GINKGO AUTOMATION

High-throughput Screening of HiBiT-tagged Proteins on Ginkgo's Reconfigurable Automation Cart (RAC) Platform

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80%

Reduction in hands-on time with integrated RAC vs. standalone lab automation

7,680

Protein samples quantified without any in-person monitoring and intervention

Only 2%

Average fold difference between normalized protein concentrations obtained from the previous, semi-automated protocol vs. the new RACs protocol (fully-automated)

High-throughput Screening of HiBiT-tagged Proteins using Ginkgo's Reconfigurable Automation Cart (RAC) Platform

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INTRODUCTION

High-throughput screening (HTS) is a widely adopted methodology in the fields of drug discovery and synthetic biology which heavily relies on the latest advances in laboratory automation. The advantages of screening thousands of samples in parallel include faster selection of samples of interest - "hits", and rejection of remaining samples from further, more costly analysis[1]. In particular, the HiBiT assay (developed by Promega) is a valuable screening method enabling quick assessment of protein expression across a large number of candidate microbial and mammalian strains.

Here, we demonstrate how Ginkgo Automation's Reconfigurable Automation Cart (RAC) platform was leveraged to fully automate the HiBiT assay, ultimately generating high-quality data at large scale - nearly 10,000 samples - and thus enabling powerful hit identification, with 80% less hands-on time versus standalone lab automation instrumentation. Additionally, we show the ease with which users can onboard new capabilities and protocols onto a RAC system. **In this application note, a new capability - plate reading, was enabled on an existing RAC system through a straightforward, single RAC addition, which took just 5 hours. This capability was added without the need for any significant lab automation system re-design.**

OVERVIEW OF THE FULLY AUTOMATED ASSAY

In the HiBiT assay, a detection reagent is added to cell culture supernatant or cell lysate containing the protein of interest (depending on whether the protein is intracellular or secreted). The protein of interest is endogenously expressed with a 11-amino acid HiBiT tag, while the detection reagent contains a proprietary LgBiT protein. This LgBiT protein binds with high affinity to the HiBiT tag to form a functional enzyme that generates bioluminescence in the presence of a furimazine substrate, which is also present in the detection reagent. The bioluminescent signal generated can be measured with a plate reader and is proportional to the HiBiT-tagged protein concentration, allowing for comparison of protein expression between different samples. The HiBiT assay is extremely sensitive, relatively straightforward and fast, lending itself well to a high-throughput protocol format[2].

