

Neo-islet formation in liver of diabetic mice by helper-dependent adenoviral vector mediated gene transfer

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Short Abstract: (50 words maximum)

We describe hepatic neo-islet formation in STZ (streptozotocin)-induced diabetic mice by gene transfer of Neurogenin3 (Ngn3) and Betacellulin (Btc) using helper-dependent adenoviral vector (HDAd) and the reversal of hyperglycemia. Our method takes advantages of helper-dependent adenoviral vectors with their highly efficient *in vivo* transduction and the long lasting gene expression.

Long Abstract: (150 words minimum, 400 words maximum)

Type 1 diabetes is caused by T cell-mediated autoimmune destruction of insulin-producing cells in the pancreas. Until now insulin replacement is still the major therapy, because islet transplantation has been limited by donor availability and by the need for long-term immunosuppression. Induced islet neogenesis by gene transfer of Neurogenin3 (Ngn3), the islet lineage-defining specific transcription factor and Betacellulin (Btc), an islet growth factor has the potential to cure type 1 diabetes.

Adenoviral vectors (Ads) are highly efficient gene transfer vector; however, early generation Ads have several disadvantages for *in vivo* use. Helper-dependent Ads (HDAds) are the most advanced Ads that were developed to improve the safety profile of early generation of Ads and to prolong transgene expression¹. They lack chronic toxicity because they lack viral coding sequences²⁻⁵ and retain only Ad *cis* elements necessary for vector replication and packaging. This allows cloning of up to 36 kb genes.

In this protocol, we describe the method to generate HDAd-Ngn3 and HDAd-Btc and to deliver these vectors into STZ-induced diabetic mice. Our results show that co-injection of HDAd-Ngn3 and HDAd-Btc induces 'neo islets' in the liver and reverses hyperglycemia in diabetic mice.

Protocol Text:

1.) Clone the therapeutic genes into HDAd shuttle vector

1.1) Clone Mouse Ngn3 and Btc cDNAs into the pLPBL1 plasmid vector that contains a ubiquitous elongation factor-1 promoter (BOS) and a poly A signal. Upon completion, verify vectors by sequence analysis and then subclone these expression cassettes into pΔ28 HDAd shuttle plasmid⁶.

1.2) Digest HDAd shuttle vectors by PmeI to release the plasmid backbone, purify DNAs by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and reconstitute with transfection-grade water.

2.) Helper-dependent adenoviral vector production

HDAd vector production involves multiple steps that need to be carefully followed for optimal results.

2.1) Transfection

2.1.1) Two days before transfection, seed 116 cells⁷ into 6-cm dish to reach 70-80% confluent on the day of transfection.

2.1.2) Three hours before transfection, remove medium and add 5 ml of fresh growth medium [MEM supplemented with 10% FBS and 1% PSG (Penicillin, Streptomycin and Glutamine, Invitrogen)].

2.1.3) Transfect 116 cells with 10μg DNA from Step 1.2), using the ProFectionR Mammalian Transfection kit from Promega according to manufacturer's instructions.

2.1.4) The next day, wash the cells with 1ml of growth medium MEM 2 times. Add helper virus (HV) at 500 vector particles (vp) /cell to 0.1 ml of PBS containing calcium and magnesium (PBS++) and overlay to the cells. Gently rock the dishes to evenly distribute the HV every 10 min.

2.1.5) After 60 min, add 1.5 ml of maintenance medium (MEM, 5% FBS, 1%PSG).

2.1.6) Add another 1 ml of maintenance medium the next day.

2.1.7) Observe cells for CPE (Cytopathic Effect - cells become rounded and detached). Greater than 80% of cells should show CPE 2 days after infection.

2.1.8) Collect crude cell lysate (CVL, cells and medium), add 10% volume of 40% sucrose and store at -80°C. The CVL is labeled as passage 0 – (CVL-P0).

2.2) Vector amplification.

2.2.1) Freeze (-80 °C)/thaw (37 °C) three times.

2.2.2) Overlay 0.5ml CVL supplemented with HV at 200 vp/cell to confluent 116 cells in 6-cm dish, and rock the dish gently every 5 min. After 30 min, add 1ml maintenance medium.

2.2.3) Add 1ml of maintenance medium next day. 2 days later, most of the cells should show CPE.

2.2.4) Collect the CVL and store at -80°C (CVL-P1) as described for step 2.1.8).

2.2.5) Repeat the procedure for 3 times to get the CVL P2-P4.

2.2.6) Extract DNA from 0.2 ml of CVL collected at P1-P4, and analyze the vector amplification by qPCR using HV- and HDAd-specific primers (Table1). Use the passage in which HDAd exponentially amplified relative to HV (P3 in Fig. 2) for the subsequent procedure.

