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METHOD PAPER

Development and forensic validation of human genomic DNA quantification kit

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Abstract

DNA quantification is an essential step for successful multiplex short tandem repeat (STR) polymerase chain reactions (PCR), which are used for confirming identities using human genomic DNA. The new DNA quantification kit, named the National Forensic Service Quantification (NFSQ) kit, simultaneously provides total human DNA concentration, human male DNA concentration, and a DNA degradation index (DI) using multiplex TaqMan fluorescent probes. The NFSQ was validated according to developmental validation guidelines from the SWGDAM and MIQE. NFSQ detected up to 0.00128 ng/μL and could detect male DNA up to a 1:8000 ratio of male to female DNA. In PCR inhibitor tests, NFSQ could measure DNA at a concentration of 200 ng/μL of humic acid and 600 μM of hematin. The NFSQ kit showed a DI value trend similar to other qPCR kits. In the reproducibility study, the coefficient of variation of the NFSQ kit was within 10%. The quantitative results of the casework samples obtained using the NFSQ kit were consistent with the STR interpretation results. The NFSQ kit can be useful in the human identification process, as it has detection capabilities similar to those of other comparable quantification kits.

Keywords DNA quantification · Forensic science · Real-time PCR · Short tandem repeat

Introduction

Human genomic DNA extracted from the evidence gathered from various crime scenes is used for human identification during forensic investigations [1–3].

Human genomic DNA from crime scene generally originates from bloodstains, semen, saliva, and touch DNA. Forensic work is executed in the following order: sample collection, DNA extraction and purification, human DNA quantification, STR (short tandem repeat) genotyping assays [4, 5], data interpretation, and comparison with reference DNA.

In this study, the NFSQ kit which was developed by National Forensic Service has been developed as a substitute for the electrophoresis and spectrophotometry assays that were previously used for DNA quantitation [6–8]. Whereas other quantitative assays were non-specific or impossible to quantify, the real-time PCR assay was used to compensate for this problem.

The NFSQ kit is designed for quantifying human DNA from crime scenes and reference evidence. The NFSQ kit can detect human DNA quantity, male DNA quantity, large DNA quantity, and internal PCR control in one reaction using the multiplex TaqMan Probe, which include three genomic targets and an internal PCR control with fluorescent dye sets. Sequence-specific probe-based detection techniques using real-time PCR assays enable accurate measurement by observing the amplified product through fluorescence in real time. The real-time PCR assay requires pairs of primers flanking the target sequence, and TaqMan probes with fluorescent materials, including reporter dyes such as FAM™, TAMRA™, JOE™, and CY5™ attached to the 5′ end of the

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TaqMan probe, and a quencher dye, which is a countercurrent material attached to the 3′ end of the TaqMan probe. In the annealing phase, the TaqMan probe specifically binds to the target DNA sequence, but the quencher inhibits the fluorescence emission of the fluorescence dye. In the extension phase, the bound hydrolysis probe is degraded by the exonuclease activity of DNA polymerase. Therefore, the quencher is released from the probe, and the quencher no longer inhibits the reporter dye, allowing the fluorescence emitted by the reporter dye to be measured [9–11].

To verify the utility of the NFSQ kit in forensic tests, we report the results of development and validation studies performed according to the Scientific Working Group on DNA Analysis Methods (SWGDAM) Validation Guidelines and MIQE Guidelines [12–14]. Furthermore, we aimed to compare the NFSQ kit with a Quantifier® Trio DNA Quantification kit (ThermoFisher Scientific, Waltham, USA), which is currently used by the NFS, and the Powerquant™ system (Promega, Madison, USA) and Investigator® Quantiplex pro kit (Qiagen, Hilden, Germany) with respect to stability.

Materials and methods

Sample collection and DNA extraction

DNA samples of selected animal species for the species specificity test (sheep, bovine, pig, chicken) were obtained from tissues, which were bought at a butcher shop, and other DNA samples (dog and cat) were obtained using buccal swabs. DNA samples from gerbil, cat, and dog were collected as forensic evidence to identify species and individual. The samples were extracted and purified using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) [15, 16]. The DNA samples were eluted with 50–100 μL of elution buffer and stored at 4 °C. All animal DNA samples were identified with 12s ribosomal RNA sequencing [17] and quantitated with a Nanodrop 2000C spectrometer, which uses UV absorbance at 260 nm (Thermo Scientific).

Male standard genomic DNA, such as 2800M, G1471 (Promega, Madison, WI, USA), and female standard genomic DNA such as K562 and 9947A (Promega, Madison, WI, USA) were purchased for validation tests. G1471 and K562 control DNAs were used for the analytical sensitivity test, the mixture test, and the reproducibility test. For the sensitivity test, G1471 control DNAs were diluted by a fivefold serial dilution.

For the PCR inhibitor and degradation tests (heating, sonication), 9947A female, 2800M male, and G1471 male standard DNA samples were used. To form degraded DNA samples, control DNA samples (2800M, 9947A) were incubated at 98 °C for 10 to 120 min or sonicated with an ultrasonic processor (Sonics & Materials) for 2 to 30 min using 30 amplitude [18–20].

Casework samples were obtained from the forensic DNA division of the National Forensic Service in the Republic of Korea. This research was approved by the Institutional Review Board (IRB) of the National Forensic Service (Approval No. 906-170118-BR-003-02). A total of 44 casework samples, consisting of blood, hair, buccal swabs, saliva, FTA (Flinders Technology Associates) card, fingernail, a cigarette, a tissue, a toothpick, and other sample types, were quantified. These casework samples were extracted and purified with a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) which classifies the types of samples in the pretreated stage, or the QIAsymphony DNA Investigator kit (Qiagen, Hilden, Germany), which is used in automated machines. Casework samples were eluted in 60 μL of elution buffer and stored at 4 °C.

PCR primers, TaqMan probes, and real-time PCR

NFSQ kit assays use a fluorescent dye set that approved effectual multiplexing such as FAM-NOVA™, TAMRA™, JOE™, and CY5™ with ROX™ passive reference dye. To set up and analyze the NFSQ kit assays, the HID Real-Time PCR Software v1.2 program was used. NFSQ kit was designed to facilitate quantification using real-time PCR with Multiplex TaqMan Fluorescent probes. There were four targets each, which consists of locus-specific PCR primers and a fluorescent dye-labeled probe, which includes a small DNA marker, a large DNA marker, a male DNA marker, and an internal positive control (IPC) marker. The 2× qPCR master mix (JS Biotech, Gyeonggi-do, Republic of Korea) includes all essential components for PCR amplification. Also, the master mix contains a ROX™ passive reference dye to stabilize the fluorescent signals from assay targets.

