# Reducing residual nucleic acid impurities in rAAV production through human nuclease overexpression

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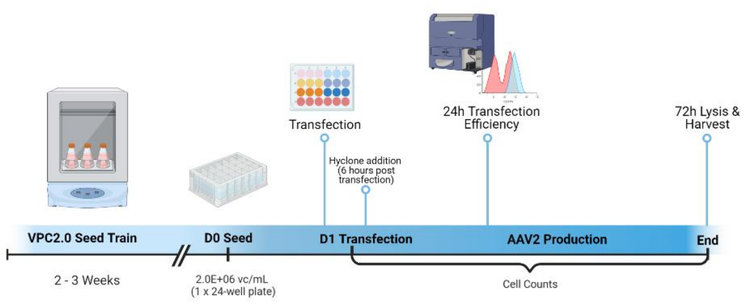
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## Introduction

*Recombinant adeno-associated virus (rAAV) serotype 2 are commonly used in gene therapy applications. Due to strong interaction of rAAV2 particles to Heparan Sulphate, producer cells are lysed during manufacturing to collect viral particles, resulting in high levels of host contaminants (such as host cell DNA, hcDNA) in the final product. Current processes rely on the addition of commercially available bacterial nucleases and extensive downstream purification, to remove impurities and meet clinical requirements (≤10 ng DNA per dose with a median fragment size ≤200 bp). This substantially increases process complexity and manufacturing costs, particularly when production is scaled to target larger patient populations. To address this challenge, here we co-expressed human nucleases (such as huDNaseI and huRNaseI) during rAAV2 and rAAV8 production and evaluated their impact on viral genome titre, viral particle titre, and residual hcDNA levels relative to standard commercial nucleases. Overall, our findings demonstrated that huDNaseI co-expression during rAAV production enables effective clearance of residual DNA while maintaining vector productivity.*

## Method

### Step 1. Co-transfect HEK293 suspension cells with plasmids containing nucleases genes alongside rAAV plasmids

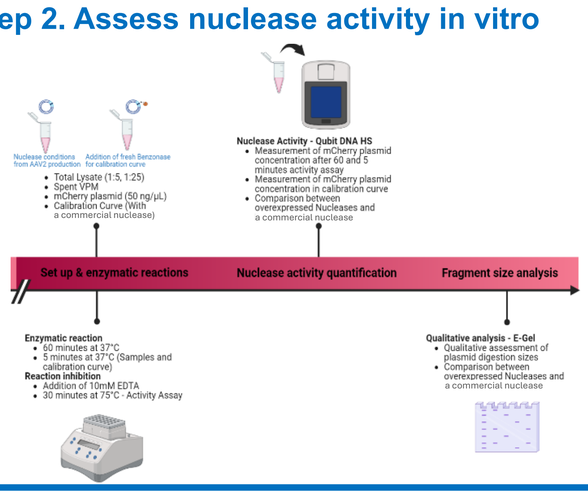



**Human DNaseI variants tested:**

- **DNaseI:** wt codon optimised
- **HuRNaseI:** used in combination with DNaseI
- **Hyperactive DNaseI** (3 residues mutated): Increased DNA-hydrolytic activity
- **Hyperactive / G-Actin resistant** (4 residues mutated): resistant to G-actin inhibition
- **Salt resistant:** resistant to salt inhibition
- **Salt / G-Actin resistant** (4 residues mutated): Resistant to salt and G-actin inhibition
- **Inactive DNase I** (7 residues mutated): used as negative control

Nuclease variants based on *Pan CQ et al, Journal of Biological Chemistry (1998).*

### Step 2. Assess nuclease activity in vitro



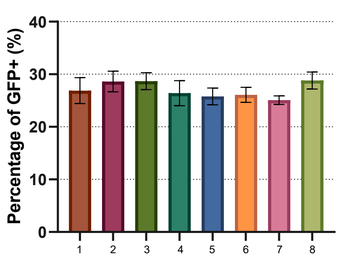

### Step 3. Measure viral titre and residual DNA

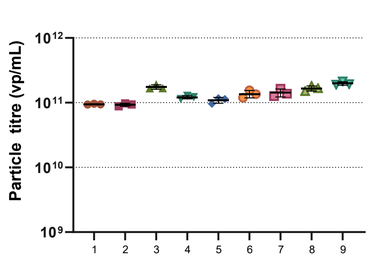
- ❑ Mini-DSP capture plates to remove residual nucleases
- ❑ Genome titre by RT-qPCR
- ❑ Particle titre by ELLA
- ❑ Residual DNA by WAKO/Picogreen and Host cell DNA qPCR (fragment size >136 bp)