2.2.7) Co-infect 90% confluent 116 cells in 15cm dish with 0.5 ml CVL and HV at 200 vp/cell. Rock the dish gently every 5 min. After 30 min, add 10 ml of maintenance medium.

2.2.8) Add 5ml of maintenance medium after 24 hours.

2.2.9) Collect cells by centrifugation at 1500 x g for 5 min 48 hours after infection.

2.2.10) Re-suspend cells in 1 ml of PBS++ containing 4% sucrose (P5) and freeze at -80°C.

2.2.11) Freeze and thaw for 3 times and mix well.

2.3) Large scale HDAd production.

2.3.1) To prepare 116 cells for infection in suspension cell culture, transfer confluent 116 cells in 8 x 15-cm dish into 3 L spinner flask and add suspension growth medium (Joklik modified MEM supplemented with 5% FBS, 0.1 mg/ml hygromycin and 1%PSG) to final 1L, and incubate in a CO₂ incubator with spinning at 60 rpm⁸.

2.3.2) Add 0.5 L of fresh medium every day for 2 days (total 2L).

2.3.3) Count cells on the third day. Cells are ready to use if reaching to total cell number of 1×10^9 .

2.3.4) Freeze/thaw P5 3 times.

2.3.5) Collect cells from 3L spinner flask by centrifugation at $1000 \times g$ for 5 mi. Save 100 mL of supernatant to re-suspend the cells.

2.3.6) Transfer cells to a 250-mL spinner flask. Add P5 and HV at 200vp/cell to cells and incubate for 1 hour at 37°C at 60 rpm.

2.3.7) Transfer cells and medium to 3L spinner flask, add 2L suspension growth medium. Transfer 1ml cell suspension to a well in a 12-well plate to observe the cells.

2.3.8) Incubate cells in spinner flask for 2 days in a CO_2 incubator at 60 rpm.

2.3.9) Collect cells by centrifugation and re-suspend with 15 ml 100 mM Tris-HCl (pH 8.0) and store at -80°C (P6) until purification.

2.4) **Vector purification.**

2.4.1) Add 1.0 mL of 5% sodium deoxycholate to the P6. Mix gently and incubate for 30 min at room temperature.

2.4.2) Add 400 μl of 2 M MgCl_2 , 300 μl of RNase A (10 mg/ml), and 300 μl of DNase I (10 mg/ml) and incubate at 37°C for 1 hour.

2.4.3) Centrifuge at $6000 \times g$ for 10 min at room temperature to collect supernatant.

2.4.4) Sterilize NVT 65 ultracentrifuge tubes (Beckman) under UV light for 1 hour in tissue culture hood.

2.4.5) Add 2.8 ml of low-density CsCl solution (1.25 g/ml), underlay 2.8 ml of high-density CsCl density solution (1.41g/ml) and then overlay 5-6 ml of supernatant to fill the tube to the neck. Use 100 mM Tris-HCl (pH8.0) to fill the tube if necessary.

2.4.6) Centrifuge at 10°C for 30 min at 50,000 rpm at 10°C with Beckman LE-80K using NVT-65 rotor.

- 2.4.7) Wipe the area with 70% ethanol for needle puncture, and collect the lower opalescent band with a 3-ml syringe equipped with 22-G needle by side puncture (Fig. 3a).
- 2.4.8) Place the collected bands into a new sterilized ultracentrifuge tubes. Fill the tubes to the neck by overlaying 1.35 g/ml CsCl density solution.
- 2.4.9) Centrifuge at 10°C at 50,000 rpm overnight. Collect the opalescent band (Fig. 3b).
- 2.4.10) Transfer the band into a dialysis cassette (Slide-a-Lyzer, 10,000 MWCO, Thermo-scientific).
- 2.4.11) Dialyze against 3L of autoclaved 10 mM Tris-HCl, pH7.2 containing 2 mM MgCl₂ and 4% sucrose at 4°C overnight.
- 2.4.12) Remove the HDAd vector from dialysis cassette. Aliquot 20 µl for physical titer and 50µl for DNA characterization. (Note: For Ngn3 vector, use 3 X P6 to obtain sufficient vector since the yield of HDAd-Ngn3 is poor compared to HDAd-Btc or HDAd-empty.)

2.5) Characterization of HDAd vectors

- 2.5.1) Determine the physical titer (vp/ml) using optical density (OD). Add 20 µl vector or 20 µl dialysis buffer to 380 µl TE buffer containing 0.1% SDS and incubate at 56°C for 20 min. Measure OD at 260nm. The physical titer = $OD_{260} \times 1.1 \times 10^{12} \times 20$ (vp/ml).
- 2.5.2) Analyze HV contamination by qPCR. Use 50µl aliquot to extract DNA using DNeasy tissue/blood DNA extraction kit (Qiagen). Dilute the DNA 1000-fold and take 5 µl for qPCR analysis using helper and vector-specific primers (Table 1). The helper contamination should be less than 1%, as shown in Fig. 4.
- 2.5.3) Use Southern blot to analyze vector structure. Perform Southern blot analysis¹⁰ using a probe for the inverted terminal repeat (ITR). The representative result is shown in Fig. 5.
- 2.5.4) Determine in vitro efficacy. Infect known number of 116 cells in a 12 well plate with HDAd vector at 1000 vp/cell in quadruplicate. Harvest the cells after 48 hours and extract RNA to determine expression of Ngn3 and Btc mRNAs by qRT-PCR (Table1). Representative results are shown in Fig. 6.

