Amplification efficiency and thermal stability of qPCR instrumentation: current landscape and future perspectives

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Abstract

qPCR is a method of amplifying and detecting small samples of genetic material in real time and is in routine use across many laboratories. Speed and thermal uniformity, two important factors in a qPCR test, are in direct conflict with one another in conventional peltier driven thermal cyclers. To overcome this, companies are developing novel thermal systems for qPCR testing. More recently, qPCR technology has developed to make it useful in point of care testing (POCT), where the test is administered and results are obtained in a single visit to a health provider, particularly in developing countries. For a system to be suitable for POCT it must be rapid and reliable. Here we compare the speed and thermal uniformity of four qPCR thermal cyclers currently available two of which use the conventional peltier/block heating method and two of which use novel heating and cooling methods.

Introduction

Quantitative polymerase chain reaction (qPCR), the method by which a small sample of genetic material can be exponentially amplified and quantitatively measured in real-time, is now a main stay of research and medical laboratories alike. As the process has evolved, the applications for it have increased rapidly, ranging from infectious diseases to paternity identification and from forensic analysis to food processing. The PCR process requires that the test samples are cycled through a temperature profile; typically 95°C, 55°C, and 72°C, multiple times. The time taken to change the temperature of the samples between these levels is a key determinant of the speed of the process and thus of the length of a test [1]. A typical 40-cycle PCR can take around 2 hours to complete and that time has struggled to keep pace with other advances in the area. So, some of the potential benefits of this extraordinary process remain limited by speed.

Thermal uniformity, the absence of which can cause discrepancies in the cycling conditions between different samples on the same plate, is directly linked to speed. The design of many PCR instruments relies on conductive blocks to connect the heating or cooling source(s) to the test samples. To heat and cool the system, heat needs to be driven in and out of the block. Heat naturally flows within the blocks to remove any temperature gradients and so, it should, over time, deliver the same conditions across all the test samples. However, block based systems are vulnerable to greater heat losses on the edges and surfaces that tend to distort the thermal distribution. The conductivity of these blocks also affects the rate of heat flow and thus affects the thermal uniformity of the samples. In addition, the larger the thermal mass of the block, the greater the amount of heat that needs to be transferred and the longer this will take. The faster the heat is driven in or out of the system, the less time the conductive block has to even out the temperature distribution and maintain the thermal uniformity. Ultimately, such a system can only maintain its thermal uniformity if the rate of change of the temperature is slower than the time taken by the conductive block to even out the temperature. To achieve quick cycle times, big temperature

gradients are applied to the block, which can lead to samples overshooting or undershooting their target temperatures. So, in these types of systems, the need for uniformity of temperature is in direct conflict with the desire for speed; they can deliver one feature or the other but not both [2].

The ABI Prism 7900HT is perhaps the industry standard peltier/block based thermal cycler. The CFX 96 (Bio-Rad) provided an upgrade to the conventional system by reducing the thermal mass of the block. Alternatives to the block based system have also been developed. The Rotor-Gene Q (Qiagen) combines a centrifugal set-up with an air based thermal system. Ensuring that samples are continuously rotated through heated air removes the edge effect to provide superior thermal uniformity. xxpress® (BJS Biotechnologies) employs a different system in which an "active heating" method is combined with a block of low thermal mass, precisely controlling the amount and location of additional heating to avoid temperature discrepancies (Table 1). Here we compare the efficiency and thermal uniformity of four of the qPCR thermal cyclers currently available which use the conventional block/peltier system or novel methods.

Methodology

qPCR

18S rRNA expression in human genomic DNA was assessed and compared by quantitative polymerase chain reaction (qPCR) using an ABI Prism 7900HT, a Bio-Rad CFX 96 System, a Qiagen Rotor-Gene Q and a BJS Biotechnologies xxpress®. Human genomic DNA was purchased from Bioline and input in concentrations of 100, 10, 1, 0.1 and 0.01ng/µL to give final concentrations of 5, 0.5, 0.05, 0.005 and 0.0005ng/µL, generating a standard curve. Eukaryotic 18S rRNA gene primers were used: forward 3'- aaacggctaccacatccaag-5', reverse 3'- cctccaatggatcctcgtta-5'. KAPA Biosytems' SYBR FAST qPCR master mix was used across all platforms using thermal profile as follows: a 20 second hot start at 95°C followed by 40 cycles of 95°C for 1 second and 60°C for 10 seconds. Heating and cooling rates and all other parameters were at the manufacturers pre-set levels.

