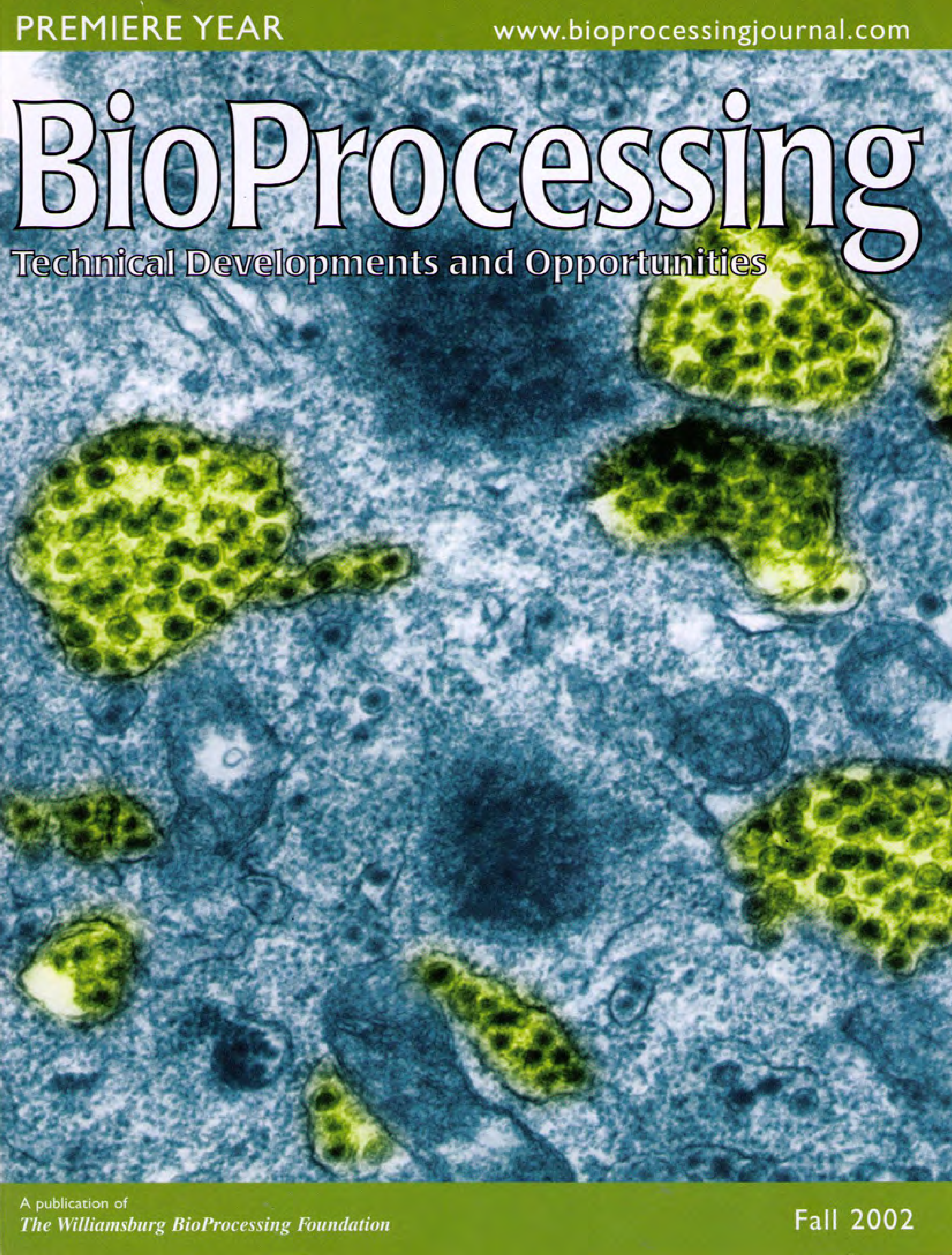
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Methods for Detection and Evaluation of Replication Competent Adenovirus (RCA)

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In the past, researchers developing gene therapy applications used replication-defective human Adenovirus 5 (Ad5) as a vector for delivering DNA sequences, almost exclusively.[1] Ad5 vectors are typically rendered replication defective by the deletion of E1a gene sequences. A complementing cell line containing the E1a gene makes it possible to produce Ad5 vectors in large scale. Of the various cell lines that have been constructed for the purpose of high-titer Ad5 production, HEK293 cells and PER.C6 cells are the most widely used.[2,3] Both cell lines are constructed by inserting Ad5 sequences into the cellular genome. More importantly, they both contain flanking regions that allow low frequency homologous insertion of E1a DNA back into the replication defective Ad5 vector, and thus making it replication competent. The resultant replication-competent Ad5 (RCA) presents a safety, quality control, and purity concern for an otherwise theoretically non-replicating, homogeneous gene delivery system.

