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Corresponding Author:	Julie R. McMullen Baker Heart and Diabetes Institute AUSTRALIA
Corresponding Author's Institution:	Baker Heart and Diabetes Institute
Corresponding Author E-Mail:	julie.mcmullen@baker.edu.au
Order of Authors:	Sebastian Bass-Stringer
	Colleen Thomas
	Clive May
	Paul Gregorevic
	Kate Weeks
	Julie R. McMullen
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TITLE:

A Step-By-Step Method to Detect Neutralizing Antibodies Against AAV Using a Colorimetric Cell-Based Assay

AUTHORS AND AFFILIATIONS:

Sebastian Bass-Stringer[1,2], Colleen J. Thomas[2,3], Clive N. May[3], Paul Gregorevic[4]*, Kate L. Weeks[1,5,6]*, Julie R. McMullen[1,2,5,6,7]*

[1]Baker Heart and Diabetes Institute, Melbourne, Vic, Australia
[2]Department of Physiology, Anatomy and Microbiology, La Trobe University, Melbourne, Vic, Australia
[3]Florey Institute of Neuroscience and Mental Health, University of Melbourne, Melbourne, Vic, Australia
[4]Department of Physiology, Centre for Muscle Research (CMR), The University of Melbourne, Melbourne, Vic, Australia
[5]Department of Diabetes, Central Clinical School, Monash University, Melbourne, Vic, Australia
[6]Baker Department of Cardiometabolic Health, The University of Melbourne, Melbourne, Vic, Australia
[7]Department of Physiology and Department of Medicine Alfred Hospital, Monash University, Melbourne, Vic, Australia

Email addresses of the authors:

Sebastian Bass-Stringer	(sebastian.bass@baker.edu.au)
Colleen J. Thomas	(colleen.thomas@latrobe.edu.au)
Clive N. May	(clive.may@unimelb.edu.au)
Paul Gregorevic	(pgre@unimelb.edu.au)
Kate L. Weeks	(kate.weeks@baker.edu.au)
Julie R. McMullen	(julie.mcmullen@baker.edu.au)

*Email addresses of the corresponding authors:

Julie R. McMullen	(julie.mcmullen@baker.edu.au)
Kate L. Weeks	(kate.weeks@baker.edu.au)
Paul Gregorevic	(pgre@unimelb.edu.au)

SUMMARY:

A comprehensive laboratory protocol and analysis workflow are described for a rapid, cost-effective, and straightforward colorimetric cell-based assay to detect neutralizing elements against AAV6.

ABSTRACT:

Recombinant adeno-associated viruses (rAAV) have proven to be a safe and successful vector for transferring genetic material to treat various health conditions in both the laboratory and the clinic. However, pre-existing neutralizing antibodies (NAbs) against AAV capsids pose 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 generates an insoluble quantifiable purple stain upon combination.

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, corresponding to viral transduction and neutralizing activity. The proportion of area colored is quantitated using a free software tool to generate neutralizing titers. This assay displays a strong positive correlation between coloration and viral concentration. Assessment of serum samples from sheep before and after 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 various 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 the release of the first commercially available AAV gene therapy drug approved by the US Food and Drug Administration[8]. Following initial successes, AAV has continued to gain traction in the basic and clinical research sectors as a vector of choice and is 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 reporter gene expression 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 concerning *in vitro* assays[14,17].

To date, *in vitro* NAb assays have mainly used luminescence (luciferase) as the reporter gene to detect neutralization. Although a light-based method has merit in many contexts, a colorimetric/chromogenic NAb assay may be 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 require 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 versus luminescent assays has yielded conflicting results. One study suggested luminescence-based ELISA assays display greater sensitivity and comparable reproducibility to colorimetric assays[22], while another 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 an hPLAP (human placental alkaline phosphatase) reporter gene (AAV6-hPLAP) to detect neutralizing activity against AAV[24]. This assay is cost-effective, time-efficient, easy to set up, and requires minimal technical skills, laboratory equipment, and reagents. Moreover, the simplicity of this assay gives it the potential to be optimized for broad applications across different types of cells, tissues, or viral serotypes.

PROTOCOL:

All aspects of animal care and experimentation were conducted following Florey Institute of Neuroscience and Mental Health guidelines and the Australian Code for the Care and Use of Animals for Scientific Purposes following Reference[25]. 1.5-3-year-old Merino ewes were used for the study. A schematic overview of the assay protocol is provided in **Figure 1**.

1. Initial preparation

1.1. For assessment in sheep: collect blood in 8 mL serum separator clot activator tubes (see **Table of Materials**), leave the blood sample at room temperature (RT) for 20-30 min, and subsequently spin down at 2,100 x *g* for 15 min. The clear supernatant that forms at the top of the tubes is serum.

1.1.1. Aliquot the clear aqueous phase into microcentrifuge tubes and store at -80 °C.

NOTE: The serum at -80 °C remains stable for ~5 years. Blood was collected from the carotid vein using a 16 G needle (tip cut-off) and syringe from conscious animals.

1.2. Heat inactivate fetal bovine serum (FBS) by placing it in a water bath at 56 °C for 30 min and swirl 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 performed in the cell culture hood[26,27]. Spray 70% ethanol on all objects and the hood before use and clean with 1% sodium hypochlorite upon completion.

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

1.5. Establish HT1080 cells (see **Table of Materials**) and passage in a 75 cm^2 square flask as described in Reference[28]. 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 of cells

2.1. Passage HT1080 cells when they reach ~80% confluency.

2.2. Pre-warm complete DMEM (prepared in step 1.4), 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.

2.3. Wash the cells in 10 mL of pre-warmed (37 °C) 1x PBS and trypsinize cells for 3-4 min in 4 mL of pre-warmed 0.05% trypsin-EDTA to detach the cells from the flask.

2.4. 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 hemocytometer and the trypan blue exclusion method[29].

2.5. Dilute the cells to a concentration of 1 x 10^5 cells/mL in pre-warmed complete DMEM. Seed 100 µL of cells/well into clear 96-well flat-bottomed plates (1 x 10^4 cells per well). Incubate the plate at 37 °C, 5% carbon dioxide (CO_2) overnight for 16-22 h.

