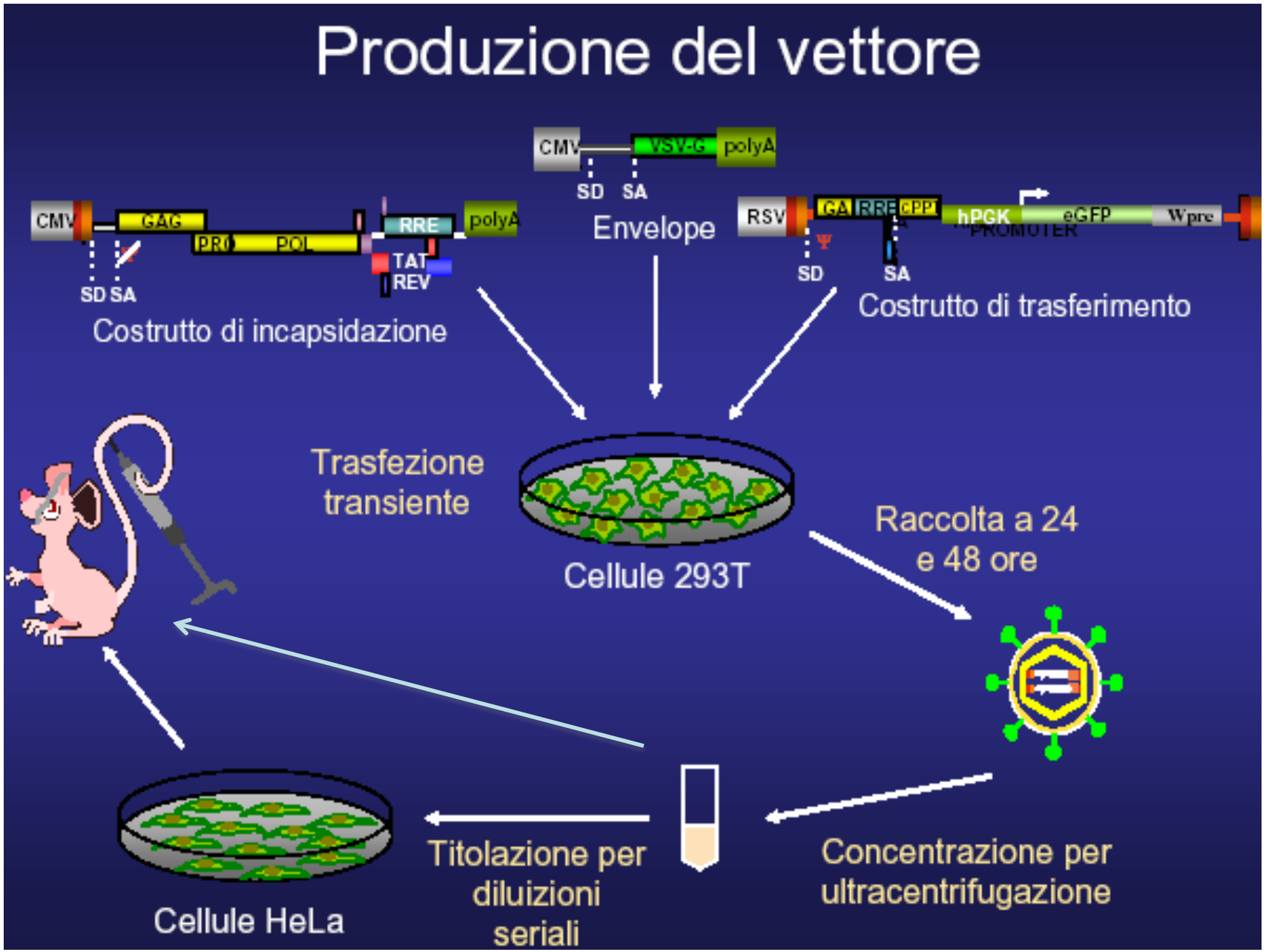
recombinant lentiviral vector production

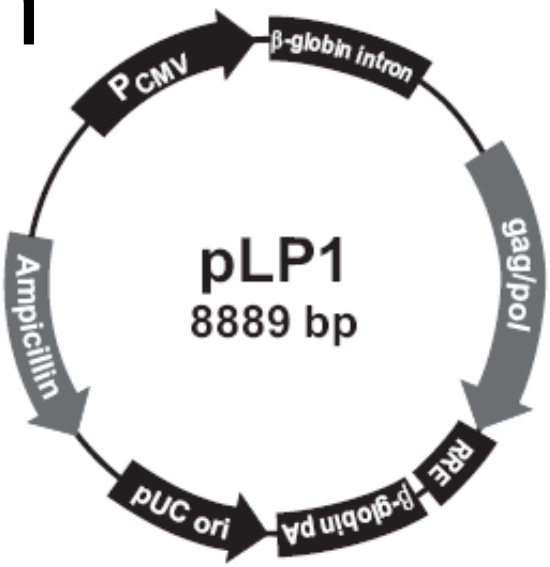
Some viral proteins are toxic, therefore stable transfected cells cannot be produced.
The packaging cells (human kidney 293) are transiently transfected with the all the vector components.
High viral titers are obtained by using the HIV vectors devoid of vif, vpr, vpu e nef.
The pseudotyping with VSV-G increases vector production although VSV-G is toxic at high level.

The production of lentiviral vectors requires the introduction, in the packaging cells, of all the vectors (the packaging, the transfer and the env constructs).

Produzione del vettore



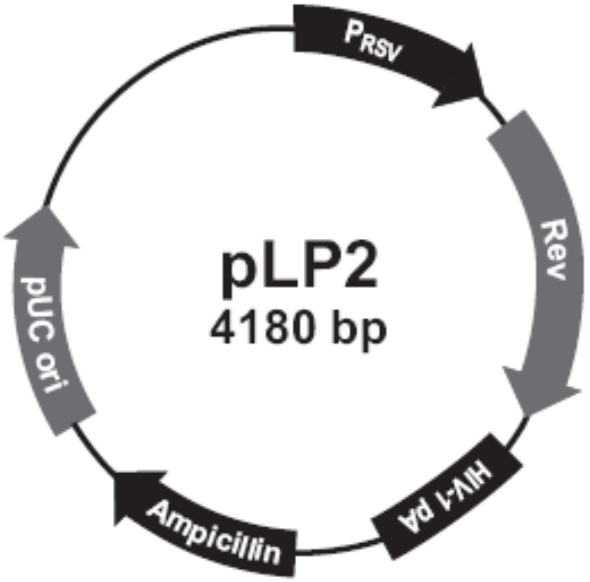
packaging vectors: trans lentiviral system



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
  TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
  gag coding sequence: bases 1355-2857
  gag/pol frameshift: base 2650
  pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

