Performance of Various TaqMan and SYBRGreen rRT-PCR Methods in Detecting the Egyptian Foot-and-Mouth Disease Viruses

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Abstract

Proficient application of the advanced technology for foot-and-mouth disease virus (FMDV) detection, still a target challenge in Egypt, not only for rapid, identification of the transboundary continuously evolving virus, but also support accurate control strategy involving manufacturing of a high-quality protective vaccine. The article discussed both two real-time RT-PCR (rRT-PCR) methods depending on either fluorophore or fluorescent dye for the precise detection of FMDV few copies. TaqMan based probe/primer overcame SYBR Green-based primer rRT-PCR by 10-fold for detecting less FMDV nucleic acid. In contrary, lower threshold cycles values with fewer nucleic acid copies biased to the fluorescent RRT-PCR with 3-4 cycles earlier. TaqMan format had better sensitivity and specificity, while SYBR Green method displayed less detection limit and precision.

Index terms— foot-and-mouth disease virus, rRT-PCR, taqman.

1 I. Introduction

oot-and-mouth disease virus (FMDV) is an ancient contagious particle as cited by Fracastorius (1478-1553) of Verona in 1514 in his classical treatise on contagions where he pointed out to the virus symptoms in oxen in Italy with pustules in the oral cavity and gradually the disease descended to the shoulders and thence to the feet (Greenwood, 1927). The virus has a linear single-stranded RNA genome. Disease is one of the most infectious diseases known in part due to rapid and massive viral replication in the host and considerable genetic variability (Tam et al., 2009 andOthman et al., 2018).

Developing countries face usually challenge against transboundary disease specially foot-and mouth-disease due to less strict measures implemented at borders, besides less sanitary condition and precaution in the quarantine areas. Thus, rapid differentiation of FMDV is imperative to take effective counter measures to control the spread of the disease. Nowadays, molecular assay, especially, real-time RT-PCR is considered a trend of fast and accurate discrimination of FMDV with reduced post-PCR processing steps.

Numbers of fluorescent and fluorophore formats were performed. Draw back was noticed such as false Negative results, where it was cited that 5' UTR probe assay greater sensitivity in the detection of type a isolates while 3Dpol target assay greater sensitivity in the detection of FMD SAT isolates **??**Calhan et al. 2002;Reid et al., 2002;Tam et al., 2009). The false result will give inaccurate data about the disease spreading status, which will result in more animals infected and losses affecting the economic welfare especially of the poor peasants in the low annual income countries that posse's individual animals breeding and not mass herd farms.

2 II. Materials and Methods

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Accordingly, the study of Taqman probe as an advanced tool for precise detection of FMDV is an alternative to SYBR Green real-time RT-PCR (RRT-PCR) format in terms of sensitivity and specificity. The fast and accurate discrimination of FMD virus is imperative for proficient containment and eradication of the disease. Therefore, the article was designed for inspection the efficiency of Taqman probe RRT-PCR in the accurate determination of FMDV. Besides, examination of two different RRT-PCR formats depending on fluorophore and fluorescent dye for sensitive and specific detection of the virus.

4 a) Viruses and samples

An archived vaccine strains viz. O/EGY/2009 iso1 (propagated on cell culture), A/EGY/2009 iso-Cai (clinical suspension) and SAT2/EGY/H1Ghb/2012, local strains of serotypes O, A and SAT2, respectively (Abu-Elnaga, 2011; EL-She hawy et al., 2011 and 2014) were used. They were exploited as the starting material for RNA extraction to optimize the real-time RT-PCR (RRT-PCR) protocols, as well as reflect the specific ability of the primers and probe to detect different FMDV serotypes found in Egypt. Other FMD viruses representing the former serotypes, previously identified by RT-PCR assay and some of them type confirmed by nucleotide sequencing were used as unknown samples in the validity of the RRT-PCR assays. These FMD viruses were clinical and culture grown isolates, O (n=10), A (n=4) and SAT2 (n=2). Furthermore, Bovine Viral Diarrhea (BVD), belonging to the family Flaviviridae, is one of the endemic viruses in Egypt that cause mucosal disease with excessive drool and lameness in chronic infection was fortunately obtained and passed versus FMDV designed specific primers and probe.