The NFSQ kit assay determined the human genomic DNA content of all samples using a 7500 Real-time PCR System (Applied Biosystems Inc. Foster City, CA, USA). To quantify standard DNA samples, G1471 human male DNA (Promega, Madison, WI, USA), which is tenfold diluted (50, 5, 0.5, 0.05 ng/μL), was used as reference material. Triplicate reactions for each standard curve sample were run per plate.

The NFSQ kit assay was performed with the standard protocol specified in the NFSQ kit. Each reaction contained 7 μL DW (Distill water), 10 μL PCR reaction mix (2×), 1 μL primer mix (20×), and 2 μL DNA sample. The total reaction volume was 20 μL. Each reaction is performed using Micro Amp™ Optical 96-well Reaction plates and Micro Amp Optical Adhesive Film (Thermo Fisher Scientific, Waltham, MA). Each primer concentration in the primer mix consisted of 500 nM. The NFSQ kit has been patented by the Korean Intellectual Property Office and will be commercialized.

A thermal cycling instrument, Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) was operated using the following thermal cycle:

$$95°C \text{ for } 3 \text{ min} \rightarrow 40 \text{ cycles} \quad \text{of} \begin{bmatrix} 95°C \text{ for } 15 \text{ s} \\ 95°C \text{ for } 45 \text{ s} \end{bmatrix}$$

Data which were evaluated from the Applied Biosystems 7500 Real-Time PCR System was analyzed using the HID Real-Time PCR Software v1.2 (Thermo Fisher Scientific, Waltham, MA).

Species specificity study

The NFSQ kit was assessed to identify species specificity of NFSQ kit primer/probe set. The NFSQ kit was tested using purified DNA of non-human species, using 2 μL of animal genomic DNA samples, including dog (4.5 ng/μL), cat (5.5 ng/μL), bovine (8.3 ng/μL), pig (13.9 ng/μL), sheep (23.5 ng/μL), chicken (46.3 ng/μL), and gerbil (14.3 ng/μL). Animal genomic DNA is identified using a 12S rRNA sequencing assay which was progressed using an Exo-SAP assay and a Dye EX assay. Microbial genomic DNA samples, which included *S. paratyphi* B (*Salmonellaparatyphi* B 3.1 ng/μL), *P. mirabilis* (*Proteus mirabilis*, 4.18 ng/μL), *S. aureus* (*Staphylococcus aureus*, 2.8 ng/μL), *E. coli* O157 (*Escherichia coli* O157, 4.0 ng/μL), *B. thuringiensis* (*Bacillus thuringiensis*, 4.02 ng/μL), *L. vaginalis* (*Lactobacillus vaginalis*, 0.224 ng/μL), *L. crispatus* (*Lactobacillus crispatus*, 4.9 ng/μL) were obtained from Bio-Resource Management for pathogens National Culture collection for Pathogens (NCCP), and the Korean Collection for Type Cultures (KCTC). These non-human DNA samples and the positive control sample (G1471 human genomic DNA) were tested using the NFSQ kit in triplicate reactions.

Analytical sensitivity study

Human Male genomic DNA G1471 (Promega, Madison, WI, USA) was quantified using a Nanodrop 2000C spectrophotometer which used UV absorbance at 260 nm (Thermo Fisher Scientific, Waltham, MA, USA) and serially diluted the DNA sample using a fivefold serial dilution (50, 10, 4, 0.8, 0.16, 0.032, 0.016, 0.0064, 0.0032, 0.00128 ng/μL) to assess the dynamic range of the NFSQ kit. DNA dilutions were analyzed in triplicate reactions using the NFSQ kit assay. The diluted samples were examined in triplicate in six runs.

The PCR inhibitor test

PCR inhibitors, such as humic acid and hematin, often appear in forensic evidentiary samples, and these can affect PCR assays which include real-time PCR for quantification and STR analysis. To assess the effect of PCR inhibitors in the NFSQ assay, an NFSQ kit was tested with the common PCR inhibitors humic acid (Sigma-Aldrich, St. Louis, MO, USA) and hematin (Sigma-Aldrich, St. Louis, MO, USA) [21, 22]. The 2800M DNA samples at concentration of 0.5 ng/μL were mixed with humic acid and hematin. DNA samples consisted of 12.5–800 ng/μL of humic acid and 62.5–1000 μM of hematin. Samples with these inhibitors were analyzed with the NFSQ kit assay using a 7500 real-time PCR system in triplicate reactions.

Detection of degraded DNA

To evaluate the detection of degraded DNA using the NFSQ assay, the degraded DNA samples were prepared using two different methods, sonication, and heating. In the case of DNA degradation with sonication, human control DNA sample (9947A) was exposed to different sonication times using an ultrasonic processor (Sonics & Materials, Newtown, USA). Concentration of control DNA sample was 0.1 ng/μL. The sonication proceeded in 2, 5, 10, 15, 20, 25, and 30 cycles, which included 1 min of sonication at 30 amplitude and 1 min of cooling at 4 °C. In the case of DNA degradation with heating, human control DNA samples (G1471) were exposed to different heating times using Gene-Amp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA). Concentration of control DNA sample was 10 ng/μL. Heating degradation test was conducted in 5, 10, 15, 20, 25, and 30 min at 99 °C. The DNA samples degraded by heat were equally diluted at a concentration of 1 ng/μL, and degradation index was measured. The degraded DNA samples were tested in triplicate with an NFSQ kit assay using an Applied Biosystems 7500 Real-Time PCR system.

Mixture study

A series of samples were produced using a mixture of human male (2800M) and female (K562) genomic DNA samples. The male genomic DNA sample concentrations were kept at 0.1 ng/μL, and the female genomic DNA samples at concentrations of 0.2, 0.5, 1, 2, 5, 10, 40, 100, 200, 800, and 1600 ng/μL were added to each male DNA sample to obtain a range of male/female ratios from 1:1 to 1:8000. The mixed DNA samples were tested in triplicate with an NFSQ kit assay using an Applied Biosystems 7500 Real-Time PCR System.

Reproducibility study

Two types of human genomic DNAs (G1471, K562) were classified based on DNA concentration. A human male genomic DNA sample (G1471) was diluted to 1, 0.5, and 0.1 ng/μL, and a human female genomic DNA sample (K562) were diluted to 1, 0.5, and 0.1 ng/μL. The DNA samples were

quantified in triplicate reactions ($N = 3$) by three different operators for each of three repeat runs with two Applied Biosystems 7500 Real-Time PCR System using NFSQ kits.

