

Novel and sensitive qPCR assays for the detection and identification of aspergillosis causing species

Running title: qPCR detection of *Aspergillus* species

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Abstract

Despite concerted efforts, diagnosis of aspergillosis is still a great challenge to clinical microbiology laboratories. Along with the requirement for high sensitivity and specificity, species-specific identification is important. We developed rapid, sensitive and species-specific qPCR assays using the TaqMan technology for the detection and identification of *Aspergillus fumigatus* and *Aspergillus terreus*. The assays were designed to target orthologs of the *Streptomyces* factor C gene that are only found in a few species of filamentous fungi. Fungi acquired this gene through horizontal gene transfer and divergence of the gene allows identification of species. The assays have potential as a molecular diagnosis tool for the early detection of fungal infection caused by *Aspergillus fumigatus* and *Aspergillus terreus*, which merits future diagnostic studies. The assays were sensitive enough to detect a few genomic equivalents in blood samples.

Keywords:

aspergillosis, *Aspergillus fumigatus*, *Aspergillus terreus*, qPCR, *Streptomyces* factor C gene.

Introduction

Certain species of the fungal genus *Aspergillus* are able to cause diseases in humans, especially in those with impaired immune system/immunocompromised patients. Besides the standard risk groups for invasive aspergillosis (IA), which include neutropenic, leukemic patients and patients receiving hematopoietic stem cell and solid organ transplantation there is a growing trend in the number of systemic invasive fungal infections associated with new advances in modern medicine using aggressive immunosuppressive regimens [1-3].

In these patients IA is very common and the mortality rate is high [4-5]. The most frequent human pathogen that causes aspergillosis is *A. fumigatus*, responsible for about 90% of all cases [6] but other *Aspergillus* species including *A. terreus*, *A. flavus*, *A. niger* may also cause the disease [7, 8]. Studies have also described *Neosartorya* [9] and *Chaetomium* [10] species causing aspergillosis.

Surviving IA depends on the early diagnosis of infection and the proper and timely initiation of antifungal therapy. Based on this there is an urgent need for standardized, reliable diagnostic methods with maximum specificity and sensitivity.

Diagnosis of IA is difficult using microbiological and histological methods that are time consuming and usually verify the disease at autopsy. Furthermore, the initiation of additional diagnostic examinations is often delayed because of a low clinical suspicion

[11]. Signs and symptoms of systemic diseases caused by *Aspergillus* species are non-specific and patients are often unable to undergo invasive diagnostic procedures.

The main non-invasive diagnostic techniques for IA include the detection of surrogate biomarkers; galactomannan [12] or β -D-glucan [13] antigens that are common cell wall components of fungal cells, or fungal DNA, usually the rRNA genes or their internal transcribed spacer regions, circulating in the blood [14].

An interesting new approach is the amplification of circulating fungal mRNA that represents viable fungal cells [15, 16]. Recently antigen-capture ELISA has been used for the detection of new fungal biomarker molecules [17, 18]. Despite these approaches, there is no generally accepted molecular method available for the early and reliable diagnosis of IA, therefore exploration of new possibilities is essential.

Here we describe novel and sensitive qPCR assays for the detection and identification of *Aspergillus fumigatus* and *A. terreus*. The assays are based on the quantification of fungal orthologs of the *Streptomyces facC* gene.

In the soil, fungi compete with filamentous bacteria such as *Streptomyces*. Interestingly, we discovered a gene in various *Aspergillus* species that has strong similarity to *facC* gene for the signaling protein factor C found in several *Streptomyces* [19]. The fact that codon usage is the same as in streptomycetes suggests that it was acquired via horizontal gene transfer [20]. Only some fungi have orthologs, allowing identification with high fidelity, while the strong sequence divergence between the orthologs in different fungal species allows speciation.

Materials and methods

Growth of the strains and isolation of genomic DNA

Aspergillus fumigatus AF293 and *Aspergillus terreus* NCCB IH2624 strains were cultivated on standard minimal nitrate medium [21]. Freshly isolated (*A. fumigatus*: 3 days, *A. terreus*: 7 days) conidia were suspended in PBS–0.01% Tween 80, counted in a Bürker chamber and used in spiking experiments.

