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Identification of universal parameters that play a critical role in rAAV production using a transient two-plasmid packaging system in the adherent HEK293T cell line

KU LEUVEN

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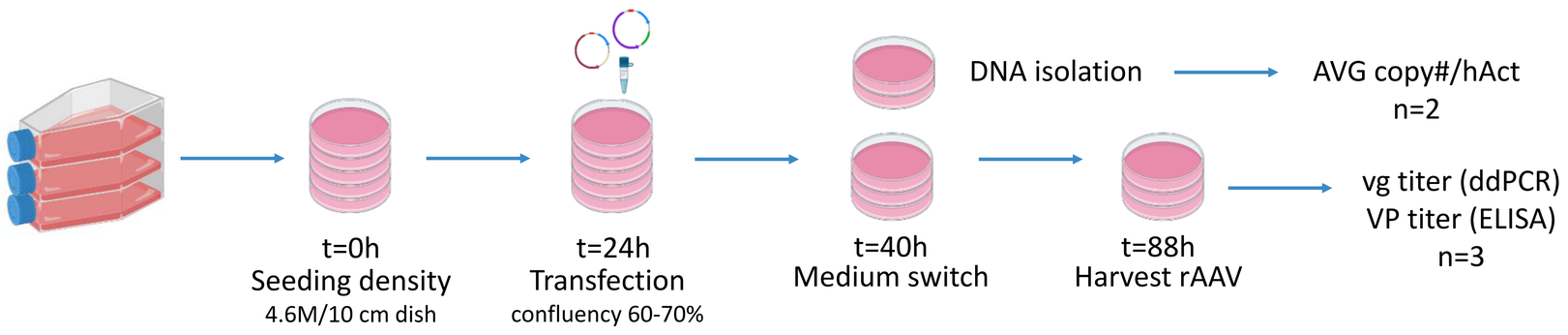
INTRODUCTION

- Adeno-associated virus (AAV) is the vector of choice for gene therapy due to its relatively good safety profile and persistence, which has led to a booming number of clinical trials
- To meet the increasing demand, challenges in manufacturing of recombinant AAV (rAAV)vectors such as low yields and low full:empty capsids ratios need to be tackled

The aim of this study was to identify critical process parameters that determine the yield and quality of rAAV production

METHODS

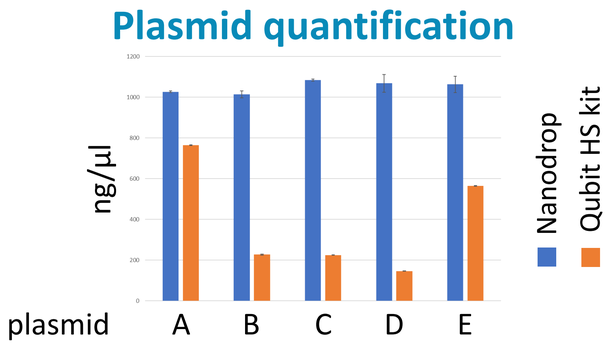
- rAAV production using a transient two-plasmid packaging system (*rep*, *cap* and Ad helper genes on same plasmid) in the adherent HEK293T cell line



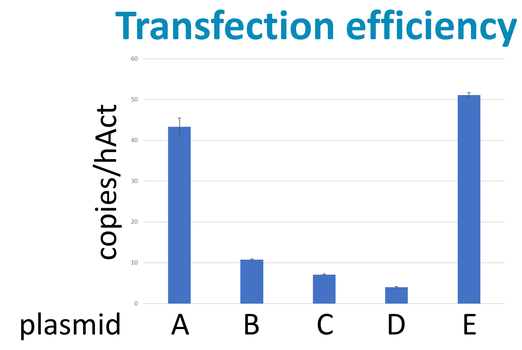
- ddPCR was used to determine 1) the intracellular average copy number of the helper and production plasmid 16h after transfection 2) vg titer 48h after medium switch

