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Corresponding Author:	Cavit Agca Sabanci University: Sabanci Universitesi Istanbul, Outside USA TURKEY
Corresponding Author's Institution:	Sabanci University: Sabanci Universitesi
Corresponding Author E-Mail:	cavit.agca@gmail.com
Order of Authors:	Iskalen Cansu Topcu Okan
	Mehri Ahmadian
	Yesim Tutuncu
	Halit Yusuf Altay
	Cavit Agca
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TITLE:

Digital Droplet PCR Method for the Quantification of AAV Transduction Efficiency in Murine Retina

AUTHORS AND AFFILIATIONS:

Iskalen Cansu Topcu Okan[1,2], Mehri Ahmadian[1,2], Yesim Tutuncu[1,2], Halit Yusuf Altay[1,2] and Cavit Agca[1,2]

[1]Molecular Biology, Genetics and Bioengineering Program, Sabanci University, Istanbul, Turkey.
[2]Nanotechnology Research and Application Center (SUNUM), Sabanci University, Istanbul, Turkey.

Email address of Co-Authors
Iskalen Cansu Topcu Okan: ctopcu@sabanciuniv.edu
Mehri Ahmadian: mehriahmadian@sabanciuniv.edu
Yesim Tutuncu: yesimtutuncu@sabanciuniv.edu
Halit Yusuf Altay: hyusuf@sabanciuniv.edu

Email addresses of Corresponding Author:
Cavit Agca: cavit.agca@sabanciuniv.edu

SUMMARY:

This protocol presents how to quantify AAV transduction efficiency in mouse retina using digital droplet PCR (dd-PCR) together with small scale AAV production, intravitreal injection, retinal imaging, and retinal genomic DNA isolation.

ABSTRACT:

Many retinal cell biology laboratories now routinely use Adeno-associated viruses (AAVs) for gene editing and regulatory applications. The efficiency of AAV transduction is usually critical, which affects the overall experimental outcomes. One of the main determinants for transduction efficiency is the serotype or variant of the AAV vector. Currently, various artificial AAV serotypes and variants are available with different affinities to host cell surface receptors. For retinal gene therapy, this results in varying degrees of transduction efficiencies for different retinal cell types. In addition, the injection route and the quality of AAV production may also affect the retinal AAV transduction efficiencies. Therefore, it is essential to compare the efficiency of different variants, batches, and methodologies. The digital droplet PCR (dd-PCR) method quantifies the nucleic acids with high precision and allows performing absolute quantification of a given target without any standard or a reference. Using dd-PCR, it is also feasible to assess the transduction efficiencies of AAVs by absolute quantification of AAV genome copy numbers within an injected retina. Here, we provide a straightforward method to quantify the transduction rate of AAVs in retinal cells using dd-PCR. With minor modifications, this methodology can also be the basis for the copy number quantification of mitochondrial DNA as well as assessing the efficiency of base editing, critical for several retinal diseases and gene therapy applications.

INTRODUCTION:

Adeno associated viruses (AAVs) are now commonly used for a variety of retinal gene therapy studies. AAVs provide a safe and efficient way of gene delivery with less immunogenicity and fewer genome integrations. AAV entry into the target cell occurs through endocytosis, which requires binding of receptors and co-receptors on the cell surface[1,2]. Therefore, the transduction efficiency of AAVs for different cell types depends mainly on the capsid and its interactions with the host cell receptors. AAVs have serotypes and each serotype can have distinct cellular/tissue tropisms and transduction efficiencies. There are also artificial AAV serotypes and variants generated by chemical modification of the virus capsid, production of hybrid capsids, peptide insertion, capsid shuffling, directed evolution, and rational mutagenesis[3]. Even minor changes in amino acid sequence or capsid structure can have an influence on interactions with host cell factors and result in different tropisms[4]. In addition to capsid variants, other factors like injection route and batch-to-batch variation of AAV production can affect the transduction efficiency of AAVs in the neuronal retina. Therefore, reliable methods for the comparison of transduction rates for different variants are necessary.

The majority of the methods for determining AAV transduction efficiency rely on reporter gene expression. These include fluorescent imaging, immunohistochemistry, western blot, or histochemical analysis of the reporter gene product[5-7]. However, due to the size constraint of AAVs, it is not always feasible to include reporter genes to monitor the transduction efficiency. Using strong promoters like the hybrid CMV enhancer/chicken beta-actin or Woodchuck hepatitis post-transcriptional regulatory element (WPRE) as an mRNA stabilizer sequence further complicates the size problem[8]. Therefore, it will also be beneficial to define the transduction rate of injected AAVs with a more direct methodology.

Digital droplet PCR (dd-PCR) is a powerful technique to quantify target DNA from minute amounts of samples. dd-PCR technology depends on encapsulation of the target DNA and PCR reaction mixture by oil droplets. Each dd-PCR reaction contains thousands of droplets. Each droplet is processed and analyzed as an independent PCR reaction[9]. Analysis of droplets enables calculating the absolute copy number of target DNA molecules in any sample by simply using the Poisson algorithm. Since the transduction efficiency of the AAVs is correlated with the copy number of AAV genomes in the neuronal retina, we used the dd-PCR method to quantify AAV genomes.

