# A step-by-step method to detect neutralizing antibodies against AAV using a colorimetric cell-based assay

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## Summary

A comprehensive laboratory protocol and analysis workflow is described for a rapid, cost effective and straight forward colorimetric cell-based assay for the detection of neutralizing elements against AAV6.

## Abstract

Recombinant adeno-associated viruses (rAAV) have proven to be a safe and successful vector for the transfer of genetic material to treat a variety of health conditions in both the laboratory and the clinic. However, the presence of pre-existing neutralizing antibodies (NAbs) against AAV capsids poses an ongoing challenge for the successful administration of gene therapies in both large animal experimental models and human populations. Preliminary screening for host immunity against AAV is necessary to ensure the efficacy of AAV-based gene therapies as both a research tool and as a clinically viable therapeutic agent. This protocol describes a colorimetric *in vitro* assay to detect neutralizing factors against AAV serotype 6 (AAV6). The assay utilizes the reaction between an AAV encoding an alkaline phosphatase (AP) reporter gene and its substrate NBT/BCIP, which upon combination generates an insoluble quantifiable purple stain.

In this protocol serum samples are combined with an AAV expressing AP and incubated to permit potential neutralizing activity to occur. Virus serum mixture is subsequently added to cells to allow for viral transduction of any AAVs that have not been neutralized. The NBT/BCIP substrate is added and undergoes a chromogenic reaction, which corresponds to viral transduction and in turn the neutralizing activity. The proportion of area colored is quantitated using a free software tool to allow for the generation of neutralizing titers. This assay displays a strong positive correlation between coloration and viral concentration. Assessment of serum samples from sheep both prior to and following administration of a recombinant AAV6 led to a dramatic increase in neutralizing activity (125 to >10,000-fold increase). The assay displayed adequate sensitivity to detect neutralizing activity in >1:32,000 serum dilutions. This assay provides a simple, rapid and cost-effective method to detect NAbs against AAVs.

## Introduction

Adeno-associated viruses (AAV) are increasingly used as vectors for the delivery of gene therapies to trial treatments for a variety of health conditions that impact the cardiovascular, pulmonary, circulatory, ocular and central nervous systems[1-5]. The popularity of AAV vectors as a leading gene therapy platform stems from their positive safety profile, long-term transgene expression and wide-ranging tissue specific tropisms[1,6]. Successful outcomes in animal studies have paved the way for over fifty AAV gene therapy clinical trials that have successfully reached their efficacy endpoints[7], as well as release of the first commercially available AAV gene therapy drug approved by the US Food and Drug Administration[8]. Following initial successes, AAV have continued to gain traction in both the basic and clinical research sectors as a vector of choice and are currently the only *in vivo* gene therapy approved for clinical use in the US and Europe[9]. Nonetheless, the presence of pre-existing neutralizing antibodies (NAbs) against AAV vector capsids remains a hindrance to both preclinical research, and the efficacy of clinical trials. NAbs are present in both naïve human and animal populations and inhibit gene transduction following *in vivo* administration of an AAV vector[1]. AAV seropositivity is an exclusion criterion for most gene therapy trials and therefore preliminary screening for host immunity is crucial in both the laboratory and the clinic. Establishing an assay that can detect the presence of NAbs against AAV is an essential step in the pipeline of any AAV gene therapy-based research project. This report focuses on AAV6 which has been of interest to researchers due to its efficient and selective transduction in striated muscle (heart and skeletal muscle)[1,10-12]. Gene therapy is considered a promising strategy for targeting the heart because it is difficult to specifically target the heart without invasive open-heart procedures.

Neutralizing activity is usually determined using either a cell-based *in vitro* or *in vivo* transduction inhibition assay. *In vivo* NAb assays usually involve administering serum from a test subject (e.g., human or large animal) into mice, followed by an AAV with a reporter gene, followed by testing for the expression of the reporter gene or corresponding antigen. *In vitro* assays determine NAb titers by incubating serum or plasma from a human or large animal in serial dilutions with a recombinant AAV (rAAV) that expresses a reporter gene. Cells are infected with the serum/virus mixture and the extent to which the expression of the reporter gene is inhibited is assessed compared with controls. *In vitro* assays are widely used for NAb screening due to their comparatively lower cost, rapidity in testing and greater capacity for standardization and validation[13,14] compared with *in vivo* assays. *In vivo* assays are often reported to have greater sensitivity[15,16], but the same claim has been made with respect to *in vitro* assays[14,17].

To date, *in vitro* NAb assays have mostly used luminescence (luciferase) as the reporter gene to detect neutralization, and although a light-based method has merit in many contexts, a colorimetric/chromogenic NAb assay may be more advantageous in some circumstances. Colorimetric assays to assess neutralization have been successfully employed for other viruses such as influenza and adenovirus[18,19]. Their attractiveness stems from their simplicity, lower cost and the requirement for only everyday laboratory apparatus and tools[20]. NAb assays that use a luminescence based reporter gene have the additional requirement of costly substrate kits, a luminometer and corresponding software for analysis[21]. This colorimetric assay has the advantage of only requiring a light microscope and a very cheap substrate. Reporting of the sensitivity of colorimetric verses luminescent assays has yielded conflicting results. One study suggested luminescence based ELISA assays display greater sensitivity and comparable reproducibility to colorimetric assays[22], whilst another study found colorimetric based ELISA assays to confer greater sensitivity[23]. Here, a detailed protocol for an *in vitro* NAb assay against AAV that utilizes the chromogenic reaction between an AAV encoding an alkaline phosphatase (AP) reporter gene and a nitro blue tetrazolium /5-bromo-4-

chloro-3-indolyl phosphate (NBT/BCIP) substrate is provided. This step-by-step protocol was developed based on a previous report that utilized a hPLAP (human placental alkaline phosphatase) reporter gene (AAV6-hPLAP) for the detection of neutralizing activity against AAV[24]. This assay is cost effective, time efficient, easy to set up and requires only minimal technical skills, laboratory equipment and reagents. Moreover, the simplicity of this assay gives it the potential to be optimized for broad application across different types of cells, tissues or viral serotypes.

## Protocol

All aspects of animal care and experimentation were conducted in accordance with Florey Institute of Neuroscience and Mental Health guidelines and the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia, 8th edition, 2013).

A schematic overview of the assay protocol is provided in Figure 1. [Place **Figure 1** here]

## 1. Initial preparation

**1.1.** For assessment in sheep: Collect blood in 8mL serum separator clot activator tubes, leave the blood sample at room temperature (RT) for 20-30 minutes and subsequently spin down at 2100 g for 15 minutes. The clear supernatant that forms at the top of the tubes is serum. Aliquot the clear aqueous phase into microcentrifuge tubes and store at -80°Celcius (C). NOTE: Blood was collected from the carotid vein using a 16-gauge needle (tip cut-off) and syringe from conscious 1.5-3 year old Merino ewes.

**1.2.** Heat inactivate fetal bovine serum (FBS) by placing it in a water bath at 56°C for 30 minutes and swirling intermittently. For precision, place a thermometer in a second bottle containing an equivalent volume of water and add it to the heat bath at the same time as the FBS bottle. Begin timing when the thermometer reaches 56°C.

**1.3.** Employ proper aseptic technique and cell culture practice for all subsequent steps that are performed in the cell culture hood [25,26]. Spray all objects and the hood itself with 70% ethanol prior to use and clean with 1% sodium hypochlorite upon completion.

**1.4.** Make complete Dulbecco's Modified Eagle Medium (DMEM) by combining high glucose (4.5g/L) DMEM (89%) with heat inactivated FBS (10%) and Penicillin Streptomycin (1%). Combine and filter using a sterile vacuum filtration (0.22 µm pore size, polyethersulfone membrane) system. Store complete DMEM wrapped in foil at 4°C.

**1.5.** Establish HT1080 cells and passage in a 75cm$^2$ square flask as described in AAV-HT1080 cell culture guidelines Catalog #240109 (https://www.chem-agilent.com/pdf/strata/240109.pdf). Create multiple frozen stocks of cells. Do not use cells after 20 passages as further passaging may influence the assay results.

