Automated Solution for Real-time Based Applications with High Reproducibility and Accuracy

Challenge

Preparation of precise and reproducible results for qPCR assays using a liquid handling platform.

Solution

CyBio FeliX as a precise pipetting system, provides superior assay reproducibility and improves the accuracy which is essential for downstream applications based on quantitative PCR (qPCR).

Introduction

The CyBio FeliX from Analytik Jena GmbH is a compact, versatile, multi-channel automated liquid handling device that offers speed, flexibility, ease-of-use, and accurate pipetting performance. This platform can be configured for various molecular biology techniques such as nucleic acid extraction, PCR/qPCR and normalization. Moreover, because it is compatible with automated liquid handling systems, (q)PCR can be regarded as a high throughput method. Indeed, manual (q)PCR setup is time-consuming, tedious and requires lots of practice. The most probable source of imprecision is the operator itself. By automating (q)PCR setup the challenge is to guarantee consistently reproducible results as well as the absence of cross-contamination. Automation of a setup saves time and effort, improves processing capacity, removes the risk of operator error and ensures batch consistency of reagents and samples. If a large number of samples has to be processed in a short time, automating (q)PCR setup can be the solution to increase the throughput while the quality of results is maintained. This application note shows the possibility to fully automate a qPCR setup on the CyBio FeliX liquid handling system. With the use of the qPCR Demo SyGreen Assay (IST Innuscreen GmbH) for the qTOWER³ from Analytik Jena GmbH we demonstrate the device reproducibility and the performance of an accurate standard curve amplification. Furthermore, the assay enables the easy and efficient demonstration of the qTOWER³ device uniformity and sensitivity for the control of experimental conditions.

Materials and Methods

Samples and reagents

- qPCR Demo SyGreen Assay (IST Innuscreen GmbH) for qTOWER³ (Analytik Jena GmbH)

including

  - Positive Control
  - Ready-to-use Mix
  - PCR-grade H_2O

Instrumentation

- CyBio FeliX Basic Unit with Enclosure (Analytik Jena GmbH)
- CyBio Head R 96/250 µL (Analytik Jena GmbH)
- 1-Channel Adapter; Head R (Analytik Jena GmbH)
- Adapter 24 tubes, passive cooling function (Analytik Jena GmbH)
- Adapter 96, passive cooling function (Analytik Jena GmbH)
- Waste Box (Analytik Jena GmbH)
- CyBio TipBox 96/250 µL with Filter Tips (Analytik Jena GmbH)
- CyBio Composer Software (Analytik Jena GmbH)
- qTOWER³ (Analytik Jena GmbH)
- qTOWER³ Software: qPCRsoft 4.1 (Analytik Jena GmbH)

Methods

The qPCR Demo SyGreen Assay for qTOWER³ was used to demonstrate the reproducibilty and the amplification of a standard curve on a qTOWER³. The data was obtained in color module 1 (470 nm/520 nm; gain = 5) of qTOWER³ for the SybrGreen channel. The CyBio FeliX used the deck layout at Figure 1 to perform all preparation steps according to the kit recommendations including predilutions.

Figure 1: Deck layout of the CyBio FeliX. Deck A is the lower deck (position 1-6) and deck B/C are the upper decks (position 7-12).

Reaction setup 1: Demonstration of reproducibilty

The CyBio FeliX Head R 96/250 µL in combination with the 1-Channel Adapter Head R, was used to transfer 20 µL of the master mix containing 4 µL diluted positive control (1:10) and 16 µL of the Ready-to-use-Mix. The master mix was dispensed in 12 wells of one row in a 96 well microplate for the demonstration of reproducibility (Figure 2).
The sealed 96 well microplate is placed in the qTOWER³ and the real-time PCR run is started with the following protocol (Table 1).

Table 1: Overview of qTOWER³ settings by qPCRsoft 4.1

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	3 min	max
2	30	Denaturation	95 °C	5 sec	max
		Annealing	58 °C	5 sec	max
		Elongation*	72 °C	15 sec	max
3	1	Melting curve*	60 °C – 99 °C	15 sec	max

* Data acquisition: Color Module 1 (470–520 nm) and gain 5

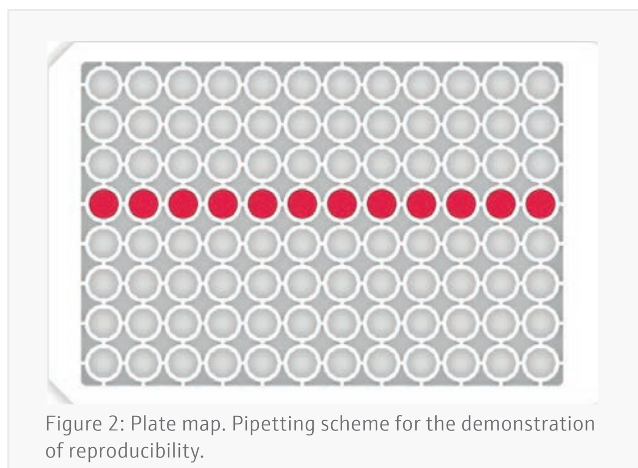


Figure 2: Plate map. Pipetting scheme for the demonstration of reproducibility.

