DNAble® Application Note

Eppendorf epMotion® 5073 & Roche LightCycler® 480 II

ENVIROLOGIX

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ABSTRACT

Genetic trait detection using nucleic-acid based amplification is widely used in agricultural life science applications. DNAble® is a rapid, isothermal amplification technology that detects specific nucleic acid sequences with molecular beacons. Similar in mechanism to PCR, DNAble has the added flexibility to work with crudely prepared samples, provides a rapid time-to-result, and does not require thermal cycling for amplification. This application note outlines a simple workflow for performing high-throughput DNAble testing using the Eppendorf epMotion® 5073 automated liquid handling (ALH) system paired with a Roche LightCycler® 480 II real-time thermocycler. As an industry standard, Artel MVS® was used to calibrate ALH pipetting accuracy prior to DNAble testing. Two DNAble assays were executed using these platforms; a synthetic singleplex assay that yields quantitative results across a dilution series, and a crude sample (soy leaf) multiplex assay that yields qualitative duplexed results across individual samples. Artel MVS results verified that both pipette heads (Span8 LiHa and 96-channel MCA) were dispensing within 5% CV across a 384-well plate. Linear regression ($R^2 > 0.93$) across a 7-log dilution series of target was used to analyze singleplex/quantitative data. Multiplex/qualitative testing across 960 soy leaf samples yielded >97% accuracy with a 95% confidence interval for both assay targets. Total time to result for multiplex testing of 384 reactions (including sample preparation, ALH pipetting, and thermodetection) was 45 minutes. In conclusion, DNAble chemistry offers a rapid method for targeted nucleic-acid amplification in crudely prepared samples that reduces time-to-result by 4-fold compared to standard PCR while maintaining industry-standard accuracy rates (95% sensitivity/specificity with 95% confidence interval).

INTRODUCTION

Genetic trait detection using nucleic-acid based amplification is widely used in agricultural life science applications including field surveillance, adventitious presence testing, and trait confirmation/purity. DNAble® is a rapid, isothermal amplification technology that uses molecular beacons to specifically detect amplified nucleic acid sequences. The DNAble system is tolerant of crude sample extracts and therefore no DNA purification is required, removing costly and time-consuming steps from the testing workflow. Since DNAble amplifies and detects target DNA sequences at a constant temperature, assay results are generated in as little as 5 minutes with excellent analytical sensitivity and specificity. The simple sample preparation combined with the rapid assay run time makes DNAble effective in low to high throughput testing environments where time to result and testing efficiency are critical for success. For these reasons, DNAble has great potential to deliver operational efficiencies in seed breeding and seed production workflows.

This application note outlines a simple workflow for performing high-throughput DNAble testing across two different assay systems using the Eppendorf epMotion® 5073 automated liquid handler and Roche LightCycler® 480 II thermodetection system.

HTP – High throughput; refers to any level of testing > 15,000 reactions per day
MM – Master Mix (resuspended liquid form of the molecular assay)
MVS – Multichannel Verification System (Artel®)
PM – Preventative maintenance (process required to ensure normal operation)
ALH – Automated liquid handler

MATERIALS AND METHODS

1 | Instrumentation & Workflow

To perform automated liquid handling (ALH) of both sample and assay reagents we used an Eppendorf epMotion 5073 equipped with one dispense head: an 8-channel air-displacement pipette used to multi-dispense both master mix and sample extracts. Disposable pipet tips from Eppendorf were used to ensure greatest compatibility between consumable and instrument. Following industry standards, optimization of liquid class in the EpMotion software was necessary to ensure accurate and reproducible pipetting; see Appendix A for specific setting recommendations. To maintain reaction temperature and capture fluorescence data resulting from amplification we used a Roche LightCycler® 480 II. Raw data was collected using LightCycler 480 v1.5.1.62 and analysis was performed in Excel 2016.