## 2. Day 1 – Plating cells

**2.1.** Passage HT1080 cells until they reach ~80% confluency as described in the AAV-HT1080 cell culture guidelines Catalog #240109 (https://www.chem-agilent.com/pdf/strata/240109.pdf).

**2.2.** Pre-warm complete DMEM, 0.05% trypsin-EDTA and 1x phosphate buffered saline (PBS) to 37°C in a water bath. Remove the growth medium from passaged cells using an aspiration system. NOTE: All aspiration in this protocol uses a vacuum system with a tube attached to a sterile 5 mL serological pipette. Wash the cells in 10 mL of pre-warmed (37°C) 1x PBS and trypsinize cells for 3-4 minutes in 4 mL of pre-warmed 0.05% trypsin-EDTA to detach the cells from the flask. Inactivate the trypsin by adding 6 mL of pre-warmed complete DMEM and pipette the cells into a 50 mL tube. Calculate the number and concentration of viable cells using a haemocytometer and the trypan blue exclusion method[27].

**2.3.** Dilute the cells to a concentration of $1x10^5$ cells/mL in pre-warmed complete DMEM. Seed 100 µl of cells/well into clear 96-well flat-bottomed plates ($1x10^4$ cells per well). Incubate the plate at 37°C, 5% carbon dioxide ($CO_2$) overnight for 16-22 hours.

## 3. Day 2 – Infecting cells

**3.1.** Remove plate/s from the incubator and use a light microscope to visually confirm cells are evenly dispersed within the wells and the confluency is roughly 50%. If cells are not within a range of 45-55% confluency, repeat the 'Day 1' protocol and adjust initial cell concentration accordingly.

**3.2.** Generate serial dilutions of the serum samples of interest in 1.5 mL microcentrifuge tubes using pre-warmed complete DMEM as the diluent. Table 1 demonstrates how to generate a dilution cascade for triplicate samples.

To perform the assay in triplicate, prepare a $7.5x10^6$ vector genomes (vg)/µl working solution of AAV6-hPLAP by diluting a virus stock solution in 1x PBS (a stock solution of AAV6-hPLAP can be provided upon request by A/Prof Paul Gregorevic, University of Melbourne, VIC, Australia, initial stock concentrations vary). Add 66 µl of the $7.5x10^6$ vg/ µl virus working solution to each tube containing 264 µl of serum/media dilution (330 µl total volume/dilution, see Table 1). NOTE: This is a robust assay which does not require perfect culture conditions. However, to accurately quantitate and ensure each assay run is reliable, it is necessary to include: i) a no serum control, ii) a no serum or virus control, and iii) a NAb positive control sample on all plates under the same experimental conditions.
The volume described (330 µl) accounts for triplicate samples +10% of the serum & virus mixture. Performing replicates of the assay is highly recommended for accurate determination of neutralizing activity.

**3.3.** Mix the virus/serum dilutions together by pipetting and place the tubes containing the virus/serum mixtures in an incubator at 37°C, 5% $CO_2$ for 30 minutes to allow potential neutralization to occur.

**3.4.** Pipette 100 µl of the virus/serum mixture to each well on the 96-well plate containing $1x10^4$ cells/well for each dilution. This will generate a final viral concentration of 15k viruses/cell multiplicity of infection (MOI) in each well. Table 2 provides an example 96-well sample plate layout for assessing samples to a 1/512 dilution.

**3.5.** Wrap the 96-well plate containing cells, serum and AAV-hPLAP in foil and place in an incubator at 37°C, 5% $CO_2$ overnight for 16-24 hours to allow AAV entry into the cells.

## 4. Day 3 - Fixing and adding substrate to cells

**4.1.** CAUTION: Paraformaldehyde (PFA) is a probable carcinogen and is toxic from skin or eye contact or inhalation. Handle in a fume hood with proper personal protective equipment as well as a facemask. Make fresh 4% PFA diluted in PBS (~7 mL required per 96-well plate) and allow to cool to RT.

**4.2.** Pre-warm an aliquot of PBS to 37°C (~25 mL/96-well plate). Cool separate aliquots of PBS (~25 mL/96-well plate) and double distilled $H_2O$ (DDW, ~25 mL/96-well plate) to 4°C. Dissolve a pellet of BCIP/NBT in 10 mL of DDW in a 50 mL conical centrifuge tube by vortexing (10 mL is enough for 2 x 96 well plates).

**4.3.** Aspirate the media from the wells of the 96-well plate using a serological pipette or similar attached to a suction based aspiration system or fume hood vacuum. Gently place the tip of the serological pipette into the well and remove the media taking caution not to disrupt the adhered cells. Add 50 µl of 4% PFA to each well using a pipette. Wrap the plate in foil and leave at RT for 10 minutes to fix the cells.

**4.4.** Wash and aspirate the cells with 200 µl of RT PBS. Repeat this step twice. **NOTE:** A multichannel pipette is an efficient option for the pipetting steps.

**4.5.** Pipette 200 µl of pre-warmed PBS into each well, wrap the plate in foil and incubate at 65°C for 90 minutes to denature endogenous alkaline phosphatase activity.

**4.6.** Aspirate wells and wash cells in 200 µl of cold (4°C) PBS. Aspirate again, wash in 200 µl of cold DDW and aspirate again.

**4.7.** Pipette 50 µl of the dissolved BCIP/NBT into each well.

**4.8.** Wrap the plate in foil and incubate at RT for 2-24 hours. NOTE: Be consistent with incubation time between runs, the time flexibility allows users to photograph wells either on day 3 or the following day.

**4.9.** Using a light microscope camera, take photos of each well using a 4x objective lens, ensuring the same exposure, white balancing and light settings are used consistently for all

assays performed.Position each well identically and ensure the edges of the well are not visible in the photos. Save photos as TIF format or similar. NOTE: Specific settings will vary between microscopes but quantitation will be most effective if the background lighting is high and consistent throughout the wells (Figure 1B).

## 5. Quantitation to determine neutralizing activity using ImageJ

**5.1.** Download and install the freely available software "ImageJ": https://imagej.nih.gov/ij/download.html

**5.2.** Open the image to be analyzed in ImageJ '**File**' > '**Open**' (Figure 2).

**5.3.** If using colored images, convert to grayscale by selecting **'Image'** > '**Type** '> '**8-bit**'.

**5.4.** Click '**Image**' > '**Adjust**' > '**Threshold**'. Adjust the threshold until all colored areas are colored in red, but the background is not. Upon addition of NBT/BCIP, colored product will deposit in the area around the cells expressing hPLAP. NOTE: It is recommended to use the same threshold setting for all images captured on the same plate.

**5.5.** Click '**Analyze**' > '**Set Measurements**' and tick the '**Area**', '**Limit to Threshold**', '**Area Fraction**' and '**Display label**' checkboxes and click '**Ok**'.

**5.6.** To determine the signal reading (percentage of coloration) of a given well, click '**Analyze**' > '**Measure**'. The '% Area' column of the pop up window displays the signal reading.

**5.7.** Perform quantitation for all sample replicates. Exclude any wells that are contaminated, show uneven cell distribution, or vary in cell density or lighting. See Supplementary Figure 1 for examples of wells that should be considered for exclusion. Typically, 3-4 wells may require exclusion from a 96-well plate. Figure 2 provides a visual representation of the quantitation process using ImageJ.

## 6. Determination of Transduction Inhibition ($TI_{50}$) titer

**6.1.** Determine the average readout from replicates (using the steps described in 5) for the:

- Media only control (baseline signal reading).
- Virus + media only control (maximum signal reading).
- Virus + serum samples of interest

**6.2.** Calculate the percentage of inhibition using the following formula:
100 - [(Test sample signal readout *(virus + serum sample of interest*) - baseline signal readout (*media only control*)) / (maximum signal readout (*media and virus only*) - baseline signal readout)) x 100] = % Transduction inhibition.[13]

**6.3.** Calculate the % transduction inhibition from all replicates of each dilution for all samples using the formula in 6.2. Determine the average transduction inhibition between technical replicates for each dilution for all samples and controls.