Accurate measurement of pDNA is crucial for process development

Plasmid quantification



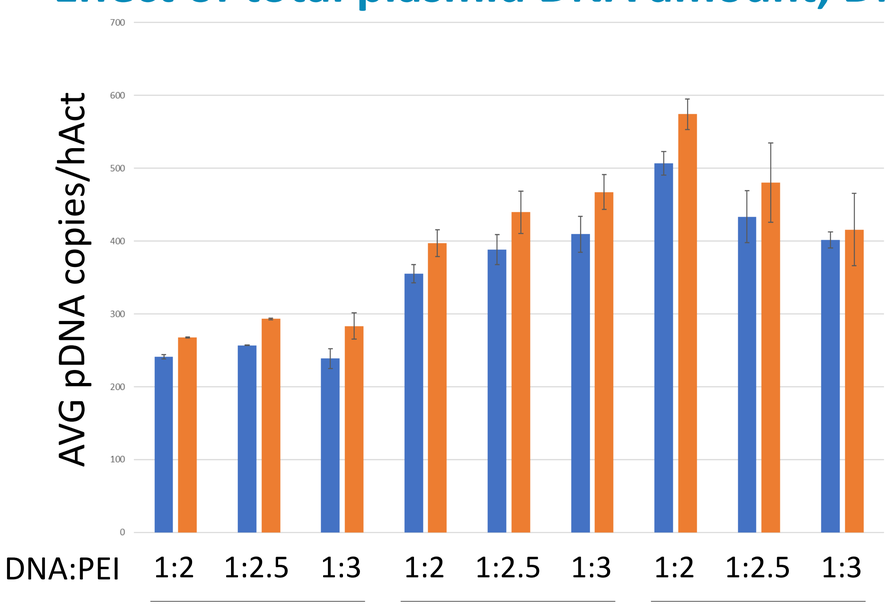
Transfection efficiency

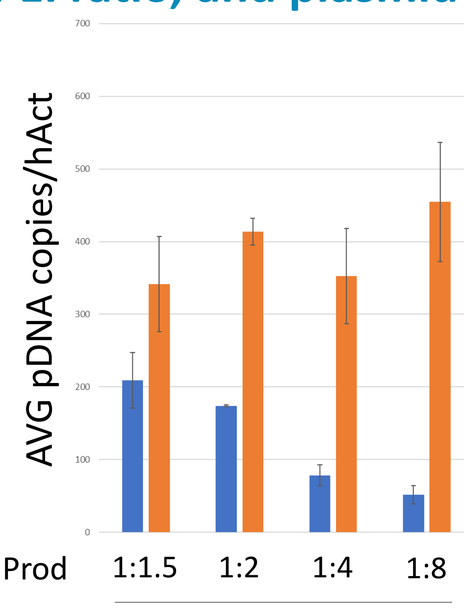


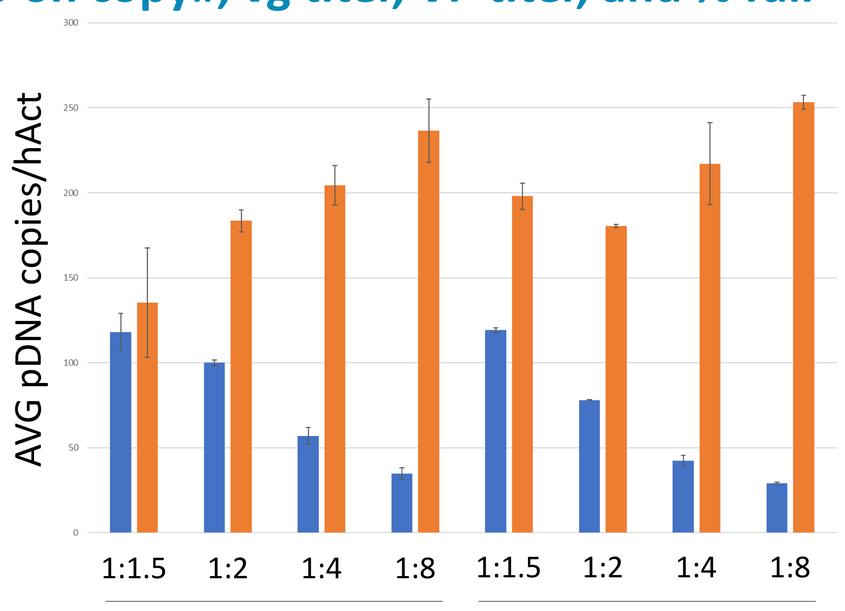
The large variation in efficiency after transfection of equimolar amounts (Nanodrop) of 5 plasmids led us to additionally implement ddPCR for copy number determination in process development

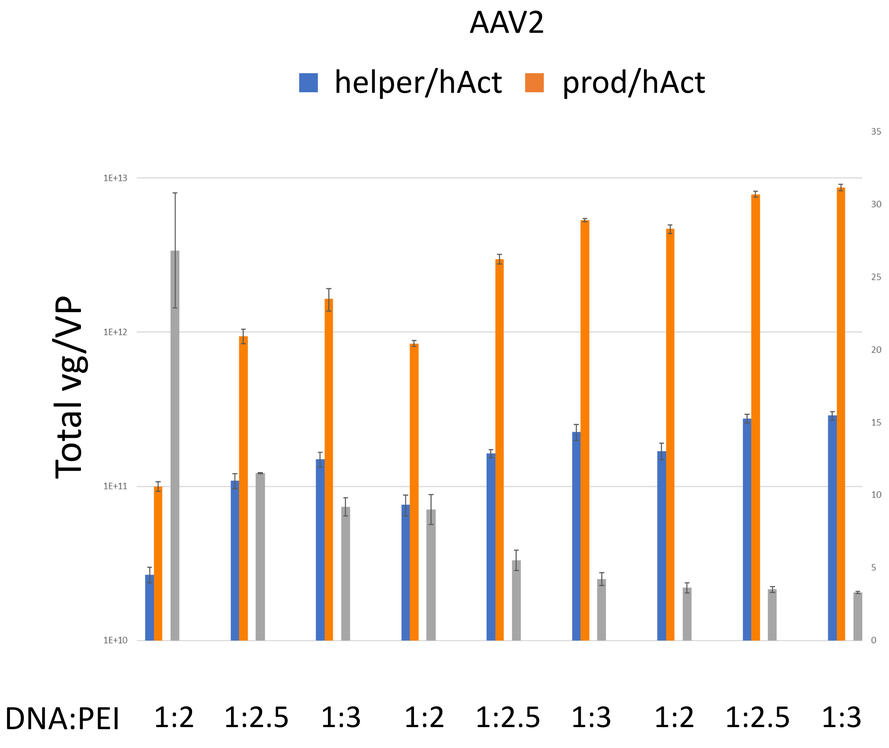
RESULTS

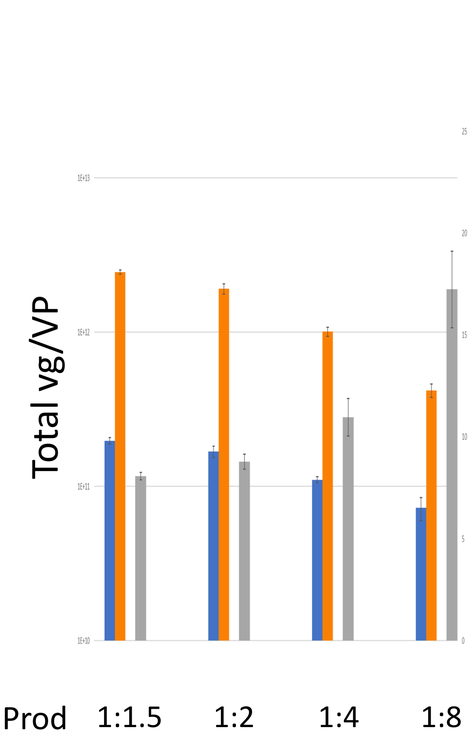
Effect of total plasmid DNA amount, DNA:PEI ratio, and plasmid ratio on copy#, vg titer, VP titer, and % full

