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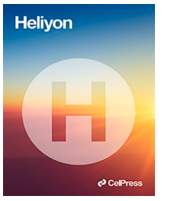

Research article

*UGT1A1*28* detection using high-resolution agarose gel electrophoresis

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ABSTRACT

A new *UGT1A1*28* detection method combining PCR and high-resolution agarose gel electrophoresis was developed. The viability of this method was demonstrated on 15 healthy adult volunteers. Subjects included 13 wild type homozygotes (86.7 %), 2 heterozygotes (13.3 %), and no mutant type homozygotes (0 %). The new *UGT1A1*28* detection method results were fully consistent with DNA sequencing. PCR and agarose gel electrophoresis are common techniques with high-resolution agarose gels available commercially. These results support the clinical viability of this method potentially reducing *UGT1A1*28* diagnosis complexity and cost.

1. Introduction

UDP-glucuronosyltransferase 1A1 (UGT1A1) is an important catalytic enzyme for drug metabolism. The UGT1A1 gene promoter region contains thymine-adenine (TA) repeats [$A(TA)_nTAA$], the number of which (n) varies between wild and mutant types. The wild type has 6 [$A(TA)_6TAA$] while the mutant type has 7 [$A(TA)_7TAA$, *UGT1A1*28*]. Due to the mutant's reduced UGT1A1 expression compared to the wild type, drug metabolism is delayed leading to more pronounced side effects including irinotecan [1–3]. Therefore, *UGT1A1*28* detection is important to ensure safe use of pharmaceuticals. Current *UGT1A1*28* detection methods include the invader method [4], DNA sequencing, fragment size analysis [5], real-time PCR [6,7], and melting curve analysis [8]. However, these each require expensive equipment and special reagents. During the development of an alternative *UGT1A1*28* detection method using photoinduced electron transfer (PeT), it was necessary to obtain a short DNA fragment [approximately 40 base pair (bp)] containing the TA repeat sequence using PCR [9]. At this time, DNA fragments of 40 bp and 42 bp were obtained for $A(TA)_6TAA$ and $A(TA)_7TAA$. Unfortunately, sufficient DNA fragment quantities for the PeT-based detection method could not be obtained. To circumvent this, we evaluated the separation of these 40 bp and 42 bp DNA fragments. Both capillary electrophoresis and polyacrylamide gel electrophoresis (PAGE) can resolve a two-base-pair difference, with PAGE sufficiently sensitive to directly detect *UGT1A1*28* [10]. In this study, agarose gel electrophoresis, a significantly simpler and cost-effective process, was trialed as one of the *UGT1A1*28* detection methods. This detection is complicated by the requirement to distinguish between small variations in TA repeats of only two base pairs. Recently, high-resolution agarose gels capable of this became commercially available. With these commercial products, the optimal agarose gel concentration for *UGT1A1*28* detection was investigated. To detect minor two-bp differences, the electrophoresed amplicons must be as short as possible. Given this, PCR conditions were adjusted to yield short amplicons in accordance with our

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previous work [9]. Finally, the newly developed detection method was demonstrated in healthy adult subjects and validated using DNA sequencing.

2. Materials and methods

2.1. Chemicals

Agarose powder (Primegel agarose PCR-sieve GAT), DNA ladders [20 bp DNA Ladder (Dye Plus)], and *Dpn*I were purchased from Takara Bio (Shiga, Japan). Taq DNA polymerase (Quick Taq HS dye mix) was purchased from Toyobo (Osaka, Japan). Pfu DNA polymerase (Pfu turbo) was purchased from Agilent Technologies (Santa Clara, CA, USA). Ethidium bromide (EtBr) was purchased from Nippon Gene (Tokyo, Japan). Other chemicals were purchased from Fujifilm Wako Pure Chemical (Tokyo, Japan). Saliva DNA collection and preservation devices and saliva DNA isolation kits were purchased from Norgen (Thorold, ON, CA). The MonoFas DNA purification kit I was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Distilled water was used for all experiments. Oligo nucleotide syntheses were outsourced to Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Instruments

Agarose gel electrophoresis was performed using a Mupid-exu (Advance, Tokyo, Japan). DNA bands were identified using a transilluminator (3UV-transilluminator Bio-doc-it imaging system equipped with a CCD camera and EtBr filter, UVP, Upland, CA, USA). PCR was performed using a thermal cycler (My cycler thermal cycler, Bio-rad Laboratories, Hercules, CA, USA). DNA sequencing was performed with the ABI PRISM 3000 sequence analyzer (Applied Biosystems, Foster City, CA, USA) or outsourced to Fasmac (Atsugi, Japan).

