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VIRAL TRANSFER TECHNOLOGY ***HOT TECHNIQUE***

A simple method for the rapid generation of recombinant adenovirus vectors

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Recombinant adenoviruses are useful vectors for basic research. When the vectors are used for delineating protein function, several viruses, each containing a mutated version of the transgene are compared at the same time. However, methods to generate multiple vectors simultaneously within a short time period are cumbersome. In this report, we show that a novel backbone plasmid, when cotransfected with routinely used shuttle vectors into HEK293 cells allowed for production of recombinant viruses in an average of 14 days. The recombinant viruses had no detectable wild-type virus contamination by A549 plaque assay and only three to 300 E1a copies per 10^9 adenovirus genomes by a sensitive PCR-based assay. Further culturing or serial amplification did not result in wild-type revertants nor did cultures show increased levels of E1a copy number by quantitative PCR. Thus, recombinant adenovirus vectors can be produced very simply, rapidly and with little to no contaminating wild-type particles. This system should facilitate the generation of multiple genetic variants by eliminating the need for time-consuming plaque purification and the need to manipulate and screen very large plasmids. We call this the RAPAd.I system. Gene Therapy (2000) **7,** 1034–1038.

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Adenovirus vectors are very important tools for deciphering the role of various proteins in biological processes *in vitro* and *in vivo*.[1–4] They are commonly used because they infect a wide variety of cell types, provide very high protein expression, and when purified show little prep-to-prep variation. The technology to generate the viruses requires only basic laboratory techniques. However, to progress from a cDNA of interest in a shuttle vector to a purified, wild-type-free virus traditionally involves many steps and requires a significant time investment.

Many laboratories continue to use standard methods of homologous recombination with shuttle plasmids and full-length Ad backbones (restricted in E1) for generation of vectors for basic research.[5] Core facilities housed within academic institutions or commercial 'kits' are widely used to streamline the process and avoid the time commitment for an individual laboratory. However, the time required to generate the vectors can range from a best-case scenario of 2 months to many months. Also, there may be wild-type contamination in the initial plaque isolation that necessitates further, time intensive, serial plaque isolations and amplification. Recent efforts have been directed at solving both the time and wild-type contamination problems. These include *E. coli* recombination methods,[6] ligation of cDNA directly into plasmids containing E1 deleted full-length viral DNA,[7–9] and an *in vitro* enzymatic recombination using Cre-loxP shuttles and backbone viral DNA.[10–12] The advantages of these systems are that the repetitive plaque isolation to purify the viral particle can be avoided because there is no[6,8,12] or limited[10] wild-type viral DNA input. Thus, there is a reduction in the amount of time it takes to progress from the transfection of viral DNA to amplified, purified virus. However, these methods also have their drawbacks. For the *E. coli* recombination using plasmids containing adenovirus genomes, the system is high fidelity, but inefficient and requires the screening of many bacterial colonies. This results in a significant time commitment even before transfection of recombinant DNA into E1-expressing cells such as HEK293 cells.[13] Similarly, ligation and recombinase methods require that several steps be completed before transfection into helper cell lines to generate virus. These recently developed methods are useful for making one or two viruses, but they are cumbersome if studies require that multiple viruses be generated.

Our goal was to develop a method to streamline adenovirus vector production with simplicity and time constraints in mind. Our previous work[5] and others' indicate that vector preparations are typically contaminated with varying amounts of wild-type virus when standard methods of homologous recombination between adenovirus backbones (digested to remove the packaging signal and E1-containing sequences) and shuttle plasmids are used. The wild-type is probably a result of the input adenovirus-DNA backbone being incompletely digested. In most cases the level of wildtype contamination is unacceptably high and the desired recombinant virus must be further isolated through repetitive, time-consuming

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plaque purification. We reasoned that an adenoviral genome devoid of sequences necessary for packaging and replication would greatly reduce or eliminate production of wild-type virus, eliminating the need for plaque purification. To test this, we generated a novel Ad backbone devoid of the left-hand ITR, the packaging signal and E1 sequences. The novel backbone and shuttle plasmids constitute the RAPAd.I system.

