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Isolation of Highly Infectious and Pure Adeno-Associated Virus Type 2 Vectors with a Single-Step Gravity-Flow Column

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ABSTRACT

One of the most promising gene transfer vectors in human clinical trials is AAV2. The quality of the vector preparations is a key element in obtaining reliable and reproducible data in preclinical studies. However, established protocols either result in impure, low infectious virus ($CsCl_2$ gradient centrifugation) or demand a high level of manual and technical skills ($CsCl_2$ gradient centrifugation, iodixanol/heparin or HPLC purification). In this study, we present an easy-to-do single-step column purification (SSCP) of AAV2 by gravity flow based on its affinity to heparin, without ultracentrifugation. Various vector preparations generated by our method reproducibly showed high titers, infectivity, and purity. *In vivo*, our single-step column-purified AAV2 vectors mediate significantly higher transduction efficiency compared with conventional protocols. Investigators still unsatisfied with previously published techniques or new to the field of AAV production may find in our method an interesting alternative.

OVERVIEW SUMMARY

We developed a single-step gravity-flow column purification method for AAV2 based on its affinity for heparin. Briefly, after production of viral particles in the packaging cell line, using triple transfection, the cell lysate containing the vector is loaded on a preequilibrated heparin column. The heparin-virus complex is then washed and AAV2 is finally eluted by a simple buffer exchange to high salt. We demonstrate that recombinant AAV2 purified by this simple and scalable method is reproducibly highly infectious *in vitro* and *in vivo*. In addition, its purity is comparable to vector obtained using protocols requiring more complex skills and equipment.

INTRODUCTION

Recombinant adeno-associated virus type 2 (AAV2) holds much promise for human gene therapy (Rabinowitz and Samulski, 1998; Bennett *et al*., 1999; Kay *et al*., 2000). The quality of AAV2 vector preparations is a key element in obtaining reliable and reproducible data (Summerford and Samulski, 1999). Purification of AAV based on $CsCl_2$ gradients was the first method described and still is the most commonly used (Toolan, 1968). However, this technique yields preparations with a considerable amount of contaminating nonviral proteins and a high ratio of genome copies versus infectious units. These factors may also vary from lot to lot and complicate the comparison of results from independent experiments. Several improved methods have been published (Grimm *et al*., 1998; Clark *et al*., 1999; Zolotukhin *et al*., 1999; Gao *et al*., 2000). One is based on a combination of ultracentrifugation in iodixanol (instead of $CsCl_2$) and subsequent affinity purification using a heparin column; two others rely on high-performance liquid chromatography (HPLC). Whereas the method published by Gao *et al.* uses a combination of a POROS HE and a POROS 50 PI column, Clark *et al.* describe an HPLC purification based on a POROS HE1/M column. Last, but not least, purification of AAV2 by use of a monoclonal antibody has been reported (Anderson *et al*., 2000). All these protocols result in AAV2 preparations that are purer and more infectious than those obtained with the conventional $CsCl_2$ protocol.

However, these improved methods still require technical skills and special equipment (ultracentrifuge or HPLC machine), which complicates the purification of larger production

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lots. A protocol would be desirable that combines the ease of plasmid column purification with the purity and infectivity of HPLC-purified virus.

In this study, we describe a single-step gravity-flow column purification protocol for AAV2, based on its affinity for heparin. This technique does not require expensive, special equipment or advanced skills, is highly reproducible, and comes close to HPLC-purified virus with respect to purity and infectivity. Briefly, the crude lysate containing the recombinant vector is directly applied to a preequilibrated heparin column. On binding of the virus to the matrix and after two washes, the vector is eluted by a change in buffer and concentrated.

This simple and fast protocol represents an interesting alternative to other published, improved AAV production methods.

MATERIALS AND METHODS

Plasmid constructs

The AAV2 packaging plasmid p600 trans and the Ad helper plasmid pΔF6 have been described (Zhang *et al.*, 2000). The sequences of the AAV2 *cis* vectors can be sent electronically on request. Plasmid DNA was purified with an EndoFree Giga Plasmid preparation kit (Qiagen, Hilden, Germany).

Vector production

For one AAV2 vector preparation, fifty 15-cm dishes of subconfluent, low-passage 293 cells (American Type Culture Collection [ATCC], Manassas, VA) were transfected by calcium phosphate (Clontech, Palo Alto, CA) with 2 mg of plasmid DNA (p600 trans, pΔF6 and the AAV2 *cis* vector). Medium was changed the following day, and cells were harvested 3 days after transfection.

