A publication of
The Williamsburg BioProcessing Foundation

July/August 2003

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development

Vol. 2 No. 4

www.bioprocessingjournal.com

QC Release Testing of an HIV-1 Based Lentiviral Vector Lot and Transduced Cellular Product

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The first HIV-based lentiviral vector to be used in humans, VRX496, is currently being tested in Phase I clinical trials (initiated in January 2003).[1,2] With each new therapeutic comes the need to establish quality control (QC) testing designed specifically for that product. The QC testing for VRX496 was developed in accordance with the Code of Federal Regulations (CFR) 21 for pharmaceutical and bulk chemical GMPs, Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993) from the Center for Biologics Evaluation and Research (CBER) at FDA, and the United States Pharmacopeia (USP) 1046 for Cell and Gene Therapy Products.[3–5]

This report describes the QC testing of lot VRX496V02-009 of our clinical grade vector, which is being used in an ongoing clinical trial evaluating the first lentiviral gene therapy vector in humans. All assays are performed according to established standard operating procedures (SOPs) and in accordance with the principles of cGMP regulations.[3]

Materials and Methods

Vector production

VRX496 is a VSV-G pseudotyped lentiviral vector with a 937-base antisense payload targeted to the envelope gene of HIV and expressed under the control of the LTR. The lentiviral vector was produced using a two-plasmid transient transfection system (Lu *et al*, submitted). Plasmid DNA was transfected into human embryonic kidney (HEK) 293 cells using the calcium phosphate precipitation method. All upstream (cell culture) and downstream (purification) work was done in a class 10,000 cleanroom. Approximately 24 and 36 hours after transfection, medium (the bulk harvest) was collected and replaced. Following collection, vector-containing medium was clarified through cartridge filters with decreasing pore diameters. The vector was subsequently concentrated by ultrafiltration, resulting in the concentrated harvest. To remove residual cellular and plasmid DNA, the vector was treated with benzonase and then applied to a gel-filtration column with Sephacryl-500 equilibrated in the storage buffer. Finally, the vector was sterile filtered and filled into plastic bags as the final fill.

Preparation of replication-competent lentivirus positive control

A viral stock of the attenuated HIV control (Fig. 3b) was produced by transfection onto HEK 293 T cells. Virus was titered by averaging three independent Tissue Culture Infectious Dose 50 ($TCID_{50}$) titer experiments on C8166-45 and MT-4 cells. Briefly, a series of four-fold dilutions of the virus stock ranging from 4^{-1} to 4^{-10} was carried out in six replications of each. Positive cultures were detected by cytopathic effect (CPE) and confirmed by p24 ELISA. $TCID_{50}$ was determined by taking into consideration both the virus dilution at which more than 50% of wells tested positive for HIV, as well as the number

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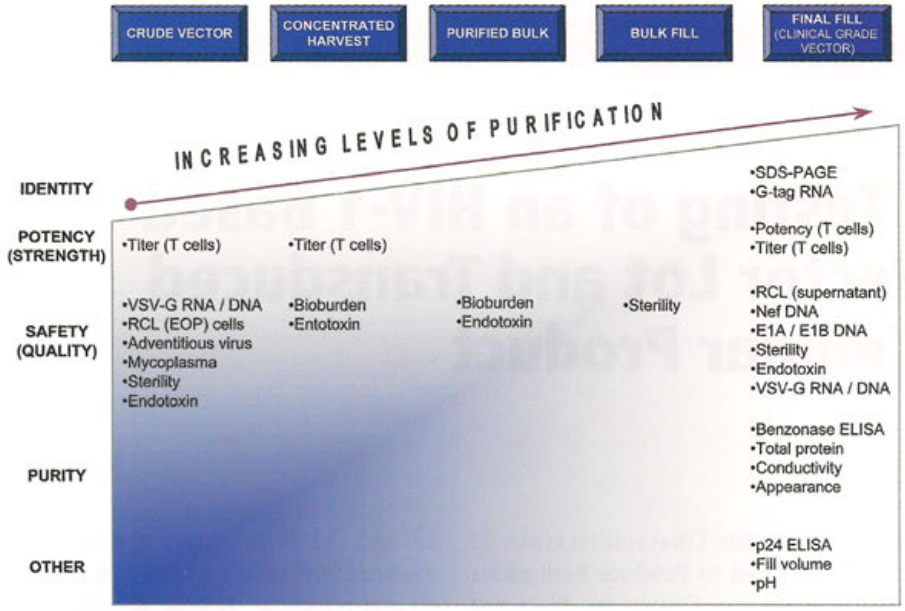

Figure 1. Overview of QC testing at various stages of vector purification. Five stages of increasing vector purification are shown: end of production/bulk harvest (EOP/BH), concentrated harvest, purified bulk, bulk fill, and final fill. Assays are categorized according to identity, potency, safety, purity, or other tests.

of wells out of six which were positive at that dilution. After averaging three independent $TCID_{50}$ assays, the titer of the attenuated HIV control was calculated to be 3.8 x 10^5 +/- 2.6 x 10^5 in C8166-45 cells and 3.9 x 10^5 +/- 2.2 x 10^5 in MT-4 cells.