Figure 1 illustrates the starting plasmids and the generation of the Ad backbone plasmid. The shuttle plasmid serves the standard purpose of 'shuttling' cDNAs of interest from a plasmid vector to a viral construct. In this case, the shuttle plasmid was also required to generate the backbone plasmid (see diagram). The most important aspect of the backbone plasmid pacAd5 9.2–100 is that 0 to 9.2 map units of the genome have been deleted, starting with the left-hand ITR. PacAd5 9.2–100 retains the critical 9.2–16.1 kb region homologous with sequences within our existing shuttle vectors. To test the RAPAd.I system, we first constructed an eGFP-expressing virus. The shuttle plasmid pacAd5RSVEGFP and pacAd5 9.2–100 were digested with *PacI* and the mixture subsequently transfected into HEK293 cells using standard calcium phospate methods as depicted in Figure 2. As a control, HEK293 cells were transfected with pacAd5 9.2–100 alone. Viral foci were noted, beginning on day 6, in only the pacAdRSVEGFP/pacAd5 9.2–100 cotransfection (Figure 2), with more foci evident over the next several days. The cells were harvested 8 days after transfection, pelleted by low-speed centrifugation, and virus liberated by three cycles of freeze/thaw. The cell lysate (1 ml in 10 mM Tris, pH 8.1) containing the recombinant virus was amplified and purified. We noted no differences between the growth characteristics of RAPAd5.RSVEGFP and viruses made using the standard methods. In the pacAd5 9.2–100 transfected plate, foci were never evident and virus could not be amplified, confirming an absence of wild-type genome input.

The resultant purified RAPAd5.RSVEGFP particles had a concentration of approximately 8×10^{12} particles/ml (1.7 ml total) and an infectious titer of 2×10^{10} IU/ml (infectious units/ml) as determined by HEK293 cell plaque assay (Table 1). A549 cell overlay and PCR methods were used to determine if any wild-type virus was detectable after the first large-scale amplification and purification. For A549 cell agar overlays serial dilutions of virus particles were applied to cells at approximately 50% confluence in 3 ml of media (MEM, 4% FBS, 1% pen/strep) and 24 h later overlayed in the same, plus 0.8% Agar Noble (Difco, Detroit, MI, USA). The plates were fed agar-containing media again (2 ml) on day 7 and 13, with the latter also containing neutral red (Sigma, St Louis, MO, USA). Plaques were counted on day 14. No wild-type viral foci were detected for RAPAd5.RSVEGFP generated using the RAPAd.I system. The lower limit of sensitivity for the assay is 1 infectious wild-type virion in 10^9 virions. This limit is largely due to cell toxicity that occurs when $>10^9$ recombinant particles are used in the primary infection before agar overlay.

As an additional test for wild-type virus, we infected A549 cells with recombinant virus at a high particle/cell ratio (10^5). After a 2-week incubation all cells on the plate were GFP-positive when examined by fluorescence microscopy, but there was no evidence of cytopathic effect (data not shown). The cells were harvested, pelleted and lysed by three freeze/thaw cycles. The lysate was used to re-infect a plate of HEK293 cells. No cytopathic effect was evident after an additional 2 weeks in culture suggesting that very low to no wild-type virus was present in the initial purified preparation.

In contrast to the A549 plaque overlay results from virus generated using the RAPAd.I system, we have found that virus produced by standard cotransfection methods with endonuclease-restricted full-length backbones[5] can contain from 1×10^4 to 1×10^6 wild-type plaques per 10^9 particles (Davidson and Anderson, unpub-