Thermal variability was assessed using qPCR by measuring amplification of 18S rRNA in a selection of wells with covering all areas of the sample plate on an ABI Prism 7900HT, a Bio-Rad CFX 96 System, a Qiagen Rotor-Gene Q and a BJS Biotechnologies xxpress. Human genomic DNA at 100ng/µL (final concentration of 5ng/µL) was used with the protocol detailed above.

Statistics

Statistical tests commonly used to determine the reliability and accuracy of a quantitative PCR assay include performing a standard curve experiment with each dilution series run in triplicate. The Cq value is plotted against the log of nucleic acid input level to generate a linear graph. The slope or gradient of this graph is used to determine the PCR reaction efficiency and a linear regression analysis with a correlation coefficient or R^2 value is included to determine the accuracy and repeatability of the standard curve. The ideal is to have a PCR reaction efficiency of 100% and an R^2 value of 1. If the efficiency is less than 90% or greater than 110%, this is unacceptable and further optimisation is required. If the R^2 value is $\leq$ 0.985, this can raise questions about assay reliability with respect to pipetting accuracy and the range of the assay [14].

Results

Amplification efficiency

A standard curve was generated by amplifying 18S rRNA in human genomic DNA at concentrations of 5, 0.5, 0.05, 0.005 and 0.0005ng/µL and plotting C_t against log concentration. Efficiency was calculated by the following equation: Efficiency = $10^{(-1/\text{slope})-1}$. Efficiency of reaction values between 90 and 110% are considered acceptable for qPCR reactions. The fastest instrument was the xxpress®, completing 40 cycles in 12 minutes (Figure 1).

Thermal variability

Thermal variability was assessed by measuring amplification of 18S rRNA in 5ng/µL human genomic DNA in a selection of wells with covering all areas of the sample plate on an ABI Prism 7900HT, a Bio-Rad CFX 96 System, a Qiagen Rotor-Gene Q and a BJS Biotechnologies xxpress (Figure 2). The average Ct, Ct spread and Ct standard deviation were for CFX: 16.0, 1.315 and 0.34; for xxpress: 13.6, 1.2 and 0.29; for Prism 7900HT: 14.4, 4.526, and 1.91; for Rotor-Gene: 16.8, 1.319, and 0.43 (Figure 3).

Discussion

qPCR instrumentation is fast evolving to meet the needs not only of the basic science but also tries to address some of the needs of the current healthcare system, in terms of diagnosis as well as prognosis. For example, qPCR technology has been widely used in the field of molecular diagnostics for a number of infectious diseases [3]. FDA-approved qPCR-based screening tests include: group A streptococcus and methicillin-resistant Staphylococcus aureus (MRSA), HIV-1, hMPV, H1N1 influenza virus to name a few [4-5]. More recently, QIAGEN received FDA approval of therascreen® KRAS RGQ PCR kit; paired with second colorectal cancer drug. KRAS mutations occur in approximately 40% of colorectal cancer patients [6-7]. Therefore, screening patients by PCR the most frequent mutations in the KRAS gene will be detected and will aid with therapeutic intervention.

Over the past decade, there has been a shift from reference hospital/centre testing into clinical/diagnostic laboratories worldwide [3]. Point of care testing (POCT) allows a test to be carried out and results obtained in a single visit to a primary or secondary care health provider [8]. In developing countries, POCT is perhaps even more effective. The need for expensive, central laboratories, highly trained technicians and a reliable method of specimen and data transport can all be removed with the implementation of a well-designed, multifunctional POCT system. Bringing the test into the clinic allows treatment to commence without delay and, in areas of high displacement, reduces the likelihood of losing patient contact before the condition has been effectively treated. This is particularly important for

communicable diseases such as HIV/AIDS, measles and typhoid fever [9]. An effective POC test in a low resource setting is inexpensive to use and maintain. The test must be easy to operate, requiring little to no training or specialist knowledge to both generate and interpret results. In a recent study of sub-Saharan Africa, only 34% of hospitals have reliable electricity access [10]. Since energy access for healthcare facilities in this region varies dramatically, and as electrical sources may be unreliable, low electrical consumption or even the ability to run on battery or solar power is desirable.