3.) Treatment of diabetic mice by HDAd-Ngn3 and -Btc

- 3.1) Induction of diabetes in mice and injection of HDAd vectors

- 3.1.1) Treat C57Bl6/J mice by intraperitoneal injection of streptozotocin (STZ, Sigma) at the dose of 125 mg/kg on two consecutive days⁹.
- 3.1.2) Fast mice for 6 hours and measure body weight and blood glucose every day. Use a One Touch glucometer for blood collected by tail snip.
- 3.1.3) Treat mice with stable hyperglycemia ($>250\text{mg/dl}$) for at least a week by a single intravenous injection of HDAd vectors via tail vein. The total vector dose is 6×10^{11} vp for all treatment groups (in 0.25 ml): 5×10^{11} vp Ngn3 + 1×10^{11} vp for combination group; 5×10^{11} vp Ngn3 + 1×10^{11} vp for Ngn3 group; and 1×10^{11} vp Btc + 5×10^{11} vp empty vector for Btc group and 6×10^{11} vp empty vector for control group.
- 3.1.4) Monitor 6 hour fasting Glucose and body weight weekly after vector treatment.
- 3.1.5) Collect blood from the saphenous vein in the leg every two weeks to assay insulin and liver enzymes (AST and ALT).
- 3.1.6) Perform glucose tolerance test (GTT) at 6 weeks after treatment. Fast mice for 6 hours and inject 1.5 g/kg of D-Glucose i.p and collect blood at 0, 15, 30, 60, 120 min for glucose and insulin.

3.2) Analysis of effects of HDAd-Ngn3+HDAd-Btc treatment.

- 3.2.1) Harvest liver and pancreas at 3 and 6 weeks after treatment. Divide into two pieces, one for snap frozen in liquid nitrogen and storage at -80°C for RNA and protein extraction and the second to fix with 10% formalin overnight for immunohistochemistry analysis.
- 3.2.2) Extract RNA by a standard protocol and analyze expression of islet specific hormones and transcriptional factors in the liver by qRT-PCR using specific primers (table1).
- 3.2.3) Extract insulin from the liver by an acid-ethanol extraction method and quantify by a commercial ELISA kit (ultra sensitive Insulin assay, Mercodia)
- 3.3.4) Perform immunostaining for islet specific hormones (Insulin, Glucagon, PP, SST) in paraffin embedded sections^{9, 10}.

Representative Results: We cloned Ngn3 and Btc cDNA into p Δ 28 vectors under ubiquitous promoter eIF2a (BOS) and generated HDAd-Ngn3 and HDAd-Btc. As shown in Fig. 2, relative HV contamination decreased significantly (implying more vector amplification and

less helper amplification) at passage 3. Therefore, we used P3 for subsequent vector production. After the first CsCl discontinuous ultracentrifugation, we collected the lowest band and then collected the opalescent band corresponding to HDAd vector in the second ultracentrifugation (Fig. 3). The purified HDAd vector had less than 1% of HV contamination (Fig. 4), indicating sufficient quality for vector infusion into mice. Further analysis included transgene expression by infection of 116 cells. The mRNA expression levels of Ngn3 and Btc were higher in vector infected cells by over 10,000-fold compared with those in non infected cells (Fig. 5). HDAd-Ngn3 and -Btc were then administered to STZ-induced diabetic mice via tail vein injection. Hyperglycemia was reversed and glucose-stimulated insulin secretion was restored in mice treated with both HDAd-Ngn3 and HDAd-Btc but not in mice treated with single gene vector or control vector. Immunohistochemistry showed insulin positive cells in the liver of mice treated with HDAd-Ngn3 and HDAd-Btc, but no insulin positive cells were observed in mice treated with control vector (Fig. 6).