5 b) RNA Extraction and Analytic Sensitivity

RNA was extracted using the QIAamp® Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. Extracted RNAs from three FMDV O were quantitated by ultraviolet (UV) spectrophotometer and used as; in-home Standard (2017 identified virus), Positive control 1 (2012 identified virus) and Positive control 2 (2009 identified virus). Two spectrophotometer instruments were used, one was classic spectrophotometer (Milton Roy 601 Spectronic 335104, USA) and the other was recent spectrophotometer (Nano Drop 2000c Spectrophotometer, Thermo Fisher Scientific, and USA). In the context of the standard, it was diluted seven times in a serial of 10-fold in Rnase-free water to obtain the Standard RNA Dilutions (SRD). Archived Stock viruses (SV) RNAs (Azab et al., 2012) were exploited in the current study to evaluate the performance of RRT-PCR assays on RNAs with long storage period. Briefly, ten-fold serial dilutions of stock virus (SV) in minimum essential medium (MEM) with Hank's salts in the range of 10 -1 -10 -8 were performed. Each dilution was exposed to RNA isolation procedures to prepare SV RNAs. Analytic sensitivity was applied on the former different RNAs preparation formats termed SRD and SV RNA. Negative controls involved: no template control (NTC) which was RNase free water; negative control 1 that was RNA from healthy BHK cells; and negative control 2 that was RNA from non-infected BHK cells showed contamination. min, 1 cycle at 95 C for 15 min and 45 cycles at 95 C for 15 s and 60 C for 1 min. For the Fluorescence RRT-PCR, the optimized reaction also contained 5% of the reaction volume RNA template and 0.6 µM from each primer. The cycling parameters were 50 C for 30 min and 95 C for 15 min; then 45 cycles consisting of 94 C for 30 s, 55 C for 30 s and 72 C for 30 s. At least one of the positive controls and negative controls was involved in every run. RNA concentration of the standard, its dilutions, and positive controls were determined by spectrophotometer using both two devices, the traditional one depend on dilution 1/100 of the measured RNA samples in 200 µl quartz cuvette (Milton Roy 601 Spectronic) and the modern one without any dilution depending on the whole volume of 2 µl (Nano Drop 2000c Spectrophotometer). The nucleic acid concentration readings for the Standard, Positive Control 1 and Positive Control 2 were 6, 4.8 and 5.75 ng/µl, respectively; while the dilutions gave minus reading (Table 1).

6 c) Real-time RT-PCR

All the extracted RNAs were tested on a fluoro metric thermal cycler (the acquired Corbett Research, Sydney, Australia) using either Quantities Probe RT-PCR Kit or QuantiTect SYBR Green RT-PCR Kit (Qiagen, Germany). The 3D Forward primer: ACTGG-GTTTT-ACAAA-CCTGT-GA; Reverse primer: GCGAG-TCCTG-CCACG-GA and labeled Taqman probe: TCCTT-TGCAC-GCCGT-GGGAC (Callahan et al., 2002 and **??**IE, 2017) was used in Taqman RRT-PCR method. The Taqman probes were labeled with a 5'-reporter dye, 6-carboxyfluorescein and a 3'-quencher, tetramer thylrhodamine (5'-FAM _3'-TAMRA). The forward/reverse primers were purchased from Bioneer, Korea; while, the Probe was designed by Metabion, Germany.

Another primer pair PoR/PoF (Shin et al., 2003), derived from the virus 3D polymerase were used in SYBR Green RRT-PCR runs. PoF (5´-CCT ATG AGA ACA AGC GCA TC -3´) and PoR (5´-CAA CTT CTC CTG TAT GGT CC -3´) was synthesized by Metabion (Germany) and involved in SYBR Green RRT-PCR protocol.