Regulatory authorities recognize the presence of RCA in non-replicating Ad5 gene therapies as a potential problem, and have already mandated its measurement.[4,5] Because of increased regulatory scrutiny and other impurity issues, considerable effort goes into generating novel cell lines containing DNA sequences that are less likely to recombine with the Ad5 vector to produce RCA.[3,7,8] Organizations manufacturing Ad5 vectors routinely measure the amount of RCA in each lot, and Ad5 production lots are routinely accepted or rejected based on their propensity to produce RCA. The standard test for RCA consists of adding the adenovirus vector stock to a cell culture that is capable of supporting Ad5 replication, but disallows replication of E1a deleted vectors. For instance, AppTec's cell culture RCA assay uses the A549 cell line, and has controls for interference. The scope of each assay is large enough to allow testing of at least $3x10^{10}$ viral particles (vp) at a non-toxic Multiplicity of Infection (MOI). Cost and turnaround time constitute the

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Table 1: Results of Positive T150 Flasks

	ADENO 5 % positive flasks												ADENO 5 with VERO Lysate % positive flasks									
	Neg		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
Tech 1	Neg	Neg	100	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100
Tech 2	Neg	Neg	100	100	100	ND	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table to demonstrate LOD of the assay in the presence and absence of a Vero cell lysate. Measures of Specificity, Precision (Repeatability and Intermediate precision) and LOD can be derived from this data. ND - data not obtained. Neg - No CPE recorded throughout entire duration of assay.

major disadvantages of this approach.

In this paper, we describe some qualification work that was performed on the cell culture-based RCA detection assay to determine its limit of detection, specificity, and precision. We also illustrate a series of PCRs designed to complement the cell-based assay for detection of RCA in Ad5 vector stocks. As an alternative, PCR might act more rapidly as a detector of RCA in Ad5 vector stocks.[9] We conclude that a combination of cell-based amplification and PCR may allow a more rapid assessment of RCA when compared with the currently employed 28-day cell culture assay.

Materials and Methods

Cells and Viruses

The A549 cell line was used for culture based Ad5 detections. Cells were obtained from the ATCC and were maintained in a medium of Ham's F-12K, 10% fetal bovine serum, and L-Glutamine. A master cell bank was prepared and screened for sterility, plus the presence of adventitious viral agents and mycoplasma. The HEK293 cell line was used for generation of genomic DNA containing the E1a sequence. HEK293 cells were also obtained from the ATCC®, and a master cell bank was screened as described above. Wild type Ad5 was used as a representative RCA. Wild type human adenovirus 5 was obtained from the ATCC®, triple plaque purified, and expanded to prepare a master virus bank (MVB). From the MVB, working virus banks were generated. Virus MVBs were screened for the presence of contaminating viruses by immunofluorescent antibody testing (IFA), and for the presence of mycoplasma and other microorganisms. Identity of the MVB was confirmed by IFA.

Culture-Based Assay

The Ad5 virus stock was titered, and the titer was confirmed over the range of 0.33 to 33 plaque forming units (PFU)/mL by three analysts on three separate set-ups. A dilution series of 0.33, 1, 3.3, 10, and 33 PFU/mL Ad5

Table 2: Onset Day CPE

	ADENO 5 Onset Day CPE Recorded												ADENO 5 with VERO Lysate Onset Day CPE Recorded									
	Neg		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
Tech 1	Neg	Neg	6	17	7	10	7	7	7	5	7	5	11	11	6	11	11	10	7	7	7	5
Tech 2	Neg	Neg	11	10	11	ND	7	7	7	7	5	7	11	24	11	10	11	10	11	10	7	7
Avg	Neg		11		9.3		7		6.5		6		14.3		9.5		10.5		8.8		6.5	

Table to demonstrate Days to Onset of CPE for the Ad5 wt doses delivered. For low doses it is apparent that the assay requires longer incubation periods before CPE is observed. ND - data not obtained. Neg - No CPE recorded throughout entire duration of assay.

