Automation of the sample preparation workflow from mix assembly to Ruby Chip loading with Nio™+

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Introduction

Nio™+ is an all-in-one digital PCR (dPCR) platform that harnesses cutting-edge microfluidic technology to integrate the dPCR workflow onto a single chip consumable, the Ruby Chip. The technology, known as Crystal Digital PCR®, partitions samples into a large array of thousands of individual droplets and amplifies nucleic acid molecules in each droplet in parallel. These reactions are tagged with fluorophores which emission is read using fluorescence light channels. The Nio™+ is a walk-away instrument that can be continuously loaded with up to 24 Ruby Chips, processing up to 384 samples per 8h shift.

The Opentrons® OT-2 is a high-precision, easy-to-use liquid handler. Its flexibility allows automation of a wide range of application workflows. This robot and its accompanying automation platform are used to automate hundreds of protocols and workflows in genomics, proteomics, cell-based assays, and drug discovery.

In this technical note:

This technical note showcases the repeatability achieved by the Nio™+ combined with the OT-2 Opentrons on a 6 colors assay for a fully automated assembly of reaction mix and Ruby Chip loading to process 96 samples.

Conclusions

- The sample preparation steps prior Crystal Digital PCR® can be fully automatized in the OT-2 Opentrons liquid handling system.
- The use of the OT-2 Opentrons liquid handling system in association with the Nio™+ leads to excellent repeatability performance, absence of cross contamination and accurate absolute quantification over the dynamic range of the Ruby Chip with a substantial gain in hands on time on the full digital PCR workflow.

The Ruby Chip is a versatile, all-in-one digital PCR chip that harnesses simplicity and flexibility. While still adhering to SBS lab standards and featuring overall compatibility with existing lab equipment. The Nio™+ is an all-in-one digital PCR instrument with continuous loading capabilities & 7-color capabilities.

Ruby Chip and the Nio™+ system

Ruby Chip

- Input volume 5 µL
- Up to 17.000 droplets per well
- Sample pooling software feature for increased sensitivity per sample
- LOD (95%) 0.80 cp / µL
- Dynamic range 5 logs
- Up to 16 Samples per chip

Nio™+

- Automated, all-in-one digital PCR platform
- High-plex with up to 7 colors, and higher-order multiplexing to go well over 20 targets
- Process up to 796 samples per 8 hour = Queue up to 8 chip plates (of 3 chips each) at random intervals, with different assays/ protocols

Mix homogenization

An OT-2 program developed by Stilla Technologies is used to automate the preparation of a reaction mix with the naica® IQ/OQ kit, for 6 Ruby Chips (96 chambers) with a sample input volume of 1 µL per chamber. This corresponds to the minimum volume that can be pipetted by the OT2-Opentrons. The sample is added to a mixture of naica® multiplex PCR MIX, primers, probes and water, hereby referred to as “the master mix”. To ensure repeatable quantification results, the two critical requirements are (i) a homogeneous reaction mix assembly and (ii) the absence of cross-contamination during mix preparation.

To verify that the robot assembles the reaction mix homogenously, a master mix is prepared by the OT2-Opentrons by automatically adding each buffer (H20, Buffer A, Buffer B, Buffer C, Buffer D) in a 1.5 mL tube. Then, the DNA positive control for the 6 targets of the naica® IQ/OQ kit is added directly by the robot into the master mix. The reaction mix is homogenized by the OT-2 and manually loaded into Ruby Chips, without any further homogenization. The dispensing of the reaction mix into the Ruby Chips is performed by aspirating the reaction mix directly below the surface of the tube, meaning that the first chip is loaded with the top part of the reaction mix and the last chip with the bottom part of the reaction mix.