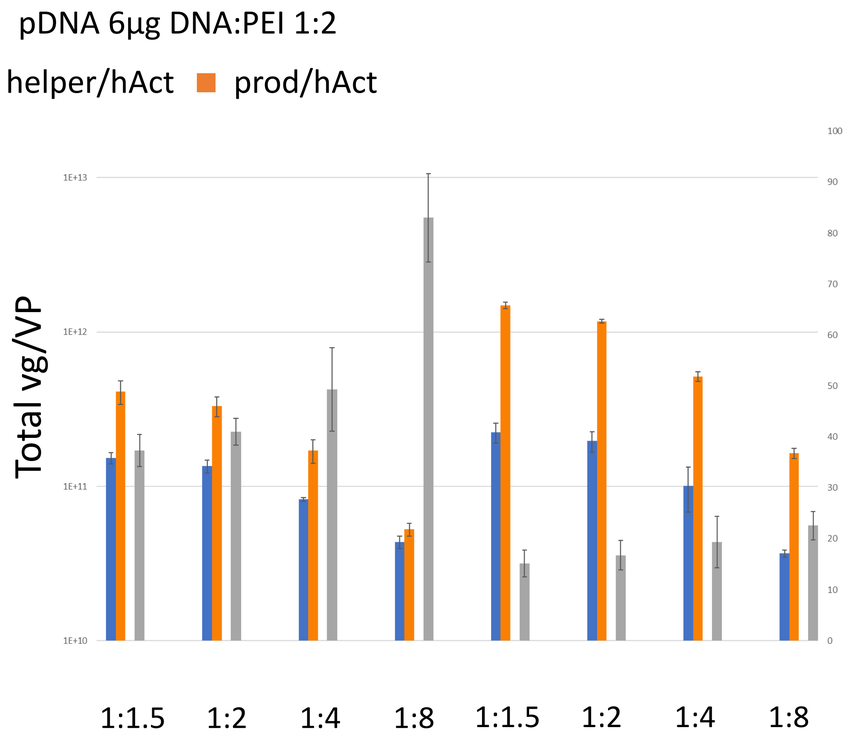












CONCLUSION

- UV absorbance-based methods for pDNA quantification alone do not form a reliable basis for rAAV production process development
- Monitoring of intracellular pDNA copy numbers allow for better insight into the transfection and production process
- Increasing the amount of PEI or the amount of total pDNA leads to higher vg titer and VP titer though reducing the % of full particles
- Decreasing the helper:production plasmid ratio gradually decreases total vg and VP titer while concomitantly increasing % full
- We observed an inverse relationship between total rAAV particle yield and percentage full across multiple serotypes

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BRAIN ORGANOIDS AS A PLATFORM TO STUDY SUBCELLULAR TRAFFICKING OF RECOMBINANT AAV VECTORS

Marlies Leysen [1], Idris Salmon [2], Sereina O. Sutter [3], Cornel Fraefel [3], Adrian Ranga [2], Benjamien Moeyaert [1], Els Henckaerts [1, 4]

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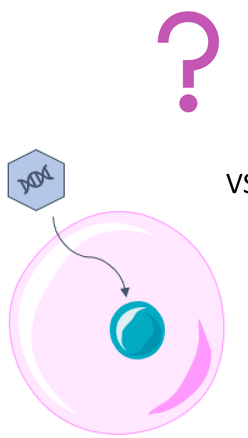
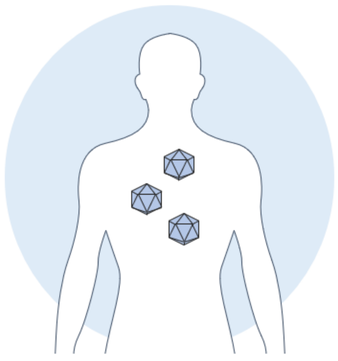
Pathways towards successful *in vivo* rAAV transduction are poorly understood



2D cell lines & primary cell cultures

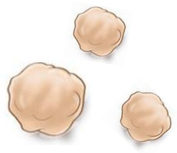
VS



In vivo

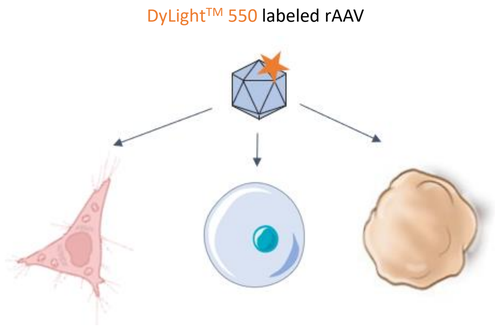
VS

- Human iPSC derived
- Physiological 3D model system



Brain organoids

Establishment of live imaging pipeline for early & late phases of rAAV transduction



HeLa hiPSC Brain organoids

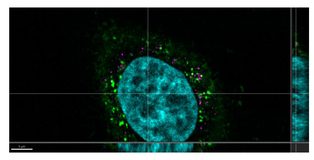
- Real-time imaging of same cohort of virus over time
- Challenging 3D structure imaging
- To study link between;
  - Amount of virus entering cells
  - Successful trafficking towards the nucleus
  - Transgene expression (e.g. GFP)

CONCLUSIONS

- **First visualization of labeled rAAV in brain organoids**
- HeLa cells, hiPSC & cells of brain organoids show heterogeneous susceptibility to viral entry
- Imaging pipeline captures poorly understood phenomena (nuclear envelope breakdown, trafficking via filopodia) of rAAV in HeLa cells

IMPACT FOR GENE THERAPY RESEARCH

This **real-time live** imaging pipeline allows us to study the **rate limiting steps for successful rAAV transduction** (eg. nuclear entry, nucleoli accumulation, transgene expression, …) in **model systems relevant for human gene therapy applications**



