CyBio® FeliX the solution for qPCR

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1. Abstract

Liquid handlers such as the CyBio® FeliX are ideal for automating sensitive applications such as qPCR; increasing reproducibility, removing the risk of manual errors and freeing up valuable researcher time. To demonstrate the capabilities of the CyBio® FeliX as an automated qPCR solution, a method for preparing qPCR reactions from starting reagents was tested using pUC19 as the DNA standard in a quantification experiment. The pUC19 DNA was subject to a 5-fold serial dilution to yield a range of concentrations between 125 pg/µl and 8 fg/ul. A Master Mix for 32 qPCR reactions was prepared and the 7 dilutions of pUC19 were run in triplicate along with three negative controls. All pUC19 dilutions gave amplification with Cq values for each dilution between 14 and 28 and CVs in the range of 0.15% to 2.19%. When the standard curve was plotted, the data had an R2 of 0.99 and a gradient of -3.38.

2. Introduction

Quantitative PCR (qPCR) enables the detection of biomarkers and expression and is a powerful tool for a range of applications. The most important advantages of qPCR are its sensitivity and specificity, along with the capability to multiplex and look for multiple targets within the same reaction. This same sensitivity however means that a qPCR experiment is vulnerable to a range of common pipetting and handling errors which can reduce the quality of results and hamper reproducibility. Automated liquid handlers are designed to perform large numbers of pipetting operations without making mistakes, however not all liquid handlers are so suitable for the precise, yet variable pipetting required in the generation of qPCR plates.

The CyBio®FeliX with a CHOICE® pipetting head is the ideal solution for setting up qPCR reactions. The CHOICE® head is capable of precisely pipetting any volume in the range of 0.5µl to 1ml, enabling the preparation of master mix from reagents, dispensing of this mix across a 96 0r 384 well plate and the careful addition of template to be carried out within the same protocol. All the tips, tubes, plates and storage required to prepare a full 96 well plate of qPCR reactions can be easily accommodated on the deck of the CyBio®FeliX, meaning the user can press start and then return to a finished plate of reactions. The CyBio®FeliX also has adaptations to reduce the risk of contamination in a qPCR workflow, including an enclosed UV unit for sterilisation and a coverless model designed to fit into a new or existing sample protection cabinet (Figure 1).

Figure 1 – A coverless CyBio®FeliX in a HEPA filter UV enclosure

To demonstrate the CyBio®FeliX's ability to prepare qPCR reactions, a method for making a standard curve by serial dilution was written and a stock of pUC19 DNA prepared for use as the standard. The CyBio®FeliX performed the serial dilutions and prepared a master mix before combining the two to make a set of reactions which where amplified in a qPCR cycler.

3. Method and Materials

Reagent	Manufacturer	Part Number
innuMIX qPCR MasterMix Probe	Analytik Jena	845-AS-1200100
dsGreen for Real-Time PCR, 100×	Lumiprobe	11010
pUC19 DNA	ThermoFisher Scientific	SD0061
pUC forward primer and pUC reverse primer	Integrated DNA technologies	N/A
qPCR water	ThermoFisher Scientific	BP2825-1

Equipment	Manufacturer	Part Number
CyBio® FeliX fitted	Analytik Jena	OL30-5015-100-24
CyBio® FeliX UV lamp	Analytik Jena	OL30-5015-954-24
CHOICE™ Head	Analytik Jena	OL3319-14-250
CHOICE™ high volume 8-channel adaptor	Analytik Jena	OL3316-11-332
CHOICE™ low volume 8-channel adaptor	Analytik Jena	OL3316-11-330
Tip rack, 1 ml	Analytik Jena	OL3317-11-140
StepOnePlus™ Real-Time PCR System	Applied Biosystems™	4376600
Waste box	Analytik Jena	844-00430-0