Discussion:

HDAds have been developed to overcome the weakness of early generation Ads and to harness for gene therapy application. However, technical challenges remain. For example, HDAd requires HV for HDAd's packaging and vector amplification is not as efficient as early generation Ads. HV is a first generation Ad and any contamination of HV compromise the effectiveness of HDAd. Therefore, highly efficient transfection and optimal conditions for each serial passage are critical. Another critical parameter for vector production is which passage (P1-P4) should be used for subsequent passage 5 that is directly used as inoculum for suspension cells. To our experience, the best results are obtained by using the passage by which HDAd vector proportion is dramatically increased in the following passage (P3 in Fig. 2). The yield of HDAd vectors depends on transgene cassettes. During vector production, both transgenes are expressed because both genes are under ubiquitous promoter. Ngn3 is a transcription factor and Btc is a growth factor, which suggests that HDAd vector expressing transcription factor which may influence cell lineage inhibits vector amplification while that expressing growth hormone helps in vector replication and packaging. In this report, we show the optimal protocol to generate high quality HDAd-Ngn3 and HDAd-Btc, and demonstrate techniques to induce and assess islet neogenesis in the livers of diabetic mice to reverse hyperglycemia.

In summary, the advantage of the HDAd-vector system for gene transfer lies in its high cloning capacity, efficient transduction and long lasting gene expression in the liver with minimum chronic toxicity as well as its nature of non-integration of vector genome into the host chromosome. The primary limitations are the complex steps involved in its generation and its in vivo application is primarily limited to the liver with the most popular Ad serotype 5.

Acknowledgments:

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Disclosures: There are no conflicts of interest to disclose.

Tables and Figures:

Figure 1: Flow chart of gene therapy of diabetic mice using helper-dependent virus system. First, Ngn3 and Btc, in a cassette driven by a ubiquitous BOS promoter, are cloned into HDAd shuttle (p Δ 28) vectors. HDAd is produced by several steps including transfection, serial passages of amplification, and a large scale infection followed by vector purification. After characterizing the quality, HDAds are injected IV by tail vein into STZ-induced diabetic mice. The effects of treatment are assessed by measuring glucose, body weight, GTT and by analyses of gene expression in the liver.

Figure 2: Determination HDAd vector amplification. DNA is extracted from passage P0 to P4 using DNA extraction kits (Qiagen). DNA is diluted 1,000-fold and 5 μ l DNA is used for real time PCR (qPCR). Helper and vector-specific primers are used. Standard curves are generated by serial dilutions (10^{-5} to 1 ng/ml) of HDAd shuttle vector plasmid and HV plasmid (top panels). Using the standard curves and the Ct values for the vector and helper virus copy number is calculated and the ratio of HDAd/HV is plotted as a percentage of the total virus (helper + HDAd). Hence, relative vector amplification is calculated as: [vector copy number / (vector + helper virus copy number)]. In the example shown (bottom panel) HDAd vector amplification plateaued at P4, while the relative HDAd/HV is increasing at P3. Therefore, P3 is selected for the subsequent step.

Figure 3: Representative HDAd vector bands after discontinuous CsCl density ultracentrifugation. HDAd vector is purified from a 3L spinner culture over sequential CsCl density gradient. (A) After first density gradient ultracentrifugation, a single opalescent vector band is visible (arrow) below opaque cell debris (CD). The opalescent band (arrow) is collected for the second density gradient centrifugation. (B) After the second density gradient ultracentrifugation, the opalescent band (arrow) is collected for dialysis.

Figure 4: Analysis of helper virus contamination. DNA is extracted from 50 μ l purified virus and helper contamination is assessed as in Fig. 2. The figure shows helper contamination of HDAd-Ngn3 and HDAd-Btc is less than 1%.

Figure 5: Analysis of structure of HDAd vector. Southern blot is performed as described previously (Oka K, et al.). Lane 1: DNA from helper virus; Lane 2: DNA from P3; Lane 3: DNA from P4; Lane 4: purified vectopr. Open arrows indicate the helper virus derived bands and the filled arrows indicate the ITR bands derived from the HDAd vector.

Figure 6: Expression level of Ngn3 or Btc in 116 cells infected with HDAd-Ngn3 or HDAd-Btc vector. 116 Cells in a 12 well plates are infected with HDAd-Ngn3 or HDAd-Btc or empty vector at 1000 vp/cell for 2 days. Cells are harvested and total RNA is extracted using Trizol reagent. qRT-PCR is performed using Ngn3- or Btc-specific primers. The relative Ngn3 or Btc mRNA expression increased by over 10,000-fold in cells infected with HDAd-Ngn3 or HDAd-Btc.

Figure 7: Gene transfer of HDAd-Ngn3 and HDAd-Btc into STZ-induced diabetic mice leads to reversal of diabetes and induction of islet neogenesis in the liver. (A) Plasma glucose and (B) body weight of STZ-induced diabetic mice treated with HDAd-Ngn3 and HDAd-Btc. (C) Plasma glucose and insulin during an IP-GTT at 6 weeks after treatment. (D) Representative insulin staining in the liver 12 weeks after treatment. * $p < 0.05$ (vs. empty vector group). The figure is reprinted from Dev.Cell 2009 Mar; 16 (3) : 358-73; Yechoor et. al., with permission from Elsevier.