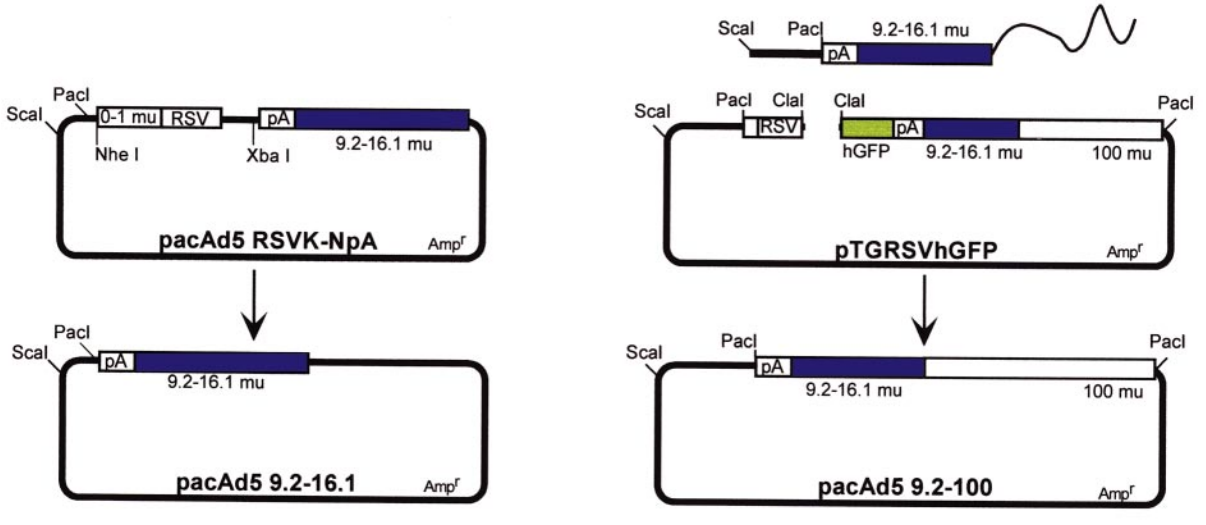

Figure 1 *Generation of plasmids required for the RAPAd.I system. To make the pacAd5 9.2–100, pacAd5RSVK-NpA was first digested with NheI/XbaI and religated to produce pacAd5 9.2–16.1. PacAd 9.2–16.1 was digested with ScaI, and co-transfected into E. coli BJ5183 cells along with ClaI restricted pTGRSVhGFP to facilitate homologous recombination between sequences within the ScaI to PacI regions. The BJ5183 E. coli support efficient homologous recombination,[6] and facilitated the generation of pacAd5 9.2–100. PacAd5 9.2–100 DNA was further amplified in E. coli DH5α and purified. The backbone is devoid of adenovirus sequences from 0 to 9.2 MU. Plasmid sequences were confirmed with the assistance of the University of Iowa DNA sequencing facility.*

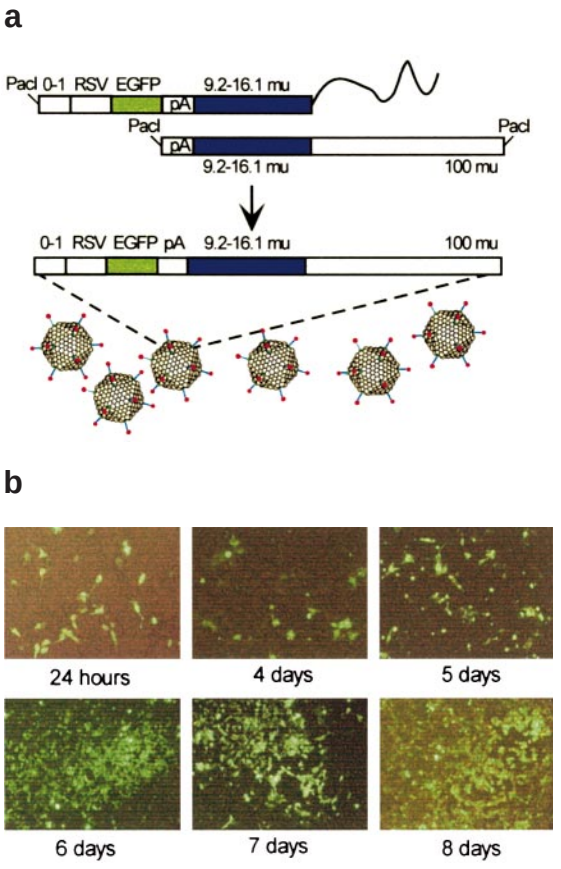

Figure 2 *Generation of recombinant adenovirus using the RAPAd.I system. (a) The shuttle plasmid pacAd5RSVEGFP was constructed by cloning the XhoI/NotI fragment from pEGFP-N1 (Clontech, Palo Alto, CA, USA) into the XhoI and NotI restriction sites of the pacAd5RSVK-NpA shuttle vector (Figure 1). PacAd5RSVEGFP and pacAd5 9.2–100 (15 and 4 μg, respectively) were digested independently with PacI (New England Biolabs, Beverly, MA, USA) and precipitated in transfection buffer (20 mM HEPES, 136 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose, pH 7.1, and a final concentration of 125 mM $CaCl_2$). The precipitate was added to 60 mm plates containing 50% confluent HEK293 cells and fresh DMEM containing 2% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech Cellgro, VA, USA). Homologous recombination in HEK293 cells lead to generation of RAPAd5.RSVEGFP. (b) EGFP expression detected after cotransfection of pacAd5RSVEGFP and pacAd5 9.2–100. Plates were examined from 1 to 8 days for the presence of viral foci by fluorescence microscopy using an inverted fluorescence microscope (Olympus IX70, Melville, NY, USA). As shown in the representative photomicrographs, RAPAd5.RSVEGFP foci produce a comet-like appearance at approximately 6 days (magnification ×10).*

lished observations). Moreover, we had detected E1a sequences by PCR amplification in 185 out of 292 recombinant lysates (Davidson and Anderson, unpublished observations). Thus, 63% of recombinant lysates generated using earlier methods required additional rounds of plaque purification. In contrast, standard PCR amplification assays were unable to detect E1a sequences in RAPAd.I system-generated virus (data not shown).

