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AUTOMATION

End-to-end High-throughput Quantitative PCR-based rAAV Genome Titering Workflow Enabled by the Ginkgo Reconfigurable Automation Cart (RAC) Platform

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23 hr

Protocol run batch execution without any in-person monitoring

7,500

qPCR reactions processed with high-quality

>100K

Sample concentrations quantified to date

End-to-end High-throughput Quantitative PCR-based rAAV Genome Titering Workflow Enabled by the Ginkgo Reconfigurable Automation Cart (RAC) Platform

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INTRODUCTION

Quantitative PCR (qPCR) is a gold-standard DNA quantification method, which uses PCR DNA amplification in the presence of either a fluorescent DNA intercalating dye or a fluorescent DNA hybridization probe to measure an unknown DNA concentration in a reaction. It is a versatile laboratory technique, which can be further adapted to e.g. quantification of miscellaneous RNA species, gene expression profiling or genotyping.

Here, we present how Ginkgo leveraged qPCR to quantify the genome titer of recombinant adeno-associated virus (rAAV) particles. We describe how we developed a fully automated, qPCR-based rAAV genomic titering workflow on our RAC platform, including upstream pre-treatment of rAAVs (including rAAVs in crude mammalian cell lysate and purified rAAVs) and downstream data analysis to maximize human hands-off time. **We further show how our approach enabled workflow runs up to ~23 hrs long and end-to-end processing of up to ~7,500 samples, without any human intervention, and how it ultimately helped Ginkgo's Operations Team quantify DNA concentrations of >100,000 samples to date.**

rAAVs are among the most popular gene therapy viral gene delivery systems currently explored in clinical trials[3]. However, persistent challenges exist in enhancing their productivity and tissue-specific delivery[4]. We believe that development of early, pre-clinical stage high-throughput screening workflows, like the one presented in this paper, can help alleviate today's bottlenecks by enabling rapid prototyping of many alternative and potentially beneficial rAAV designs[1].

FULLY AUTOMATED BIOLOGICAL & DATA ANALYSIS WORKFLOW

The fully automated workflow consisted of the following five main biological protocol and data analysis steps (see **FIG. 1**). These were previously performed in a partially manual and partially automated manner (relying on two benchtop liquid handling workstations — Beckman Coulter Echo 525 and HighRes Biosolutions Prime — and a manual data retrieval and analysis process):

1. **DNase I rAAV sample pre-treatment:** Acoustic dispensing of a 2.5 µL rAAV sample (Echo 525, Beckman Coulter) and bulk dispensing of a 22.5 µL DNase I rAAV sample pre-treatment mix (Multiflo FX, Agilent), followed by a 15 min 37℃ incubation in a thermal cycler (Automated Thermal Cycler, Thermo Fisher Scientific)
2. **Proteinase K rAAV sample pre-treatment:** Bulk dispensing of a 5 µL proteinase K rAAV sample pre-treatment mix into 25 µL of a DNase I-pre-treated rAAV sample (from step 1) (Multiflo FX, Agilent), followed by a 15 min 56℃ incubation in a thermal cycler and a 15 min 95℃ enzyme heat inactivation (Automated Thermal Cycler, Thermo Fisher Scientific)
3. **Tween 20 rAAV sample dilution:** Bulk dispensing of a 45 µL 10% v/v Tween 20 diluent into empty 384-well Echo Qualified Polypropylene Microplates (Multiflo FX, Agilent) and a 5 µL DNase I and proteinase K-pre-treated rAAV sample (from step 2) mix-in, for a 10-fold rAAV sample dilution (Bravo, Agilent)
4. **qPCR reaction setup & thermal cycling:** Acoustic dispensing (generating 3 qPCR technical replicates) of 2.5 µL Tween 20-diluted unknown rAAV samples (from step 3), as well as untreated qPCR standards, and qPCR positive and negative controls into target 384-well qPCR microplate wells (Echo 525, Beckman Coulter), followed by bulk dispensing 7.5 µL of a qPCR reaction master mix (Multiflo FX, Agilent) and quantitative thermal cycling - 1 cycle of 95°C for 15 min (prolonged heat denaturation helps with the final release of genomic DNA from the rAAV capsids), 40 cycles of 95°C for 15 sec, 60°C for 30 sec and fluorescence readout (CFX Opus, BioRad)
5. **Data retrieval & analysis:** Automated retrieval of vendor software-generated output qPCR data files (upon qPCR thermal cycling completion) via Ginkgo's Events Processing Pipeline (EPP), and automated data analysis via Ginkgo's custom data analysis pipeline