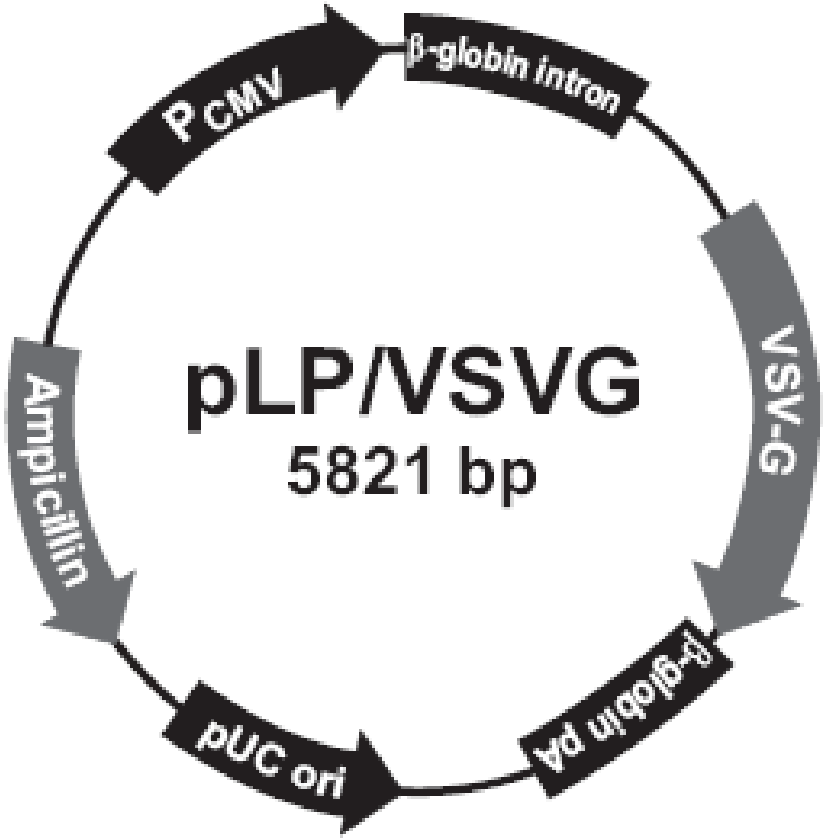
C=complementary strand



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
  TATA box: bases 200-207
  Transcription initiation site: base 229
  RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

envelope vector

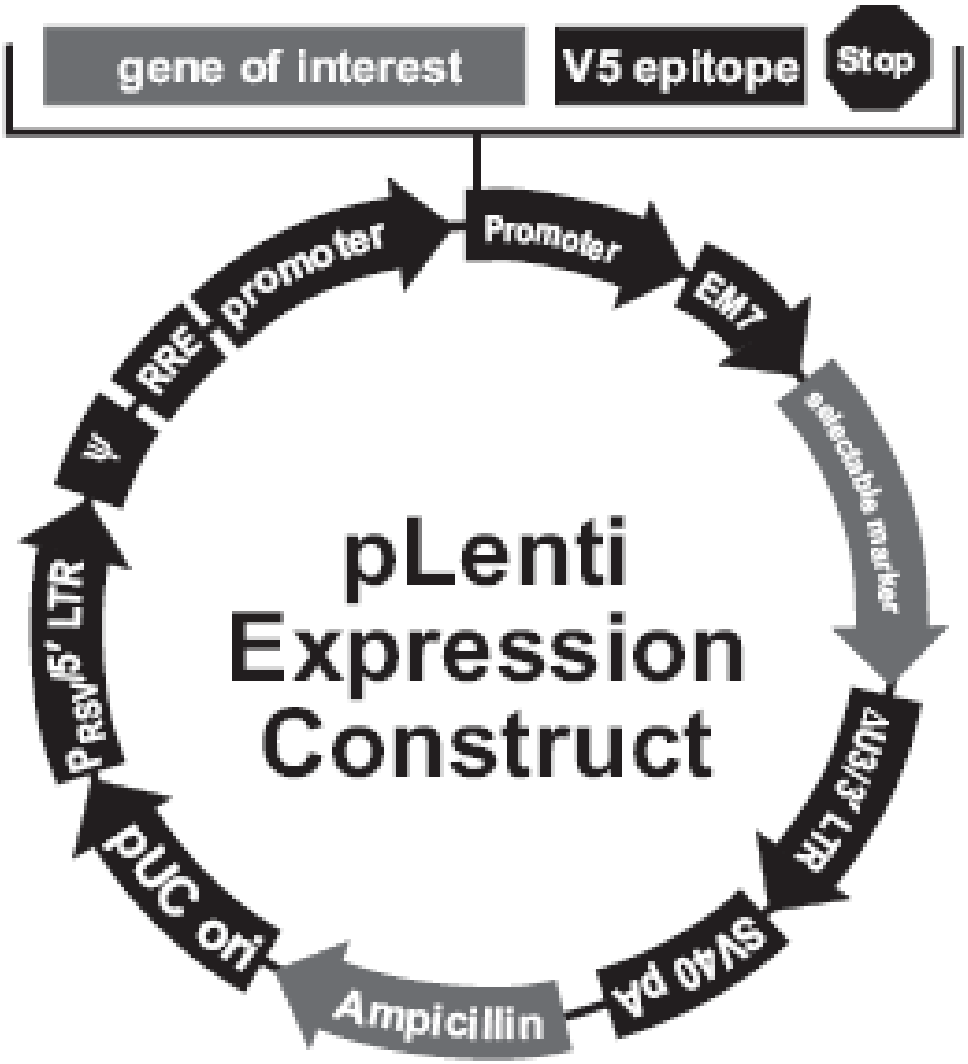


Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

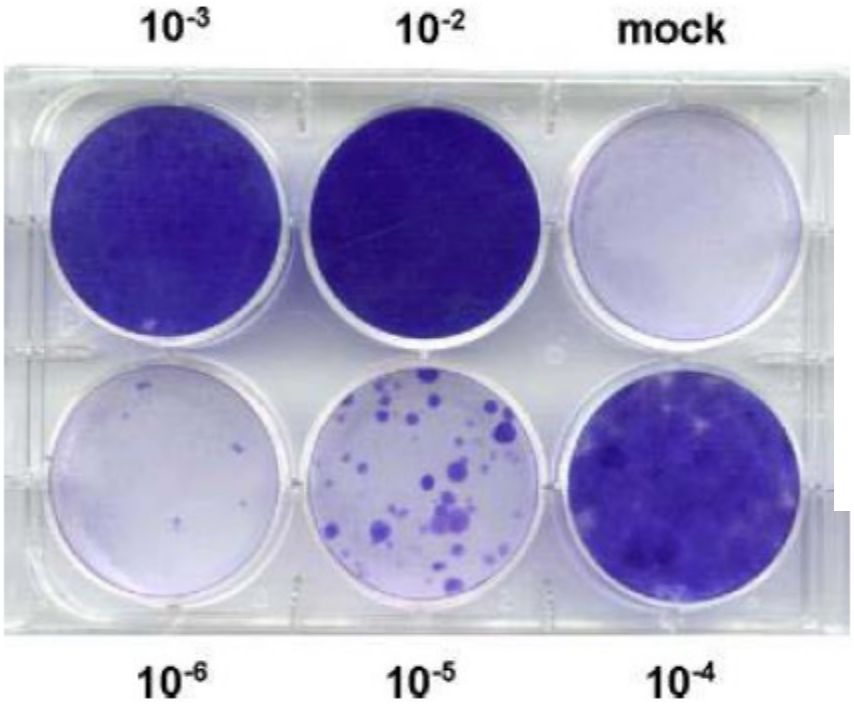
C=complementary strand

transfer vector



Titering Your Lentiviral Stock

When titering pLenti lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 1 - 5 x 10^5 (for unconcentrated virus) up to 2 x 10^7 (for concentrated virus) transducing units (TU)/ml.