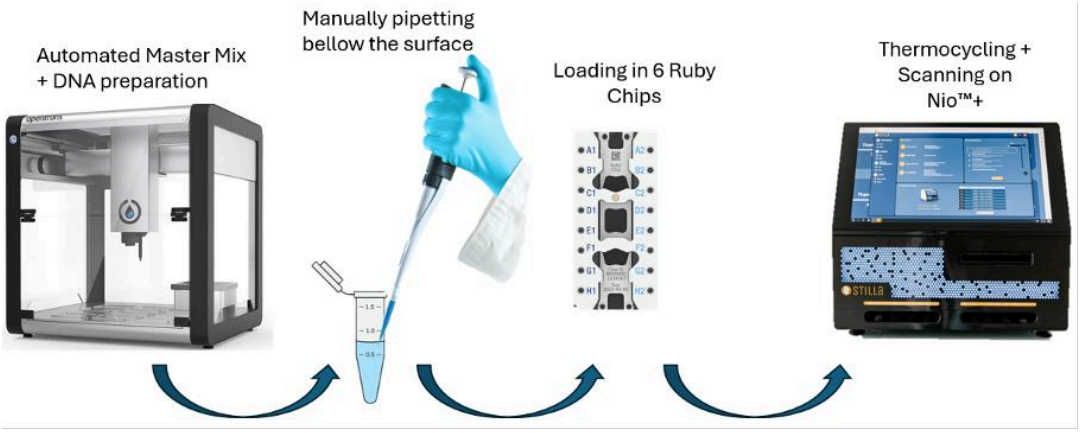
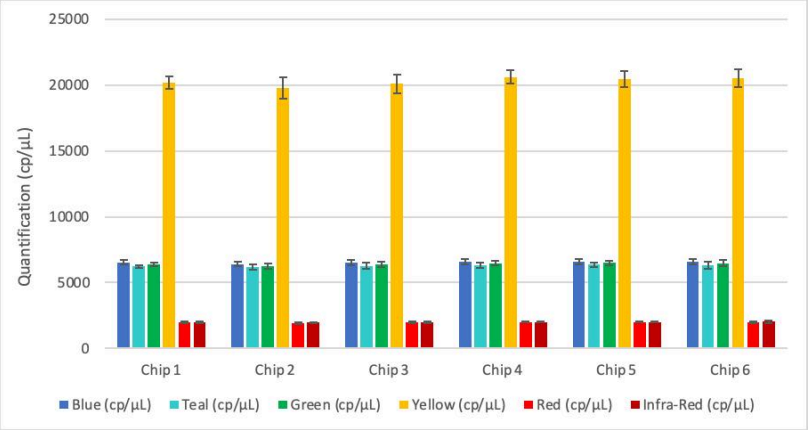


Figure 1: Master mix automated homogenization testing workflow

Differences in quantification between the chips would highlight an inhomogeneous distribution of the DNA molecules in the reaction mix. The final concentration of each target is chosen to minimize the relative measurement uncertainty to accurately assess the deviation, if any.

By using a one-way ANOVA test for each channel, no significant difference is observed between the 6 chips (p-Value > 0.05). The quantification in each chip does not depend on the level of aspiration in the reaction mix tube, demonstrating the homogeneity of the reaction mix as prepared in the OT-2.

Figure 2 : Quantification obtained in 6 channels of the Nio™+ with the same mix manually distributed from its top part in chip 1 to its bottom part in chip 6. The error bars indicate the standard deviation.