3. Day 2 – Infecting the cells

3.1. Remove plate/s from the incubator and use a light microscope to confirm that cells are evenly dispersed within the wells and that the confluency is ~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 the generation of a dilution cascade for triplicate samples.

3.2.1. To perform the assay in triplicate, prepare a 7.5 x 10^6 vector genomes (vg)/µL of working solution of AAV6-hPLAP (see **Table of Materials**) by diluting a virus stock solution in 1x PBS.

3.2.2. Add 66 µL of the 7.5 x 10^6 vg/µL of virus working solution to each tube containing 264 µL of serum/media dilution (330 µL of total volume/dilution, see **Table 1**).

NOTE: This is a robust assay that does not require perfect culture conditions. However, to accurately quantitate and ensure each assay run is reliable, it is necessary to include the following: (1) a no serum control, (2) a no serum or virus control, and (3) 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 and virus mixture. Performing replicates is highly recommended for the accurate determination of neutralizing activity.

3.3. Mix the virus/serum dilutions by pipetting and place the tubes containing the virus/serum mixtures in an incubator at 37 °C, 5% CO_2 for 30 min 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 1 x 10^4 cells/well for each dilution.

NOTE: 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 h to allow AAV entry into the cells.

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

4.1. Pre-warm an aliquot of 1x 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 (see **Table of Materials**) in 10 mL of DDW in a 50 mL conical centrifuge tube by vortexing (10 mL is enough for 2 x 96 well plates).

4.2. 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.

4.2.1. Add 50 µL of 4% PFA to each well using a pipette. Wrap the plate in foil and leave it at RT for 10 min to fix the cells.

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.

4.3. Wash and aspirate the cells with 200 µL of RT 1x PBS. Repeat this step twice.

NOTE: A multichannel pipette is an efficient option for the pipetting steps.

4.4. Pipette 200 µL of pre-warmed PBS into each well, wrap the plate in foil and incubate at 65 °C for 90 min to denature endogenous alkaline phosphatase activity[30].

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

4.6. Pipette 50 µL of the dissolved BCIP/NBT (prepared in step 4.1) into each well.

4.7. Wrap the plate in foil and incubate at RT for 2-24 h.

NOTE: Be consistent with incubation time between runs; the time flexibility allows users to photograph wells either on day 3 or the following day.

4.8. 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.

4.8.1. Position each well identically and ensure the edges of the well are not visible in the photos. Save photos in 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 the neutralizing activity using ImageJ

5.1. Download and install the freely available software "ImageJ" (see **Table of Materials**).

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

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

5.4. Click on **Image** > **Adjust** > **Threshold**. Adjust the threshold until all colored areas are colored in red, but the background is not. Upon adding NBT/BCIP, the 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 on **Analyze** > **Set Measurements** and tick the **Area**, **Limit to Threshold**, **Area Fraction,** and **Display label** checkboxes and click on **Ok**.

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

5.7. Perform quantitation for all sample replicates. Exclude any contaminated wells, wells showing uneven cell distribution, or wells varying in cell density or lighting.

NOTE: 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 step 5) for the following: (1) Media-only control (baseline signal reading). (2) Virus + media only control (maximum signal reading). (3) 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 the 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[9].

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 plated onto 1 x 10^4 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 1 x 10^4 HT1080 cells/well provided ~50% cell confluency in each well of a 96-well plate. To determine the optimal viral concentration for the assay, an rAAV encoding an hPLAP (human placental alkaline phosphatase) reporter gene (AAV6-hPLAP)[31] 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 (1.5 x 10^8 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 1,50,00 (n = 6, r = 0.995, P<0.001). This concentration adequately displayed the reporter gene signal above the background in the presence of high concentrations of NAbs (low plate coloration) while 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 trialed 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 (step 6). Assessing log_{10} dilutions, the anti-AAV6 mAb displayed a TI_{50} titer of ~10 ng/mL (1 ng total mAb), while 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 G 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 1.88×10^6 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, 1.88×10^6 to 7.5×10^7 neutralized AAV particles/µL of serum) (**Figure 4A**).

Subsequently, direct cardiac injection (n = 5) of AAV6 was performed at doses ranging between 5×10^{12} and 3×10^{13} vg to naïve sheep. Briefly, sheep were anesthetized as previously described[32]. The heart was exposed from the left lateral position. The pericardium was opened, and AAV was administered *via* 10-40 ~20 µL 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 the anesthetic was removed. Serum was collected from all animals before and six to eight weeks after AAV administration from the pre-cannulated right jugular vein using a 16 G needle cut-off and syringe (~5 mL 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 before AAV administration ranged from 1/4 to 1/80 (3.75×10^6 to 7.5×10^7 neutralized AAV particles/µL of 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 (5 x1 0^{12} vg) displayed a 1/2048 TI_{50} titer (dashed blue line, 1.92×10^9 neutralized AAV particles/µL serum) and the remainder of the doses (1-3 x 10^{13} vg) displayed TI_{50} titers ranging from 1/12000 to >1/32768 (1.13 x1 0^{10} to >3 x 10^{10} neutralized AAV particles/µL of serum).

FIGURES AND TABLE LEGENDS:

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 the 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 the background area is not (this threshold should be consistent across an entire plate). (**E**) Select the 'Analyze' dropbox, click on 'Set measurements' and tick 'Area', 'Area fraction', 'Limit threshold' and 'Display label', and click on 'OK'. (**F**) Click on '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 and assessment of assay efficacy against an anti-AAV6 monoclonal antibody (mAB). (**A**) The 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 shown 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 is 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 ~10 ng/mL concentration. n = 3 replicates per dilution. Data represent mean ± SEM.

Figure 4: Example of neutralizing antibody (Nab) assay results using ovine serum samples. (**A**) Adeno-Associated Virus (AAV) neutralizing serum samples were collected from 11 naïve sheep, measured in 2-fold serial dilutions ranging from 1/2 to 1/512. Colored lines represent samples with low and high neutralizing activity; grey lines represent the nine additional 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 was 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.

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 was assessed before and after receiving a 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.

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

DISCUSSION:

This report describes a colorimetric assay that assesses the extent of AAV neutralization in a given serum sample by evaluating a chromogenic reaction corresponding 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[24,33-35]. Its merit stems from its time and cost-effectiveness, accessibility, ease of setting up and performing while 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[34] (**Supplementary Figure 2**). NBT/BCIP is an hPLAP substrate that is initially dephosphorylated by alkaline phosphatase and sequentially undergoes oxidization to form a dimer, resulting in an insoluble product that is a vibrant purple color[36].