Table 1 : Primer sequences

name	forward primer	reverse primer
helper	GACCATCAATCTTGACGACC	ATGTCGCTTTCCAGAACCC
vector	TTGGGCGTAACCGAGTAAG	ACTTCCTACCCATAAGCTCC
Ngn3	AAGAGCGAGTTGGCACTCAG	TCTGAGTCAGTGCCCAGATG
Btc	GCACAGGTACCACCCCTAGA	TGAACACCACCATGACCACT

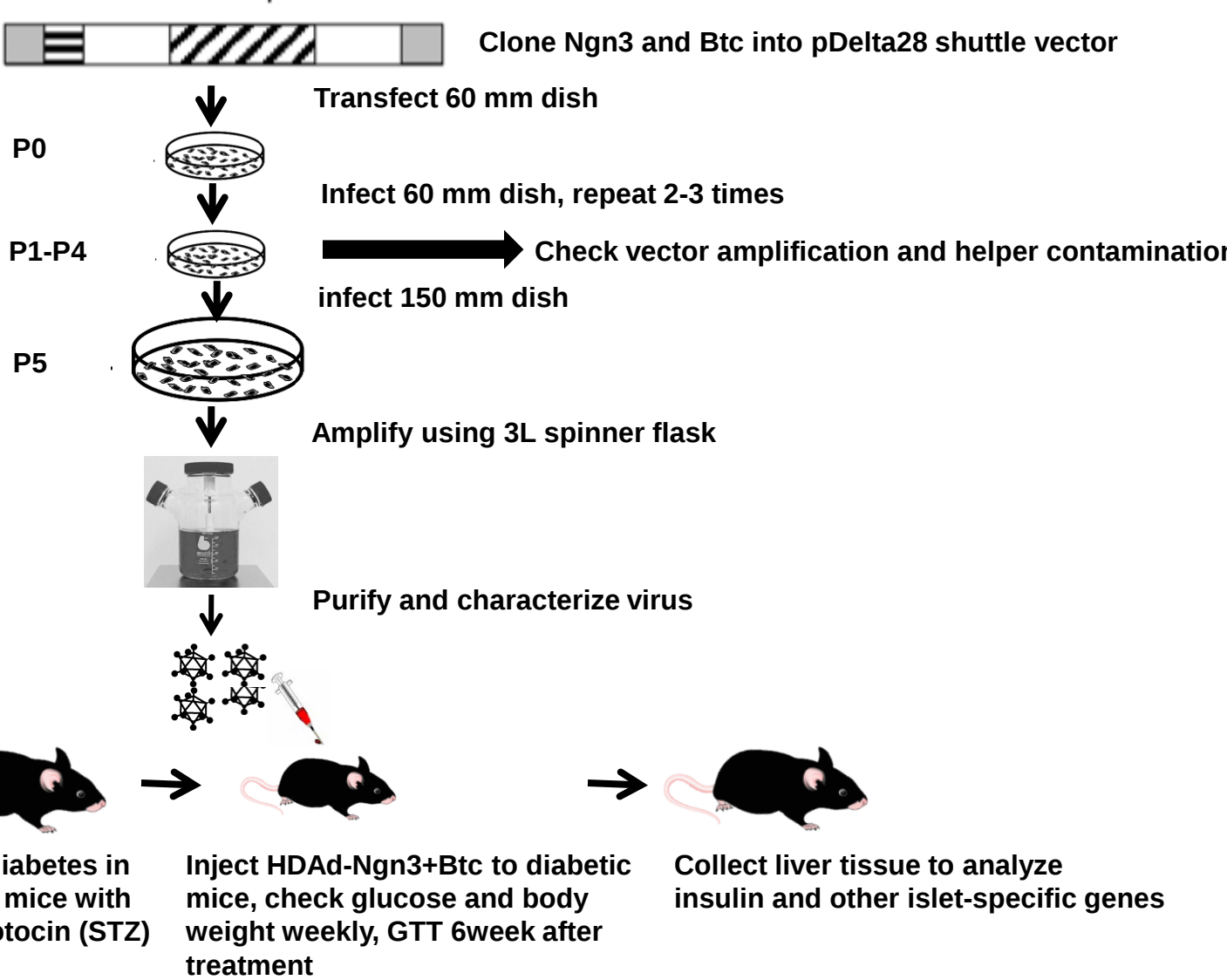
Table2: specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
MEM powder	invitrogen	61100087	
Hygromycin B	Sigma	H0654-1G	
MEM EAGLE JOKLIK	Sigma	M0518-10L	
Glass spinner flasks	Corning	4500-3L	
Glass spinner flasks	Corning	4500-250	
Slide A-lyzer casset	PIERCE CH	PI66380	
Tube optiseal poly allomer ,11.2ml	Beckman Coulter	362181	
Cesium chloride 1kg	JT4042-2	VWR	
Beckman LE-80K	Beckman Coulter	Optimal LE-80K ultracentrifuge	