Here we demonstrate that new technologies in qPCR instrumentation like xxpress®, perform equally well –or even better- when compared to conventional qPCR instruments, in terms of amplification efficiency and thermal uniformity. However, a major advantage is that this instrument can deliver 40 cycle qPCR in less than 10 minutes. Rapid testing can be lifesaving. For example, rapid diagnostic tests can help in the diagnosis and management of patients who present with signs and symptoms compatible with influenza. These technologies can cut down the time for conventional viral cell cultures from 3-10 days to less than minutes [11]. Infections with MRSA are known to be associated with considerable morbidity and mortality [12]. Current sample preparation/testing times based on blood samples can take up to 5 hours. However, in an emergency situation this process might be too long; if the patient admitted is positive for MRSA and therefore has the potential to infect others. Equally, an early diagnosis of tuberculosis will assist not only in appropriate treatment initiation but also limit the spread of this highly contagious disease [13]. Having a test that could be administered either at admission to the clinic, or even in an ambulance on the way to the hospital, and would only take 10 minutes could be of real benefit. Moreover, given the unreliability of electricity in the developing world, diagnostic instrumentation that is rapid is vital.

To this date, qPCR-based diagnostics is often associated with high cost, time consuming procedures, scientists and clinicians trained on qPCR analyses, lack of specificity and sensitivity or even standardisation for certain tests. Looking into the future (Figure 4), a

standardised, rapid, scalable, affordable, and easy-to-use qPCR as a POCT will provide an invaluable platform in the field of diagnostic/prognostic testing that will complement the current conventional methods including microscopy, cell cultures and immunological-based methods.

Disclosure

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Figure legends

Table 1. Ramp rate and thermal uniformity of qPCR instruments.

Figure 1. Amplification efficiency of 4 qPCR instruments.

Figure 2. Thermal variability upon amplification of 18s rRNA using 5ng/µL of human genomic DNA. Panel a: CFX; Panel b: xxpress, Panel c: PRISM7900HT, and Panel d: Rotor-Gene.

Figure 3. Average Ct of all instruments**.**

Figure 4. Current and future applications of qPCR testing. CTCs: circulating tumour cells, SNPs: Single nucleotide polymorphisms, TDM: Therapeutic drug monitoring, miRNA: micro RNA.

qPCR Platform	Thermal System	Advertised fastest ramp rate	Advertised thermal uniformity
ABI 7900 HT	Block/Peltier	1.5°C per second	± 0.5°C (measured 30 seconds after the clock starts)
BioRad CFX 96	Block/Peltier	3.3°C per second (average Ramp Rate)	±0.4°C (well-to-well within 10 sec of arrival at 90°C)
Qiagen Rotor-Gene	Air	15°C per second (Peak Ramp Rate)	±0.02°C
BJS Biotechnologies xxpress	Resistive heating	10°C per second	±0.3°C

Table 1.