**6.4.** Calculate the 50% transduction inhibition titer ($TI_{50}$ titer) of a sample of interest by determining the lowest dilution of the sample that yields 50% or greater transduction inhibition of hPLAP activity. e.g., if a 1/8 dilution of a sample has greater than 50% transduction inhibition based on the calculation performed in 6.2 (and a 1/4 dilution does not) report the $TI_{50}$ titer as 1/8.

## 7. Determination of neutralized AAV particles

**7.1.** Calculate the number of neutralized AAV particles per µl of serum for a given sample by employing the following formula:

- ((MOI x cell count/well) / (volume of serum / dilution factor of $TI_{50}$ titer)) / 2 = neutralized AAV particles / µl of serum. NOTE: Dividing by 2 accounts for the $TI_{50}$ measuring 50% of neutralized particles.

- For a sample that gives a $TI_{50}$ titer of 1/4 (25% serum, 75% diluent) in which the assay used 80 µl of undiluted serum and an MOI of 15k was plated onto $1x10^4$ cells, the following calculation would be used: ((15000 x 10000) / (80/4)) / 2 = $3.75x10^6$ neutralized particles / µl of serum.

## Representative results

*Transduction assay to establish the optimal viral dosage for plate coverage*

HT1080 cells, a well-established fibrosarcoma cell line, were selected for this assay. A concentration of $1x10^4$ HT1080 cells/well provided ~50% cell confluency in each well of a 96-well plate. To determine the optimal viral concentration for the assay, a rAAV encoding a hPLAP (human placental alkaline phosphatase) reporter gene (AAV6-hPLAP) was added in triplicate at a range of concentrations of vg containing particles per cell (MOI: 0, 150, 500, 1500, 5000, 15000, 50000 & 150000 (Figure 3A)). An MOI of 15000 ($1.5x10^8$ vg/well) conferred 36% plate coloration and was selected as the optimal viral dosage. A positive correlation was observed between coloration and viral concentrations for all MOI between 0 and 15000 ($n$=6, $r$=0.995, $P$<0.001). This concentration adequately displayed the reporter gene signal above background in the presence of high concentrations of NAbs (low plate coloration), whilst not losing sensitivity due to color saturation in the absence of NAbs (high coloration, Figure 3B).

The efficacy of the assay when exposed to neutralizing elements was trialled using serial dilutions of an anti-AAV6 mouse monoclonal antibody (mAb) in triplicate. The standard approach of using the first dilution to display 50% or more transduction inhibition ($TI_{50}$) was applied to determine the

neutralizing titer of a given sample (see Methods). Assessing $log_{10}$ dilutions, the anti-AAV6 mAb displayed a $TI_{50}$ titer of ~10 ng/mL (1 ng total mAb), whilst a concentration of 500 ng/mL (50 ng total Ab) and above completely inhibited reporter gene expression (Figure 3C).

*Assessment of NAbs against AAV6 in sheep samples*

Serum was collected from the carotid vein using a 16 gauge needle (tip cut-off) and syringe from conscious naïve healthy adult sheep (1.5-3 year old Merino ewes) (*n*=11) and screened to determine the $TI_{50}$ titer using the colorimetric NAb assay. Two-fold serial dilutions ranging from a 1/2 to a 1/512 dilution were assessed for each serum sample in duplicate or triplicate. The 1/2 dilution contained a total of 40 µl of serum, which corresponded to a concentration of $1.88x10^6$ AAV particles per µl of serum. The degree of AAV neutralization varied within the naïve sample population, with $TI_{50}$ titer values ranging from as low as 1/2 (blue line) to as high as 1/80 (green line, $1.88x10^6$ to $7.5x10^7$ neutralized AAV particles/µl serum) (Figure 4A).

Subsequently, direct cardiac injection (*n*=5) of AAV6 was performed at doses ranging between $5x10^{12}$ and $3x10^{13}$ vg to naïve sheep. Briefly, sheep were anaesthetized as previously described[28]. The heart was exposed from the left lateral position, the pericardium opened and AAV administered via 10-40 ~20µl injections into the left ventricular myocardium (anterior) in the region around the second branch of the left anterior descending coronary artery (LAD). The pericardium, intercostal muscle, subcutaneous tissue and skin were closed and anaesthetic removed. Serum was collected from all animals prior to and six to eight weeks after AAV administration from the pre-cannulated right jugular vein using a 16 gauge needle cut-off and syringe (~5ml serum/animal). The colorimetric NAb assay was employed to determine the change in NAb titer following AAV6 administration. Post-AAV serum samples were screened in triplicate as 2- to 4-fold serial dilutions ranging from a 1/2 to a 1/32768 dilution. Assay results indicated that AAV inhibition $TI_{50}$ titer values prior to AAV administration ranged from 1/4 to 1/80 ($3.75x10^6$ to $7.5x10^7$ neutralized AAV particles/µl serum; Figure 4B). Following AAV cardiac injection a clear and consistent contrast in the NAb titer was observed compared with pre-AAV serum titers (Figure 4B, Table 3). The lowest AAV dose ($5x10^{12}$ vg) displayed a 1/2048 $TI_{50}$ titer (dashed blue line, $1.92x10^9$ neutralized AAV particles/µl serum) and the remainder of the doses (1-$3x10^{13}$ vg) displayed $TI_{50}$ titers ranging from 1/12000 to >1/32768 ($1.13x10^{10}$ to $>3x10^{10}$ neutralized AAV particles/µl serum).

[Place **table 3** here]

## Figures Legends and Table titles

**Figure 1:** Schematic diagram of NAb assay protocol. **A)** Visual representation of the NAb assay illustrating the primary steps involved in the three-day protocol. Briefly, cells are grown and plated overnight. The following day, serial dilutions of serum are prepared, incubated with AAV and then incubated with the cells overnight. The next day, cells are fixed, washed, incubated, combined with substrate and incubated again followed by imaging and quantitation. **B)** Representative images of a minimum signal control (complete AAV inhibition), a maximum signal control (no inhibition) and an ovine serum sample with ~50% signal inhibition. Scale bar = 0.5 mm.

**Figure 2:** Steps for determining percentage coloration using ImageJ software. **A)** Open the image to be analyzed with ImageJ software. **B)** Convert the image to 8-bit grayscale. **C)** Open the threshold window **D)** Adjust the maximum threshold so all colored areas are covered but background area is not (this threshold should be consistent across an entire plate). **E)** Select the 'Analyze' drop box, click 'Set measurements' and tick 'Area', 'Area fraction', 'Limit threshold' and 'Display label' and click 'OK'. **F)** Click 'Measure' to measure the covered area. The % area indicates the proportion of the image that was colored. This can then be used with the control samples to determine the $TI_{50}$ titer.

**Figure 3:** Optimization of viral dose & assessment of assay efficacy against an anti-AAV6 monoclonal antibody (mAB)**. A)** Proportion of coloration (% of total area) for individual wells at different multiplicities of infection (MOI) and representative images (below) displaying the corresponding chromogenic reaction between the alkaline phosphatase (hPLAP) and NBT/BCIP for each viral dose (left). Percentage plate coverage is also displayed in tabulated form (right). Each data point represents a technical replicate (*n*=3 replicates per MOI). **B)** A representation of the correlation between coloration and MOI shown in figure 3A. The red dotted line represents the highest concentration tested that did not affect the linear correlation between coloration and viral concentration. **C)** Neutralizing activity against AAV6 from an anti-AAV6 monoclonal antibody at $log_{10}$ dilutions. 50% inhibition of AAV6 transduction ($TI_{50}$) is observed at a concentration of ~10 ng/mL. *n*=3 replicates per dilution. Data represent mean ± SEM.

**Figure 4:** Example of neutralising antibody (Nab) assay results using ovine serum samples. **A)** Adeno-Associated Virus (AAV) neutralizing activity of serum samples collected from 11 naïve sheep, measured in 2-fold serial dilutions ranging from 1/2 to 1/512. Colored lines are representative samples with low and high neutralizing activity, grey lines represent the other nine samples. The dotted line represents 50% transduction inhibition ($TI_{50}$) and the corresponding $TI_{50}$ titers for the low (blue) and high (green) representative samples. **B)** AAV neutralizing activity of serum samples collected from five sheep before and after receiving a dose of AAV via direct cardiac injection. Neutralizing activity assessed in 2-fold serial dilutions ranging from 1/2 to 1/32768. Each color represents serum from a different animal, filled lines represent pre-AAV administration and dotted lines represent post-AAV administration. *n*=2-3 replicates per dilution for each sample. Data represent mean ± SEM.