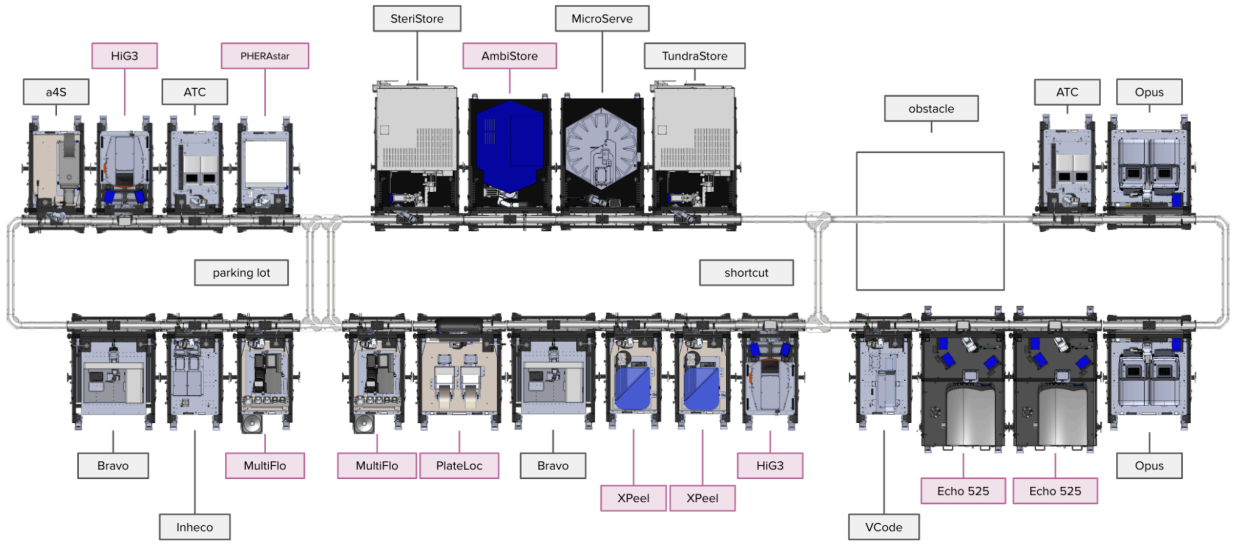


Fig 1 Internal Ginkgo RAC System Used to Fully Automate the HiBiT Assay; Devices relevant to the assay onboarding, optimization and execution are highlighted in pink (plate reading - PHERAstar, BMG Labtech; centrifugation - HiG3, BioNex; ambient temperature random access storage - AmbiStore, HighRes Biosolutions; acoustic dispensing - Echo 525, Beckman Coulter; microplate seal peeling - XPeel, Azenta; microplate sealing - PlateLoc, Agilent; bulk dispensing - MultiFlo FX, Agilent); Each RAC accommodates one device type (i.e. multiple copies of the same device type can be installed onto one RAC); RACs are connected via the MagneMover LITE (Rockwell Automation) transport system.

Prior to launching protocols on our RAC system (see Fig 1), “sample” and “standards” source microplates were loaded into the ambient storage RAC. Here, “sample” source microplates contained *K. phaffii* (formerly *P. pastoris*) cell supernatant with 4 different ~60 kDa HiBiT-tagged

proteins, while the “standards” source microplate contained a HiBiT control protein dilution series. In order to calculate unknown protein sample concentrations through the derived standard curve interpolation, standards were technically replicated in each “assay” destination microplate, with 2 replicates per standard concentration.

The **fully automated** HiBiT assay consisted of the following main steps (see Fig 2):

1. **Sample and standards transfer and dilution in the destination assay microplate** - 5 µL of PBS buffer were bulk dispensed (using Agilent MultiFlo FX) into all 384 wells of empty destination assay microplates (Revvity ProxiPlates™). Sample and standards source microplates were centrifuged at 1000 x g for 30 s (using BioNex HiG3), followed by an acoustic dispense of 100 nL of samples and standards into the newly PBS-filled destination assay microplates (with Beckman Coulter’s Echo 525). Sample source microplates were transferred one-to-one to destination assay microplates, while one standards source microplate was transferred to all destination assay microplates.
2. **Addition of the detection reagent and incubation of destination assay microplates** - 5 µL of pre-prepared and room temperature detection reagent were bulk dispensed (using Agilent MultiFlo FX) into all wells of all destination assay microplates generated in Step 1, followed by a 1000 x g centrifugation for 30 s (using BioNex HiG3) and a 10 minute room temperature incubation.
3. **Quantification of output bioluminescence on a plate reader** - bioluminescence was measured in each well of all destination assay microplates (using BMG Labtech PHERAstar FSX). Output data was automatically extracted and uploaded as sample metadata to Ginkgo’s Laboratory Information Management System (LIMS) via Ginkgo’s Event Processing Pipeline (EPP).

