for the amplification phase, with cells transfected with VSV-G to determine the concentration of VSV-G positive cells (i.e. cells that have productively been infected by an RCV) in a larger population of cells. Here, the lowest concentration that could be detected was 1 VSV-G containing cell in 1.4 x 10^5 cells in total.

Sastry et al. (2003) used pMDG ranging in concentrations from 5 up to 5x10^5 copies in a background of 0.1 microgram DNA from uninfected 293 cells. Detection was possible even in the lowest concentration of 5 copies by regular PCR as well as by RT-PCR. Additionally, the authors described the LoD of the *psi-gag* PCR. In brief, they prepared 10-fold serial dilutions of a plasmid encoding for a GFP-labeled and attenuated HIV strain (R7-GFP) with plasmid concentrations ranging from 1 to 10^5 copies in a background of 0.1 microgram DNA from uninfected 293 cells. By doing so, they were able to detect a plasmid concentration as low as 10 copies. Similarly, they used the virus itself whereby they could detect viral concentrations of 0.1 IU/ml or more.

Table 3 Limit of detection for RCV molecular assays described in literature

Vector	Assay	Sample tested	Limit of detection	Reference
Lentiviral	Psi-gag PCR	pHIV-GFP (plasmid)	10 copies	Sastry et al. (2003)
		R7 HIV-GFP (virus)	0.1 IU / ml	Sastry et al. (2003)
	VSV-G PCR	VSV-G plasmid in human genomic DNA	+/- 5 copies / 1.4 x 10^5 cells	Escarpe et al. (2003)
		VSV-G transfected cells mixed with C8166 cells	+/- 1 VSV-G containing cell / 1.4 x 10^5 cells	Escarpe et al. (2003)
		pMDG	+/- 5 copies	Sastry et al. (2003)
		Final vector batch	5 copies / reaction well	McGarrity et al. (2013)
		VSV-G plasmid in nuclease free water with or without human genomic	10 copies	Skrdlant et al. (2017)
		DNA extracted from transduced cells	10 copies / ml	Wiltshire et al. (2021)
	PERT	Pure HIV RT	100 molecules of HIV RT ≈ 1 to 10 HIV particles	Sastry et al. (2005)
		R7 HIV-GFP (virus)	0.1 IU / ml	Sastry et al. (2005)
		Recombinant HIV RT	10^4 pU recombinant HIV-1 RT ≈ 20 viral particles	Vermeire et al. (2012)
Gamma-retroviral	PERT	Pure MoMLV RT	100 molecules of HIV RT ≈ 1 to 10 MoMLV viral particles	Sastry et al. (2005)

A detection limit of 5 copies / reaction well was reported for a VSV-G qRT-PCR by McGarrity et al. (2013). They applied the assay after the amplification phase in C8166 cells either exposed to end of production cells or final vector product. No specifications were made on the volume of a reaction well.

In the digital droplet PCR as referred to above, Wiltshire et al. (2021) could detect a concentration of 10 copies per microliter.

Extensive information on the LoD for the PERT assay was described by Sastry et al. (2005). They used purified HIV or Moloney murine leukemia virus (MoMLV) RT in serial dilutions containing 10^2 (0.02 fg) up to 10^7 (2000 fg) RT molecules. Each concentration was used to reverse MS2 substrate RNA. Subsequently, an RT PCR was performed. The LoD was 100 molecules of RT. For HIV, the authors indicated that this would correspond to approximately 1 to 10 infectious viral particles, as

HIV-1 may contain 10 to 100 RT molecules per virion. In view of RCL, the PERT assay was also performed with the GFP labeled, attenuated R7 strain of HIV. Here, the detection limit was 0.1 IU / ml, which is similar as for the *psi-gag* PCR (Sastry et al., 2003).

Skrdlant et al. (2017) reported data on the frequency of detection rather than of the LoD. Test articles were created containing 10, 8, 6, 5, 4, or 3 copies of VSV-G DNA sequence per reaction. The frequency of detection of VSV-G sequences spiked into nuclease-free water ranged from 100% for 10 copies to 66.67% for 3 copies. When human genomic DNA was spiked into the sample, the frequency of detection was lower, ranging from 83.33% for 10 copies to 33.33% for 3 copies. Importantly, when adding DMSO at a final concentration of 3% the frequency of detection in samples with human genomic DNA increased, supporting the use of DMSO to improve the reproducibility of detection of low levels of VSV-G DNA in the presence of genomic DNA.

Apart from the LoD, several authors reported on additional parameters of RCV assays. The results are summarized in Table 4. First, reproducibility was addressed, thereby referring to repeating a procedure in 3 or more independent experiments, with no specification of what was accepted as "independent". Second, linearity was described, aiming at a value $R^2 \geq 0.98$ (Bustin et al., 2009). Finally, several papers addressed the accuracy of the RCV assay.

