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Measuring Immune Responses to recombinant AAV Gene Transfer

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Abstract

Following AAV-based gene transfer, the occurrence of adaptive immune responses specific to the vector or the transgene product is a major roadblock to successful clinical translation. These responses include antibodies against the AAV capsid, which can be neutralizing and therefore prevent the ability to repeatedly administer the vector, and $CD8^+$ cytotoxic T lymphocytes, which can eliminate transduced cells. In addition, humans may have both humoral and cellular pre-existing immunity, as a result from natural infection with parent virus or related serotypes. The need for assays to detect and measure these anti-capsid immune responses in humans and in experimental animals is profound. Here, ELISPOT, immunocapture (ELISA), and neutralization assays are explained and provided in detail. Furthermore, such techniques can readily be adapted to monitor and quantify immune responses against therapeutic transgene products encoded by the vector genome.

Keywords

AAV; Viral capsid; $CD8^+$ T cells; ELISPOT; IFN-γ; ELISA; antibody; neutralization

1. Introduction

Among vector systems that allow efficient *in vivo* gene transfer, recombinant adeno-associated virus vectors (rAAV) hold great potential. However, untoward immunes responses against the viral capsid and/or transgene products have emerged as serious obstacles for successful clinical translation, possibly resulting in prevention or elimination of therapeutic gene expression.

In animal models, rAAV-based gene transfer often resulted in deleterious humoral as well as cellular immune responses towards heterologous and, in some instances, even autologous gene products (1-5). The potential of such responses depends on several parameters including vector construct and dose, viral serotype with its ability to transduce antigen-presenting cells (APCs), the purity of viral preparation, and genetic and non-genetic factors in the recipient. Moreover, the route of vector delivery appears to be a key factor, even able to shift the balance from an immunogenic to a tolerogenic response towards the transgene product as shown following intrahepatic rAAV administration (6-11). Aside anti-transgene responses, animal studies have also shown formation of neutralizing antibodies against

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vector particles following rAAV delivery that prevents re-administration. In humans, high prevalence of anti-AAV preexisting immunity is an additional challenge for rAAV gene transfer. Preformed neutralizing antibodies are present in most individuals with the highest prevalence for AAV2 serotype (12, 13). Low titers of these preexisting antibodies have been shown to neutralize and to abrogate gene transfer efficiency using several AAV serotypes, even when high-dose vector is administered (14-16). Moreover, antibodies against AAV in humans have been shown to be associated to anti-capsid T cell responses including both CD4+ helper and CD8+ cytotoxic T cell (CTL) responses. Importantly, in recent clinical trials using rAAV of serotypes 1 and 2 (17-19), specific capsid CTLs have been detected, and their activation was reported to be dependent on the vector dose (17). In a clinical trial on treatment of hemophilia, these CTLs have been hypothesized to eliminate transduced hepatocytes *in vivo*. Supportive evidence was recently obtained using an *in vitro* killing assay (20). Interestingly and for still unclear reasons, this key issue has not been identified in animal studies even when AAV-specific CTLs have been detected.

Therefore, the evaluation of the immune status of potential recipients of AAV vector gene therapy trials prior and after gene transfer is critical and should include monitoring antibodies as well as T cell responses. Specific and sensitive assays for the detection and the quantification of such responses have been developed. This chapter provides protocols used to detect and measure anti-capsid antibodies in serum of any species using both an ELISA technique as well as an AAV neutralizing transduction assay. The ELISA technique allows rapid detection of most anti-AAV antibodies of even low avidity. The neutralizing assay is longer but allows a more relevant functional analysis of the antibodies; in fact both assays are likely complementary and both may have clinical relevance. In terms of cellular responses against capsid, the chapter also describes an ELISPOT assay for IFN-γ producing cells that utilizes AAV capsid-derived peptides recognized by CD8+ cells from human or mouse origin.

2. Materials

2.1. Isolating White Blood Cells for IFN-γ Secretion. (see Note 1)

1. PBS: Sterile phosphate-buffered saline.
2. Hanks Balanced Salt Solution (Invitrogen, Carlsbad, CA).
3. Culture Media: RPMI 1640 Medium [+] L-Glutamine (Invitrogen, Carlsbad, CA) with 25mM HEPES (Sigma, St. Louis, MO), 10% Heat Inactivated Fetal Bovine Serum (Invitrogen, Carlsbad, CA), and 1% Penicillin – Strepomycin Solution (Mediatech, Manassas, VA).
4. Red Blood Cell Lysis Buffer (ebiosciences, San Diego, CA).
5. 70μm nylon cell strainer (BD Biosciences, San Jose, CA).
6. Ficoll Paque Plus (GE Healthcare, Piscataway, NJ).
7. Trypan blue (Invitrogen, Carlsbad, CA).