Consumable	Manufacturer	Part Number
1000 µl tips, filter sterile	Analytik Jena	OL3811-25-939-F
250 µl tipbox, filter sterile	Analytik Jena	OL3811-25-935-F
50 µl tipbox, filter sterile	Analytik Jena	OL3811-25-937-F
Tube rack 2.0 ml for 24 tubes	Analytik Jena	844-00136-0
Ergo Freeze Cooling Block Erg (96-well cooling block without rack and lid)	4titude	SP-0451
MicroAmp™ Optical 96-Well Reaction Plate	Applied Biosystems™	N8010560
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	Applied Biosystems™	4346906
Optical Adhesive covers	Applied Biosystems™	4360954
1.5 ml tubes		

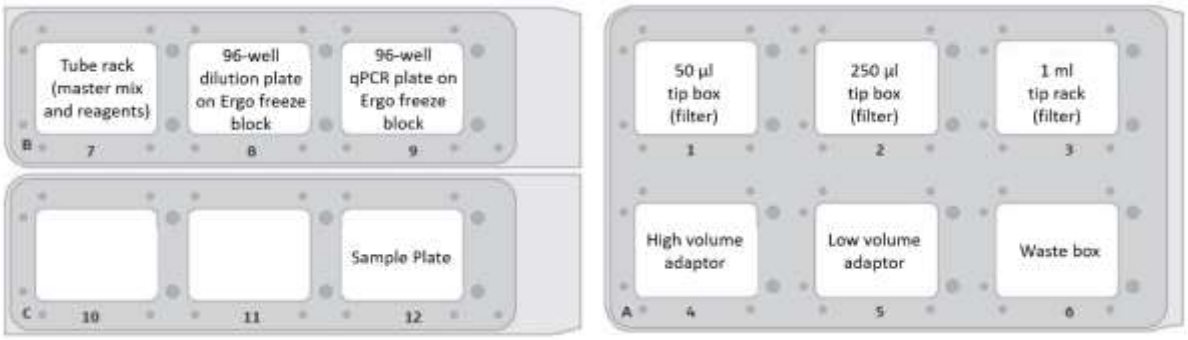


Figure 2 Deck Map for setting up qPCR reactions on the CyBio®FeliX including space for Serial Dilution of a DNA standard, preparation of Master Mix from Reagents and set up a full plate of reactions.

The pUC19 plasmid was chosen as a sample material for serial dilution because it was commercially available and its use as a standard for qPCR has been published [1]. The forward and reverse primer sequences and the qPCR cycling conditions were taken from the publication. A method was written for performing the serial dilution, generating the master mix and making the reactions. While all three stages are covered separately below, they can be run individually or together as a single method with all reagents, consumables and plates held on a single CyBio®FeliX deck (Figure 2).

3.1. Serial Dilution of DNA to generate a standard curve

The serial dilution was performed in the deep well v-bottomed reaction plate on Position 8 of the FeliX deck as follows:

- The deck of the CyBio®FeliX was loaded with all labware and reagents required for the serial dilution (Figure 2); 250µl tips on Position 2, High Volume adaptor in Position 4, Waste Box on Position 6, Tube rack with water tube on Position 7 and the Serial Dilution plate on Position 8 of the CyBio®FeliX deck.
- pUC19 DNA was firstly diluted by hand down to the starting concentration (125pg/µl) and 80µl of material was dispensed into well A1 of the serial dilution plate. Subsequent steps are all automated on FeliX:
- 80µl of water was transferred from the water tube in the tube rack on Position 7 into wells B1 to H1 of the serial dilution plate.
- A fresh tip was picked up and 20µl was aspirated from well A1 without prewetting.
- The 20µl from well A1 was dispensed into well B1 and the contents of well B1 were subject to a high intensity ‘vortex like’ mix:
    - 10 cycles of 75µl mixes at a speed of 400µl/s
    - Aspiration steps at 0.5mm above the bottom of the well
    - Dispense steps at 7.5mm above the bottom of the well
- The tip was ejected into the waste bin and a fresh tip picked up
- The serial dilution process above was repeated using the same pipetting technique down to well G1.
- The plate was sealed and stored at 4°C for later use