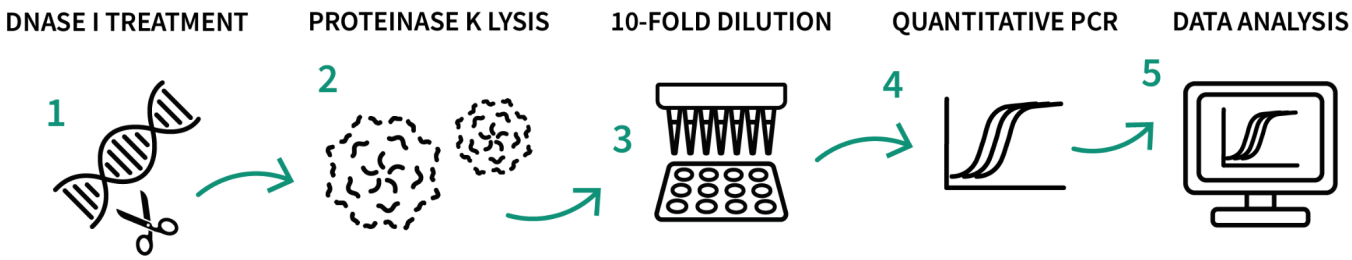

FIGURE 1. **Fully Automated rAAV Genomic Titering Workflow**

FIG. 2 shows Ginkgo's Automation Control Software (ACS) biological workflow scheduling results: the biological part of the workflow was divided into 2 independently re-usable ACS protocols, which were altogether **executed in ~23 hrs, processing 7,488 samples (2,240 mammalian cell lysate technical replicates x 3 qPCR reaction technical replicates = 6,720 unknown concentration rAAV samples and 768 qPCR standards and controls) without any in-person monitoring**. The ACS protocol runs ran outside our Operations Team working hours with no runtime errors, **demonstrating the potential of our RAC platform in maximizing scientific output not only per working shift, but also per 24-hour day, thus reducing the scientific discovery cycle turnaround times.**