**Supplementary Figure 1:** Visual examples of different reasons for excluding sample wells. **A)** The presence of contamination as can be seen by clumps of in the centre. **B)** High cell density. **C)** Uneven lighting of well (left image), corresponding thresholding of the same well displaying excess coverage in bottom right corner (right image). **D)** Technical replicate images, image on left representing results with normal cell density, image on right reflecting results with low cell density. **E)** Cells unevenly distributed across a well.

**Supplementary Figure 2:** Simplified plasmid map displaying hPLAP insert. CMV: Cytomegalovirus promoter. hPLAP: human placental alkaline phosphatase. SV40: Simian virus 40 polyadenylation signal. ITR: Inverted terminal repeat sequences.

**Table 1**: Volumes of serum and diluent required to generate serial dilutions of serum in triplicate.

**Table 2:** Example 96-well plate layout for assessing naïve serum samples in dilutions ranging from 1/2 to 1/512. Higher dilutions are incorporated into the assay if assessing a sample known to be positive for AAV NAbs (post administration samples) or if a higher titer is required. MO (-C): Media only control. VO (+C): Virus and media only control. mAb: Monoclonal antibody against AAV (NAb positive control).

**Table 3:** Impact of AAV exposure on neutralizing activity. Neutralizing activity for sheep assessed both before and after receiving direct cardiac injection of a rAAV6 at varying doses. The dose received pre and post $TI_{50}$ titers and fold change following administration are displayed.

## Discussion

This report describes a colorimetric assay which assesses the extent of AAV neutralization in a given serum sample by evaluating a chromogenic reaction that corresponds to the degree of *in vitro* viral transduction. The development of the protocol was based on the known chromogenic reaction between the enzyme alkaline phosphatase and NBT/BCIP which has been widely utilized as a staining tool for the detection of protein targets in applications such as immunohistochemistry, and as a reporter tool for evaluating viral transduction[24,29-31]. Its merit stems from its time and cost effectiveness, accessibility, ease of setting up and performing, whilst still demonstrating a high degree of efficacy. The rAAV6 employed in this assay (AAV-hPLAP) carries the reporter gene human placental alkaline phosphatase (hPLAP) and is driven by a cytomegalovirus (CMV) promoter[30] (Supplementary Figure 2). NBT/BCIP is a hPLAP substrate that is initially dephosphorylated by alkaline phosphatase and sequentially undergoes oxidization to form a dimer which results in an insoluble product that is a vibrant purple color[32].

In selecting the optimal MOI for this assay, the aim was to establish a viral concentration that would provide sufficient expression of the AAV reporter gene through virus-cell binding, and in conjunction with the NBT/BCIP substrate, provide coloration within a range that could be accurately measured. An MOI of 15000 was selected, as it was the highest concentration tested that did not affect the linear correlation between coloration and viral concentration. Higher concentrations (50 000 and 150 000 MOI) caused the concentration-color response curve to plateau, indicating color saturation (Figure 3B). Assessment of viral MOIs between 0 and 15000 (n=6) and their corresponding level of coloration resulted in $r$=0.995 ($P$<0.001), validating the sensitivity of the assay by establishing a very strong positive correlation between viral concentration and reporter-gene driven coloration. Given potential variability in factors such as cell culture conditions, laboratory technicians, techniques and equipment as well as variations in viral batches, it is recommended that any new user perform a preliminary trial assessment of the optimal MOI when establishing a NAb assay.

The MOI chosen for a given NAb assay is major contributing factor in the overall titer observed for a given serum sample. If an MOI of 5000 instead of 15000 had been selected, a 3-fold difference in titer value would be anticipated. This has historically been problematic in the field of AAV gene therapy, as different pre-clinical and clinical trials have implemented AAV NAb assays with MOI values ranging from less than 1000 to as high 25000[33], meaning any kind of cross-study comparison of NAb titers for a given AAV serotype is of little to no value. It has recently been suggested that reporting titers as neutralized AAV particles per µl of serum can provide values that are more comparable across different studies[9,34]. There are numerous other factors that may contribute to

variation in titers between studies, such as the choice of cell line, reporter gene, incubation times and culture conditions. To facilitate the standardization of AAV NAb assays, both the titer values and neutralized AAV particles/µl of serum have been reported.

It is important to include a serial dilution of a known NAb sample on every plate to act as both a positive control and a common sample between plates. This is important to identify any possible variability between separate runs. A neutralizing monoclonal antibody against the AAV of interest is an ideal positive control and standard, but a serum sample known to be positive for NAbs is also acceptable. The efficacy of the assay was validated by demonstrating that a monoclonal antibody specific to intact AAV6 particles (ADK6) can quantitatively inhibit transduction in a concentration-dependent manner.

Based on the manufacturer's material data sheet, the BCIP/NBT substrate system produces the insoluble blue-purple product within ~10 min, and is very stable. However, it is noted that procedures may affect the length of incubation time. Based on prior reports, time frames of 1-24h have been used[35,36]. For this assay, it is important that incubation times are consistent between runs. The time flexibility, allows users to photograph wells either on day 3 or the following day.

Preclinical trials using large animal models provide a crucial stepping-stone between the laboratory and the clinic due to the physiological resemblance that animals such as sheep and pigs share with humans[1,37,38]. Historically, the vast majority of candidate AAV gene therapies that have made it to clinical trials have undergone preliminary trials in large animals[1]. Multiple studies have demonstrated that both humans and a range of large animals including sheep, pigs, dogs, rabbits and non-human primates can harbour neutralizing antibodies against AAV6 as well as many other AAV serotypes [39,40]. This highlights the importance of preliminary screening for NAbs prior to trials in both large animal models and humans. NAb status of serum samples from sheep that had no previously known exposure to AAV were assessed, of which 10 of 11 displayed $TI_{50}$ titers <1/30 (<$3x10^7$ neutralized AAV particles/µl serum), whilst one displayed a $TI_{50}$ titer of 1/80 ($7.5x10^7$ neutralized AAV particles/µl serum). Five of the sheep went on to receive direct cardiac muscle injection of rAAV. Amongst all samples, administration of AAV dramatically changed the neutralizing activity, with fold change increases ranging from a 125 to a >10,000 fold in $TI_{50}$ titer values between pre and post AAV exposure (Table 3). Of note, the lowest AAV6 dose administered ($5x10^{12}$ vg) corresponded to the lowest post-AAV $TI_{50}$ titer value ($TI_{50}$ titer 1/2000) and fold change increase (125 fold). While no clear evidence of pre-existing NAbs within the 11 naïve sheep was observed at levels which would prevent AAV transduction (all $TI_{50}$ titer values <1/100), data from the 5 sheep which received a direct injection of AAV would suggest a cut off for NAb positivity would be >1/1000 (based on pre- and post-AAV values). The stark difference between the pre- and post-NAb titer and the capacity to detect titers >1/32,000 provides further validation of the efficacy and sensitivity of the assay. Of note, establishment of a cut-point at which a sample is deemed positive for neutralizing activity is an important subsequent step when determining a threshold for NAb positive animals. This can be determined by assessing the variability in a group (~n ≥30) naïve samples from a specific population of interest. This allows for the establishment of criteria to determine a statistically derived cut-point in which a sample is deemed positive for neutralizing activity. Alternatively, including positive control samples from animals both pre- and post-AAV administration, as shown in Figure 4B can provide a clear indication of the positive titer range for neutralizing activity. Recommendations regarding the establishment of cut-point thresholds as well as validation and optimization of *in vitro* neutralizing assays have been described extensively in the literature[41-44].