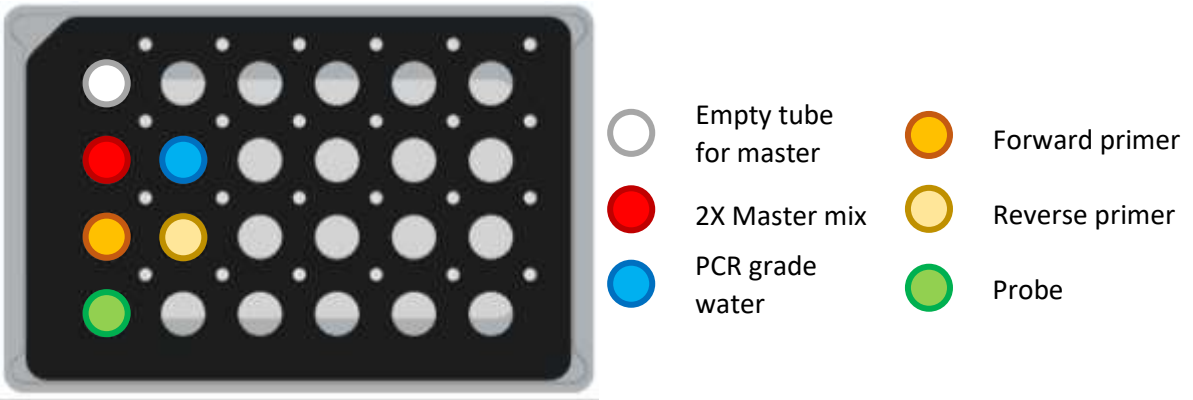


Figure 3 – Reagents supplied in the tube rack and their positions. For the generation of master mix, all the reagents necessary were supplied in 1.5ml Eppendorf tubes. If more than 48 reactions are required, a second empty tube should be place in the slot next to the displayed empty tube.

3.2. Preparation of Master Mix for all Reactions

Master mix was prepared in 1.5ml tubes within the tube rack on Position 7 of the FeliX deck as follows:

- All reagents required for the generation of Master Mix were put in 1.5ml Eppendorf tubes and loaded into the Tube Rack (Figure 3).
- The deck of the CyBio®FeliX was loaded with all labware and reagents required for the preparation of Master Mix (Figure 2); 250µl tips on Position 2, 1ml tips on Position 3, High Volume adaptor in Position 4, Waste Box on Position 6 and the tube rack on Position 7 of the CyBio®FeliX deck.
- All transfers were carried out with appropriate tips – i.e. the smallest tip with enough capacity to transfer the correct volume
- 81µl of water was transferred from the water tube to the prepared master mix tube
- 28µl of the forward primer was transferred from the forward primer tube to the prepared master mix tube.
- 28µl of the reverse primer was transferred from the reverse primer tube to the prepared master mix tube.
- 28µl of probe was transferred from the probe tube to the prepared master mix tube
- 280µl of 2X master mix was transferred from the 2X master mix tube to the prepared master mix tube.
- Reusing the 1ml tip from the previous step, the finished master mix was mixed with the following settings:
- 334µl mixing volume for 15 cycles
- 600µl/s pipetting speed
- Aspirating at 0.5mm above the tube bottom
- Dispensing at 7.5mm above the tube bottom
- 50µl blowout above the liquid and dab off after mixing.
- The generated master mix was immediately used for qPCR reaction set up

3.3. Reaction Set Up

The qPCR reactions were immediately set up using the prepared master mix generated in the previous step. The sample material to be tested in this experiment was the serially diluted DNA (section 3.1) which would normally be used for quantification and the sample plate was thus omitted.