Casework samples study

Casework samples were categorized into those taken from the human body and those collected at crime scenes. To verify the performance of the NFSQ kit, these samples were quantified by NFSQ kits and a Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Waltham, MA) to compare the concentrations of human DNA. In addition, to confirm the status of these DNA samples, the AmpF/STR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA) and GloberFiler PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA) were used for STR amplification.

Comparisons with other quantification kits

To compare the performance of the newly developed NFSQ kit with other kits, the degradation index tests were conducted using each kit. The Quantifiler® Trio DNA Quantification kit, the PowerQuant™ system, and the Investigator® Quantiplex pro kit were compared with the NFSQ kit, and each kit was performed according to the manufacturer's instructions [23–25].

The Quantifiler® Trio DNA Quantification kit, the PowerQuant™ system, and the Investigator® Quantiplex pro kit were performed according to each user guideline. The sensitivity test was performed using standard DNA diluted fivefold on a Nanodrop 2000C spectrophotometer, which used an ultraviolet absorbance at 260 nm (Thermo Fisher Scientific, Waltham, MA) to assess the dynamic range of NFSQ kit. The degradation index test was performed using DNA samples destroyed by ultra-sonication and heating in a heat block. The degradation index was measured according to the time of ultra-sonication and heating, respectively.

STR amplification

Short tandem repeat analysis was performed as part of the degradation index test and casework test. The AmpF/STR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA) and the GloberFiler PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA) were used for STR amplification using 44 casework samples and six degraded DNA samples. The final reaction volume was 25 μL, and amplification was performed using the Gene-Amp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions.

STR results analysis

After STR amplification, 1 μL of amplicons was mixed with 10 μL Hi-Di™ formamide (Thermo Fisher Scientific, Waltham, MA) and 0.1 μL of LIZ™ 500 size standard (for the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit) and LIZ™ 600 size standard (for the GloberFiler PCR Amplification Kit). The mixture was subjected to capillary electrophoresis on the 3500xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA) and analyzed using Gene Mapper® ID-X v1.4 software (Thermo Fisher Scientific, Waltham, MA).

Results and discussion

Selection of quantification target loci and standard curve

The multiplex TaqMan fluorescent probes of the NFSQ kit included a small DNA marker (106 bp) that spanned *HLA-DRA* (HLA class II histocompatibility antigen, DR alpha chain) gene to provide whole DNA quantity, and a male DNA marker (85 bp) that spanned SRY (Sex-determining region Y) region to confirm the presence of male DNA, and a large DNA marker (189 bp) that spanned *HLA-DOA* gene (HLA class II histocompatibility antigen, DO alpha chain), an internal positive control (IPC) marker (387 bp) that spanned WSSV (White Spot Syndrome Virus) region. The concentration of small and male DNA and the status of the evidence could be determined using these markers (Table 1).

The NFSQ kit was designed with a large DNA marker and a small DNA marker of single copy target loci from the autosomal gene, *HLA*, and a male DNA marker of single copy target loci from the SRY region. The HLA-DRA and DOA regions were selected because many *HLA* genes, including the HLA-DRA and DOA regions, have already been studied and their details are available in the international Immuno GeneTics project (IMGT)-HLA database. Additionally, unlike the alpha chain of other MHC class II molecules, the HLA region is practically invariable [26, 27].

Table 1 The markers of NFSQ. NFSQ kit configurations contain small DNA, large DNA, and male DNA and IPC markers

Marker	Marker size	Region
Small DNA	106 bp	HLA DRA
Large DNA	189 bp	HLA DOA
Male DNA	85 bp	SRY
IPC	387 bp	WSSV

The calibration curve was drawn using HID Real-Time PCR Software v1.2 (Thermo Fisher Scientific, Waltham, MA) (Fig. 1). The small DNA marker had a slope − 3.098 and $R^2 = 0.999$, the male DNA marker had − 3.183 and $R^2 = 1$, and the large DNA marker had a slope − 3.164 and $R^2 = 0.999$.

Species specificity and analytical sensitivity

The species specificity study was conducted using purified genomic DNA samples of several animals and bacteria to determine if the probe we designed specifically detected human genomic DNA. The DNA samples from different animal species were tested in triplicate reactions.

Seven samples of animal DNA, including dog, cat, bovine, pig, chicken, sheep, and gerbil, and 7 samples of microorganism DNA including *B. thurigiensis*, *L. vaginalis*, *L. crispatus*, *P. mirabilis*, *S. aureus*, *E. coli* O157, and *S. paratyphi* B were used for the species specificity study. All of the DNA samples were tested in triplicate reactions.

Except for the G1471 human male DNA sample, animal DNA and microbial DNA samples were not detected by small, large, and male markers of the NFSQ kit. Conversely, the DNA of each species was amplified (mean C_Ts < 25) by the IPC marker. Human DNA markers in the NFSQ kit do not recognize animal and microbial DNA, and the markers are specific to human genomic DNA. There were several trials and errors in the process of selecting the marker region and the target sequence. To improve the sensitivity of the male DNA marker, we attempted to utilize the DYS14 region (multi-copy loci) as the male DNA marker. However, animal DNA was detected and amplified by the DYS14 region (data not shown). Therefore, SRY region, a single-copy locus, was used for human male DNA marker and it was highly specific to the human male DNA.

Concentration of DNA samples collected from crime scene evidence varies wildly. To examine the limit of quantitation (LOQ) and the sensitivity of the kit, serial dilutions of human male genomic DNA (G1471, Promega) were quantified with the NFSQ kit using a 7500 Real-time PCR System (Applied Biosystems Inc. Foster City, CA, USA). Standard DNA was diluted based on spectrophotometry (50, 10, 4, 0.8, 0.16, 0.032, 0.016, 0.0064, 0.0032, 0.00128 ng/μL). The diluted DNA samples were amplified for 40 cycles with the NFSQ kit. The experiments were performed in triplicate with 6 runs. The DNA samples above an expected concentration of 0.00128 ng/μL were detected by all the markers of the kit (Table 2). However, lower limit of quantitation (LLOQ) is defined as the minimum concentration at which CV is less than 25% [28]. And, limit of detection (LOD) is defined as the lowest concentration at which 95% of the positive samples are detected [14]. Additionally, low-copy PCRs are stochastically limited, and LODs of < 3 copies per PCR are not possible [14]. Therefore, according to Table 2 data, the LOD of NFSQ markers is 0.0064 ng/μL. The LLOQ of small marker was 0.0064 ng/μL, and LLOQ of large, male marker was 0.016 ng/μL. IPC was detected in all of the DNA samples (data not shown). The most suitable DNA concentration recommended for STR identification is 0.5–2 ng/μL [1]. Considering the requirements for human identification, the NFSQ kit exhibited sufficient sensitivity.