Genomic DNA was isolated from liquid cultures grown in minimal medium at 37 °C (*A. fumigatus*) and 25 °C (*A. terreus*) at 220 rpm for 18 h. The mycelium was disrupted in liquid nitrogen [21] and DNA isolated using the Genomic DNA Purification Kit (Thermo Scientific, Maryland, USA) according to the manufacturer's instructions.

DNA extraction from biological samples spiked with conidia

After the disruption of the fungal cell walls with the MagNA Lyser instrument using ceramic Green Beads for 90 sec at 5000 rpm (Roche Applied Science) fungal DNA was isolated by automated extraction with the MagNA Pure LC instrument. 1200 µl of whole blood samples from healthy volunteers were spiked with 10^6 to 10^1 *Aspergillus*

fumigatus or *A. terreus* conidia. Samples were bead beaten with glass/ceramic beads and 1000 µl lysate was further processed for DNA extraction using the MagNa Pure LC Total Nucleic Acid Isolation Kit – Large Volume (Roche Applied Science) according to the manufacturer's recommendations. DNAs were eluted in a volume of 50 µl.

***A. fumigatus* and *A. terreus* TaqMan primers and probes**

A. fumigatus and *A. terreus* genomic DNA was used as template in the assays. There are two *facC* orthologous genes in *A. fumigatus* located on chromosomes 3 and 5. The copy on chromosome 3 (AFUA_3G14910 FacC-like extracellular signaling protein) was targeted with the following qPCR assay: forward primer: 5'-CAAGCAGCCGGAGTTGGA-3'; reverse primer: 5'-ACTGTCCATACGCTGCATAACC-3'; hydrolysis probe 5'-FAM-ACGCTGTCGTTGACTTT-MGB (MGB1 assay). These primers amplified a 62 bp DNA fragment.

The copy on chromosome 5 (AFUA_5G00540 FacC-like extracellular signaling protein) was targeted with the following qPCR assay: forward primer: 5'-AATCCCCGACTCTCCACGAT-3'; reverse primer: 5'-TCCGCCAGAGGTCATACGA-3'; Hydrolysis probe 5'-FAM-CGACCTCACCAAACC-3'-MGB (MGB2 assay). These primers amplified a 56 bp

DNA fragment. In the dual detection reaction both copies of *facC* (located on chromosomes 3 & 5) were detected (MGB3 assay).

Only a single copy of the *facC* orthologous gene (ATEG_03536 hypothetical protein similar to factor C protein precursor) is present in *A. terreus*. This was targeted using the following qPCR assay: forward primer: 5'-CGAATGGATACGGGAAGAAGCT-3'; reverse primer: 5'-CGAGCGAGGCATCGGTATG-3'; hydrolysis probe 5'FAM-TTGCCATTGACAAACTT-MGB (MGB4 assay). These primers amplified a 78 bp DNA fragment.

Assay conditions

The assays were run in 20 µl reactions containing: 10 µl TaqMan® Universal PCR Master Mix (2x) (Applied Biosystems); 1 or 2 µl TaqMan® gene expression assay(s) (20x Applied Biosystems) containing the forward and reverse primers (900 nM) and the FAM-labeled TaqMan-MGB probe (250 nM); 8 or 9 µl of DNA template. qPCR was performed on 7500 Real Time PCR platform (Applied Biosystems) with the following reaction parameters: Stage 1: UNG inactivation; 50°C, 2 min. Stage 2: denaturing DNA and Taq enzyme activation; 95°C, 10 min, Stage 3: 55 cycles of 95°C, 10 sec, 60°C 1 min. Negative controls (NTC, non-template control) were 8 or 9 µl PCR grade water added to the reaction mix instead of sample. Each sample was run in triplicate and

according to ISHAM EAPCRI consensus at least one positive result was enough to consider the sample as positive.

Analytical sensitivity and specificity

Serial dilutions of *A. fumigatus* or *A. terreus* genomic DNA (gDNA) was used as template to determine the analytical sensitivity of the assays. The concentrations of gDNA ranged from 17 ng to 85 fg which is equivalent of about 10^6 to 5 genomic equivalents (GE); assuming that 1 GE of *A. fumigatus* and *A. terreus* corresponds to 17 fg.