2.2. ELISPOT (Enzyme-linked immunospot) Detection of IFN-γ Secreting Cells

1. Murine or Human IFN-γ Development Module Kit (R&D Systems, Minneapolis, MN) containing IFN-γ capture and detection antibodies.

[1]Described white blood cell source is from either murine splenocytes or human peripheral blood mononuclear cells (PMBCs).

2. BCIP/NBT substrate (R&D Systems, Minneapolis, MN): 5-Bromo-4-Chloro3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tertazolium Chloride (NBT) in organic solvent.
3. Streptavidin-AP (R&D Systems, Minneapolis, MN)
4. Specific $CD8^+$ T cell epitope (Anaspec, San Jose, CA).
5. SEB: Staphlococcal enterotoxin B (Sigma, St Louis, MO).
6. PBS: phosphate-buffered saline.
7. Wash buffer: 0.05% Tween 20 in PBS.
8. Blocking buffer: 1% Bovine Serum Albumin (BSA) and 5% Sucrose in PBS.
9. Reagent Diluent Concentration (R&D systems, Minneapolis, MN): filter sterilized PBS with 10% BSA.
10. Tissue culture incubator.
11. dH_2O: Sterile deionized water.
12. 96-well immunospot plate (Millipore, Billerica, MA). (*see* Note 2)
13. ELISPOT Reader. (*see* Note 3)
14. Optional: multi-channel pipette or automated plate washer.

2.3. Antibody ELISA (Enzyme-linked immunosorbent assay) to rAAV2 Humoral Response

1. Intact (DNA containing or empty) rAAV particles of the same serotype used for gene transfer.
2. Experimental host serum (i.e. from rAAV infected mice or humans).
3. Mouse immunoglobulin standard (IgG2a, Sigma, St. Louis, MO)
4. Anti-mouse IgG2a-HRP (horseradish peroxidase conjugate; Southern Biotech, Birmingham, Alabama). (*see* Note 4)
5. Coating Buffer: 13mM Na_2CO, 88mM $NaHCO_3$.
6. Wash Buffer: 0.05% Tween 20 in PBS.
7. Dilution Buffer: 5% BSA (bovine serum albumin), 0.05% Tween 20 in PBS.
8. dH_2O: Sterile deionized water
9. OPD (*o*-Phenylenediamine dihydrochloride) Tablets (Sigma, St. Louis, MO).
10. Microtiter Plates (Corning, Lowell, MA); or suitable alternative.
11. Adhesive sealing film (EXCEL Scientific, Victorville, CA).
12. Microplate reader (Biorad, Hercules, CA) or suitable alternative.

[2]There is a range of immunospot plates with different binding membranes; consult technical support to suit individual needs.
[3]In the absence of an ELISPOT reader, a dissecting microscope can be used to count spots.
[4]Mice typically produce immunoglobulin of IgG2a subclass against rAAV particles. However, other subclasses such as IgG3 have also been described and can be tested for and quantified using analogous reagents for the alternate subclass (murine immunoglobulin standards and detecting antibody) (24). In humans, the dominant response is IgG1 (i.e. the human equivalent to IgG2a, reflecting a Th1-driven response), while IgG2, 3, and 4 may also be found (25). Respective analogous reagents to measure such titers in humans are available. The identical assay can be used to determine antibody titers against the transgene product by coating the microtiter plate with the respective protein instead of with vector particles (8, 26, 27).

13. Optional: Multi-channel pipette or automated plate washer.
14. Optional: Stop solution for HRP development; 3 M HCl or 3 M H_2SO_4

2.4. Neutralizing Antibody Assay

1. rAAV2-CMV-LacZ particles (*see* Notes 5 and [6])
2. Wild type adenovirus type 5 (wt Ad5) particles.
3. Experimental host serum (i.e. rAAV2 infected mice, non human primates or humans).
4. HeLa cell line (*see* Note 6).
5. Sterile trypsin.
6. DMEM: Sterile Dulbecco's Modified Eagle Medium medium.
7. FCS: Heat inactivated Fetal Calf Serum FCS.
8. PBS: Sterile phosphate-buffer saline PBS.
9. X-Gal staining buffer: 50ng/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for colorimetric detection of β-galactosidase enzyme activity (Promega), 0.1% $MgCl_2$, 1% potassium ferricyanide, 1% potassium ferrocyanide in PBS (*see* Note 7).
10. 0.5% Glutaraldehyde in PBS.
11. dH_2O: Sterile deionized water.
12. Sterile 24 well cell culture plates.