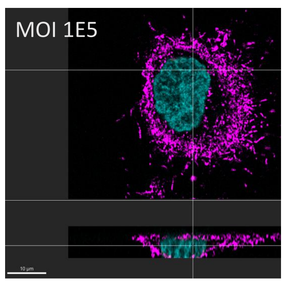
- Zeiss LSM880 confocal microscope (Airyscan)
- 37°C/ 5% CO_2
- 25x/ 40x objective

HeLa

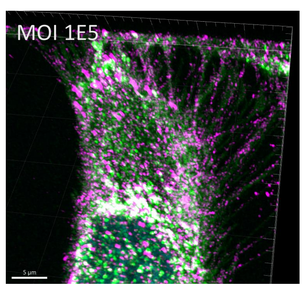
Live imaging/ HeLa cells / ~3h30 post transduction/ 40x objective

Hoechst/CellMask Deep Red/rAAV2 x DL550

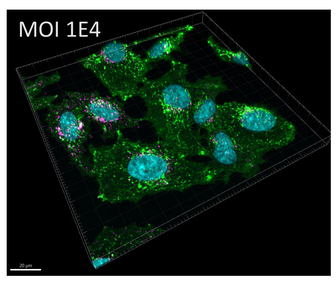
Nuclear envelope invagination

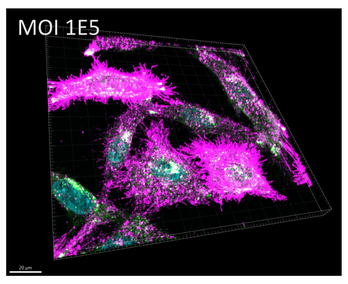


rAAV trafficking via filopodia

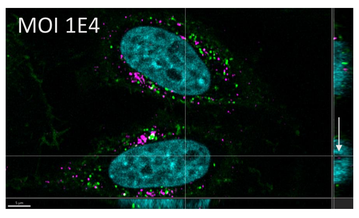


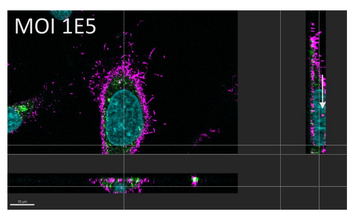
Some HeLa cells are more susceptible to viral entry





Trafficking dynamics might be MOI dependent





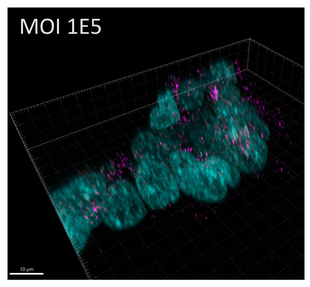
After adding rAAV, internalization of the virus is synchronized in HeLa & hiPSC by placing cells @4°C for 30 min. Afterwards, cells are washed to remove unbound virus & imaged @37°C, 5% CO_2.

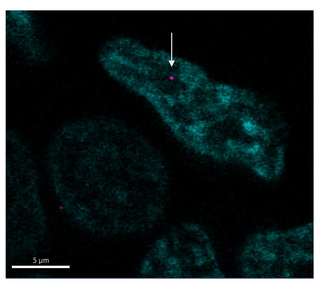
hiPSC

Live imaging/ hiPSC/ ~1h30 post transduction/ 40x objective

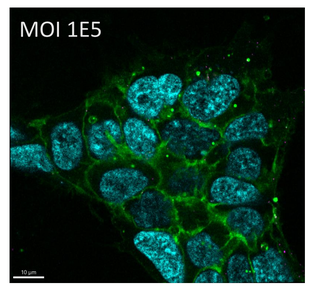
Hoechst/ CellMask Deep Red/rAAV2 x DL550

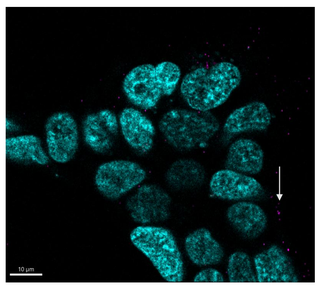
Less susceptible, though effective viral entry in hiPSC





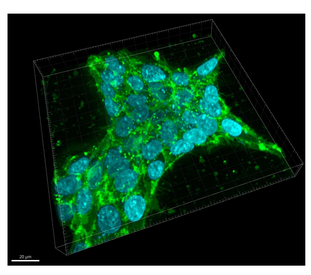
Some hiPSC cells are more susceptible to viral entry

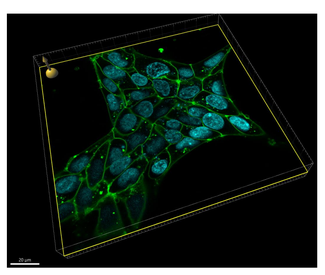




Staining allows cell segmentation analysis

Hoechst/CellMask Deep Red/PBS



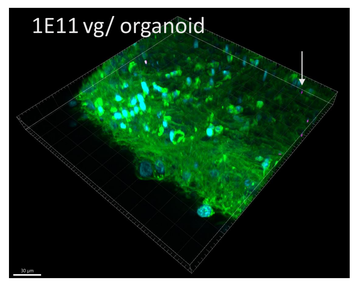


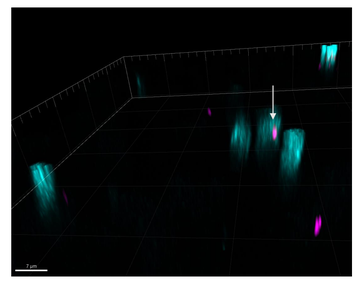
Brain organoid

Live imaging/ organoids/ ~2h post transduction/ 25x objective

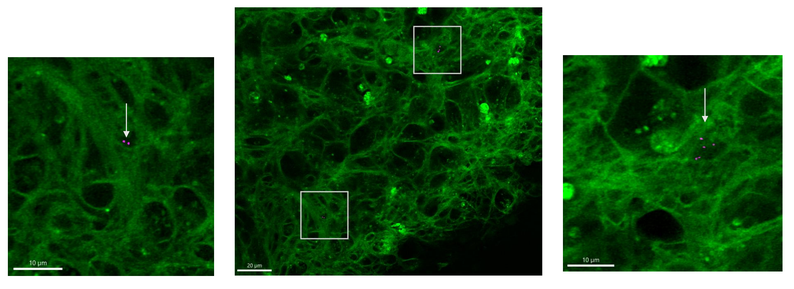
Hoechst/ CellMask Deep Red/rAAV9 x DL550