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Fig. 1



HDAd Virus
production

Treatment of
diabetic mice

Fig. 2

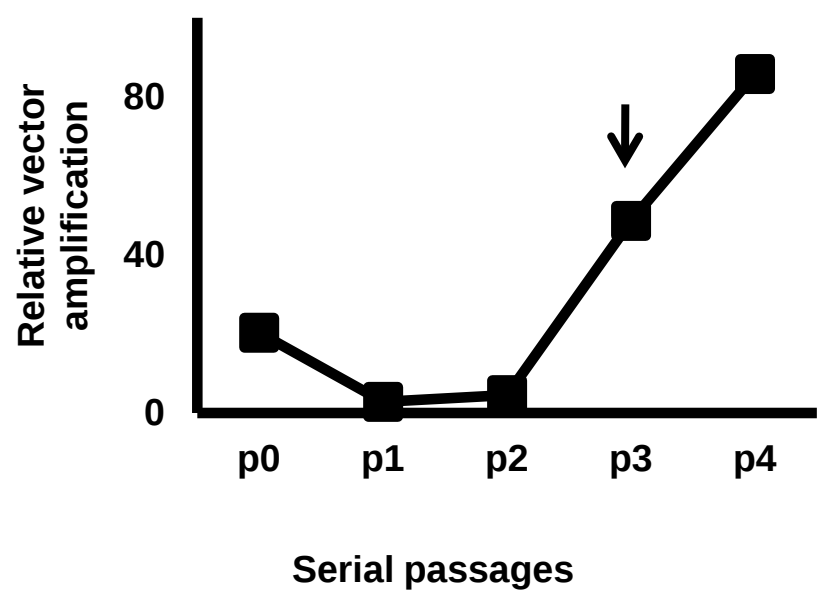
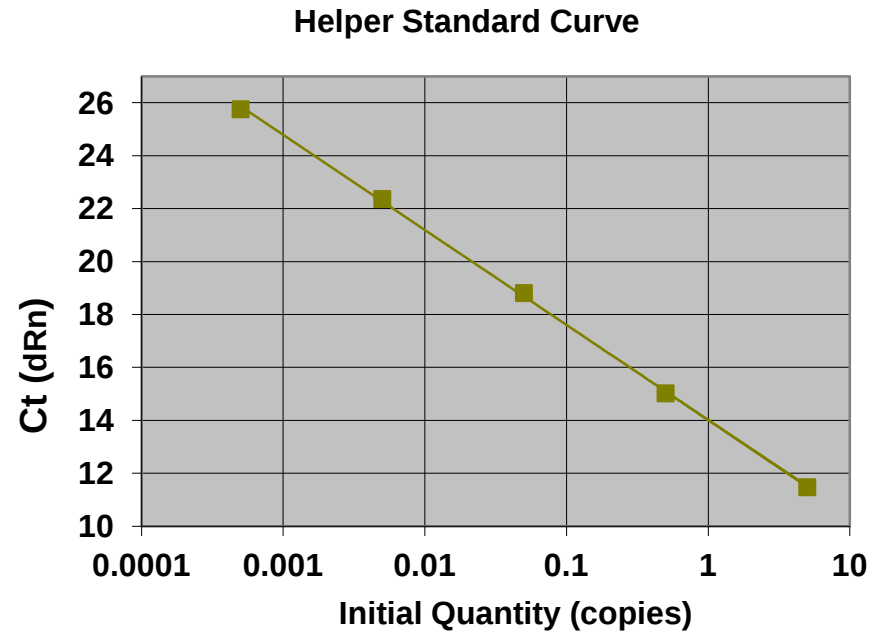
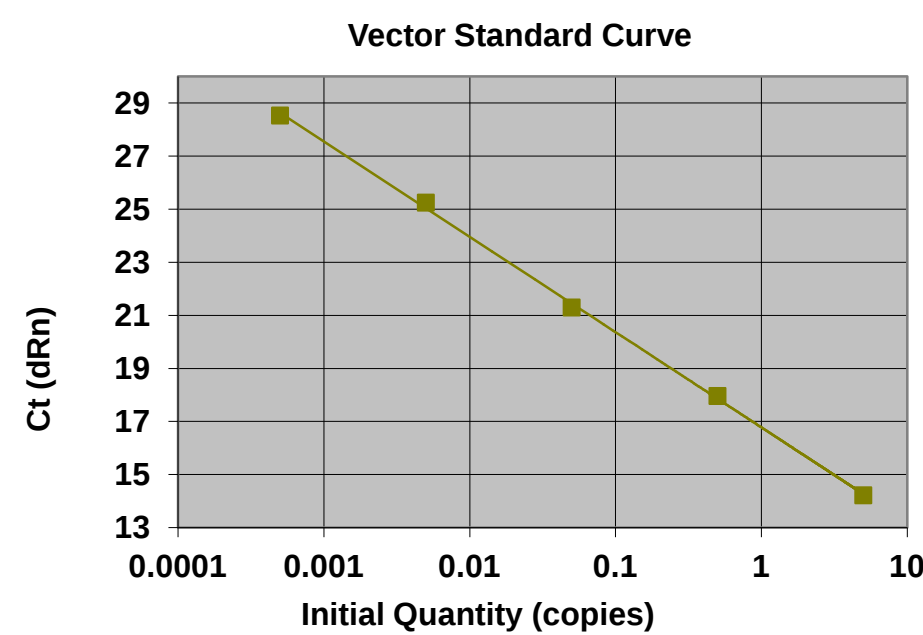