- The deck of the CyBio®FeliX was loaded with all required plates, adaptors and labware for the reaction set up (Figure 2): 50µl tips on Position 1, 250µl tips on Position 2, High Volume adaptor on Position 4, Low Volume adaptor on Position 5, Waste Box on Position 6, Tube Rack carrying master mix tube on Position 7, plate carrying serially diluted DNA and water on Position 8 and qPCR plate on Position 9.
- The CHOICE® High Volume adaptor was loaded and a fresh 250µl tip picked up.
- Master mix was transferred to the first 3 columns of wells in the qPCR plate by multi-dispensing:
    - The 120µl was aspirate from the tube and 10µl immediately dispensed back into the tube.
    - 16µl of the prepared master mix was dispensed into each well until the volume remaining in the tip fell below 30µl.
    - The remaining master mix was returned to the source tube in the tube rack and the maximum volume picked up again.
    - This process was repeated until all the wells in the first 3 columns were filled.
    - Any remaining master mix was returned to the tube.
- The tip was then dropped and the CHOICE® Low Volume adaptor picked up.
- 4µl of serially diluted DNA or PCR grade water was transferred into each well of the first 3 columns of the qPCR plate by the following method:
    - A full fresh column of 50µl tips were picked up
    - 4µl of serially diluted DNA or negative control were transferred from the first column of the serial dilution plate into a column of the qPCR plate.
    - The sample material and master mix were then briefly mixed (2 cycles, 10µl).
    - The tips were then dropped into the waste box.
- Following sample transfer, the qPCR plate was immediately sealed.

3.4. Quantitative PCR

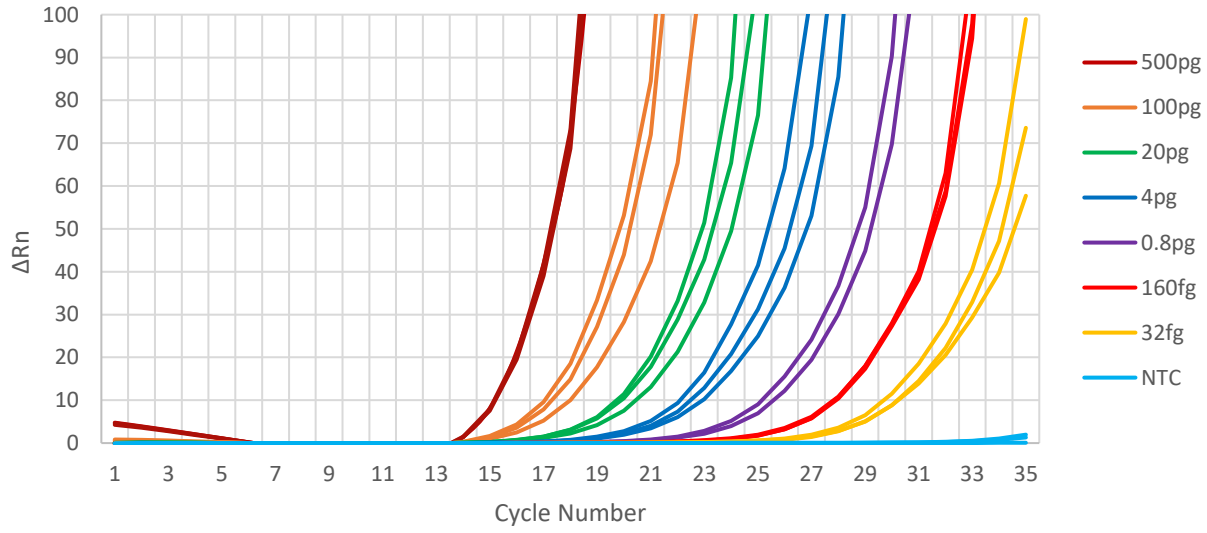
The plate was taken to qPCR cycler and the qPCR run started with the following settings:

- Initial denaturation: 95°C for 80 seconds
    - 35 cycles of:
        - 95°C for 20 seconds
        - 60°C for 40 seconds
- Readings were taken in the green FAM channel after each annealing/extension step.