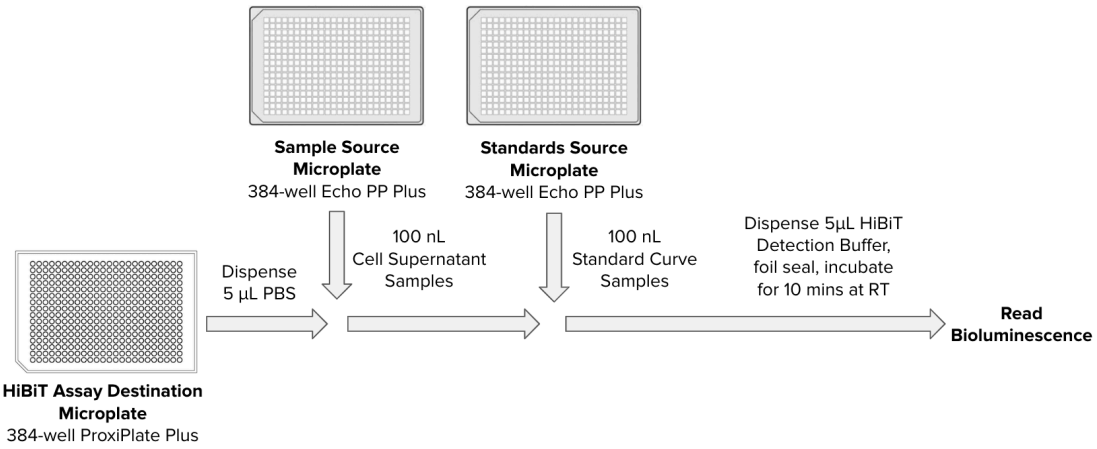


Fig 2 Steps of the Fully Automated HiBiT Assay; Samples and standards are acoustically dispensed into pre-filled assay destination microplates, followed by the HiBiT Detection Buffer addition and bioluminescence readout.

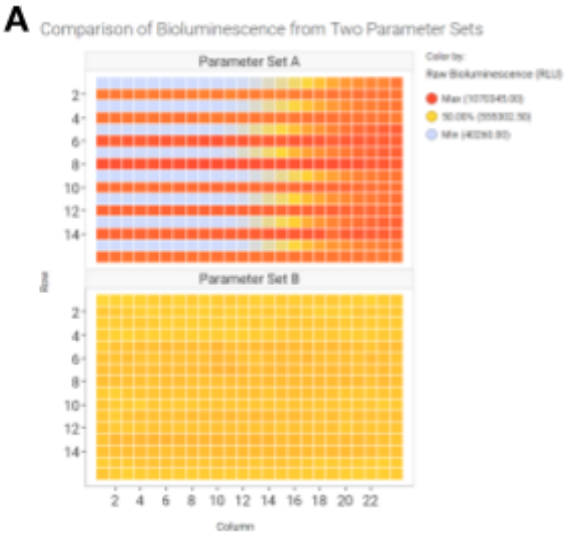
THE RAC PLATFORM ENABLES RAPID NEW CAPABILITY AND ASSAY ONBOARDING AND OPTIMIZATION

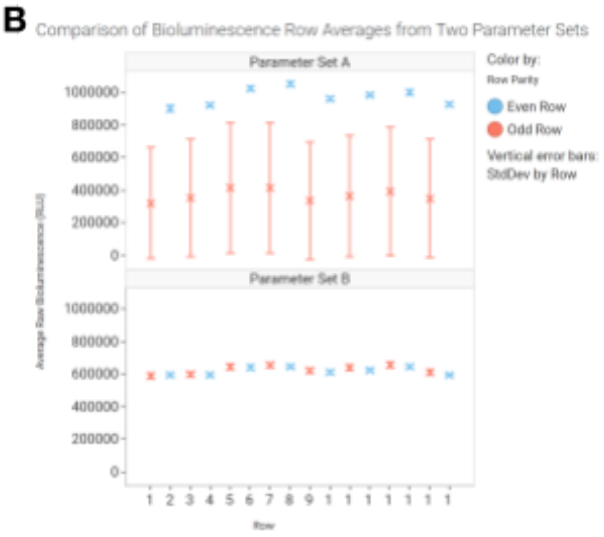
In order to onboard the HiBiT assay and other plate reading-based protocols onto our existing RAC system at Ginkgo - originally built to serve qPCR and NGS library prep protocols - we simply added one additional RAC to the pre-existing RAC system, which contained the BMG Labtech's PHERAstar FSX plate reader (see Fig 1). **It took 3 hrs to physically add the new RAC to the existing RAC system and an additional 2 hrs to complete RAC system reconfiguration online, from within the Catalyst ACS (Automation Control Software) Configuration Center. Neither a lab automation system re-design nor robotic arm re-teaching was required.**

Ginkgo's RAC platform uniquely features not only hardware, but also software modularity, which was key in enabling rapid HiBiT assay onboarding and optimization. In particular, the protocol writing experience is streamlined by grouping granular driver and transport commands into operations, which can then be linked together to describe the protocol. This abstracts away the complexity of programming automation, enables reuse between protocols, and allows targeted validation of protocol steps.