In the plate above, the colony counts were:

- Mock: no colonies
- 10^{-2} dilution: confluent; undeterminable
- 10^{-3} dilution: confluent; undeterminable
- 10^{-4} dilution: confluent; undeterminable
- 10^{-5} dilution: 46
- 10^{-6} dilution: 5

Thus, the titer of this concentrated lentiviral stock is 4.8 x 10^6 TU/ml (*i.e.* average of 46 x 10^5 and 5 x 10^6).

TU	5	46	TU/ml4,8 x 10^6

Vector: Lenti6/V5-GW/lacZ
Host cells: HT1080

Retrovirus safety concerns

Safety

Produzione di particelle competenti per la replicazione (RCR):
La ricombinazione omologa/ectopica/NHEJ avviene sempre nel DNA
Steps che portano alla formazione di RCR:
I- ricombinazione tra i vettori nella cellula packaging
II - co-packaging di RNA diversi nella stessa particella virale e successivo trasferimento nella cellula bersaglio

ex vivo transduced bone marrow progenitor cells that had been inadvertently exposed to high titer RCR contained in the retroviral vector material to severely immunosuppressed Rhesus monkeys. In this setting, 3/10 animals developed lymphomas and died within 200 days (Donahue et al 1992).

Reshus monkey- midollo osseo trattato ex vivo con vettore MoMLV
3 dei 10 animali trattati hanno sviluppato "T-cell lymphoma"
2 dei 3 hanno mostrato RCR derivate da ricombinazione con i vettori che formavano il sistema di packaging/env
1/3 ha mostrato RCR derivanti da ricombinazione con un retrovirus endogeno

Donahue, R. E., S. W. Kessler, D. Bodine, K. McDonagh, C. Dunbar, S. Goodman, B. Agricola, E. Byrne, M. Raffeld, R. Moen, J. Bacher, K. M. Zsebo, and A. W. Nienhuis. 1992. Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. J. Exp. Med. 176:1125-1135.

Lentivirus-2 safety concerns

Safety: RCR

Donahue, R. E. et al. 1992. Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. J. Exp. Med. 176:1125-1135.

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Reshus monkey- midollo osseo trattato ex vivo con vettore MoMLV
3 dei 10 animali trattati hanno sviluppato "T-cell lymphoma"

- 2 dei 3 hanno mostrato RCR derivate da ricombinazione con i vettori che formavano il sistema di packaging/env

- 1/3 ha mostrato RCR derivanti da ricombinazione con un retrovirus endogeno

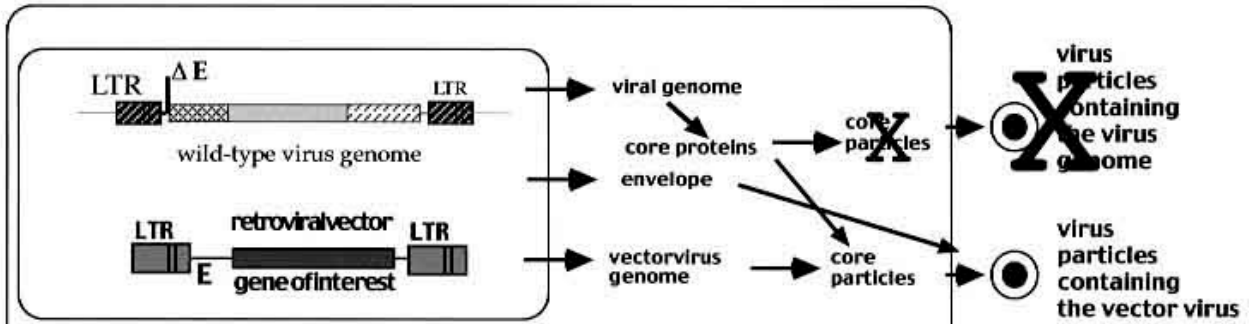
RCR

RCR, REPLICATION COMPETENT RETROVIRAL particle can form by:

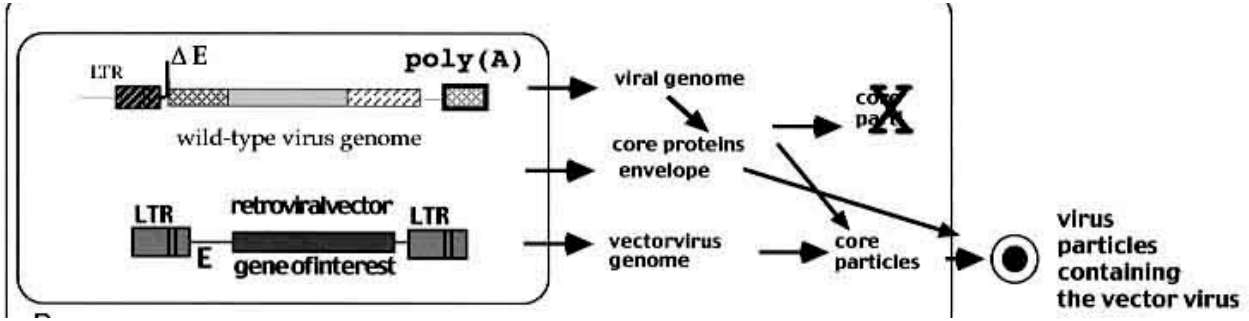
- Homologous recombination among the vectors composing the recombinat-vector system

- Recombination beteween the recombiant-vector system and endogenous provirus –

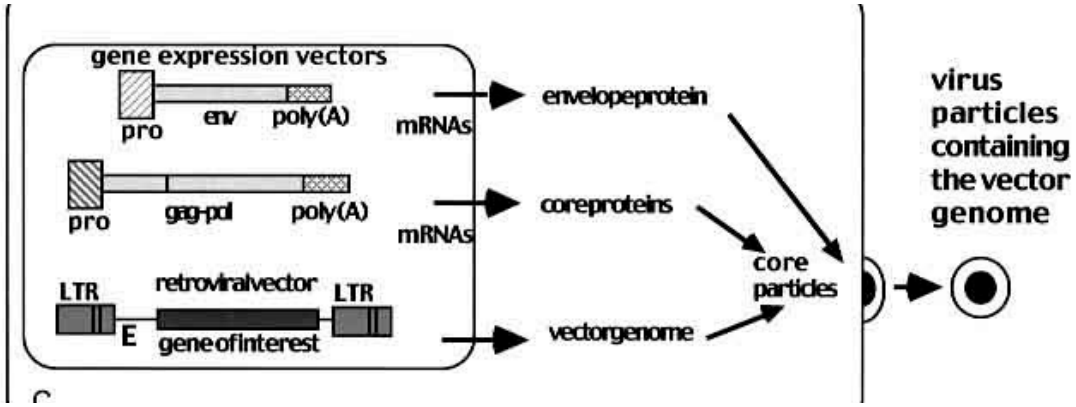
The vector system dictates number of recombination events



one recombination step is required to generate a replication competent retrovirus (RCR)



two recombination steps are required to generate a replication competent retrovirus (RCR)



three recombination steps are required to generate a replication competent retrovirus (RCR)

Guidance for Industry

Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors

U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research
November 2006

THE CELLS TO BE USED

Use of a cell bank system is recommended in order to ensure an adequate and consistent supply of vector producer cells. The Master Cell Bank (MCB) is a collection of cells of uniform composition derived from a single tissue or cell. The Working Cell Bank (WCB) is derived from one or more ampules of the MCB, expanded by serial subculture to a specified passage number

THE AMOUNT TO BE TESTED

To ensure that retroviral vector supernatants used in human clinical trials are free of RCR, the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) recommends that 5% of each vector supernatant lot, and 10^8 or 1% of the end-of-production cells be tested for RCR (US Food and Drug Administration, 1993).