In selecting the optimal MOI for this assay, the aim was to establish a viral concentration that would sufficiently express 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 15,000 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 1,50,000 MOI) caused the concentration-color response curve to plateau, indicating color saturation (**Figure 3B**). Assessment of viral MOIs between 0 and 15,000 (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 a major contributing factor in the overall titer observed for a given serum sample. If an MOI of 5,000 instead of 15,000 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 preclinical and clinical trials have implemented AAV NAb assays with MOI values ranging from less than 1,000 to as high 25,000[37], 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 more comparable values across different studies[9,38]. Numerous other factors may contribute to variation in titers between studies, such as the choice of the 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 essential 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 that is 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 datasheet, 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 1-24 h have been used[30,39]. For this assay, incubation times must be consistent between runs. The time flexibility allows users to photograph wells either on day 3 of the protocol 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[1,40,41]. Historically, most 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 harbor neutralizing antibodies against AAV6 as well as many other AAV serotypes [42,43]. This highlights the importance of preliminary screening for NAbs before trials in both large animal models and humans. NAb status of serum samples from sheep that had no previously known exposure to AAV was assessed, of which 10 of 11 displayed TI_{50} titers <1/30 (<3 x 10^7 neutralized AAV particles/µL of serum). In contrast, one displayed a TI_{50} titer of 1/80 (7.5 x 10^7 neutralized AAV particles/µL of 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 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 (5 x 10^{12} vg) corresponded to the lowest post-AAV TI_{50} titer value (TI_{50} titer 1/2000) and fold change increase (125 fold). In comparison, no clear evidence of pre-existing NAbs within the 11 naïve sheep was observed at levels that would prevent AAV transduction (all TI_{50} titer values <1/100). Data from the 5 sheep that received a direct AAV injection 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. Establishing a cut-point at which a sample is deemed positive for neutralizing activity is an essential subsequent step when determining a threshold for NAb positive animals. This can be determined by assessing the variability in a group (~n ≥ 30) of naïve samples from a specific population of interest. This allows for establishing criteria to choose a statistically derived cut-point in which a sample is deemed positive for neutralizing activity. Alternatively, positive control samples from animals both pre- and post-AAV administration, as shown in **Figure 4B**, can indicate the positive titer range for neutralizing activity. Recommendations regarding establishing cut-point thresholds and

validation and optimization of *in vitro* neutralizing assays have been described extensively in the literature[44-47].

The technique involves certain limitations. The use of the microscope camera to image wells is helpful because it provides very high-quality images that can accurately differentiate the degree of neutralization. However, microscopy can be labor intensive and may not be practical if processing many (>100) samples. 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. It has been suggested that high AAV doses may evade neutralizing activity or that AAV transduction may occur in the presence of NAbs[48-50]. This assay uses a moderately high MOI (15,000); however, the sensitivity does not appear to be impeded as it can 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 can be adjusted and optimized to perform with different AAV serotypes with relative ease. The AAV6-hPLAP vector can be provided for this assay upon request.

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DISCLOSURES:

The authors have nothing to disclose.

