Internal Validation Guide of the Amp F_STR Identifiler PCR by Using Quantifiler™ Y Human Male DNA Quantification for Use Forensic Laboratories

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Abstract

Fifty samples of buccal swabs were collected from male oral cavity, then genomic DNA was extracted from each sample by two methods, the manual organic phenol chloroform methods and prepfiler forensic extraction kit methods.

The concentration of genomic DNA extracted was measured by using Quantifiler® Duo Kit throughout detection the SRY (FAM™-labeled probe), RPPH1(VIC®-labeled probe) and an Internal Positive Control-IPC (NED™-labeled probe).

Results showed that the concentration of genomic DNA in buccal swab was ranged between 0.26 and 9.422 ng/ul. these samples work with a serial dilution decimal, as well as PCR .Then introduced to the device 3130xl Genetic Analyzer 16-capillary array system, The results showed that the concentrations is preferable which is located between 25-50 pg, as this did not appear in the concentrations of any problems and the emergence of the peaks clearly Compared with the ALLELIC ladder and positive control, After the election concentrations were chosen Thresholds, Peak amplitude thresholds.

Keywords: DNA, STR, Quantitative PCR, Internal validation.

Introduction

The field of human identification has progressed significantly in last years with the development of highly discriminating PCR-based DNA typing systems. The use of STR loci and highly sensitive instrumentation based on fluorescence detection enables rapid genetic profiling from minute amounts of biological material. As such, STR DNA typing analysis has been extensively used in forensic casework to establish the presence of a perpetrator's DNA in crime scene evidence. Biological evidence recovered at crime scenes often consists of mixtures of bodily substances originating from more than one individual, so mixed STR profiles are frequently encountered in criminal investigations. Profile interpretation in such cases normally follows a systematic approach (1–2).

Three major characteristics of the STR DNA typing process can impact on mixture interpretation:

1. Stutter peaks, which are minor peaks typically one repeat unit length shorter than that of their parent nominal allele peak and generated through strand slippage during amplification (3).
2. Sporadic imbalances in allele peak height or peak area ratios at heterozygous loci can also impact on mixture interpretation. However, imbalances can be triggered by stochastic effects when amplifying too little DNA or degraded DNA, or by the inability to remove PCR inhibitors during DNA extraction. These allelic imbalances can complicate the interpretation of mixed profiles if allele peaks from one contributor cannot be reliably identified because of significant signal imbalances.
3. The unpredictability concerning the number of shared alleles between contributors can also reduce the ability to identify individual profiles in a mixture (3).

STRs in a single reaction became available in the late 1990s/ early 2000s (4-5). PCR allowed the generation of genetic information from minute amounts of DNA; multiplexing of primers allowed the generation of genetic data from multiple sites from the same aliquot of DNA thus reducing sample consumption; fluorescent primers assisted multiplexing and new automated typing systems; and the use of STRs improved the chances of profiling poor

quality samples. As the desire for higher discrimination power between individuals arose, the number of loci targeted by a single multiplex increased and there are now a number of commercially available, well-validated kits, incorporating 15-16 highly variable STR loci (plus amelogenin), such as Power Plex® ESX and ESI systems (6) and Amp FlSTR® NGM (7). These new kits also include improvements in primer design, buffer composition and amplification conditions which improve the analysis of trace samples (6-7).

Quantitative PCR (qPCR) has displaced hybridization based methods for human-specific DNA quantification in forensic applications. qPCR has reduced the rate of false-negative STR results due to lack of sensitivity and increased the objectivity of data interpretation by providing a numerical output rather than requiring a visual comparison of band intensities. However, some current qPCR methods do not allow simultaneous quantification of total human and male DNA or do not have a level of sensitivity that consistently exceeds that of subsequent STR assays. A number of quantification procedures have been developed and are traditionally used to provide human specific DNA quantification, such as hybridization techniques as for example such as Quantifiler® Human DNA Quantification Kit and Quantifiler® Y Human Male DNA Quantification Kit (Applied Biosystems, foster City, CA) and most recently Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, foster City, CA), for simultaneous quantification of total human and human male DNA proved to be very useful not only for human DNA quantification, but also for detection of DNA degradation and inhibitor's (8,9). These are generally considered time-consuming, labour intensive, not suitable for automation and also low sensitive for STR genotyping systems (2), Real Time PCR (q-PCR) methods based on Taqman probes were developed in a single multiplex reaction, allowing the use of an additional internal template control to detect PCR inhibitors and Multiplex typing systems must be optimized to the point where they meet certain performance standards.