For Fluorogenic RRT-PCR, cycling conditions were: 1 cycle at 50 C for 30

The specificity of the primers/probe for detection of different Egyptian FMDV serotypes was achieved. Besides, negative controls gave neither threshold cycle (C T) values nor PCR positive band. However, when specificity

assay was repeated to confirm the former result, the same conclusion was obtained, but for negative controls, something was changed. In detection limit run, negative controls gave higher C T values around and above the cut off (?40), in addition to, no or very difficult seen bands on agarose gel electrophoresis (Fig. 1 and 2). Sensitivity assay of the Taqman and SYBR green methods illustrated the range of RNA detection reached six hundred attograms (ag) RNA per microliter (µl) for Taqman, while failed to achieve the previous value in using SYBR Green. Using Taqman Probe, the standard virus and its serially 10-fold dilutions showed a 3.3-3.7 increment increasing of C T values between undiluted virus until its 10 -5 dilution (undiluted & 5 dilution series), whereas, there was a lull in the C T values at dilutions 10 -6 and 10 -7 . Likewise, using SYBR Green protocol, the standard virus and its serially 10-fold dilutions gave a 3.3-4.4 increment variations of C T values between undiluted virus until its 10 -5 dilution (undiluted & 5 dilution series). Furthermore, dilution 10 -6 recorded a 2.6 increment from the previous one, while dilution 10 -7 did not produce C T value.

Investigation, the archived stock virus (SV) RNA, fluorogenic and fluorescent signals with positive results were obtained (Fig. 3 and 4). Signals C T values were in direct proportion to dilutions from 10 -1 to 10 -6 using Taqman method, while from 10 -1 to 10 -5 using the SYBR Green assay. Furthermore, the detection limit extended to 10 -8 for the probe and 10 -7 for the SYBR methods. Melt curve peak (Tm) illustrated the specific amplification giving the expected peak, affirmed by yielding the fragment size on agarose-based electrophoresis. The negative samples controls did not exhibit the anticipated specific former Tm of the primers with either no or border C T values around the concluded negative cut off, 40.

7 IV.

8 Discussion

Two different operators measured the nucleic acid concentrations using the traditional Milton Roy 601 Spectronic on three successive days, whereas, one operator quantified them using the modern NanoDrop 2000c Spectrophotometer at the same day. The accuracy and sensitivity of the NanoDrop were better; specifically it was read in ISO certified lab. Thus, its read was the dependable in the current article. A point of interest, during evaluation the sensitivity of the former instruments (especially for the NanoDrop) in quantification the standard RNA dilutions, were unreliable read beyond 1ng/µl and plateau results in dilutions 10 -1 -10 -7 . To investigate the reason of limitations of the NanoDrop spectrophotometer to give a trustable reading in the 10-fold serial dilutions, its manual was revised, and its specification illustrated that its detection limit is two ng/?L dsDNA. relatively expensive price fridge. Fortunately, the following was applied to minimize the degradation of the nucleic acid stored at -20 C. Fridge had a well-arranged cabinet to allow empty aeration spaces. The cabinet door cautiously handled with rapid open/close action, usually ranged between 15-60 seconds, to avoid the escape of the chilled air. The temperature usually ranging between -18 to -22 C, according to the year season. Also, the former fridge exposed to pause periods (~15 to 30 min) of the Freon freezing cycle along the day that downsize the temp to -7 to -13 C due to the effect of the pre-programmed switch of built-in timer and not the voluntarily switched thermostat.

