A Cost-saving Human DNA Quantification System using CSF1PO TaqMan probe

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ABSTRACT

The aim of this research was to develop a DNA quantification system which could quantify human DNA using real-time PCR. Primers and TaqMan probes were chosen from a research which targeted CSF1PO locus. The result would also be used to determine not only quantity of DNA but also quality of DNA. Conventional PCR mastermix was also used instead of TaqMan universal mastermix to reduce the cost of analysis. According to the result, the 67-bp CSF1PO primers multiplex quantification system was successfully optimized. The quantification system could quantify amount of DNA as low as 0.046 ng. DNA recovered from mock crime scene samples was tested and the Ct values were compared to that of the Quantifiler® Human DNA Quantification Kit. The efficiency of 2 quantification kits was similar but the cost of the 67-bp CSF1PO primers multiplex quantification system was twice cheaper than that of commercial quantification kit.

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์พัฒนาวิธีวัดปริมาณสารพันธุกรรมมนุษย์ที่สามารถบอกได้ทั้งปริมาณและคุณภาพของสารพันธุกรรมโดยใช้ชุดไพรเมอร์และโพรบที่ออกแบบจากตำแหน่ง CSF1PO ร่วมกับชุด IPC สำหรับการตรวจหาปริมาณสารพันธุกรรมในสภาพจริง โดยใช้องค์ประกอบของปฏิกิริยาลูกโซ่โพลีเมอเรสแทนน้ำยา TaqMan universal mastermix เพื่อลดค่าใช้จ่าย ผลการทดลองแสดงให้เห็นว่าปฏิกิริยาลูกโซ่โพลีเมอเรสเพื่อตรวจหาปริมาณสารพันธุกรรมในสภาพจริงวัดปริมาณดีเอ็นเอได้ต่ำสุด 0.046 นาโนกรัม ค่า Ct ของข้อมูลแต่ละจุดบนกราฟมาตรฐานของ 67-bp CSF1PO primers multiplex system นั้นมีค่าใกล้เคียงกับค่าที่ได้จาก Quantifiler® Human DNA Quantification Kit การวัดปริมาณดีเอ็นเอจากตัวอย่างจำลองด้วย 67-bp CSF1PO primers multiplex system พบว่าประสิทธิภาพสามารถเทียบเคียงได้กับชุดน้ำยาวัดปริมาณดีเอ็นเอที่มีการขายในท้องตลาด และมีค่าใช้จ่ายถูกกว่าครึ่งหนึ่งของการใช้ชุดน้ำยาวัดปริมาณดีเอ็นเอในท้องตลาด

Keywords: DNA Quantification, TaqMan probe, Degraded DNA
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Introduction

DNA evidence plays an important role in the legal system. In casework, DNA evidence can be used to establish a linkage between a criminal or suspect and the crime scene. DNA evidence could also be used in paternity testing to prove a relationship between two people, or to identifying an unknown dead body to their relatives. Nine to 24 loci of Short Tandem Repeats (STRs) which are highly polymorphic and abundant in human genome are simultaneously amplified to generate a DNA profile. There are many commercial STR typing kits available in the market. The target sizes of amplified STRs ranged from 100 base pairs to 350 base pairs. The optimal quantity of DNA needed for amplification is 1-2 ng in a reaction. Excessive DNA template results in an off-scale peak or pull-up peak in the DNA profile so the interpretation is difficult. Insufficient DNA template may result in allele drop out so incomplete profile or no DNA profile may be obtained (Butler, 2005). Therefore, DNA quantification is important in the process of DNA typing. The technique that is widely used in routine forensic DNA analysis is real-time PCR-based quantification because of its sensitivity and specificity. Fluorescent-labeled probe or called TaqMan probe is designed for specifically binding to the DNA target between 2 PCR primers. During the extension step, probes are hydrolyzed and the fluorophores will then be released from the hydrolyzed probes. Fluorescent signal is increased during the amplification process and the level of fluorescent intensity can correlate to the amount of DNA template (Tevfik, 2006). A number of commercial quantification kits are available but the price is too expensive. Moreover, the DNA target is not in the set of STRs loci *i.e* human telomerase reverse transcriptase (hTERT) in Quantifiler® Human DNA Quantification Kit. The target does not represent the STR locus so it could not be used to predict the stage of DNA degradation or quality of DNA [1]. In this study, the primers and probes were chosen from Swango *et al* (2006 & 2007). The system is designed to amplify 67-bp fragment on the CSF1PO region which is one of core STR loci in DNA typing kits. This quantification system is designed not only to quantify the amount of human DNA but also to assess the quality of DNA. This locus is often the first locus that drops out when analyzing highly degraded DNA because the size of CSF1PO locus in STR typing kit is 300-400 bases which is the largest locus.