Effective nuclear entry in organoids



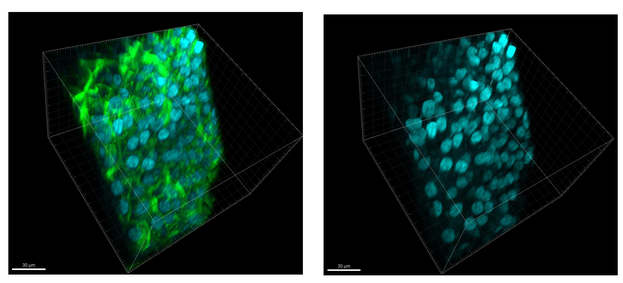


Some cells of brain organoids are more susceptible to viral entry



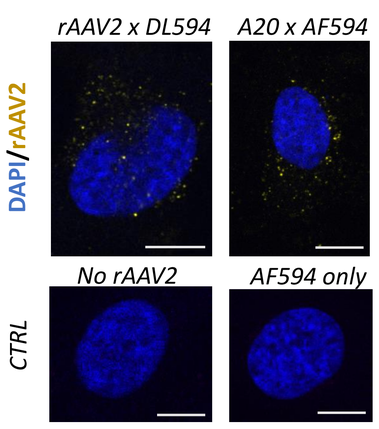
Staining allows nuclear segmentation analysis

Hoechst/CellMask Deep Red/PBS

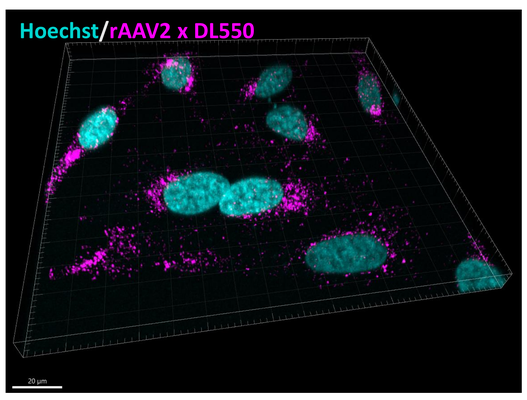


Successful fluorescent labeling of rAAV

IF/HeLa cells/ MOI 1E5



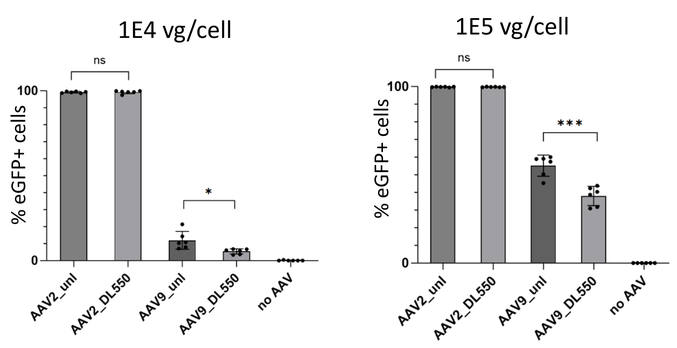
Live imaging/ HeLa cells/ 3h30 post transduction/ MOI 1E4



Scale bars: 10 µm

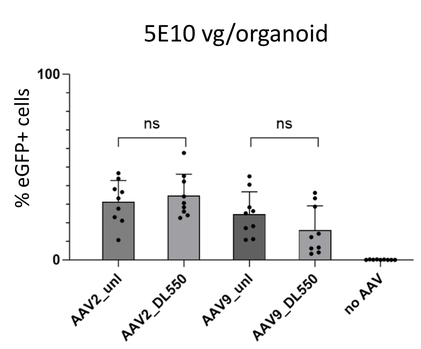
Transduction check post-labeling

HeLa cells



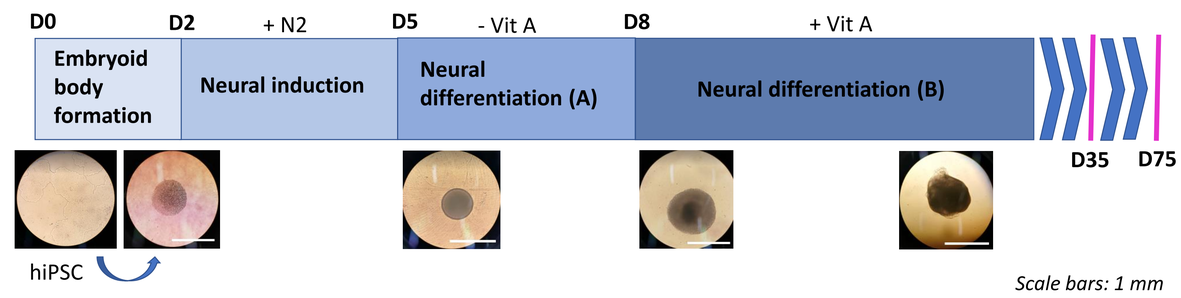
Unpaired Student's t-test, two-tailed, n=6. The data are presented as mean ± SD. eGFP readout 48h post transduction.

Organoids



Unpaired Student's t-test, two-tailed, n=9. The data are presented as mean ± SD. eGFP readout 9 days post transduction.