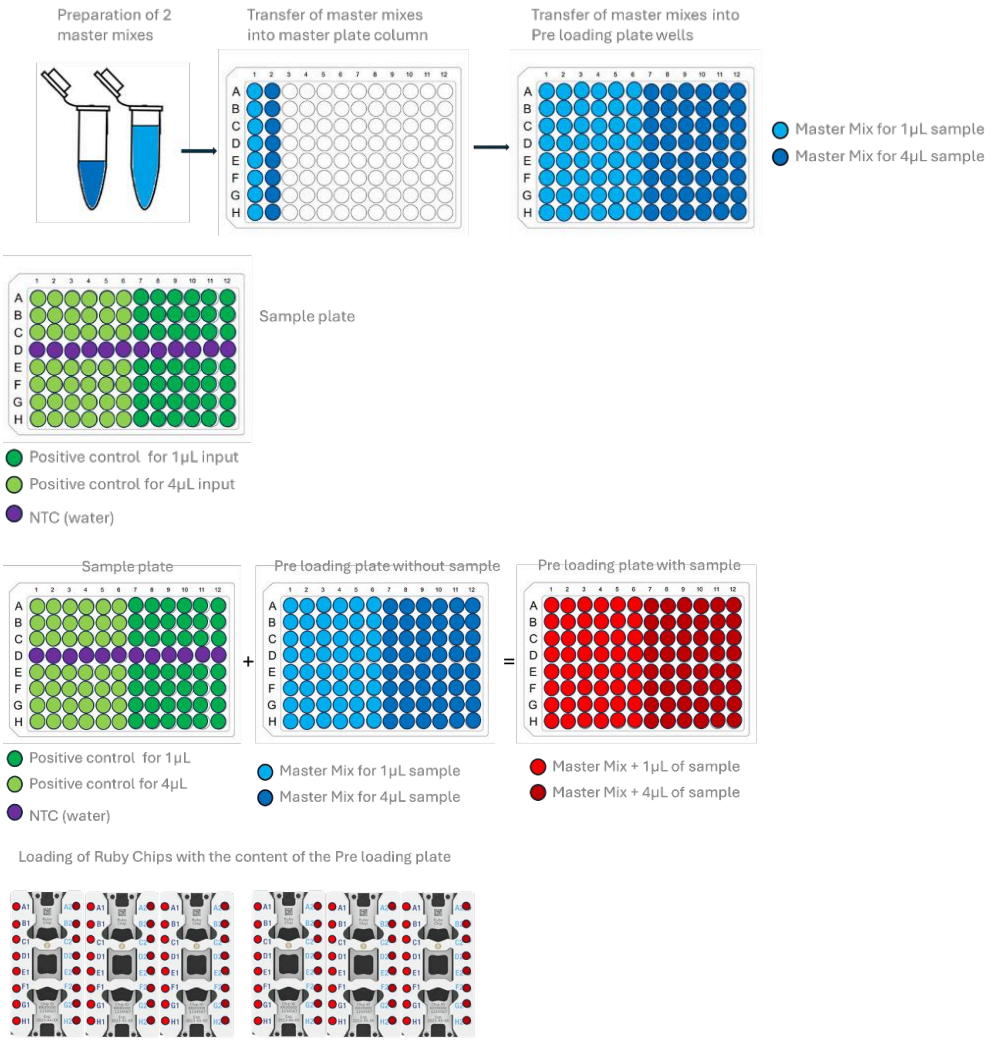


Absence of cross contamination and repeatability over the dynamic range of the Ruby Chip

To be useful in routine operation, the association of the OT-2 Opentrons with the Nio™+ must not bring cross contamination between samples and must show repeatable results between replicates. To assess this, two master mixes for 48 reactions each are prepared by the OT-2 Opentrons. One is assembled to leave room for 1 µL of sample per Ruby Chip chamber, mimicking a use case where the availability of sample is limited. The second one is assembled to leave room for 4 µL of sample per Ruby Chip chamber, mimicking a use case where a large sample volume needs to be analyzed. The sample mixture contains 6 independent DNA targets with final concentrations equal to 7400, 29000, 1850, 12800, 110 and 455 cp/µL and detected in the blue, teal, green, yellow, red and infra-red channel respectively. These final concentrations are chosen to cover a large part of the Ruby Chip chamber's dynamic range.

Once the OT-2 Opentrons deck is loaded with the proper labware and the sample plate, all liquid handling steps, from master mixes preparation to Ruby Chip loading, are fully automated. First, the two master mixes are assembled in tubes and then distributed into two columns of an Opentrons Tough 0.2 mL 96-Well PCR Plate, Full Skirt (SKU: 991-00076). The content of each well of these columns is then redistributed into 6 wells of a line of a second plate, the "Pre loading plate splitting it in two half: the left half for the 1 µL mix and the right half for the 4 µL mix. The sample plate is split in two parts as well: the left and right half containing 1 and 4 µL of sample respectively. Each 96 sample from the sample plates is mixed with its corresponding master mix of the Pre loading plate. Lastly, the 96 reaction mixes from the Pre-loading plate are loaded into 6 Ruby Chip, alternating a column from its left part (1 µL sample volume input) and a column from its right part (4 µL sample input volume). As such, a total of 42 replicates of samples and 6 non-template controls are prepared per sample volume input. As the sample contains 6 targets at different concentrations, this creates 42 data points for six final concentrations between 110 and 29000 cp/µL.