Objectives of the study

To develop an in-house and cost-saving quantification system that is accurate, and able to predict on quality of DNA being analyzed.

Methodology

Human DNA samples

Two types of fresh human DNA were used in this study which are Human DNA Standard from Quantifiler® Human DNA Quantification Kit (Applied Biosystems, USA), and human genomic DNA. Human genomic DNA was extracted from buccal swabs by using Wizard® SV genomic DNA purification system (Promega, USA) according to the manufacturer's instruction.

Another set of DNA samples was obtained from mock crime scene samples including used toothbrushes (Bandhaya *et al*, 2008), bloodstain and seminal stain (Sutthapodjanarux, 2010). These samples were used to imitate

samples usually found in the crime scenes. The DNA samples were extracted from 10 bristles collected from toothbrushes used for 1, 14, and 30 days. Bloodstains and seminal stain samples were deposited on tiles and cotton sheets. The samples were left at room temperature for 1 week and at 100°C in hot air oven for 24 hours before extraction. These mock crime scene DNA samples were extracted using QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction. The result of DNA quantification of the mock crime scene toothbrush using The 67-bp CSF1PO primers multiplex quantification system would be compared to the previous quantification using Quantifiler® Human DNA Quantification Kit (Applied Biosystems, USA) to observe the efficiency of the 67-bp1PO CSF1PO primers multiplex quantification system.

DNA Quantification

Quantifiler® Human DNA Quantification Kit

Quantifiler® Human DNA Quantification Kit (Applied Biosystems, USA) was used to compare its efficiency with the new quantification system. The total volume of each PCR reaction was 25 µL containing 10.5 µL of primer mix, 12.5 µL of the reaction mix, and 2 µL of DNA extracted sample. The thermal cycling condition was used according to the manufacturer's instruction. Standard curves were generated from 5 different concentrations of Quantifiler® Human DNA standards consisting of 16.7 ng, 5.56 ng, 1.82 ng, 0.21 ng, and 0.023 ng.

Human DNA quantification using CSF1PO TaqMan Probe

The sequences of the primers and probes sequences were obtained from Swango *et al* (2006 & 2007). The quantification system targeted an intron region of human c-fms proto-oncogene for the CSF-1 receptor gene on chromosome 5 (5q33.3-34). List of primers and probes is shown in Table 1. The PCR reaction was carried out in the total volume of 25 µL (Tevfik, 2006). The PCR reaction contained 1x PCR buffer, 0.2 µM dNTP, 1.5 µM $MgCl_2$, and 1 unit AmpliTaq Gold® DNA Polymerase (Applied Biosystems, USA). The concentrations of the primers and probes were varied throughout the research which would be stated further in the paper. The thermocycling condition used was 95ºC for 10 minutes in the initial denaturation step then 45 cycles of 95ºC for 15 sec and 60ºC for 1 minute for annealing and extension steps, respectively. Reactions were amplified using ABI PRISM®7500 Sequence Detection System (7500 SDS) and analyzed by SDS Software Version 1.0 (Applied Biosystems, USA). C_t value of each PCR reaction was observed and analyzed. C_t values above 40 were considered as a negative result.

Table 1 Sequences of primers, probes and oligonucleotides. (Swango *et al* (2006 & 2007))

Target	Primer/ probe	5' Sequence 3'	T_m (°C)	Length (base)	Target size (base pairs)
CSF1PO	Forward	GGGCAGTGTTCCAACCTGAG	64	20	67
	Reverse	GAAAACTGAGACACAGGGTGGTTA	70	24	
	Probe	**VIC**-CAACCTGCTAGTCCTT-**MGB-NFQ**	48	16	
IPC	Forward	AAGCGTGATATTGCTCTTTCGTATAG	72	26	77
	Reverse	ACATAGCGACAGATTACAACATTAGTATTG	80	30	
	Probe	**NED-** TACCATGGCAATGCT-**MGB-NFQ**	44	15	
	Oligo nucleotide	AAGCGTGATATTGCTCTTTCGTATAGTTACCATGGC AATGCTTAGAACAATACTAATGTTGTAATCTGTCGC TATGT	210	77	