Limitations: The use of the microscope camera to image wells is useful in that it provides very high-quality images that can accurately differentiate the degree of neutralization. However, microscopy can be labour intensive and may not be practical if processing a very large number of samples. The use of a high-resolution flatbed or plate scanner may provide a more rapid approach to imaging the wells if the quality and lighting of images can be maintained. High MOI's have been reported to reduce assay detection sensitivity and it has been suggested that high AAV doses may be able to evade neutralizing activity, or that AAV transduction may occur in the presence of NAbs[45-47]. This assay uses a moderately high MOI (15000), however, the sensitivity does not appear to be impeded as it has the capacity to detect neutralizing activity at very high dilutions (>1:32,000) (Figure 4B). Lastly, as this assay uses a viral vector, appropriate approval by a governing body is generally required to use recombinant AAV, this will differ from country to country.

In summary, this *in vitro* assay provides a rapid, cost effective, easily accessible, and simple method to detect the presence of neutralizing antibodies against rAAV6. This assay has the capacity to be adjusted and optimized to perform with different AAV serotypes with relative ease. The AAV6-hPLAP vector can be provided for the purpose of this assay upon request.

## Acknowledgments

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## Disclosures

The authors have nothing to disclose.

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Figure 1

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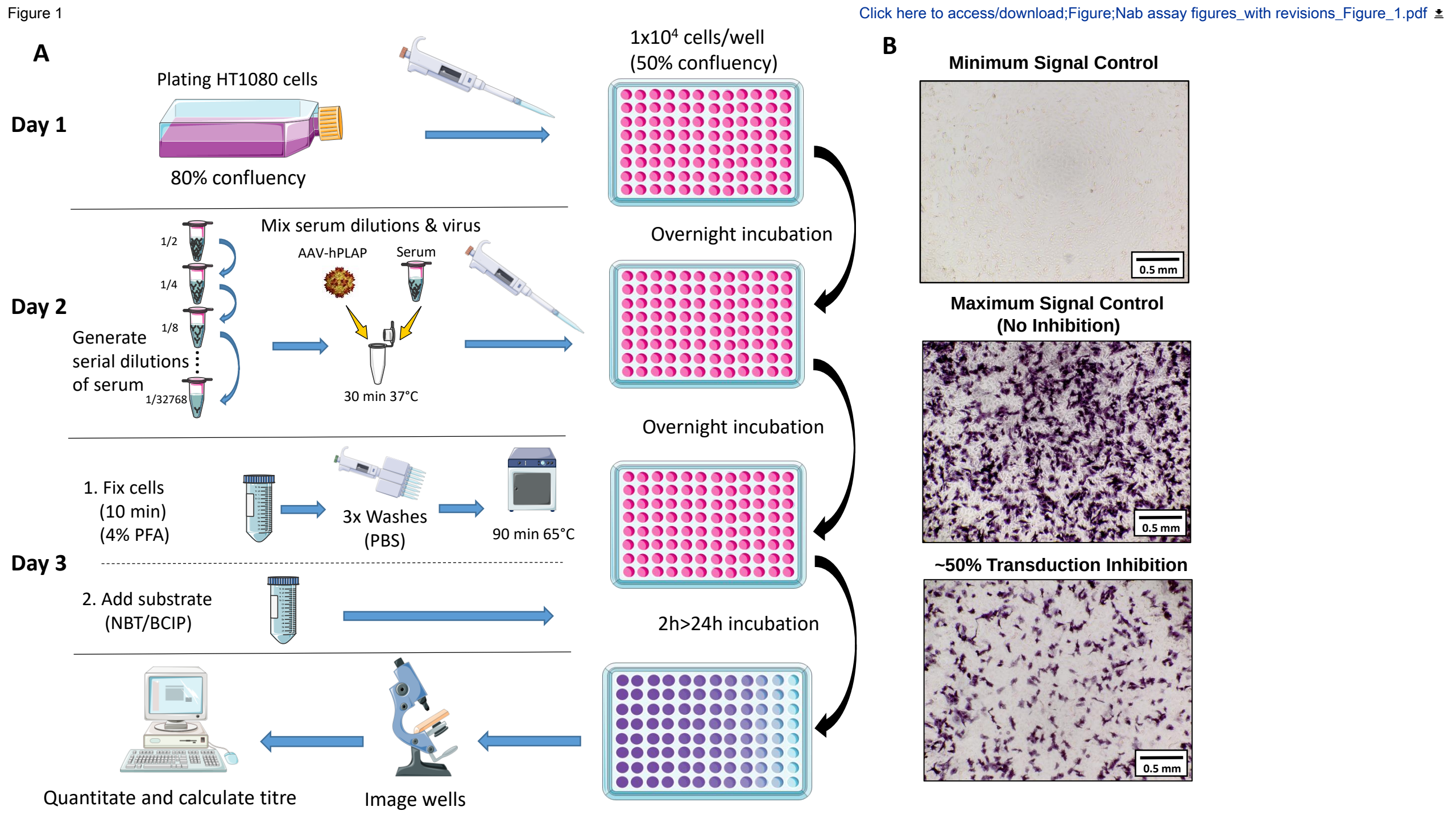

Figure 2

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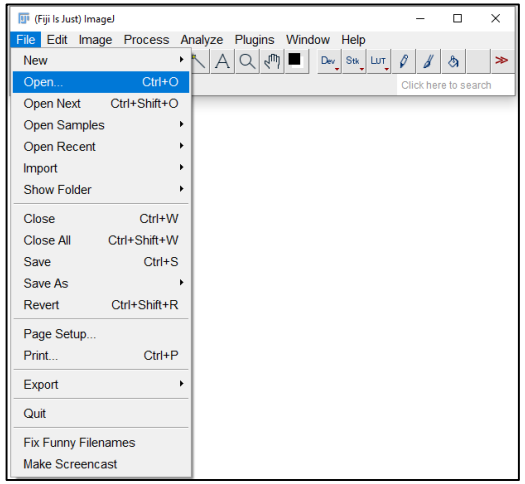


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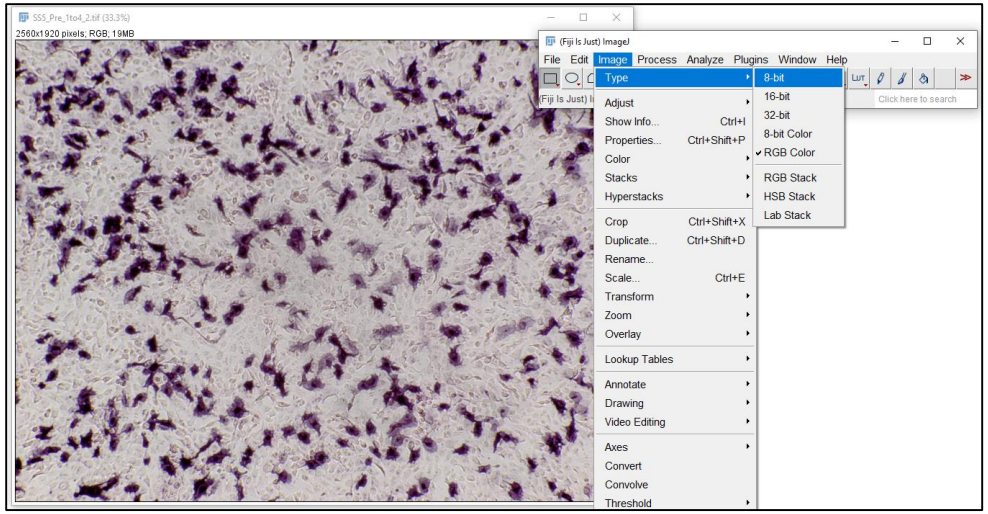