Table 3: Termination (+3/+4 CPE) Day

	ADENO 5 Termination Day (showing +3/+4 CPE)												ADENO 5 with VERO Lysate Termination Day (showing +3/+4 CPE)									
	Neg		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL		0.33 PFU/mL		1.0 PFU/mL		3.3 PFU/mL		10.0 PFU/mL		33.0 PFU/mL	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
Tech 1	Neg	Neg	13	18	12	10	11	10	11	10	11	10	29	19	13	17	12	11	11	10	11	10
Tech 2	Neg	Neg	20	24	13	ND	11	10	11	10	11	10	18	26	18	11	11	11	11	10	11	10
Avg	Neg		18.8		11.7		10.5		10.5		10.5		23		14.8		11.3		10.5		10.5	

Table to demonstrate Days to Termination. ND - data not obtained. Neg - No CPE recorded throughout entire duration of assay.

Table 4

Adenovirus sample	Dose (particle or pfu)	RCA Response	*Response to 1-3 PFU spike (2-10 flasks assayed)	*Response to 9-10 PFU spike (2-10 flasks assayed)
1	8×10^{10} pt	negative	9/10 positive **	positive
2	4.8×10^{10} pt	positive	positive	positive
3	1.0×10^{9} PFU	positive	positive	positive
4	1.0×10^{11} pt	positive	positive	positive
5	1.0×10^{9} PFU	negative	positive	positive
6	1.0×10^{11} pt	positive	positive	positive
7	5×10^{8} pt	positive	positive	positive
8	5×10^{10} pt	positive	positive	positive
9	3×10^{9} PFU	negative	positive	positive
10	1.0×10^{8} pt	positive	positive	positive
11	1.0×10^{8} pt	negative	positive	positive
12	1.0×10^{11} pt	negative	8/10 positive **	positive
13	1.8×10^{10} pt	negative	positive	positive
14	1.8×10^{10} pt	negative	positive	positive
15	3×10^{10} pt	negative	positive	positive
16	1×10^{10} PFU	negative	negative	positive
17	3×10^{8} PFU	negative	positive	positive
18	9.1×10^{8} PFU	positive	positive	positive
19	3×10^{9} pt	negative	positive	positive
20	3×10^{10} pt	negative	positive	positive
21	1×10^{10} PFU	negative	negative	positive
22	2×10^{11} pt	negative	negative	positive

Table to demonstrate Specificity and Intermediate Precision of the assay.
Multiple different adenovirus vector samples have been included in the RCA assay at different times with no significant reduction in detecting the positive spikes.
*Positive or negative, all flasks demonstrated the same response (with 2 to 10 flasks tested normally).
**If a number is indicated, only a portion of the total tested was observed as positive.

was inoculated onto subconfluent monolayers of A549 cells. The inoculation was performed twice, by each of two analysts, to generate intermediate precision data. All cultures were fed after a 60-90 minute incubation, and then every three to four days. Cultures were observed on a daily basis for CPE (cytopathic effect), and observations were recorded. As appropriate, subcultivation was performed on days 7, 14, and 21. Cultures with a CPE level greater than +3 (75-90% of cells show cytopathic changes by microscope examination) were discarded.

As a measure of assay impurity specificity, a Vero cell lysate was spiked into the Ad5 dilution series. The Vero lysate was used to mimic a representative master cell bank sample. Development of CPE in the Vero lysate cultures was evaluated in parallel with control cultures containing no lysate.

Cultures were followed for the length of the experiment and the following information was recorded:

a) Limit of Detection (LOD): the lowest inoculum of virus showing CPE

b) Days to onset of CPE

c) Days to termination: cultures reaching +3 CPE

With two analysts performing the experiments on two separate occasions, measurements for Intermediate Precision, Repeatability, Impurity Specificity, and Limit of Detection could be derived.