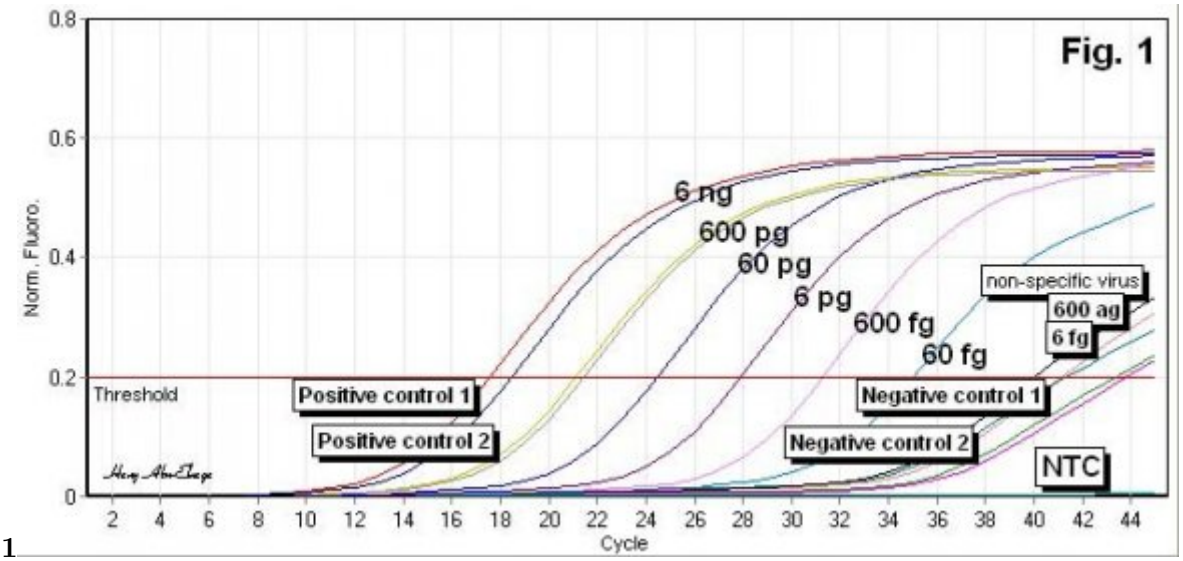
Taqman probe was specific to FMD viruses as revealed by the test carried versus FMD viruses from each virus serotype found in Egypt. Besides no cross-amplification was occurred with one of FMDV, mucosal disease, bovine viral diarrhea, in Egypt. However, in repeating the sample on the agar gel electrophoresis, a very faint band appeared from Taqman assay as well as an obvious weak band was shown from SYBR Green test. Nevertheless, when analysis the Quantitative values obtained from the former two RRT-PCR methods, the concentration value of BVD RNA from fluorescent emission was 2.4 fg/µl, while from fluorogenic signal was two Fg/µl, in addition to, C T values were 37 and 40, respectively. The RRT-PCR value results were somewhat acceptable because it illustrated a bare detection of BVD.

Six-years before the current assay, detection limit of the SYBR Green RRT-PCR PCR for the stored Stock virus (SV) RNA was extended to 10 -7 dilutions using 20% RNA template/rxn volume (Azab et al., 2012). In context, the current article achieved the previous result for SV RNA detection that extended to dilution 10 -7 using SYBR methods, while over passed to dilution 10 -8 using Taqman method, taking in consideration that the RNA template input in this paper was 4x lesser. This result was very satisfactory on a long-term preserved RNA. The stored SV RNA for 6-years in fridge was exploited in the current study to evaluate the performance of a long period storage on RNAs in -20 C fridge, which is an economical price household appliance, in comparison to storage at -70C using a Lastly, Taqman based probe/primer overcame SYBR Green-based primer RRT-PCR by 10-fold for detecting less FMDV nucleic acid. In contrary, early lower C T values with fewer nucleic acid copies biased to the fluorescent RRT-PCR with 3-4 cycles fewer. The Taqman method had better sensitivity and specificity, while SYBR Green method displayed less detection limit and precision. Higher C T values with negative control pose in future to try or search for other probes or another fluorogenic chemistry that might give no or negligible C T value with any negative control to improve the performance of detection avoiding any diminished cross amplification that might occur.