3. Methods

3.1. Isolating White Blood Cells for IFN-γ Secretion

3.1.1 Murine splenocyte isolation

1. Remove spleen from mouse or rat in a sterile environment and immerse in a container with ice cold Hanks Media and store on ice.
2. Pulverize spleen on cell strainer, collect cellular content in Hanks Media as it flows through the screen, rinse cell strainer with Hanks Media and bring volume up to 20 – 40 mL in a 50-mL tube with Hanks Media.
3. Centrifuge isolated splenocytes and red blood cells at 4°C (350g for 10 minutes).
4. Carefully pour off supernatant, break up the cell pellet, resuspend in red blood cell lysis buffer (2 to 5 mL) and incubate at room temperature for 5 minutes.
5. Wash with 20 - 40 mL of Hanks Media and centrifuge white blood cell fraction of bulk splenocytes at 4°C (350g for 10 minutes).

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[5]Other transgenes (i.e luciferase (13), Green Fluorescent Protein, GFP) can be used for this type of assay and specific detection methods can also be performed (i.e flow cytometry for GFP+ cells).

[6]The assay can be adapted to other rAAV serotypes. Optimal MOI may need to be determined for each serotype. Using Hela or Huh7 hepatoma cell line, a recent study showed that maximal transduction was reached at a MOI of 5000 vector genomes [Vg]/cell for AAV2, 5 and 6, at MOI of 10,000 Vg/cell for AAV1 and 8, and at a MOI of 100 000 Vg/cell for AAV9 (13).
Aside MOI, optimal cell line must also be tested for specific serotypes. For instance, even if the assay can be performed with HeLA cell line, it is not optimal for rAAV8 serotype. Boutin et *al.* showed that HeLA cell were best transduced with recombinant AAV1, 2, 5, 6 and 9, whereas Huh7 cell line was more suitable for serotype 8 (13). In another study, Huh7 cells were used for serotypes 1, 2, 7 and 8 (12).

[7]This solution is stable 1 month in dark at 4°C.

6. Carefully pour off supernatant, break up cell pellet and resuspend in complete RPMI 1640 media (containing 2 μg/mL of the peptide antigen that encodes the specific T cell epitope) at a concentration of 1.0×10^7 cells/mL.
7. Determine cell count concentration using a 1:10 dilution of trypan blue to exclude dead cells.

3.1.2 Human PBMC isolation

1. Pipette 25 mL of human blood collected with anti-coagulant onto 12.5 mL of Ficoll-Paque Plus solution.
2. With centrifuge set to "0" acceleration and "0" brake, centrifuge at 500g for 30 minutes at room temperature.
3. Isolate the white blood cell fraction (*see* Note 8), transfer to 50-mL tube and bring volume to 50 mL with PBS and centrifuge at room temperature (350g for 10 minutes).
4. Carefully pour off supernatant, break up cell pellet, resuspend in 5 to 10 mL of red blood cell lysis buffer, and incubate for 5 minutes at room temperature.
5. Add 25 mL of Hanks buffer and centrifuge PBMCs at room temperature (350g for 10 minutes).
6. Carefully pour off supernatant and resuspend in complete RPMI 1640 media (containing 2 μg/mL of the peptide antigen that encodes the specific T cell epitope) at a concentration of 1.0×10^7 cells/mL.
7. Determine cell count concentration using a 1:10 dilution of trypan blue to exclude dead cells.

3.2. ELISPOT (Enzyme-linked immunospot) Detection of IFN-γ Secreting Cells

3.2.1. Prepare ELISPOT plate to culture IFN-γ secreting white blood cells. (see Note 9)

1. Pre-wet and coat a 96 well ELISPOT plate 24 hours prior to isolating white blood cells from murine splenocytes or human PBMCs.
2. Pre-wet plate with 15 μL/well of 35% ethanol for 15 seconds and flick out ethanol (DO NOT VACUUM) by inverting plating. (*see* Note 10)
3. To coat plate, prepare 1:60 dilution (*see* Note 11) of IFN-γ capture antibody in PBS, add 100 μL/well of diluted antibody to 96-well plate and incubate overnight 4°C.
4. Remove capture antibody and wash wells 3 times with wash buffer using multi-channel pipette or automatic plate washer and remove excess wash buffer by inverting and blotting the plate on a paper towel.
5. Block membrane with 200 μL/well of blocking solution for 2 hours at room temperature.
6. Repeat step 3 to remove blocking buffer and wash the plate.