This architecture helped us expedite the process of protocol onboarding and optimization on the newly configured RAC system. As an example, during protocol development, we used standalone instrument operations to test different bulk dispensing parameters to rapidly resolve

a row-wise liquid handling inaccuracy pattern (see Fig 3). A bulk dispense operation was used to sequentially dispense a HiBiT reaction buffer and a diluted HiBiT control protein to multiple assay microplates, with a different set of parameters used per each assay microplate, so that any spatial data artifacts identified could be traced back to unit bulk dispenser parameters. A single RAC, disabled from any ongoing integrated RAC system protocol execution, was used during this protocol development and controlled via a web application (see Fig 3C and 3D). Subsequently, the assay microplates were processed with a generic plate reading protocol on the RAC system, with plate reader measurements automatically uploaded to the data warehouse. Since individual instrument operations perform identically to operations within our end-to-end HiBiT protocol, finalized protocol development parameters were simply copied to the full production protocol parameter set with confidence that the instrument behavior would be identical.




B
Comparison of Bioluminescence Row Averages from Two Parameter Sets
Parameter Set A
Parameter Set B
Color by:
Row Parity
Even Row
Odd Row
Vertical error bars:
StdDev by Row
Average Raw Bioluminescence (RLU)
1000000
800000
600000
400000
200000
0
1 2 3 4 5 6 7 8 9 1 1 1 1 1 1 1 1
Row


C
Recipes
Runner
Cycle Runner
Devices
Camera
Data Files
Extensions
RUN

Fig 3 Rapid Optimization of Bulk Dispensing Parameters Facilitated by Software Modularity of Ginkgo's RAC Platform; A row-wise bulk dispensing inaccuracy pattern is rapidly resolved by isolated testing and optimization of bulk dispensing parameters (**A & B**), via the MultiFlo FX ACS Application (**C**) and standalone-mode RAC execution of the corresponding instrument operations (**D**); The bioluminescence plate reader data is automatically extracted and uploaded into Ginkgo's LIMS, as sample metadata, via Ginkgo's Event Processing Pipeline, and instantaneously viewable via a TIBCO Spotfire web dashboard (**A & B**).

THE FULLY-AUTOMATED ASSAY INCREASES WALKAWAY TIME AND YIELDS COMPARABLE RESULTS TO THE PREVIOUS SEMI-AUTOMATED ASSAY

There is a large variety of robotic instruments available that can automate individual steps of a given protocol, such as liquid handlers or acoustic dispensers. However, scientists still need to operate these instruments, set up, remove, and transport labware, and troubleshoot runtime errors. This semi-automated form of scientific work, where instruments are used in a "standalone mode", leads to scientists not being able to walk away from the protocol execution to engage in other valuable work. **The RAC platform enables fully-automated, "integrated mode" protocol execution, where timely execution of individual protocol steps and labware transport is fully controlled by an overarching software and hardware**

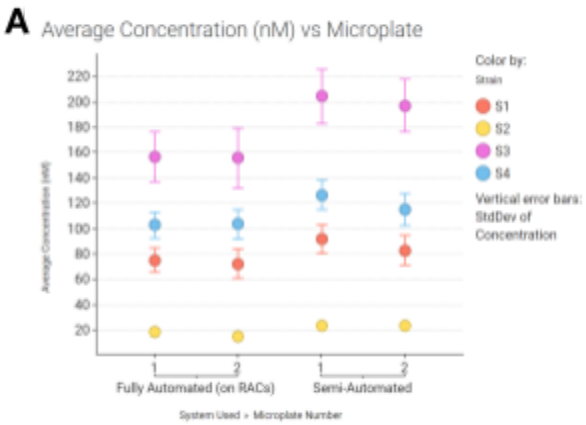
infrastructure. In addition, Ginkgo Automation engineers provide remote monitoring and proactive error resolution services (as part of the Catalyst Flow subscription), enabling scientists to truly walk away from protocol execution and confidently expand past typical working hours.