Here, we describe a dd-PCR methodology to calculate the transduction efficiency of AAV vectors from retinal genomic DNA[6,10]. First, AAVs that express tdTomato reporter were generated using the small scale protocol, and titered by the dd-PCR method[11]. Secondly, AAVs were intravitreally injected into the neuronal retina. To demonstrate the transduction efficiency, we first quantified tdTomato expression using fluorescent microscopy and ImageJ software. This was followed by the isolation of genomic DNA for the quantification of AAV genomes in injected retinas using dd-PCR. Comparison of tdTomato expression levels with the transduced AAV genomes quantified by the dd-PCR showed that the dd-PCR method accurately quantified the transduction efficiency of AAV vectors. Our protocols demonstrated a detailed description of a dd-PCR based methodology to quantify AAV transduction efficiencies. In this protocol, we also show the absolute number of AAV genomes that are transduced after intravitreal injections by simply using the dilution factor

after genomic DNA isolation and the dd-PCR results. Overall, this protocol provides a powerful method, which would be an alternative to reporter expression to quantify transduction efficiencies of AAV vectors in the retina.

PROTOCOLS:

All experimental protocols were accepted by the Sabanci University ethics committee and experiments were conducted in accordance with the statement of 'The Association for Research in Vision and Ophthalmology' for the use of animals in research

1. Small scale AAV production[12]

1.1. Culture HEK293T cells using 15 cm plates in complete 10 mL of DMEM/10% FBS until 70-80% confluency.

1.2. Prepare the transfection mixture with 20 µg of helper plasmid (pHGT1-Adeno1), 7 µg of capsid plasmid and 7 µg of AAV2-CBA-tdTomato-WPRE vector and 136 µL of PEI solution (1 mg/mL) in 5 mL of DMEM.

1.3. Add the 5 mL prepared transfection mixture into to a cell culture dish containing 10 mL of culture media and incubate the transfected cells for 48-60 h at 37 ˚C.

1.4. Collect the media at 48-60 h post-transfection and digest it with DNase I at a final concentration of 250 U/mL for 30 min at 37 ˚C.

1.5. Centrifuge the digested media at 4000 x *g*, 4 ˚C for 30 min and then filter it with a 0.22 µm syringe filter into the pre-wetted regenerated cellulose membrane for 100 kDa.

1.6. Centrifuge the regenerated cellulose membrane at 4000 x *g*, 4 ˚C for 30 min and discard the media.

1.7. Wash and centrifuge the regenerated cellulose membrane with PBS containing 0.001% Pluronic F-68 three times at 4000 x *g*, 4 ˚C for 30 min. Discard the PBS at each step.

1.8. Collect concentrated AAVs from the top part of the regenerated cellulose membrane and aliquot for further uses.

1.9. Digest 5 µL of freshly made AAV with DNase I (0.2U/µl) for 15 min at 37 ˚C for titering. This is followed by 10 min at 95 ˚C incubation for both inactivating the DNase I and degrading the viral capsids.

1.10. Prepare a 10-fold serial dilution from digested AAVs using 0.05% Pluronic F-68. Use AAV2-ITR and WPRE primers for titration (**Table 1**). The titers were calculated by multiplying dd-PCR results with the dilution factors. Results were converted to genome copy/mL (GC/mL).

NOTE: AAV concentrations for small scale AAV production are expected to be around 1 x 10^{12} GC/mL. This protocol is not applicable for AAV strains that do not efficiently release AAVs into media like AAV2[13].

2. Intravitreal injection of AAV

2.1. Preparation of equipment

2.1.1. Before starting, prepare 10 µL microsyringe with a 36 G blunt needle. Rinse five times with 70% EtOH, five times in ddH_2O, and finally five times with PBS.

2.1.2. Load the appropriate amount of AAV into the microsyringe for each injection.

2.1.3. Prepare the surgical needle (suture, silk, 6/0), tape, forceps, and scissors.

2.2. Intravitreal injection

2.2.1. Apply 1 drop of 0.5% tropicamide and 2.5% phenylephrine hydrochloride (e.g., Mydfrin) containing eye drop before anesthesia to dilate pupils.

2.2.2. Anesthetize mice with isoflurane 5% (1 L/min) and continue with 1.5% isoflurane (1 L/min) during the procedure. A small isoflurane chamber is used for the first induction.

2.2.3. Verify the depth of anesthesia by the loss of righting reflex, the withdrawal reflex, and tail pinch response.

2.2.4. Apply 0.3% tobramycin and 0.1% dexamethasone sterile ophthalmic solution to each eye before the injection procedure. Application of eye drop prevents dryness and exerts anti-inflammatory and anti-bacterial effects.

2.2.5. Use the surgical hook to stabilize the upper eyelid. Slightly pull back to expose the dorsal part of the eye. Tape the hook to the bench to hold it in position.