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[8] White blood cell fraction is the white ring below the upper plasma layer.
[9] Culture isolated white blood cells the day of isolation for ELISPOT analysis.
[10] Pre-wetting plate helps minimize background after detection.
[11] A 1:60 dilution is recommended, but optimization of both capture and detection antibodies maybe required.

7. After washing, rinse the plate with 200 μL/well of complete RMPI 1640 media and remove the rinse media when IFN-γ secreting cells are ready to be cultured.

3.2.2. Stimulate activated CD8+ rAAV specific IFN-γ secreting cells. (see Note 12)

1. Remove rinse media from ELISPOT plate by flicking the plate while inverted and blot remaining rinse media on a paper towel
2. Add 1.0×10^6 cells/well, which is 100 μL of the isolated white blood cells re-suspended at a concentration of 1.0×10^7 cells/mL in the appropriate stimulation media and incubate for 24 to 36 hours (*see* Note 13) at 37°C in a 5% CO_2 cell culture incubator.
3. Experimental stimulation media consists of the complete RPMI 1640 media with 2 μg/ml of appropriate $CD8^+$ epitope (*see* Table 1 for examples) to determine the frequency of capsid-specific IFN-γ secreting cells (18, 20-23).
4. Negative control stimulation media consists of complete RMPI 1640 media without epitope to determine background level of IFN-γ secreting cells.
5. A non-specific, positive control stimulation media consists of the complete RMPI 1640 culture media with 5 μg/ml of a highly immunogenic superantigen, Staphylococcal enterotoxin B, to ensure that IFN-γ secreting cells are present and to determine the maximal frequency of IFN-γ secreting T cells.
6. An internal positive control can be used by the addition of recombinant IFN-γ protein at 2 ug/mL to the culture media alone, without cells, to verify that there are no procedural errors and to ensure the quality of the reagents. This entire well will appear completely bluish- purple after color development.

3.2.3. Detection of IFN-γ Protein Secreted from Stimulated CD8+ rAAV Specific T-Cells

1. Remove stimulation media and wash 3 times with wash buffer as described above.
2. Dilute 10X Reagent Diluent Concentrate in sterile dH_2O and use to prepare a 1:60 dilution of IFN-γ detection antibody, add 100 μL/well of diluted antibody to 96-well plate and incubate overnight at 4°C.
3. Remove detection antibody and wash 3X as described above.
4. Dilute 10X Reagent Diluent Concentrate in sterile dH_2O and use to prepare a 1:60 dilution of Streptavidin-AP, add 100 μL/well of diluted Streptavidin-AP and incubate for 2 hours at room temperature.
5. Remove Streptavidin-AP and wash plate three times as described above.
6. Add 100 μL/well of BCIP/NBT solution. Cover and develop the plate in the dark for 10 to 30 minutes at room temperature. (*see* Note 14)

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[12] $CD8^+$ T-cell responses specific for the transgene product can be assessed with the same assay using the appropriate $CD8^+$ T cell epitope. Similarly, the assay can be used to measure $CD4^+$ T cell responses using peptides encoding the respective epitopes. In this case, frequencies of IFN-γ secreting cells are determined to measure Th1 responses, while for Th2 responses, IL-4 or IL-5 producing cells are typically measured.

[13] Begin with a 32 hr incubation time but optimization maybe necessary. Optimizing incubation time can be done by using naïve isolated white blood cells and the positive control stimulation media with 5 ug/mL Staphylococcal enterotoxin B. Too short of a time period may result in minimal spot formation and differences in the experimental groups may not be accurately assessed while excessive incubation time may cause saturation of spot formation.

[14] Watch development carefully to prevent over-development, which will lead to high background and make it difficult to accurately count the spots that have formed.

7. After development remove BCIP/NBT, rinse THOROUGHLY with dH_2O (*see* Note 15) and allow plate to air dry at room temperature.