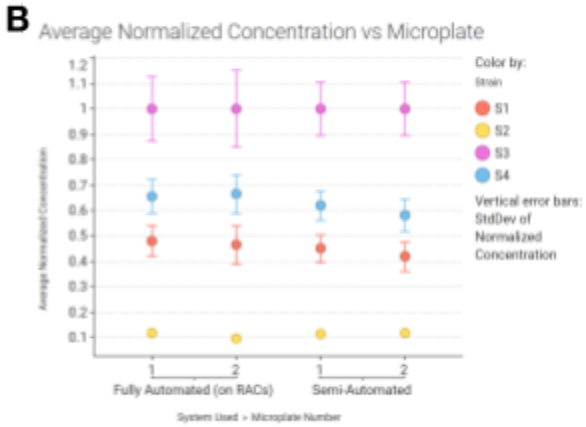
Transitioning from semi- to fully-automated assay execution requires careful comparison of data quality between the two modes of execution. To conduct such a comparative study, we prepared four positive control *K. phaffii* (formerly *P. pastoris*) cell cultures (S1, S2, S3 and S4), each expressing a different HiBiT-tagged protein, and one negative control cell culture (S5). The corresponding supernatants, alongside blank PBS samples, were re-arrayed in a checkerboard pattern into one sample source microplate, which was next assayed both on the RAC system (fully-automated execution) and on manually-operated robotic instruments (semi-automated execution), producing two destination assay microplates per execution mode.

To assess measurement agreement between the two datasets, a Bland-Altman analysis was conducted. The measurements from each dataset were paired by sample well and microplate processing order, and the geometric mean and ratio of each pairing were plotted on the x and y axis, respectively. The geometric mean is an estimation of the true value, while the ratio is an estimation of the measurement error - thus, the Bland-Altman analysis allows us to confirm no biased relationship between the two[3]. Bland and Altman (1986) and Iversen *et al.* (2012) proposed that two datasets demonstrate good agreement if they meet the following criteria: the Minimum Significant Ratio (MSR, the smallest statistically significant ratio) is less than 3, the Mean Ratio (MR, the average fold difference between the two datasets) is between 0.67 and 1.5, the min and max Limits of Agreement (LsA, within which 95% of the ratios should fall, if they are normally distributed) are between 0.33 and 3, and there are no unexpected trends[4].

In our assessment, the MSR was 1.40, the MR was 1.26 (i.e. the average fold difference between the two datasets was 26%), the min and max LsA were 0.9 and 1.76 (96% of measurement pairs had ratios between 0.9-1.76, indicating the expected normal distribution), and no bias was observed in the Bland-Altman plot (see Fig 4C). Statistics obtained from our assessment thus revealed good agreement between the two execution modes. **Additional normalization of all protein concentrations in each assay microplate to the average concentration of the highest-expressing strain (S3) in the same microplate yielded an MR of 1.02 (representing a 2% average fold difference between the two normalized datasets), demonstrating high reproducibility in relative protein expression levels measured by the two assay execution modes.** The technical replicate %CVs per strain and per assay microplate were all ≤16.2% (N = 56). No signal was detected in any of the negative control (S5)

or blank samples, indicating no cross-contamination. **Therefore, the fully-automated assay execution on RACs generated results consistent with the previous semi-automated assay, at the same time unlocking meaningful savings in scientist hands-on time (~2 hours in this case).**





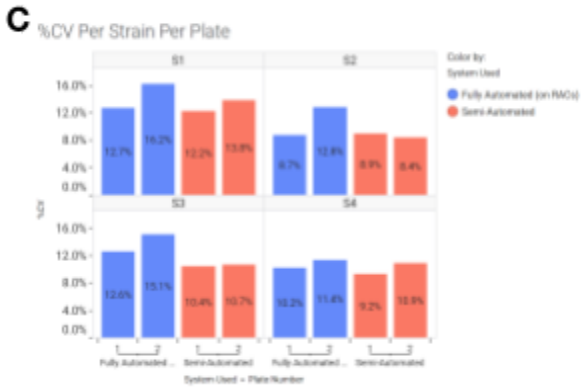


Fig 4 Measurement Agreement Analysis between the Two Execution Modes - Fully- and Semi-automated; Average technical replicate HiBiT-tagged protein concentration per strain, per assay microplate (N=56) (**A**); Average HiBiT-tagged protein concentrations per strain, per assay microplate (N=56), normalized to the in-microplate average S3 strain HiBiT-tagged protein concentration (**B**); %CVs of technical replicate HiBiT-tagged protein concentrations measured per strain, per assay microplate (N=56) (**C**); Bland-Altman plot (**D**).