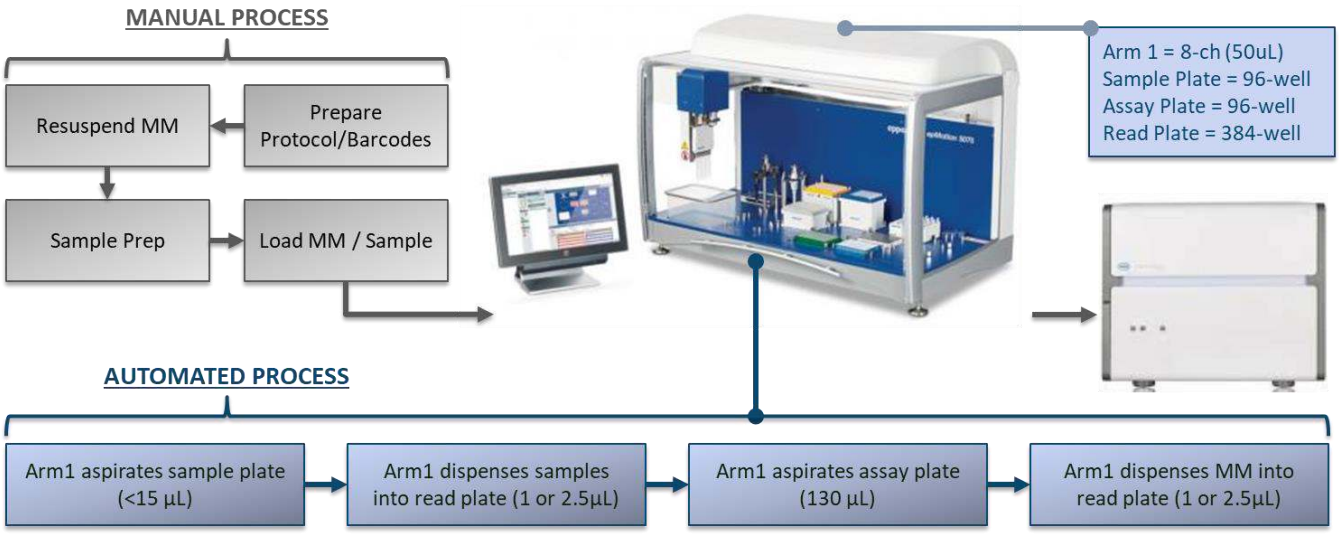

Figure 1. High-throughput workflow for DNAble using Tecan Freedom EVO and Roche LightCycler® 480 II.

2 | DNAble® Assay Reagents

Two DNAble assay systems were tested using the epMotion 5073 ALH paired with the LightCycler 480 II. The first was a singleplex, quantitative assay using a synthetic oligonucleotide target and the second was a multiplex, qualitative assay using freshly prepared crude soy leaf extract. The singleplex assay produces a standard curve across a seven-log dilution series of synthetic target in less than eight minutes of amplification whereas the multiplex assay produces absence/presence results in crudely prepared samples within fifteen minutes. For both assays, DNAble reagents were prepared as lyophilized material requiring only the addition of Reaction Buffer to resuspend active components.

3 | Samples for Testing

For singleplex assays, known concentrations of oligonucleotide were used to generate a seven-log dilution series ranging from $1x10^6$ copies/µL to $1x10^0$ copies/µL. For the multiplex assays, Soy leaf tissue known to be Trait A positive, Trait B positive, or Trait A/B positive were used. Leaf samples (collected as punches) were lyophilized (for shipping only; not required) and processed on-site by the addition of 200µL of MB15 extraction buffer followed by heating at 95°C for 5 minutes in a standard heat block. For users lacking the proper format heat block, an alternative heating step has also been validated, which utilizes a simple dry-oven set to 95°C. Crude extracts must be cooled to room temperature before being used for testing to ensure pipetting accuracy and experimental reproducibility.

4 | Artel® Volumetric Evaluation (MVS)

The Artel MVS® system was used to assess the volumetric accuracy of dispensing 2.5µl of MasterMix or sample using the 8-channel pipette tool on the epMotion 5073. All testing used Range B solution (Artel) to determine accuracy of dispense across a 384-well plate, resulting in 4 replicates per channel (MCA). All plates were read using the Artel MVS® System utilizing MVS® Data Manager 3.2 Advanced software. Volumetric verification via the Artel MVS system was crucial to ensure proper pipetting accuracy as a function of dispense pattern, liquid class characteristics, and environmental conditions.

RESULTS

5 | Artel® Volumetric Evaluation (MVS)

Dispense Head	Tip Type	# of Replicates	# of Tests	Target Volume (uL)	Mean Volume (µL)	Inaccuracy (%)	CV (%)
8-channel	Eppendorf Filtered/10uL	48 per channel	384	5.00	5.11	2.20 %	1.52 %
8-channel	Eppendorf Filtered/10uL	48 per channel	384	2.50	2.52	0.77 %	2.92 %

Table 1. Artel MVS summary of volumetric accuracy testing for 8-channel pipette tool on epMotion 5073.