Table 4 Other validation data of RCV molecular assays described in literature

Vector	Assay	Validation parameter	Outcome	Reference
Lentiviral	Psi-gag PCR	Reproducibility	Reproducible	Sastry et al. (2003)
	VSV-G PCR	Reproducibility	Reproducible	Sastry et al. (2003) Skrdlant et al. (2017) Wiltshire et al. (2021)
		Linearity	Linear	Skrdlant et al. (2017) Wiltshire et al. (2021)
		Accuracy	Accurate	Wiltshire et al. (2021)
	PERT	Reproducibility	Reproducible	Sastry et al. (2005) Vermeire et al. (2012)
		Linearity	Linear	Sastry et al. (2005) Vermeire et al. (2012)

4.6 Positive and negative controls

4.6.1 Positive controls

For **RCL assays**, various publications described the use of an attenuated HIV-1 virus devoid of accessory genes as a positive control (Cornetta et al., 2020; Cornetta et al., 2011; Sastry et al., 2005). Since this attenuated virus expresses the native HIV-1 envelope, it should be mentioned that it may be less suitable as positive control for assays purely based on the detection of heterologous envelop proteins such as VSV-G, or for cellular assays where positive control virus and potentially formed RCL have different abilities to enter the cells due to different Env proteins.

Other publications described the use of the attenuated HIV virus that encoded a heterologous Env protein (e.g. VSV-G) (Corre et al., 2016; Escarpe et al., 2003; Farson et al., 2001) and thus more closely mimicked a pseudotyped vector and potential RCL raised. However, adding a heterologous Env to a replication competent lentivirus may lead to an increase in risk. Even though not demonstrated in practice, recombination of such control virus could potentially result in a pathogenic and replication competent virus with broad tropism (EMEA, 2005; Farley et al., 2015; Iggo and Richard, 2015). From a biosafety point of view, not including the genetic sequence of a heterologous Env protein broadening host or cell tropism is thus preferred.

Alternatively, pure HIV RNA could be used as positive control in molecular assays (Iggo and Richard, 2015; Sastry et al., 2005). Use of purified HIV RT was included as a positive control for a PERT assay described by Sastry et al. (2005). Spiking with VSV-G encoding (genetic) material (Sastry et

al., 2003; Skrdlant et al., 2017) has been described as a suitable positive control in VSV-G based assays and could also be used for other heterologous envelop proteins.

For **RCR assays**, the use of the 4070A amphotrophic MLV was most frequently reported as positive control for a variety of RCR assays (Chang and Yee, 2012; Miskin et al., 2006; Sastry and Cornetta, 2009; Sastry et al., 2005). Other positive controls included RD114 virus and GALV virus for a S+/L- assay, as well as purified MoMLV RT for a PERT assay (Sastry et al., 2005).

4.6.2 Negative controls

Negative controls mainly consist of uninfected medium, both for RCL and RCR testing (Cornetta et al., 2018; Cornetta et al., 2020; Cornetta et al., 2011; Farley et al., 2015; Miskin et al., 2006; Sastry et al., 2005; Skrdlant et al., 2017). For the *psi-gag* PCR, Sastry et al. (2003) also described the use of genomic DNA of uninfected cells as a negative control.

5 Testing for RCV in practice

5.1 Regulation and guidance

5.1.1 COGEM

A total of 34 COGEM advices were identified relating to R&D with lentiviral or gammaretroviral vectors, of which 30 were deemed relevant in view of RCV testing. Of these, 16 referred to R&D in the context of clinical trials and 14 concerned more fundamental R&D activities (Annex 8.1). Of these, 28 full text documents were publicly available. A summary of the latter is briefly discussed below.

Nineteen generic or activity specific COGEM advices referred to **HIV based vectors**. The majority involved vectors generated with a production system of the **3rd generation** and **SIN** characteristics. For this type of vectors, COGEM highlighted that testing of the virus batch for RCVs has no added value in view of the potential environmental risk. Additionally, COGEM indicated that for a finished investigational medicinal product (i.e. transduced cells) generated using such vectors no RCV testing is required (CGM/190729-01). Only data (e.g. plasmid maps or a description of the components present on the transfer plasmid, packaging plasmids and pseudotyping plasmids) must be supplied demonstrating the use of a third generation SIN lentiviral system. Hereby, it was referred to the negligible likelihood for RCV formation as described in literature. However, all institutes requesting COGEM advice for R&D in the context of clinical trials clearly indicated that RCV testing had been performed, even though not specifically requested by COGEM. The latter can be explained by requirements imposed by other regulatory frameworks such as EMA and FDA (see below). Also, for more fundamental R&D activities, reference to RCV testing was made in part of the advices, despite the use of SIN vectors produced in a 3rd generation production system. Where described, the type of assays used was diverse and mainly included an amplification phase followed by a structural assay.

When **HIV based vectors** are used of a **lower generation**, COGEM referred to the performance of validated assays such as a p24 ELISA during/after an amplification phase (CGM/090331).

In the 10 generic or activity specific advices related to **gammaretroviral based vectors**, COGEM requested a sensitive and validated RCV assay irrespective of the vector design. Where specified, assays mainly included marker rescue assays or PERT after an amplification phase. Specifically for the PERT assay, a recent COGEM advice (CGM/220303-01) refers to a cut-off value of Ct > 40 that can be used to conclude on the absence of RCR.

In all other advices, it was referred to a variety of detection limits. Values were difficult to interpret as no further background on experimental design was provided in most of the COGEM advices. Whether or not RCV have actually been detected was mostly not specified, although it was frequently indicated that a vector batch can only be released if the outcome of the RCV testing is negative.

5.1.2 SBB

In advices issued by the Belgian Biosafety Server, it is requested to test a production lot of vectors for RCV prior to its first use, unless the provider performed such check or the likelihood for RCV is negligible (e.g. SIN vectors generated using a 3rd generation production system). No recommendation is provided on the type of assay to be performed (personal communication).