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686

Figure 1

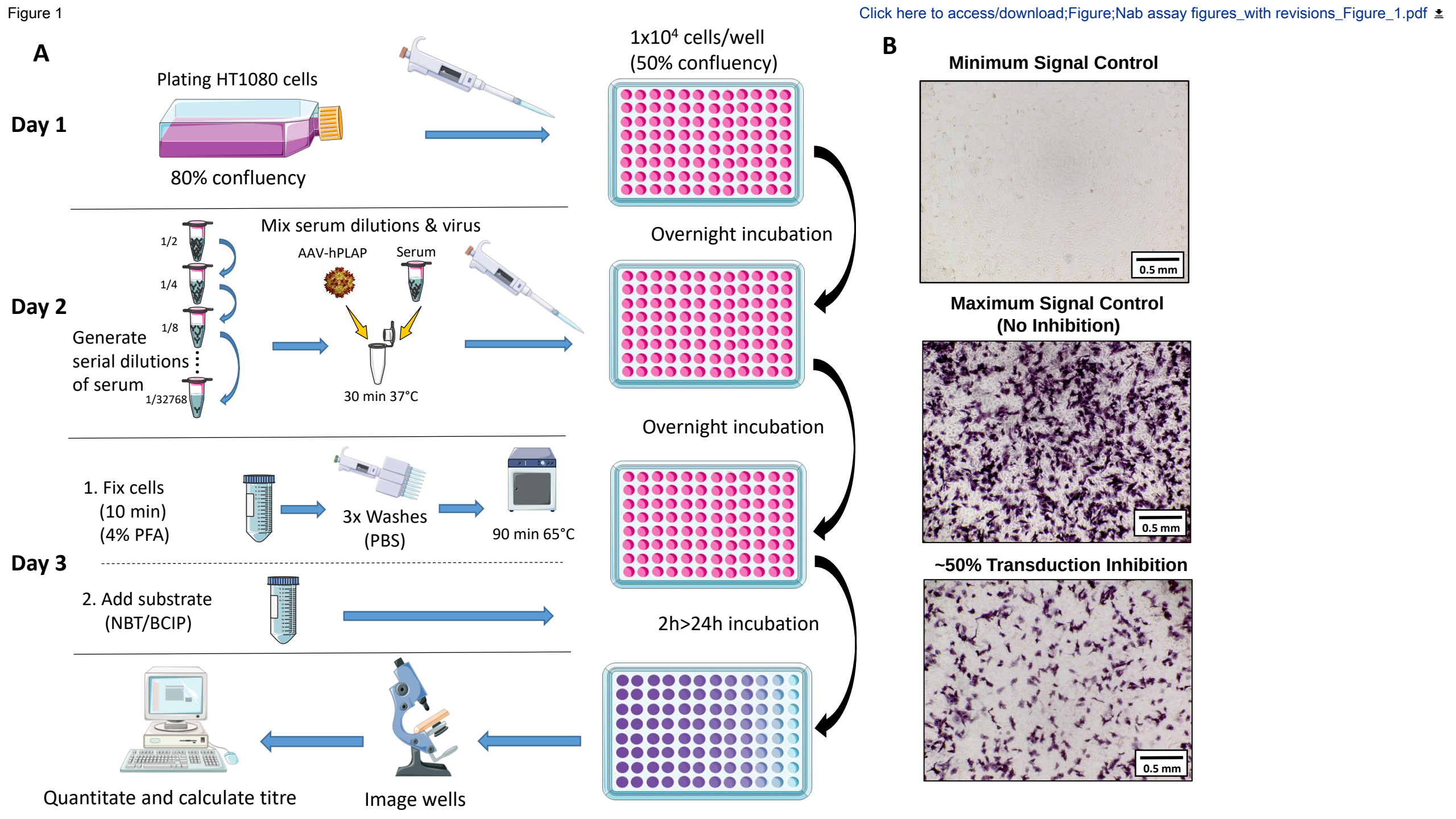

Figure 2

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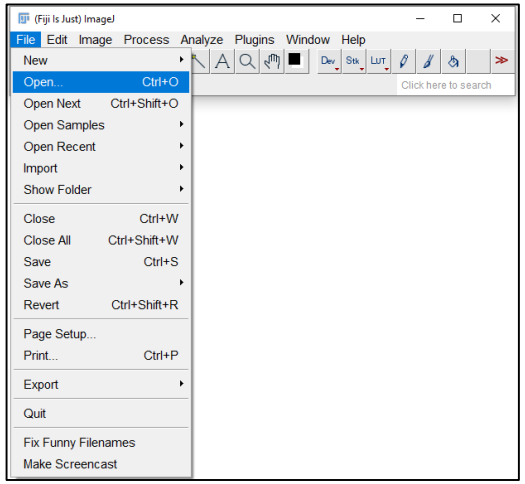
A



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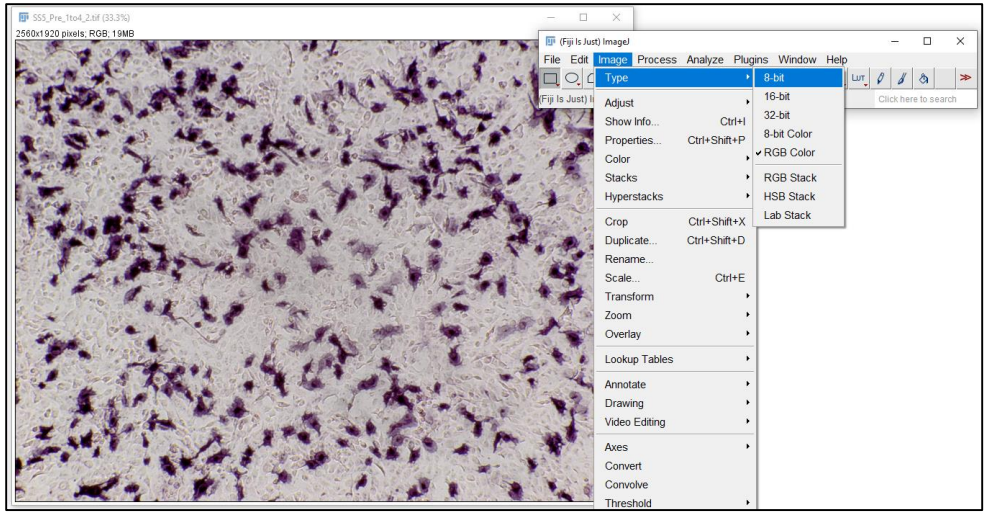

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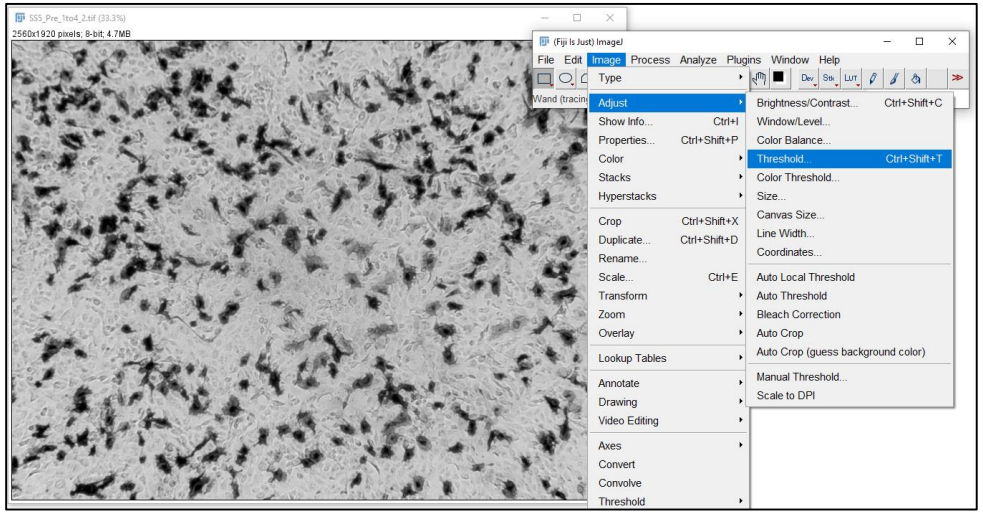

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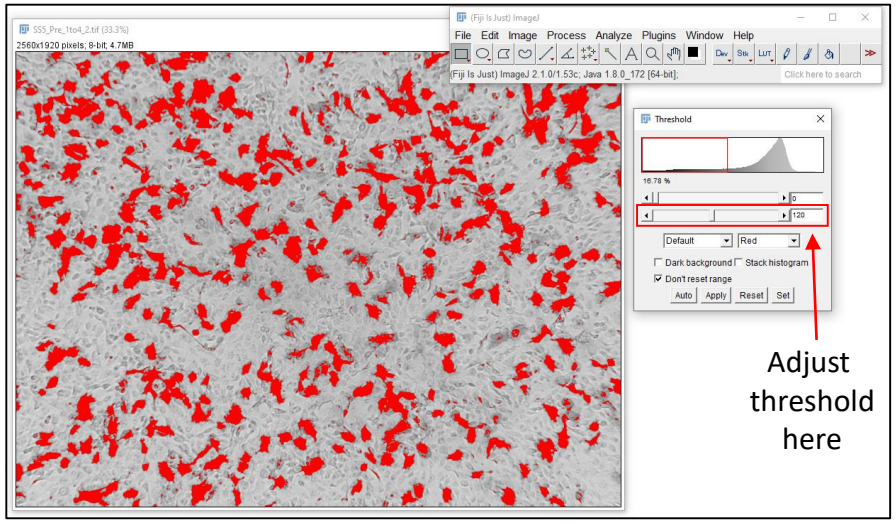