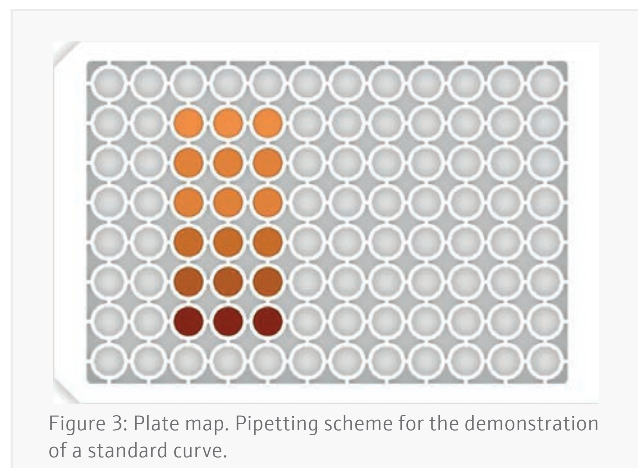


Figure 3: Plate map. Pipetting scheme for the demonstration of a standard curve.

Reaction setup 2: Demonstration of a standard curve

The demonstration of the sensitivity is conducted by a dilution series over a concentration range of 10^7–10^2 copies and measured in triplicates (18 reactions). For each dilution a master mix tube which consists of Ready-to-use Mix and diluted template is prepared and 20 µL each are distributed in triplicates in a 96 well microplate (Figure 3).

The sealed 96 well microplate is placed in the qTOWER³ and the real-time PCR run is started with the protocol of Table 1. After the qPCR run, a melting curve was performed to identify any potential off-target amplification.

Results and Discussion

The CyBio FeliX equipped with the pipetting Head R 96/250 µL performed all steps for preparing a set of qPCR reactions. The demonstration of reproducibility is performed in 12 wells within one row of the 96 well microplate. The preparation of the reproducibility test by the CyBio FeliX took about 6 minutes. Figure 4 represents a successful run to demonstrate the device reproducibility. The optimal performance of the device is shown by the Ct-values standard deviations (SD) of 0.05 and a homogenous saturation of fluorescence intensity.

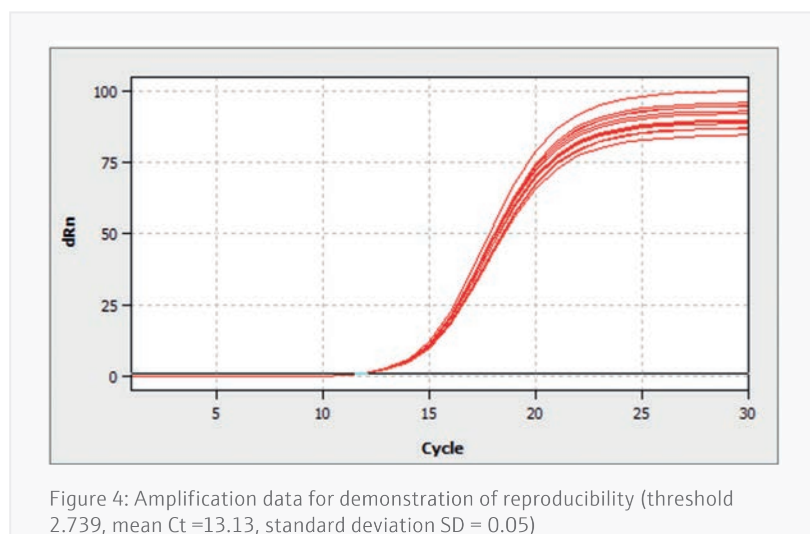


Figure 4: Amplification data for demonstration of reproducibility (threshold 2.739, mean Ct =13.13, standard deviation SD = 0.05)

The measured triplicates for the ten-fold dilution series to demonstrate the sensitivity are shown in the amplificationblot in Figure 5. An optimal dilution series is characterized by a Ct-value shift of 3.3–3.6 per decreasing concentration steps. The individual Ct-values of the triplicates of each concentration has low standard diviations indicating that the CyBio FeliX performs the preparation of the standard curve accurate and reproducible (Table 2). The preparation procedure by the CyBio FeliX took about 18 minutes.