6 | DNAble® Verification & Validation

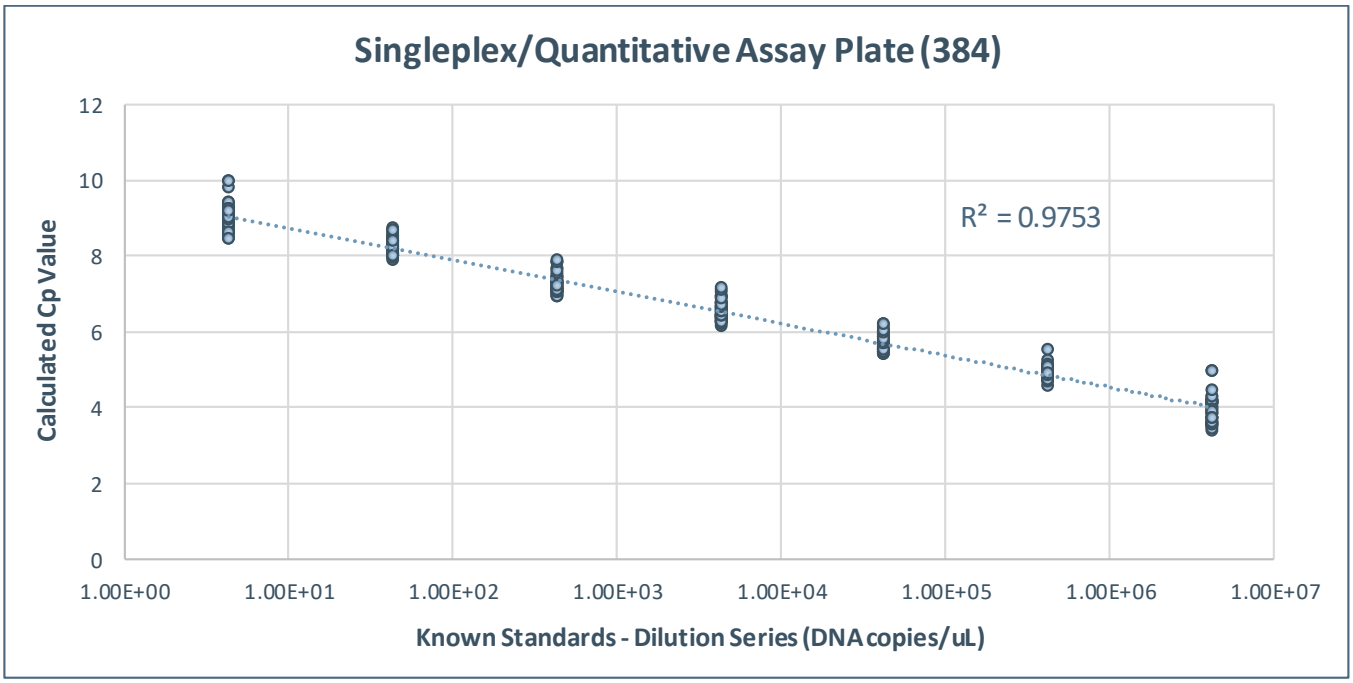

Figure 1. Representative standard curve of Singleplex/Quantitative Assay results for one 384-well plate. Samples were tested as a 7-log dilution series of synthetic target. Regression analysis determined the proportion of variance at each dilution level and linearity was quantified via R^2 value. The average R^2 value across all ten, 384-well test plates was 0.937.

Trait/Sample Condition	Biological Replicates	Technical Replicates	Total # of Rxns	ALLELE A (RR2) Accuracy (No CI)	ALLELE A (RR2) Accuracy (95% CI)	ALLELE B (CONV) Accuracy (No CI)	ALLELE B (CONV) Accuracy (95% CI)
NTCs	240	4	960	98.4%	97.6%	100%	99.7%
Conventional (-/-)	240	4	960	99.8%	99.4%	100%	99.7%
Heterozygous (+/-)	240	4	960	100%	99.7%	99.4%	98.8%
RR2 (+/+)	240	4	960	100%	99.7%	100%	99.7%

Table 2. Multiplex/Qualitative Assay Results for ten 384-well plates. Samples were tested as 24 biological replicates per condition, with four technical replicates, per test plate. Both assays in this multiplex system achieved >97% accuracy with a 95% confidence interval across all ten test plates.