TaqMan PCR (Applied Biosystems, Branchburg, NJ, USA) was used to quantitate the number of copies of E1a in RAPAd5.RSVEGFP particles. Viral DNA was isolated from purified adenovirus by standard protease treatment and ethanol precipitation methods. Samples (100 ng, 200 nM final concentration, approximately 3×10^9 adenovirus genomes), primers (900 nM), and probe (200 nM final) were tested for E1a sequences using a Perkin Elmer ABI PRISM 7700 Sequence Detection System (Foster City, CA, USA). Real-time fluorescence of the probe was monitored. Forward and reverse primer sequences were: 5′-CCA GTG ACG AGG ATG AA-3′ (bases 951–970 in the adenovirus type 5 genome) and 5′-CCG TAT TCC TCC GGT GAT AAT G-3′ (bases 1031–1052), respectively. The E1a-specific probe 5′-ACA AGA CCT GCA ACC GTG CCC-3′ (bases 1010–1030) (MegaBases, Evanston, IL, USA) contained the fluorescent reporter dye FAM at the 5′ end and the quencher TAMRA at the 3′ end. A plasmid containing the Ad5 E1a gene was used to generate standard curves, with linearity from 10^0 to 10^5 E1a sequences per microliter.

Table 1 Assay for wild-type adenovirus after serial amplification of RAPAd5.RSVEGFP

Amplification round	*Titer*[a]		*E1a copy number*
	HEK 293 cells	*A549 cells*	
2	2×10^{10}	<1	3
3	1×10^{10}	<1	1
4	5×10^{10}	<1	4
10	6×10^{10}	<1	39
15	2×10^{10}	<1	59

[a]Titer is expressed as IU/ml in HEK293 cells. A549 cell titer is wild-type titer in 10^9 particles. The limit of detection is one wild-type virion in 10^9 total particles.
E1a copy number is per 10^9 adenovirus genomes. The linear range of the assay is from 1 to 10^5 copies of E1a.
All assays were performed at least twice.

Results from TaqMan E1a PCR assays showed that the RAPAd5.RSVEGFP particles had three E1a genomes per 10^9 adenovirus genomes (Table 1). In contrast, viruses generated by plaque isolation using standard cotransfection methods[5] with restricted wild-type backbone and shuttle plasmid DNA had approximately 30 000 copies/10^9 genomes as detected by TaqMan PCR (data not shown).

Some studies suggest that serial amplification of first generation adenoviruses in HEK293 cells can allow for generation of wild-type adenovirus which can eventually overgrow the recombinant viruses.[14] The wild-type virus could arise from recombination of sequences in the recombinant virus backbone with homologous sequences within the 4100 bp left-hand region of the adenovirus genome integrated into HEK293 cells.[13] Alternatively, wild-type virus could be present in low to undetectable levels in the initial isolate; serial amplification would enable amplification of both wild-type and recombinant virus, with the former at a growth advantage. To test if virus made using the RAPAd.I system allowed for these possibilities, we serially amplified our RAPAd5.RSVEGFP and assayed for wild-type virus at several stages (Table 1). RAPAd5.RSVEGFP was serially amplified by taking the lysate from one harvest and using that to re-infect a fresh plate of HEK293 cells. The lysate from this plate was then harvested and subsequently used for the next infection. Virus particles purified from lysates harvested after 2, 3, 4, 10 and 15 rounds of amplification were tested for E1a containing sequences by Taq-

Man PCR titer and replication competent wild-type virus by A549 plaque assay. The results listed in Table 1 indicate an absence of infectious wild-type particles as determined by A549 plaque assay, even after 15 serial amplifications. E1a copy number as analyzed by TaqMan PCR also remained remarkably low. Together, the data suggest there was no significant increase in wild-type virus upon amplification. There were also no noticeable differences in the growth properties and particle yields between the various preparations.