2.3. Construction of control plasmids

2.3.1. Extraction of genomic DNA

The present study was approved by the Ethics Committee of the Health Sciences University of Hokkaido (approval number: 23P002). Human genomic DNA was extracted from oral mucosa epithelial cells of healthy adult volunteers who agreed to donate genomic DNA after explanation of the study's purpose. Oral mucosa cells were collected with a cotton swab from each subject by swabbing for 1 min and suspended in 600 μL PBS. After centrifugation (2000×*g* for 3 min), to the precipitate was added 100 μL of lysis buffer (PBS contained 0.2 mg/mL proteinase K, 1 % SDS) then heated at 60 °C for 1 h. To this mixture was added 10 μL of 3 M sodium acetate, then 200 μL of cold ethanol was added and allowed to cool on ice for 10 min. After centrifugation (2000×*g* for 3 min), the precipitate was dried, then precipitate was dissolved in 200 μL of TE and used immediately as a PCR template.

2.3.2. Cloning of UGT1A1 promoter region

Partial *UGT1A1* region (100 bp) underwent amplification. The 25 μL PCR reaction mixture contained 13 μL of Quick Taq HS dye mix, genomic DNA (approximately 20 ng/μL), forward (5′-ATAGTCACGTGACACAGTCA-3′) and reverse (5′-CTTTGCTCCTGCCAGAGGTT-3′) primer (each 0.2 μM), and distilled water. PCR conditions are as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 2 min. The amplicon was ligated into a modified plasmid [11], and *E. coli* JM109 cells were transformed with the construct by $CaCl_2$-madiated transformation [12] and stored at −80 °C. The resulting plasmid was subjected to DNA sequencing to identify the six TA repeats, and designated pA$(TA)_6$TAA.

2.3.3. Site-directed mutagenesis

Site-directed mutagenesis was performed to construct the A$(TA)_7$TAA control plasmid for pA$(TA)_6$TAA according to the previous work [13]. The 50 μL reaction mixture contained Pfu turbo (0.05 U/μL), pA$(TA)_6$TAA (approximately 1 ng/μL), forward (5′-GCCATATATATATATATATAAGTAGG-3′) and reverse (5′-CCTACTTATATATATATATATATGGC-3′) primer (each 1 μM), dNTPs (each 0.25 mM), and distilled water. Thermal cycling conditions are as follows: initial denaturation at 95 °C for 1 min, followed by 16 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 1 min, and extension at 68 °C for 13 min. After 16 cycles, a final extension at 68 °C for 13 min was performed. The 45 μL of reaction mixture contained 4 μL of 10 × T buffer, *Dpn*I (0.2 U/μL), and distilled water, with the mixture then maintained at 37 °C for 1 h. After template plasmid digestion, *E. coli* JM109 cells, were transformed using the construct and stored at −80 °C. The resulting plasmid was subjected to DNA sequencing, identifying the seven TA repeats, and designated pA$(TA)_7$TAA.

2.4. Investigation of PCR condition

PCR conditions were compared by changing annealing temperature. The 50 μL PCR mixture contained 26 μL of Quick Taq HS dye mix, pA$(TA)_6$TAA (approximately 1 ng/μL), forward [5′-ATTGGTTTTTGCCATATATA-3′ (sequence annealing to TA repeat is underlined)] and reverse (5′-CCCTCTCCTACTTATATATA-3′) primer (each 0.1 μM). PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 30–55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 2 min. The lowest annealing temperature possible for the thermal cycler used here was 30 °C. The