5.1.3 ZKBS

The document ‘General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability: Stable and transient gene expression using γ-retroviral and lentiviral vectors’ (ZKBS, 2020), does not refer to specific RCV testing methods. However, it highlights that the containment level for activities with gammaretroviral and lentiviral vectors depends, amongst other, on whether a contamination with RCV must be assumed. This risk for contamination with RCV is mentioned for the production of eco-, ampho- or xenotropic gammaretroviruses, but is not to be assumed for production of lentiviral vectors provided that a packaging system of the 2nd, 3rd or newer generation is used.

5.1.4 EMA

For replication deficient viral vectors in general, **EMA** states in its **'Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products'** that demonstration of replication incompetence begins with a clearly documented strategy to render the viral vector replication incompetent (EMA, 2018). The possibility of any recombination events leading to RCV or replication via *trans* regulation should be discussed. The absence of RCVs is then tested on the drug substance, intermediates where appropriate, as well as any packaging/producer cell lines. Screening for RCVs should be in accordance with Pharmacopoeial recommendations, using a suitably sensitive detector cell line and appropriate passage numbers. Also, controls and testing should be conducted to prevent infection and/or contamination of the packaging cell line by wild-type, helper or hybrid viruses which might lead to the formation of replication competent recombinant viruses during production. No further guidance is provided on the type of assays to be used, except that it is essential to establish the suitability and the sensitivity of the tests. Hereby, it is mentioned that the LoD must be such that the test provides assurance of the safety of the vector product. Also, the appropriateness of the permissive cell type(s) used in the assays for replication competent virus should be established.

When it comes to specific testing methods, the **EMA 'Guideline on development and manufacture of lentiviral vectors'** provides examples of suitable RCL testing (EMEA, 2005). This includes infection of a susceptible cell line and serial passaging of successive cell supernatants to achieve RCL amplification, followed by a p24 Gag immunocapture assay or *gag* RNA PCR assay. Where VSV-G is the pseudotyping envelope protein, it is also appropriate to use a VSV-G immunoassay and/or molecular assay for VSV-G DNA or RNA. Alternatively, quantitative RT assays or a marker rescue assay can be considered. It is noticed that the limit of quantification (LoQ) required for RCL tests should be established according to the proposed LV dose and to the size of the production lot. No indications on the values for LoD or LoQ for any of the proposed assays are provided. Ideally, the capacity of tests to detect one RCL in a vector dose should be proven.

Finally, the **EMA 'Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells'** highlights that no additional testing is required for cells genetically modified with the viral vectors, provided that the generation of RCVs during manufacturing is ruled out by an appropriate risk assessment and testing as mentioned above (EMA, 2020).

5.1.5 European Pharmacopoeia

For retroviral vectors, **Chapter 5.14** of the **European Pharmacopoeia** refers to testing of purified harvest or final vector lot with suitable methods, such as amplification on permissive cells followed by detection of a viral antigen (e.g. p24 by ELISA) or by marker-rescue assay (Ph. Eur., 2019).

Also, producer cells should be tested by means of e.g. a co-cultivation for several cell doublings of the producer cells with a permissive cell line, followed by detection (either by observation of a cytopathic or haemadsorbing effect on indicator cells like PG4 S+L-, by detection using indicator cell lines by nucleic acid amplification techniques or by marker-rescue assay). Positive controls are to be included in each assay to monitor its sensitivity. No further guidance is provided on the type of controls or on values to be obtained in terms of sensitivity.

5.1.6 FDA

Most extensive and recent recommendations on RCV testing are provided by the FDA in their Guidance document for Industry entitled 'Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up' (Version January 2020) (FDA, 2020). Table 5 outlines the FDA recommendations for the material to be tested for RCV during production, as included in the 2020 Guidance document.

Whereas previous FDA guidelines [14] also recommended testing of the Working Cell Bank, such is no longer included in the 2020 Guidance document, provided that the MCB has been appropriately qualified according to the recommendations included in the 2020 Guidance document.

[14] Briefing Document - Testing for Replication Competent Retrovirus (RCR)/Lentivirus (RCL) in Retroviral and Lentiviral Vector Based Gene Therapy Products - Revisiting Current FDA Recommendations' and 'Guidance for Human Somatic Cell Therapy and Gene Therapy', Version March 1998

Table 5. FDA recommendations for the material to be tested for RCV during production

(based on 'Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up', Version January 2020) (FDA, 2020)

Material to be tested	Frequency of testing	Testing for expected RCR - cells and supernatant [1]	Testing for ecotropic RCR - cells and supernatant
MCB	One-time		
• Derived by transduction with ecotropic vector		Yes	Yes [2]
• Derived by transfection of retroviral vector plasmid		Yes	Not applicable
Vector Harvest Material	Product release		
• End of Production cells (EoP) [3]		Yes	Not applicable
• Vector supernatant		Yes	Not applicable

[1] RCR testing should be based on the type of vector envelopes used
[2] If the MCB was produced using a retroviral vector pseudotyped with an envelope distinct from the clinical vector product, for example, an ecotropic MLV, the potential exists for introduction of an RCR with that distinct envelope. Even though an ecotropic MLV RCR may present a minimal direct safety risk to humans, the presence of any replication-competent genome in the Vector Producer Cell (VPC) MCB is problematic because of the increased probability of generating an RCR with a human host range through recombination with elements within the VPC
[3] Cells from which a single bulk harvest of retrovirus-containing supernatant is taken or cells from which the last of a serial set of supernatant harvests is taken