2.2.6. Remove conjunctiva (less than 1 mm^2) with the curved iris scissors to expose the sclera of the eye under the dissecting microscope. Use a fresh and sterile insulin syringe (30 G) to puncture the sclera.

2.2.7. Insert the needle of the microsyringe through the same puncture using a micromanipulator. Place the tip of the needle behind the lens in the middle of the eyecup.

2.2.8. Inject 1 µL of AAV2/BP2 and AAV2/PHP.S vectors having 1.63 x 10^{12} GC/mL and 1.7 x 10^{12} GC/mL concentrations slowly into the vitreous of the eye. Injection volume control is done manually. Leave the needle in place for 1 min and slowly withdraw the needle.

2.2.9. Release the eyelid and apply anti-inflammatory and anti-bacterial topical gel treatment containing 0.3% tobramycin and 0.1% dexamethasone to eyecup. Monitor and evaluate the mice in the following days according to the score sheet in terms of appearance (bright eyes, groomed coat, hunching), behavior (activity, immobilization, self-mutilation), and body weight on a scale of 0 to 3. Each condition has a score from 0-3 and a total score of 3 or above is a termination criterion.

2.2.10. Place the animals in the cage after recovery.

3. Fluorescence and fundus imaging

3.1. Strain the mouse and dilate the pupil by placing drops of 0.5% tropicamide and 2.5% phenylephrine hydrochloride into each eye.

3.2. Anesthetize the animal with the above procedure with isoflurane. Assess the depth of anesthesia carefully by pinching the paws.

3.3. Place the mouse on the imaging stage and verify the proper dilation by checking through the fundus camera. Apply topical gel (0.2% carbomer 980) onto the surface of the eyes to protect eyes from dehydrating under anesthesia and to use it as a coupling gel for imaging.

3.4. Manipulate the imaging stage to line up with the nosepiece of the objective. Adjust the stage as needed to center the mouse eye. Once the eye is centered, slowly move the objective until it makes contact with the eye.

3.5. Perform fundus and fluorescence imaging on both eyes to follow up tdTomato expression at 1 week and 2 weeks after intravitreal injection (**Figure 3B**)[14]. Take fundus and fluorescence images at identical settings for comparison of reporter expression.

4. Retina isolation

4.1. Euthanize animals after fundus and fluorescence imaging by CO_2 inhalation for retina collection at 2 weeks time point.

4.2. Shift the eyeball forward with the help of a 13.5 cm splitter forceps.

4.3. Remove the lens together with the leftover vitreous by applying gentle pressure with the forceps.

4.4. Cut the connection of the eyeball to the optic nerve with precision curved forceps and gently squeeze the retina with the same curved forceps.

4.5. Transfer dissected retinas into a microcentrifuge tube.

4.6. Snap freeze retina by placing the tubes into the liquid nitrogen.

5. Tissue genomic DNA isolation

5.1. Isolate genomic DNA with a commercialized Proteinase K digestion-based tissue genomic DNA kit.

5.2. Digest retina at 55 °C, 200 x *g* for 30 min with Proteinase K, which is already supplied in the kit.

5.3. Perform RNase incubation step, wash steps with wash buffer I and II, and finally elution step according to the manufacturer's protocol.

5.4. Add an extra spin step after the last wash to remove residual ethanol.

5.5. Measure the concentration of genomic DNA and store samples at -20 °C.

6. Droplet digital PCR analysis of mouse retina samples for quantification of viral genomes

6.1. Dilute genomic DNAs from injected retinas in 0.05% Pluronic F-68 solution to reach a final concentration of 1 ng/µL for each sample.

6.2. Perform separate reactions for WPRE and 18S using target specific primers with a final concentration of 125 nM for each primer.

6.3. Perform droplet generation in a 20 µL of PCR reaction mix including 1 ng of genomic DNA, 125 nM primers, and 2x evagreen supermix.

6.4. Load the sample mix in the middle part of the cartridge for droplet generation.

6.5. After sample loading to cartridge was done, add 70 µL of the droplet generation oil into the bottom row of the cartridge.

6.6. Put the gasket on top of the cartridge and place it into the droplet generator. Make sure that there is no gap between the gasket and the cartridge.

6.7. Gently take approximately 40 µL of droplets that are generated in the top well. Add the droplet solution to the semi-skirted 96-well PCR reaction plate.

6.8. Seal the PCR plate with PCR plate sealer by using aluminum sealing foil.

6.9. Place PCR plate into 96-well heat-sealed thermal cycler. Use the PCR protocol; 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min and 4 °C infinite

hold. Apply 2 °C/s ramp rate at each cycle to ensure that the droplets reach the correct temperature for each step during the cycling

6.10. Place PCR plate into the droplet reader to quantify droplets using dd-PCR software. A template is set up using specific settings for evagreen.

6.11. Analyze data using a 1-D plot graph with each specimen for fluorescence intensity vs droplet number. The threshold value is arranged so that the droplets above the threshold are assigned as positive and the below ones as negative. This is followed by the application of the Poisson algorithm to determine the starting concentration of target DNA in units of copies/µL. The ratio of WPRE versus 18S is calculated for measuring AAV transduction efficiency.