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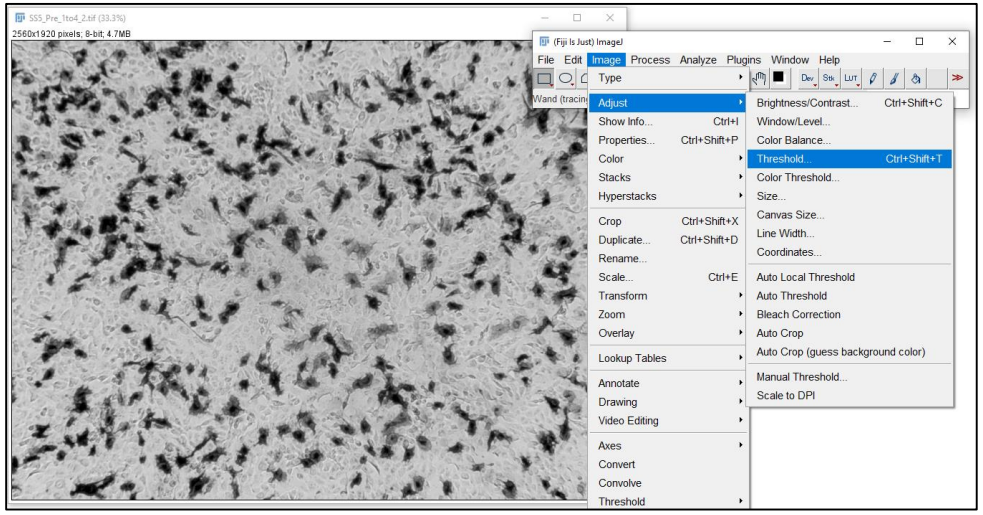


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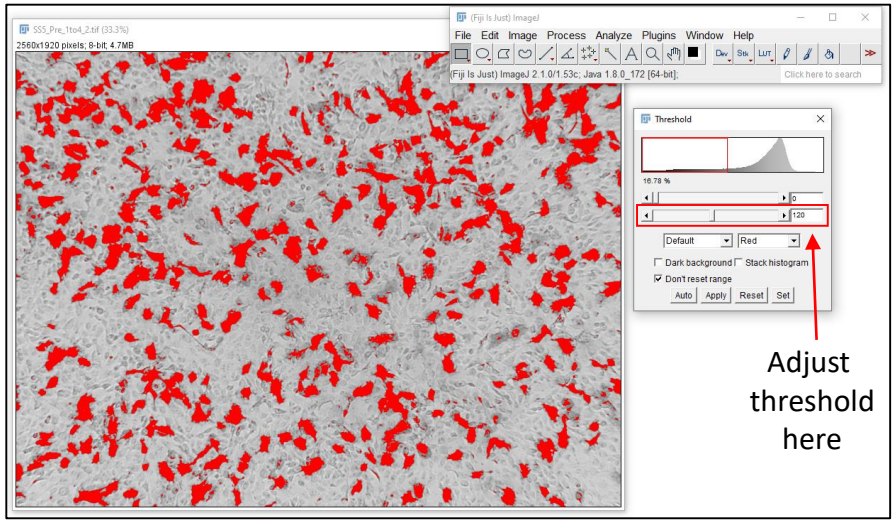


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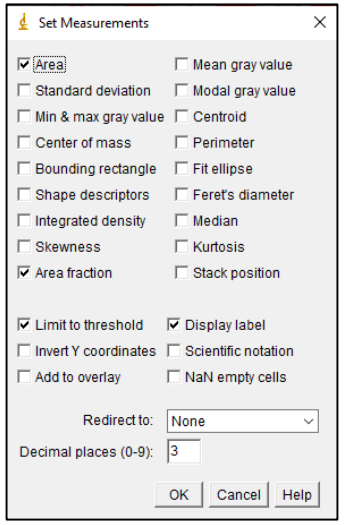


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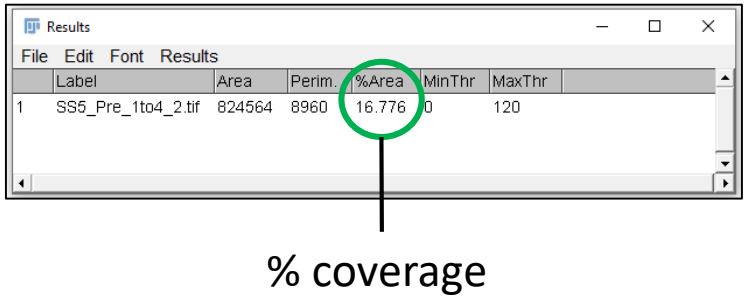


% coverage

Figure 3

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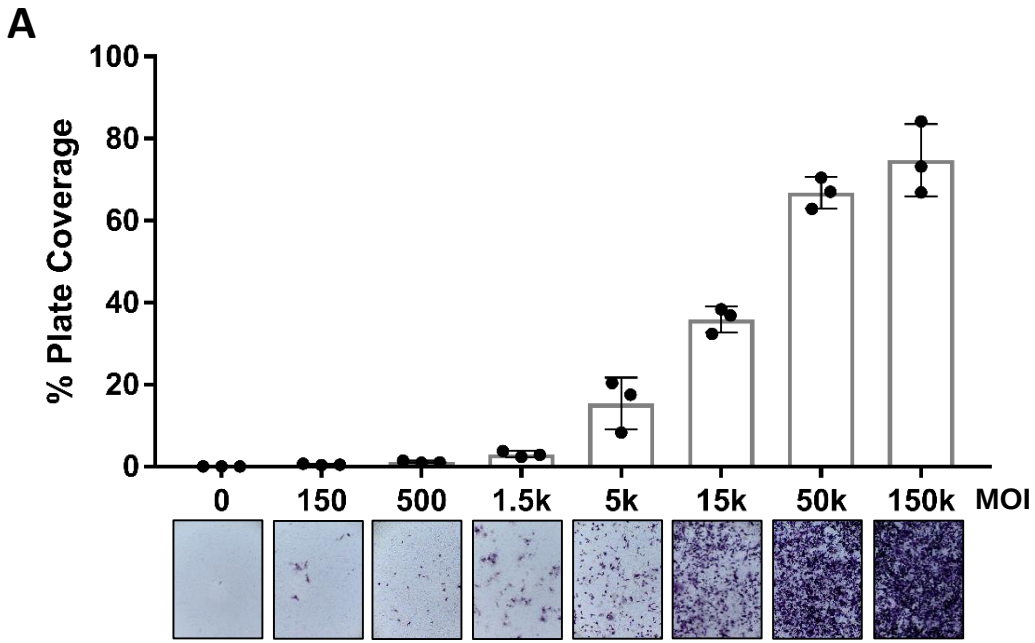
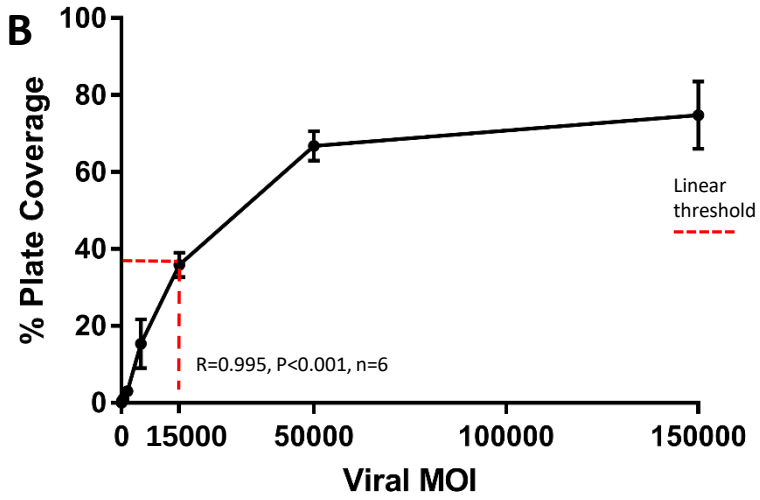



| MOI | Plate coverage (% of plate area) |
|---|---|
| 0 | 0.01 |
| 150 | 0.4 |
| 500 | 1.0 |
| 1500 | 3.0 |
| 5000 | 15.4 |
| 15000 | 35.9 |
| 50000 | 66.8 |
| 150000 | 74.8 |