THE FULLY-AUTOMATED ASSAY ENABLES INCREASED ASSAY SCALE, AT THE SAME TIME ENSURING ASSAY QUALITY

Having confirmed that the new fully-automated assay generates data consistent with the previous semi-automated assay, we next focused on testing its performance at large scale, typical for a high-throughput screen. We prepared a new sample source microplate with the same sample contents and layout as in the prior experiment, and used it to generate 20 destination assay microplates (totalling 7,680 assay measurements). **The setup and cleanup of the large scale assay on the RAC system took < 25 min, with no in-person monitoring or intervention needed during runtime. This represents an 80% reduction of hands-on time compared to semi-automated execution, which requires 2 hours of hands-on time for a run of equivalent scale. The average execution time per assay microplate was 20 min, with the processing of assay microplates being parallelized. We confidently ran the assay past typical working hours, thanks to the extensive coverage of our ticketless, proactive error resolution and troubleshooting service, Catalyst Flow.**

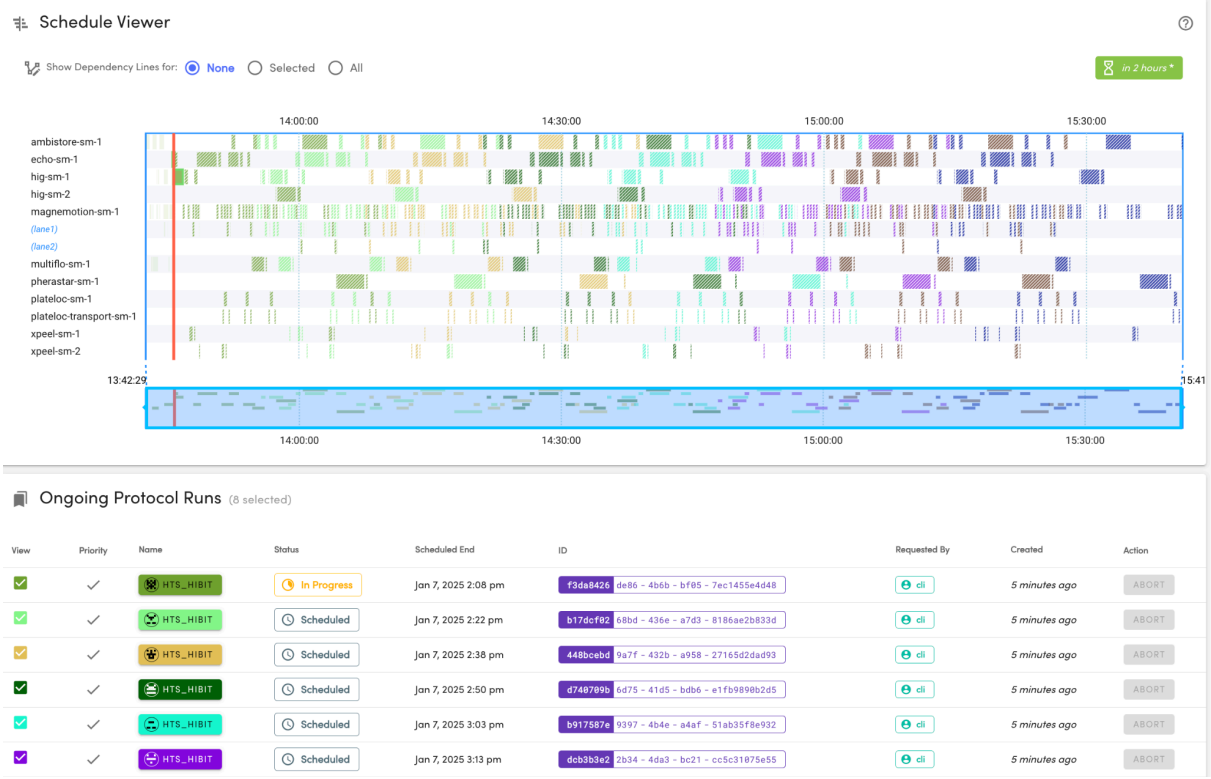
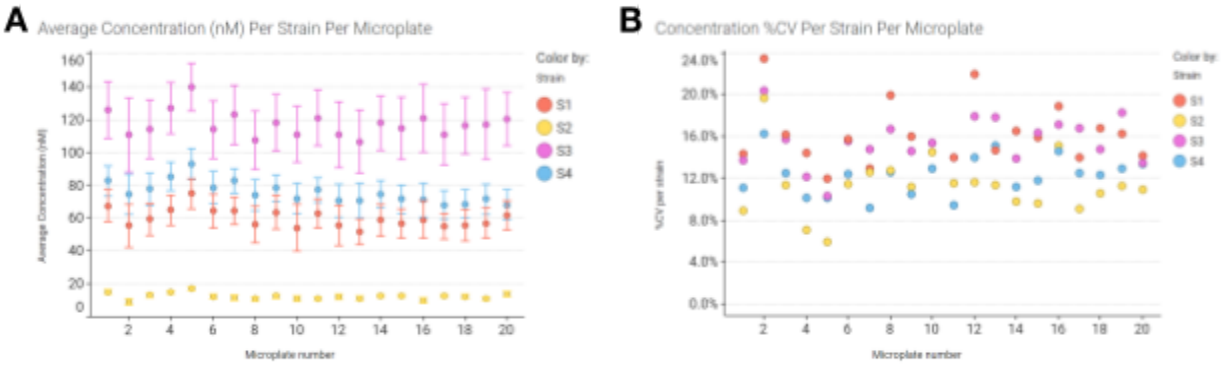