Vector purification

$CsCl_2$ and iodixanol/heparin purifications were performed as described (Zolotukhin *et al.*, 1999; Zhang *et al.*, 2000). For the single-step column purification (SSCP) protocol, cells contained in one 15-cm dish were resuspended in 2.5 ml of Dulbecco's modified Eagle's medium (DMEM) (Cellgro; Mediatech, 10-013-CM [Herndon, VA]), frozen, and thawed two times and incubated with 0.1 mg each of DNase I and RNase A (Roche Biochemicals, Mannheim, Germany) for 30 min at 37°C. After 15 min of centrifugation at 3000 rpm in a Sorvall RT6000D centrifuge at 4°C, the supernatant was transferred into a new tube, incubated with 0.5% (final concentration) deoxycholic acid (Sigma D6750, lot 19H0696; Sigma, St. Louis, MO) for 30 min at 37°C, and sequentially filtered through a 5-μm pore size filter (Millipore, SLSV R25 LS [Bedford, MA]) and a 0.8-μm pore size filter (Millipore, SLAA O25 LS). The cleared crude lysate was then applied on a heparin column prepared in the following way: 8 ml of a heparin–agarose suspension (Sigma H6508) was pipetted into a 2.5-cm-diameter glass column (Sigma C4669) equipped with a Luer Lock (Sigma S7396). After the agarose suspension solution flowed through, a filtration membrane (Sigma S7271) was placed on top of the agarose bedding. The matrix was afterward equilibrated with 25 ml of phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, Gaithersburg, MD). After closing the Luer Lock, the filtered crude lysate was applied to the column. The lock was then opened so that the flow speed was 1 drop/sec. After all the lysate went through the column (which requires several loadings), the matrix was washed twice with 25 ml of PBS, pH 7.4, plus 0.1 *M* NaCl (i.e., PBS with a final concentration of 0.254 *M* NaCl). The virus was then eluted with 15 ml of PBS, pH 7.4, plus 0.4 *M* NaCl. The eluate was concentrated to about 1 ml with a Millipore Biomax-100K NMWL filter device (UFV2BHK40) by centrifugation. To adjust the NaCl concentration to physiological levels, the filter device was refilled with PBS, pH 7.4, and the virus was concentrated to 1 ml again. After removal of the virus-containing solution, the membrane of the filter device was washed three times with 100 μl of PBS, pH 7.4, which was added to the main part of the recombinant AAV2.

It is possible to reuse each of the affinity column components. The glass column, Luer Lock, and sample diffusion disk have been sterilized and reutilized by the authors. Theoretically the resin can be reused as well after a thorough wash (i.e., 1 *M* NaCl in PBS). Nevertheless, because of the relatively low cost of heparin and the possibility of a residual cross-contamination, we discourage the recycling of matrix.

For our protocol, we consider critical four steps: (1) two (instead of three) rounds of freeze and thaw (the third round decreases the yield of infectious virus by 27 ± 3.2%; our unpublished data); (2) the removal of cellular debris before adding deoxycholic acid; (3) addition of deoxycholic acid to the lysate (otherwise the virus does not bind to heparin); and (4) the use of appropriate PBS.

Vector characterization

Genome copies were determined by Real-Time PCR, using TaqMan (Perkin-Elmer Biosystems, Foster City, CA) (Gao *et al.*, 2000). Transducing units were assessed by limiting dilution of virus on 84/31 cells (Gao *et al.*, 2000). The number of green fluorescent protein (GFP)-positive cells was determined by fluorescence-activated cell sorting (FACS), the number of LacZ-positive cells by histochemical 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining.

Purity of viral preparations was assessed by loading 10^{10} genome copies on sodium dodecyl sulfate (SDS)-polyacrylamide gel (4–12% acrylamide gradient gel; Novex/InVitrogen, Carlsbad, CA) run under reducing conditions. Protein was detected by Coomassie staining.

For analysis of transduction efficiency *in vivo*, the tibialis anterior muscles of immunocompetent C57BL/6 mice (three animals per group) were injected with 10^6 to 10^7 genome copies of AAV2 CMV *lacZ* (Fisher *et al.*, 1997), purified either by $CsCl_2$ or SSCP. Muscles were harvested 28 days after injection and either lysed for subsequent β-galactosidase (β-Gal) enzyme-linked immunosorbent assay (ELISA) analysis (Roche Biochemicals) or frozen, cut, and stained for LacZ expression (Fisher *et al.*, 1997).