PCRs

PCRs 1-3 are qPCRs performed on a Roche Lightcycler™. PCR 2 uses primers located within the *E1a* region

Primer AA at 138 nt and primer D at 583 nt of E1a region generates an amplicon of 446 bp. Primer G at 4141 nt of E1 region and primer F at 4760 nt of E2 region generates an amplicon of 620 bp. Primer AA at 138 nt of E1a region and primer F at 4760 nt of E2 region generates an amplicon of 4.6 kbp. Primer Hx5 at 19063 nt and primer Hx3 at 19412 nt of hexon gene region generates an amplicon of 350 bp.

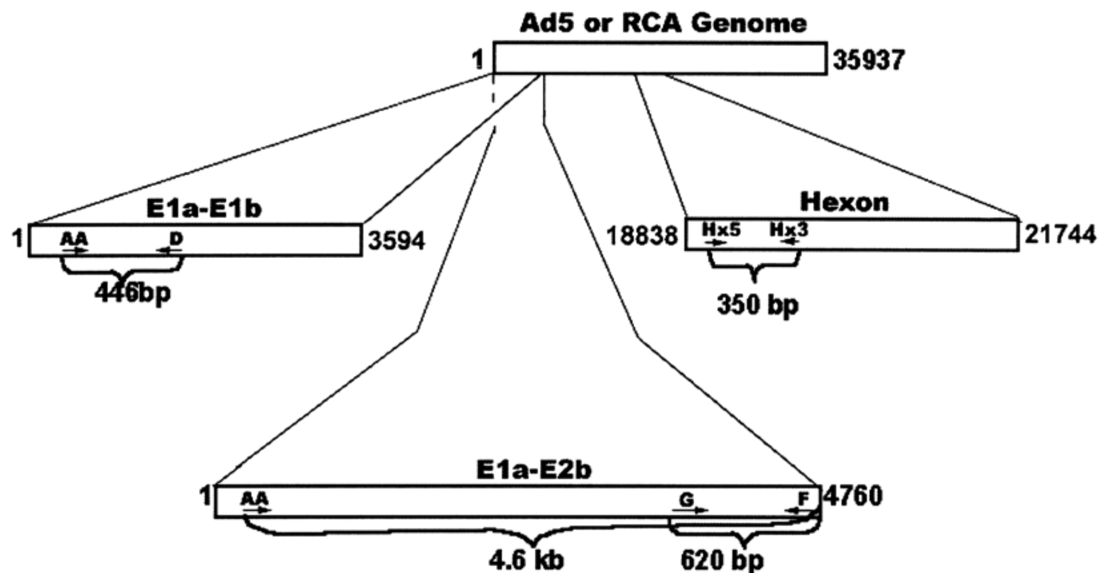

Figure 1: Schematic Diagram of PCR designs.

of the Ad5 genome, and generate a PCR amplified DNA product of 446 bp. The Hexon qPCR 3 uses primers located within the *hexon* region of the Ad5 genome, and generates a PCR amplified DNA product of 350 bp. The H-Alu qPCR uses primers recognizing the repetitive human Alu sequences, and generates a PCR amplified DNA product of 256 bp. The above specific amplicons are detected during the PCR cycling by FITC labeled donor probes, and by LC 640 labeled acceptor probes that bind specifically to the target amplicon DNA sequences.

PCRs 4 and 5 are endpoint PCRs performed on a DNA Tetrad™ Engine (MJ Research). PCR 4 uses primers (primers G and F) which span the E1 and E2 region of the Ad5 genome, and generates a PCR amplified DNA product of 620 bp. PCR 5 uses primers (primers AA and F) located within the E1a and E2 region of the Ad5 genome, and generates a PCR amplified DNA product of 4.6 kbp. After PCR amplification, amplicons were separated by ethidium-stained agarose gel electrophoresis, and were then examined by UV transillumination.

PCR reagents and method specifics

A master mix of reagents containing the appropriate primers, probes, dNTPs, DEPC-water, $MgCl_2$, reaction buffer, and enzyme mix was employed for the qPCR assays. Negative controls included sentinel reagent controls and controls spiked with non-specific DNA. Known copy number spikes were used to control for sample inhibition. Positive DNA corresponding to 101-105 viral particles or 100-2x10^2 pg (1, 5, 10, 20, 50, 100, and 200 pg) genomic DNA was prepared in order to provide a linear standard line from which linear regression measurements could be made.