Sastry L, Cornetta K. **Detection of replication competent retrovirus and lentivirus**. Methods Mol Biol. 2009;506:243-63. doi: 10.1007/978-1-59745-409-4_17. PMID: 19110631.

- Murine oncoretroviruses have been known to develop recombinations leading to RCR (Replication Competent Retrovirus);
- RCL (Replication Competent Lentivirus), has not been reported with the commonly used lentiviral vector systems, although theoretically possible;
- A question that is still debated is whether vector product should be screened for a true RCL, or should signs of recombination events between vector and packaging plasmids (without complete generation of RCL) be grounds for rejecting a vector product

HOW TO TEST RCR/L

CELL CULTURE BASED ASSAY TO DETECT RCR

Virus is detected using

- indicator cell line. The PG-4 cell line is S+/L−, it contains the murine sarcoma virus genome (S+) but lacks the murine leukemia virus genome (L−). When infected by a murune leukimia virus these cells are induced to a transformed phenotype.
- Virus is detected using the marker rescue assay . The permissive cell line contains a retroviral vector with a “marker” gene (neomycin). If RCR are present it will package both the RCR genome and the rescue marker

MOLECULAR AND BIOLOGICAL ASSAYS FOR DETECTION OF RCL

Virus is detected in C8166 (Indicator) cells, which are cultured for an additional seven days and analyzed for the presence of viral markers.

- Sensitive assays have been developed for detection of RCL indicative markers: p24gag antigen (by ELISA),
- viral reverse transcriptase (by product enhanced reverse transcriptase or PERT),
- psi-gag sequences (by PCR)
- the psuedotyping envelop VSV-G (by PCR)

The amplification step

The test material is placed on a permissive cell line, and the cells are passaged for a minim um of 3 weeks (amplification phase)

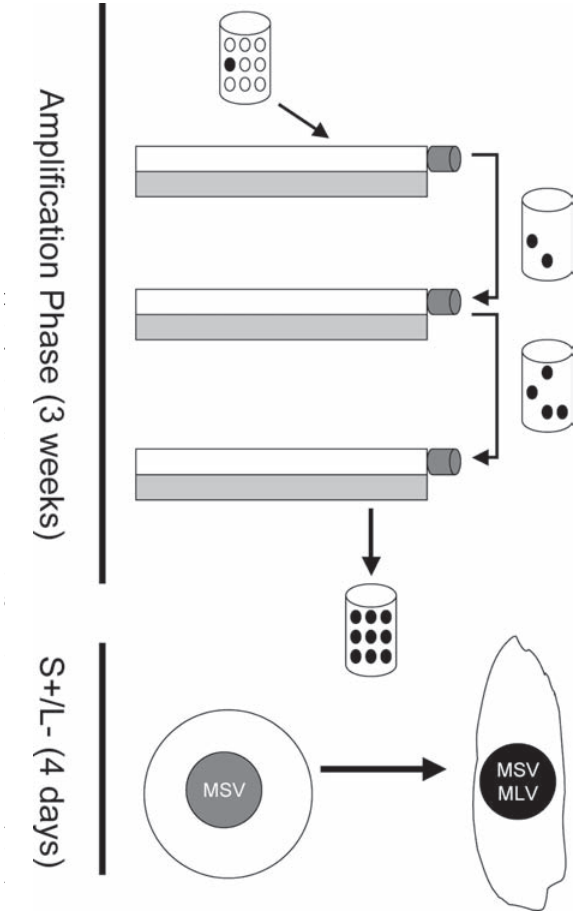
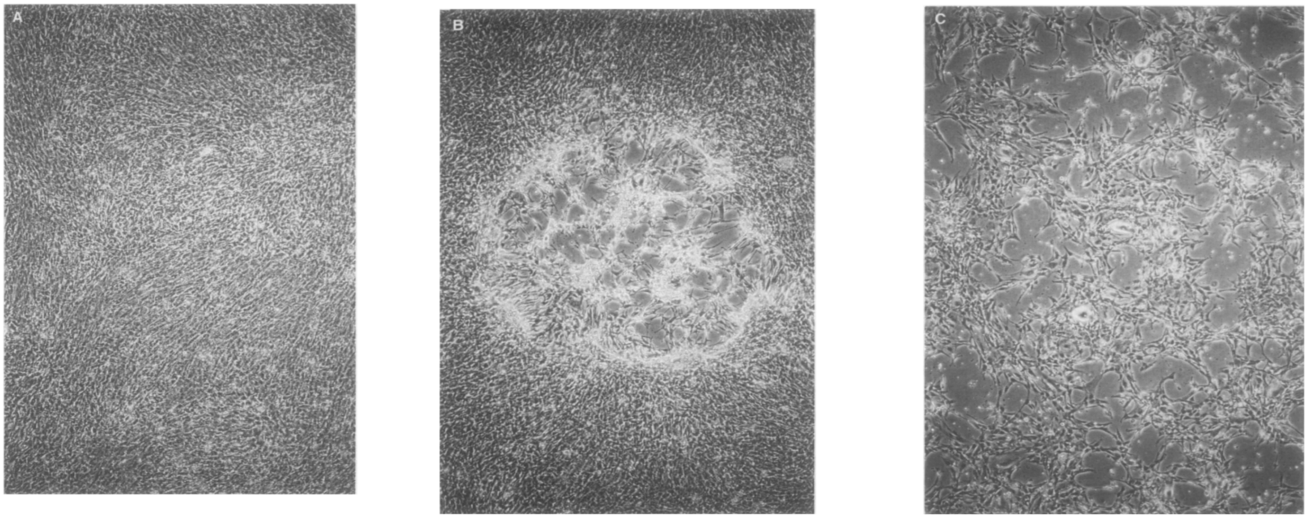


Fig. 1. The detection of replication competent retrovirus (rcr) using biologic assays. Amplification Phase: Test material depicts retroviral vector supernatant in which a small portion of the replication defective vector material (*open ovals*) is contaminated with RCR (*filled ovals*). Biologic assays often utilize a 3-week amplification phase in which a permissive cell line is used to increase the titer of any RCR present in the test material. In the Marker Rescue Assay, the cell lines used in the amplification phase contains an integrated retroviral vector that expresses a marker gene (such as a drug resistance gene). If RCR is present, it will "rescue" the marker vector and the cell supernatant will contain RCR along with virions capable of conferring drug resistance to naïve cells in the Indicator Phase. The S^+/L^- assay also has an amplification step, but in this case the indicator cell line detects RCR directly. The indicator cell is termed an S^+/L^- cells since it contains the murine sarcoma virus (MSV), which will transform the cell phenotype but only in the presence of a murine leukemia virus (MSV) RCR.

monitoring RCR: fosuc forming assay

MLV (murine leukemia virus) is measured using a focus-forming assay on PG-4 S^+L^- cells which contain a replication-defective murine sarcoma virus. Superinfection by MuLV rescues the sarcoma virus resulting in transformation and the formation of foci

PGL-4 S+L- assay: it is based upon morphological changes in the PG-4 indicator cells. In this cells, following MLV infection syncitia are formed leading to foci.