RESULTS

Previous reports have revealed that $CsCl_2$-purified AAV2 vectors show a high ratio of genomic copies to transducing units (Clark *et al.*, 1999; Zolotukhin *et al.*, 1999). To test whether this is due to the $CsCl_2$ itself, we incubated in two independent ex-

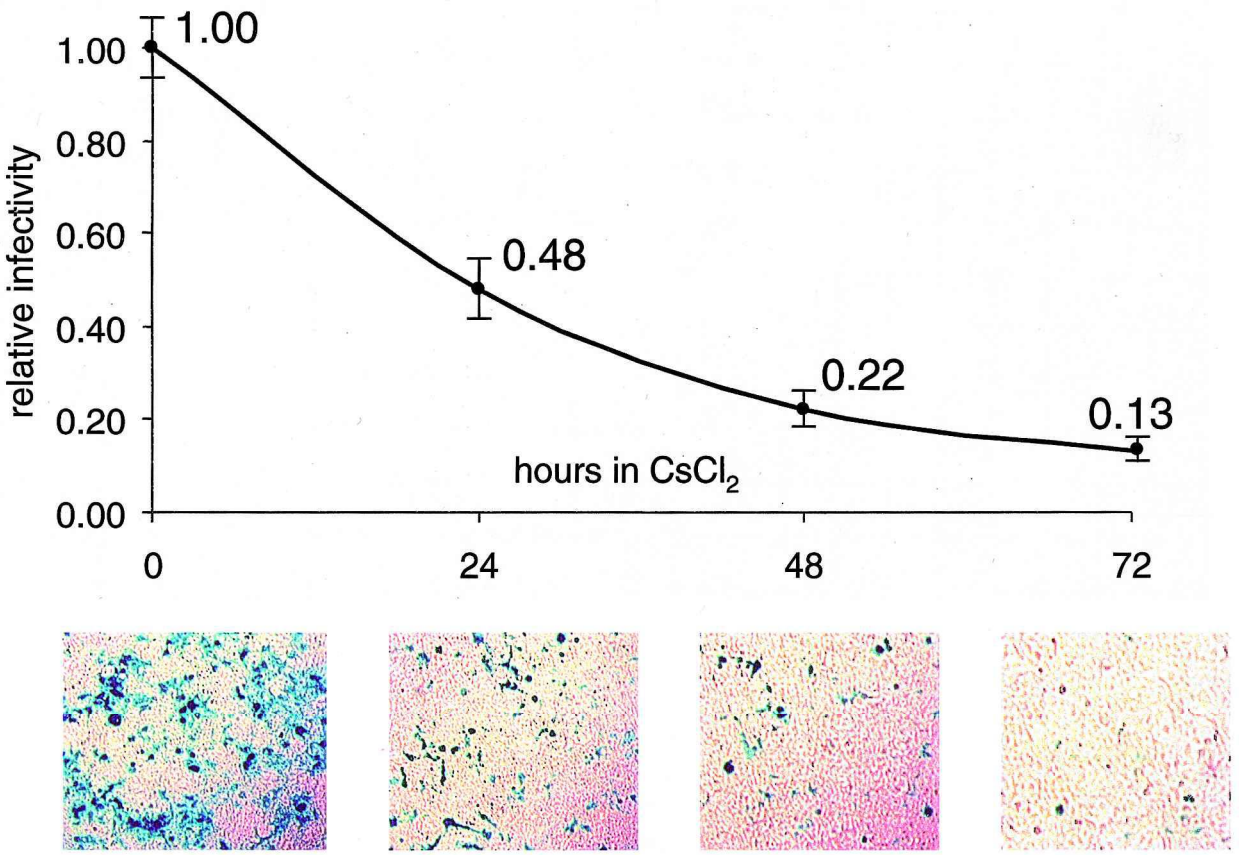

FIG. 1. Stability of AAV2 in $CsCl_2$. A total of 10^{10} genomic particles of AAV2 CMV *lacZ* was incubated in either $CsCl_2$ (1.4 g/cm^3; for 24, 48, or 72 hr) or PBS (for 72 hr, designated 0 [0 hr in $CsCl_2$]) at 4°C. 293 cells were then infected at an MOI of 1 and stained after 3 days for gene expression. The experiment was performed twice, each time in duplicate. *Top*: The relative infectivity compared with virus in PBS over time. *Bottom*: LacZ-positive cells transduced with virus incubated for 0, 24, 48, and 72 hr in $CsCl_2$.