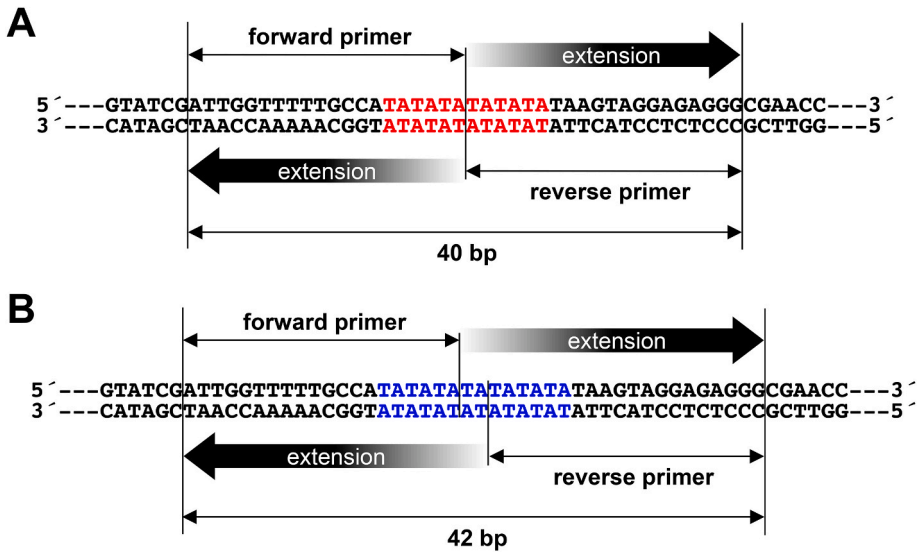

Fig. 1. Primers for short chain DNA fragment amplification.
DNA fragments of 40 bp (**A**) and 42 bp (**B**) were amplified from pA$(TA)_6$TAA and pA$(TA)_7$TAA, respectively, using specific primers.

PCR mixture was electrophoresed using 5 % agarose gel (*see materials and methods, section **5***).

2.5. Examination of conditions for agarose electrophoresis

Agarose gels (2–5%, length 6 cm × width 5.3 cm × thickness 0.9 cm) were prepared with 0.5 × TB buffer (50 mM Tris, 48.5 mM boric acid) containing EtBr. Electrophoresis was performed with a constant 135 V (upper voltage limit of the device) for 1.5 h. After electrophoresis, the gel image was captured by a CCD camera under UV-B (302 m) irradiation using a transilluminator.

2.6. Implementation in healthy volunteers

The present study was approved by the Ethics Committee of the Health Sciences University of Hokkaido (23P002). Prior to saliva collection, all volunteers were informed of the study's purpose and agreed to provide saliva. To demonstrate the detection method, genome DNA was extracted from saliva produced by healthy adult subjects. Saliva was collected using a saliva DNA collection and preservation device (Norgen) according to the manufacturer's protocol. DNA was then isolated from saliva using a saliva DNA isolation kit (Norgen) according to the manufacturer's protocol. Initially, a DNA fragment of approximately 540 bp was amplified using genomic DNA as template. The 50 μL PCR mixture contained 26 μL of Quick Taq HS dye mix, genomic DNA (10 ng/μL), forward (5′-CGTCCTTCTTCCTCTCTGGTAACAC-3′) and reverse (5′-CGTCTCTGATGTACAACGAGGCGTC-3′) primer (each 0.1 μM), and distilled water. The first PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 2 min. After the first PCR, the product was purified using Monofas DNA purification kit I.

The second PCR was carried out as above (*see above section **4***) but used the product obtained from the first PCR (approximately 0.4–0.6 ng/μL) as template and an annealing temperature of 30–35 °C. Electrophoresis of the second PCR product was as described above (*see above section **5***) except for gel size (length 6 cm × width 10.8 cm × thickness 0.9 cm).

2.7. DNA sequencing

An approximately 540 bp DNA fragment containing TA repeat was amplified using genomic DNA and purified as described above (*above section **6***). The DNA sequencing using a specific primer (5′-CCCTGCTACCTTTGTGGACTGACAGC-3′) was outsourced to Fasmac (Atsugi, Japan).

3. Results and discussion

3.1. Investigation of PCR conditions

*UGT1A1*28* detection requires a two-bp resolution capability between the wild-type and mutant TA repeats. To enhance the likelihood of identifying this small difference, it is beneficial for the difference to be large relative to the entire molecule. For example, it is generally easier to identify a difference of two bp in a 100 bp molecule than two bp in 1000 bp. Therefore, amplicons for agarose

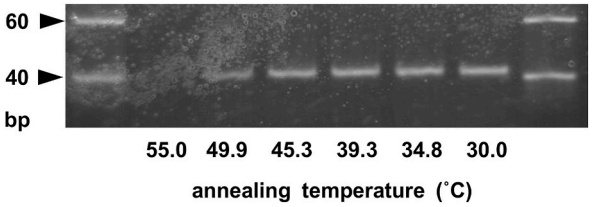

Fig. 2. Effect of annealing temperature on amplification.
Amplicons obtained using various annealing temperatures (30–55 °C) were electrophoresed. PCR was performed with pA(TA)$_6$TAA as described above (*see materials and methods, section **4***). Agarose gel electrophoresis was performed using 5 % agarose gel as described above (*see materials and methods, section **5***). Twenty bp DNA Ladder (Dye Plus) was used.