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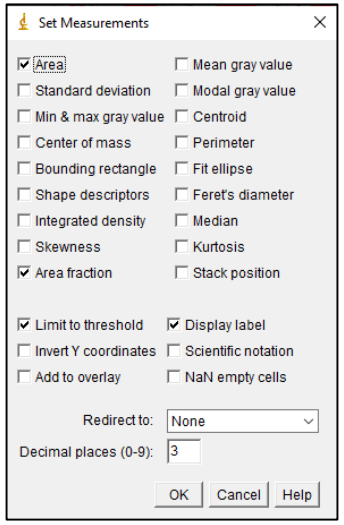

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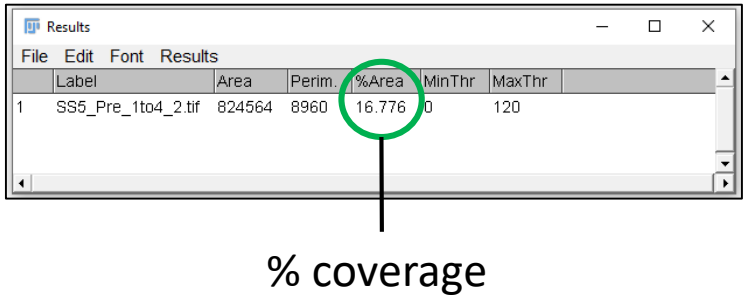
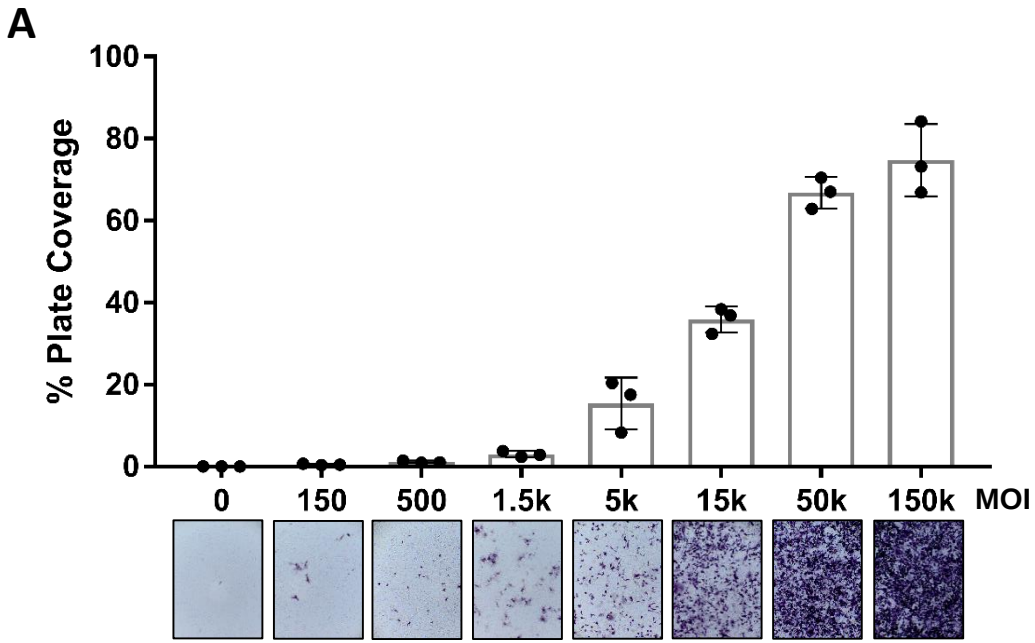
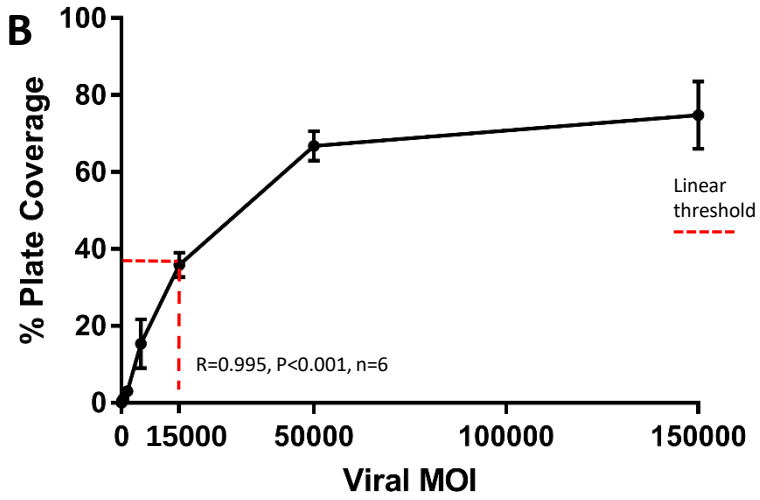