For the endpoint PCRs, a master mix of reagents containing the appropriate primers, dNTPs, buffer, water, $MgCl_2$, and the *Taq* DNA polymerase was used. The above negative controls, as well as three levels of positive DNA (usually 10, 100, and 1000 vp) were used to determine the assay's Limit of Detection.

Table 5: PCR assays for detection of RCA

PCR Assay	Source of DNA		
	Adenovirus Vector	HEK293 DNA	RCA/wtAd5
1. H-Alu qPCR	*	Detectable (LOD = 1 pg)	*
2. E1a qPCR (138-583 nt) (primers AA / D)	*	Detectable (LOD = 100 pg)	Detectable (LOD = 10 VP)
3. Hexon qPCR (19063-19412 nt)	Detectable (LOD = 10 VP)	*	Detectable (LOD = 10 VP)
4. Endpoint PCR (4141-4760 nt) (primers G / F)	Detectable/Not detectable depending on construct	*	Detectable (LOD = 10 VP)
5. Endpoint PCR (138-4760 nt) (primers AA / F)	*	*	Detectable

Summary Table of PCR Data.
Where the LOD is given in the table, the data have been generated experimentally.
*indicates that the sequence should not be present and an amplification signal will therefore be undetectable. VP = viral particles.

Results and Discussion

The data in Table 1 (page 26) indicate that the detection of CPE in the cell-based assay has an LOD of < 0.33 PFU/mL, and that the presence of a Vero cell lysate has a minor effect on detection limits. Since different operators, who were performing replicates on different days, generated similar results, we know the assay has a high degree of repeatability and intermediate precision. Table 2 (page 27) shows a different data set obtained from the same experiments. These data address the lag time until the onset of CPE for the various experimental conditions. This parameter was used as an indirect measure of specificity, as time until CPE onset is generally less with higher PFU. The data indicate that there is some inhibitory effect of Vero lysate, as indicated by delayed onset of CPE at the 3.3 PFU/mL level.

A different parameter, Days to Termination (Table 3, page 27), was also measured and gave a reasonable inverse correlation to the Ad5 titer used. Using the Vero lysate, there was a slight "delay" experienced in the termination day recorded for the lower virus concentrations (1.0 PFU/mL and 0.33 PFU/mL) but no difference was noted at the higher concentrations.

Thus, the use of wild type (wt) Ad5 as a representative RCA has resulted a sensitive and precise cell culture-based assay for RCA detection. Data from 22 representative Adenovirus vector samples, tested between 1998 and 2001, are summarized in Table 4 (page 28). The Adenovirus lots were derived from different sources and had a range of titers and purities. Data in Table 4 demonstrates the repeatability of the assay.

Despite the great sensitivity of the cell culture assay, it is run in its standard format as a 28-day test and therefore suffers from an extended turnaround time. PCR was thus considered a possible alternative method that could generate results in a shorter time. The design of PCR primers for RCA is complicated by several factors:

1) The presence of E1a gene sequences in residual HEK293 or PER.C6 DNA, which force the design of primer sets that straddle E1a, and Ad5 DNA, in locations expected for E1a re-insertion. An E1a PCR alone may pick up residual DNA.

2) The possible cryptic insertion of a E1a sequence, by recombination, into unexpected locations in Ad5 vectors, and thus rendering them replication competent but undetectable by standard straddling E1a/Ad5 primer sets.

