# Assessment of an Automated, Multi-dimensional Digital PCR Workfow for the Rapid Evaluation of Recombinant AAV Genome Integrity

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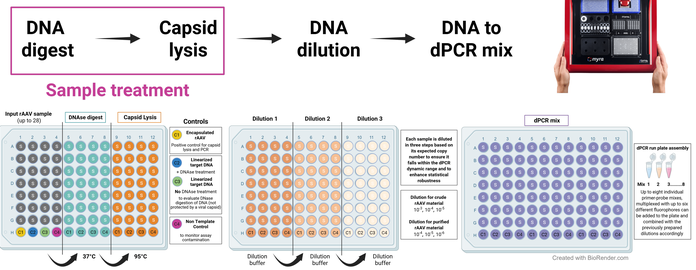
## Introduction

Digital PCR (dPCR) is considered the gold standard for rAAV genome quantifcation and plays a crucial role in monitoring improvements in virus production. Additionally, quantification of multiple targets along the rAAV genome enables assessment of genome integrity in the same dPCR run. Generating reliable and reproducible results with dPCR is essential - not only for comparing different samples within a single run but also for ensuring consistency across different runs, time points, and operators.

However, to achieve accurate measurements, standardized and gentle handling of samples during DNAse treatment, capsid lysis, and dilution is critical. We observed DNA fragmentation after capsid lysis is signifcantly affecting integrity assessment, leading to underestimation of intact genomes.

To address this, we developed an automated robotic workflow using the BMS Myra that covers DNA digestion, capsid lysis, sample-specifc dilutions, and the final dPCR reaction preparation. Our protocol allows for multiplexing with genome-specifc dPCR assays suitable for direct processing on the QIAcuity instrument for rAAV quantification and genome integrity assessment.

## Robotic workflow




## Assessments

- **Human vs. Robot Performance**
  Direct comparison of accuracy between manual and automated workflow.
- **Accuracy of Genome Integrity Assessment**
  Evaluation of genome integrity by dPCR using control samples with defined fragmentation percentages.
- **rAAV Quantification and Genome Integrity**
  Assessment of rAAV dilution accuracy and genome integrity before and after sample treatment.

## Results

### Human vs. Robot Performance

Three different rAAV samples were diluted and measured to compare the performance of the Myra robot versus a trained operator.

We observed no differences between the robot and the operator within the normal range of deviation observed in previously performed dPCR assays (around 10%)

**a) rAAV quantification in independent samples**

| | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| | copies/mL | | |
| Human | $2.31\ 10^{10}$ | $2.08\ 10^{10}$ | $2.65\ 10^{10}$ |
| Robot | $2.43\ 10^{10}$ | $1.91\ 10^{10}$ | $2.76\ 10^{10}$ |
| Difference | -5.2% | 8.2% | -4.2% |

**b) Difference in quantification over several dilutions**

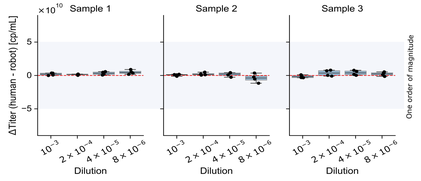



**Figure 1. a)** Table with quantification of three independent rAAV samples in copies/mL. dPCR quantification was performed either by a trained operator or the Myra robot. **b)** The difference in quantifications measured by a trained operator vs the Myra robot over different dilution factors.

### Accuracy of Genome Integrity Assessment

A transgene cassette encoding EGFP flanked by rAAV ITRs was digested with restriction enzymes to separate the DNA into three fragments corresponding to the CMV promoter, EGFP coding sequence, and polyA signal — each targeted by dPCR assays. The fragmented DNA was then mixed with intact (non-fragmented) DNA at defined ratios, ranging from 0% to 100% fragmentation in 10% increments.

Our results show accurate percentages of intact DNA across all ratios, which correlate consistently with estimated values in both 2 target (2D) and 3 target (3D) dPCR.