REPRESENTATİVE RESULTS:

Small scale AAV production is a fast and efficient method that provides vectors for intravitreal injections (**Figure 1**). Small scale AAV production usually gives titers within the range of 1 x 10^{12} GC/ml which is sufficient to detect reporter expression in the retina (**Figure 2**). Titering of AAV using dd-PCR gives consistent results. ITR2 and WPRE specific primers are routinely used and the starting concentration of each target molecule was calculated with dd-PCR software by modeling as a Poisson distribution. Calculations were finalized by multiplication of dilution factors and converted to genome copy per mL. AAVs that are produced for this protocol had titers of 1.63 x 10^{12} GC/mL and 1.7x 10^{12} GC/ml for AAV2/BP2 and AAV2/PHP.S[7,15], respectively. For comparison, identical tdTomato expression constructs were used for both strains. We injected approximately equal titers of AAV for the quantification of AAV transduction efficiency. After injection of 1 µL from the aforementioned concentrations of AAVs, animals were imaged at 1 week and 2 week time points. A fundus and fluorescence imaging system was used for both AAV2/BP2 and AAV2/PHP.S injected retinas generating similar reporter expression profiles except for retina #1 (**Figure 3A,B**). TdTomato expression was quantified using ImageJ software for average gray value (mean fluorescence intensity) in order to cross compare transduction efficiency and reporter expression (**Figure 3A,B**). This is basically the sum of the gray values of all the pixels in the selection divided by the number of pixels[16]. Fluorescent images of Retina #1 showed only a small area of tdTomato expression, most likely due to backflow or leakage after intravitreal injections. Consistent with this finding, fluorescence intensity of retina #1 was 0.9 which was the lowest compared to all retinas that were intravitreally injected and imaged. Mean fluorescence intensity was between 4 – 11. This showed that the quantification of fluorescent images of tdTomato expression was successful and correlated with the images that were shown (**Figure 3A,B**).

After imaging of retinas, genomic DNA was isolated from injected retinas at 2-week time point. dd-PCR was performed using WPRE primers for AAV genome quantification. Mouse 18S was used for the normalization of AAV genomes to the mouse genome (**Figure 3C**)[17]. Consistent with the mean fluorescence intensity and images, retina #1 had a very low AAV genome copy number relative to 18S, 0.011 fold lower compared to retina #2. Moreover, retinas #2, 3, 4, and 5 give similar fold level differences. The fold differences compared to retina #2 for retinas #3, 4, and 5 were 1.75, 0.55, and 0.99, respectively. Among those, retina #3 both gave the highest fluorescent intensity and the AAV genome copy number. This already showed that quantification of

transduced AAV genomes with dd-PCR correlates with the fluorescence intensity and thus to tdTomato expression that is observed. The dd-PCR method also allowed us to do absolute quantification of transduced AAVs. Absolute quantification of the total AAV genome per retina was calculated simply by multiplying the total number identified from dd-PCR and the dilution factor for genomic DNA. This yielded similar results compared to 18S normalized AAV genome transduction efficiency **(Figure 3D).**

FIGURE LEGENDS:

Figure 1: Flow chart of the experimental procedures. Small scale AAV production is followed by dd-PCR -based AAV titering. AAVs are intravitreally injected into the adult retina. Follow up of animals were performed using a fundus and fluorescent imaging system in order to analyze the reporter expression. Fundus and fluorescent images were taken at 1 week and 2-week time points. Genomic DNA is isolated from injected retinas for analysis with dd-PCR to quantify the total AAV genome that is present in the injected retinas.

Figure 2: AAV titering using the dd-PCR method. (**A**) Dd-PCR method benefits from encapsulated PCR reactions within a droplet. After PCR reaction using evagreen chemistry, positive and negative droplets for the target gene were analyzed to determine the absolute number of target DNAs within a solution. (red boxes in green droplets). (**B**) Representative dd-PCR data for AAV titering. Several batches of AAVs were titered using ITR2 primers. Positive and negative droplets are separated above and below the threshold (purple line).

Figure 3: Quantification of transduced AAV genomes in the retina by dd-PCR. 16-week-old wild type animals were intravitreally injected with AAV2/BP2 and AAV2/PHP.S vectors having 1.63 x 10^{12} GC/mL and 1.7x 10^{12} GC/mL concentrations, respectively. Both AAVs had the identical tdTomato expression construct and injection volume for all AAVs was 1 µL. Injected retinas **(1-7)** were followed up with fundus and fluorescence imaging. Images that are taken were quantified using Image J software. Despite the capsid difference, all animals had a fluorescence mean intensity value (*average gray value*) ranging between 4 to 11 except retina #1 which had the lowest intensity, 0.9, and weak tdTomato expression. Red and gray columns are AAV2/BP2 and AAV2/PHP.S injected retinas, respectively (**A-B**). dd-PCR was performed using WPRE primers for AAV genome and 18S primer for normalization at 2-week time point. Retina # 1 also showed distinctively lower AAV copy numbers per 18S copies (**C**)**.** Total AAV genomes per retina were also calculated using WPRE copy number and dilution factor for genomic DNA isolation (**D**). Red and gray squares are AAV2/BP2 and AAV2/PHP.S injected retinas, respectively.