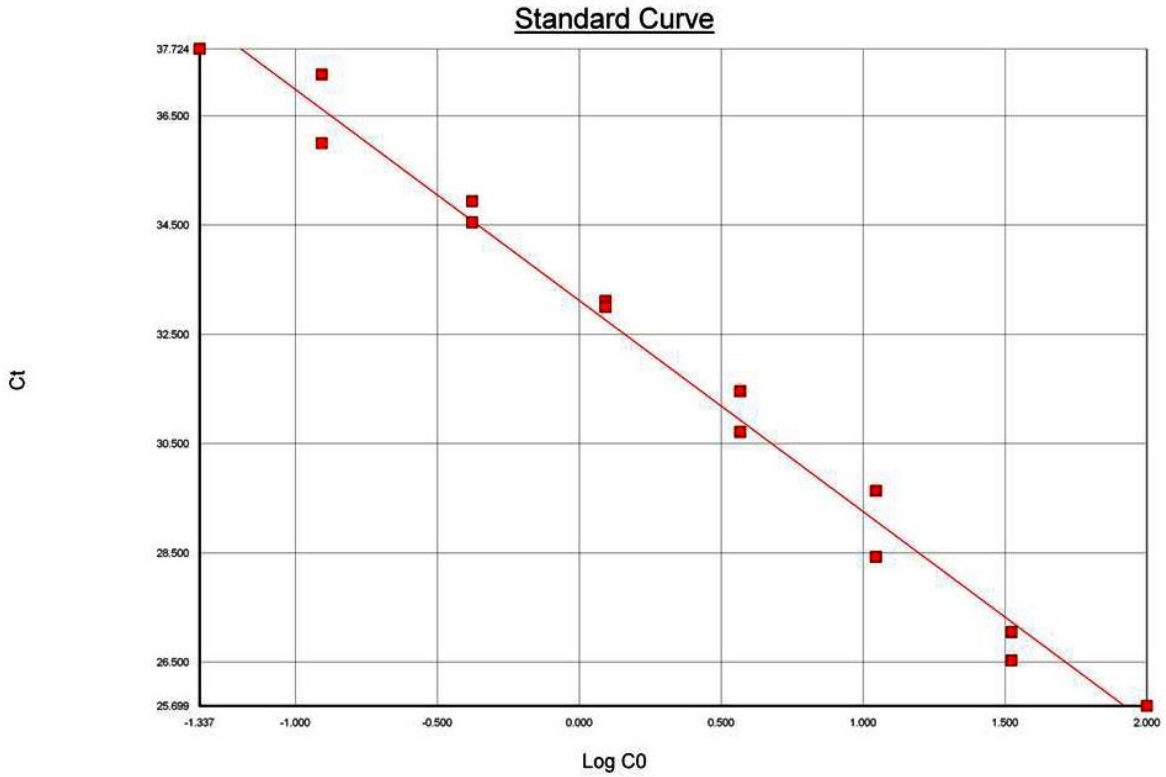
Results

Optimization of multiplex qPCR for human DNA quantification

The primer concentrations were varied ranging from 50 nM - 600 nM amplifying 2 ng of Quantifiler® Human DNA standard (Table 2). According to the result, the optimal concentration for the 67-bp CSF1PO primers and probe set was 100 nM. The optimal concentration of the primer was chosen from the concentration which gave the earliest C_t. After obtaining the optimal concentration of the primer, 8 different concentrations of Quantifiler® Human DNA standard ranged from 50 ng to 0.023 ng were used to generate the standard curve of the quantification system. The R^2 of the standard curve of the 67-bp CSF1PO quantification system was 0.99 (Figure 1). As for IPC, 100nM of primer and probe was used in the sensitivity test to investigate the best amount of oligonucleotide used for amplification. The amount of oligonucleotide suitable for amplification would be determined by C_t, since IPC is the internal positive control of the reaction so it would be best if the C_t is around half the total of 45 cycles. According to the result, the best amount of oligonucleotide template to be used for the reaction was 0.0001 since its C_t was at 21.6 (Figure 2).