T cell titer and potency assay

Normal human blood was obtained from AllCells (Berkeley, CA). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient separation and $CD4^+$ T lymphocytes were subsequently purified by positive selection using the magnetic activated cell sorting (MACS) system (Miltenyi Biotec, Auburn, CA). Purity was determined to be above 95% by flow cytometric staining for CD4/CD3. $CD4^+$ T lymphocytes were cultured in X-Vivo 15 containing gentamycin and 10% human serum. For transduction, T lymphocytes were plated at 1 x 10^6 cells per well in a 24-well plate. Plates were precoated with Retronectin™ (Takara Bio Inc, Japan) at a concentration of 1.5 µg/cm^2. The vector was loaded onto the plates at 5%, 10%, and 20% of culture medium 30 minutes prior to addition of cells. We then added immobilized CD3/28 beads at a ratio of three beads per cell and 100U/ml IL-2 (Chiron, Emeryville, CA). All cultures were carried out in triplicate. T lymphocytes were cultured for three days, washed three times to remove vector, and replated in 3 ml of media. At day five, an additional 1 ml of media was added, and at day seven the beads were removed. For titer, 1 x 10^5 cells from each culture at day seven were tested for DNA by TaqMan PCR using primers specific to a 186-base tag region derived from the green fluorescent protein (GFP) (Lu *et al*, submitted). Cultures were infected at a multiplicity of infection (MOI) of 0.001 with the NL4-3 strain of HIV (ABL Inc., Kensington, MD). Non-transduced cells and cells transduced at 20% with a positive control vector were used for challenge controls. Cultures were then followed for 15 days and passaged twice weekly. Supernatants were taken at each passage for p24 determination by ELISA.

Adventitious virus, mycoplasma, sterility, and endotoxin testing

These tests were carried out by BioReliance's contract testing laboratory (Rockville, MD). Briefly, mycoplasma testing was carried out in accordance with the 1993 CBER Points to Consider and 21 CFR 610 using an agar isolation assay and a Vero cell culture assay. Adventitious viruses were tested for in accordance with the 1993 Points to Consider.[4] Three cell lines — MRC-5, Vero, and HeLa — were treated with vector supernatant and monitored for CPE. At days 14 and 28, culture supernatants were tested for hemagglutination on chicken, guinea pig, and rhesus monkey erythrocytes. Sterility was tested by broth inoculation in accordance with 21 CFR 610. Finally, endotoxin was tested using a chromogenic *Limulus Amebocyte Lysate* (LAL) assay.

ELISA

Virion concentration was measured by p24 ELISA according to the manufacturer's recommendations (ABL Inc.). Benzonase ELISA was performed according to the manufacturer's instructions using the Benzonase ELISA kit II (EM Industries, Inc., Gibbstown, NJ). Bovine serum albumin (BSA) in the cell product was tested using a quantitative sandwich ELISA from Bethyl Laboratories (Montgomery, TX).

Total protein

Total protein was measured using the Bio-Rad DC Protein Assay Kit (Hercules, CA). This colormetric assay is a modified Lowry method that determines protein concentration following detergent solubilization. Absorbance was read at 750 nm on a spectrophotometer.

TaqMan PCR

TaqMan PCR was performed on an ABI Prism 7900HT Sequence Detector. RNA was isolated according to the Boom procedure, and 1 X 10^6 cells or 200 µl of test article were tested in each run.[6] Forward and reverse primers were added at a final concentration of 300 mM, and a detection probe was added at a final concentration of 500 nM in the presence of RT-PCR TaqMan Universal Mix and RNase inhibitor (Applied Biosystems, Foster City, CA). An RNA standard curve ranging from 10^0 to 10^7 copies was run in each reaction alongside an isolation negative control and a no template control. An internal positive control provided by ABI was run in each vesicular stomatitis virus (VSV)-G

RNA detection reaction to control for reagents and machine function. All reactions were run in triplicate.

Nef, g-tag, and E1A/E1B PCR

Nucleic acids were isolated from 1 x 10^6 cells (1 x 10^5 for *g-tag*) according to the Boom procedure for nucleic acid isolation[6]. For *nef* and *g-tag*, RT-PCR was performed in a single reaction using C. *therm* polymerase in the presence of forward and reverse primers. The reaction was first carried out in a GeneAmp 9700 at 60° C for 30 minutes. DNA PCR was then performed at 95° C for 5 minutes, then 40 cycles at 94° C for 1 minute, 53° C for 1 minute, 72° C for 1 minute, followed by a final 10 minutes at 68° C. Gel products were visualized on 3% high-resolution agarose gel.

Determination of the proviral copy number

Primers and probes for the TaqMan PCR analysis were designed for the HIV-1 gag p24-coding region. Three x 10^6 cells were tested in a total of 27 replicates. If there are no positive signals among the 27 replicates then there are fewer than 1.3 copies of HIV *gag* DNA present per replicate at the 95% upper confidence limit. Proviral copy numbers per cell from pre-expansion cells are compared to post-expansion cells to determine if there is a statistically significant increase as determined by the student-paired t-test.

Nickel test

Nickel ions in the final cell product were tested by graphite furnace absorption spectroscopy at Galbraith Laboratories (Knoxville, TN).

Results

Characterization of clinical -grade vector

According to CFR 21, the role of QC is to ensure the identity, strength, quality, and purity of the drug product. Figure 1 lists our QC tests categorized by these groups. The vector was tested at several stages from the initial harvest (bulk harvest) to the final purified clinical-grade preparation (final fill). Most QC tests are performed only on the final