The specificity of the assays was tested by adding 200 ng human genomic DNA to serial dilutions of *A. fumigatus* or *A. terreus* gDNA ranging from 17 ng to 17 pg

The analytical specificity of the assays was further tested against a panel of several *Aspergillus* and other fungal species as well as bacterial strains listed in Table I. Some of these strains are known to contain factor C orthologs.

PCR linearity and efficiency

To study the correlation between the threshold cycle number (C_q) of the qPCR and the genomic load standard curves were obtained by plotting C_q values against the log of genome number. PCR efficiency was calculated from the slopes of the curves by the following equation: $E = [10^{(-1/\text{slope})}] - 1$.

Results and Discussion

Aspergillus species involved in IA have *Streptomyces facC* orthologs

Factor C was described as an extracellular signaling protein that affects morphological differentiation and antibiotic production in *Streptomyces*. Proteomic studies recently showed that it compensates for a mutation that led to almost complete abolishment of production of A factor and regulates the production of many sporulation specific proteins [22,23].

At the time of the sequencing of its gene it was an orphan gene [19]. DNA hybridization experiments and the accumulation of genome sequencing data revealed only a few homologous genes in *Streptomyces* and fungal strains but the gene is far from being widely distributed even in these taxa [20]. Because of the high GC content of the fungal orthologous genes we hypothesize that the gene was acquired by the fungal strains by horizontal gene transfer from streptomycetes in their common ecological niche. It is reasonable to suppose that these filamentous soil microorganisms eat each other – streptomycetes possess batteries of chitinases that would help them to digest fungal walls, and many fungi make antibiotics active against bacterial cell wall biosynthesis, including β -lactams; and the fungal genes for β -lactam biosynthesis are themselves likely to have been acquired by lateral transfer [24]. Acquisition of the *facC* gene could

help filamentous fungal species in the competition with streptomycetes perhaps by increasing the rate of sporulation. In the fungal strains the *facC* genes diverged that makes them suitable for species specific identification.

Analytical sensitivity and specificity of the assays

The analytical sensitivity of the qPCR assays is shown in Figures 1. and 2. The limit of detection of single *facC* ortholog-specific assays was 127.5 fg (7.5 GE), while the analytical sensitivity of *A. fumigatus*-specific, dual-copy assay hybridizing to both chromosome 3 and 5 *facC* orthologs was 85 fg (5 GE).

The limit of detection for the *A. terreus* assay was 170 fg (10 GE).

The analytical sensitivity of the *A. fumigatus* and *A. terreus* assays was unaffected by the presence of high excess (200 ng) of human gDNA (data not shown).

Neither of the *A. fumigatus* assays cross reacted with *A. terreus* DNA or human genomic DNA nor did the *A. terreus* assay cross reacted with *A. fumigatus* genomic DNA or human genomic DNA (results not shown). Testing the assays against a panel of DNA from a variety fungi and bacteria revealed no cross reactions (Table I) even in species that are known to contain factor C or *facC* orthologous genes such as *Streptomyces albidoflavus* 45H, *Penicillium chrysogenum*, *Streptomyces albus* R-55, or *Streptomyces flavofungini*.

PCR linearity and efficiency

Standard curves derived from qPCR analysis of serial dilutions (17 ng to 85 fg) of gDNA were used to determine the linearity and efficiency of each assay. The PCR efficiencies for the *A. fumigatus* assays were 102.8%, 113.9% and 106.6% when the *facC* copy on chromosome 3, the *facC* copy on chromosome 5, or when both copies were detected. The efficiency of the *A. terreus* assay was 108.4%. The efficiency values of the qPCR assays are shown in Table II.

These data are within the acceptable range.

Testing the analytical sensitivity on blood samples

The usefulness and efficiency of the MGB3 and MGB4 assays was tested on 1200 μ L blood samples spiked with conidia. Although the blood of patients contains mycelial fragments rather than conidia in these experiments to measure the number of cells precisely we used conidia that are more difficult to disrupt. To test the assays in parallel experiments we also used mycelia spiked blood and both assays worked properly. The assays performed well in the range of 10^6 to 10 conidia. The efficiency values of the *Aspergillus fumigatus* and *A. terreus* specific assays were; 89, 88% and 102.6%. R² values were 0.968 and 0.940 respectively (data not shown).