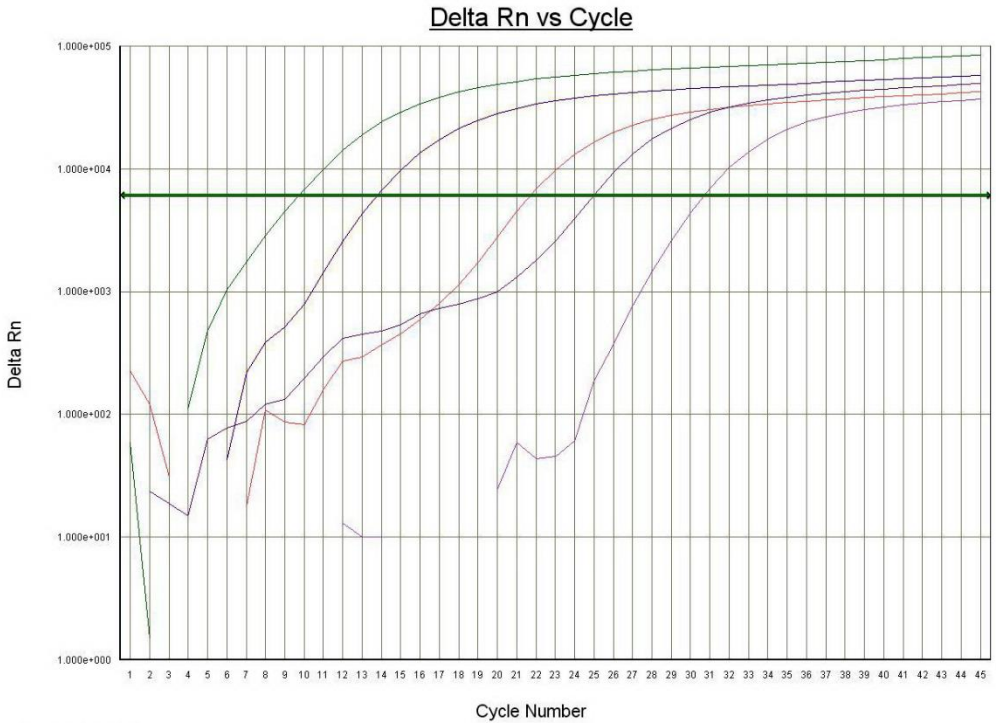
Table 2 Average C_t of concentration test of 67 bp primer set from 2 repeats

Primer concentration (nM)	Ave. C_t
50	37.80
100	37.25
250	27.50
400	26.85
600	26.95



DNA concentration (ng)
50.000
16.700
5.560
1.860
0.620
0.210
0.068
0.023

Figure 1 Standard curve of 67 bp primer and probe set with R^2 = 0.99, slope = -3.860, and intercept 33.120 using 8 different DNA concentrations ranging from 50.000-0.023ng.



Concentration IPC Oligonucleotide (ng)	C_t
0.01	9.70
0.001	13.70
0.0001	21.60
0.00001	25.00

Figure 2 Amplification plot and C_t values of IPC template concentration testing

Mutiplex qPCR

In this experiment, the quantification system was combined with the IPC primers and probe as an internal control. The concentration of 67-bp CSF1PO primers and probe was 100 nM, as for the IPC primers and probe concentration was 100 nM. The reaction was tested by using 2 ng of Quantifiler® Human DNA standard. The C_t of IPC for the multiplex reactions was 19, however C_t of the 67-bp CSF1PO primers was undetected. The CSF1PO primers and probe concentration was then increased to 400 nM and the C_t was obtained. The 67-bp CSF1PO primers and IPC primers multiplex were then used to create a standard curve using 8 different DNA concentrations ranging from 50 ng to 0.023 ng. According to the result, the R^2 of the standard curve was 0.994403 (Figures 3 and 4). The multiplex reaction was then used to quantify concentration of 2 different human genomic DNA with 2 repeats. The 67-bp CSF1PO primers multiplex quantification system was able to successfully quantify the human DNA from buccal swab samples (Table 3).