8. The visible spots that have formed can be counted either manually with a dissecting microscope or with an ELISPOT reader (*see* Fig. 1). Results are typically reported as the average number of spot forming units (SFU) per 10^6 cells plated.

3.3. Antibody ELISA (Enzyme-linked immunosorbent assay) to rAAV2 Humoral Response

3.3.1. Preparing Standard curve for measuring antibody titers

1. Prepare the first point of standard curve using purified mouse IgG2a (Sigma) at a concentration of 2000 ng/ml in coating buffer and then make 1:2 serial dilutions using coating buffer as diluent to get a total of 8 points with the final point being at a concentration of 15.625 ng/ml (*see* Note 4 and Fig. 2).

2. Add 50 μL/well of each standard concentration in duplicate to microtiter plate.

3.3.2. Measuring rAAV2 antibody titers

1. After the standard curve wells have been established, coat the remaining wells with 8.0×10^7 intact rAAV viral particles of the respective serotype in 50 μL aliquots using coating buffer. Cover the plate with adhesive sealing film and incubate microtiter plate overnight at 4°C.

2. Wash plate 3X with 200 μL/well of wash buffer using multi-channel pipette or automated plate washer. Blot inverted microtiter plate on a paper towel after washing to remove residual wash buffer.

3. Block microtiter plate using 200 μL/well of dilution buffer at room temperature for 2 hours and then wash plate as previously described.

4. Make a 1:20 dilution (*see* Note 16) of experimental host serum using dilution buffer. Add 50 μL of diluted experimental host serum in duplicate. Incubate at 37°C for 2 hours and then wash plate as previously described. For standard curve wells just add 50 μL of dilution buffer alone. Additionally, blank wells and positive control wells should be used. Dilution buffer alone should be added to blank wells, and known anti-rAAV positive sera should be used as a positive control to ensure that there have been no procedural errors and to verify that efficient reagents are being used.

5. Make a 1:2000 dilution (*see* Note 17) of the rabbit anti-mouse IgG2a-HRP secondary antibody in dilution buffer. Add 100 μL/well of the diluted HRP-labeled secondary antibody to the microtiter plate and incubate at 37°C for 2 hours than wash plate as previously described.

6. For HRP-catalyzed color reaction, dissolve OPD tablets in 20 mL of dH_2O and add 200 μL/well to plate.

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[15] If the plate is not thoroughly rinsed, the residual BCIP/NBT can cause over-development of plate while drying. Additionally, the thin plastic sheet on the bottom of the plate that protects the membrane can now be removed manually with pliers so the bottom of the membrane can be rinsed as well.

[16] This dilution may need to be optimized depending on antibody titers in sera (i.e very high levels may need to be diluted more and very low levels may need to be diluted less). Absorbance values should be within the linear range of the standard curve.

[17] This dilution may need to be optimized to suit individual needs.

7. Allow plate to develop for 5 to 15 minutes (*see* Note 18) followed by measurement of absorbance at 450 nm wavelength using a microtiter plate reader. Alternatively, 100 μL/well of stop solution can be added to the plate and the absorbance can be measured later.

3.4. Neutralizing Antibody Assay

1. On day 1, prepare 24 well cell culture plate(s) with 2×10^5 HeLa cells (*see* Note 19) in 500μl DMEM 10% FCS per well.
2. On day 2, inactivate experimental, and possibly control sera (*see* Note 20) by heating them at 56°C for 30 minutes.
3. Prior to the assay, remove HeLa adherent cells from 3 wells using trypsin, count and calculate the average number of cells per well.
4. Aspirate the medium from the wells.
5. Add 500μl of DMEM 2% FCS containing wt Ad5 preparation at a Multiplicity of Infection (MOI) of 8 infectious particles/cell
6. Incubate at 37°C for 2 hours.
7. During the incubation of the cells with the wt Ad5
   - **7.1** Prepare dilutions of the experimental serum (i.e 1:5, 1:10, 1:20, 1:50, 1:100, 1:200) in DMEM 2% FCS medium (*see* Note 21).
   - **7.2** Add to serum dilutions rAAV2-CMV-LacZ viral particles. The number of AAV particles should achieve in the well a MOI of 4000 particles. The final volume per well of each diluted serum/rAAV should be 500μl.
   - **7.3** Incubate the serum with rAAV at room temperature for 30 minutes to allow AAV contact with neutralizing antibodies.
8. Aspirate the wells and add 500μl of diluted serum/rAAV per well.
9. Incubate the plate at 37°C for 24 hours.
10. Aspirate the wells and fix the cells by adding 500μl of glutaraldehyde 0.5% in PBS per well.
11. Incubate at room temperature for 5 minutes.
12. Wash the wells 1X with deionized water (dH_2O).
13. Add 500μl of X-Gal solution per well.
14. Incubate at 37°C for 6 hours (*see* Note 22).