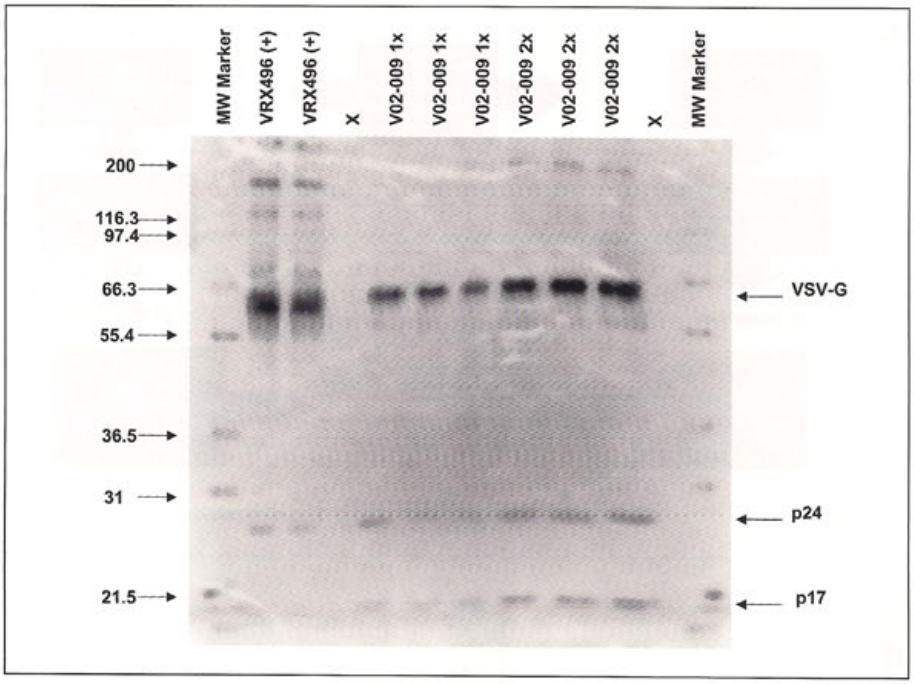

Figure 2. SDS-PAGE visualizing the final fill clinical vector lot. Approximately 1 μg and 2 μg of protein as measured by p24 in the final fill from clinical vector lot VRX496V02-009 was loaded in triplicate onto the gel for visualization. A previous vector lot was loaded as a positive control for comparison.

Table 1. Summary of QC procedure specifications and results from clinical Lot VRX496V02-009

Test	Specification	Result
titer	>/= 1 x 10^7/ml	pass
p24	0.1–10ug/ml	pass
total protein (Lowry)	</= 0.70 mg/ml	pass
SDS-PAGE	matches standard profile	match
benzonase	</= 100 ng/ml (0.1 ppm)	<100 ng/ml
fill volume	>/= 40 ml	40 ml / 41 ml[2]
pH	7.0–7.4	pass
conductivity	4–7 mS/cm	pass
appearance	colorless, clear to slightly turbid	clear
endotoxin	< 100 EU/ml	pass
adventitious virus	ND	ND
mycoplasma	ND	ND
sterility	no growth	no growth
wt-HIV	ND	ND
g-tag RNA	present	present
RCL	ND	ND

[1]TU=transducing units. [2]Two bags tested. [3]ND=not detectable.