Figure 3A Figure 4A

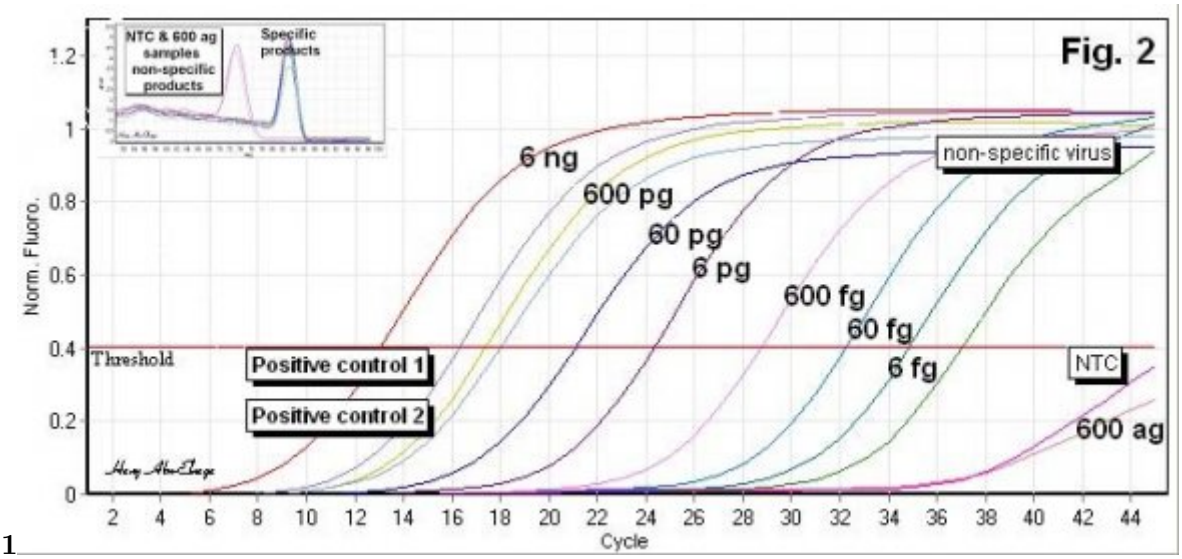
[1]

[1]



1

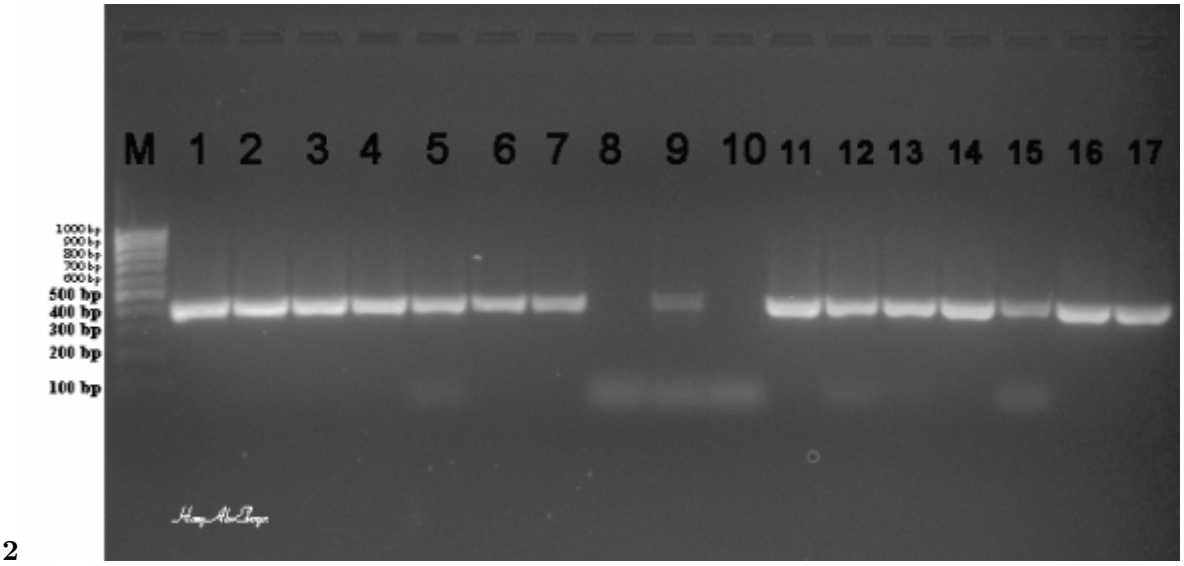
Figure 1: 1



2A1

Figure 2: Figure 1A Figure 2A Figure 1 :

Figure 3:



2

Figure 4: Figure 2 :