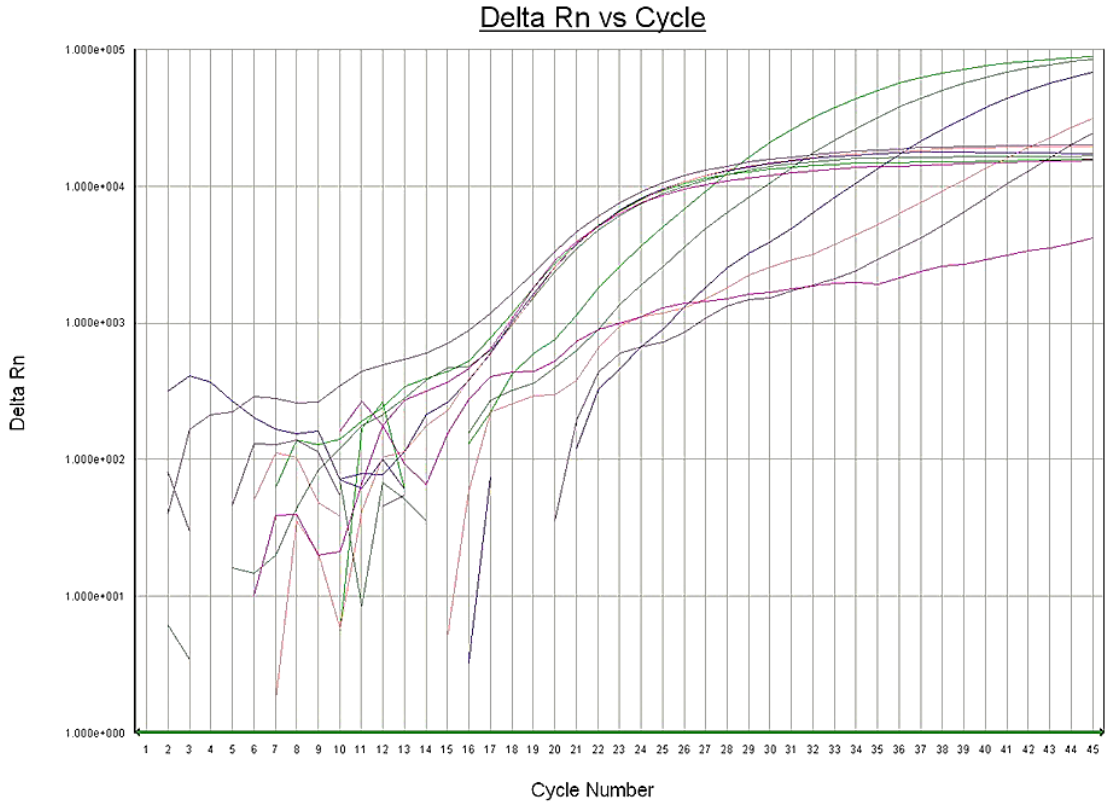


Figure 3 Real-time PCR amplification plot of multiplex 67-bp CSF1PO primer pair quantification system using 8 different DNA concentrations ranging from 50.000-0.023 ng