Vector supernatant assays should include culture of supernatant on a permissive cell line in order to amplify any potential RCR present. Also, EOP cell testing should be accomplished by co-culture with a permissive cell line in order to amplify any potential RCR present. The recommendation to test both supernatant and EOP is based on data and experience reported at the 1997 FDA/NIH Gene Therapy Conference, where it was reported that RCR in vector production lots was not always consistently detected in both vector supernatant and EOP cells. Typically, RCR culture assays have included a minimum of five passages. Assay optimization may allow for fewer passages if demonstrated to achieve similar assay sensitivity. RCR assays should support virus entry, amplification, and particle production specific to vector design, meaning that certain production systems may require more than one amplification cell line. The amplified material may then be detected using a suitable RCV assay (e.g. PG-4 S+L-, PERT, psi-gag or VSV-G pcr, or commercially available p24 ELISA). Any alternative methods should be developed in consultation with the FDA's Center for Biologics Evaluation and Research (CBER). Data on sensitivity, specificity and reproducibility should be provided to support the use of alternative methods. When large volumes or high titer retroviral vector preparations are used, interference in RCR detection has to be taken into account and more sensitive detection methods may have to be developed.

In terms of amounts of material to be tested, FDA recommends testing at least 5% of the total supernatant of the vector harvest material thereby ensuring a 95% probability of detection of RCR if present at a concentration of 1 RCR/dose equivalent. A more detailed explanation of the rationale and the mathematical formulas applied to achieve this level of testing confidence is found in the Appendix 1-1 of the Guideline. For EOP cells, it is recommended that 1% or 10^8 (whichever is less) pooled vector-producing cells is tested by co-culture with a permissive cell line.

Finally, the FDA emphasizes that all assays should include relevant positive and negative controls to assess specificity, sensitivity, and reproducibility of the detection method employed. For example, a standard virus stock can be used for determination of the volume in which a single RCR can be determined and for determination of inhibitory effects on detection of RCR. Gammaretrovirus RCR standard virus stocks have been developed, their infectious titers have been determined, and are commercially available. Standard virus stocks have not yet been developed for other retrovirus vectors. For recommendation on developing an in-house standard virus stock FDA refers to its Guidance 'Analytical Procedures and Methods Validation for Drugs and Biologics; Guidance for Industry' (Version July 2015) (FDA, 2015).

5.2 RCV assays, a survey amongst stakeholders

In addition to researching published information, this study also aimed to include experience with and daily practices of RCV testing from different stakeholders. Such practical experience is important to put the findings from literature in perspective. In total, 20 potential contributing organizations were identified, covering a diversity in terms of type of activity (research, production, application, RCV testing) and type of organization (academic, industry, CRO). They were contacted by targeted mailing, phone and/or teleconference. A broad call for interest was launched via social media (LinkedIn) and via the discussion platform of EBSA.

Thirteen organizations that were solicited either did not respond or indicated not to complete the survey due to various reasons (no interest or not relevant for their activity, no time, not willing to share knowhow and/or information considered to be proprietary, of no concern since working with 3rd generation SIN vectors).

In total, 7 organizations completed the survey. All of the contributing organizations were involved in research. In addition, some were also performing clinical application of vectors/transduced cells (3/7 organizations) and/or offered vector production as a service (3/7 organizations). Table 6 provides an overview of the **type of activities** performed. All production activities involved small scale (max. 300 ml).

Table 6. Type of activities for the organizations responding to the survey

Type of activity	Number of organizations performing the activity / total number of organizations
Production of vectors	7 / 7
Use of vectors for transduction of cells *in vitro*	7 / 7
Use of vectors for transduction of cells *in vivo*	5 / 7
Clinical application of vectors	1 / 7
Clinical application of transduced cells	3 / 7

Six out of 7 organizations that completed the survey performed activities with lentiviral vectors (HIV and one facility SIV), 3 out of 7 organizations used a murine gammaretrovirus (MMoLV). The production systems used to generate the vectors were of the 2nd or 3rd generation, either with or without SIN properties. A variety of proteins were used for pseudotyping.

RCV testing was performed by 3/6 organizations working with **lentiviral vectors**, the 3 additional organizations handling lentiviral vectors indicated not to perform RCV testing based on a risk assessment, i.e. using a production system of 3rd generation, SIN. One production organization additionally highlighted that in case of HIV-based vectors generated with a production system of the 2nd generation, their clients are advised to perform RCV testing upon receipt of transduced cells. Only if unexpected cell death is observed, the production organization will perform RCV testing on the vector batch.

RCV testing for MMoLV-based vector batches was performed by 1 out of 3 organizations. One organization indicated not to perform RCV testing since, based on a risk assessment, the likelihood for RCV was deemed negligible and all activities are performed in a containment level 2 environment. One organization did not further specify the rationale for not performing RCV testing.