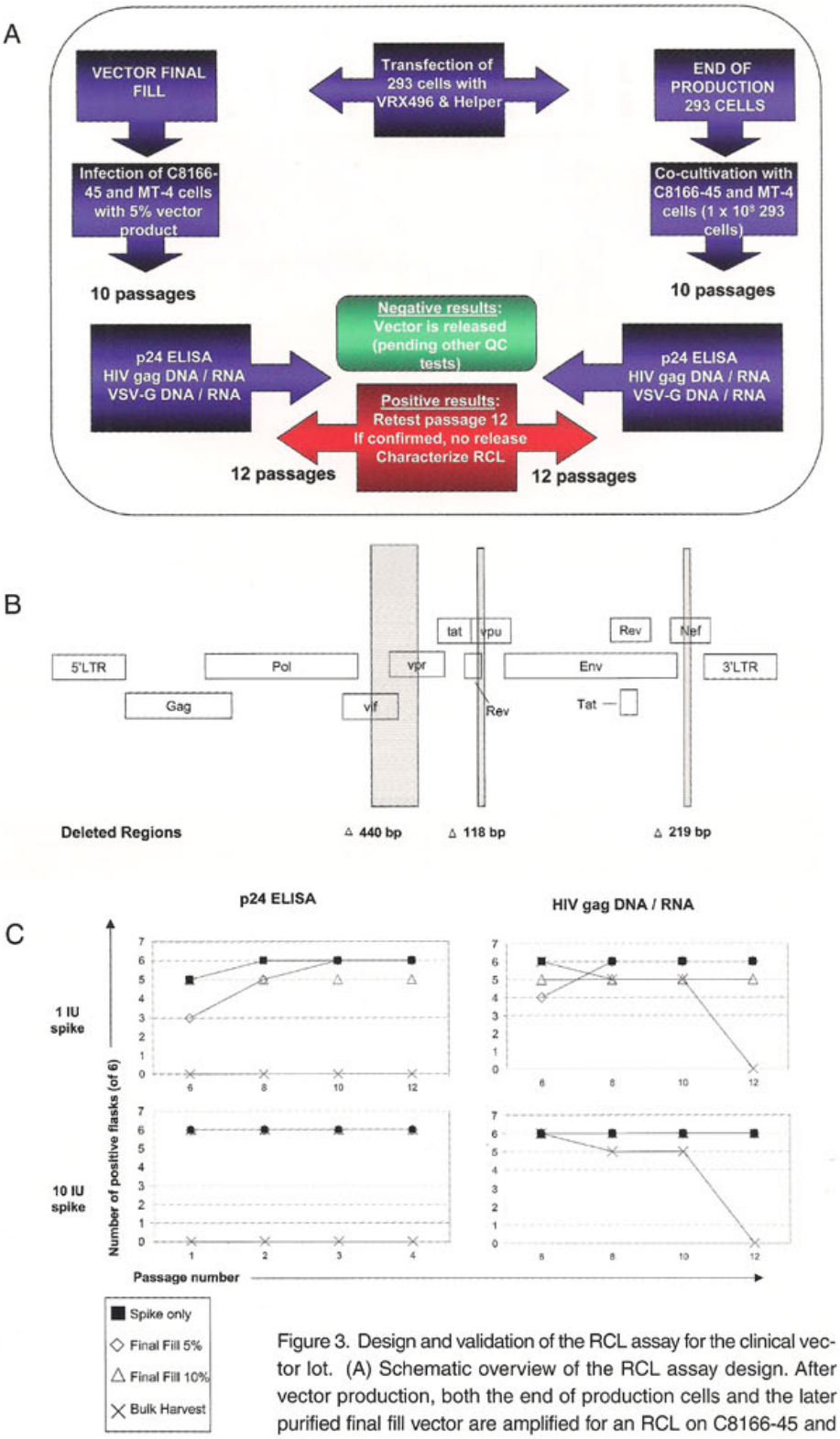

Figure 3. Design and validation of the RCL assay for the clinical vector lot. (A) Schematic overview of the RCL assay design. After vector production, both the end of production cells and the later purified final fill vector are amplified for an RCL on C8166-45 and MT-4 cells for 10 passages, then detection assays are performed. During testing of passage 10, cells are carried to passage 12. If any positive results are detected at passage 10, testing will be repeated on passage 12 samples for confirmation. The assay design is similar for testing of the cellular product, except that only C8166-45 cells are used. (B) Cartoon of the attenuated HIV positive control for the RCL assay. Deleted regions are shaded and the size of the deletion is shown below the shaded area. (C) Qualification of the sensitivity of 1 or 10 infectious units (IU) attenuated HIV spike detection in the presence of vector at various concentrations. Attenuated HIV was detected by p24 ELISA (left panels), or by HIV *gag* DNA/RNA RT-PCR (right panels). Six flasks were tested in each group.

fill because it is the final product used to transduce patient cells. However, certain tests are performed earlier to maximize detection of contaminants.

For visual identification, the vector product was analyzed on a 10% SDS-PAGE gel under reducing conditions. After purification, the envelope protein from VSV, and the HIV capsid protein p24 and matrix protein p17 can be observed (Fig. 2). Table 1 shows several of the QC assays, the corresponding assay specifications, and the results for our clinical lot VRX496V02-09, which is being used in an ongoing Phase I clinical trial. The RCL assay, and detection of *nef*, *g-tag*, and E1A/E1B DNA are discussed below.

RCL assay

One primary concern for retrovirus-based gene delivery vectors is the development of a replication-competent lentivirus (RCL). Typically this would be tested for on the end of production (EOP) cells and the bulk harvest. However, our vector product is expected to be inhibitory to a putative RCL, so we tested the EOP cells from the bulk harvest, but tested the vector separately from the final fill. The rationale for this method of testing is discussed later in this section and in Figure 3c.

Detection of a putative RCL was performed *in vitro* by direct testing on C8166-45 and MT-4 cells (Fig. 3a). The assay begins with incubating the test article with the indicator cell lines for 10 passages (five weeks) to allow sufficient amplification of a putative RCL to facilitate detection. It is difficult to design an assay for detection of an RCL, as it remains a theoretical possibility and has an unknown structure. However, certain assays are likely to detect an RCL derived from a VSV-G pseudotyped HIV-based vector. These include detection of VSV-G envelope DNA/RNA, HIV *gag* DNA/RNA regions not present in VRX496, and HIV p24-gag protein, an indicator of virion production. Therefore, at the end of each amplification cycle (5 weeks) each of these three tests were performed to minimize the chance of false positive and false negative results. As the passage 10 tests were

being conducted, the indicator cells were passaged twice more to passage 12. In the event that any test yields a positive signal, then passage 12 will be tested to confirm the result. It is expected that any signal detected in passage 10 should increase by passage 12.

Although there are no specific guidelines for detection of an RCL, there exist Guidance for Industry documents for replication-competent retrovirus (RCR) testing provided by CBER.[7] VRX496 is produced by a transient transfection of HEK 293 cells with the two-plasmid system of vector and helper DNA (Lu *et al*, submitted). We tested at least 1 x 10^8 EOP cells (transfected) in the RCL assay, which is in accordance with the FDA RCR guidance. Following production, VRX496 was extensively purified. After purification, approximately 6.34% of the final vector product, known as the final fill (FF) was tested on C8166-45 and MT-4 cells for a total amount tested of 160 ml. Each 80-ml test sample represents one dose of vector product that would be used to transduce one entire lot of autologous T cells (approximately 2 x 10^8 cells) from an HIV-1 infected patient. This amount also satisfies guidance documents that specify testing of at least 5% of the total vector product used to transduce patient cells.

An attenuated HIV unable to express the HIV-1 accessory proteins vif, vpr, vpu, and nef was used as the positive control for our vector RCL assay (Fig. 3b). This is an appropriate control because none of these HIV-1 accessory genes are expressed in the vector or packaging construct, and therefore would not be present in an RCL generated during vector production. This attenuated control was measured for $TCID_{50}$ on the indicator cell lines as described in the methods.

Before choosing C8166-45 and MT-4 cells, we tested several T cell lines well known for their permissiveness to HIV replication (C8166-45, SupT1, MT-4, Molt 4, and H9). Each cell line was infected with the attenuated HIV control at approximately 80 infectious units (as determined by $TCID_{50}$ on C8166-45 cells) per flask of 1 x 10^7 cells and tested for sensitivity to replication of the attenuated control as detected by p24 production. This should indicate the cell line most capable of amplifying a putative RCL (Table 2). We found that the C8166-45 and MT-4 cultures produced the highest levels of p24 in the shortest amount of time. Therefore, C8166-45 and MT-4 cells represent the best choice for RCL indicator cell lines.

The potential RCL causing the most concern is a replication-competent HIV that has acquired the VSV-G envelope protein, because this could increase HIV's host cell range. Therefore, detection of VSV-G in the RCL assay is a critical testing parameter. To ensure that our indicator cells are indeed permissive to a VSV-G-expressing virus, we validated the ability of our VSV-G pseudotyped vector to transduce these cells. When VSV-G pseudotyped vector was added to cultures at 20%, C8166-45 and MT-4 cells were transduced at approximately 13.7 +/- 2.6 and 16.5 +/- 5.4 copies per cell respectively. This experiment demonstrates the permissiveness of these cells to entry by a VSV-G pseudotyped virus.