Table 1: dd-PCR primer sequences. Sequences of forward and reverse primers for WPRE, ITR2, and mouse 18S.

DISCUSSION:

In this protocol, we generated two AAV vectors that have different capsid proteins and then titered them accordingly. One of the most crucial steps of this protocol is to produce sufficient amounts of AAVs that will yield detectable reporter expression after the transduction[12,13].

Titering of AAVs is also an important factor to adjust dosages of AAV for intravitreal injections. Once these important criteria are achieved, it is feasible to quantify the transduction efficiency of AAVs by dd-PCR methodology.

Many laboratories are using the quantitative PCR (qPCR) method for AAV titering. qPCR-based absolute quantification method requires a standard curve that has dilutions of known amounts of target DNA[18]. However, dd-PCR does not require a standard curve as it directly quantifies the total number of target molecules within a given sample by detecting the positive droplets that have at least one copy of target DNA. Since dd-PCR is an end-point analysis and no standard curve is required, the efficiency problems that occurred during the qPCR reaction are of less concern for dd-PCR like low-efficiency PCRs or the quality of standard curves [9]. The dd-PCR methodology can be applied to samples that are already prepared for qPCR. As it was already mentioned in the Methods section for AAV titering, dd-PCR require diluted samples. This is a critical step since sample concentration should be adjusted in a way that there are sufficient negative droplets to perform the Poisson algorithm. In other words, it is not possible to perform the dd-PCR reaction with samples with too high concentrations of target DNA that do not yield negative droplets.

For both AAV titering and transduction efficiency measurements, different target sets are possible to use. For AAV titering, we mainly use AAV2 ITR specific primers due to the AAV2 backbone in our constructs. It is also possible to use WPRE and other gene-specific targets depending on the AAV construct that has been analyzed.

To evaluate the transduction efficiency of AAVs in the neuronal retina, we applied the dd-PCR method using whole retina samples to quantify the total amount of AAV genomes per retina. We assessed the accuracy of methodology by using an AAV vector that expresses tdTomato reporter. Comparison of dd-PCR results with tdTomato expression levels correlated except the two outliers. Retinas #6 and #7 yielded excess AAV genome copies despite showing similar fluorescence intensities compared to retinas #2,3 4 or 5. This may be because these retinas have higher transduction rates with limited expression levels or may in part be related to enduring AAV particles or vector DNA within the vitreous or neuronal retina[19,20]. Overall, this was the only limiting factor for our method and can easily be identified among other samples. We also quantified the total number of AAV genomes per retina which was one of the strengths of this methodology. This allows the comparison of data between different laboratories and batches of animals. Therefore, this method can easily be applied to assess the transduction efficiency of a new serotype, variant or batch to batch variation for AAV vectors that have no reporter expression and help us to cross-compare data at different settings. This is critical for AAV vectors that do not allow the expression of a reporter due to size constraints.

This method also provides a basis for other types of sensitive assays that utilizes target DNA. Using the identical protocols, we can quantify mitochondrial genome copy numbers with appropriate primers. Further improvements are also possible including dissociation of retina and flow cytometry sorting steps to quantify the target DNA in either single cells or batches of cells.

Moreover, it is also feasible to assess the correction efficiency of base editing using this method[21,22], which is also critical for several retinal diseases and gene therapies.

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DİSCLOSURES:

The authors have nothing to disclose.