Thus, the RAPAd.I system allowed for generation of a reporter virus within 2 weeks and the virus produced contained virtually no contaminating E1a sequences or replication-competent virus. This compares favorably with current methods that take much longer.[5,15–20] The RAPAd.I system uses two components, a standard shuttle plasmid and the RAPAd.I backbone. The method is straightforward and requires very limited 'hands on' time from shuttle/backbone cotransfection to the isolation of virus particles. The RAPAd5.RSVGFP particles produced had roughly equivalent infectious titers as the standard viral genome/shuttle plasmid recombination method used frequently in many laboratories. Our current virus particle isolation methods yield approximately 10 ml of 1×10^{12} particles/ml. Thus, approximately 160 ml of wild-type-free virus particles (approximately 2×10^{14} total particles) would be produced by the fifteenth serial amplification, an amount likely to be more than adequate for most *in vitro* and *in vivo* gene transfer studies.

As stated, wild-type Ad genomes may arise from contaminating input viral genomes, or from recombination of overlapping Ad sequences found in HEK293 cell genomes. Cells harboring adenovirus E1 with limited surrounding Ad sequences have been made to decrease this latter possibility.[21–23] However, our serial amplification results suggest that reversion to replication competent virus occur very rarely if at all in HEK293 cells. This is important since most investigators do not have access to the alternative cell lines, or cannot justify the cost of their use. Thus, for most transgenes placed into adenovirus vectors, standard HEK293 cells can be used. However, when making recombinant viruses that express pro-apoptotic transgenes, a fragmented cellular genome may increase the potential for homologous recombination between overlapping adenovirus sequences within the genome and recombinant virus sequences. For such transgenes, anti-apoptotic agents or the use of cell lines containing E1 sequences only may be required to avoid generation of wild-type virus.

To test the reproducibility and utility of the RAPAd.I system, 61 different virus constructs were produced over 4 months, four to six constructs at a time. Each contained a cDNA sequence encoding a distinct transgene. The virus production was similar to the pilot reporter virus; multiple viral foci were evident and virus-producing cells could be collected after an average of 8 days following transfection into HEK293 cells. All 61 recombinants were found to be functional for the desired protein product (data not shown). Most importantly, the quantities of E1a copies per 10^9 adenovirus genomes ranged from $\leqslant 1$ to 300 as tested by TaqMan PCR, and A549 wild-type foci assays indicated that the amplified, purified viruses contained <1 wild-type particle in 10^9 total particles (Table 2).

Table 2 Assay for wild-type adenovirus in RAPAd.I virus preparations

Virus	*A549 titer*[a]	*E1a copy number*[b]
RAPAd5.RSVEGFP	<1	3
RAPAd5.CMVsSCLCN3	<1	14
RAPAd5.GLUT4EGFP	<1	182/1[c]
RAPAd5.CMVUCP2	<1	33/3[c]
RAPAd5.CMVCGRPIII	<1	21/8[c]
RAPAd5.CMVGPXIII	<1	300/38[c]
RAPAd5.cav-1-myc	<1	71/1[c]
RAPAd5.CMViNOS	<1	<1
RAPAd5.CMVSODI113T	<1	<1
RAPAd5.CMVFas	<1	2
RAPAd5.RSVmtAeq	<1	<1
RAPAd5.RSVmMLC	<1	<1/<1[c]
RAPAd5.PE-hENDO	<1	<1

[a]A549 titer is the number of detectable foci 14 days after inoculation of virus preparations.
[b]E1a copy number is the number of copies of E1a in 10^9 viral genomes.
[c]The second number indicates the E1a copy number detected in a second, independent assay.

The RAPAd.I method would not be limited to E1a-deleted viruses, since alternative backbones can easily be moved into this system. For example, modifications to E3 and E4 could be made to the pacAd5 9.2–100 backbone. Deletions in E3 or E4 would provide additional space for larger expression cassettes, or could be used to insert a separate expression cassette. Moreover, the novel backbone we describe could provide appropriate helper functions to allow for generation of adenovirus vectors fully devoid of adenovirus sequences except the ITRs and packaging signal ('gutless' vectors[24]) or adeno-associated virus vectors.[25,26] Similar to the recombinant adenovirus preparations reported herein, 'gutless' or adeno-associated virus vectors prepared with pacAd5.2–100 as helper should have low to no contaminating wild-type adenovirus genomes.

In summary, we have developed an easy, reproducible system for generating recombinant adenovirus. The novel backbone, when transfected into HEK293 cells along with standard shuttle plasmids containing various transgenes, allowed for the generation of 61 purified, high titer recombinant adenoviruses in 4 months. Importantly, wild-type genomes are not detected by A549 plaque assay and do not overtake recombinant genomes after multiple serial amplifications. We speculate that the methods and reagents reported will greatly facilitate investigators' abilities to make single or multiple viruses rapidly for testing protein structure, function or trafficking.

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