Table 7. Overview of the RCV testing for organizations using lentiviral vectors

Characteristics of RCV testing	Specification of RCV testing by organization and vector type			
	Organization 1 - Lentiviral vector	Organization 2 - Lentiviral vector	Organization 3 - Lentiviral vector	Organization 4 - MMoLV-based vector
Type of activities	R&D, Vector / transduced cell production as service	R&D, Clinical application, Vector / transduced cell production as service	R&D, Clinical application	R&D, Vector production as service
Timepoint of testing	Transduced cells prior to release to clients	End of production	End of production cells Final filled vector batch	End of production
Amplification prior to testing	Yes	Yes	Yes	Yes
Type of assay used	p24 ELISA	p24 ELISA	PERT	Marker rescue assay (e.g. GFP, transfer of G418 resistance)
Positive control	Detergent inactivated wild type HIV	Attenuated HIV devoid of accessory genes	Wild type HIV	Supernatant of MEL cells containing Rauscher sarcoma virus
Negative control	Lysis buffer or medium	Culture medium of uninfected cells	Culture medium of uninfected cells	No
Assay parameters checked				
- Limit of blanc	-	-	-	-
- Limit of detection	-	1 TCID50	RT activity detected in 1 out of 3 flasks inoculated with 1 TCID50/culture	-
- Limit of quantitation	Yes (9 pg/ml)	-	-	-
- Accuracy	Yes (not specified)	Ratio between 70 and 130%	-	-
- Sensitivity	-	-	-	-
- Repeatability	Yes (max. twofold difference in OD values allowed)	CV max of 1.41	Performance of positive control consistent over 2 occasions by one operator	-
- Within-lab reproducibility	Yes (not specified)	-	Performance of positive control consistent over all runs irrespective of the operator	-
- Between-lab reproducibility	-	-	-	-
- Linearity	-	OD standard 62.5 pg/mL >0,500 (p24 test)	-	Dilution series with positive control
- Robustness	-	100 %	-	-
Advantages	Sensitive (pg range) Relatively easy to perform Suitable for HIV and SIV	Sensitive (due to amplification phase)	Not specified	Easy to perform Robust
Disadvantages	Labor intensive (amplification phase) Regular runs required Differences between ELISA kits of different providers No functional assay	Labor intensive (amplification phase) High number of parallel cultures to be performed	Not specified	Handling of replication competent positive control virus
RCV detected	No	No	No	Yes
Rationale for RCV testing	Historical use, Risk assessment	Risk assessment, Legal requirements	Legal requirements	Historical use

Table 7 provides a detailed overview of the RCV testing specifications for each of the 4 organizations that did perform testing. Importantly, all included an amplification step. The types of assays used to detect putative RCV varied from organization to organization. For the p24 ELISA, used for HIV-based vectors, the relevance of demonstrating a decrease in p24 signal over a specified period of time was highlighted. One institution even indicated that cells transduced with lentiviral vectors were only transferred to other departments or clients when no p24 signal is detected anymore, even if this implied having the cells longer in the production facility.

Interestingly, one organization mentioned they detected replication competent viruses in gammaretroviral vector preparations. The RCV formation was related to the presence of endogenous retroviral sequences in the murine host or production cells, highlighting the importance of using (non-murine) cells free of such sequences.

Detailed validation data were provided by some organizations. Taken the diversity of assays and controls into account, no comparison could be made. This difficulty was also reflected in the information obtained via literature.

Finally, depending on the type of activities performed by the organizations, different rationales were presented as to why RCV testing was performed. Only for those organizations involved in clinical application of vectors or vector-transduced cells, regulatory requirements were indicated. Other organizations performed RCV testing based on a risk assessment (e.g. production system of the lentiviral vector, SIN characteristics, likelihood of endogenous retroviral sequences, …).

6 Conclusion

This study was set up to collect information on test systems used to detect RCVs, potentially generated during the production of lentiviral and gammaretroviral vectors.

Lenti- and gammaretroviral vectors have been used for many years in R&D and clinical application. However, as they are derived from wildtype viruses, different safeguards have been designed and combined to ensure that the hazardous features, such as the capacity to replicate, are removed and cannot be reconstituted. Indeed, one of the major concerns for safe use of these vectors is the formation of RCV, in particular the reconstitution of primary RCV during the production phase. Since their first use, specific safeguards have therefore been implemented in vector production systems to eliminate the possibility of RCV generation, as summarized in Section 3 of this report. For each of these safeguards, there is adequate information to conclude that they significantly reduce the likelihood for RCV formation. Even though many design considerations are of a theoretical nature, this review confirmed that none of the scientific publications or questionnaire respondents have detected RCV, provided production cells are free of endogenous retroviruses.

Nevertheless, as long as insufficient information is available to estimate the theoretical risk, performance of assays to check for the absence of RCV in vector batches or in cells transduced with the vectors has been recommended and is for many clinical applications even mandatory. A variety of assays have been developed to test for the absence of RCV.

1 **The absence of RCV is a key determining factor for reducing the risk level of activities with lenti- or retroviral vectors. In that perspective, different safety features have been combined in the design of the vectors to make formation of RCV extremely unlikely. In addition, a variety of assays has been developed to test for the presence of RCV.**

International guidelines with specific recommendations for RCV testing have been established for medicinal applications. In this study, examples from EMA and FDA were described that impose technical objectives for mandatory demonstration of RCV absence at different stages of vector production. Even more, whereas limited data are available in literature on the actual performance and reliability of the assays, developers are required to provide detailed information and validation records on the methods that are used for each product. Alternative approaches (e.g. a risk assessment based on vector design supporting that the likelihood for presence of RCV is negligible) have been accepted and may become more regular as more experience becomes available.