## Results

### 1. Human nucleases successfully over-expressed in rAAV2 production

#### Seven nuclease variants were co-transfected during rAAV2 production



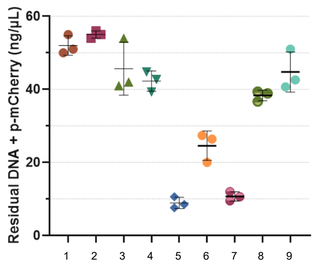

1. DNaseI
2. Hyperactive DNaseI
3. DNaseI + HuRNaseI
4. Inactive DNaseI
5. Hyperactive / G-Actin resistant
6. Salt resistant
7. Salt / G-actin resistant
8. mCherry only
9. mCherry + Commercial nuclease 1

For 60 min Activity assay:

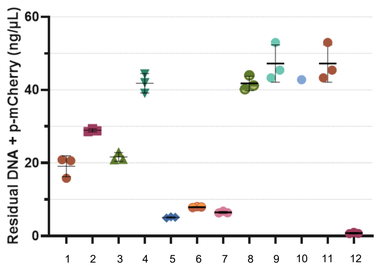
10. Undigested mCherry
11. *Commercial nuclease 1
12. *Commercial nuclease 2

#### Nucleases activity assessed in post-lysis rAAV2 harvest material

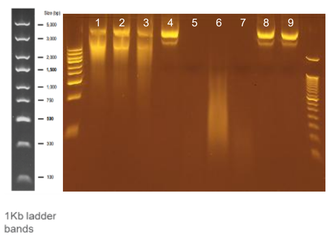
Activity in Lysate (5 mins) - 500 mM NaCl

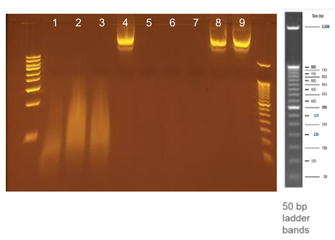



Activity in Lysate (60 mins) - 500 mM NaCl




**60 minutes incubation with 0.05 U/µL commercial nucleases was performed adding fresh enzyme to spent medium and digesting known quantity of mCherry plasmid*

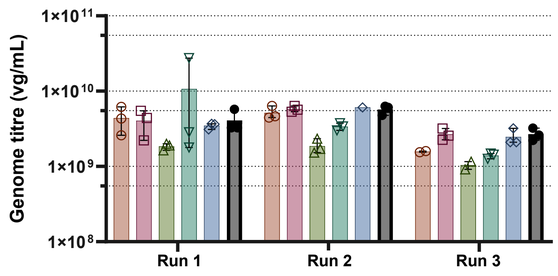


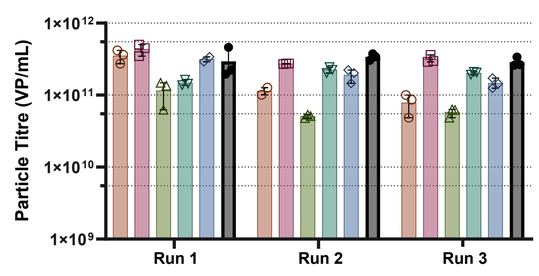




- Functional nucleases were produced from all variants tested during rAAV2 production
- Based on nuclease activity results in vitro, the DNaseI and Hyperactive / G-Actin resistant variants were selected for full characterization

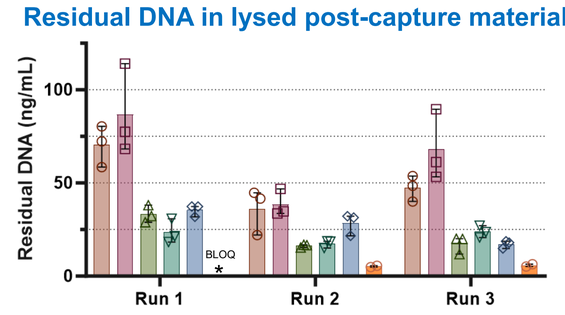
### 2. Short-listed nucleases effectively clear residual DNA in rAAV2 sup

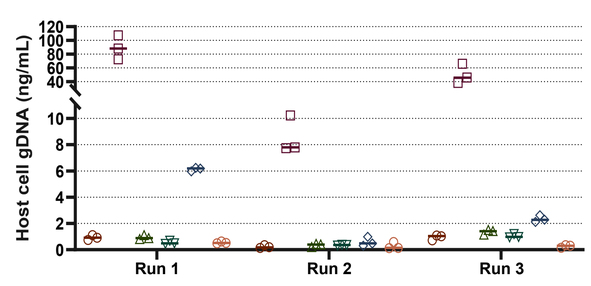
#### rAAV2 productivity in lysed post-capture material