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Figure

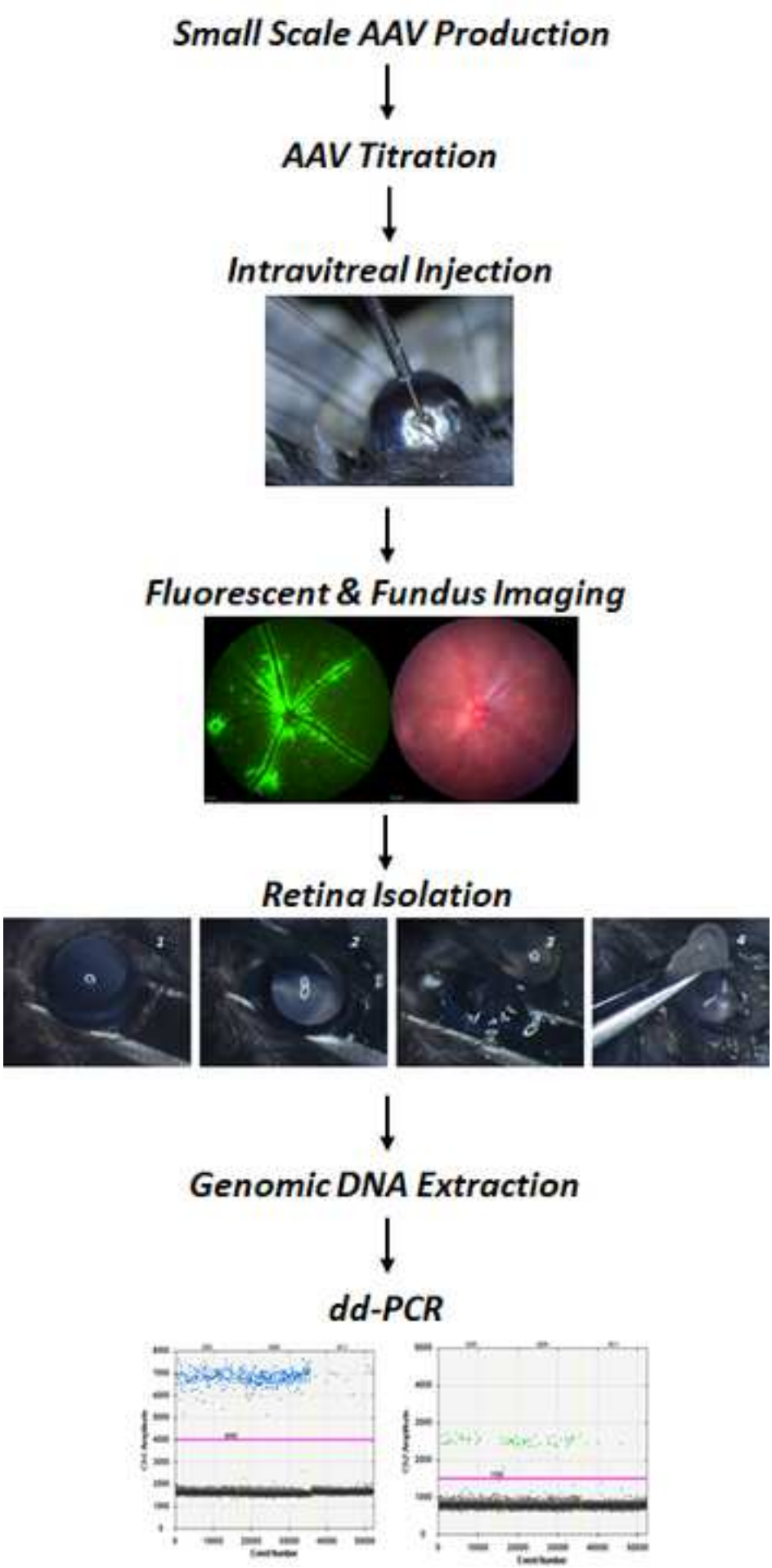

Figure 2

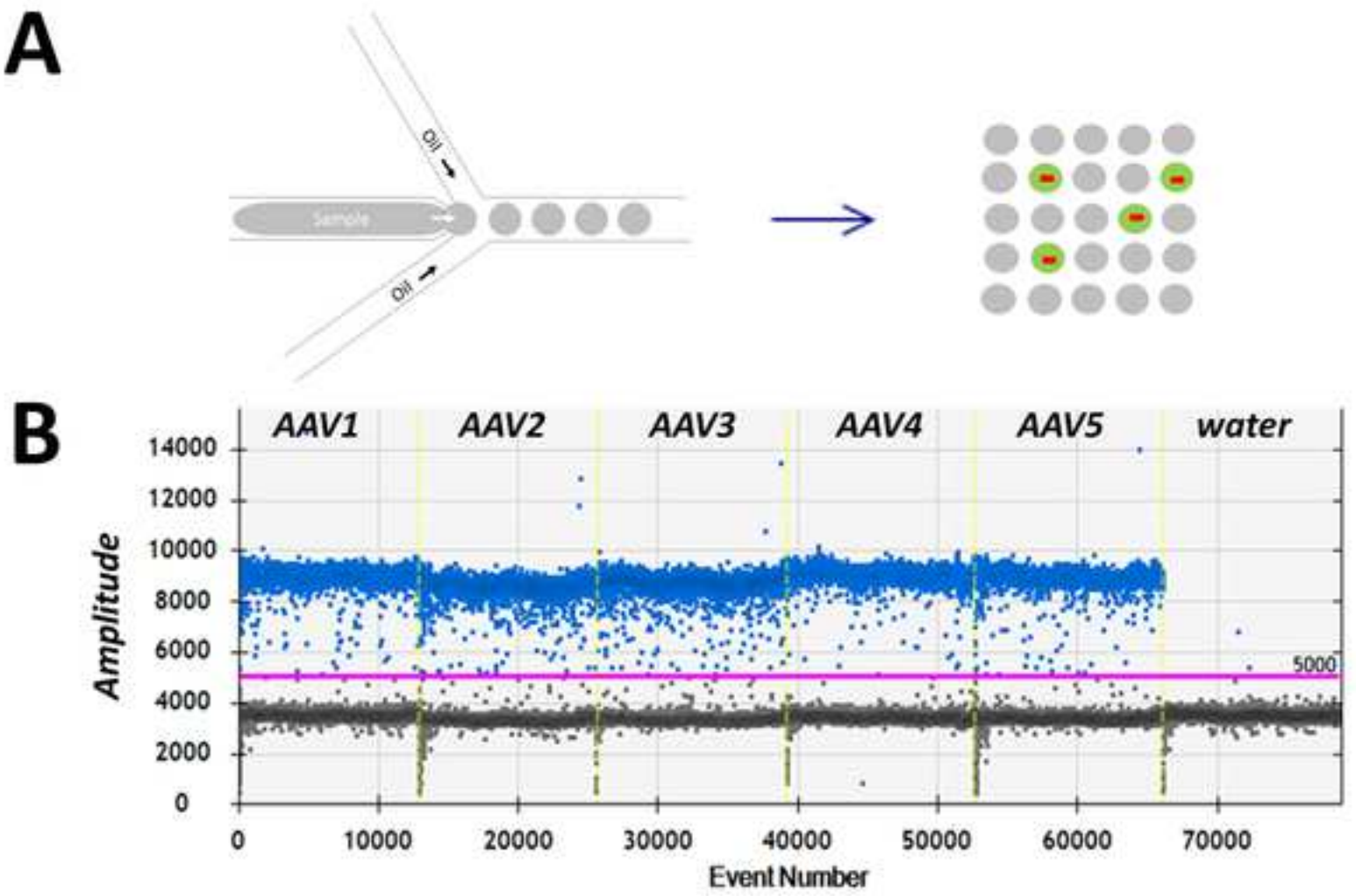

Figure 3

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