% coverage

Figure 3



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



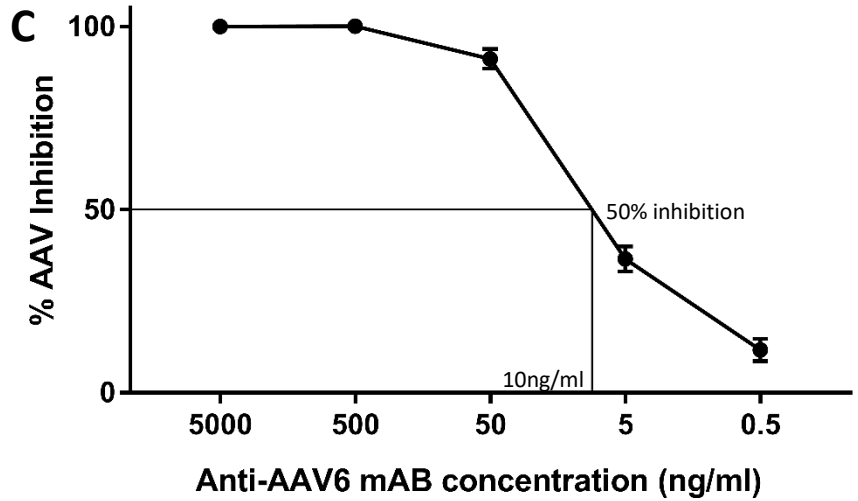
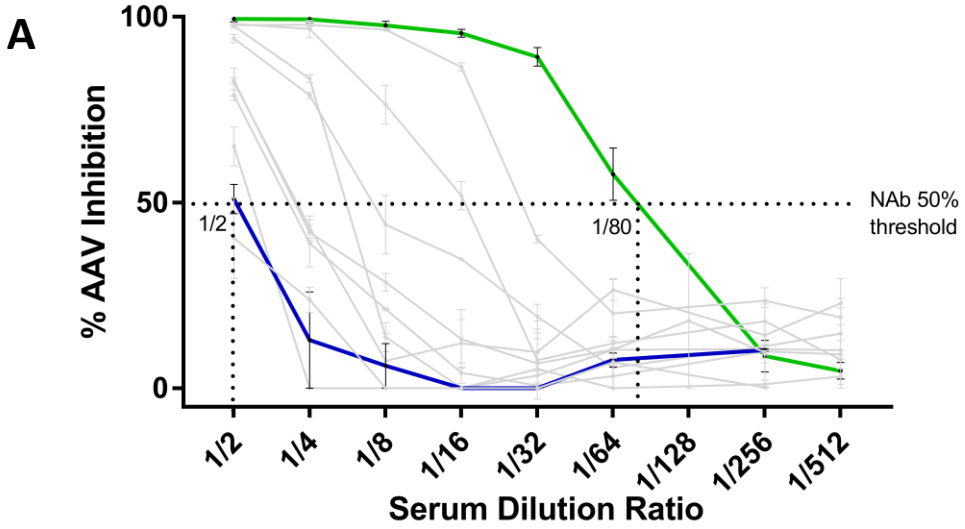


Figure 4



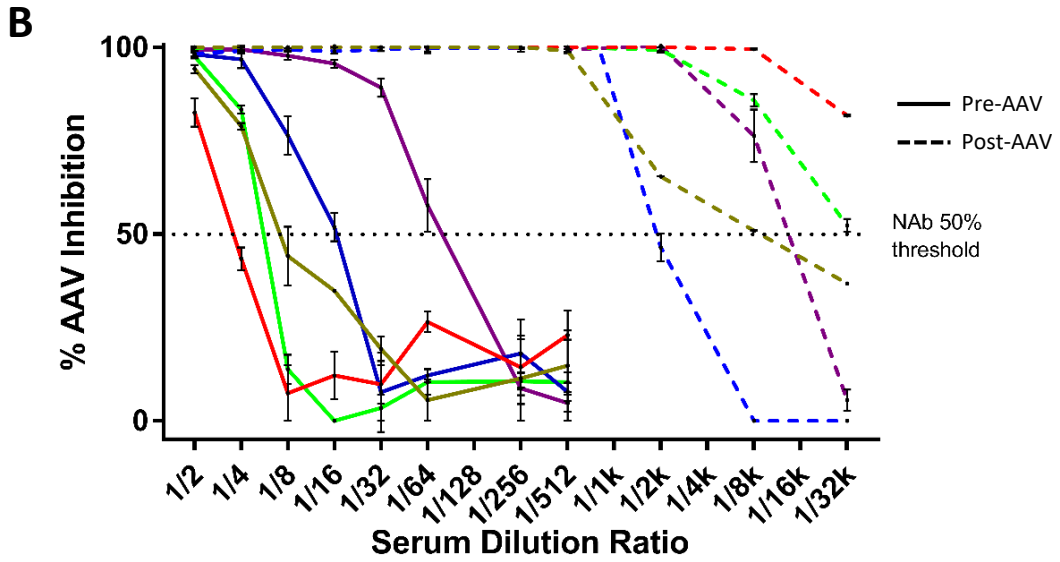


Table 1

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

	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

e #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;63419_Table 3.xlsx

Sheep ID	Administration status	Dose received (vg)	NAb Titer (TI_{50})
Sheep 1	Pre-AAV	$1x10^{13}$	1/5
	Post –AAV		1/32000
Sheep 2	Pre-AAV	$1x10^{13}$	1/80
	Post –AAV		1/16000
Sheep 3	Pre-AAV	$5x10^{12}$	1/16
	Post –AAV		1/2000
Sheep 4	Pre-AAV	$2x10^{13}$	1/4
	Post –AAV		>1/32000
Sheep 5	Pre-AAV	$3x10^{13}$	1/8
	Post –AAV		1/12000

AAV neutralized / µL serum	Fold change Pre *vs.* Post
$5.6x10^{6}$	6400
$3x10^{10}$	
$7.5x10^{7}$	200
$1.5X10^{10}$	
$1.5x10^{7}$	125
$1.9x10^{9}$	
$3.8x10^{6}$	>10000
$>3x10^{10}$	
$7.5x10^{6}$	1700
$2.3x10^{10}$	

Editorial comments:

Changes to be made by the Author(s):

Responses to the editorial comments are shown in red.

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5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Vacuette, Millipore, Agilent 141 Technologies –All trademark symbols and commercial products have been removed (except in the table of materials).
6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. –Text is updated in the protocol to be in the imperative tense, and otherwise are added as a note.
7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. –More detail has been added to the protocol steps, describing how each step is performed or referencing published material.
8. Please add more details to your protocol steps:

Step 1.1: Please include the age/sex/strain of the animals used. Please mention how the serum samples from the animals were collected. Age and sex of sheep and method for collecting serum has been added. A citation will also suffice. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm) RPM has been changed to (x g).

Step 1.4: Please mention the pore size and the material of the filtering unit. Pore size and filtering material has been included.

Step 3.3: Please mention what virus solution was used and what was the source of the solution. If obtained commercially, please include the details in the Table of Materials. –The concentration of the stock virus for our experiment has been added to the protocol (but this may differ from batch to batch). The source of the virus is in the table of materials and it is mentioned that it is available upon request in the protocol.