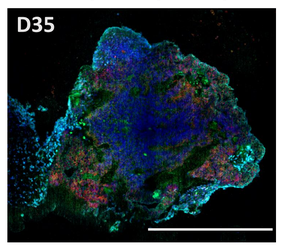
Brain organoid differentiation protocol



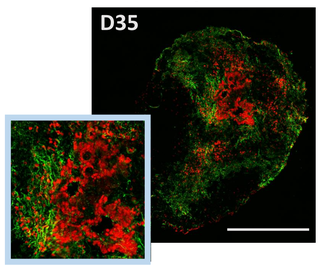
Scale bars: 1 mm

Brain organoids contain cell types of human fetal brain development

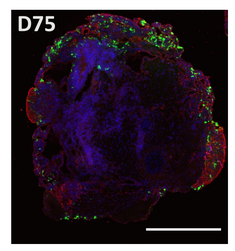
Hoechst/TBR1/PAX6/CTIP2



TUJ1/SOX2



Hoechst/NeuN/eGFP/GFAP

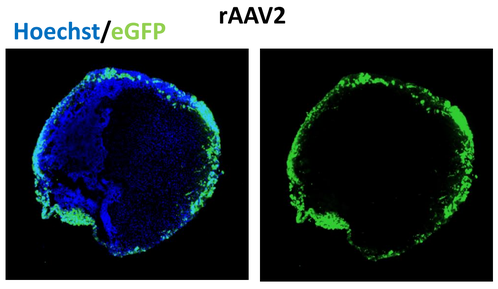


Scale bars: 500 µm

Effective brain organoid transduction

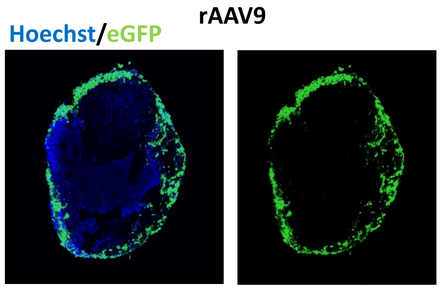
Hoechst/eGFP

rAAV2



Hoechst/eGFP

rAAV9



Brain organoid slices/ 5E10 vg per org

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[2] *Laboratory of Bioengineering and Morphogenesis, Biomechanics Section, Department of Mechanical Engineering, KU Leuven, Belgium/* [3] *Institute of Virology, University of Zurich, Switzerland*
Thanks to the VIB BioImaging Core for training, support and access to the instrument park

Validation of a ddPCR protocol for the quantification of AAV genomes in purified vector samples

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INTRODUCTION

- AAV (adeno-associated virus) is a non-pathogenic virus that is used as a delivery vehicle to transfer therapeutic genes into a patient's cells
- **Accurate quantification of AAV genome copies is essential** for the optimization of the production and purification processes, preclinical studies, and clinical dosage of AAV-based gene therapy products
- Viral genome titers can be quantified through quantitative PCR (qPCR) or droplet digital (dd)PCR. ddPCR is preferred over qPCR because of the independence of the amplification efficacy, no need for a standard curve, higher precision, and robustness.

AIM

We aimed to validate a ddPCR protocol for the quantification of AAV viral genomes in purified samples

VALIDATION PARAMETERS

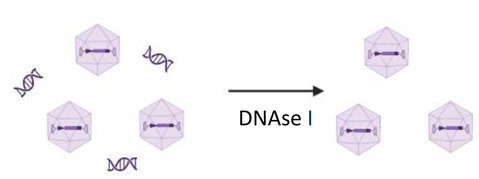
The following parameters were evaluated according to the ICH Q2(R1) guidelines:

- Precision
- Specificity
- Linearity
- Limit of detection
- Limit of quantification
- Range

METHODS

SAMPLES

- 3 serotypes (AAV2, AAV9 and an engineered serotype) and 3 different primer/probe sets for different targets were evaluated
- To eliminate unpackaged contaminant DNA, samples were treated with DNAse I for 1 hour at 37°C



- A total of 4 sets of quality control (QC) samples were prepared, each set containing 5 AAV concentrations. Each set was measured 3-5 times

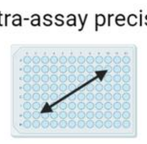
		QC1	QC2	QC3	QC4	QC5
SET 1	Copies/µL	5	10	100	5000	8000
SET 2	Copies/µL	5	10	100	5000	8000
SET 3	Copies/µL	5	10	100	5000	8000
SET 4	Copies/µL	5	10	100	5000	8000

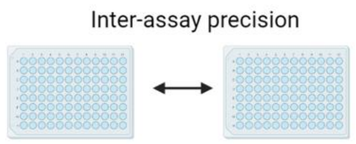
AAV GENOME QUANTIFICATION BY ddPCR

- Samples were diluted in dilution buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.001% BSA, 0.2 ng/µL sssDNA, 0.1% Pluronic F-68) and added to the Master Mix (909 nM forward primer, 909 nM reverse primer, 227 nM probe, 1x ddPCR supermix for probes (no dUTP, Biorad))
- Droplets were made according to Biorad's ddPCR application guide, PCR amplification was performed, and droplets were read using the QX200 ddPCR system (Bio-Rad)

The assay allows for precise, specific and reproducible quantification of AAV9 genomes

PRECISION





copies/µL	5	10	100	5000	8000
run 1	5%	14%	3%	6%	4%
run 2	10%	3%	6%	5%	4%
run 3	10%	34%	3%	5%	3%
run 4	7%	3%	1%	5%	6%
run 5	10%	8%	4%	11%	5%

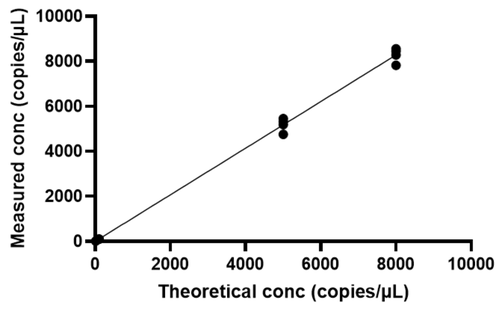
copies/µL	5	10	100	5000	8000
Set 1	14%	23%	7%	8%	3%
Set 2	7%	6%	2%	7%	4%
Set 3	10%	7%	4%	6%	4%
Set 4	4%	8%	3%	6%	7%