There are several governing bodies that ensure that high typing and analysis standards are maintained. Among these is the International Society for Forensic Genetics (ISFG), the Scientific Working Group on DNA Analysis Methods (SWGDAM), and the European DNA Profiling Group (EDNAP). These organizations have proposed guidelines for the use and validation of multiplex PCR typing systems. Some common validation exercises include: (1) establishing that the typing system is sensitive and performs consistently using freshly prepared and stored DNA, (2) that identical results are obtained irrespective of the type of tissue from which DNA was extracted, (3) that the systems yield consistent results in several laboratories, and (4) that the system performs well when used to analyze samples similar to those encountered in forensic casework (10) Validation plays a vital role in the forensic community since there is a constant influx of new DNA technology being developed (11). There are two types of validation processes; i.e. a developmental validation and an internal validation.

Developmental validation is the more thorough of the two methods and it is used to determine the reliability and limitations of a novel method (12). This entails the determination of: accuracy, precision, reproducibility, species specificity sensitivity, stability, PCR parameters and simulated casework studies (12). SWGDAM has recommended a set of developmental validation guidelines which are available fromwww.cstl.nist.gov/strbase/validation/SWGDAM.

Internal validation the purpose of an internal validation is solely to demonstrate the ability of a laboratory to perform a previously developmentally validated procedure (12). Both developmental and internal validations determine whether the analytical procedure will be adequate for its intended use (11).

Materials and Methods

DNA Extraction and Quantitation Fifty samples of buccal swabs were collected from human oral cavity and two methods for DNA extraction were used to isolate DNA: organic phenol chloroform manual (13,14) and

prepfiler forensic extreaction kit (Applied Biosystems).The quantity of human DNA was determined by two methods the Quantifiler TM human DNA quantitation kit (Applied Biosystems) and nano drop Q3000 UV spectrophotometer. DNA in this samples used for sensitivity studies was prepared by serial dilutions using for sensitivity studies was prepared by serial dilutions using Table (1).

Table (1)
Concentration of DNA and serial dilution for ten sample.

Number of sample	*Concentration of DNA ng/ul*	*Serial Dilution*									
		10 pg+D.W		*25pg+D.W*		*50pg+D.W*		*75pg+D.W*		*100pg+D.W*	
Sample 1	4.774	0.209	99.79	0.26	49.73	0.52	49.48	0.79	49.21	0.94	49.06
Sample 2	1.27	0.78	99.21	0.984	48.01	1.97	48.03	2.95	47.05	3.54	46.46
Sample 3	2.91	0.34	99.6	0.42	49.57	0.86	49.14	1.29	48.71	1.55	48.45
Sample 4	9.422	0.11	99.89	0.13	49.87	0.27	49.73	0.40	49.60	0.53	49.47
Sample 5	2.87	0.35	99.65	0.44	49.56	0.87	49.13	1.31	48.69	1.74	48.26
Sample 6	5.9	0.169	99.83	0.21	49.79	0.42	49.58	0.64	49.36	0.85	49.15
Sample 7	0.26	3.85	96.15	4.81	45.19	9.62	40.38	14.42	35.58	19.23	30.77
Sample 8	0.844	1.18	98.82	1.48	48.52	2.96	47.04	4.44	45.56	5.92	44.08
Sample 9	0.535	1.87	98.13	2.34	47.66	4.67	45.33	7.01	42.99	9.35	40.65
Sample 10	1.02	1.02	0.49	1.23	48.77	2.45	47.55	3.68	46.32	4.90	45.10