Conclusions

Despite recent advances in diagnosis and treatment, aspergillosis is still a leading cause of death in immunocompromised individuals. Although the array of DNA-based molecular diagnostic tests that outperform the conventional methods has expanded over the past two decades, these platforms were not included in the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) consensus definitions because of the lack of standardization. It is clear that there is an urgent need to develop effective and reliable diagnostics for IA due to the continuing growth of publications working at incorporating DNA based applications into routine clinical diagnostics to reduce costs and to improve sensitivity and specificity respectively [25-31] and supporting diagnostic driven therapy.

The species level identification of two of the most common causes of aspergillosis is also of great importance, since it is a prerequisite of the targeted antifungal therapy because different species respond differently to a given antifungal treatment. This also addresses a significant drawback of empirical therapies that assist the spread and development of drug-resistant fungi; additionally they expose the infected patient to toxicity and other severe side effects [32].

In the case of *A. fumigatus* and *A. terreus* more than 10 independent isolates were tested and proved to contain the target *facC* gene. Furthermore in an international EAPCRI specificity panel study our assays proved to be specific for these two species. We believe that this is an indication that the *facC* orthologs are present in these species without sequence variation. Although only one isolate was tested from the other fungal strains we suppose that the gene is really absent from them e.i. testing many more isolates would also give similar results.

The methods described in this study presents sensitive, species-specific, cost-effective qPCR assays for the detection of IA. The absence of false positive results may be explained by the rare occurrence of the investigated *facC* gene in fungi and by the relatively high guanine and cytosine (G+C) content of the gene. Although they are not multi-copy assays, their analytical sensitivity (5 to 10 GE) matches that of assays based on the detection of multi-copy rRNA genes [33]. In a direct comparison of the sensitivity our home-brewed real-time PCR *A. fumigatus* assay superseded the gold standard galactomannan assay.

Since the assays are not based on the detection of fungal ribosomal RNA gene, they may also be used to verify results based on the detection of rRNA genes. This combined identification may improve the reliability of the identification of *Aspergillus* species.

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Table I. Testing of species-specific real-time PCR assays against genomic DNAs from fungi, bacteria and human

Strain Tested	Factor C ortholog	Target Gene		
		AFUA_3G14910	AFUA_5G00540	ATEG_03536
<i>Aspergillus fumigatus</i> Af293 and 8 other clinical isolates	Present	+	+	-
<i>Aspergillus terreus</i> NCCB IH2624 and 2 other isolates	Present	-	-	+
<i>Aspergillus nidulans</i>	Absent	-	-	-
<i>Aspergillus niger</i>	Absent	-	-	-
<i>Aspergillus brasiliensis</i>	Absent	-	-	-
<i>Aspergillus lentulus</i>	Absent	-	-	-
<i>Aspergillus flavus</i>	Absent	-	-	-
<i>Aspergillus tamaritii</i>	Absent	-	-	-
<i>Aspergillus pseudotamaritii</i>	Absent	-	-	-

<i>Aspergillus tubingensis</i>	Absent	-	-	-
<i>Candida albicans</i>	Absent	-	-	-
<i>Trichoderma resei</i>	Absent	-	-	-
<i>Penicillium chrysogenum</i>	Present	-	-	-
<i>Escherihia coli</i>	Absent	-	-	-
<i>Streptomyces albidoflavus</i> 45H	Present	-	-	-
<i>Streptomyces griseus</i> 52-1	Absent	-	-	-
<i>Streptomyces lividans</i> 1326	Absent	-	-	-
<i>Streptomyces albus</i> R-55	Present	-	-	-
<i>Streptomyces flavofungini</i>	Present	-	-	-
<i>Homo sapiens</i>	Absent	-	-	-

Table II. Efficiency (%) and the coefficient of determination of the assays

Assay	Equation (y)	Slope	Efficiency (%)	Coefficient of Determination (r^2)
MGB1	$-3.2569x+43,173$	-3.2569	102.8	0.9987
MGB2	$-3.0274x+41,363$	-3.0274	113.9	0.9979
MGB3	$-3.1558x+41,456$	-3.1558	106.6	0.9972
MGB4	$-3.1353x+43,105$	-3.1353	108.4	0.9953

Figure captions

Figure 1.

Determination of the analytical sensitivity of the TaqMan assays.