Fig. 3

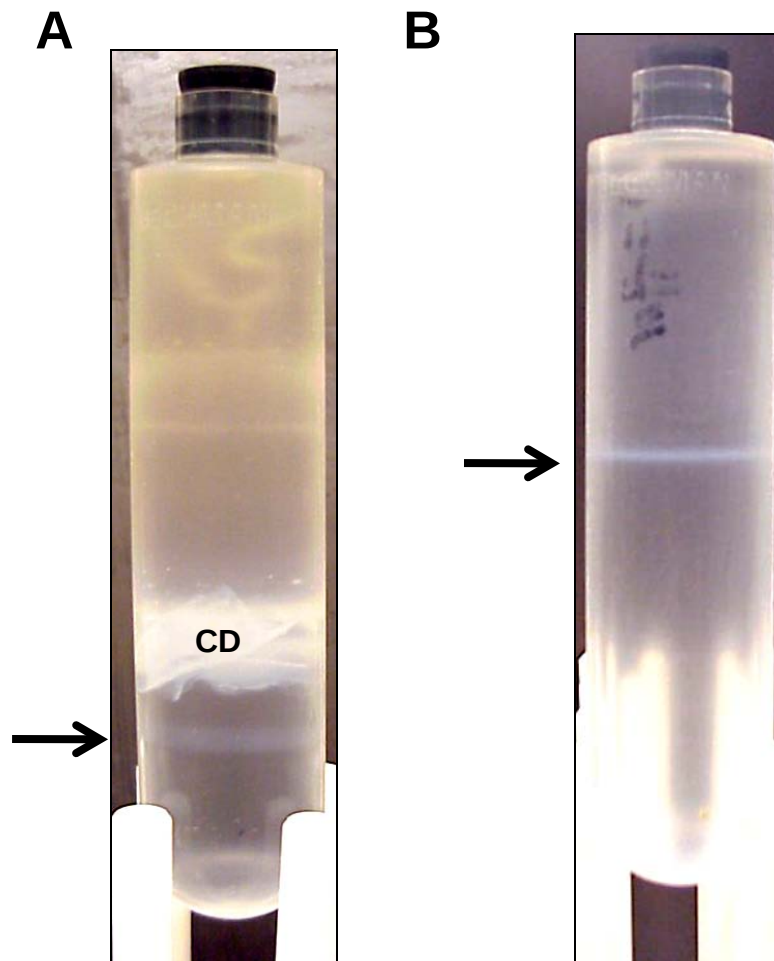


Fig. 4

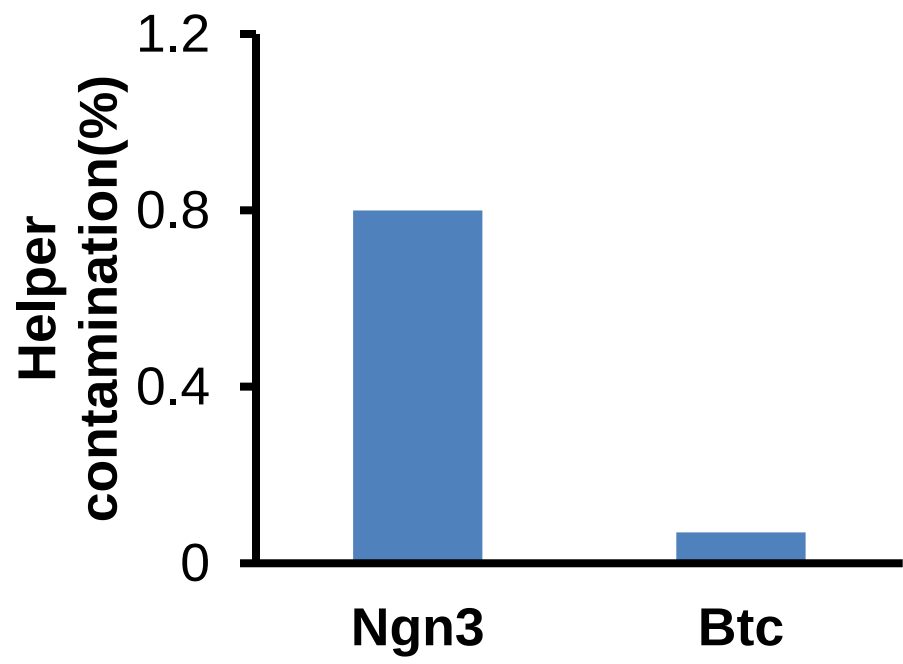