Real-Time PCR Amplification

A multiplexed TaqMan ® was assembled that amplifies SRY (FAM™-labeled probe), RPPH1 (VIC®-labeled probe) and an Internal Positive Control-IPC (NED™-labeled probe) (Table (2)). Assays were designed using the TaqMan® Gene Expression (16). Amplification reactions contained 2ul of sandards Dilution series (Std) starting concentration from 50 ng/ul (Std1) to 0.023 ng/ul or 23 pg/ul (Std8) sandards Dilution showed Table (3), Real-Time PCR reactions: Quantifiler duo primer mix, Quantifiler Duo pcr reaction Mix, Quantifiler Duo DNA standard and Quantifiler Duo Dilution Mix in the reaction use 10.5 ul of Quantifiler Duo Primer Mix, 12.5 ul of Quantifiler Duo Reactin Mix, and 2.0 ul of DNA sample. Real-Time PCR program of cycler condition Table (4).

Table (2)
Configuration of the Quantifiler® Duo Kit.

Target	*Marker*	*Size*	*Dye*
Human DNA	RPPH1 (Ribonuclease P RNA component H1)	140 bp	VIC®
Human Male DNA	SRY (Sex determining Region Y)	130 bp	FAM™
IPC	Artificial Template	130 bp	NED™

Table (3)
Preparation of DNA standards.

Standard	*Concentration (ng/ul)*	*Minimum Amounts*	*Dilution factor*
Std1	50.000	10 ul (200 ng/ul stock)+30ul Quantifiler Duo DNA dilution buffer	4X
Std2	16.700	10 ul(Std1)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std3	5.560	10 ul(Std2)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std4	1.850	10 ul(Std3)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std5	0.620	10 ul(Std4)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std6	0.210	10 ul(Std5)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std7	0.068	10 ul(Std6)+ 20ul Quantifiler Duo DNA dilution buffer	3X
Std8	0.023	10 ul(Std7)+ 20ul Quantifiler Duo DNA dilution buffer	3X

Table (4)
Real-Time PCR program.

Temp	*Time*	*Cycles*
50	2mine	1
95	10mine	1
95	15sec	40
60	1mine	40

Amplification for STRs

Fifty autosomal STR markers (the 13 CODIS core loci and D19S433 and D2S1338) were typed along with amelogenin using the Applied Biosystems AmpFiSTR® Identifiler™ kit (3) 1±2 ng of target DNA following the protocols described in the User's Manual (applied Biosystems). The samples were amplifyed using verity PCR System (applyed Biosystems). The standard thermal cycling parameters were :1cycle 95℃ for 11 minutes ; 28 cycle of 94 ℃ for 1minuts , 59 ℃ for 1 minutes ,72 ℃ for 1 minutes and Final Extension 60℃ for 60 minutes. (1ul) of PCR product. was mixed with 8.7 ul of Hi-Di™ Formamide and 0.3 $\mu\ell$ of GeneScan ™ 500 LIZ® size standard (Applied Biosystems). Separation and detection of PCR products were accomplished with ABI Prism® 3130xl Genetic Analyzer 16-capillary array system (Applied Biosystems) according to the manufacture´s recommendation. Following data collection, samples were analyzed using Data Collection v. 2.0 software (Applied Biosystems, Foster City, CA, USA) and samples were analyzed by GeneMapper1 v. 3.2 software (Applied Biosystems, Foster City, CA, USA).

Results and Discussion

Fifty sample was measured using multiplex real-time PCR assay was assembled that amplifies sex determining region Y(SRY), ribonuclease P RNA component H1 (RPPH1) and a synthetic oligonucleotide sequence that served as an Internal Positive Control (IPC). Amplification reactions were performed on a 7500 fast Real-Time PCR System and the data were analyzed with the 7500 fast System SDS software v2.0.5 (Applied Biosystems, Foster City, CA). During real-time PCR, accumulation of the PCR product is monitored with each cycle. In the H-Quant assay, accumulated PCR product is measured by the fluorescence resulting from the binding of Human genomic DNA from donors was used to generate in a single reaction two standard curves for the human and the human male-specific targets with the DNA concentration

ranging from 50 ng/µl to 23 pg/µl in three-fold increments. The eight concentration points are 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µl and 2.0 µl of each sample were tested with the multiplex assay. IPC artificial template was included in each reaction to obtain the CT value of about 30 across the whole standard curve. The multiplexed assay performed well across a large dynamic range and is well suited for quantification of human samples. The standard curve plots and parameters are illustrated in Fig.(1), Fig.(2) and in Table (5). The detected Ct of approximately15 cycles for the lowest DNA concentration (0.023 ng/µl).