- (a) Assay MGB1, target gene *Aspergillus fumigatus facC* ortholog on chromosome 3.
- (b) Assay MGB2, target gene *Aspergillus fumigatus facC* ortholog on chromosome 5.
- (c) Assay MGB3, target gene *Aspergillus fumigatus facC* orthologs on chromosomes 3 & 5.
- (d) Target gene *Aspergillus terreus facC* ortholog.

Normalized fluorescence values as a function of cycle numbers are shown. Samples are a serial dilution of genomic DNA from *A. fumigatus* (a, b and c) or *A. terreus* (d): 1 = 17 ng; 2 = 1.7 ng; 3 = 170 pg; 4 = 17 pg; 5 = 1.7 pg and 6 = 85 fg.

Figure 2.

Real-time PCR linearity and dynamic range.

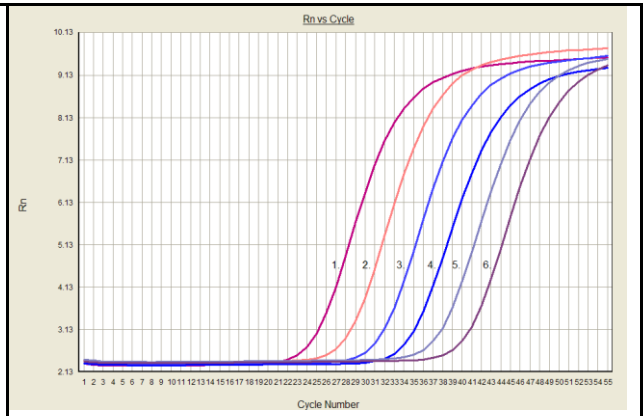
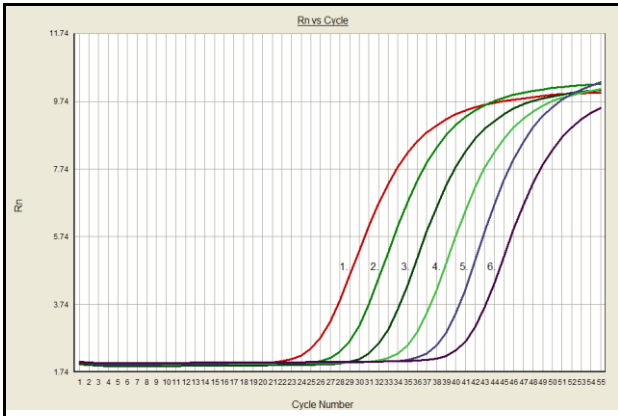
The linearity of the assays was achieved over a 5-log range of input from 17 ng to 85 fg gDNA.

Aspergillus fumigatus MGB1 assay detecting the *facC* gene ortholog located on chromosome 3. LoD: 127.5 fg.

Aspergillus fumigatus MGB2 assay detecting the *facC* gene ortholog located on chromosome 5. LoD: 170 fg.

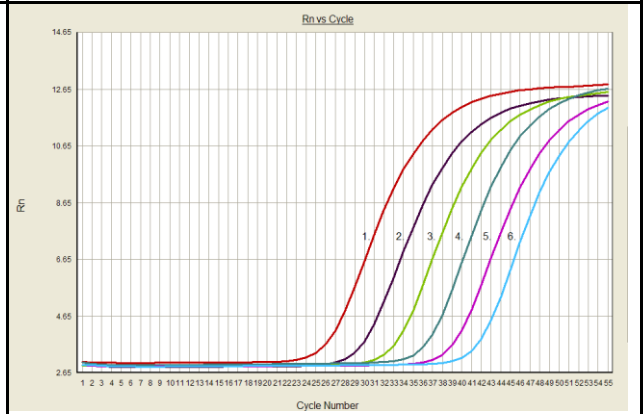
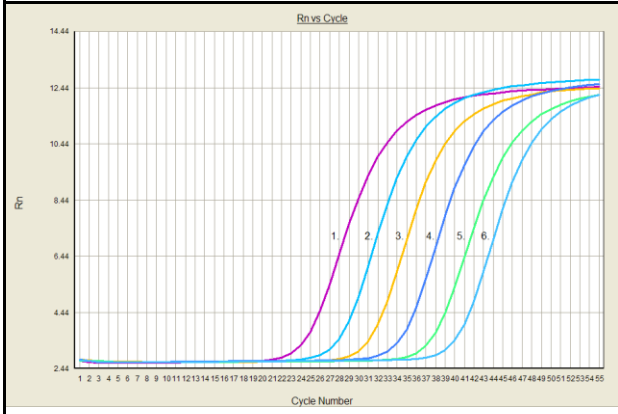
Aspergillus fumigatus MGB3 assay detecting the *facC* orthologs located on chromosome 3 & 5. LoD: 85 fg.