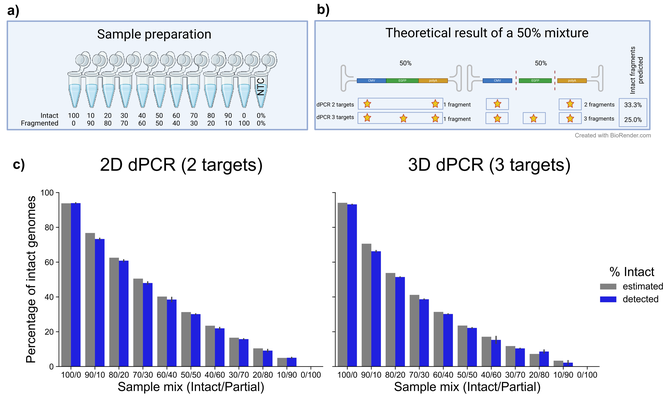



**Figure 2. a)** Sample preparation for ratios of intact vs fragmented DNA. **b)** Theoretical results for 2D (2 targets) and 3D (3 targets) dPCR in a 50% mixture. **c)** Estimated vs detected percentages of the prepared sample ratios in 2D and 3D dPCR assays.

### rAAV Quantification and Integrity

A single rAAV sample was diluted to $1.78x10^{11}$ copies/mL and then further serially diluted in a matrix using the Myra robot before and after sample treatment (DNAse treatment/capsid lysis). This was done to determine quantification and integrity consistency across sample dilutions before or after sample treatment.

**a) Concentration of purified rAAV diluted pre- and/or post sample treatment**

Pre DNAse and capsid opening dilution

| Post DNAse and capsid opening dilution — Factors | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 |
|---|---|---|---|---|---|---|---|---|
| 1 | 17788 | 8424 | 4520 | 2143 | 1028 | 506 | 266 | 131 |
| 1:2 | 7770 | 3894 | 2023 | 1022 | 482 | 228 | 133 | 52 |
| 1:4 | 3495 | 1681 | 878 | 465 | 214 | 116 | 60 | 28 |
| 1:8 | 1701 | 807 | 462 | 248 | 106 | 53 | 26 | 9 |
| 1:16 | 929 | 430 | 217 | 106 | 56 | 30 | 11 | 4 |
| 1:32 | 404 | 230 | 120 | 52 | 29 | 10 | 4 | 2 |
| 1:64 | 250 | 102 | 61 | 25 | 11 | 4 | 3 | 2 |
| 1:128 | 117 | 48 | 25 | 11 | 6 | 3 | 2 | 1 |

copies/µL

**b) rAAV quantification and genome integrity**

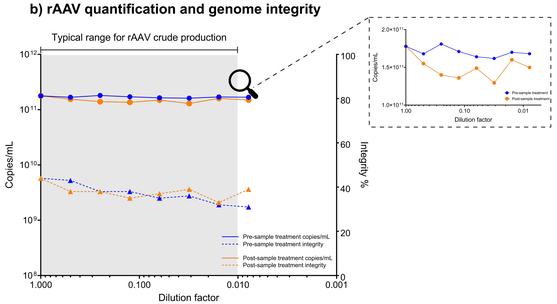



**Figure 3. a)** Table with raw concentrations of the same rAAV sample before or after sample treatment (DNAse and capsid lysis) over several dilutions. **b)** The final quantification and genome integrity of the same rAAV sample over several dilutions before or after sample treatment.

## Conclusions

Reduced hands-on time and ensured full traceability via automated logging. Results matched those from manual processing.

2D and 3D dPCRs reliably quantified intact-to-fragmented DNA ratios. As fragmentation can occur during sample treatment, a robotic approach may minimize variability in integrity assessments.

Consistent genome and integrity quantification across dilutions performed before or after capsid lysis, confirming method accuracy.