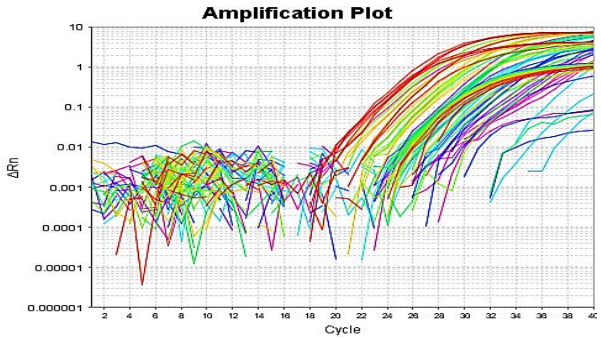

Fig.(1) :Amplification Plot – Standard DNA dilutions , controls and human sample.

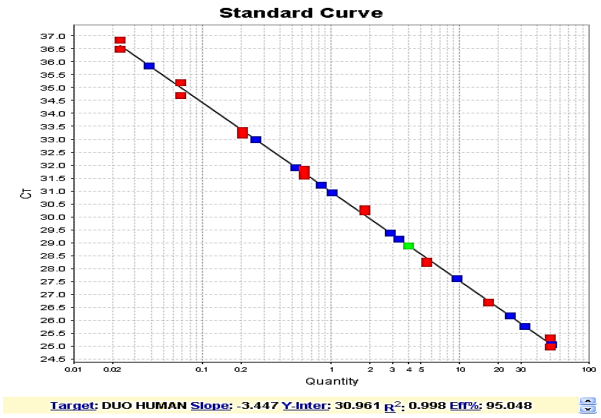

Fig.(2): Standard curves , IPC ,CT values and samples for the Quantifiler® Duo Kit.

Table (5)
Standard curve parameters.

	Value	*Typical Slope (range)*
R2	0.998	<98
Slope	-3.34	-3.0_-3.6
Efficiency	95.045	90_105%
Y-Intercept	30.961	1

Fifty sample were measured by Real Time PCR. These samples work with a serial dilution decimal, as well as work PCR Then introduced to the device 3130xl Genetic Analyzer 16-capillary array system, Knowledge for the purpose of concentrations the best that they appear peaks clearly and without artifacts.

Which will be based later in the laboratories Forensic DNA Center for Research and Training (FDNAC) in Al- Nahrain University. The results showed that the samples diluted samples with concentrations less than 25pg did not show their peaks clearly with the disappearance of some peaks than that the concentrations used are not good. The results of the samples with concentrations higher than 50pg appearance same artifacts such as stutter. high peaks and Unincorporated Dye. While concentrations is preferable which is located between 25-50, as this did not appear in the concentrations of any problems and the emergence of the peaks clearly Compared with the allelic ladder and positive control Fig. (3). These results focus adopted 25-50 pg in FDNAC labrotory Fig.(4).