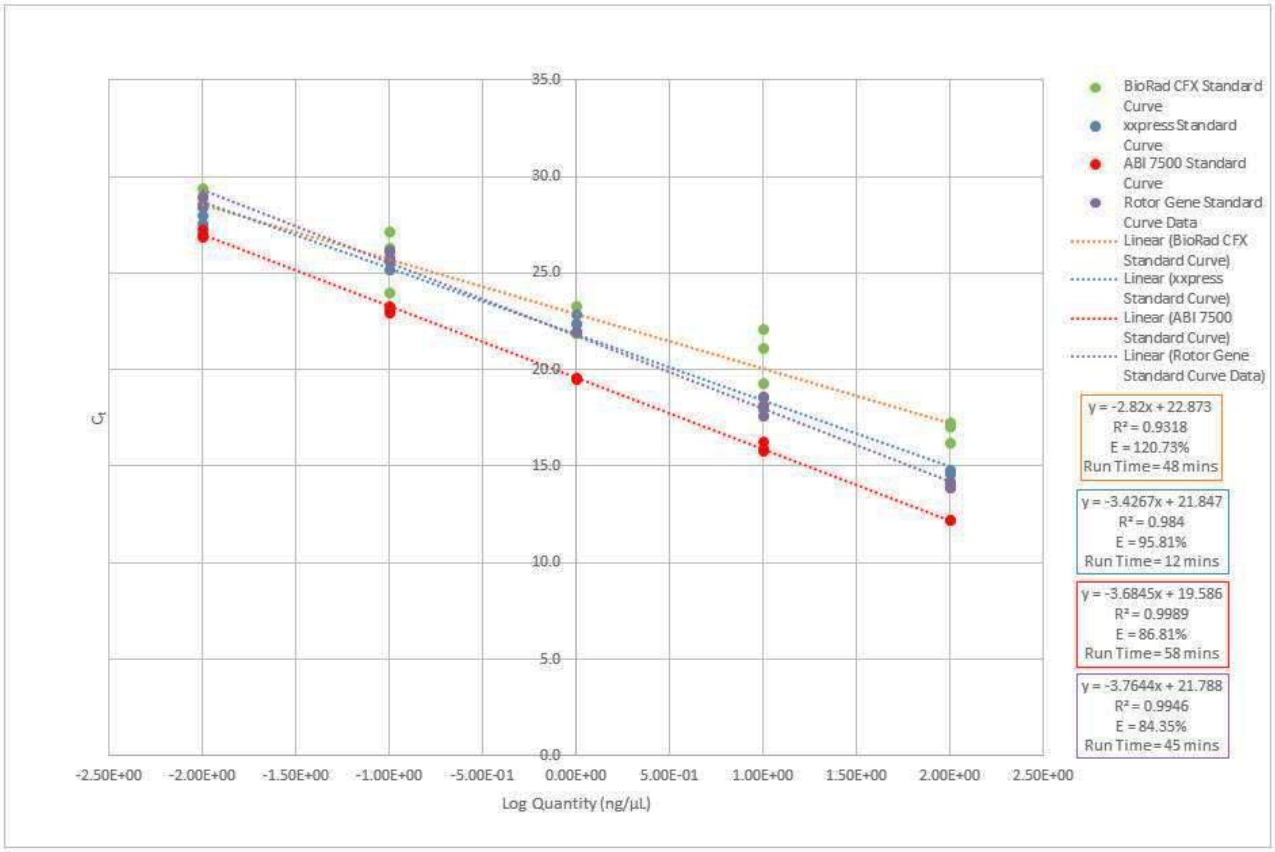
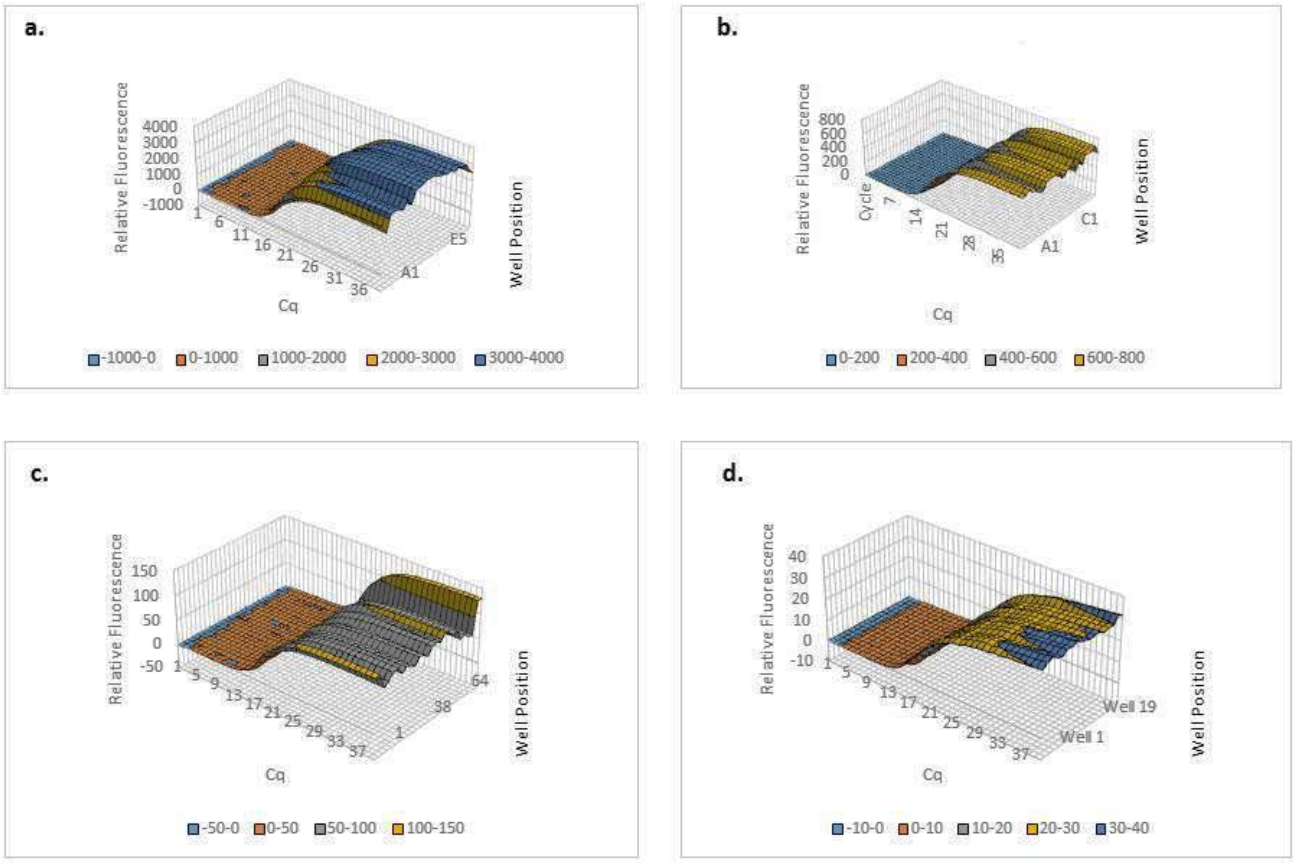