In contrast, only a limited number of regulatory guidance documents were identified addressing the need for RCV testing for production of vectors in the context of R&D. This may be explained by the fact that application of vectors for R&D only occurs in a controlled, contained environment. In other words, containment measures are implemented stringently to avoid exposure to and dispersal of the vectors. There seems to be consensus among risk assessors that for SIN vectors generated with a production system of the 3rd generation the chance for RCV testing is negligible, and thus, that testing is not required. Nevertheless, demonstrating the absence of RCVs in R&D is relevant for transduced cells in order to allow researchers to manipulate these cells at a lower containment than required for the vectors themselves. Similarly, it was highlighted that an evaluation of remaining replication-deficient vector particles was equally important, especially in case of transgenes with a risk for human health and/or environment. The latter however remained outside the scope of this study.

2

For applications with (investigational) medicinal products consisting of or generated with retro- or lentiviral vectors, RCV testing is in many cases mandatory, unless the competent authorities allow alternative approaches.
In R&D environment, RCV testing is not performed routinely and only when deemed necessary based on a risk assessment.

Assays to detect RCVs can roughly be divided into (1) structural assays, i.e. assays that are based on the detection of structural elements such as genetic sequences (e.g. qRT-PCR) or proteins (e.g. immune assays) of a putative RCV, and (2) functional assays, i.e. assays that reveal the presence of a biologically functional RCV. Each assay has specific strengths and weaknesses as outlined in Section 4. A major point of attention for the structural assays is that a positive outcome does not necessarily correlate with the presence of RCV. Indeed, the structural components identified (e.g. genetic sequences, proteins) may also be present in replication-deficient vector particles.

As compared to structural assays, functional assays will only provide a positive outcome when RCV are present since the biological function to be tested is not present in the replication-defective vector particle. Still, care is to be taken in view of interpretation as the biological function may be intrinsically present in the host cell or introduced via endogenous retroviruses. Using a read out that cannot be naturally detected in the host cell may offer an advantage.

Amplification in cells prior to performing the structural or functional assays is useful to increase the number of putative RCVs and decrease the number of vector particles, in consequence increasing the sensitivity of an assay. Also, amplification will allow to determine the kinetics of a structural parameter. Where it is expected that the structural parameter will decrease when it originates from a vector particle due to washing and dilution, the parameter will increase when it originates from an RCV that is replicating in course of time. The amount of RCVs may however be low, thus a clear understanding of the limitations of the assays is important. On the other hand, amplification does require time and thus costs which especially in the context of clinical application is a clear disadvantage. For this reason, Martineau et al. (1997) proposed that a PCR-based rapid alternative RCR or RCL assay may be used as a lot release test for the transduced cell product, but that a cell culture based RCR or RCL assay is initiated at the same time for the purpose of retrospective confirmation.

3

RCV assays are either based on structural elements or on functional aspects of RCV. Each assay has different advantages and disadvantages. Including an amplification phase prior to testing increases sensitivity for all assays since the amplification phase increases the number of RCVs and decreases the number of (interfering) vector particles. Amplification may be the only way to discern between RCV and vector particles, although it significantly increases the required time and effort.

Despite the frequent use of RCV assays and determination of critical parameters such as LoDs, it is difficult to compare the results and to establish their biological significance. The LoD will indicate the lower level that can be detected, yet - as pointed out before - this cannot be interpreted as complete absence. In order to determine the biological significance of 'no detection of a parameter' *versus* 'no detection of a parameter above the LoD', one would need to compare the assay lower limit with the minimal dose that would be required for a successful infection leading to RCV propagation. Such

information is for many organisms not available and is an important element of uncertainty. Such uncertainty can be included in the risk assessment.

While absence of evidence is not evidence of absence, confirming the absence of a rare occasion of RCV formation remains delicate. It requires correct interpretation of an assay to ensure that a negative result does indeed refer to RCV absence and not to an incapability of the assay to measure RCV, even at low levels. Nevertheless, repetitive demonstration of no significant detection increases the confidence in the assumption of the highly exceptional nature of RCV creation with advanced production systems.

Another point of attention in relation to biological relevance is the fact that detecting a parameter indicative for recombination, in particular for a structural assay, does not necessarily mean that recombination resulted in a functional RCV. This is clearly illustrated in the study by Sastry et al. (2003) who showed that *psi-gag* recombinants were routinely detected by means of the PCR assay but no evidence was found of *psi-gag* transfer to naïve cells with only the latter being a relevant parameter proving the presence of functional RCV. In this perspective, one might argue that performing more than one assay based on a different methodology may be useful. Even though each individual test may not be proven to be fully reliable, using multiple tests based on different (functional and/or structural) aspects of the RCV may provide a solid testing strategy. This was illustrated by Marcucci et al. (2018) who observed that samples provided a positive signal in a molecular assay, but when retested with a biological assay the samples turned out to be RCV negative.

Advanced vectors are designed to reduce the likelihood of RCV formation and the advanced assays are intended to confirm that no RCV is present. As a consequence, in the absence of cases where RCV have been demonstrated, it is impossible to determine the likelihood of the remaining - so far theoretical - risk.