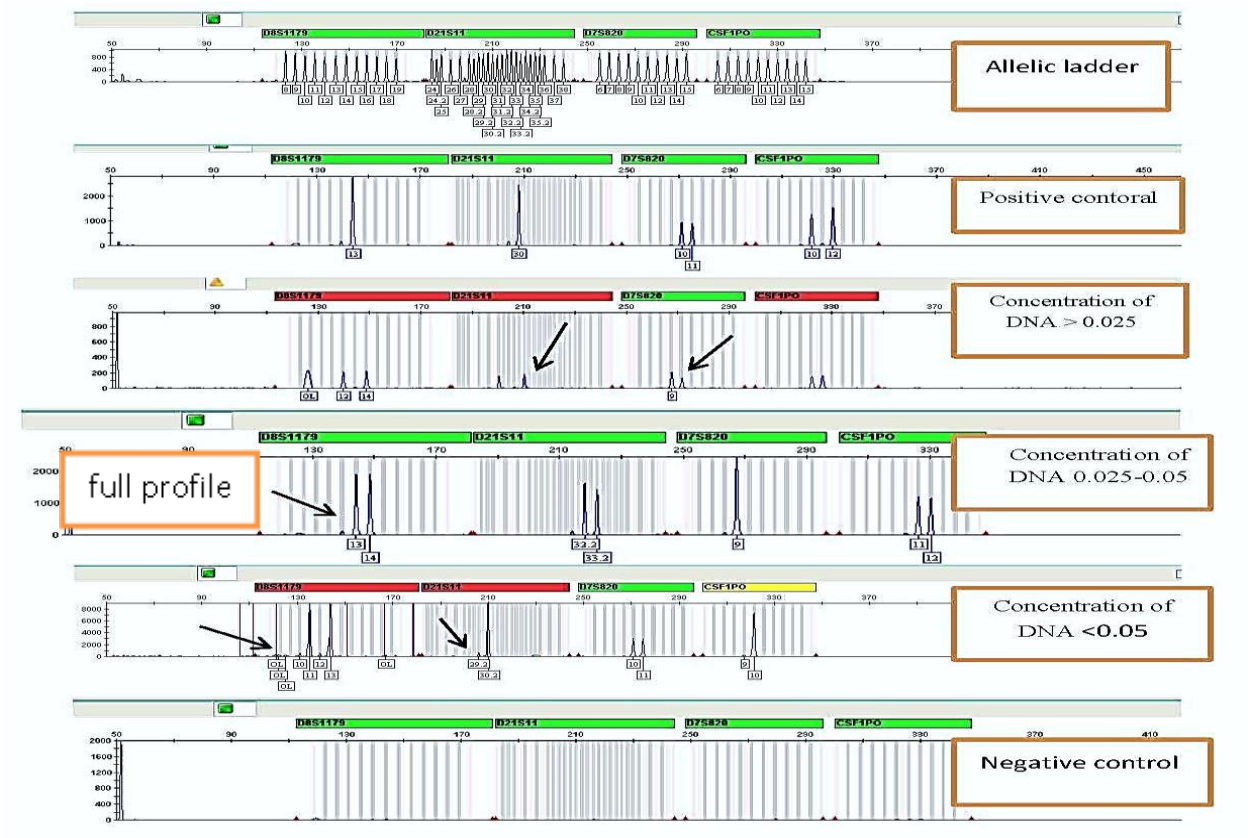

Fig.(3) :Allelic ladder, positive control, serial dilution of DNA and negative control.