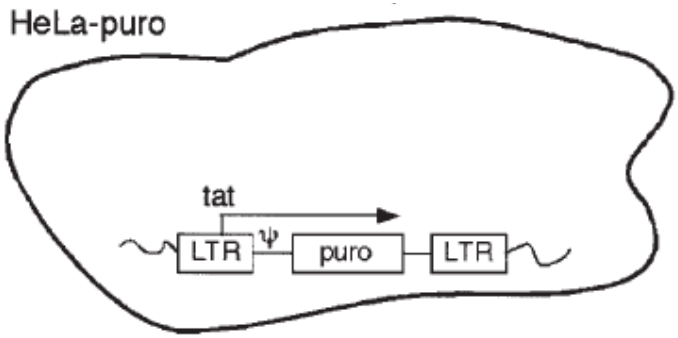
Photomicrogrphs (all at 40X magnification) comparing morphological differences in PG-4 S^+L^- 7 days after inoculation with (A) medium. (B) positive control, MLV supernatant. or (C) high titer RCR-negative vector supernatant.

Infection of indicator cell lines

Monitoring vector recombintion

Infection of indicator cell lines harboring puromycin resistance gene under control of HIV-1 LTR

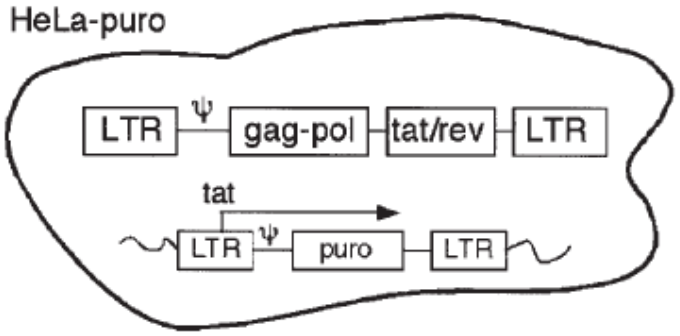
This assay is based on a cell line containing a stably integrated copy of the puromycin resistance gene (HeLa-puro) introduced by transduction with a lentiviral vector. Since the puromycin gene was placed under control of the HIV-1 LTR, it is inducible by Tat expression.



A) Supernatants from cultures of 293T cells infected with a second generation lentiviral vector (10^7 infectious units [IU])

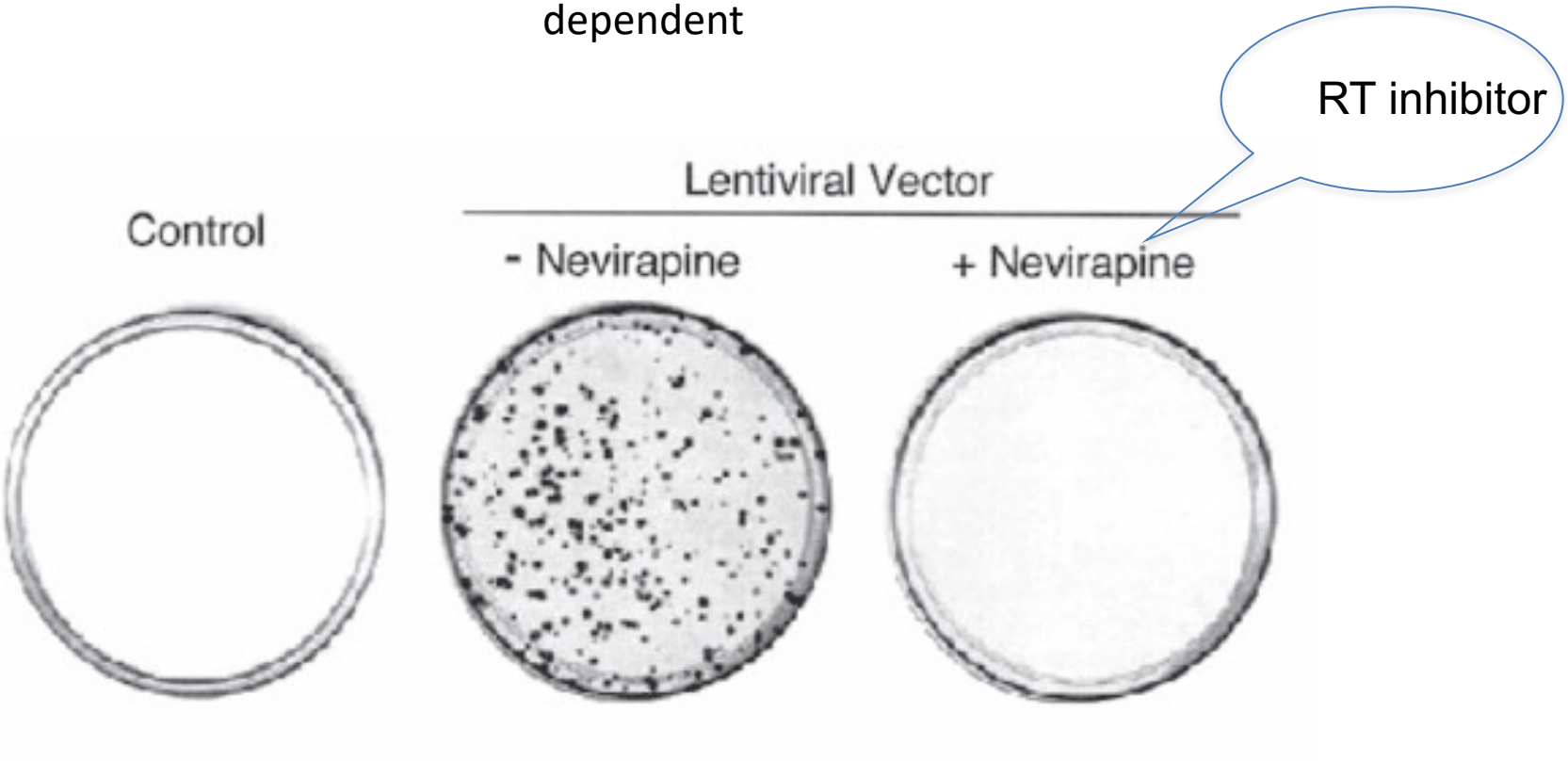
B) SN is inoculated in 293T cells previously co-transfected with the Env construct (pVSV-G) and the tat-expressing vector (pCMV-tat).