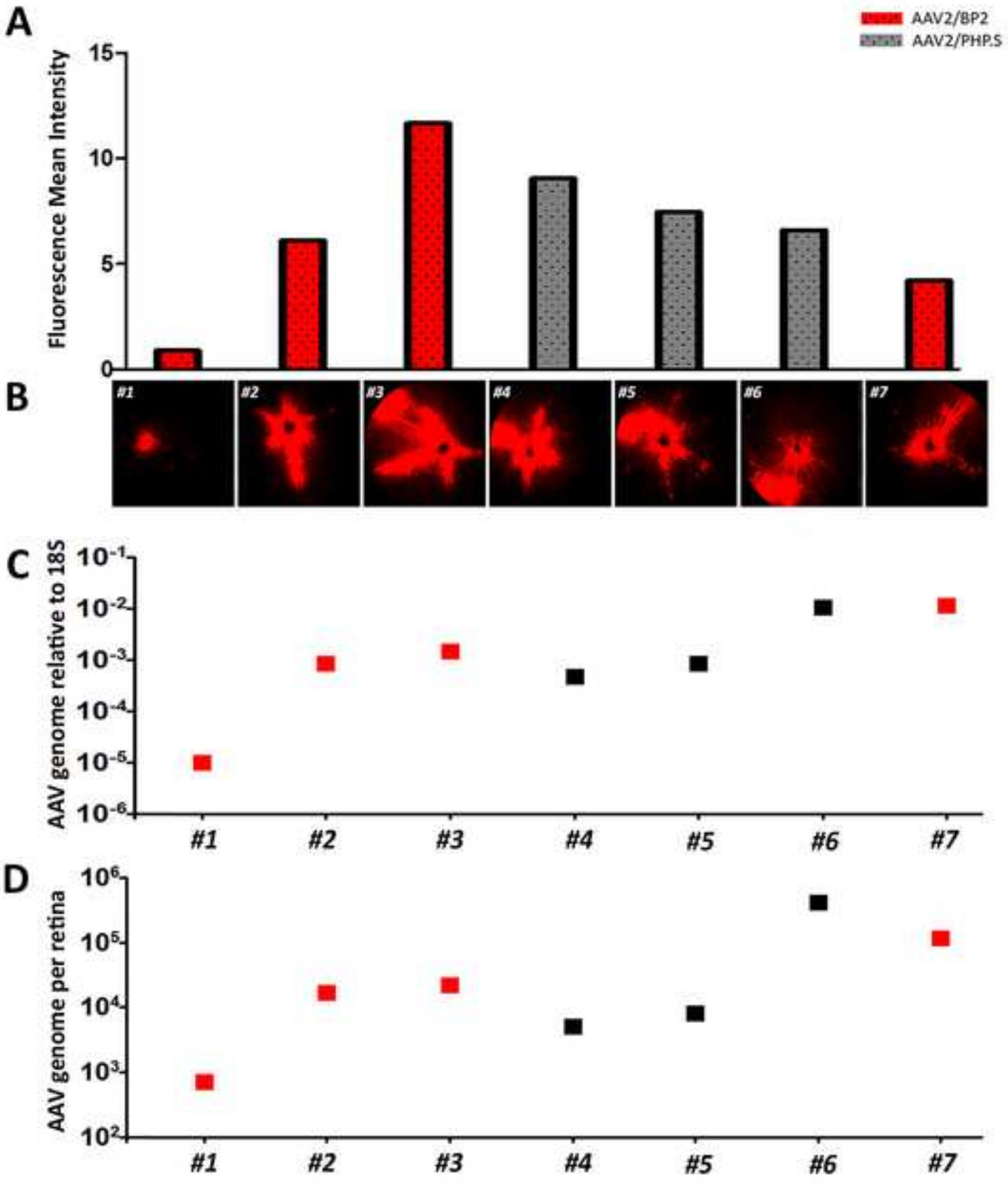

	ddPCR
ITR2 F	
ITR2 R	
WPRE F	
WPRE R	
18S F	
18S R	

Primers
GGAACCCCTAGTGATGGAGTT
CGGCCTCAGTGAGCGA
GGCTGTTGGGCACTGACAA
CCAAGGAAAGGACGATGATTTC
GGCCGTTCTTAGTTGGTGGA
CCCGGACATCTAAGGGCATC