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[18] Color development should be such to achieve a linear range of immunoglobulin concentration as a function of the absorbance (on a semi-log plot). Therefore, the time of color development has to be optimized. Alternatively, the plate can be measured several times over 5 to 15 minute range in order to identify an optimal readout

[19] Huh7 and 2V6.11 embryonic kidney cell lines have also been described for neutralizing assays for AAV2 serotype (12, 25).

[20] Several controls are recommended to validate the assay: C1) cells alone; C2) cells + wt Ad5; C3) cells + rAAV2; C4) cells + wt Ad5 + rAAV2 (X-Gal positive control); and if possible C5) cells + wt Ad5 + rAAV2 + control neutralizing negative serum (also X-Gal positive control) and C6) cells + wt Ad5 + rAAV2 + control neutralizing positive serum (X-Gal negative coloration control). In animal experimental studies, internal neutralizing negative serum can be obtained before rAAV immunization.

[21] This is recommended serum dilution but it may need to be adapted to suit individual needs and depending on levels of rAAV2 antibodies (i.e very high levels may need to be diluted more, up to 1:5000).

[22] Other variants of incubation with X-Gal are 2 hours at 42°C or overnight at 30°C.

15. Wash the wells 1X with deionized water (dH_2O) and read the X-Gal coloration (blue cells) by light microscopy (*see* Fig. 3 and Notes 23, [24]).

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[23]The plate can be stored at 4°C for further reading.

[24]This assay is semi-quantitative (blue cell count using miscroscope) and does not allow precise determination of neutralizing antibody titers. For more precise quantification, the colorimetric detection of beta-galactosidase may be assessed by bioluminiscence after cell lysis and measurement in a microplate luminometer as described elsewhere (12, 25). In assays using luciferase transgene, similar luminescent quantification is described (13).

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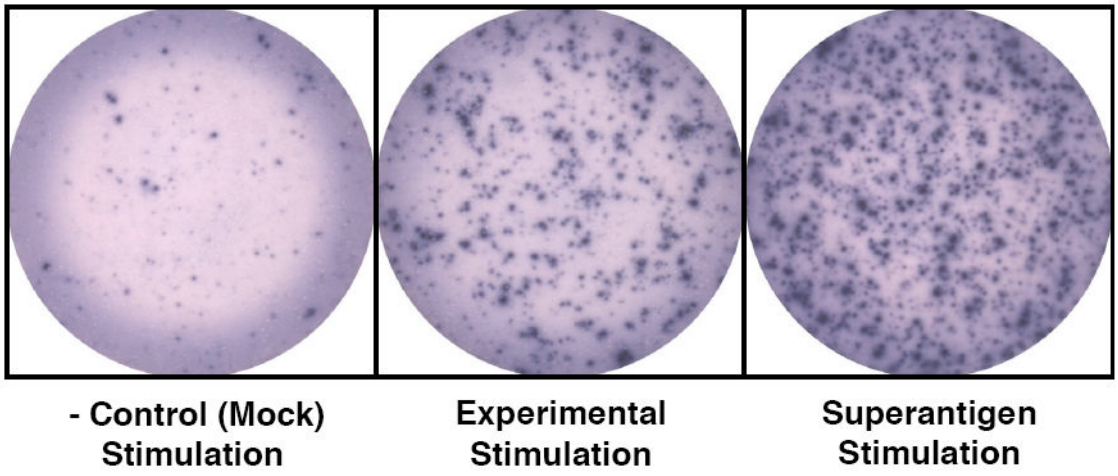

Fig. 1.
Examples of ELISPOT wells with SFUs (Spot Forming Units) after color development.