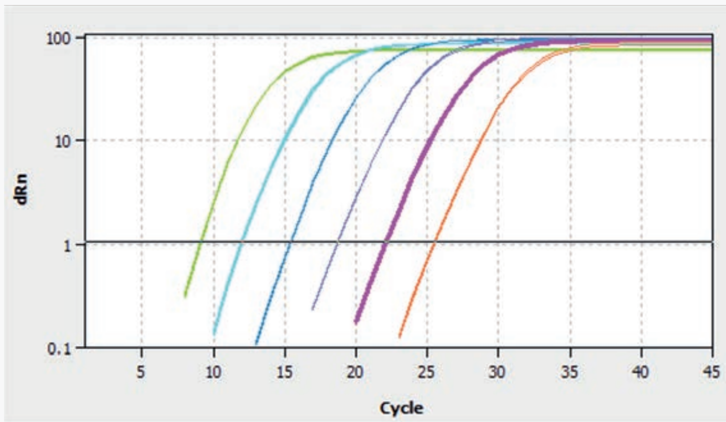
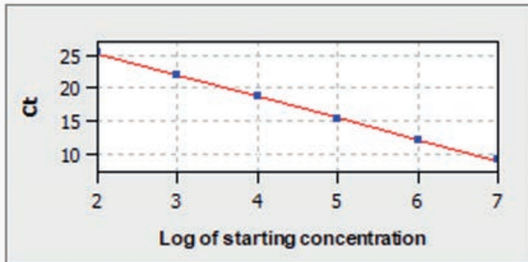


Figure 5: Amplificationblot (logarithmic scale) with the data of a ten-fold dilution of 10^7 - 10^2 copies measured in triplicates.

The PCR-efficiency was determined at 100% (slope = -3.30) with an R^2 value of 0.999 (Figure 6) by linear regression analysis of Ct-values that were plotted against the logarithmic concentrations.



	FAM
R^2	0.99949
Slope	-3.30004
Offset	31.95044
PCR-efficiency	1.00922

Figure 6: Standard curve with R^2, slope and PCR-efficiency

Table 2: The results of the experiment, exported out of the qPCR soft 4.1

Well	Ct	Concentration copies	Mean Ct	Mean concentration copies	Standard deviation Ct
A3	9.08	10^7	9.06	8637666.21	0.02
A4	9.07	10^7	9.06	8637666.21	0.02
A5	9.03	10^7	9.06	8637666.21	0.02
B3	12.05	10^6	12.02	1091457.4	0.04
B4	12.05	10^6	12.02	1091457.4	0.04
B5	11.98	10^6	12.02	1091457.4	0.04
C3	15.29	10^5	15.30	110541.97	0.02
C4	15.31	10^5	15.31	110541.97	0.02
C5	15.32	10^5	15.31	110541.97	0.02
E3	18.69	10^4	18.68	10537.45	0.01
E4	18.68	10^4	18.68	10537.45	0.01
E5	18.66	10^4	18.68	10537.45	0.01
F3	22.07	10^3	22.08	977.1	0.05
F4	22.14	10^3	22.08	977.1	0.05
F5	22.04	10^3	22.08	977.1	0.05
G3	25.45	10^2	25.45	93.2	0.04
G4	25.41	10^2	25.45	93.2	0.04
G5	25.49	10^2	25.45	93.2	0.04

The analysis of the melting curve shows the expected fragments at 82.87 °C and that no unexpected fragments were amplified (Figure 7).

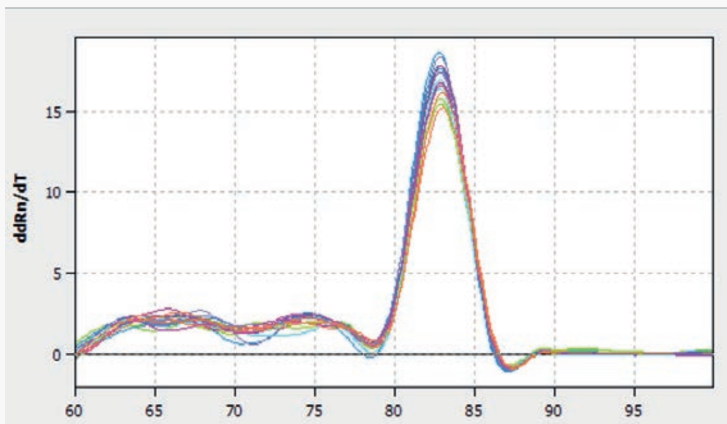


Figure 7: Melting curve of all selected products (Tm = 82.87 °C; standard deviations Tm = 0.08)

Conclusion

Automation offers high consistency, throughput and accuracy of the results. By reducing human intervention using an accurate pipetting system, the CyBio FeliX from Analytik Jena GmbH, a superior assay reproducibility without cross-contamination was achieved, which guarantees reliable results. The automation of the qPCR Demo SyGreen Assay allows the simple and efficient demonstration of reproducibility and/or the amplification of a standard curve on the qTOWER³ that can be realized with a PCR-efficiency of 100% and an R^2 value of 0.999.

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