The sensitive RT-TaqMan PCR assay was used as one of the ways to measure the positive control and a putative RCL in the supernatant of the indicator cell lines. Specifically, HIV *gag* and VSV-G RNA were analyzed after passage of the vector on the indicator cell line. The assay was also used directly to detect residual VSV-G DNA in the vector and cell product. The "Boom" extraction method was used to extract RNA and DNA from cell supernatants.[6] No DNase digestion step was used to specifically determine RNA versus DNA detection because it significantly reduces assay sensitivity. Because our specifications for release of the vector product after RCL testing require no positive detection of HIV *gag* or VSV-G DNA/RNA sequences after biological amplification in an indicator cell line, the DNAse treatment step is unnecessary. When the assay is used to directly detect total residual VSV-G DNA/RNA in the vector product, a value is provided on the Certificate of Analysis for information as requested by the FDA. Full length VSV-G DNA has been detected in all process development lots to date, indicating that the signal is coming from residual plasmid DNA. There is no specification for detection of VSV-G DNA/RNA in the final vector product; however, it is required that these sequences are not detected in the final cell product.

The sensitivity of the RT-TaqMan assay was established by determining the minimum number of copies of HIV *gag* or VSV-G sequences detected after the extraction process. First, standards were obtained by PCR amplification of

Table 2. Comparison of indicator cell lines for sensitivity to RCL amplification

Cell Line p24 pg/ml[1]

	Day 3	Day 7
C8166-45	6	18,983
SupT1	4	1,560
MT-4	4	42,657
Molt 4[2]	<LDL[3]	<LDL
H9	<LDL	<LDL

[1]Average value from two T75 flasks.

[2]Molt-4 clone #8.

[3]LDL=lower detection limit.

Table 3. Overview of experimental design for determination of RCL assay detection sensitivity and determination of test article inhibitory effects on detection

Test article	10^7 cells per flask	
Attenuated HIV spike	**1**	**10**
bulk harvest 50 ml/flask	6 flasks	6 flasks
final fill (10%) 5 ml/flask	6 flasks	6 flasks
final fill (5%) 2.5 ml/flask	6 flasks	6 flasks
spike alone	6 flasks	6 flasks
cells alone	1 flask	1 flask
total flasks	25 flasks	25 flasks

plasmid DNA coding for HIV gag or VSV-G using primers containing the T7 promoter for RNA transcription. RNA was digested with DNase I to remove any residual DNA, then column purified and examined for purity by gel and OD260 for copy number. Known copies of HIV *gag* or VSV-G RNA standards were diluted from 10^7 copies to 1 copy and spiked into the reaction.

To further validate the sensitivity of the assay for nucleic acids isolated from cells, 10, 33, or 100 copies of VSV-G RNA were spiked into Boom lysis buffer. Then 200 µl of cell culture medium was added to mimic the isolation procedure in cells. RNA was extracted from the samples, and was subsequently used in each replicate at one-tenth the original volume. Three runs of three replicates (a total of nine replicates) were tested and each replicate was scored for the presence or absence of amplified sequence. The data was plotted for copy number against the percentage of negative replicates, and a best-fit line was generated (data not shown). Signals from spiked samples could be distinguished from negative controls at a sensitivity of less than 10 copies per reaction. Controls included a no template control and an isolation negative control, and the number of controls tested per run equaled the number analyzed for each test sample. A valid assay requires that the coefficiency value (R^2 value) of the standard curve be 0.985 or higher and all the negative controls be negative.

A unique issue for VRX496, an anti-HIV vector product, is that it is inhibitory for the attenuated HIV-positive control because the anti-HIV antisense sequences present in the vector are targeted to envelope sequences present in the attenuated HIV. Therefore, a concern was that testing of the vector product for an RCL might itself reduce the sensitivity of the detection assay. To examine this possibility, we tested (on indicator cell lines) the effect of increasing levels of vector treatment (5% FF, 10% FF, and the bulk harvest respectively) on detection of three different spike doses