Fig 5 The Catalyst ACS Schedule and Protocol Run Queue View; The fully-automated assay execution consisted of 20 protocol runs, each processing one destination assay microplate, to allow flexible “continuous flow” protocol run batch scheduling (respecting pre-defined time constraints) and management, via the Catalyst ACS Scheduler and Broker.

Data analysis showed consistent and high-quality output data for all standard, positive and negative control samples in all destination assay microplates (see Fig 6). Strain rankings were also consistent in every plate. Per-microplate, per-strain technical replicate %CVs were < 20% in 90% of microplates (N=56). The correlation coefficient for all microplate standard curves was ≥ 0.97, implying high-accuracy and high-precision liquid handling of each standard curve replicate on the RAC system (see Fig 6C). Negative control and blank samples again did not exhibit any signal, confirming no cross-contamination during the run.

In order to estimate microplate-to-microplate reproducibility within a batch run of a protocol, and to confirm the absence of any unwanted trends, we calculated the MR and MSR of the absolute concentrations, as well as the S3 strain-normalized concentrations, between three pairs of microplates: microplate 1 (processed first) and microplate 10, microplate 10 and microplate 20 (processed last), and microplate 1 and microplate 20. The results, summarized in Fig 6D, show that the RAC system produced consistent data at the beginning, middle, and end of our run. **Altogether, the output HiBiT-tagged protein concentration data and quality metrics demonstrate that the HiBiT assay developed and executed on the RAC platform can reproducibly assess protein concentrations across large batches of samples.**



C

Assessment of within-run reproducibility	MR (absolute protein concentrations)	MR (S3 strain -normalized protein concentrations)	MSR (equal between absolute and normalized data)
microplate 1 vs microplate 10	1.22	1.08	1.48
microplate 10 vs microplate 20	0.9	0.98	1.48
microplate 1 vs microplate 20	1.11	1.06	1.41