#### Residual DNA in lysed post-capture material

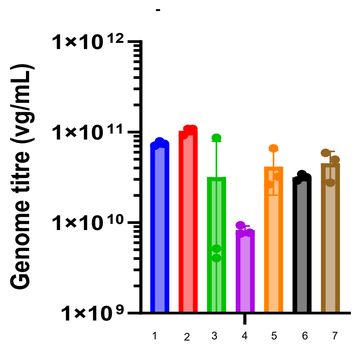


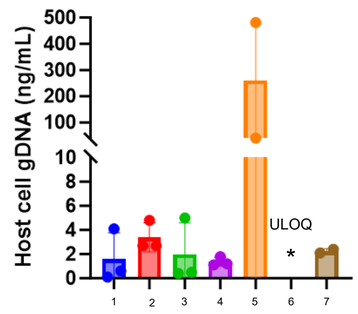




- The Hyperactive / G-Actin resistant effectively cleared residual DNA but it negatively impacted genome titre.
- The DnaseI variant consistently showed best performance for preserving rAAV2 titre and clearing residual DNA.

1. DNaseI
2. DNaseI + RNaseI
3. Hyperactive / G-Actin resistant
4. Hyperactive / G-Actin resistant + RNaseI
5. mCherry only
6. mCherry + Commercial nuclease 2

### 3. Nucleases can be co-expressed from the pHelper plasmid



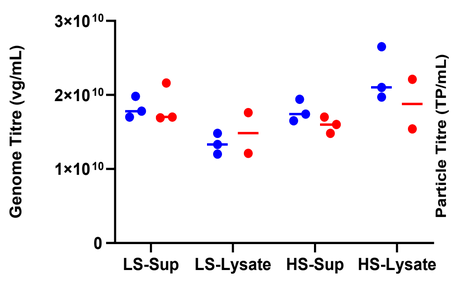

**Ratio (ITR:pHelper-DNaseI:RepCap)**

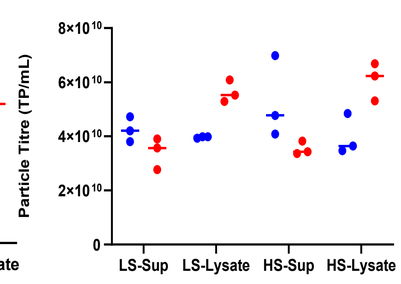
1. 2 : 9 : 3
2. 2 : 3 : 3
3. 2 : 1 : 3
4. 2 : 0.333 : 3
5. 2 : 0.111 : 3
6. pHelper-mCherry (2 :1:3)
7. pHelper-mCherry (2:1:3) + Commercial nuclease 2

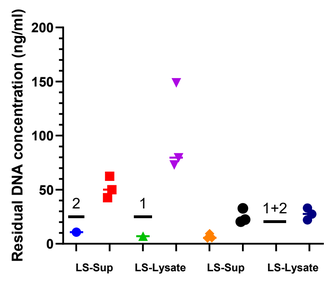
- The DNaseI was cloned into a mini-pHelper construct (Helper-DNaseI) to evaluate co-expression from one plasmid and transfected in VPC2.0 at different plasmid ratios, to identify the best condition for rAAV2 production. A pHelper-mCherry was used as control.
- The best performance was obtained with a ratio of 2:3:3 (ITR-pHelper-DNaseI-RepCap) as demonstrated by genome titre (pre-capture) and DNA clearance in post-lysis harvest culture.

### 4. Proof-of-concept of rAAV8 production with Helper_DNaseI construct

- Helper_DNaseI showed no negative impact on rAAV8 productivity.in 24 deep well plates
- Secreted DNaseI effectively removed residual DNA within the culture supernatant as demonstrated by WAKO/Picogreen analysis.




LS: Low salt concentration
HS: High salt concentration
Sup: Supernatant

pHelper-DNaseI  pHelper-mCherry

1 = Below lower limit of quantification (LLOQ)
2 = CV > 20%

## Conclusion

- ❑ Human nucleases can be co-overexpressed during rAAV2 and rAAV8 production without negatively impacting viral productivity..
- ❑ Post-capture host cell DNA analysis and PicoGreen residual DNA analysis indicated that the secreted nuclease effectively removed residual DNA and was comparable to commercial nucleases.
- ❑ These findings support the use of Helper_coHuDNAseI in a continuous harvest perfusion platform, where the secreted nuclease can treat the harvest product without negatively impacting productivity. This will reduce the cost of good, by replacing the commercial nuclease with the secreted nuclease produce by the cells.

Innovate UK

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***If you are interested in collaborating with us,*** *please, contact our team at info@ct.catapult.org.uk or ele.zucchelli@ct.catapult.org.uk*