periments (each performed in duplicate) 10^{10} genome copies of AAV2 CMV *lacZ* in either $CsCl_2$ (1.4 g/cm^3) or in the original buffer (PBS) for up to 72 hr at 4°C. 293 cells were then infected at a multiplicity of infection (MOI) of 1 and stained after 3 days for gene expression (Fig. 1). The number of LacZ-positive cells decreased by a factor of 2 each day the virus was incubated in $CsCl_2$. After 72 hr (corresponding to the time it takes to perform two rounds of $CsCl_2$ ultracentrifugation), only about 13% of the original virus was still infectious. We, therefore, developed a protocol that does not rely on $CsCl_2$ gradient centrifugation, but on the ability of AAV2 to bind to heparin (Summerford and Samulski, 1998), and compared it with established protocols. We focused our comparison on the most widely used $CsCl_2$ centrifugation and iodixanol/heparin purification protocol (Zolotukhin *et al.*, 1999), as the necessary reagents are commonly available and no special equipment (HPLC machine) is required.

A preparation of 300 15-cm dishes of 293 cells were triple-transfected with an AAV2 CMV eGFP *cis* plasmid, the packaging plasmid, and the Ad helper plasmid. Cells were harvested 3 days later, and two sets of 50 plates were independently pu-

TABLE 1. COMPARISON OF $CsCl_2$-, IODIXANOL-, AND SSCP-PURIFIED VECTORS: GENOME COPIES AND TRANSDUCING UNITS

Method	*GC*	*TU*	*Ratio GC/TU*	*Average GC yield*	*Average TU yield*	*Average GC/TU*
$CsCl_2$ 1	8.0×10^{12}	7.2×10^{10}	111			
$CsCl_2$ 2	6.6×10^{12}	1.8×10^{11}	37	7.3×10^{12}	3.9×10^{10}	74
Iodixanol 1	1.35×10^{12}	3.4×10^{10}	40	1.8×10^{12}	1.1×10^{11}	35
Iodixanol 2	2.2×10^{12}	7.5×10^{10}	30			
SSCP 1	6.24×10^{12}	6.0×10^{11}	10.4	5.7×10^{12}	5.5×10^{11}	8.4
SSCP 2	5.2×10^{12}	8.3×10^{11}	6.3			

Abbreviations: GC, genome copies; TU, transducing units.

TABLE 2. REPRODUCIBILITY OF VECTOR PREPARATIONS BY SSCP

Vector	*Size (kb)*	*Genome copies*	*Transducing units*	*Ratio*
AAV2 CMV *lacZ*	4.9	1.8×10^{12}	1.5×10^{10}	120
		2.7×10^{12}	2.1×10^{10}	144
		4.5×10^{12}	5.6×10^{10}	81
		1.4×10^{12}	1.1×10^{10}	129
AAV2 CMV eGFP	2.7	6.0×10^{12}	6.0×10^{11}	10
		3.6×10^{12}	3.1×10^{11}	12
		1.4×10^{13}	1.3×10^{12}	11
		1.1×10^{13}	1.4×10^{12}	8
AAV2 TBG *lacZ*	5.1	4.8×10^{12}	ND	ND
AAV2 TBG insulin	2.4	3.7×10^{12}	ND	ND
AAV2 TBG OTC	3.1	1.4×10^{12}	ND	ND

Abbreviations: ND, not determined; TBG, thyroxine-binding globulin promoter; OTC, ornithine transcarbamoylase.

rified according to each protocol (Table 1). Similar genome copy yields, higher than with the iodixanol protocol, were obtained with our method and the $CsCl_2$ method. However, the ratio of genome copies to transducing units was about 10-fold higher with the $CsCl_2$ technique, meaning that about 10 times more genome copies are required to infect the same number of cells. A similar estimate of infectivity was obtained by infectious center assay (ICA [Gao *et al.*, 2000]; data not shown). The reported infectivity of HPLC preparations (Clark *et al.*, 1999; Gao *et al.*, 2000) is equivalent to SSCP-generated vectors.