35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Ct
-2.50E+00
-2.00E+00
-1.50E+00
-1.00E+00
-5.00E-01
0.00E+00
5.00E-01
1.00E+00
1.50E+00
2.00E+00
2.50E+00
Log Quantity (ng/µL)
BioRad CFX Standard Curve
xxpress Standard Curve
ABI 7500 Standard Curve
Rotor Gene Standard Curve Data
Linear (BioRad CFX Standard Curve)
Linear (xxpress Standard Curve)
Linear (ABI 7500 Standard Curve)
Linear (Rotor Gene Standard Curve Data)
y = -2.82x + 22.873
R² = 0.9318
E = 120.73%
Run Time = 48 mins
y = -3.4267x + 21.847
R² = 0.984
E = 95.81%
Run Time = 12 mins
y = -3.6845x + 19.586
R² = 0.9989
E = 86.81%
Run Time = 58 mins
y = -3.7644x + 21.788
R² = 0.9946
E = 84.35%
Run Time = 45 mins

Figure 1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Relative Fluorescence
4000
3000
2000
1000
0
-1000
1
6
11
16
21
26
31
36
Cq
A1
E5
Well Position
-1000-0
0-1000
1000-2000
2000-3000
3000-4000
b.
Relative Fluorescence
800
600
400
200
0
Cycle
7
14
21
28
35
Cq
A1
C1
Well Position
0-200
200-400
400-600
600-800
c.
Relative Fluorescence
150
100
50
0
-50
1
5
9
13
17
21
25
29
33
37
Cq
1
38
64
Well Position
-50-0
0-50
50-100
100-150
d.
Relative Fluorescence
40
30
20
10
0
-10
1
5
9
13
17
21
25
29
33
37
Cq
Well 1
Well 19
Well Position
-10-0
0-10
10-20
20-30
30-40