Assay	Method	Sample Prep	Liquid Handling	Manual Labor	Detection	Total Time
Singleplex Quantitative	N/A	5 min	15 min	2 min	8 min	30 min
Multiplex Qualitative	Heat Block	15 min	15 min	2 min	15 min	47 min
Multiplex Qualitative	Incubator	50 min	15 min	2 min	15 min	82 min
Standard PCR	DNA Purification	60-120 min	12 min	2 min	50 min	124-184 min

Table 3. Process, Throughput, and Cost Comparison between Singleplex, Multiplex, and PCR workflows. Significant time savings in a commercial environment can be realized with DNAble due to the rapid sample preparation and reduced thermodetection requirement.

DISCUSSION

In conclusion, DNAble chemistry offers a rapid method for targeted nucleic-acid amplification in crudely prepared samples that reduces time-to-result by 4-fold compared to standard PCR while maintaining industry-standard accuracy rates (>95% sensitivity/specificity with 95% confidence interval). This time advantage over standard PCR, along with the cost savings associated with crude sample preparation, leads to significant gains in a commercial testing environment where efficiency is key. Additionally, DNAble technology provides flexibility in point-of-detection for field-based applications; users are now able to survey fields in real-time, producing actionable data that can be leveraged within the same day as opposed to sending out samples for third party and/or laboratory-based testing.

REFERENCES

1. J.T. Bradshaw, T. Knaide, A. Rogers, R. Curtis, JALA, 2005, 10 (1), 35-42. "Multichannel Verification System (MVS): A Dual-Dye Ratiometric Photometry System for Performance Verification of Multichannel Liquid Delivery Devices."

APPENDIX

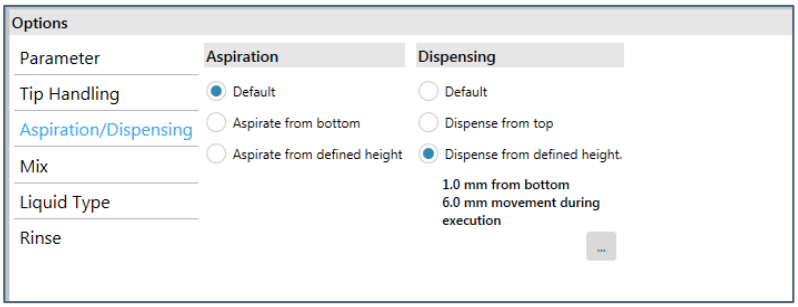

Figure S1. Optimized Aspiration/Dispensing Settings for DNAble on EpMotion 5073.

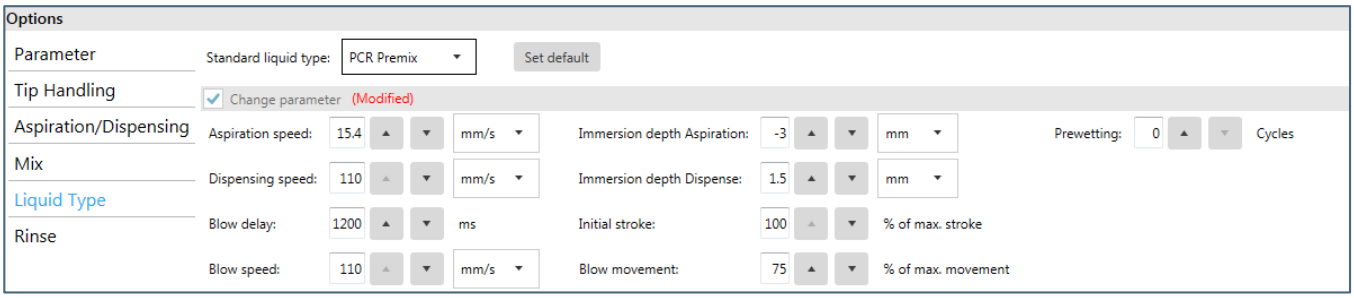

Figure S2. Optimized Liquid Type Settings for DNAble on EpMotion 5073.

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