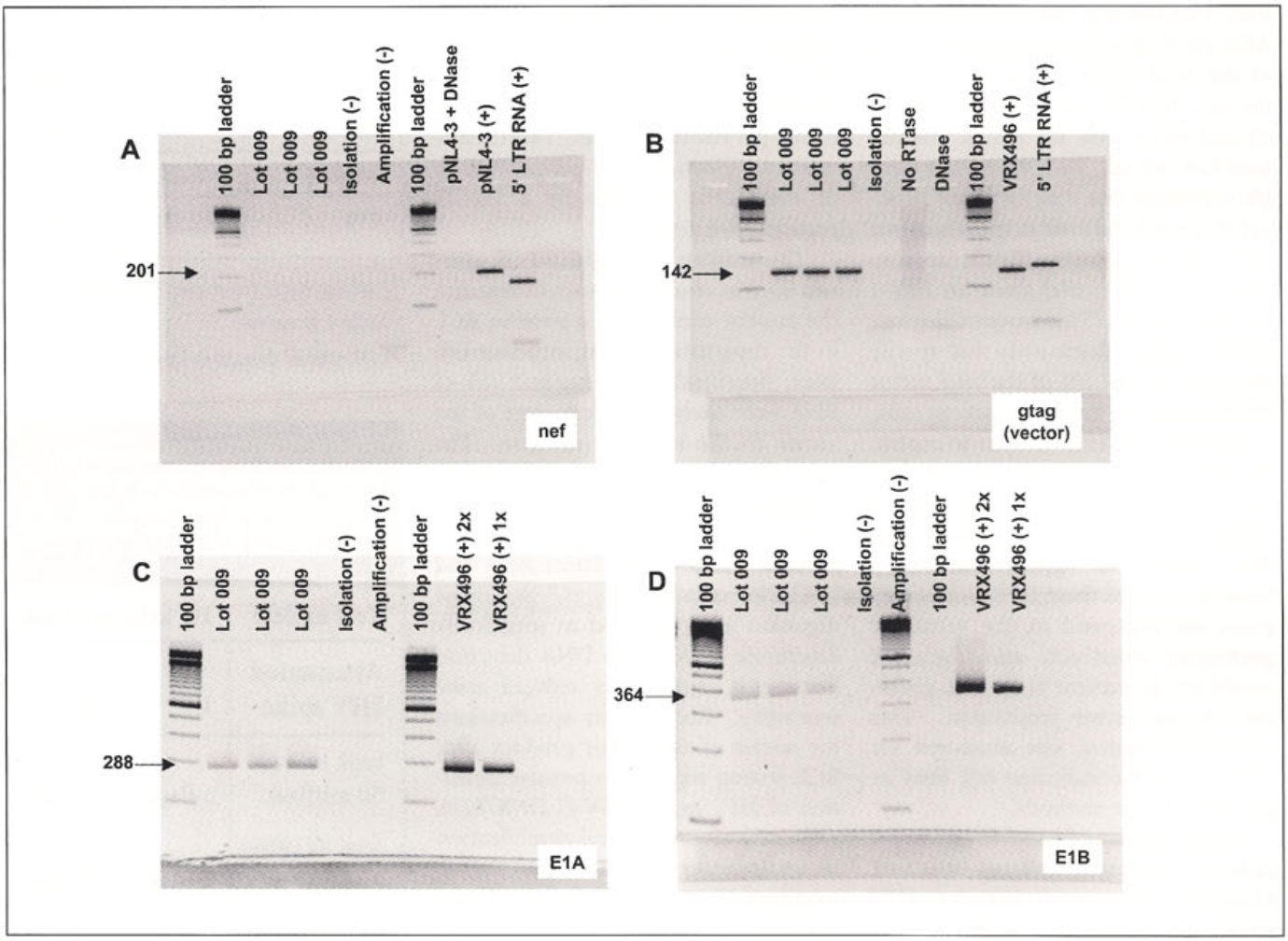

Figure 4. Detection of DNA sequences in the final vector lot. (A) Detection of wt-HIV by *nef*-specific primers. A 201-base fragment is amplified. Controls include DNase treated pNL4-3 HIV DNA, pNL4-3 DNA alone, and RNA from the 5' LTR of pNL4-3. (B) Identification of the vector lot using vector-specific *g-tag* primers generating a 142-base fragment. A vector DNA control and 5' LTR RNA control are used. (C) Detection of producer cell DNA using primers to the HEK 293 cell-specific E1A gene, which amplifies a 288-base band. (D) Detection of producer cell DNA using primers specific to the HEK 293 cell-specific E1B gene, which amplifies a 364-base band. For E1A and E1B producer cell DNA assays, an earlier lot of VRX496 was used as the positive control because it was positive for producer cell DNA sequences. E1A and E1B have never been detected in the final cellular product.

with the attenuated positive control (Table 3). Detection of the positive control was performed by p24 ELISA and by RT-PCR for the HIV *gag* RNA/DNA (without DNase treatment) at passages 6, 8, 10, and 12 (weeks 3–6). At the lowest spike (1 infectious unit), no inhibition was detected in the 5% final fill, 10% final fill inhibited detection of attenuated HIV marginally, and the bulk harvest inhibited all detection (Fig. 3c). At the highest spike (10 infectious units), 10% or 5% final fill did not inhibit amplification of the positive control, but treatment of cells with bulk harvest continued to inhibit detection 100%. Although testing of the vector supernatant would normally be performed on the bulk harvest, in this case testing of the bulk harvest could mask detection of a putative RCL. Therefore, we tested 5% of the final fill and infected with 1 infectious unit spike per flask for the control. Interestingly, when measuring HIV *gag* levels in the bulk harvest, a positive signal was detected early on in culture, which disappeared later in culture. This is a result of residual HIV *gag* sequences left over from the helper plasmid from production, which are removed by benzonase treatment during vector purification prior to the final fill. The RCL assay was carried for 10 passages because, between passage eight and 10, we continued to observe increases in detection of the positive control (refer to Figure 3c, I IU spike, and 5% final fill).

To date, we have tested four clinical vector lots for the presence of an RCL and each has been negative for detection of a putative RCL (Table 4). Since all detection assays were negative for each vector lot at passage 10, no testing was performed on passage 12.

Table 4. RCL assay results from four vector lots

Vector Lot Number	VRX496V01-006	VRX496V02-007	VRX496V02-009	VRX496V03-001
p24	ND[1]	ND	ND	ND
HIV *gag* RNA/DNA	ND	ND	ND	ND
VSV-G RNA/DNA	ND	ND	ND	ND
passages	10	10	10	10

[1]ND=not detected.

Table 5. QC release assays for transduced autologous T cell product

Test	Specification
copy number	0.5–5 copies/cell
RCL	ND[1]
VSV-G DNA/RNA	ND
BSA ELISA	</= 1 ug/ml
E1A / E1B	ND
provirus pre- and post-expansion	Post-expansion </= pre-expansion
endotoxin	< 1 EU/ml
mycoplasma	ND
number of residual beads	< 100 per 3 x 10^6 cells
cell viability	> 70%
nickel test	< 200 ug/10^{10} cells

[1]ND=not detected.