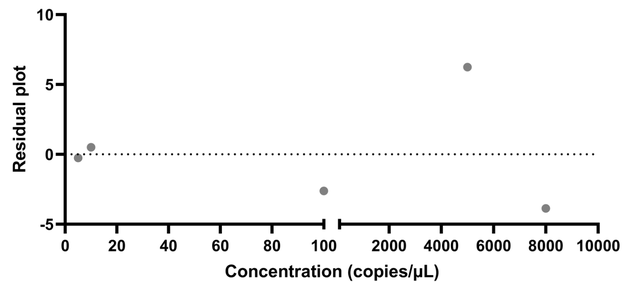
The coefficient of variation (%CV) between the 4 sets of QC samples per run (intra-assay precision, left table) and %CV of each QC sample of each set between the 5 runs (inter-assay precision, right table)

LOQ and LOD

- Limit of detection (LOD) = 1.29 copies/µL
- Limit of quantification (LOQ) = 5 copies/µL

LINEARITY AND RANGE





- Linearity is shown in the range from 5 to 8000 copies/µL
- A random pattern is observed in the residual plot

SPECIFICITY

- 40 out of 40 (100%) NTC had a value lower than the LOD of 1.29 copies/µL
- The presence of spiked-in plasmid or HEK293T DNA does not impact the AAV concentration

Similar results were obtained for other serotypes and primer/probe targets

Serotype	AAV9	AAV2	Engineered serotype	AAV9	AAV9
Primer/probe target	**GFP**	**GFP**	**GFP**	**mKate**	**polyA**
Precision	✓	✓	✓	except 5 copies/µL	except 5 copies/µL
intra-assay precision	≤14% CV *	≤13% CV *	≤17% CV	≤9% CV	≤9% CV
inter-assay precision	≤14% CV *	≤14% CV *	≤14% CV	≤16% CV	≤18% CV
Linearity	✓	✓	✓	✓	✓
Specificity	✓	✓	✓	✓	✓
Limit of detection (copies/µL)	1.29	1.36	1.81	2.59	2.76
Limit of quantification (copies/µL)	5	5	5	10	10
Range (copies/µL)	5-8000	5-5000**	5-8000	10-8000	10-8000

* except one outlier
** higher was not evaluated

CONCLUSION

- A ddPCR protocol for the quantification of AAV viral genomes in purified samples was validated for 3 different serotypes (AAV2, AAV9 and an engineered serotype) and 3 different primer/probe targets
- The high precision and low limit of quantification allows for reproducible AAV genome quantification in an adequate concentration range
- Reliable viral genome titration will aid the development of AAV-based gene therapy products

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Nanopore sequencing of recombinant AAV vectors for the characterization of DNA content

KU LEUVEN

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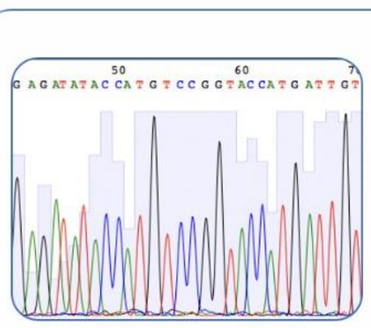
INTRODUCTION

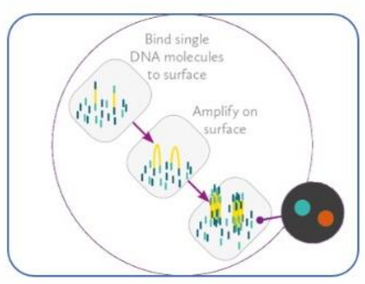
- Recombinant adeno-associated virus (rAAV) is used as a vector to deliver therapeutic transgenes.
- However, during the production process, contaminant DNA such as rearranged vector DNA, host cell genomic DNA, or plasmid DNA can be packaged.
- Nanopore sequencing allows analysis of long DNA sequences without amplification, in a short time and at low cost compared to other sequencing platforms.



Sanger

Advantages:
+ High accuracy
+ Low costs (<20 amplicons)
+ Turnaround time

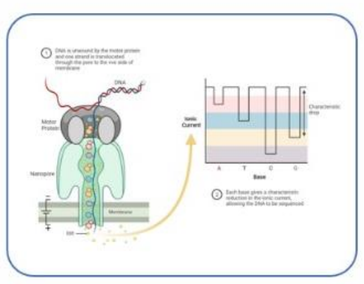
Disadvantages:
- Capacity
- High cost (>20 amplicons)
- Low throughput
- Short read sequencing



Illumina

Advantages:
+ Wide applicability
+ High throughput
+ Higher capacity with sample multiplexing

Disadvantages:
- Expensive/big machines
- Short read sequencing
- Coverage and mapping
- Structural variant detection