Fig. 5

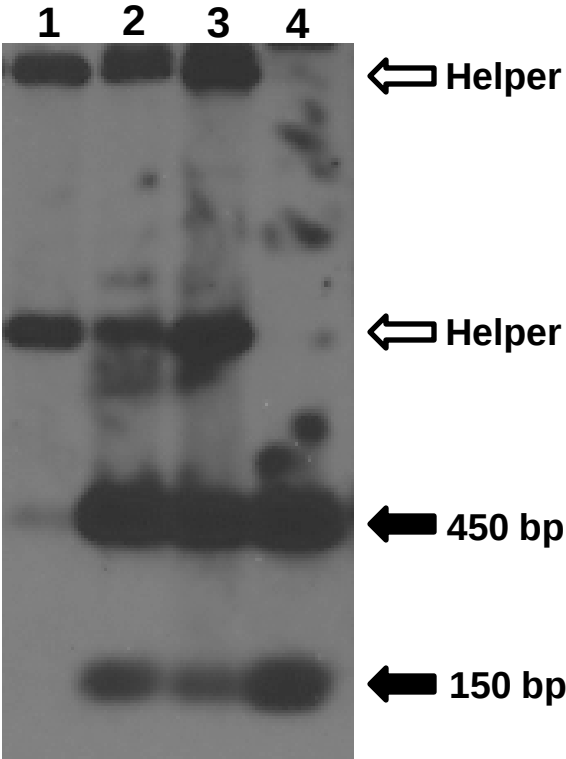


Fig. 6

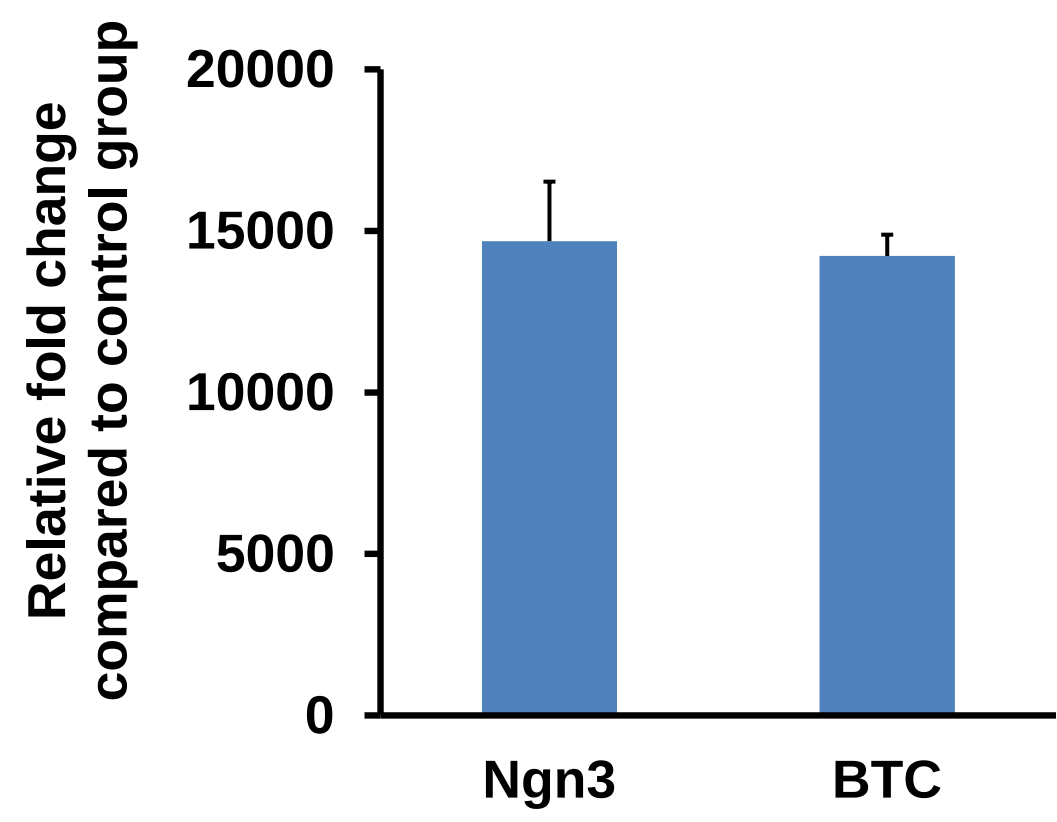


Fig. 7