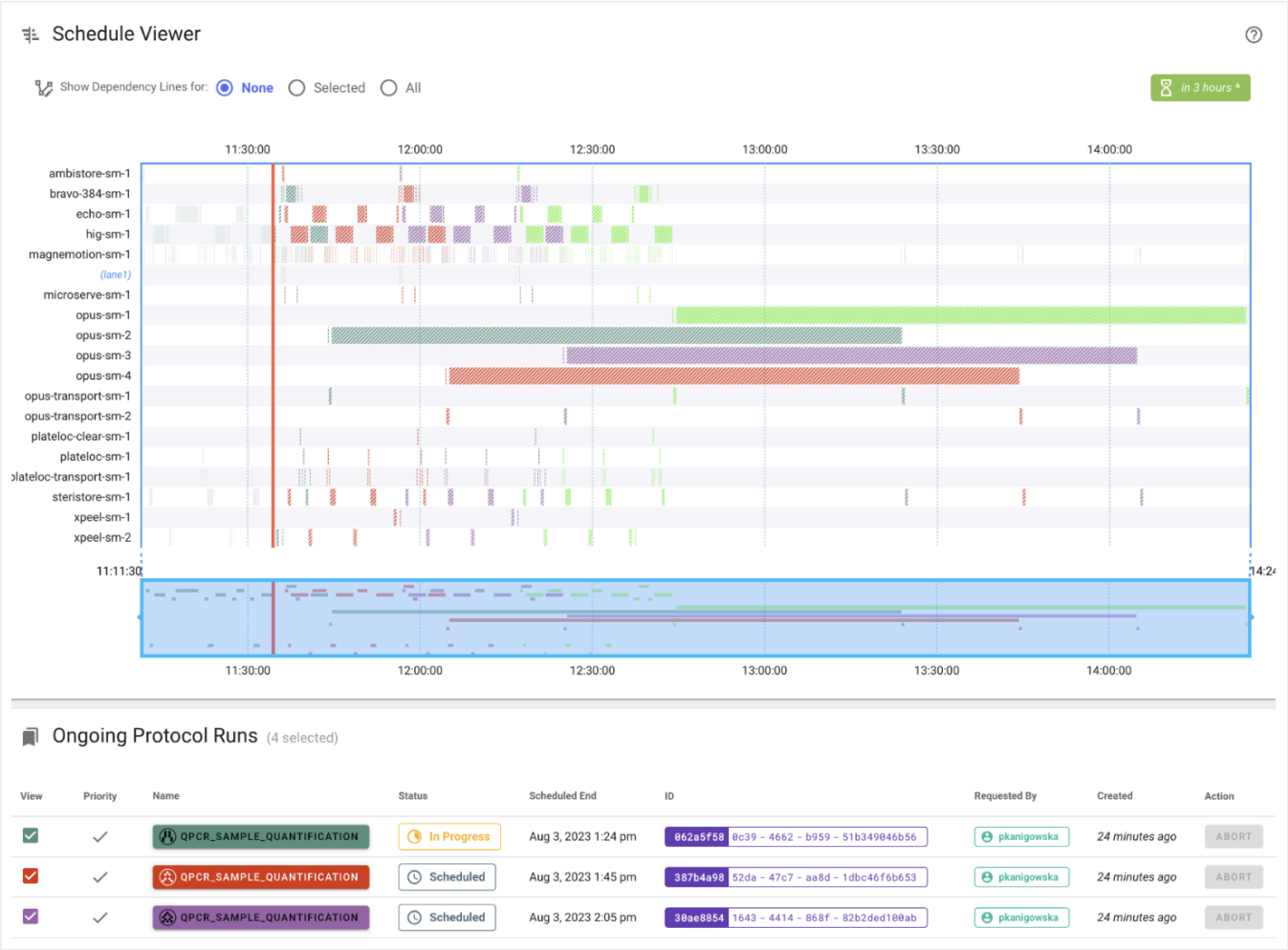

FIGURE 2. Automated Control Software (ACS) Schedule Viewer Here displaying a subset of "qPCR sample quantification" ACS protocol runs being scheduled. The RAC system harbored four (4) qPCR machines in total.

Ginkgo's RAC platform extends beyond simple robotic execution of different biological protocol steps, offering additional functionalities, which can greatly improve user's qPCR quantification experience. For instance, upstream, our ACS BioRad CFX Opus qPCR instrument driver enables fully customizable, "on the fly" qPCR instrument input file creation via straightforward specification of ACS protocol inputs, as opposed to manual input file setup and import onto qPCR instrument's PC. Downstream, upon each qPCR instrument run completion, our cloud-based Events Processing Pipeline (EPP), enables processing biologically-relevant events automatically - in this case extracting, associating with the correct Ginkgo LIMS samples and uploading the vendor

software-generated output qPCR data to Ginkgo's internal two-stage qPCR data analysis engine (performing "primary" qPCR standard curve and controls QC and "secondary" rAAV genome titer calculations, with the qPCR data analysis results being stored in Ginkgo's internal data lake).

QUANTITATIVE PCR STANDARDS & CONTROLS QUALITY CONTROL

All 24 qPCR standard curve slopes ranged between -3.3 and -3.2, which corresponded to 100% and 105% qPCR reaction efficiencies, respectively (see **FIG. 3**). The qPCR standard curve coefficients of determination (R2) were all ≥0.997, which indicated high accuracy of our manual qPCR standard 10-fold serial dilution, low variability of RAC system-generated qPCR standard technical triplicates, and overall high RAC system liquid handling quality. All qPCR positive and negative controls passed our