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Table of Materials

Materials for JOVE ATMP_alphabetically ordered.xlsx

Dear Editors,

We would like to thank editors for reviewing our protocol. Please find enclosed our 2nd revised version of manuscript entitled,

`Digital droplet PCR method for quantification of AAV transduction efficiency in murine retina`

We followed the editors' recommendation, took this opportunity to respond to each point of editors, and revised the manuscript accordingly. We provided an explanation of our revisions below.

For editorial comments, we have done minor revisions as suggested in comment boxes in the text as well as narrations in video.

1- We change the formats throughout the MS where it is necessary as suggested.

2- ddPCR is changed to dd-PCR due to commercial term issue as requested.

3- Necessary citations are added as requested.

4- We have added documentation for our animal permits from Sabanci University.

5- We clarify the cell culture section 1.4 as requested.

6- We explained the scoresheet scaling as requested.

7- Representative results and discussion have been revised as suggested.

8- For editorial suggestion related to RT-PCR,

Unfortunately, in our lab we do not isolate gDNA and totalRNA simultaneously. We normally do these techniques separately. I also think solely quantifying transcript levels without gDNA will not be useful for transduction efficiency unless they are done simultaneously, since there are variety of parameters that effect transcript levels including promoters, RNA stability, possible translational regulations and etc. However, gDNA quantification from the whole retina is a solid representative of total numbers AAV particles that are transduced or still present. Our main aim is to have an absolute quantification of AAV genome copy numbers thus total number of AAV particles in an AAV injected retina. Simultaneous gDNA and total RNA isolation, cDNA preparation and RT-PCR also are not related to the protocol we proposed. We also explained the issue in our first revision (Please see below from revision).

`2-) ***On the other hand,. The reviewer suggests including measurement of the transduction efficiency by examining the copy number of the transcripts by ddPCR.***

We do not feel an analysis of this sort would add much value. tdTomato protein levels and hence the Tdtomato transcripts levels originating form AAVs should correlate with the observed tdTomota fluorescence levels. Another technical difficulty performing this comparison would be to isolate gDNA and RNA with a more efficient set up and would require extensive optimization. `

For video comments, we have done all the revisions that are suggested except for the scale bar.

1- In our first revision we already explained the issue below. Micron IV has round optics since the retina is a sphere and a scale bar is not feasible. Please check our previous reply below.

`1-) For all images taken with a microscope, please include a scale bar.

The fundus microscope has a special round optics to be able to image retina which is a hemisphere. Therefore, scale bar is not applicable to our retina images.`

2- Spelling is corrected for *`DNAse should be spelled as DNase.*

3- Narration is fixed for *2:24: the narration is cut here – Please include centrifuge for 3 times….*

4- The sign is corrected for *`2.59, 4.28: % sign should be after the number: 0.5% and not %0.5.`*

5- Commercial terms were blurred where available *` 4.39: We cannot have commercial terms in the video. Please blur "Phoenix" both in the instrument and in the software, and BioRad from the ddPCR section.`*

6- Spelling is corrected for *`4:53 Please subscript 2 in CO2.`*

7- We have removed droplet generator form the figure 2. *`Figure 2 A: Image of the ddPCR machine … is this copyrighted? If yes, please include permission to use this.`*

Video Comments:

1- We have added cross-dissolve before. However, when the background is very similar at the next scene, it gives the impression of sudden transition. The other transition problems are solved. *`Transition seems too sudden which looks distracting or confusing to the viewers, please consider increasing the footage duration by half a second to address it if possible`*

Graphics Comments:

1- We have highlighted parts for *`• 7:51 - 8:13 Please highlight the parts as the narrator speaks, it will be good for the viewers to understand it better.*

Audio Comments:

1- Audio problem is solved. for *` • 2:35 - 2:51 Audio level seems a bit low, please consider maintaining audio level between -12dB to -6dB.`*
2- Audio problem is solved for *`• 2:43 There is a noise at this timestamp and after that, the audio level goes down substantially, please address this.``*
3- Audio problem is solved for *`• 7:30 Audio seems to start mid-word, please check it once and address it if possible.`*
4- Duration of the footage is increased for *`• 7:51 Please let the sentence complete by increasing the duration of the footage.`*
5- Audio problem is solved for *`• Level of both the Audio channels isn't the same, please consider matching the audio levels of both the channels (L+R).`*

We believe that we responded adequately to all concerns and hope that you will find the 2nd revised version of our manuscript acceptable for publication in JOVE.

Regards,