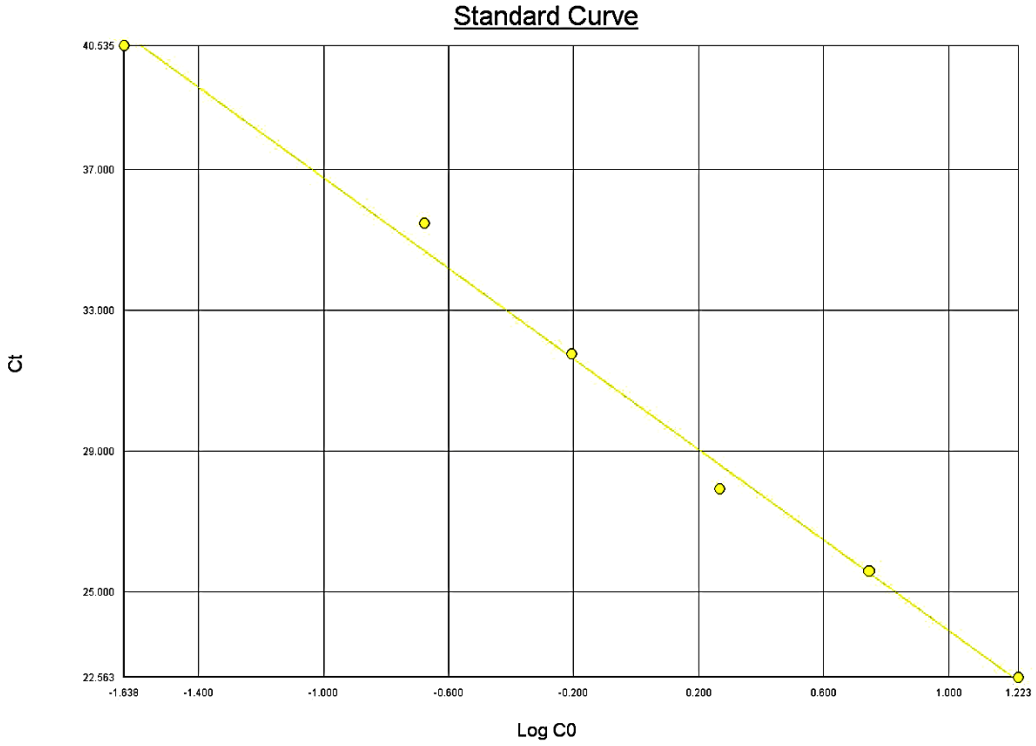


Figure 4 Standard curve of multiplex 67-bp CSF1PO primer pair quantification system with R^2 = 0.994, slope = -6.440, intercept = 30.330

Table 3 Average C_t and DNA concentration of different human genomic DNA from buccal swabs using multiplex 67-bp CSF1PO primer pair quantification with 2 repeats.

Human DNA sample	Target	Ave. C_t	Total (ng/µL)
1	CSF1PO	26.5450	5.95
	IPC	19.4296	
2	CSF1PO	26.8853	5.19
	IPC	19.0018	

The efficiency of 67-bp CSF1PO multiplex quantification system was compared to that of the Quantifiler® Human DNA Quantification Kit. Five different Quantifiler® Human DNA concentrations were used as the templates and the C_t values were observed. The concentrations were 16.7 ng, 5.56 ng, 1.82 ng, 0.21 ng, and 0.023 ng. The result showed that the C_t values obtained for both quantification systems were similar with each other. This means that the efficiency and sensitivity of the 67-bp CSF1PO multiplex quantification system were almost similar to the commercial Quantifiler® Human DNA Quantification Kit (Table 4).

Table 4 C_t values of each concentration of the standard curve for each quantification system

Concentration of Quantifiler® Human DNA Standard (ng)	Multiplex 67-bp CSF1PO Primer Pair Quantification System	Quantifiler® Human DNA Quantification Kit
16.700	24.27	24.76
5.560	26.14	26.25
1.820	28.95	28.05
0.210	31.76	30.58
0.023	34.48	33.36

Multiplex Testing on Mock Forensic Sample Type

The 67-bp CSF1PO multiplex quantification system was used to quantify DNA recovered from mock samples. First the 67-bp CSF1PO multiplex quantification system was tested by quantifying DNA recovered from 10 bristles of toothbrush. According to Bandhaya *et al* (2008), the result from toothbrush used for 14 days quantified using Quantifiler® Human DNA Quantification Kit the sample contained 2.355 ng/µL, whereas using multiplex of 67 bp and IPC to quantify the sample the result was 1.624 ng/µL. As for the toothbrush used for 1 day and 30 days, the result using Quantifiler® Human DNA Quantification Kit were 0.006ng/µL and 0.845 ng/µL, respectively. For the 1-day and 30-day samples using the 67-bp multiplex quantification system, the result was undetermined in both samples. The result of the previous research suggested that the samples that contained the highest amount of DNA were 14 days followed by 30 days and the lowest was 1 day. The reason why the 67-bp CSF1PO multiplex

quantification system was not able to quantify the samples from 1 day and 30 days because the DNA in the samples were all degraded since they were kept for almost 6 years already. However, the 14-days samples contained a larger amount DNA, despite the fact that some of the DNA was degraded (Table 5). The 67-bp CSF1PO multiplex quantification system was also tested with other types of samples, including bloodstain and seminal stain samples (Sutthapodjanarux, 2010). The bloodstain and seminal stain samples were spoted on 2 different types of surface which were tiles and cotton sheets, then the samples were placed at 25°C for 1 week and at 100°C for 24 hrs. The 67-bp multiplex quantification system was able to successfully quantify all of the blood and seminal stain crime scene samples (Table 6).