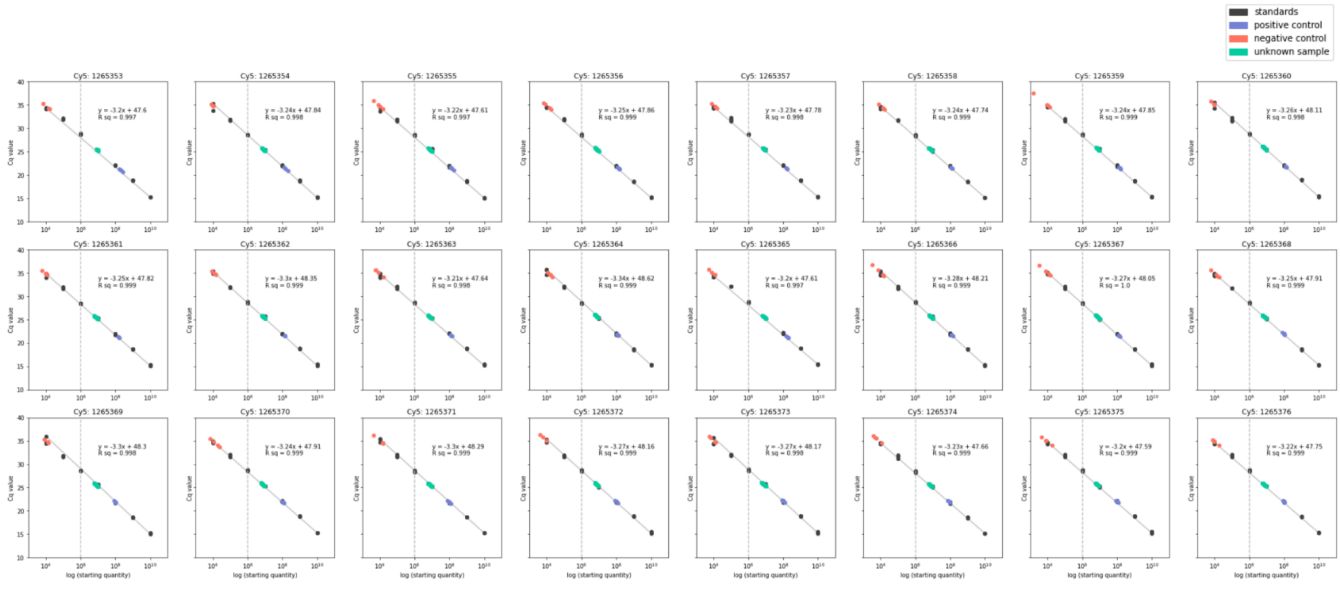

FIGURE 3. qPCR Standard Curve and Positive & Negative Controls QC in All 24 Microplates All qPCR standard curves and positive / negative controls are passing our quality control.

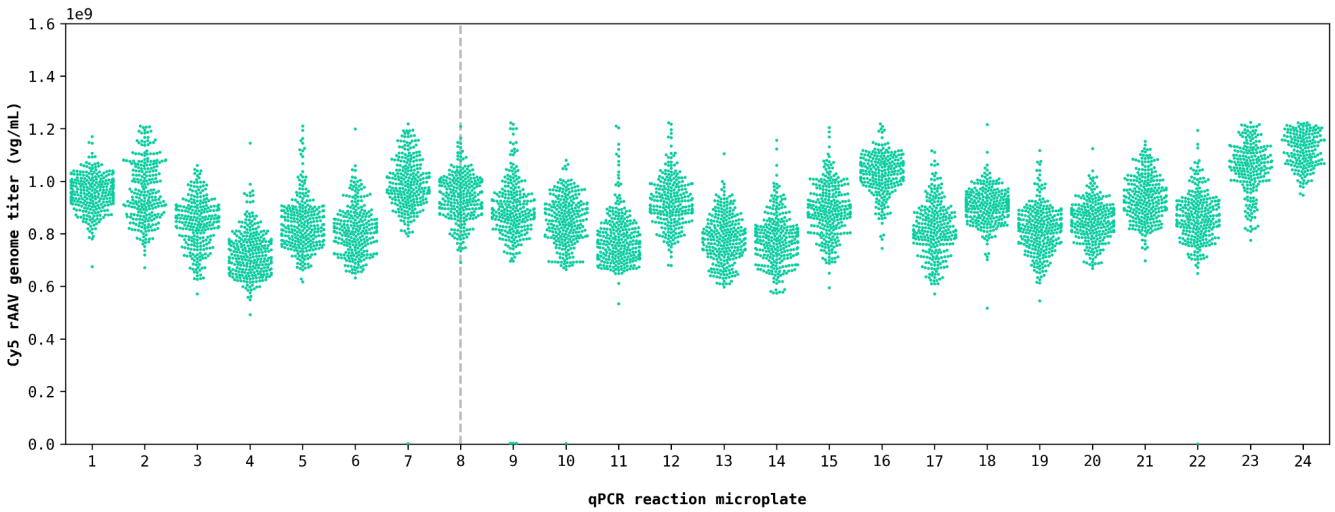

FIGURE 4. rAAV Genomic Titer Trends across Microplates qPCR reaction microplate data organized across the x-axis, rAAV genome titers on the y-axis; The dashed vertical line indicates the largest qPCR workflow run size reliably achievable on an alternative integrated lab automation system at Ginkgo.

internal QC criteria (each negative control Cq value greater than the lowest concentration standard Cq values and each positive control within the qPCR standard curve Cq value range). We did not observe any declining qPCR standard quality trends in the course of our workflow runs, which **additionally highlighted timely and error-free RAC system execution, as well as beneficial effects of including temperature-controlled (here 4℃ temperature) input / output RACs within the RAC system design. Temperature-controlled storage is particularly valuable for enabling large workflow runs, which require more attention to sample and reagent stability.**

rAAV TITERING PRECISION

High-throughput screening assays require high precision to differentiate between signal and noise. Therefore, our experiment sought to assess precision of different fully automated biological protocol steps (rAAV sample pre-treatment and DNA payload qPCR quantification), as well as identify any potential microplate processing biases.