Step 4.2: How was the aspiration done? Was a pipette used? –Details regarding aspiration

have been added to the protocol.
Step 5: Please also ensure that the button clicks for the software steps are bolded throughout. –Button clicks have been bolded.
Step 5.1: Please provide the weblink for the source of ImageJ in the Table of materials. – The weblink for ImageJ is in the table of materials under the comments column.
Step 6.2/7.1: Please include a citation for the equation used. –Citation is included for equations used unless the equation is a standard formula.
9. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Steps to include have now been highlighted. This could be reduced if too lengthy.
10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight. –Steps are logical and include imperative text.
11. Please submit each Figure individually in a high-resolution format to your Editorial manager account. Also please ensure that all the Figures are in correct orientation. Figures submitted as individual PNG.
12. All Tables should be uploaded separately to your Editorial Manager account in a .xls or .xlsx file. –Uploaded tables as individual excel files.
13. Please include a paragraph on the limitations of the method in the Discussion section. –A limitations paragraph has been incorporated into the discussion.
14. Please do not abbreviate the journal names in the References. The reference list has been updated to include full journal name for all references.

Reviewers' comments:

Responses to reviewer's comments are shown in italics.

Reviewer #1:
Manuscript Summary:
manuscript contains useful protocol for detecting neutralizing anti-AAV6 activity in biological samples. I had not major comments.

We were pleased the reviewer thought our protocol was useful.

Major Concerns:
none

Minor Concerns:
As a relatively minor recommendation, consider clarifying what is meant by "colored area" when describing detection step. This can be done by stating that upon addition of substrate, colored product will deposit in the area around the cells expressing enzyme.

This additional information has been added to page 7.

- Also, in the abstract consider providing data supporting acceptable assay performance

We agree this is a good point and have added data from sheep pre- and post-AAV which supports acceptable assay performance into the abstract.

Reviewer #2:
Manuscript Summary:
The manuscript describe an interesting protocol for NAb levels assessment in vitro by using an AAV encoding an alkaline phosphatase (AP) reporter gene and its substrate NBT/BCIP. Determination of NAb has been a challenge for the field, and the lack of agreement between the centers regarding the multiples variables that can affect the current methods have made difficult to compare any available method or results. One of the major difficulties of the current methods is the need of specific instruments with high cost (no accessible for small or medium level centers). The protocol describe here relies in a free imaging software as ImageJ which clearly facilitates its implementation in any lab or center.

We were pleased that the reviewer recognised that the protocol described is accessible to more labs/centres than many current methods.

Major Concerns:
my major concern is the necessity of an almost 'perfect' culture scenario and readout to avoid any misreading interpretation.

We thank the reviewer for raising the point and appreciate the concern. While we think it is good practice to use optimal cultures we do not think a "perfect" culture scenario is essential given the significant shifts in the curves we identified in sheep pre- and post-AAV.

However, to avoid misinterpretation we have incorporated controls so that plates can be excluded if required e.g. if the positive and negative control did not work.

Firstly, a number of controls including: i) virus only, ii) a neutralizing antibody positive sample, and iii) a no virus sample is included on every plate, and serial dilutions are generated for every sample.

Secondly, the transduction inhibition readout is calculated using the virus only (maximum signal) and no virus samples (minimum signal) for every plate, accounting for between-plate variation.

Thirdly, all samples are run in duplicate or triplicates and the mean value for each is determined accounting for variability between wells on a given plate.

The text has been updated in section 3.2.

Minor Concerns:
none

Reviewer #3:
Manuscript Summary:
In this study the authors managed to establish a protocol to measure Nab titer against AAV6 carrying alkaline phosphatase (AP) as a reporter gene. The protocol based on applying a colorimetric assay which detects color changing after adding the substrate (NBT/BCIP). The authors carried on with an in vivo experiment to inject AAV6 in 11 sheep and measured pre/post injection Nab titer.

Major Concerns:
I have no major concerns.

We were pleased the reviewer had no major concerns.

Minor Concerns:
In this protocol serum samples collected from sheep were used as test samples. It is not clear what was the rational for measuring Nab against AAV6.

We have now added more information to provide rationale for using AAV6.

Additional text added to the introduction is highlighted below in blue and page 3.

"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.

In the introduction (or discussion) part, the authors may include more details regarding the technical advantages of colorimetric assay comparing to other assays, is it more sensitive, reliable? In addition, they may briefly compare their approach with similar approaches, e.g. PMID: 27910044.

We agree that further details are helpful for the reader, and we have inserted the reference recommended, as well as some additional references. The following text has been added to the Introduction (page 3).

"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]"

Please clarify that you collected blood from the sheep in 8 ml Vacuette tubes in step 1.1 of initial preparation(to obtain serum after centrifugation)?

This has been clarified in 1.1. However, to meet editorial requirements, it was not possible to include "Vacuette" because commercial names cannot be included. But this has been included within the table of materials.

At day 1 step 2.3 the authors didn't mention the plate's type they used (clear, white or black plate) as the same plate is used later for imaging.

This detail has now been provided.

At day 3 step 4.7: What does incubation time (2-24h) depend on? Is the time point the incubation is ended based on the color development? if yes, does the time also vary based on the antibodies concentration?

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. We have added more detail on page 12.

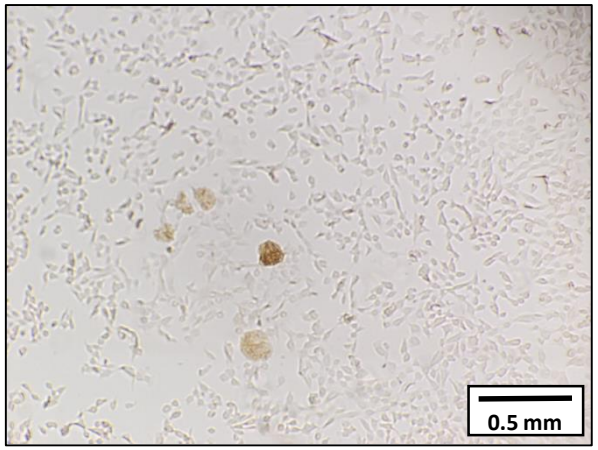
At day 3 step 4.8 the authors used an imaging method to quantify Nab titer. Did authors consider measuring color emission from cell lysate and then normalize it to the number of cells to avoid a cell overlay effect?