With these constraints in mind, we

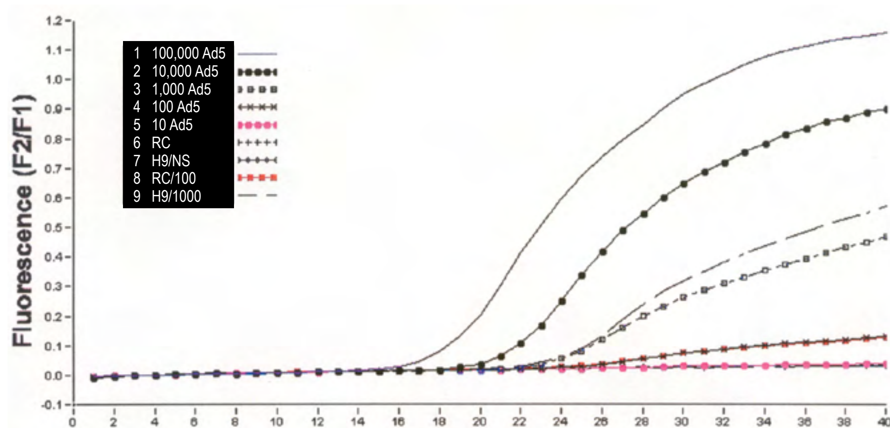

Figure 2 Quantification of E1a DNA sequences by qPCR.
Ad5: Wild type Ad5 viral DNA; RC: PCR Reagent Control; H9: human T cell DNA; NS: Non-Spike Positive Controls: Reagent control spiked with 100 VP Ad5 DNA; 0.5 mg H9 DNA (Negative control DNA) spiked with 1000 VP Ad5 DNA

designed a series of PCRs that is summarized in the schematic of Figure 1 (page 28), and in Table 5 (page 29).

1) PCR 1 detects human DNA and gives a measure of the absolute mass of residual human DNA in a sample.
2) PCR 2 detects E1a sequences and generates a signal from wild type Ad5 DNA RCA (at an LOD of approximately 10 vp), and from residual HEK293 DNA (at an LOD of 100pg or approximately 18 genomes).
3) PCR 3 detects hexon DNA and is a measure of total Ad5 DNA copies, regardless of their replication competency.
4) PCR 4 detects RCA and wild type Ad5 DNA, and is designed to straddle the 3' re-insertion site of E1.
5) PCR 5 detects RCA and wild type Ad5, and is designed to amplify E1a sequences in an Ad5 background, as opposed to a HEK293 or PER.C6 background.

Neither PCR 4 nor PCR 5 will detect HEK293 or PER.C6 DNA, since at least one primer is located within an Ad5 sequence that is absent in HEK293 and PER.C6 genomic DNA.

Using these assays, the effective detection of RCA by PCR is tied to the PCR detection limit. In a normal Ad vector stock, there can be more than 10^{12} vp per dose, however a normally accepted amount to test is $3x10^{10}$ vp. Since $3x10^{10}$ vp will theoretically generate 1.2 µg DNA, each PCR must detect one copy in a background of 1.2 µg to ensure RCA is detected with the same sensitivity achieved with the cell culture-based assay. In the PCRs performed at AppTec, samples are assayed in triplicate and controls are performed in triplicate, spiking 0.5 micrograms non-homologous DNA in each.

The data in Table 5 indicate that we have developed a series of PCRs that can determine the level of E1a in residual HEK 293 DNA, and thus exclude this from the level of E1a present in an Adenovirus (wt or RCA). Then the presence of E1a, in its expected location for wt or RCA, can be determined by confirmatory PCRs 4 and 5. In most cases, the LOD of the PCR method is 10 vp, which indicates the method's sensitivity is close to that of the cell culture assay (0.33 PFU/mL, assuming a VP/PFU ratio of 30). The PCRs may thus be useful for confirming the presence of RCA. However, the method as a whole suffers from the caveat that PCRs 4 and 5 will not identify E1 sequences that may have recombined in unexpected, or cryptic, sites in the vector genome, although the E1a PCR 1 should pick these up if they have not rearranged. It is also unclear if the PCR set can detect helper dependent E1 positive particles.[5]

The cell culture-based assay described here is very sensitive, and it allows the detection of extremely low levels of contaminating replication competent adenovirus. AppTec routinely confirms the presence of adenovirus in its RCA assay by an indirect immunofluorescence assay (IFA). However, if a sample is contaminated with a replicating virus other than wtAd5 or RCA, the A549 cells may exhibit CPE and complicate the interpretation of results. One advantage of PCR is its ability to amplify only those sequences with exact, or very close, homology to the primers and probes used. Conversely, this specificity can lead to PCR assays that miss recombined RCA variants with point mutations, or with small deletions at the primer or probe locations. In conclusion, a combination approach allows for quantitation of RCAs with possibly greater accuracy. Therefore, the prudent researcher should employ a combination of biological (cell-based) and molecular (PCR-based) assays to confirm the presence or absence of RCA.

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