4

There remains uncertainty on the link between the outcome of an RCV assay on the one hand and the biological relevance in view of RCV formation on the other hand. Additional research on the biological relevance of an assay outcome and providing a robust set of validation data would be necessary to complete the framework for risk assessment of the likelihood of RCV formation and to determine the relevance of RCV testing.

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8 Annexes

8.1 Overview of relevant COGEM advises referring to RCV testing

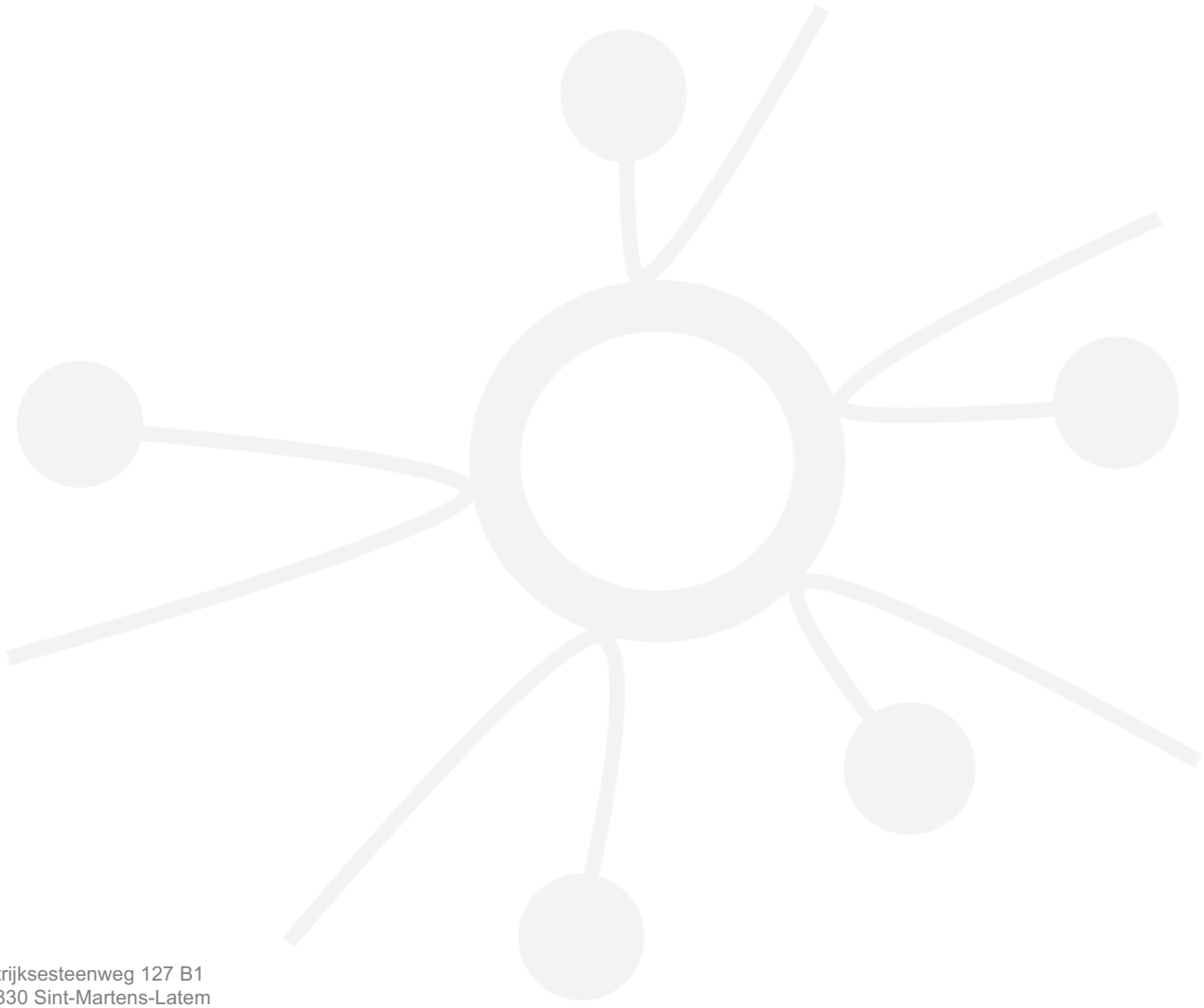
Application	Vector type	Reference	Title	Vector characteristics		RCV testing performed
				Production system 3rd generation	SIN	
Clinical	HIV-based	CGM/160229-01	Klinische studie met lentiviraal getransduceerde T-cellen tegen B-cel maligniteiten	Yes	Yes	Yes
		CGM/180612-01	Klinische studie met lentiviraal getransduceerde T-cellen (JCAR017) tegen B-cel maligniteiten	Yes	Yes	Yes
		CGM/181206-01	Klinische studie met lentiviraal getransduceerde T-cellen (KITE-585) tegen B-cel maligniteiten	Yes	Yes	Yes
		CGM/181231-01	Klinische studie met lentiviraal getransduceerde T-cellen (JCAR017) tegen B-cel maligniteiten	Yes	Yes	Yes
		CGM/190624-03	Klinische studie met lentiviraal getransduceerde T-cellen (JNJ-68284528) tegen B-cel maligniteiten	Yes	Yes	Yes
		CGM/190726-01	Klinische studie met lentiviraal getransduceerde T-cellen (bb2121) ter behandeling van multipel myeloom	Yes	Yes	Yes
		CGM/190729-01	Generieke milieurisicobeoordeling van klinische studies met ex vivo retro- en lentiviraal getransduceerde cellen	Generic advice		
		CGM/190805-01	Klinische studie met lentiviraal getransduceerde CD34+ cellen ter behandeling van RAG-1-SCID en RAG-2-SCID	Yes	Yes	Yes
		CGM/191114-01	Klinische studie met lentiviraal getransduceerde CD34+ cellen ter behandeling van cerebrale adrenoleukodystrofie (CALD)	No	Yes	Yes
	Gammaretroviral-based	CGM/110831-01	Klinische studie met retroviraal getransduceerde humane T- lymfocyten	N.a.	No	Yes
		CGM/110913-01	Klinische studie met retroviraal getransduceerde humane T- lymfocyten in leukemiepatiënten	N.a.	No	Yes
		CGM/111012-03	Aanvullende informatie over de klinische studie met retroviraal getransduceerde humane T-lymfocyten	N.a.	No	Yes
		CGM/161130-01	Klinische studie met getransduceerde T-cellen tegen B-cel maligniteiten	N.a.	Not specified	Yes