Mixture study

To evaluate the male specificity of the NFSQ kit, mixed samples of male and female DNA were analyzed by the NFSQ kit

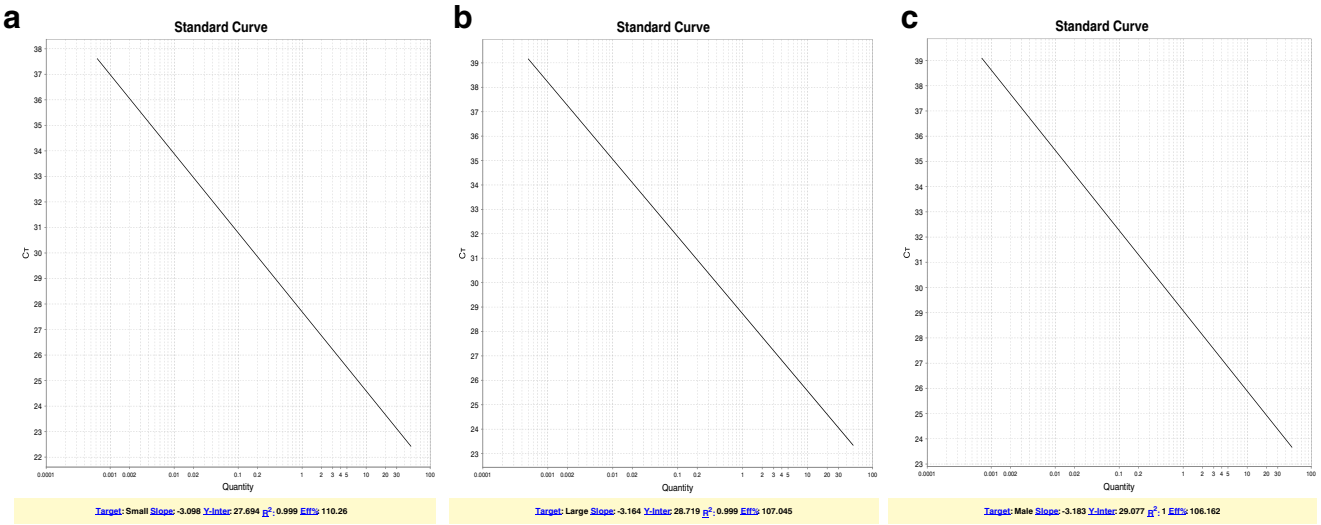

Fig. 1 Standard curve of each DNA marker. (**a**) Small DNA marker. (**b**) Large DNA marker. (**c**) Male DNA marker

Table 2 Sensitivity study results of NFSQ DNA quantification kit. The sensitivity study was conducted six times in triplicates per sample. The mean, the standard deviation, and the coefficient of variation (CV) of each sample were calculated from the overall quantitative values

Expected DNA Conc. (ng/μL)	Target marker											
	Large DNA marker				Male DNA marker				Small DNA marker			
	Mean Conc. (ng/μL)	SD (ng/μL)	CV (%)	Pos. Reps (*N* = 18)	Mean Conc. (ng/μL)	SD (ng/μL)	CV (%)	Pos. Reps (*N* = 18)	Mean Conc. (ng/μL)	SD (ng/μL)	CV (%)	Pos. Reps (*N* = 18)
50	50.04	3.30	6.59	18/18	57.06	4.55	7.98	18/18	50.02	3.78	7.56	18/18
10	8.74	0.71	8.16	18/18	7.23	0.54	7.49	18/18	8.14	0.47	5.77	18/18
4	4.19	0.251	5.98	18/18	3.38	0.29	8.59	18/18	3.64	0.26	7.06	18/18
0.8	0.919	0.090	9.74	18/18	0.737	0.071	9.58	18/18	0.741	0.062	8.32	18/18
0.16	0.140	0.0140	10.01	18/18	0.150	0.016	10.49	18/18	0.160	0.016	10.23	18/18
0.032	0.0368	0.0062	16.81	18/18	0.0369	0.0057	15.53	18/18	0.02454	0.0047	19.14	18/18
0.016	0.02266	0.00463	20.42	18/18	0.02241	0.00524	23.38	18/18	0.01486	0.00291	19.62	18/18
0.0064	0.00563	0.00151	26.75	18/18	0.00536	0.00141	26.30	18/18	0.00424	0.00094	22.12	18/18
0.0032	0.00330	0.00117	35.49	16/18	0.00389	0.00132	33.80	17/18	0.00343	0.00129	37.68	18/18
0.00128	0.00120	0.000441	36.87	14/18	0.00249	0.000858	34.42	7/18	0.00147	0.000646	43.94	15/18

Pos. Reps number of target positive reactions out of three replicates, *Conc.* concentration, *SD* standard deviation, *CV* coefficient of variation

in triplicate reactions. Since a female DNA does not have a complementary region to the male DNA marker, mixture DNA can be classified by comparing small DNA concentration and male DNA concentration. Mixed samples were prepared with 2800M and K562 standard DNA in mixed ratios ranging from 1:1 to 1:8000 (male/female). The male DNA was kept at a constant level of 0.1 ng/μL.

Table 3 shows the changes in the quantitative values according to the differences in the ratios of the mixed samples. DNA samples of 1:1 to 4000 ratios were quantified using the male DNA marker at 0.11 ± 0.008 ng/μL. Although 1:8000 mixed samples gave a mean male DNA target result of 0.076 ng/μL, the concentration of the male DNA was also measured as similar to the original value of 0.1 ng/μL. The male DNA was easily detected by NFSQ, including the 1:8000 DNA sample.

Stability study (PCR inhibitors and degradation index)

To evaluate how the newly developed kit is affected by PCR inhibitors, hematin, and humic acid, known inhibitors of PCR assays were used in this study. 2800M standard male DNA was prepared with a fixed concentration of 0.5 ng/μL without the PCR inhibitors. Humic acid was added to each sample at concentrations ranging from 0 to 800 ng/μL. Furthermore, another set of samples were prepared with hematin at concentrations ranging from 0 to 1000 μM.