Aspergillus terreus MGB4 assay detecting the *facC* gene ortholog in the *A. terreus* genome. LoD: 170 fg.



a.,

b.,



c.,

d.,

Figure 1.

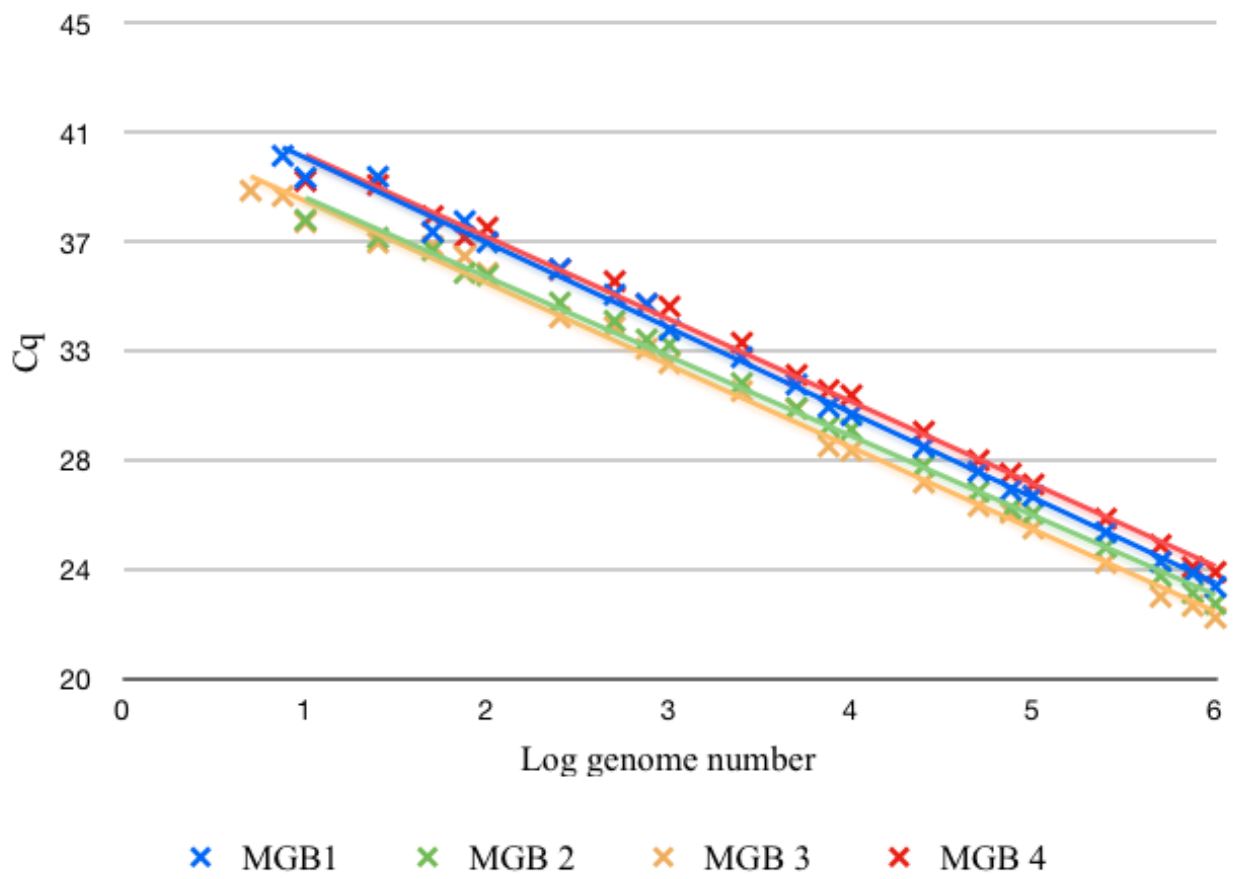


Figure 2.