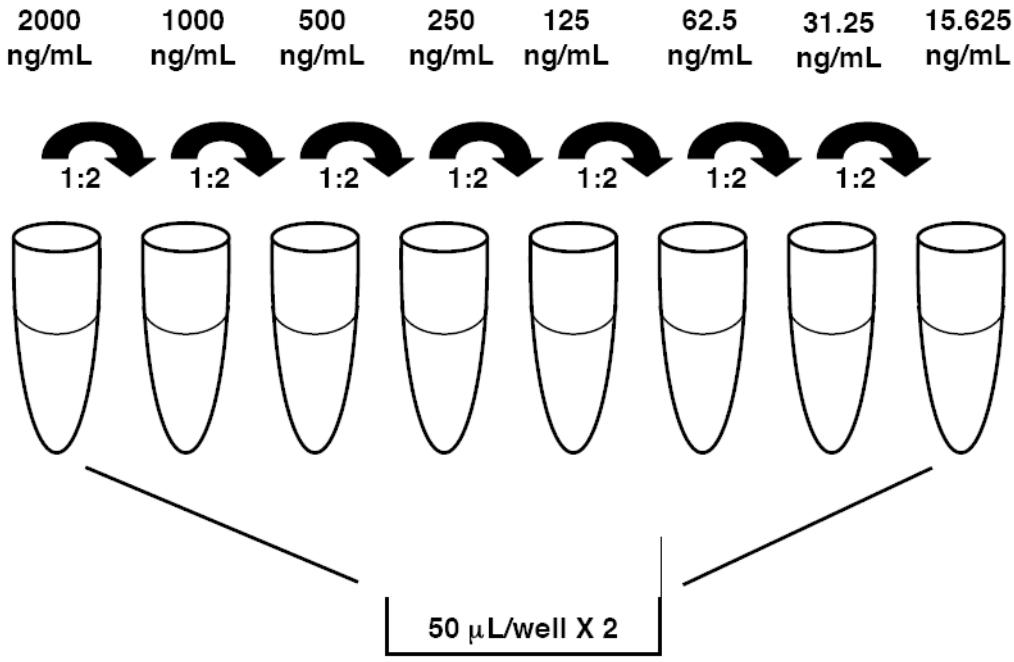

Fig. 2.
Schematic to preparing immunoglobulin standard used for ELISA by serial dilution.

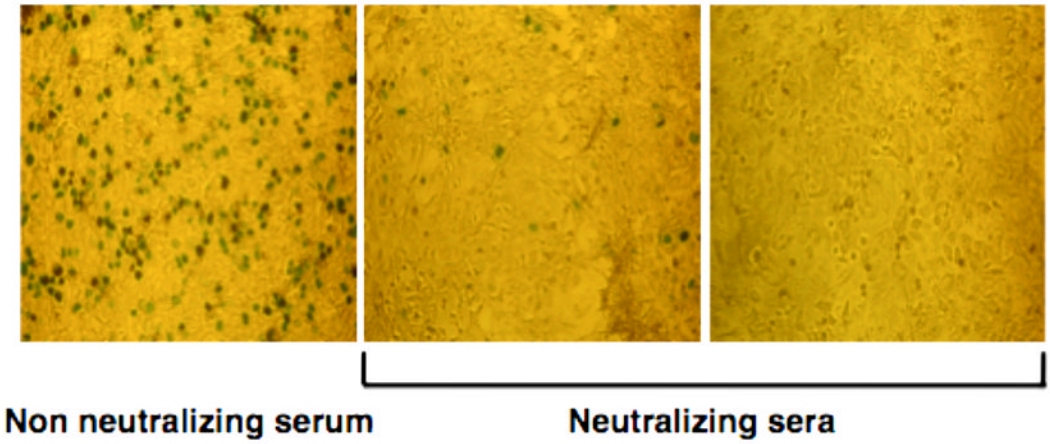

Fig. 3.

Examples of X-Gal coloration detected by microscopy. Left panel: absence of neutralization.Blue spots correspond to transduced cells.Left and middle panels: neutralizing sera with different amounts of neutralizing antibodies. Far right panel, the serum abrogates completely AAV-mediated transduction.

Table 1

Examples of dominant $CD8^+$ rAAV epitopes

Host	AAV Serotype	Peptide Sequence	MHC I restriction
Mouse; BALB/c (*H*-2^d)	AAV2	VPQYGYLTL	L^d
	AAV8	IPQYGYLTL	L^d
Mouse; C57BL/6 (*H*-2^b)	AAV2	SNYNKSVNV	K^b
	AAV8	NSLANPGIA	D^b
Human (HLA-A)	AAV2	SADNNNSEY	HLA-A*0101
Human (HLA-B)	AAV2	VPQYGYLTL	HLA-B*0702