Figure 2

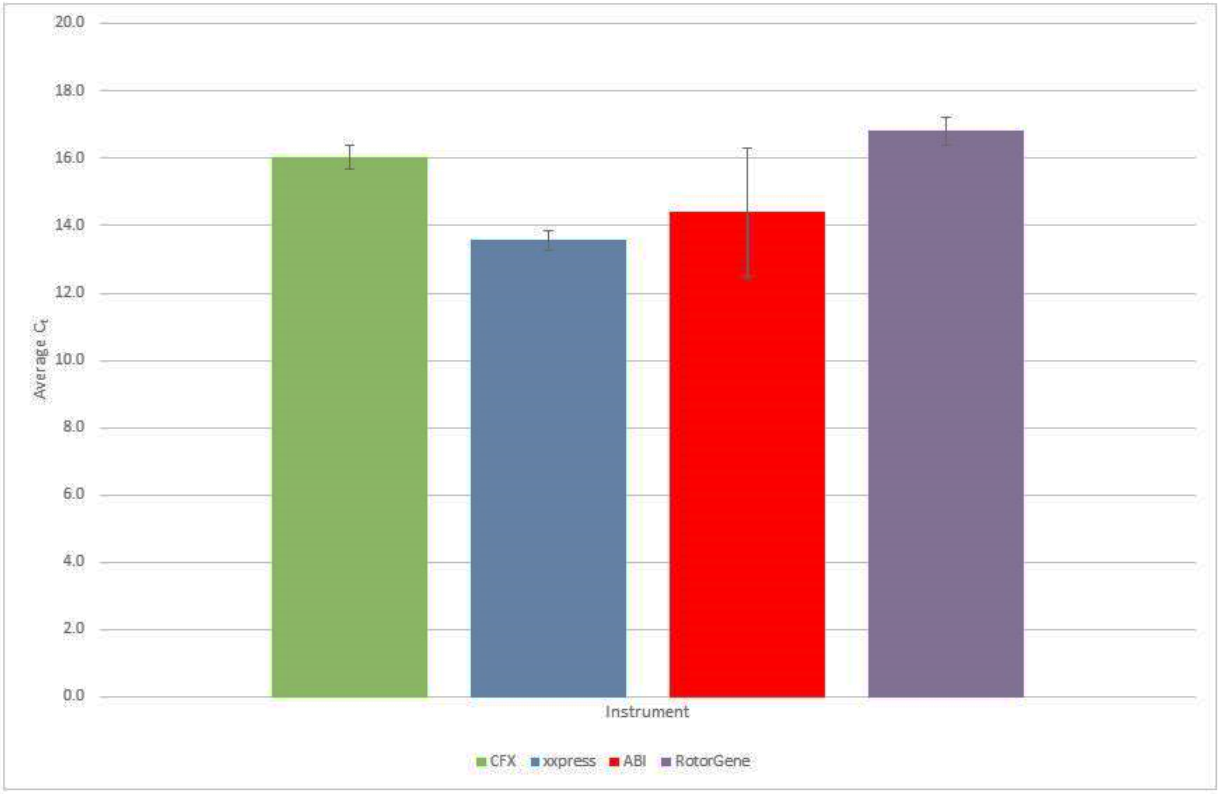
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Average Ct
Instrument
CFX
xxpress
ABI
RotorGene

Figure 3

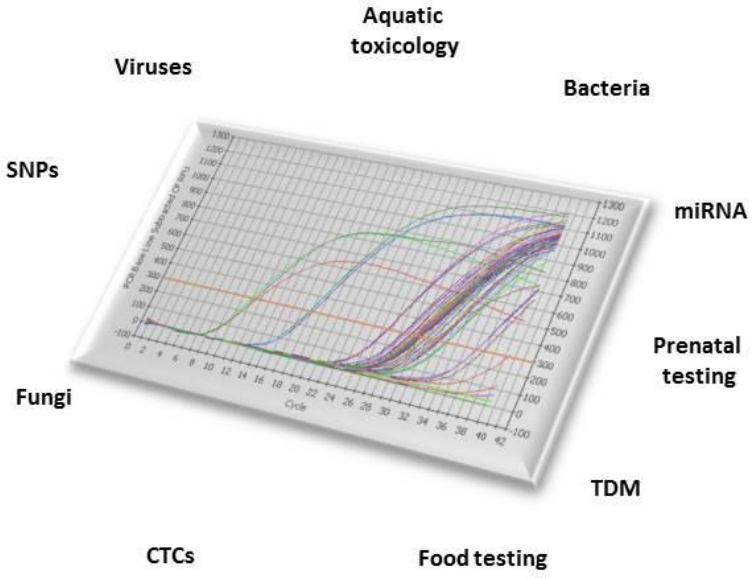
Aquatic toxicology
Viruses
Bacteria
SNPs
miRNA
Prenatal testing
Fungi
TDM
CTCs
Food testing

Figure 4