Table 4 shows the results of the newly developed quantification kit for samples containing PCR inhibitors. When humic acid contained in the soil was added, the NFSQ kit was able to amplify all the DNA targets up to the sample treated with 200 ng/μL. In the sample treated with 400 ng/μL, the NFSQ kit started flagging inhibition, and a larger DNA sample was quantified as 0.001 ng/μL, and male DNA was quantified as 0.03 ng/μL, but small DNA was not detected. The NFSQ kit failed to quantify all of the DNA concentrations containing humic acid at 800 ng/μL or above. The degradation index increased from 0.94 to 2.08 as the concentration of humic acid increased (Table 4).

When the hematin contained in methemoglobin was added, all the targets of DNA in the NFSQ kit were detected up to the sample treated with 600 μM of hematin. In samples treated with 600 μM hematin, the NFSQ kit flagged inhibition in one of three samples, small DNA was quantified as 0.031 ng/μL, large DNA was quantified as 0.005 ng/μL, and male DNA was quantified as 0.104 ng/μL. From over 750 μM of treatment, the NFSQ kit failed to quantify all of the DNA concentration and had flagging inhibition in all triplicate reactions. The degradation index increased from 1.06 to 5.79 as the concentration of hematin increased (Table 4). Furthermore, as the concentration of PCR inhibitor increased, the resulting DNA concentration was measured as slightly higher than the original concentration of the DNA sample. In the PCR inhibitor study using the NFSQ kit, the performance of the NFSQ kit in the presence of PCR inhibitors was similar to that of Quantifiler® Trio DNA Quantification kit, which is being used in the NFS [21, 29].

The NFSQ kit can also be used to evaluate the status of the sample DNA. The degradation index (DI) is used as an indicator of the percentage of DNA that has been degraded, which is measured as the ratio of small DNA concentration to large DNA concentration. The degradation index study was

Table 3 Results of mixture study. The average DNA concentration measured with small DNA marker and large DNA marker of NFSQ kit. The mean quantity results for male DNA and small DNA marker are shown

Sample (male/female)	Mean DNA Conc. (ng/μL)		
	Expected male DNA conc.	Male DNA conc.	Small DNA conc.
1:0	0.1	0.112	0.105
1:1	0.1	0.101	0.282
1:2.5	0.1	0.105	0.371
1:5	0.1	0.112	0.615
1:10	0.1	0.118	1.034
1:25	0.1	0.114	2.379
1:50	0.1	0.133	4.405
1:100	0.1	0.101	24.207
1:500	0.1	0.118	53.116
1:1000	0.1	0.156	128.124
1:4000	0.1	0.115	362.319
1:8000	0.1	0.076	799.217

conducted in two types of experiments, ultrasonication, and thermal degradation. In the case of DNA degradation by ultrasonication, the concentration of standard DNA samples (2800M and 9947A) was prepared as 0.1 ng/μL, and changes in the degradation index and changes in DNA concentration measured at each marker were observed depending on the sonication time (0–30 min).

Figure 2a shows the mean results of the DNA concentration and the degradation index obtained by the NFSQ kit with the 9947A DNA, which was degraded by sonication. All samples were tested in triplicate reactions. When the DNA samples were not degraded by sonication, a DI value of 0.9704 was measured. As the sonication time increased, the degraded DNA concentrations were decreased, and the DI values were increased. When the sonication treatment time was 30 min, the concentration of large DNA decreased to 0.0063 ng/μL, and the DI value increased to 7.6455. At the same time, the concentration of small DNA also decreased to 0.0477 ng/μL.

In addition, the STR amplification results were also compared to confirm the degradation status of the samples used in this test. The STR result was detected when sonication was applied for 30 min. The peak of the 100 bp marker was observed to be approximately five times larger than the peak of the marker for 200 bp (data not shown).

In the case of DNA degradation by heating (98 °C), male genomic DNA samples (G1471) were degraded by time-dependent heating (0–120 min). All of the degraded DNA samples using heat were diluted to a concentration of 1 ng/μL, and the degradation index values were measured.

Figure 2b shows the change in the DI value and the target DNA concentration according to the heat treatment time measured by the NFSQ kit. When no heat was applied to the sample, the concentration of large DNA was measured at 1.078 ng/μL, and the DI value was found to be 0.952. When the heating time was 2 h, the concentration of large DNA was measured as 0.191 ng/μL, and the degradation index value

Table 4 Results of standard DNA added PCR inhibitors. The assay was processed with 2800 M human genomic DNA and different levels of humic acid and hematin PCR inhibitor. The mean quantity results for each target are shown

PCR inhibitor	PCR inhibitor Conc.	NFSQ kit results (ng/μL)			Degradation index	IPC flag
		Small DNA Conc.	Large DNA Conc.	Male DNA Conc.		
Control	–	0.419	0.430	0.417	0.98	N
Humic acid	12.5 ng/μL	0.431	0.461	0.462	0.94	N
	25 ng/μL	0.396	0.461	0.415	0.86	N
	50 ng/μL	0.565	0.607	0.542	0.93	N
	100 ng/μL	0.751	0.664	0.597	1.13	N
	160 ng/μL	0.808	0.516	0.763	1.57	N
	200 ng/μL	0.901	0.413	0.785	2.08	N
	400 ng/μL	Undet.	0.001	0.030	–	Y
	800 ng/μL	Undet.	Undet.	Undet.	–	Y
Hematin	62.5 μM	0.555	0.525	0.465	1.06	N
	125 μM	0.492	0.518	0.492	0.95	N
	250 μM	0.827	0.642	0.773	1.29	N
	400 μM	0.977	0.292	0.669	3.44	N
	600 μM	0.031	0.005	0.104	5.79	Y/N
	750 μM	Undet.	Undet.	Undet.	–	Y
	1000 μM	Undet.	Undet.	Undet.	–	Y