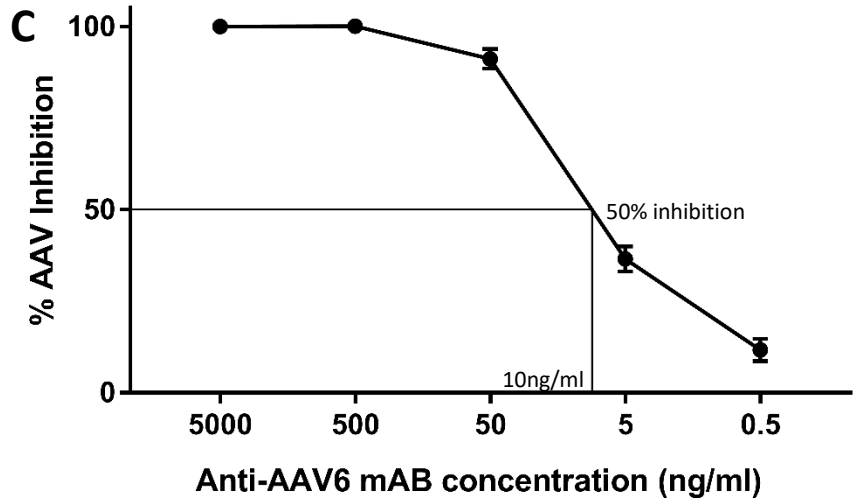



Figure 4

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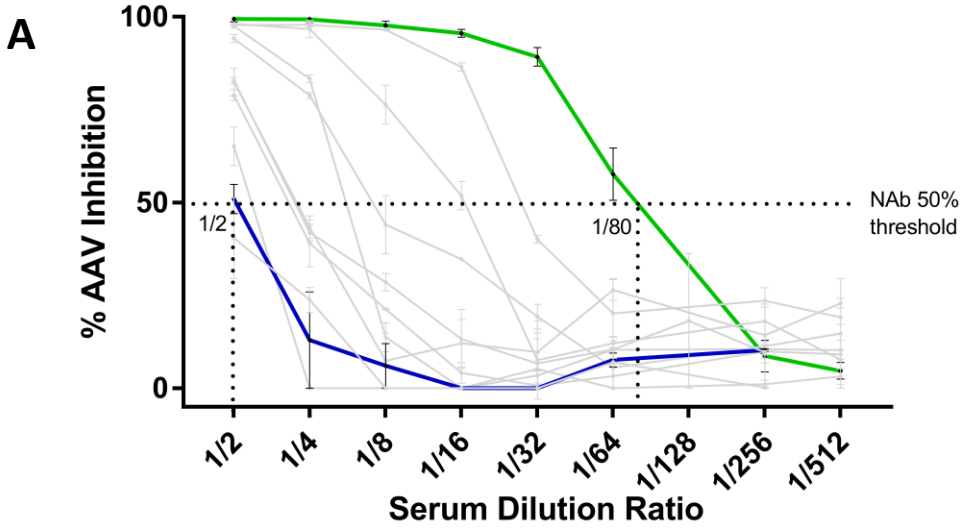


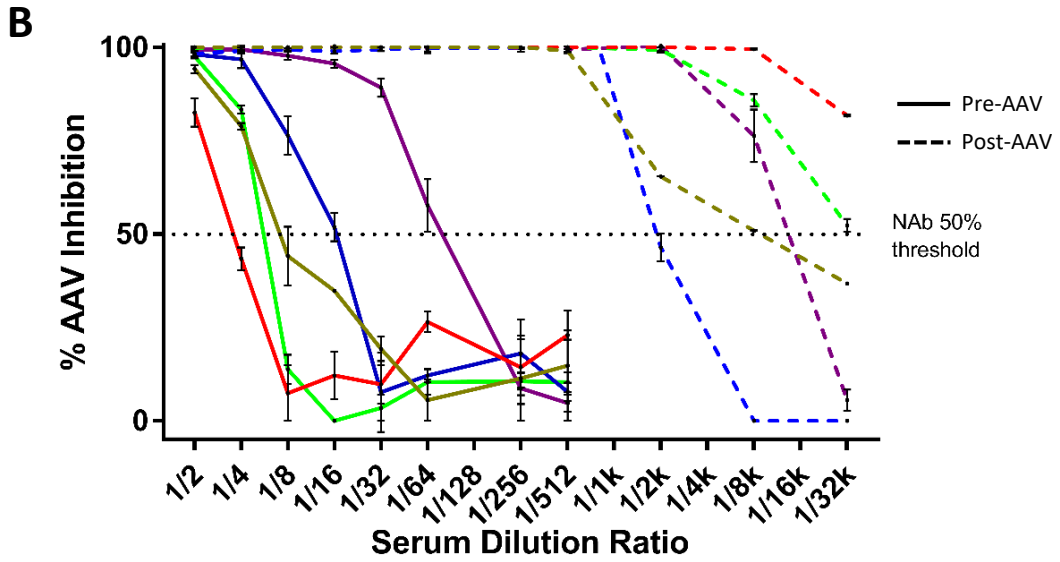

Table 1

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| Dilution cascade label | Dilution | 3 x sample (240 µL) + 10% buffer volume (24 µL) | Ratio of serum:media |
|---|---|---|---|
| Dilution 1 (D1) | 1/2 | 264 µL serum 264 µL media | 50:50 |
| Dilution 2 (D2) | 1/4 | 264 µL D1 + 264 µL media | 25:75 |
| Dilution 3 (D3) | 1/8 | 264 µL D2 +264µL media | 12.5:87.5 |
| Dilution 4 (D4) | 1/16 | 264 µL D3 +264 µL media | 6.25:93.75 |
| Dilution 5 (D5) | 1/32 | 264 µL D4 +264 µL media | 3.13:96.87 |
| Dilution 6 (D6) | 1/64 | 264 µL D5 +264 µL media | 1.56:98.44 |
| Dilution 7 (D7) | 1/128 | 264 µL D5 +264 µL media | 0.78:99.22 |
| Dilution 8 (D8) | 1/256 | 264 µL D5 +264 µL media | 0.39:99.61 |
| Dilution 9 (D9) | 1/512 | 264 µL D7 + 264 µL media | 0.2:99.8 |
| Dilution 10 (D10) | 1/2048 | 132 µL D8 + 396 µL media | 0.05:99.95 |
| Dilution 11 (D11) | 1/8192 | 132 µL D9 + 396 µL media | 0.01:99.99 |
| Dilution 12 (D12) | 1/32768 | 132 µL D10 + 396 µL media | 0.003:99.997 |

Table 2

Click here to access/download;Table;Table 2_Nab_assay_26_9_21.xlsx

| | Serum sample #1 | | | Serum sample #2 | | | Serum sample | |
|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| A | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/2 |
| B | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 |
| C | 1/8 | 1/8 | 1/8 | 1/8 | 1/8 | 1/8 | 1/8 | 1/8 |
| D | 1/16 | 1/16 | 1/16 | 1/16 | 1/16 | 1/16 | 1/16 | 1/16 |
| E | 1/32 | 1/32 | 1/32 | 1/32 | 1/32 | 1/32 | 1/32 | 1/32 |
| F | 1/64 | 1/64 | 1/64 | 1/64 | 1/64 | 1/64 | 1/64 | 1/64 |
| G | 1/256 | 1/256 | 1/256 | 1/256 | 1/256 | 1/256 | 1/256 | 1/256 |
| H | 1/512 | 1/512 | 1/512 | 1/512 | 1/512 | 1/512 | 1/512 | 1/512 |

| #3 | Mono AB (mAB), controls and extra samples | | |
|---|---|---|---|
| **9** | **10** | **11** | **12** |
| 1/2 | 50 ng MAb | 50 ng MAb | 50 ng MAb |
| 1/4 | 5 ng MAb | 5 ng MAb | 5 ng MAb |
| 1/8 | 0.5 ng MAb | 0.5 ng MAb | 0.5 ng MAb |
| 1/16 | MO (-C) | MO (-C) | MO (-C) |
| 1/32 | VO (+C) | VO (+C) | VO (+C) |
| 1/64 | Sample #1 1/512 | Sample #1 1/512 | Sample #1 1/512 |
| 1/256 | Sample #2 1/512 | Sample #2 1/512 | Sample #2 1/512 |
| 1/512 | Sample #3 1/512 | Sample #3 1/512 | Sample #3 1/512 |