C) after few days of incubation the culture supernatant (SN) possibly containing RCR, is collected



A) HeLa-puro cells are inoculated with the culture SN; cells are cultivated under selection with puromycin

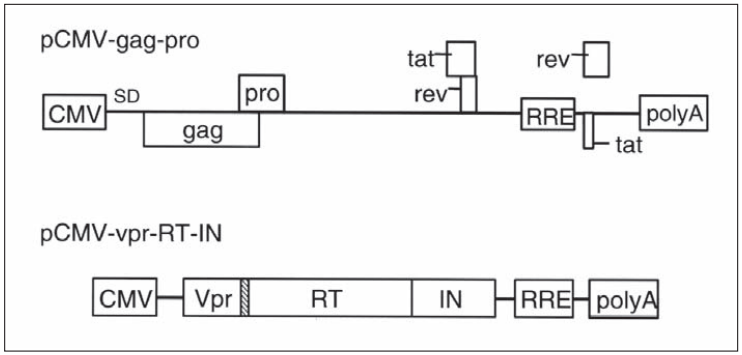
The detection of puromycin resistant cell colonies is reverse transcriptase-dependent

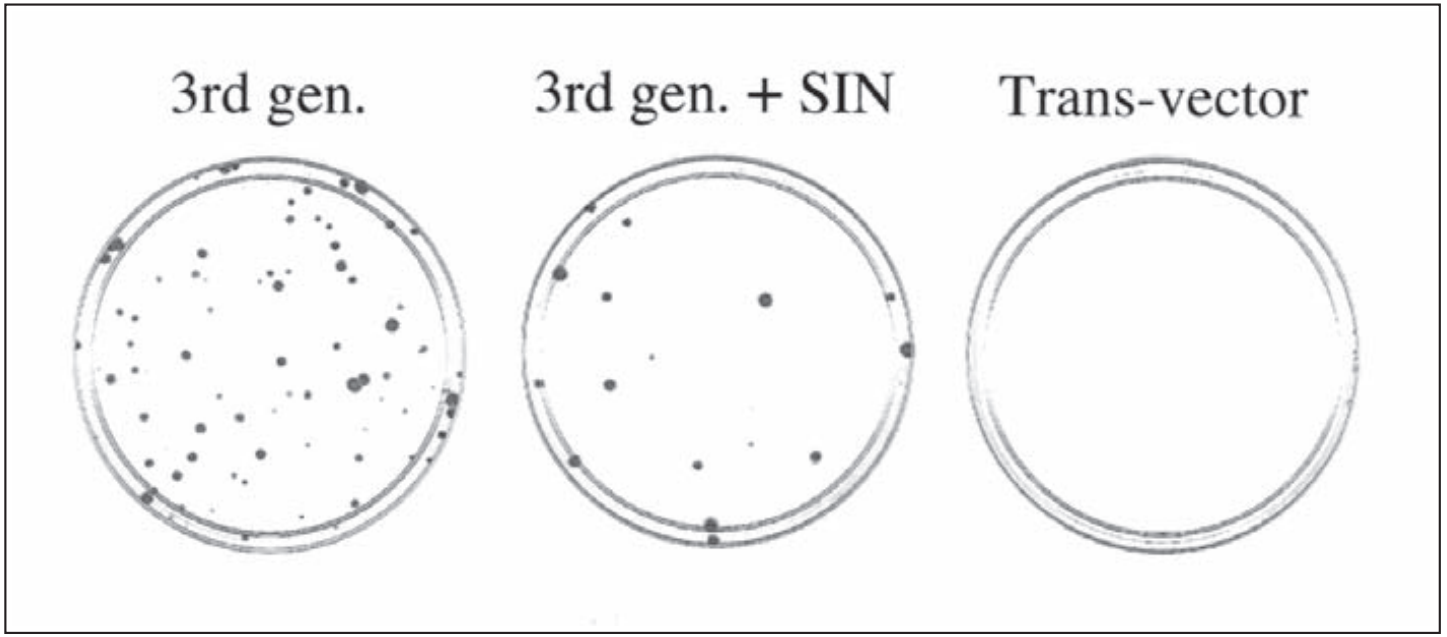


The non-nucleoside reverse transcriptase inhibitor, Nevirapine, completely blocked colony formation, indicating that the formation of lentiviral vector recombinants was dependent on the function of the HIV-1 reverse transcriptase.

recombination in third generation and third generation SIN and trans lentivaral vector lentival vectors

While the third generation and SIN vectors transferred puromycin resistance to naive cells, the trans-vector did not

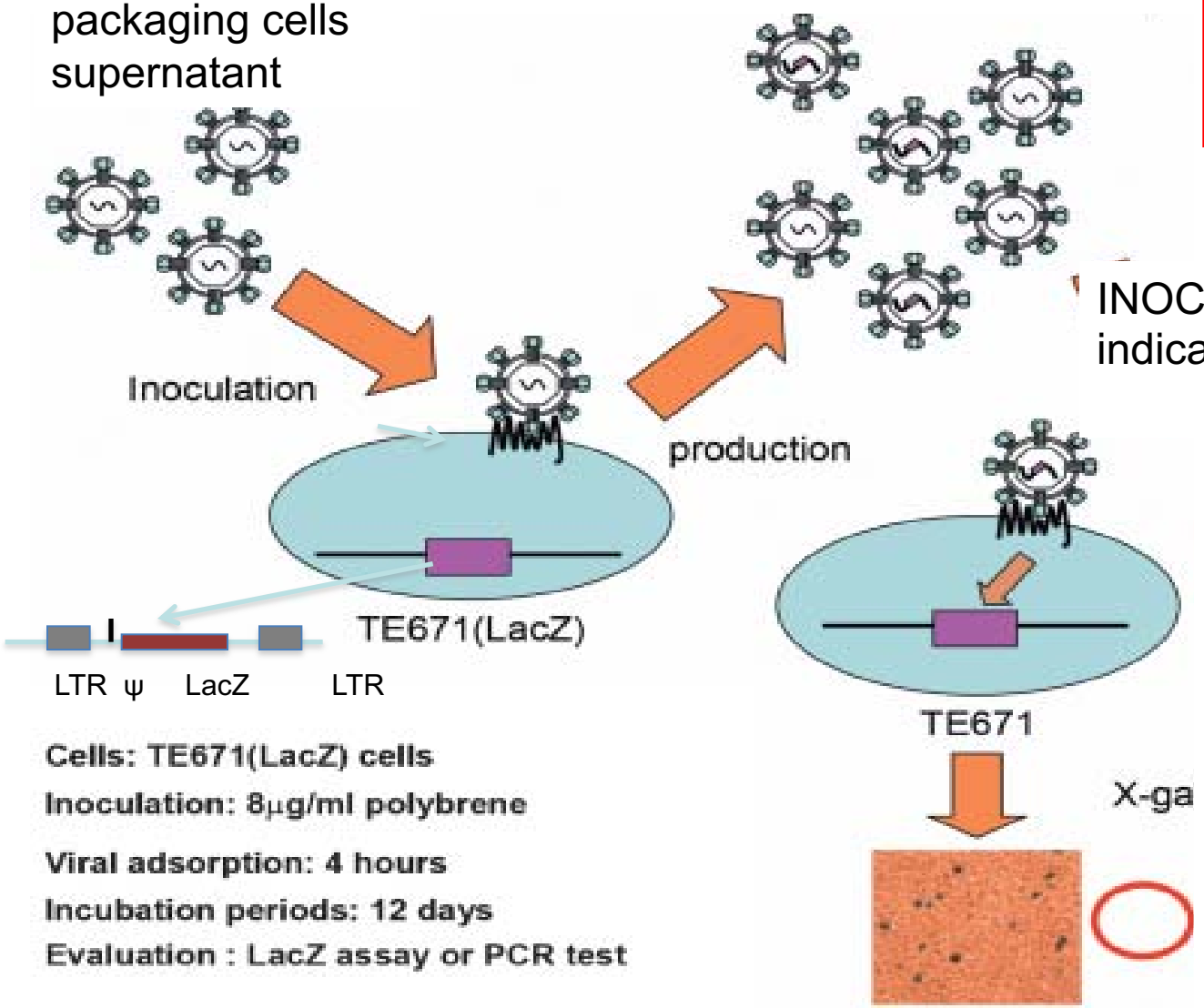




Marker rescue assay

samples from packaging cells supernatant

it is important that the cells used to rescue the marker are free of endogenous retrovirus