Table 5 Summary of DNA concentration from toothbrush quantified using Quantifiler® Human DNA quantification

Samples	**Multiplex 67-bp CSF1PO Primer Pair Quantification System**	**Quantifiler® Human DNA Quantification Kit** [4]
1 day	Undetermined	0.006 ng/µL
14 days	1.624 ng/µL	2.355 ng/µL
30 days	Undetermined	0.845 ng/µL

Kit and the 67-bp CSF1PO multiplex quantification system.

Table 6 Average amount of DNA extracted bloodstain and seminal stain samples quantify using the 67-bp CSF1PO multiplex quantification system.

Type of sample	**Temperature**	**Surface**	**Ave. amount of DNA (ng)**
Bloodstain (24 hours)	100 ºC	Cotton sheet	44.630
		Tile	3.820
Bloodstain (1week)	25 ºC	Cotton sheet	97.040
		Tile	170.180
Seminal stain (24 hours)	100 ºC	Cotton sheet	42.280
		Tile	64.020
Seminal stain (1week)	25 ºC	Cotton sheet	0.162
		Tile	102.630

Discussion and Conclusions

In this research, an in-house human quantification system has been developed to quantify and reflect the quality of the DNA samples. A conventional real-time PCR reaction was used instead of a commercial TaqMan Universal MasterMix. The result was a success, since the primers and probes were able to produce similar result using conventional real-time PCR reaction as in the paper. In singleplex reaction, the result suggested that the optimal

concentration of 67-bp CSF1PO primers and TaqMan probe was 100 nM. If there were too much primers or probes in the reaction then it would only be a waste of resource, since there was a limited amount of target for the primers to amplify. The standard curve of the quantification system was created by using 8 different concentrations of human DNA. The R^2 of the quantification system was over 0.99, hence it could be said that the quantification system was successfully created. However when the quantification system was combined with IPC using similar condition, no signal was produced. The concentration of CSF1PO primers and probe of the quantification system was then varied with a fix concentration of IPC primers and probe.

In the 67-bp CSF1PO multiplex quantification system, the concentrations of the 67-bp CSF1PO primer and probe were 400 nM and 100 nM, respectively. The concentration of IPC probe and primers were 100nM, respectively. From this optimal PCR condition, the standard curve the 67-bp CSF1PO multiplex quantification system was compared to that of Quantifiler® Human DNA Quantification Kit to determine the efficiency. The C_t values of the stand curve from 2 systems were very similar.

Extracted DNA from 3 different types of mock crime scene samples-was then quantified by using the 67-bp CSF1PO multiplex quantification system. The result showed that the 67-bp CSF1PO multiplex quantification system was able to quantify most of DNA recovered from the mock samples. The amount of DNA recovered from 10 bristles of toothbrush used for 14 days can be quantified by this multiplex quantification system. The amount of DNA was 0.731ng lower than that of DNA quantified by Quantifiler® Human DNA Quantification Kit. However, the DNA recovered from 1 and 30-days are not able to quantify by this quantification system. By comparing the previous study, amount of DNA recovered from 1-day and 30-days were lower than that of DNA recovered from 14 days. This may be due to the fact that those samples were kept in the freezer for 6 years so they were more likely to degrade overtime.

For DNA recovered from the bloodstain and seminal stain samples, the result suggested that 67-bp CSF1PO multiplex quantification system was compatible with crime scene samples. The efficiency of the 67-bp CSF1PO multiplex quantification system was almost similar to those of the commercial quantification kits. Also considering the price per reaction of the 67-bp CSF1PO multiplex quantification system, it was a lot cheaper than using commercial quantification kits or the condition given in the previous research paper. The price per reaction of Quantifiler® Human DNA Quantification Kit was approximately 133 Baht per reaction. In this study, the conventional PCR mastermix was used instead of the TaqMan universal mastermix so the price was cheaper. In the 67-bp CSF1PO multiplex quantification system consists of PCR buffer and $MgCl_2$, Ampli*Taq* Gold DNA polymerase, dNTP, primers and probe were needed and the total cost was approximately 75 Baht. This was almost half the price of commercial quantification kits so a fair amount of cost could be save.

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