To test the reproducibility of our method, we generated AAV2 virus preparations, each from 50 triple-transfected 15-cm dishes, with various expression cassettes and genome sizes (Table 2). High yields (genome copies and transducing units) were obtained reproducibly, independent of the *cis* vector design. In these experiments up to 1.4×10^{13} particles of AAV2 could be purified and concentrated using 8 ml of agarose–heparin suspension. An upper limit of virus binding to heparin could not be determined with the amount of virus used in our experiments so far. In addition, the purity of some preparations was examined by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2): 10^{10} genome copies were loaded per lane along with the same amount of $CsCl_2$-purified particles. The most abundant proteins in all the SSCP lots were VP1, VP2, and VP3. In addition, three minor bands, identical in each lane, could also be detected. Altogether, the purity of SSCP-produced vectors is similar to that reported for iodixanol/heparin (Zolotukhin *et al.*, 1999) and HPLC (Clark *et al.*, 1999; Gao *et al.*, 2000).

To confirm *in vivo* the higher infectivity of the SSCP-purified vectors observed in cell culture, various vector doses of both $CsCl_2$- and SSCP-generated preparations were administered to the muscle of C57BL/6 mice (Fig. 3A). Twenty-eight days after injection, the muscles were harvested, and the relative β-galactosidase expression was assessed by ELISA. With increasing viral dose, up to 5-fold higher, gene expression could be detected with the SSCP-prepared virus compared with

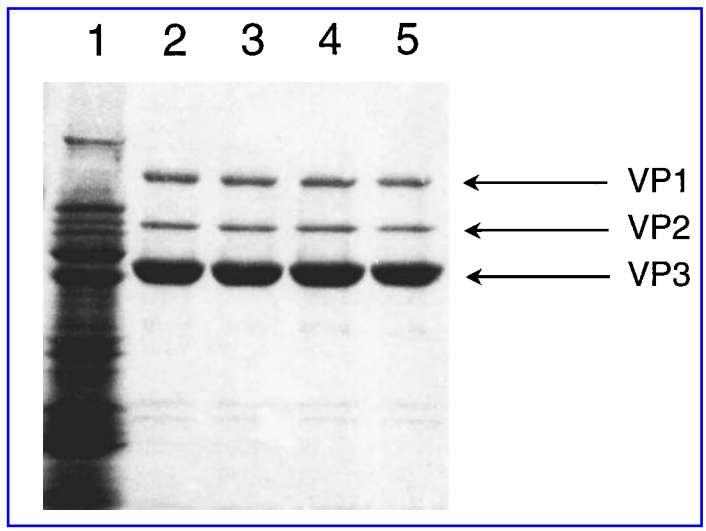

FIG. 2. Comparison of $CsCl_2$- versus SSCP-purified vectors: purity. A total of 10^{10} genome copies was loaded and separated on a reducing SDS-polyacrylamide gel (4–12% acrylamide gradient gel). Protein was detected by Coomassie staining. Lane 1 shows recombinant AAV2 CMV eGFP virus purified with CsCl; lanes 2 to 5 were loaded with four different preparations of the same vector purified by SSCP.