Figure 3 : Fully automated workflow, from the mix preparation to Ruby Chip loading in the OT2-Opentrons. The sample plate is manually prepared prior starting the workflow.



The Limit of Blank (o) is the maximum concentration that is plausible with a 1 – probability (typically 95% for = 5%). For both sample input volume conditions, 1 and 4µL, the quantification in the 6 NCTs is lower than the o established for the naica® IQ/OQ kit used (see table 1). This demonstrates the absence of cross contamination in the negative controls.

Table 1: Average concentrations obtained fo the 6 NTC replicates for both 1 and 4 µL of water volume input.

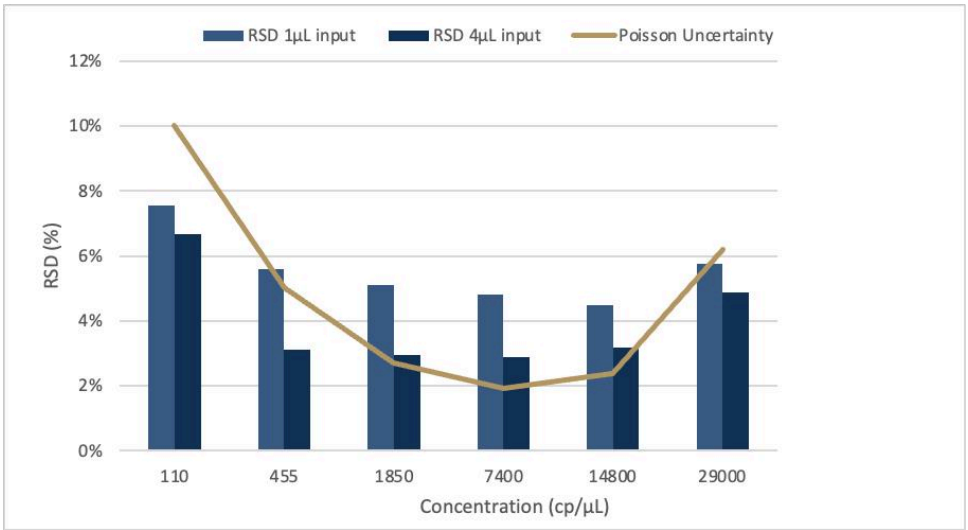
Condition	Blue (cp/µL)	Teal (cp/µL)	Green (cp/µL)	Yellow (cp/µL)	Red (cp/µL)	Infra-Red (cp/µL)
1µL NTC	0.39	0.00	0.16	0.00	0.00	0.09
4µL NTC	0.15	0.00	0.19	0.05	0.00	0.06
naica® IQ/OQ *LoB*	1.45	0.25	0.30	0.53	0.31	0.67

For both sample volume inputs and for each target, the repeatability is assessed by measuring the relative standard deviation (RSD) on the 42 replicates. Assuming that the distribution of the concentrations obtained follows a normal distribution, 95% of the observations are expected to be contained in the interval defined by the average concentration observed plus or minus two standard deviations. The measured RSD at a given target concentration corresponds to the theoretical uncertainty (or "Poisson uncertainty") plus the experimental uncertainty. The Poisson uncertainty of the dPCR method, CI95%, is described as the relative half-width of the 95% confidence interval on the concentration estimation. The CI95% provides insight on the precision of the measurement and is provided by Nio™ Analyzer software together with each concentration result. Thus, when the sources of experimental variability are minimized, the RSD of the observed concentration is expected to tend towards half the CI95%.

The RSDs range from 2.90% up to 7.57% and remains below 10% on the range of final concentrations assessed.

As expected, the RSD is higher than the Poisson uncertainty in the middle of the dynamic range, where the experimental variability is overriding whereas it is lower at the lower and higher-ends of the dynamic range, where the Poisson uncertainty becomes dominant because of sampling and partitioning errors respectively.

Figure 1 : Relative standard deviation of the 42 replicates for each condition of sample volume input, compared to the Poisson uncertainty (CI95%) of the measurement at the six target concentrations.



The 4 µL sample input volume condition shows less variability than the 1 µL condition which is in accordance with the OT-2 pipette P20 Multichannel specification as the higher the volume pipeted, the higher the precision (from 10% for 1 µL pipetted to 1.5% for 20 µL).