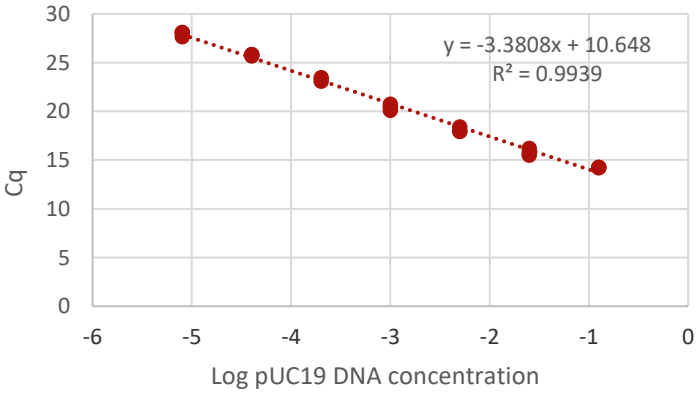
4. Results and Discussion

The CyBio®FeliX with CHOICE® pipetting head carried out all the steps in preparing a set of qPCR reactions. To generate sample material for qPCR analysis, a 7-step, 5-fold serial dilution of PUC19 was preformed inside a 96-well v bottomed plate, while only diluent was transfered into the final well in the column, for use as a No Template Control (NTC). After this, the CyBio® FeliX was given a tube rack containing all the reagents for making up the master for the qPCR reactions, including the primers, probe and 2X InnuMix qPCR master mix. The required volume of each component was combined into a single tube and thoroughly mixed ready for use in the reactions. This was dispensed to all active reaction wells in a PCR plate. Finally, the CyBio® FeliX transferred the prepared serial dilutions and no template control to the PCR plate in triplicate. The whole process from start to finish took less than 25 minutes.

A



B



C

pUC19 conc. (pg/µl)	Average Cq	CV
125	14.24	0.15%
25	15.81	2.19%
5	18.10	1.42%
1	20.42	1.56%
0.2	23.27	1.13%
0.04	25.79	0.33%
0.008	27.94	0.91%

Figure 4 - Amplification results for the serially diluted pUC19 plasmid DNA. The ΔRn at each cycle for all 32 samples is shown in A, with all 29 reactions which contained template showing amplification. The quantity of DNA is indicated for each curve, and the blue lines are the No Templates Control (NTC) wells. The Standard curve was plotted as the log of the concentration against the Cq for each sample in B, with the line of best fit plotted and R^2 calculated. Data for the precision of the qPCR reactions is shown in the data table in C, in which the average Cq and Coefficient of variation is displayed as a percentage for each dilution concentration.

All 29 wells which contained sample material amplified while there was no amplification of the No Template Controls (Figure 4A). The Cq values for each individual reaction were calculated and then plotted against the Log of the pUC19 DNA concentration in each well to give a standard curve (Figure 4B). A trendline fitted to this data had an R^2 value of over 0.99, and had an intercept of -3.38, suggesting a PR efficiency of over 95% - suitable for almost all qPCR applications. The coefficient of variation was calculated for each concentration and is displayed in the table in Figure 4C. This values never rose above 2.2% and illustrates the high precision of the CyBio FeliX pipetting.

5. Conclusion

The results from running the qPCR demonstrated that within a single method, a CyBio®FeliX can prepare a qPCR master mix, perform a serial dilution of a DNA standard and then combine these to make a set of qPCR reactions accurately, precisely and without contamination. This makes the CyBio®FeliX an ideal instrument for preparing qPCR reactions for a variety of qPCR experiments and applications. While the method demonstrated in this note was used to set up 32 reactions, it can generate a whole 96 well of qPCR reactions in under 40 minutes without user intervention. If an entirely automated system for qPCR is needed, the CyBio®FeliX can be combined with a CyBio®Carry and the qTower Auto to make a fully walk away reagents to qPCR robotic solution (Figure 5).

Figure 5 – Example of a fully integrated qPCR workstation. The workstation contains a CyBio®FeliX for preparing qPCR reactions, a CyBio®Carry transport system and 2 qTower Auto automated qPCR thermocyclers. The workstation can be loaded with reagents and left to prepare and run qPCR reactions without user intervention

6. References

[1] C. Chang, D. Mau-Hsu, K. Chen, C. Wei, C. Chiu and T. Young, “Evaluation of digital real-time PCR assay as a molecular diagnostic tool for single-cell analysis,” *Science Reports,* no. 8, p. 3432, 2018.