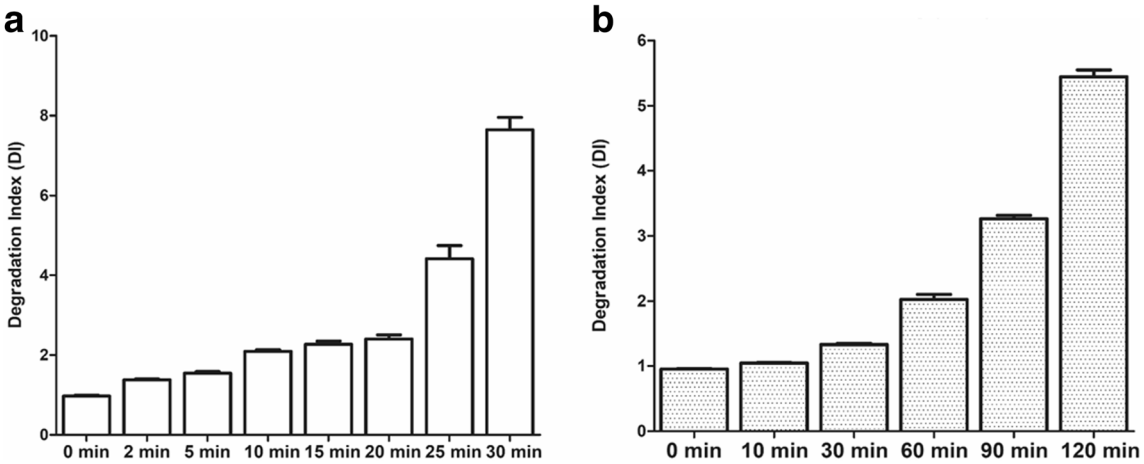

Fig. 2 Degradation index results depend length of sonication and heat treatment. The mean degradation index results are shown. (**a**) Degradation with 9947A female DNA using ultra-sonication. (**b**) Degradation with G1471 male DNA using heating. The DI value was calculated from the quantification results using the ratio (small DNA concentration/large DNA concentration)

was measured as 5.445. The concentration of large DNA was decreased, and the degradation index value was increased as the heat application time increased.

The STR amplification results were also compared to confirm the degradation status of the samples used in this test. The STR result was partially detected when heating was applied for 90 min. Since the G1471 male DNA sample was extracted from the blood of several males, several peaks appeared in one region. The peak for the 100 bp marker was observed to be approximately four times larger than the peak for the 200 bp marker (data are not shown). To determine whether there is a problem or error with the degraded DNA samples, PCR was conducted using an STR assay to check the DNA length, but no problem was found in the sample.

To evaluate the performance of the NFSQ kit, degradation index studies of the NFSQ Kit and other commercial kits including Quantifiler® Trio, the Powerquant™ system and Investigator® Quantiplex pro kits were compared. The degradation study was performed using 2800M male standard DNA at a concentration of 0.1 ng/μL. DNA degradation was performed by ultra-sonication using 30 pulses and heating at 98 °C. In the case of DNA degraded by ultra-sonication (Fig. 3), the NFSQ kit measured a DI value of 4.7937 in the sample undergoing ultra-sonication for 30 min, and the NFSQ kit showed the third highest DI value among the kits. The Investigator® Quantiplex Pro kit showed a DI value of 11.1520 in the same sample, which was the highest DI value. Moreover, the Quantifiler® Trio kit showed the lowest value with a DI value of 2.2213 for the sample.

To confirm the results with the DNA samples degraded by heating, a DNA degradation index of heat-treated DNA samples was measured using quantification kits (Fig. 4). The DI value measured by the NFSQ kit was 4.7948 for the sample heated for 90 min, and the DI value was significantly increased from the sample subjected to heat for 60 min. The Quantifiler® Trio DNA Quantification kit showed a DI value of 5.8223 for the sample heated for 90 min, and the DI value was also significantly increased from the sample subjected to heat for 60 min. The Investigator® Quantiplex Pro showed a DI value of 5.8713 for the sample with heating for 90 min, and DI value was also significantly increased from the sample subjected to heat for 60 min. The Powerquant™ system showed that a DI value was

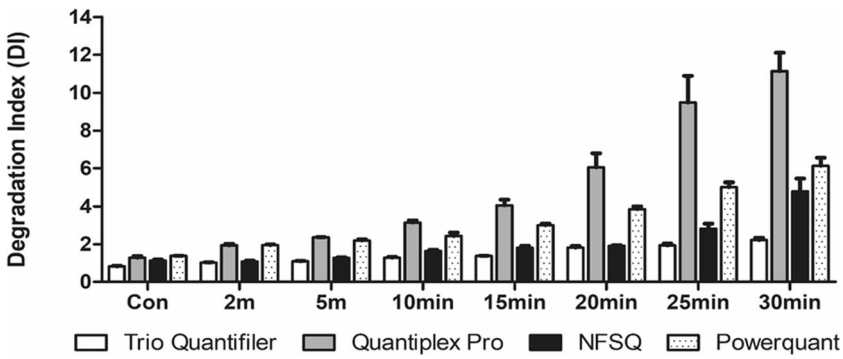

Fig. 3 Degraded DNA sample series results. The degradation index results using quantification kits depend on ultra-sonication degradation times. The mean degradation index results are shown

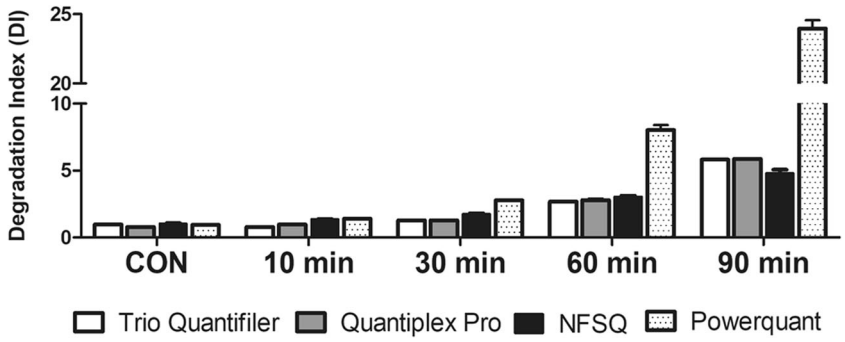

Fig. 4 Degraded DNA sample series results. Degradation index results using quantification kit depend on time with the method of heating degradation. The mean degradation index results are shown

17.494 for the sample heated for 90 min, and the DI value was significantly increased compared with the sample subjected to heat for 60 min.

In the DI analysis, the validation of the other commercial kits was performed with DNA artificially degraded by sonication and heating in the same manner [19, 30, 31]. DI values increased with the time of the degradation. Comparison of the DI values of each kit showed similar rates of increase in DI, but different DI values. Because of the size and region of the degradation of DNA markers and small DNA markers, the criterion for the DI value varied, but the increasing tendency was similar. Degraded DNA samples prepared by sonication showed very low DI growth rates only in the Quantifier® Trio DNA Quantification Kit. In some previous studies, it was confirmed that the DI value of a degraded DNA sample was lower when measured using the Quantifiler® Trio kit [32]. In the case of degradation by heating, the DI value of the NFSQ kit was relatively low compared with that of the commercial kits, because the degraded DNA target sequence is shorter and the small DNA target sequence is longer than those of the other kits [13].