Application	Vector type	Reference	Title	Vector characteristics		RCV testing performed
				Production system 3rd generation	SIN	
		CGM/171013-02	Klinische studie met TEG001 ter behandeling van hematologische en solide tumoren	N.a.	No	Yes
		CGM/180103-02	Klinische studie met retroviraal getransduceerde T-cellen tegen hematologische maligniteiten	N.a.	Not specified	Yes
		CGM/190722-01	Klinische studie met retroviraal getransduceerde T-cellen gericht tegen MAGE-C2 maligniteiten	N.a.	No	Yes
R&D	HIV-based	CGM/041103-01	Handelingen met lentivirale getransduceerde cellen in een ruimte zonder inperking	Yes	Not specified	Yes
		CGM/050330-01	Transplantatie van lentiviraal getransduceerde beenmergcellen in apen	Yes	Yes	Yes
		CGM/050427-01	Implementatie van een lentivirus-afgeleid genoverdracht systeem: Metingen van lentiviraal getransduceerde zoogdiercellen onder een microscoop in een ruimte zonder inperking	Yes	Yes	No
		CGM/050527-01	Lentiviraal gemedieerde transductie van hematopoietische stam/voorlopercellen	Yes	Yes	Yes
		CGM/050613-01	Handelingen met lentivirale getransduceerde zoogdiercellen in een ML-I ruimte gebruikmakend van FACS en fluorescentie- microscoop	Yes	Yes	No
		CGM/051129-01	Handelingen met lentivirale getransduceerde zoogdiercellen	Yes	Yes	No
		CGM/051215-01	Handelingen met lentivirale vectoren getransduceerde zoogdiercellen		Generic advice	
		CGM/090331	Inschaling van laboratoriumwerkzaamheden met lentivirale vectoren		Generic advice	
		CGM/210218-01	Heroverweging inschaling werkzaamheden met replicatiedeficiënte lenti- en gammaretrovirale vectordeeltjes onder Ingeperkt Gebruik		Generic advice	
		CGM/210503-01	Heroverweging inschaling werkzaamheden replicatiedeficiënte lenti- en gammaretrovirale vectordeeltjes		Generic advice	
	Gammaretroviral-based	CGM/020301	Identificatie en functionale karakterisering van genen verantwoordelijk voor de groeicontrole van menselijke cellen	N.a.	Not specified	Yes
		CGM/070405-02	Omlaagschaling van handelingen met retroviraal getransduceerde cellen	N.a.	Not specified	Yes
		CGM/210503-01	Heroverweging inschaling werkzaamheden replicatiedeficiënte lenti- en gammaretrovirale vectordeeltjes		Generic advice	
		CGM/220303-01	Advies over protocol voor de detectie van replicatie-competent retrovirus	N.a.	Not specified	Yes

8.2 Potential parameters for an RCV assay [15]

Parameter	Meaning
Limit of blank (LoB)	Highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested
Limit of detection (LoD)	Lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte
Limit of quantitation (LoQ)	Lowest concentration at which the analyte cannot only be reliably detected but at which some predefined goals for bias and imprecision are met
Accuracy (or trueness)	Agreement between the value found and an expected reference value
Sensitivity	Capability of the assay to differentiate similar organisms or analytes or other interference from matrix elements that could have a positive or negative effect on the assay value
Specificity	Ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.
Repeatability (or intra-assay precision)	Closeness of the results obtained with the same sample (or subsamples of the same sample) using the same measurement procedure, operators, measuring system, operating conditions and location over a short period of time
Within-lab reproducibility (or intermediate precision)	Precision obtained within a single laboratory over a longer period of time, takes into account more changes than repeatability (e.g. different operators, batches of reagents, equipment)
Between-lab reproducibility	Ability of an assay to provide consistent results (when testing the same samples) in different laboratories
Linearity	Ability (within a given range) of the assay to return values that are directly proportional to the concentration (amount) of the analyte in the sample
Robustness	Capacity to remain unaffected by small but deliberate changes in test conditions
Range	Interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity

[15] ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology (CPMP/ICH/381/95); EMA 'Guideline on development and manufacture of lentiviral vectors'; Broeders et al., 2014; Bustin et al., 2009



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