INOCULATION into indicator cells

X-gal staining

Cells: TE671(LacZ) cells

Inoculation: 8μg/ml polybrene

Viral adsorption: 4 hours

Incubation periods: 12 days

Evaluation : LacZ assay or PCR test

cells: free of endogeous retrovirus

RCR/RCL in service

Creative Biolabs: https://www.creativebiolabs.net/

BIO-RAD laboratories: https://www.bio-rad.com/

Creative Biolabs

Generally, retroviral vectors are manufactured by collecting supernatants following transient or stable production from cultured cells. RCR may develop at any step during manufacturing, from the initial transfection or transduction steps through production of the retroviral vector supernatant. In addition, if the retroviral vector is used for *ex vivo* genetic modification of cells, the expansion of *ex vivo* transduced cells i culture provides the potential for amplification of an RCR contaminant that may be below the level of detection in the retroviral vector supernatant. Therefore, current recommendations include material testing multiple stages of product manufacture. Figure 1 depicts the recommendations for RCR testing and patient monitoring.

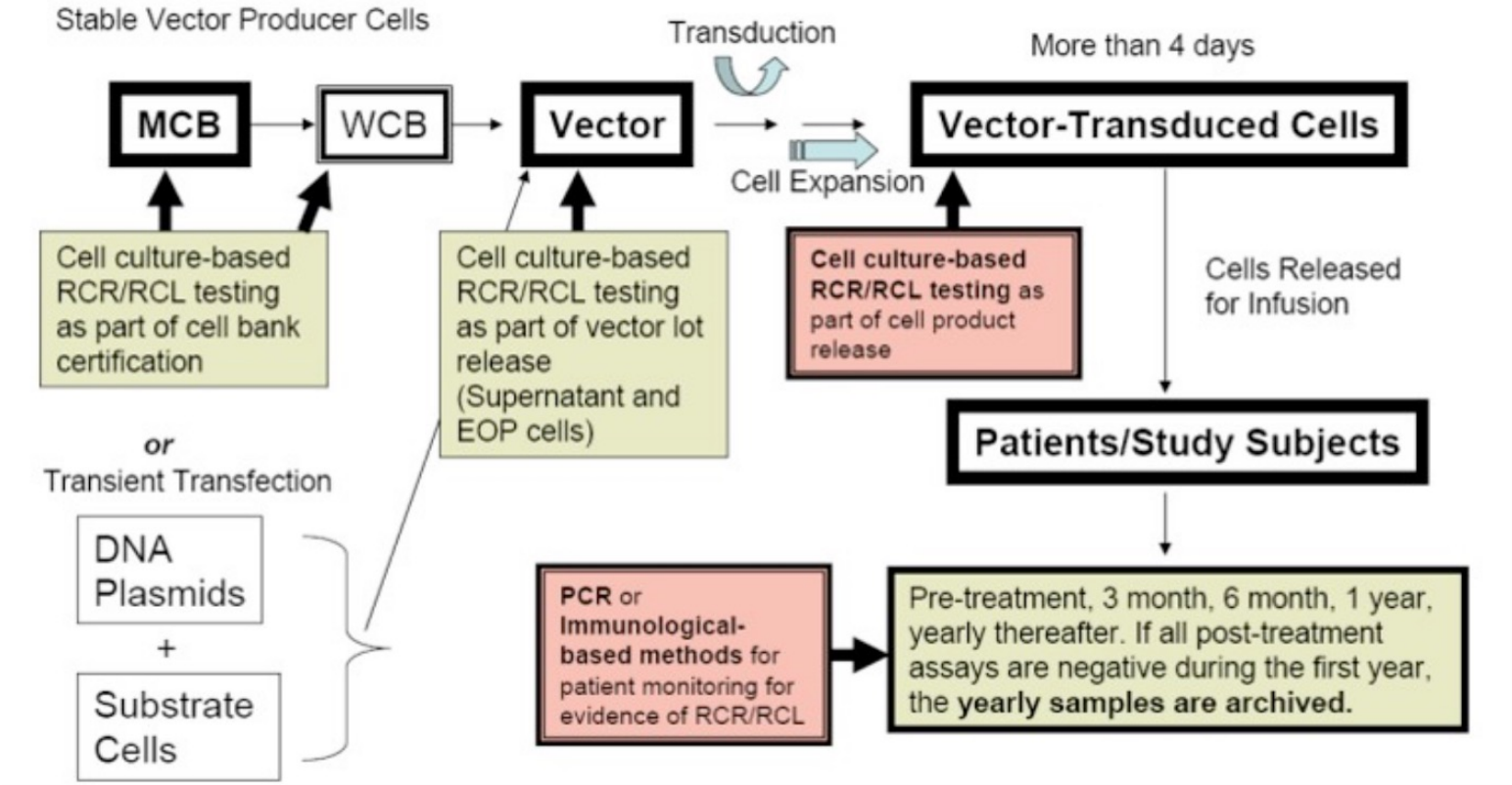


Fig.1 RCR/RCL Testing Schedule for Retroviral or Lentiviral Vector Transduced Cell Products.

MCB, master cell bank
WCB, working cell bank

Cell culture based testing

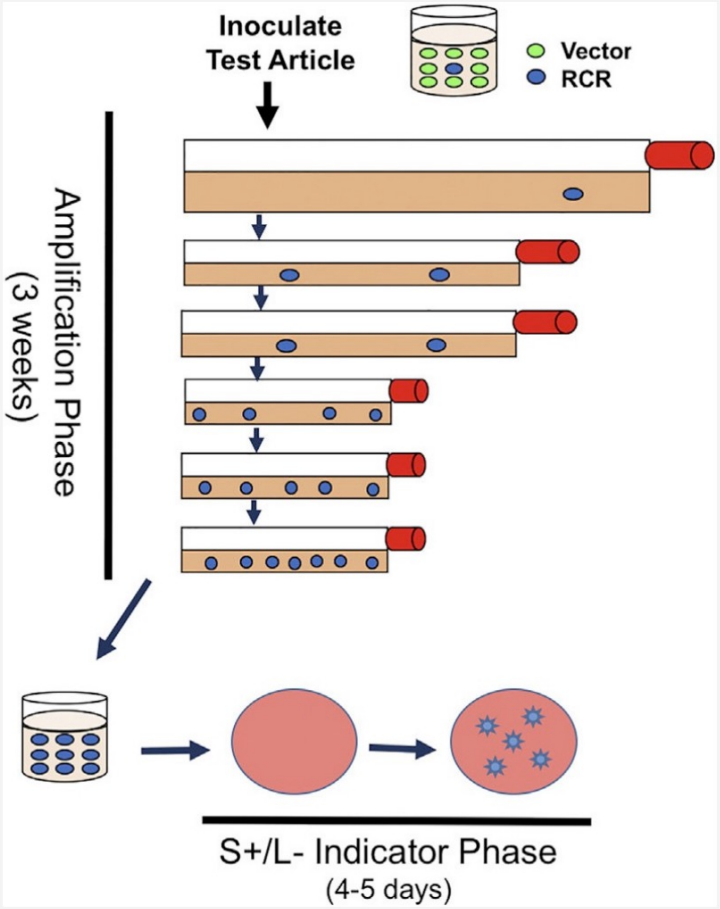


Fig.2 Schematic Representation of RCR Assay. (Kenneth *et al*. 2018) 8)

MOLECULAR AND BIOLOGICAL TESTING - RCL

To maximize the assay sensitivity, a cell culture based RCL assay with an appropriate permissive cell line is used to allow viral amplification and end point detection. Our RCR assay uses human T-cell line (C8166) that is permissive for the infection and growth of HIV-1. To detect RCL, sensitive end-point assays have been developed including an ELISA-based p24 Gag antigen assay, a product enhanced reverse transcriptase (PERT) assay that involves the vector's reverse transcriptase, and a PCR-based assay that detects Psi-Gag or VSV-G Env.