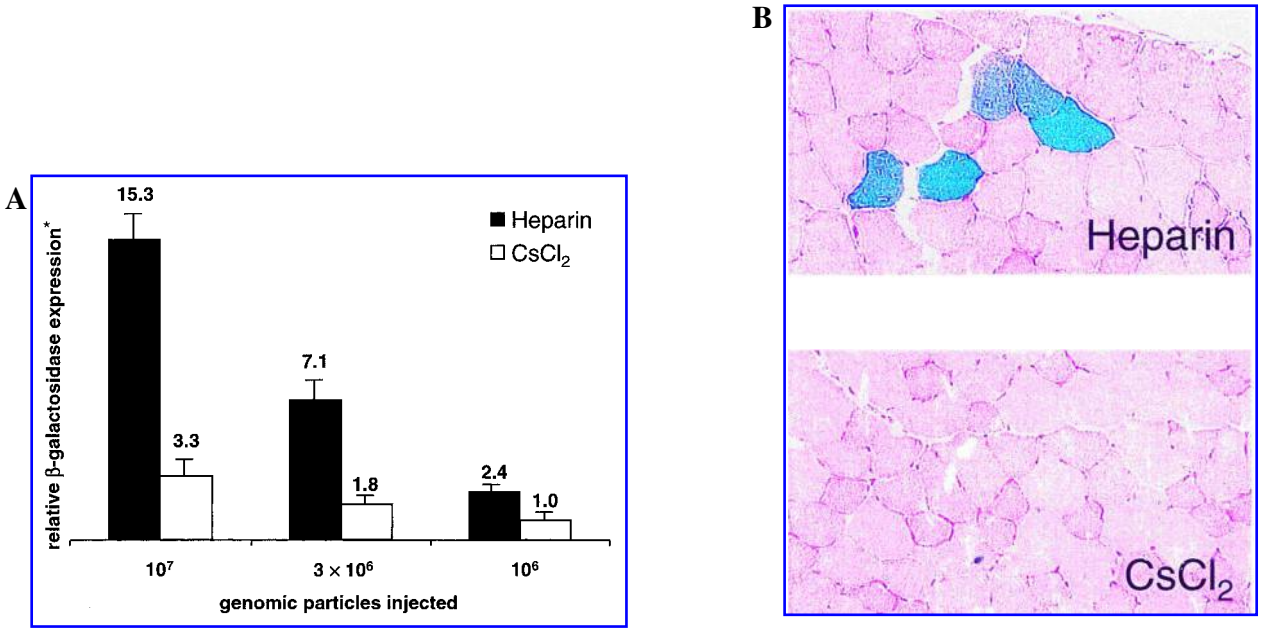

FIG. 3. Comparison of $CsCl_2$- versus SSCP-purified vectors: transduction *in vivo*. The tibialis anterior muscles of immunocompetent C57BL/6 mice were injected, harvested, and analyzed as described in Materials and Methods. (**A**) β-Galactosidase expression relative to transduction of muscle tissue with 10^6–10^7 genome copies of $CsCl_2$-purified AAV2 CMV *lacZ* (mean value of three animals per group). (*β-Galactosidase expression with 10^6 genomic particles of $CsCl_2$-purified virus set to 1.) (**B**) X-Gal staining of transduced muscles.

$CsCl_2$-purified virus. Only on administration of virus produced by our technique could cells be visualized by the less sensitive X-Gal staining (Fig. 3B).

DISCUSSION

Purification of AAV2 on the basis of its affinity for heparin (Summerford and Samulski, 1998) has arisen as an alternative to $CsCl_2$ ultracentrifugation(Table 3). In this report, we describe an improvement on existing affinity purification protocols (Tamayose *et al.*, 1996; Grimm *et al.*, 1998; Clark *et al.*, 1999; Zolotukhin *et al.*, 1999; Anderson *et al.*, 2000; Gao *et al.*, 2000) in terms of simplicity. Briefly, the virus producer cells are lysed in DMEM, and the crude lysate is directly applied to a preequilibrated column, packaged with commercially available heparin–agarose. On binding of the virus in the presence of 0.5% deoxycholic acid, the matrix is washed with PBS plus 0.1 *M* NaCl and the virus is then eluted with PBS plus 0.4 *M* NaCl. Whereas in the single-column HPLC protocol virus is eluted with a linear NaCl gradient (Clark *et al.*, 1999), we use a simple buffer exchange to high salt. Our protocol does not require more technical skills or equipment (in particular, no ultracentrifuge or HPLC equipment) than a commercially available column purification of plasmid DNA. Virus can be purified within one afternoon, reducing the total production time by at least 2 days compared with the still most commonly used $CsCl_2$ purification method. In addition, affinity purification of AAV2 al-

TABLE 3. SUMMARY

	$CsCl_2$ protocol	*Iodixanol/heparin protocol*	*HPLC protocol (heparin)*	*SSCP protocol*
Total time:	6 days	4 days	4 days	4 days
Gradient centrifugation:	Yes	Yes	No	No
Special equipment:	Ultracentrifuge, refractometer	Ultracentrifuge	HPLC machine	No
Cost for purification in US$:	185	ND	ND	120
Ratio genome copies/Transducing units:	~75	~35	~10	~10
Purity[a]:	4	1–2	1	1–2
Difficulty in learning the technique[b]:	3	2–3	4	1–2

Abbreviation: ND, not determined.

[a]Grade for purity: 1, low level of cellular contaminants as detected by SDS-PAGE; 4, high level of cellular contaminants as detected by SDS-PAGE.

[b]Grades for difficulty in learning the technique: 1, easy; 4, difficult.

lows the recovery of virus without the major cellular contaminants present when a physical separation method such as $CsCl_2$ density centrifugation is used. More importantly, as AAV2 is unstable in $CsCl_2$, our method yields highly infectious virus with ratios of genome copies to transducing units of up to 6. Similar results are reported for HPLC and iodixanol/heparin (Clark *et al.*, 1999; Zolotukhin *et al.*, 1999; Gao *et al.*, 2000).

In conclusion, the single-step column purification of AAV2 described here should prove a valuable tool for investigators either struggling with existing protocols or seeking a convenient way to improve the potency of their AAV2 preparations.

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