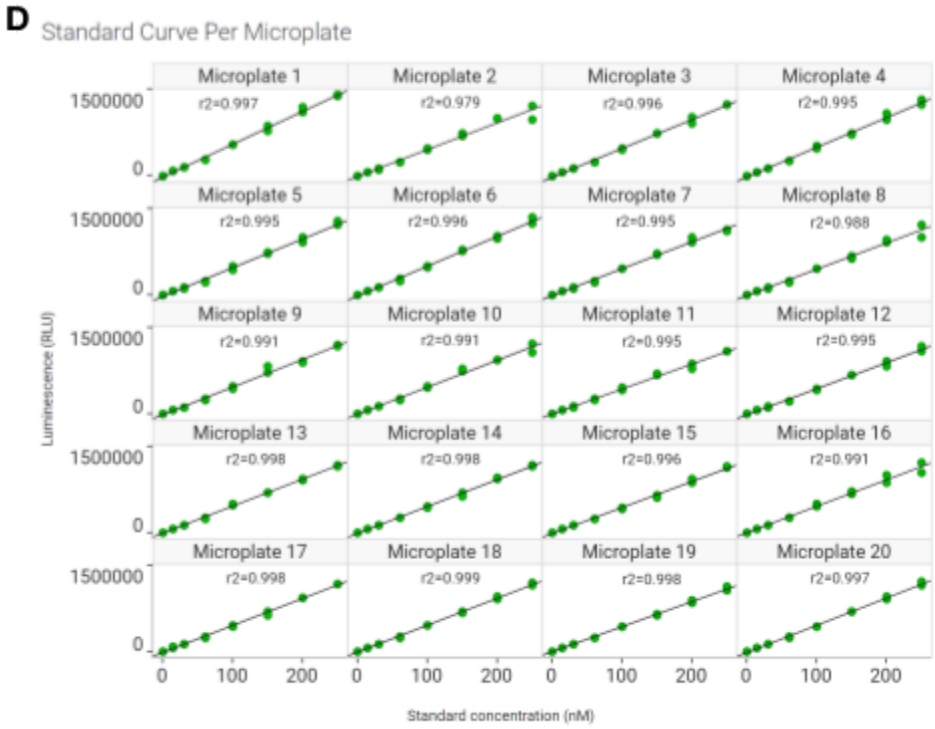


Fig 6 Data Analysis of a 20-microplate HiBiT Assay on the RAC Platform; Average technical replicate

HiBiT-tagged protein concentration per strain, per assay microplate (N=56) (**A**); %CV of technical replicate HiBiT-tagged protein concentrations per strain, per assay microplate (N=56) (**B**); MSR and MR of representative pairs of microplates (**C**); Standard curve per each microplate, and the corresponding quality metrics (coefficient of determination) (**D**).

CONCLUSION

Here, we demonstrated how Ginkgo's RAC platform can be rapidly adapted to execute fully-automated, large-scale, plate reader-based HTS assays, generating high-quality datasets, on par with previous semi-automated execution. The fully automated assay showcased here is applicable to various related HiBiT protein tagging technology assays, assessing protein internalization, secretion and degradation, and more broadly, assays relying on similar plate reader-based protocol logic (e.g. AlphaLISA or HTRF assays).

The initial HTS assay onboarding and optimization process was significantly accelerated by Ginkgo's hardware and software stack modularity (**with a new plate reader RAC addition to enable this new workflow taking only 5 hours**). In a large 20-plate run, we obtained consistent HiBiT-tagged protein concentration estimates, unbiased by processing order, and no runtime errors were encountered. Ginkgo Automation's ticketless error troubleshooting and resolution service (Catalyst Flow) gave additional confidence in executing protocol runs without any in-person oversight and past working hours. **Overall, RACs led to ~80% reduction in hands-on time when executing a HiBiT assay processing 7,680 samples, delivering both high-quality data and valuable time savings.**

GLOSSARY

Catalyst Automation Control Software (Catalyst ACS): comprises the protocol editor and launcher, system configuration center, scheduler, and live-updating run dashboard.

Catalyst Flow: ticketless, remote error troubleshooting and resolution service provided by Ginkgo.

MATERIALS

- Beckman Coulter Echo Qualified 384-well Polypropylene 2.0 Plus Microplate (001-14622)
- Revvity ProxiPlate 384-shallow well Plus (6008280)
- Teknova PBS buffer (P5275)
- Promega HiBiT Control Protein (N3010)
- Promega Nano-Glo HiBiT Detection System (N3040)
- *K. phaffii* cell culture supernatant containing 60 kDa HiBiT-tagged protein

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