T-cell line C8166 permissive for HIV1 infection and growth

RCL Detection

- P24 ELISA
- Detection of the vector Reverse Transcritase

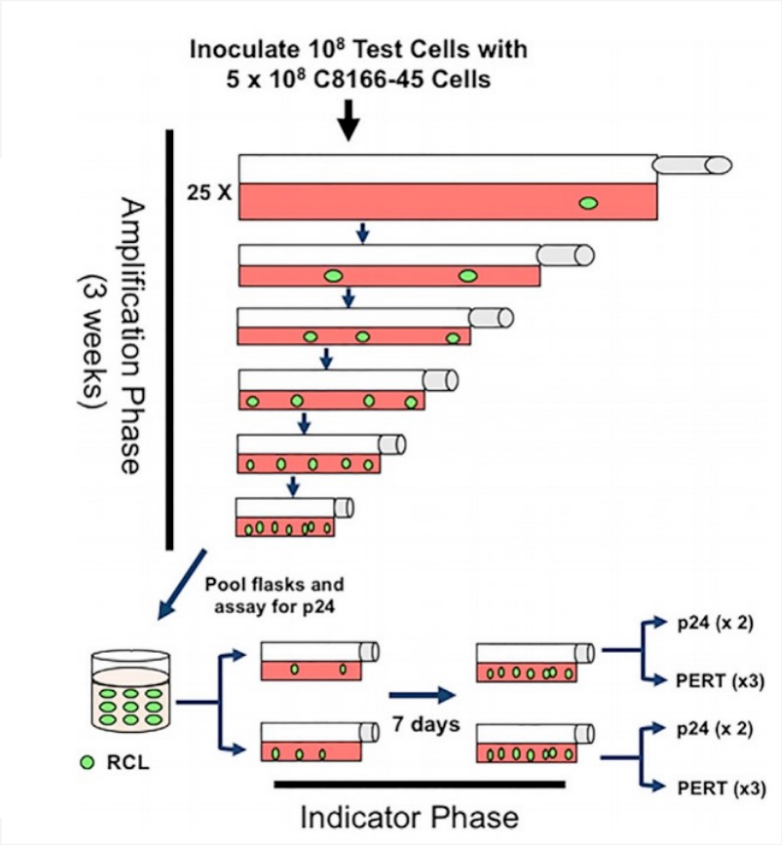
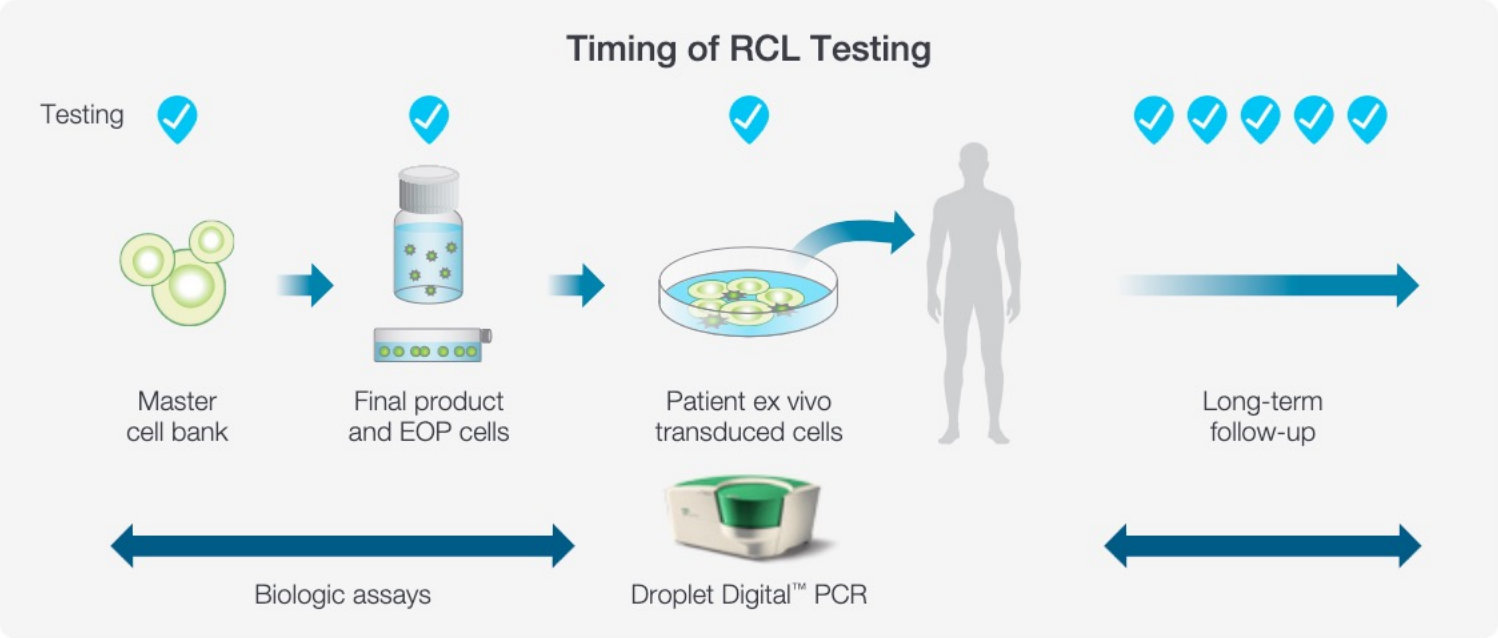


Fig.3 Schematic Representation of the RCL Assay. (Kenneth *et al*. 2017)

BIO-RAD Vericheck



EOP, end of production.

Vericheck ddPCR Replication Competent Lentivirus Kit #12019529

Overview

The Vericheck ddPCR Replication Competent Lentivirus (RCL) kit provides a rapid, cost effective, sensitive, and fully validated solution for the detection of RCL.

Prezzo di listino: **Verifica**

Quantità: 1 offerta Elenco preferiti

Sastry L, Cornetta K. Detection of replication competent retrovirus and lentivirus. Methods Mol Biol. 2009;506:243-63. doi: 10.1007/978-1-59745-409-4_17. PMID: 19110631.

Cornetta K, Lin TY, Pellin D, Kohn DB. Meeting FDA Guidance recommendations for replication-competent virus and insertional oncogenesis testing. Mol Ther Methods Clin Dev. 2022 Dec 2;28:28-39. doi: 10.1016/j.omtm.2022.11.009. PMID: 36588821; PMCID: PMC9791246.