Detection of wt-HIV, g-tag, and E1A/E1B sequences

To ensure that our vector product is not contaminated with wild type (wt)-HIV, we performed RT-PCR on our final vector product for wt-HIV using primers against the *nef* gene, which is not present in either the vector or helper constructs used in production. The sensitivity of this reaction was determined to be 10 copies per reaction. This limit of detection is not a statistical determination, but rather a more conservative limit representing that 10 copies of the positive control could be detected in each lane for each run (data not shown). We did not detect any wt-HIV in our final vector product (Fig. 4a).

To distinguish our vector from wt-HIV in HIV-infected patients, we inserted a 186-base tag sequence (*g-tag*) derived from GFP into the vector backbone. To identify the vector product, we performed RT-PCR for this gene. We detected a strong *g-tag* signal in our final vector product (Fig. 4b).

Because HEK 293 cells carry integrated copies of E1A and E1B, qualitative DNA-PCR assays have been developed to detect any residual E1A and E1B host cells as a way to detect residual producer cell DNA in the vector preparation. The advantage to using the HEK 293 cell-specific E1A and E1B genes as a marker of producer cell contamination is that, if present in the vector product, the final cellular product may also be tested to ensure such sequences have been removed. If a ubiquitous human gene were used in this assay, a follow-up assay on transduced autologous T cells using the same primers would not be possible because the test would always turn positive. We detected residual producer cell DNA in Lot VRX496V02-009 (Fig. 4c,d). Therefore, we will also test the cell product after transduction with this vector prior to release. The sensitivity of these reactions was determined to be 10 copies per reaction. Detection

of these sequences in the vector product is for information only and is not a release criterion. For the cellular product, however, no detection of producer cell DNA is a requirement for release (Table 5). We have not detected E1A or E1B in the final cellular product.

Determination of vector potency

We determined the potency of Lot VRX496V02-009 in a primary T cell challenge assay (Fig. 5). VRX496V02-009 inhibited HIV replication more than 1,000-fold over challenged mock-transduced cells. In conjunction with this assay, the vector titer was performed, and was determined to be 6.6 x 10^7 transducing units (TU)/ml. For a valid assay, cells must expand at least five-fold by day seven after culture initiation prior to challenge, the untransduced challenged cells must produce >10,000 pg/ml of p24 per 1 x 10^6 cells by day 15, and the positive control must exhibit >80% inhibition of p24 as measured by p24 ELISA.

Testing of the transduced autologous T cell product

A second round of QC testing on the VRX496-transduced cellular product was performed prior to patient dosing. The same principles of identity, strength, quality, and purity apply to this product. A summary of the tests performed on the autologous T cell product and the respective specifications is presented in Table 5. We are in the process of completing the QC testing on our first clinical lot of autologous T cells transduced with VRX496.

The RCL assay performed on the cellular product is the same design as for the vector product, involving a 10-passage amplification process on indicator cells using an attenuated HIV as a positive control. MT-4 cells began to die around passage three when infected at an MOI of 10 or higher in untransduced cells, and around passage five in cells transduced at 5% with vector, so there was a concern that endogenous HIV from patient lymphocytes would destroy these cells and disrupt the ability to detect an RCL (data not shown). C8166-45 cells did not exhibit extensive cell death as a result of HIV infection. Therefore, only C8166-45 cells are used as indicator cells in the RCL assay for the cellular product.

In accordance with the guidance documents for RCR testing, 1 x 10^8 cells from the final expanded cell product and 300 ml of final cell culture supernatant were tested for RCL on C8166-45 cells.[7] Therefore, we limited the RCL detection criteria to detection of VSV-G RNA/DNA, because wt-HIV replication from patient cells could lead to a positive p24 and/or HIV *gag* reading not associated with an RCL.

Final cell product was also directly tested for VSV-G DNA. Since VSV is an RNA virus, detection of VSV-G DNA would be unique to a retroviral recombinant. There is no guidance for direct testing of VSV-G DNA. In our validation assays, we statistically determined in limiting dilution assays that the limit of detection of our VSV DNA TaqMan was 0.4 molecules of VSV-G in 3 x 10^6 cells. We calculated the required cell number to test substituting cell number for volume in the Appendix 1-1 equation in the guidance documents, which is shown below:[7]

$$Vt = -(1-c)\ln(1-p)$$

Here, if p=0.95 and applying 1/2.5 x 10^5 VSV-G DNA molecules per cell (from the 95% upper confidence limit of detection), the suggested minimum number of cells to be tested is 7.5 x 10^5 in order to meet the 95% confidence for probability of detection. Thus, 3 x 10^6 cells exceed this specification.

We further performed an additional test not specifically requested in a regulatory document: We directly analyzed 3 ml of the final cell supernatant for VSV-G RNA by TaqMan RT-PCR. Although we cannot analyze 300 ml of the cell supernatant for reasons of practicality (this would require more than 9000 replicates), the 3 ml can provide direct evidence for the presence or absence of VSV-G molecules in the final cell product added to the RCL biological amplification assay. To date, we have completed two RCL assays on transduced cellular product, and no RCL has been detected.

Four tests are specific for release of the cellular product: residual stimulatory beads, cell viability, HIV provirus copy number per cell, and residual nickel ions. During the transduction and expansion process, beads coated with T cell stimulatory antibodies are added to the culture and removed before formulating the product. To ensure proper bead removal, the cell product is examined for residual beads. Following transduction and expansion, the cell product is frozen until the patient is ready for dosing. Just before dosing, the