Table 3

Click here to access/download;Table;Table 3_Nab_assay_26_9_21.xlsx

| Sheep ID | Administration status | Dose received (vg) | NAb Titer ($TI_{50}$) | AAV neutralized / µl serum | Fold change Pre vs Post |
|---|---|---|---|---|---|
| Sheep 1 | Pre-AAV | $1x10^{13}$ | 1/5 | $5.6x10^{6}$ | 6400 |
| | Post –AAV | | 1/32000 | $3x10^{10}$ | |
| Sheep 2 | Pre-AAV | $1x10^{13}$ | 1/80 | $7.5x10^{7}$ | 200 |
| | Post –AAV | | 1/16000 | $1.5X10^{10}$ | |
| Sheep 3 | Pre-AAV | $5x10^{12}$ | 1/16 | $1.5x10^{7}$ | 125 |
| | Post –AAV | | 1/2000 | $1.9x10^{9}$ | |
| Sheep 4 | Pre-AAV | $2x10^{13}$ | 1/4 | $3.8x10^{6}$ | >10000 |
| | Post –AAV | | >1/32000 | $>3x10^{10}$ | |
| Sheep 5 | Pre-AAV | $3x10^{13}$ | 1/8 | $7.5x10^{6}$ | 1700 |
| | Post –AAV | | 1/12000 | $2.3x10^{10}$ | |

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**Table of Materials**
JoVE_Materials table_23_9_21.xls

Supplemental Figure 1

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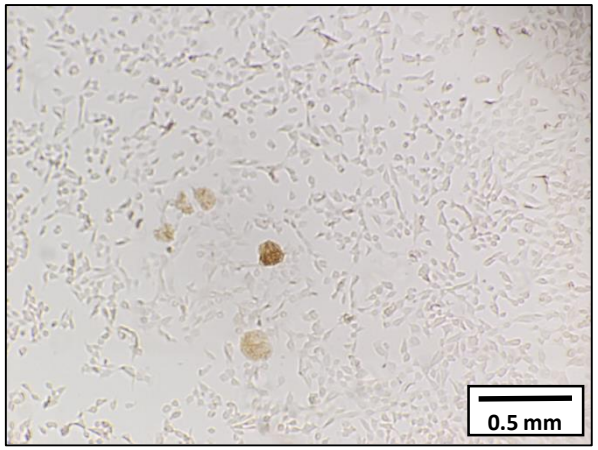
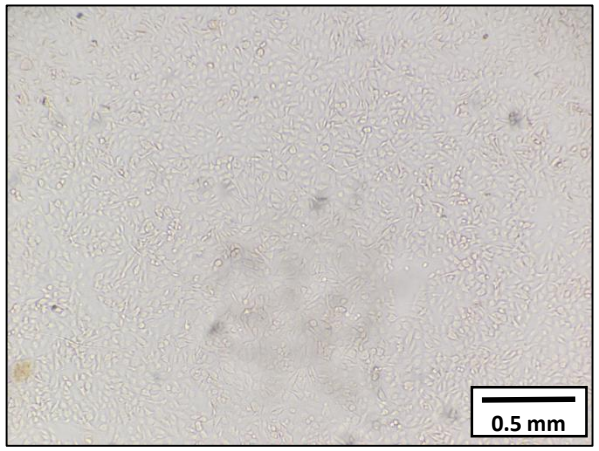
A



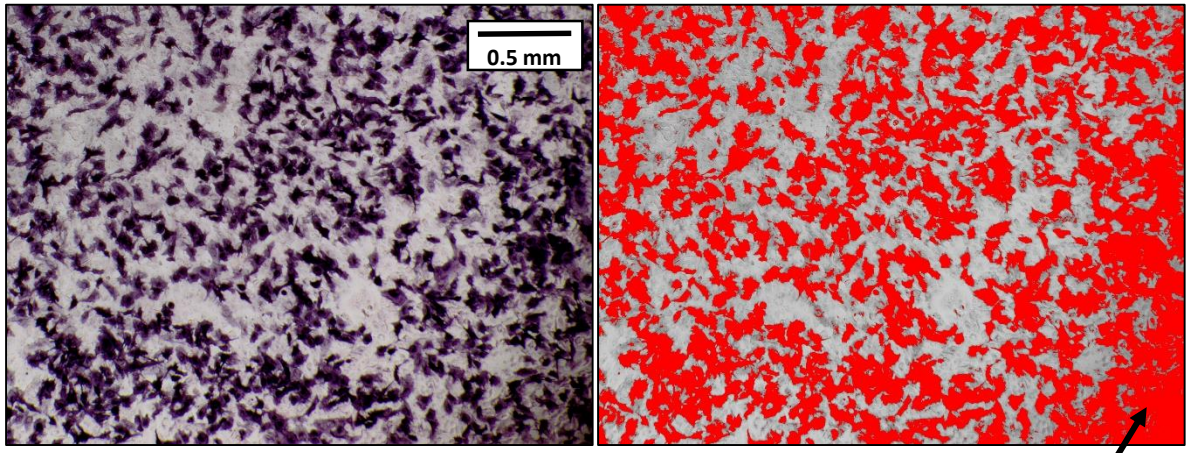

Contamination

B




High cell density

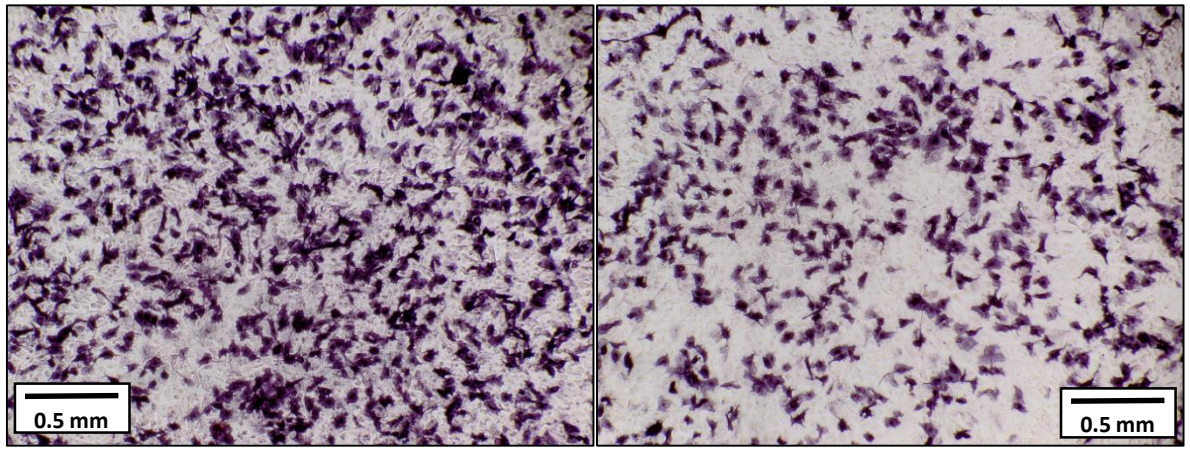
C




Lighting imbalance

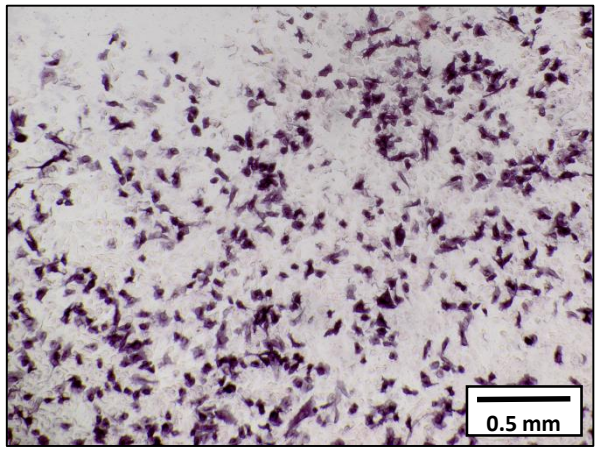
Oversaturated section

D




Technical replicates: Left Image: Normal, Right image: Low cell density

E




Uneven cell distribution

Supplemental Figure 2

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