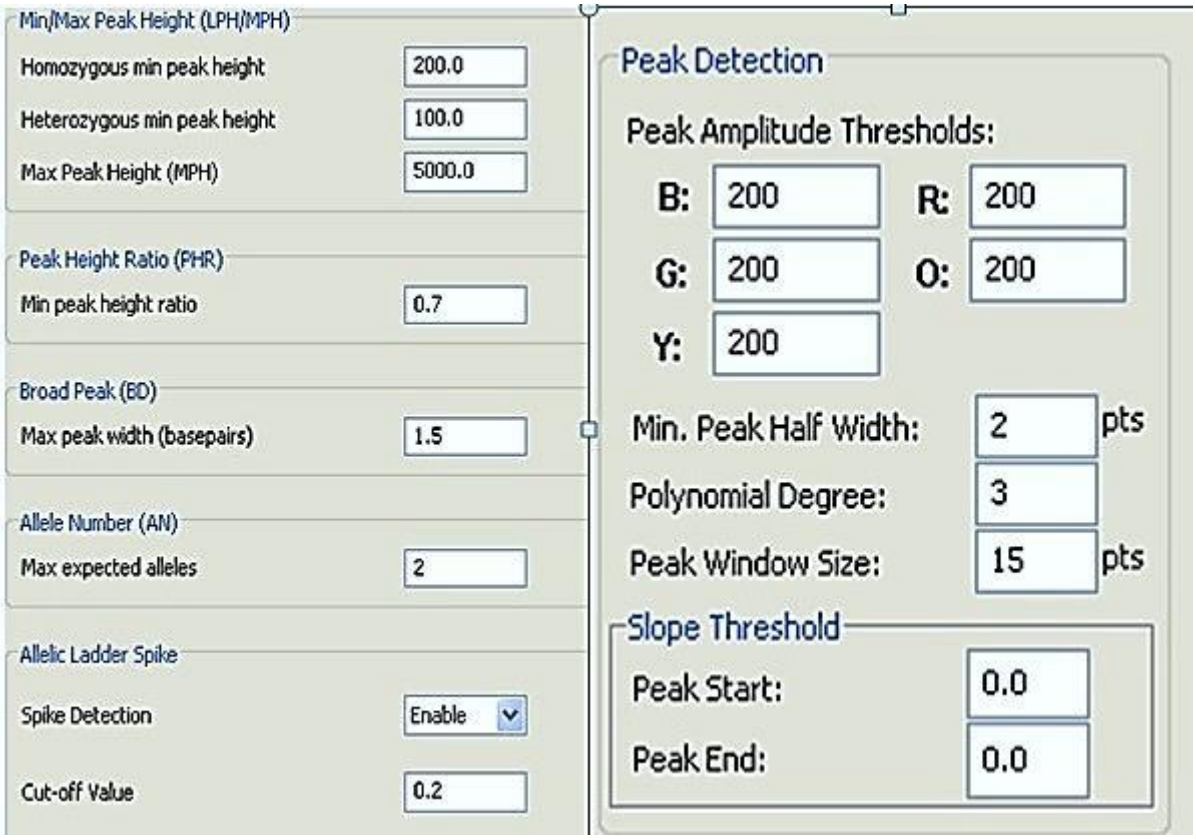

Fig.(4): Revaeals that ALL showing Thresholds, peak amplitude thresholds, Homozygous min peak height, Heterozygotes min peak height, Max peak height (MPH) and Peak height ratio (PHR).

Conclusions

Quantification of human DNA in forensic samples is essential for defining input DNA needed for obtaining interpretable STR profiles. The most accurate method of choice for forensic DNA quantification is real-time PCR. We have developed a multiplex real-time PCR assay for the simultaneous quantification of human and human male DNA with IPC in forensic samples. The assay is efficient, specific, sensitive and robust. The results

Correlate well with the AmpFℓSTR® Identifiler® and Yfiler® kit performance in terms of predicting the generation of interpretable STR profiles for inhibited DNA samples and male/female DNA mixtures. The Quantifiler® Duo DNA Quantification Kit is a useful tool for the quantitative and qualitative assessment of DNA in forensic type biological samples.

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الخلاصة

جمعت خمسين عينة من اشخاص اصحاء بواسطة مسحة الفم اجري لها استخلاص بطريقتين الاولى هي التقليدية (فينول كلوروفورم) والطريقة الثانية هي استخدام عدة جاهزة من شركة(Applied Biosystems). بعد استخلاص العينات اجري لها قياس التركيز بواسطة عدة (QuantifilerTM human DNA quantitation kit) التي تتكون من ثلاث بروبات لغرض قياس تركيز DNA. اظهرت نتائج قياس تركيز العينات بانه يتراوح من ٠,٢٦- ٩,٤٢٢ بيكو غرام. اجريت لها تخافيف عشرية لغرض اختيار التركيز الامثل قبل ادخالها في جهاز الترحيل في الانابيب الشعرية اظهرت النتائج ان افضل تركيز اعطى نتائج خالية من المشاكل التي عادة تصاحب العمل في المجال الجنائي هو من ٢٥-٥٠ بكيوغرام مقارنة مع العينة القياسية. ثم بعدها اختيار خط العتبة الذي ظهرت عندة نتائج جيدة وكذلك اختيار ارتفاع العمود الامثل اذ يمكن اعتمادها في مجال عمل المختبرات الجنائية.