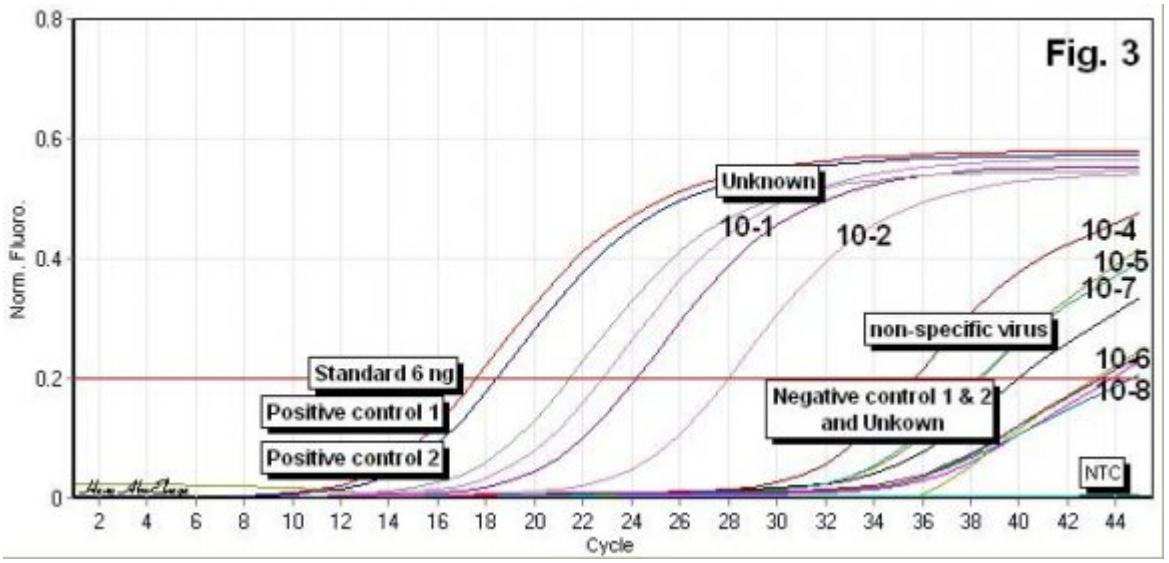


Figure 5: Figure 3B

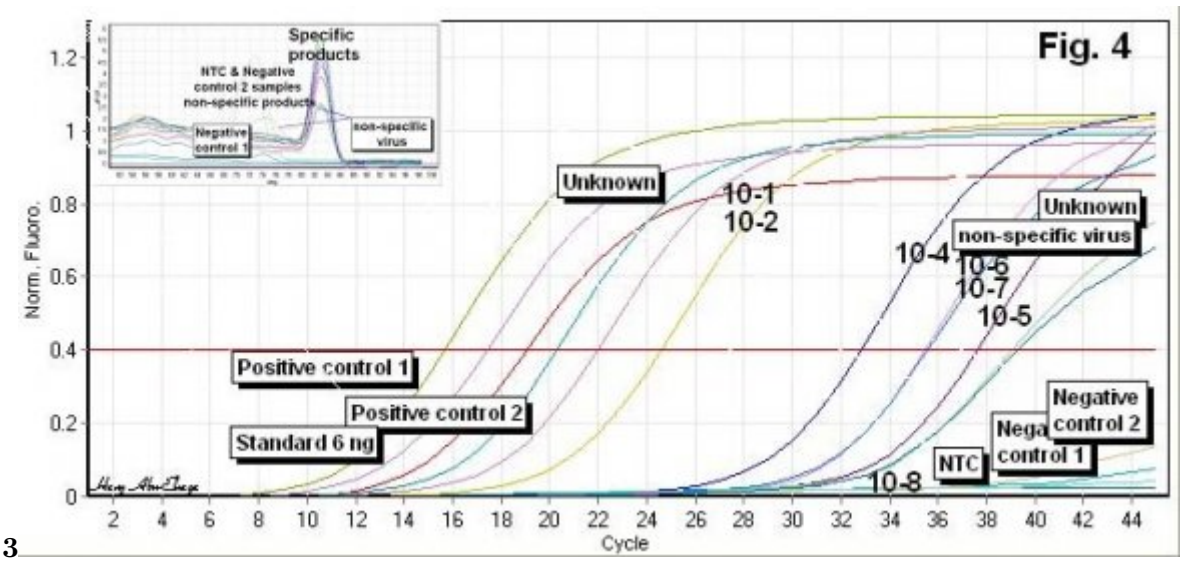


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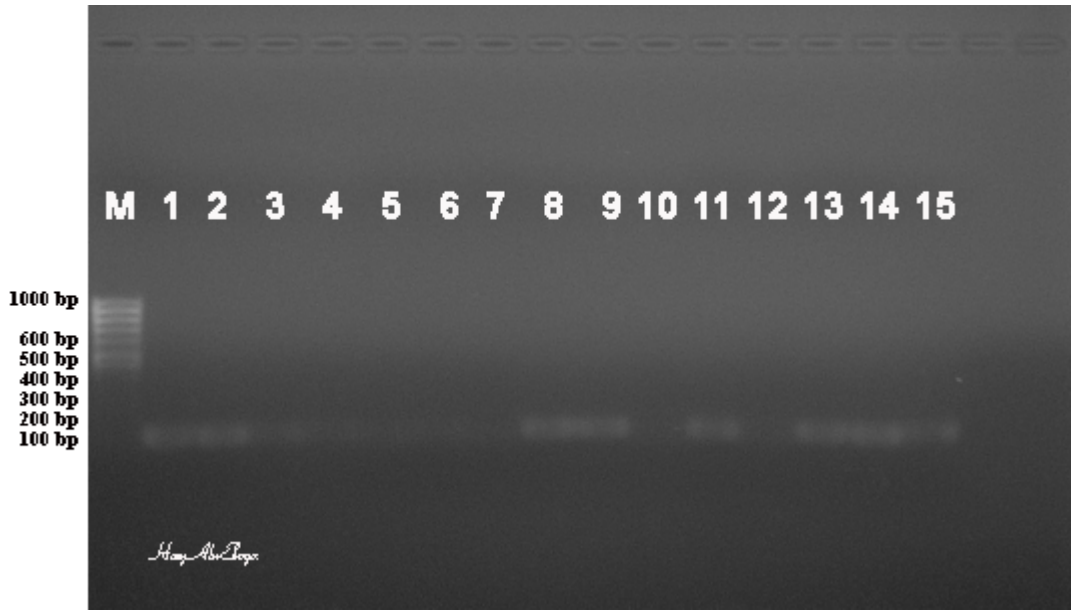
Figure 6: Figure 3 :



4B4

Figure 7: Figure 4B Figure 4 :

1

No.	Sample	Milton Roy 601 Spectronic 1 st day reading	2 nd day reading	3 rd day reading	NanoDrop 2000c Spectrophotometer 1 st reading	2 nd reading	3 rd reading	Average mean
1	Standard	52	88	20	4.3	4.9	8.8	~6
2	Standard, 1 st dilution (10 -1)	ND	ND	24	-1.2	-2.1	ND	-1.65
3	Standard, 2 nd dilution (10 -2)	ND	ND	12	-2.6	ND	ND	-2.6
4	Standard, 3 rd dilution (10 -3)	ND	ND	44	-2.5	ND	ND	-2.5
5	Standard, 4 th dilution (10 -4)	ND	ND	12	-3.2	ND	ND	-3.2
6	Standard, 5 th dilution (10 -5)	ND	ND	4	-3.4	ND	ND	-3.4
7	Standard, 6 th dilution (10 -6)	ND	ND	4	-3.3	ND	ND	-3.3
8	Standard, 7 th dilution (10 -7)	ND	ND	12	-3.3	ND	ND	-3.3
9	Positive Control 1	40	156	32	5.4	4.2	ND	~4.8
10	Positive Control 2	108	104	20	5.7	5.8	ND	~5.75

ND=not done, reading measurement were in the unit of ng/µl

Figure 8: Table 1 :

.1 Conflict of Interest:

The authors declare that they have no conflict of interest.

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