We agree this method may also work but was not one we considered or tested because we observed a very strong positive correlation between virus MOI and % colouration. We have not tested whether the reaction will be as consistent/effective in lysate verse adhered cells. We are not aware of similar colorimetric cell based assays quantifying results in this way but are happy to incorporate references and discussion if the reviewer is aware of such references.

Supplemental Figure 1

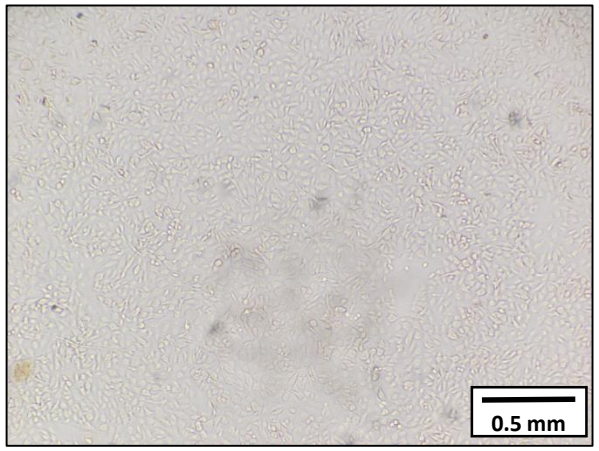
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Nab assay figures_with revisions_Supp_fig_1.pdf

A



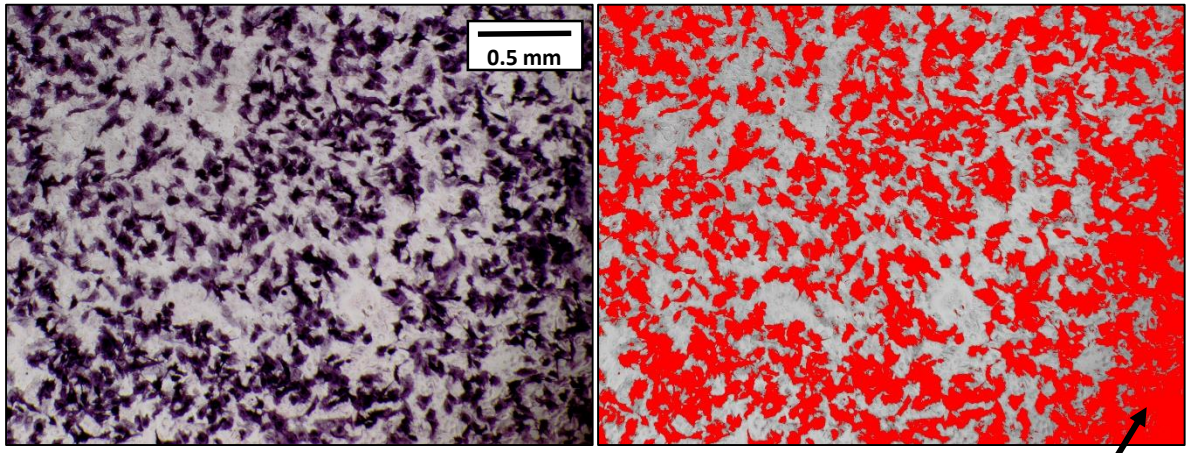
Contamination

B



High cell density

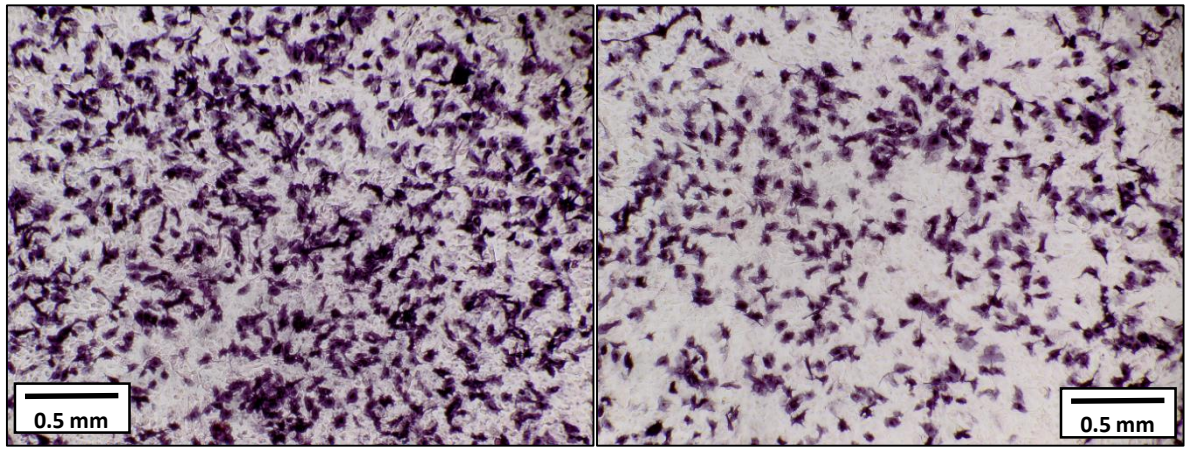
C



Lighting imbalance

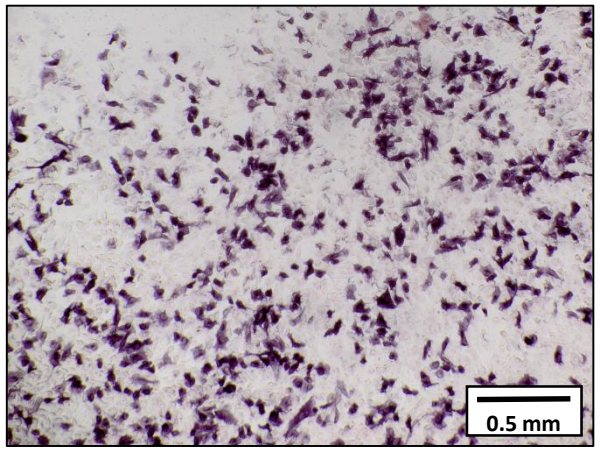
Oversaturated section

D



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

E



Uneven cell distribution

Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Nab assay figures_with revisions_Supp_fig_2.pdf