Reproducibility study

To evaluate the reproducibility of the NFSQ kit, two types of human control genomic DNA (male G1471 and female K562) which were quantified and prepared using nanodrop measurements were diluted to 0.1 and 1 ng/μL, and quantified using the NFSQ kit. The samples of each concentration were prepared in triplicate named #1, #2, and #3. The samples were quantified in triplicate reactions by three different operators with two real-time PCR machines ($N = 54$). Table 5 shows the results of the reproducibility test with G1471 male and K562 female genomic DNA with the mean concentrations and the

Table 5 Results of reproducibility test with G1471 male genomic DNA and K562 female genomic DNA. Three samples of equal concentration were performed in triplicate by three different operators with two different real-time PCR machines using NFSQ kit

Sample	Sample number	Mean quant (ng/μL)			Average within-run variability			All run variability		
					Quant CV (%)			Quant CV (%)		
		Large	Small	Male	Large	Small	Male	Large	Small	Male
G1471 1 ng/μL	#1	1.223	1.197	1.298	5.773	7.054	9.311	7.507	8.000	9.495
	#2	1.224	1.202	1.184	7.457	8.519	7.513			
	#3	1.130	1.152	1.138	6.740	8.157	5.825			
G1471 0.1 ng/μL	#1	0.119	0.122	0.123	11.809	8.531	9.396	9.536	9.008	9.357
	#2	0.119	0.125	0.123	8.372	7.156	9.303			
	#3	0.116	0.121	0.119	8.233	11.148	9.450			
K562 1 ng/μL	#1	1.050	1.047	Undet.	11.634	7.815	–	9.160	9.483	–
	#2	1.033	1.112	Undet.	8.319	9.030	–			
	#3	1.048	1.087	Undet.	7.393	10.778	–			
K562 0.1 ng/μL	#1	0.116	0.120	Undet.	10.500	8.967	–	8.665	9.451	–
	#2	0.113	0.128	Undet.	7.724	6.237	–			
	#3	0.114	0.121	Undet.	7.754	11.796	–			

CV coefficient of variation

Table 6 Casework samples study. The table shows results of casework samples performed using NFSQ, Trio Quantifiler, and STR kits. The quantification results are analyzed by each marker, and Degradation Index value is calculated with small and large DNA quantity. STR result shows resulting overall profile quality

		NFSQ kit				Trio Quantifiler			
Sample number	Sample	Small DNA Conc. (ng/μL)	Large DNA Conc. (ng/μL)	Male DNA Conc. (ng/μL)	Degradation Index	Small DNA Conc. (ng/μL)	Male DNA Conc. (ng/μL)	STR kit	STR profile
1	Blood	1.9016	1.6895	Undet.	1.1255	1.62	Undet.	ID Plus	Full/ss
2	Blood	1.8779	1.5283	Undet.	1.2288	1.68	Undet.	ID Plus	Full/ss
3	Blood	0.8254	0.8050	Undet.	1.0253	0.83	Undet.	ID Plus	Full/ss
4	Saliva	1.2604	1.0018	1.0256	1.2581	1.583	2.135	GF	Full/mix
5	Buccal swab	4.3648	4.0469	Undet.	1.0786	4.699	0.017	GF	Full/ss
6	Buccal swab	11.4677	10.0718	Undet.	1.1386	15.315	Undet.	GF	Full/ss
7	Lip (saliva)	1.4563	0.8455	Undet.	1.7224	1.191	Undet.	GF	Full/ss
8	Skin	0.0247	0.0159	Undet.	1.5477	0.026	Undet.	GF	Full/ss
9	Hair	0.0365	0.0030	Undet.	12.2919	0.048	Undet.	GF	Partial/ss
10	Hair	0.0200	0.0116	Undet.	1.7143	0.019	Undet.	GF	Partial/ss
11	Hair	0.0178	0.0101	Undet.	1.7675	0.011	Undet.	GF	Partial/ss
12	Hair	0.0044	Undet.	Undet.	–	0.001	Undet.	GF	Partial/ss
13	Hair	0.0010	Undet.	Undet.	–	0.001	Undet.	GF	ND
14	Nail	0.5487	0.3333	Undet.	1.6460	0.387	0.009	GF	Partial/mix
15	Nail	0.0623	0.0609	Undet.	1.0229	0.051	Undet.	GF	Full/ss
16	Nail	0.5350	0.4974	0.1166	1.0756	0.453	0.041	GF	Full/mix
17	Vaginal fluid	0.7610	0.8856	0.1510	0.8592	0.699	0.15	GF	Full/mix
18	Vaginal fluid	0.1659	0.0871	0.0020	1.9037	0.117	0.005	GF	Full/ss
19	Vaginal fluid	57.7695	41.5094	0.0062	1.3917	48.017	0.004	GF	Full/mix
20	Vaginal fluid	38.7306	23.3452	0.0004	1.6590	39.835	0.0001	GF	Full/ss
21	FTA card	1.1844	1.4365	1.0761	0.8245	1.165	1.039	GF	Full/ss
22	FTA card	3.0477	2.3756	2.1991	1.2829	2.753	2.999	GF	Full/ss
23	FTA card	4.4904	4.0147	5.8898	1.1185	4.672	5.682	GF	Full/ss
24	FTA card	9.9308	9.7941	10.2128	1.0140	10.709	11.708	GF	Full/ss
25	FTA card	6.1353	4.5537	4.4505	1.3473	6.158	4.922	GF	Full/ss
26	Can	0.0502	0.3351	0.0639	0.1498	0.032	0.019	GF	Full/mix
27	Cigarette	1.2427	0.4980	1.0140	2.4952	1.11	1.34	ID Plus	Full/ss
28	Cigarette	3.1007	2.0765	0.0020	1.4932	2.88	Undet.	ID Plus	Full/ss
29	Cigarette	0.8148	0.3920	0.9120	2.0786	0.5	0.59	ID Plus	Full/ss
30	Glass	0.1947	0.1660	0.0995	1.1731	0.123	0.112	GF	Full/ss
31	Glass	0.0908	0.0695	0.1239	1.3062	0.045	Undet.	GF	Full/ss
32	Syringe	0.0237	0.0295	0.0308	0.8035	0.015	0.022	GF	Full/ss
33	Tissue	0.0891	0.0422	0.0240	2.1086	0.04	0.01	ID Plus	Partial/mix
34	Tissue	0.7904	0.5703	0.2349	1.3859	0.808	0.259	GF	Full/mix
35	Tissue	2.5929	1.9514	0.0448	1.3288	2.509	0.005	GF	Full/ss
36	Handle (touch DNA)	0.0710	0.0187	0.0859	3.7953	0.05	0.04	ID Plus	Partial/mix
37	Mask	0.0639	0.0502	0.0335	1.2721	0.065	0.031	GF	Partial/mix
38	Sun glasses	0.0037	Undet.	0.0011		0.001	Undet.	GF	Partial/ss
39	Toothpick	0.1677	0.0812	0.2687	2.0640	0.158	0.255	GF	Full/ss
40	Window (bloodstain)	0.3577	0.2948	0.0015	1.2134	0.441	0.001	GF	Full/ss
41	Wall (bloodstain)	0.2803	0.2439	0.0001	1.1492	0.241	0.0001	GF	Full/ss
42	Wall (bloodstain)	0.8653	0.7510	0.0159	1.1522	0.574	0.018	GF	Full/ss
43	Wall (blood stain)	0.1225	0.0610	0.1403	2.0072	0.083	0.053	GF	Full/ss
44	Wall (blood stain)	0.1527	0.1066	0.1218	1.4317	0.108	0.119	GF	Full/ss