The mean rAAV genome titer estimated, across 24 microplates and 6,720 unknown genome titer rAAV samples (qPCR and rAAV sample pre-treatment technical replicates) was 8.90×10^8 viral genomes (vg) / mL, while genome titer %CV was 13% (across the corresponding 10th - 90th percentile non-outliers). 2,239 out of 2,240 qPCR

technical triplicate groups exhibited %CVs ≤ 25% (see **FIG. 4**). We did not observe any major intra-microplate effects, which can e.g. arise from sample evaporation around microplate edges (see **FIG. 5**). **Overall, these results indicate that Ginkgo's RAC system-based workflow has higher precision (with more hands-off time) than recently published studies of similar, manually processed samples[2].**

CONCLUSIONS

High-throughput rAAV genome titering workflows can further accelerate rAAV-based gene therapy advancements. **This paper demonstrates how our RAC platform can be leveraged to develop such workflows and robustly execute them at a large-scale, with minimal human involvement.**

FIGURE 5. rAAV Genomic Titer Trends within Microplates Microplate quadrants 1-3 (excluding column 24) are populated with unknown DNA payload concentration samples - qPCR data shown (quadrant 4 and column 24 are populated with qPCR standards and positive / negative controls, respectively - qPCR data not shown); Here, qPCR data of the 6th microplate processed is shown (mean genome titer = 8.49×10^8 vg / mL, %CV = 8.71, no observable intra-microplate effects).

REFERENCES

1. Kontogiannis, T., Braybrook, J., McElroy, C., Foy, C., Whale, A.S., Quaglia, M. and C Mark Smales (2024). Characterization of AAV vectors: A review of analytical techniques and critical quality attributes. Molecular Therapy - Methods & Clinical Development, [online] 32. doi:https://doi.org/10.1016/j.omtm.2024.101309.

2. Meierrieks, F., Kour, A., Pätz, M., Pflanz, K., Wolff, M.W. and Pickl, A. (2023). Unveiling the secrets of adeno-associated virus: novel high-throughput approaches for the quantification of multiple serotypes. Molecular Therapy - Methods & Clinical Development, [online] 31. doi:https://doi.org/10.1016/j.omtm.2023.101118.

3. Pupo, A., Fernández, A., Low, S.H., François, A., SuárezAmarán, L. and Samulski, R.J. (2022). AAV vectors: The Rubik's cube of human gene therapy. Molecular Therapy, [online] 30. doi:https://doi.org/10.1016/j.ymthe.2022.09.015.

4. Wang, J., Gessler, D.J., Zhan, W., Gallagher, T.L. and Gao, G. (2024). Adeno-associated virus as a delivery vector for gene therapy of human diseases. Signal Transduction and Targeted Therapy, [online] 9(1), p.78. doi:https://doi.org/10.1038/s4139202401780w.

MATERIALS

- 8 384-well Echo Qualified Polypropylene Microplates pre-filled with technical replicate mammalian cell lysates - HEK293-derived suspension mammalian cell line subjected to a triple, PEI-based transfection (with a cis-acting plasmid encoding a 4.2 kb inverted terminal repeats (ITR)-flanked target gene expression cassette, and trans-acting rAAV9 Rep/Cap and helper plasmids) and lysed (72 hrs post-transfection), using 0.2% v/v Triton X-100
- 1 384-well Echo Qualified Polypropylene Microplates pre-filled with purified rAAV9 qPCR standards (104 - 1010 vg / mL), and positive and negative controls - untransfected HEK293-derived suspension mammalian cell line lysate spiked with purified rAAV9 particles and nuclease-free water, respectively
- 8 empty 384-well PCR microplates (for DNase I and proteinase K rAAV sample pre-treatment)
- 8 empty 384-well Echo Qualified Polypropylene Microplates (for Tween 20 rAAV sample dilution)
- 24 empty 384-well qPCR microplates (for qPCR reaction setup and thermal cycling)
- DNase I rAAV sample pre-treatment mix (0.2 U / µL DNase I and 1X DNase I buffer - from Thermo Fisher Scientific, 0.1% v/v Pluronic F-68)
- Proteinase K rAAV sample pre-treatment mix (0.5 mg / mL proteinase K - from Thermo Fisher Scientific, 0.2% v/v SDS)
- 10% v/v Tween 20 rAAV sample diluent
- qPCR reaction master mix (1X TaqMan™ Fast Advanced Master Mix - from Thermo Fisher Scientific, rAAV particle DNA payload ITR regions-targeting 0.5 µM fwd & rev primer and Cy5 fluorescent dye-labeled probe mix - from Integrated DNA Technologies)