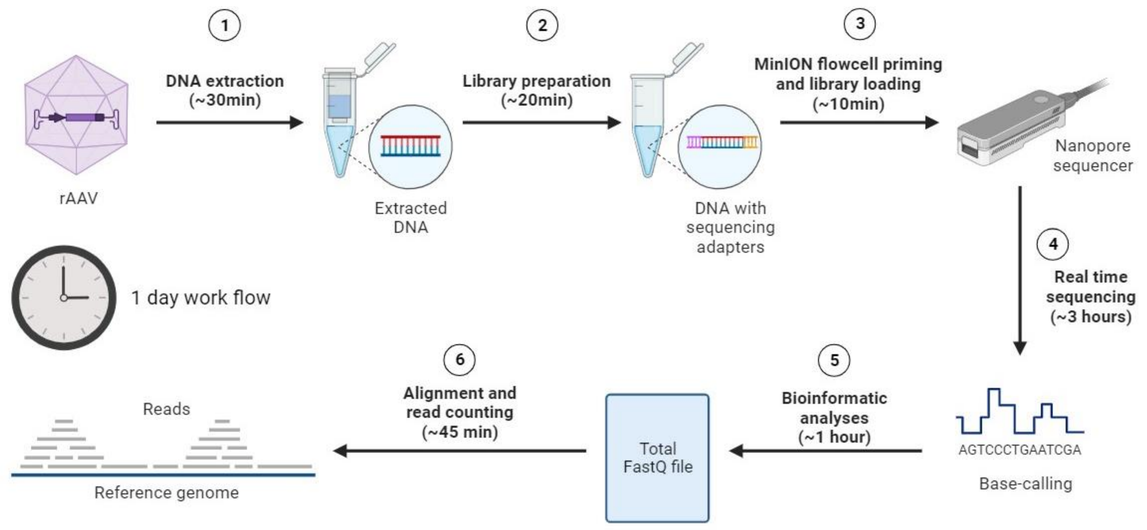
Nanopore

Advantages:
+ Affordable
+ Long read sequencing
+ Coverage and mapping
+ De novo assembly
+ Structural variant detection

Disadvantages:
- Variability (flow cell quality)
- Accuracy (homopolymers)

METHODS

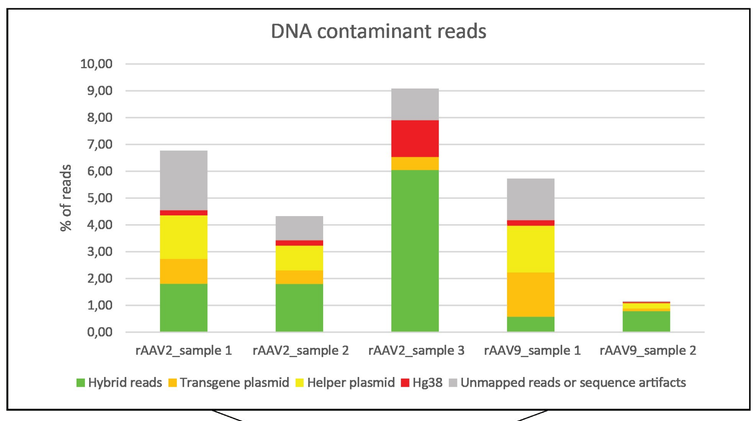
- 3 AAV2 and 2 AAV9 batches were analyzed via nanopore sequencing.
- DNA was isolated from heat-denatured capsids with the QIAquick PCR Purification Kit (QIAGEN), as per the manufacturer's instructions.
- The libraries were prepared with the Rapid Sequencing Kit and loaded onto MinION flow cells (R9) for sequencing and were base called using the ONT's MinKNOW software.
- Sequence reads were aligned and analyzed using a custom Python script and visualized using IGV (Integrative Genome Viewer).

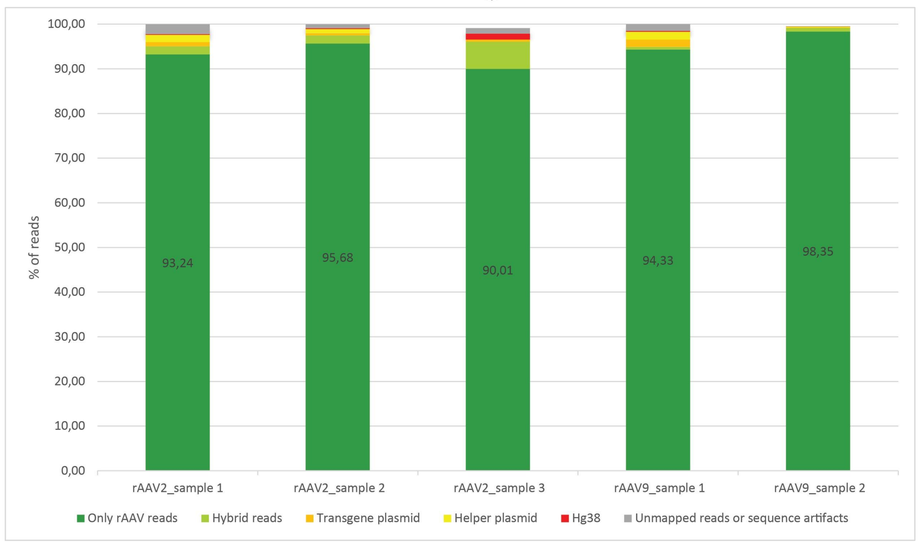


AIM

The aim of this study was to develop a customized nanopore sequencing pipeline for the analysis of the DNA content of AAV preps.

Nanopore sequencing allows for quantitative analyses of DNA contaminations in purified AAV vectors





Vector reads

Our database shows that for all the reads that passed the length quality threshold of ≥1,000bp, between 90% and 98% mapped exclusively against the AAV genome.

Hybrid reads

0.6%-1.8% were classified as hybrid reads – reads that could be mapped to the AAV genome and either to the transgene plasmid, the helper plasmid(s) or the human genome (hg38).

Contaminants

The remaining reads that did not map against the vector genome were considered to be real contaminants, with a range between 0.36%-5.15% mapping solely to the transgene plasmid backbone, helper plasmid or hg38, as well as a small fraction of sequencing artifacts.

Future perspectives

- Our customized nanopore sequencing pipeline allows for qualitative and quantitative DNA content analysis of AAV, including contaminant sequences.
- This technique dramatically simplifies sample preparation and reduces turnover times compared to other NGS characterization methods.
- In a next step, we aim to validate the pipeline to assess the accuracy of the data to determine possible sources of bias. Additionally, we will optimize our protocol to analyze AAV content without prior fragmentation to more accurately obtain full-length resolution of encapsidated genomes.

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