Conc. concentration, *Undet.* undetermined, *ID Plus* Identifiler Plus, *GF* GloberFiler

coefficient of variation. Table 5 also shows the mean concentration and the coefficient of variation for the each sample of the same concentration.

In the case of the 1 ng/μL concentration of the G1471 human male genomic DNA sample, the coefficient of variation for all of the samples was calculated as 8.001% for the small DNA marker, 7.507% for the large DNA marker, and 9.495% for the male DNA marker. In the case of the 0.1 ng/μL concentration of the G1471 human male genomic DNA sample, the coefficient of variation for all samples was calculated as 9.008% for the small DNA marker, 9.536% for the large DNA marker, and 9.357% for the male DNA marker. In the case of the 1 ng/μL concentration of the K562 human female genomic DNA sample, the coefficient of variation for all samples was calculated as 9.483% for the small DNA marker, and 9.160% for the large DNA marker. In the case of the 0.1 ng/μL concentration of K562 human female genomic DNA sample, the coefficient of variation for all samples was calculated as 9.451% for the small DNA marker, and 8.665% for the large DNA marker. The coefficient of variation for the concentrations of all samples was within 10%. The average coefficient of variation between the three experimenters was relatively low. In the reproducibility study, the male DNA markers, large DNA markers, and small DNA markers showed a variation coefficient of less than 10%, which demonstrates stable reproducibility as compared with other commercially available kits. According to other studies, commercial kits showed a variation coefficient of 10–15% in a reproducibility study [21, 33, 34].

Casework samples study

Real-time PCR assays are an essential method used to quantify the concentrations of DNA samples from various crime scenes and for obtaining satisfactory STR results in the forensic workflow. In this study, 44 casework DNA samples were analyzed using the NFSQ kit. These quantity results were compared with the results of the Quantifiler® Trio DNA Quantification kit and the STR results using the GlobalFiler™ PCR amplification kit (Life Technologies, Carlsbad, California, USA) and the AmpFLSTR Identifiler Plus® PCR amplification kit (Life Technologies, Carlsbad, California, USA). The types of samples included peripheral blood, buccal swabs, saliva, skin, hair, fingernail, vaginal fluid, FTA card, can, cigarette, glass, syringes, tissue, and touch DNA from handles, a mask, sunglasses, and blood stains from a wall and a window.

Table 6 shows the quantification of casework samples using the NFSQ kit and the Quantifiler® Trio DNA Quantification kit and results of the STR profile. A total of 35 samples showed clear peaks at all loci in the STR results. A total of eight samples showed partial detection of loci, and one sample showed no peaks detected (ND) at all loci. When comparing the concentration of DNA samples from the body quantified by the Quantifiler® Trio DNA Quantification kit and NFSQ kit, the quantity of NFSQ was higher than that of the Quantifiler® Trio DNA Quantification kit, and the difference between the two quantification kits was within 30% at a concentration above 0.01 ng/μL. When the STR assay results appeared as partial profiles or no peak detected at all loci (ND), or when the DNA concentration was less than 0.01 ng/μL, the difference between the results of the kits was more than 30%. In some of the DNA samples collected from the body, female DNA and male DNA were often mixed. The male DNA sample results were very different when measured using the Quantifiler® Trio DNA Quantification kit because the samples contained low-concentration DNA.

Since the samples collected at crime scenes represent many samples collected from touch DNA or outdoor locations, low concentrations of DNA were extracted on average, and the DI values tended to be high. The DNA concentration of the two quantification kits differed by less than 38% except for two samples, and the male DNA quantities were within 35% except for four mixed samples and one blood stain sample.

In the casework sample test, comparing the STR profile and of the DNA samples collected from various crime scenes, it was confirmed that the quantitative results of NFSQ were reliable. When the concentration of DNA samples was higher than 0.01 ng/μL, the STR results were interpreted as a full profile. If the concentration of the DNA sample was lower than 0.01 ng/μL, or the DI was high, such as the DNA extracted from touch DNA and hair, the STR profile is not interpreted or is only partially confirmed.

Conclusions

Human genomic DNA quantification is an essential step in conducting a smooth STR assay during human DNA identification. Real-time PCR assays are commonly used to quantify DNA samples. The NFSQ DNA Quantification kit using a real-time PCR assay was recently developed, not only to give total human DNA and male DNA from crime scene evidence but also to indicate the condition of the DNA samples.

In this study, we developed the human DNA quantification kit using a small DNA marker from the *HLA-DRA* gene, a large DNA marker from the *HLA-DOA* gene, and a male DNA marker from the *SRY* gene. We evaluated specificity, accuracy, stability, reproducibility, and produced a casework study of newly developed quantification kit according to developmental validation guidelines from the Scientific Working Group on DNA Analysis Methods (SWGDAM). Additionally, the NFSQ kit was compared with other commercial DNA quantification kits. NFSQ kits showed sufficient specificity, sensitivity, reproducibility, and stability, which were applicable during human identification. The performance of the NFSQ kit was comparable to other commercial

kits. Furthermore, the locally developed NFSQ kit costs approximately one third less than that of other commercial kits, implying that approximately three times the present workload can be handled at the present cost when using the NFSQ kit. Therefore, the NFSQ kit can replace other commercial quantification kits.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards by Institutional Review Board (IRB) of National Forensic Service (Approval No. 906-170118-BR-003-02).

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