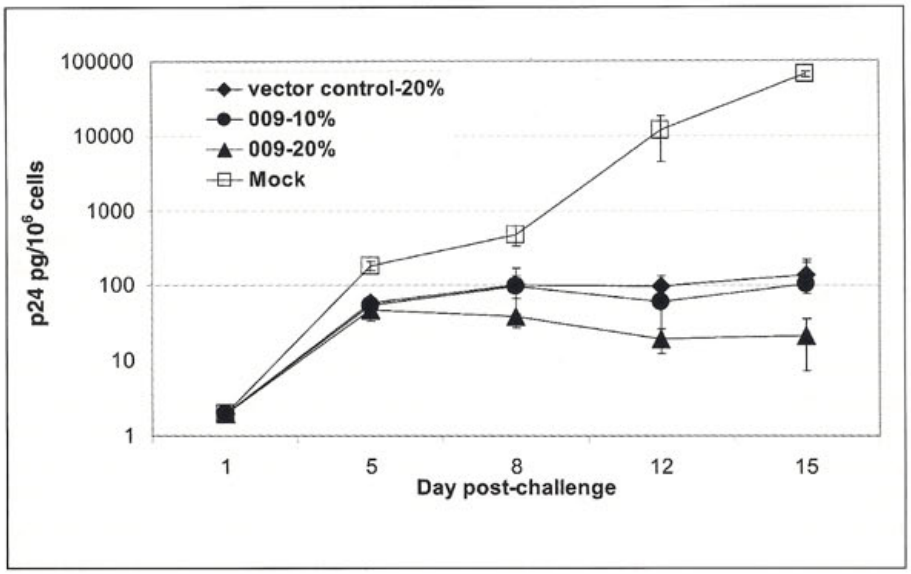

Figure 5. Determination of potency of the final vector lot. Primary $CD4^+$ T lymphocytes were transduced with control vector or the final vector lot test article, and then subsequently challenged with HIV. Untransduced challenged cells served as the positive control for replication.

viability of the cell product is determined by trypan blue exclusion. As a safety precaution, transduced cells are tested for HIV provirus copy number pre and post expansion by quantitative TaqMan PCR for HIV *gag* to ensure there is no increase (it could pose a health risk to the patient.) Finally, the purification process for the patient cells involves a column that contains nickel. Although cells undergo extensive washing and dilution after purification, they are still tested for residual nickel ions to ensure safety.

Discussion

This paper presents, for the first time, the QC release criteria for a clinical grade lentivirus-based vector and lentivirus vector-transduced cellular product. These criteria were developed in close consultation with the FDA, and also in accordance with existing regulatory documents.[2–5,7] The sample results presented in this paper are from the vector lot that is being used in the ongoing Phase I clinical trial examining the safety and tolerability of vector-transduced autologous T cells for treatment of HIV.[1,2] The vector being tested in this trial is a VSV-G pseudotyped HIV-1-based vector containing a 937 base antisense payload directed against the HIV-1 *env* gene, which is produced using a two-plasmid transient transfection production system.

During the recent Annual Meeting of the American Society of Gene Therapy (ASGT), a working group meeting for lentiviral vectors was held to discuss, in part, the requirements for a valid RCL detection assay.[8] Results from this discussion are expected to be published in fall of this year in *Molecular Therapy* as part of a joint effort between Cell Genesys, VIRxSYS Corporation, and FDA. Specifically, it was recommended that the most sensitive cell line for amplification of a putative RCL should be used as the indicator cell line, that test articles should be amplified on this cell line before conducting detection assays, that the positive control should be detected in the presence of the test article, and that multiple endpoints should be tested for detection of a putative RCL. Many of the elements of our RCL assay are consistent with the ideas set forth in this meeting. We found C8166-45 cells to be the best indicator cells in terms of amplification of attenuated virus and cell viability. We investigated detection of the spiked attenuated positive control in the presence of our vector product to determine at what concentration our product could be tested without inhibiting detection of the control. Finally, after a five-week amplification of the test article, we tested for an RCL using both biological and molecular assays.

An additional point brought up in the working group meeting was that an RCL should be transferable, thus allowing partial recombinants to be distinguished from true replication-competent recombinants. To incorporate this into the assay would require a final passaging step of the supernatant only (no cells). We did not have a problem with false positive detection resulting from partial *gag-pol* recombinants, perhaps in large part due to a stop codon we have in the *gag* region of our vector as an additional safety feature (Lu *et al*, submitted). In addition, we tested for VSV-G RNA/DNA that would only be present in an RCL and not in a partial recombinant, thereby serving as a distinguishing factor between true and partial recombinants. Therefore, we do not believe we need a final passaging step.

In the guidance documents, it is recommended that the EOP cells be directly tested for RCR.[7] Therefore we tested our EOP cells directly in the RCL assay. However, testing of the EOP cells was originally intended for use on a packaging cell line, where the cells making the vector would be well represented at the end of production. Because our vector is made using a transient transfection process, most of the surviving EOP cells are those that had not been efficiently transfected (most of the transfected cells die by the end of production.) Therefore the EOP cells are unlikely to harbor an RCL. In the future, this test may be more informative as progress is made toward a packaging cell line for lentiviral vectors.

Finally, we validated the permissiveness of our indicator cell lines to entry by a VSV-G pseudotyped replication-incompetent vector. In each assay, we used an attenuated HIV as a replication control for these cells. Recently, the design of another lentiviral RCL assay was published, which uses a VSV-G pseudotyped attenuated HIV-positive replication control.[9] This report presents the best available positive control, since VSV-G-mediated viral entry and lentivirus replication can be controlled for in each assay. We do not feel it is necessary to incorporate this control into our current assay because permissiveness of the cells to VSV-G-mediated entry has already been shown.

The vector examined in this paper, VRX496V02-009, will be the first lentivirus-based vector to be evaluated in humans. We hope that these assays will serve as a useful template for subsequent lentivirus vector-based gene therapy clinical trials.

ACKNOWLEDGEMENTS

We would like to thank Jody Baller, Lan-Fei Chang, Eden Deausen, Bing Jiang, Lauren Korshalla, Peter Manilla, Randal Merling, Kemi Ogunjimi, Susan Sun, and Sanjaya Thapa for their contributions to this manuscript.

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