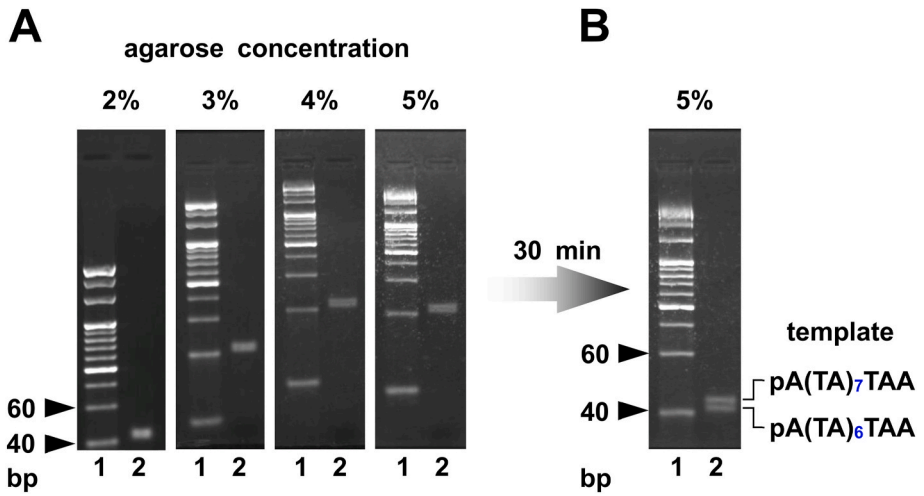

Fig. 3. Separation of 40 and 60 bp DNA and amplicons at various agarose gel concentration.
(**A**) Twenty bp DNA Ladder (lanes **1**) and amplicon mixture (lanes **2**) were electrophoresed for 1h using different agarose gel concentrations (2–5%) containing EtBr. (**B**) The 5 % agarose gel was electrophoresed for an additional 30 min. Amplicons were obtained by PCR as described above (*see materials and methods, section **4***). Images were obtained using Bio-doc-it imaging system equipped with a CCD camera and EtBr filter under UV-B (302 nm) irradiation.

gel electrophoresis must be as short as possible. Thus, short DNA fragment amplifying primers were designed for the 40 bp wild type and 42 bp mutant sequence (Fig. 1). The forward primer sequence is 5′-ATTGGTTTTTGCCATATATA-3′ and the reverse primer sequence is 5′-CCCTCTCCTACTTATATATA-3′. The forward and reverse primers contain adenine (A) and thymine (T) proportions of 75 % and 65 %, respectively. The A–T pair has fewer hydrogen bonds than the guanine (G)–cytosine (C) pair, resulting in lower annealing temperatures. The certificate analysis report stated a Tm of 36 °C for the forward primer and 41 °C for the reverse primer. PCR was examined at lower than typical annealing temperatures (≤55 °C) in detail. In this study, the lowest annealing temperature possible for the thermal cycler was 30 °C. Thus, annealing temperatures in the range 30–55 °C were investigated using the temperature gradient function of the thermal cycler. At about 40–55 °C, the short DNA amplicon band became lighter with temperature decreases of 5 °C, which ceased between about 35 and 30 °C (Fig. 2). Based on this, the subsequent annealing temperature was set to 30–35 °C.

3.2. Examination of agarose gel concentration

To visually distinguish *UGT1A1**28 by agarose gel electrophoresis, the amplicons, short DNA chains of 40 bp and 42 bp, must be sufficiently separated. Fortunately, high-resolution agarose gels are now commercially available. Primegel agarose LMT PCR-sieve GAT is considered suitable for separating short DNA fragments and was used. Also, to evaluate agarose gel resolution using constant DNA concentrations, a commercially available DNA ladder was used. The commercially available DNA ladder had bands every 20 bp. Additionally, amplicon mixtures of pA(TA)$_6$TAA and pA(TA)$_7$TAA (namely, a mixture of 40 bp and 42 bp fragments) were also electrophoresed. Using these DNAs, the optimal agarose gel concentration (2–5%) for the 40 bp and 60 bp band resolution was evaluated. Although higher agarose gel preparation concentrations (>6 %) are reported using an autoclave [14], the standard microwave oven method of melting the gel was used. Partial bubble removal was achieved using repeated dissolution–solidification in an Erlenmeyer flask. After 1 h of electrophoresis, separation of the 40 bp and 60 bp DNA ladder markers was observed for all concentrations (Fig. 3A, lanes **1**). Upon subjecting an amplicon mixture derived from pA(TA)$_6$TAA (40 bp) and pA(TA)$_7$TAA (42 bp) to electrophoresis, slight amplicon separation was observed using 4–5% agarose gel (Fig. 3A, lanes **2**). Subsequently, with prolonged

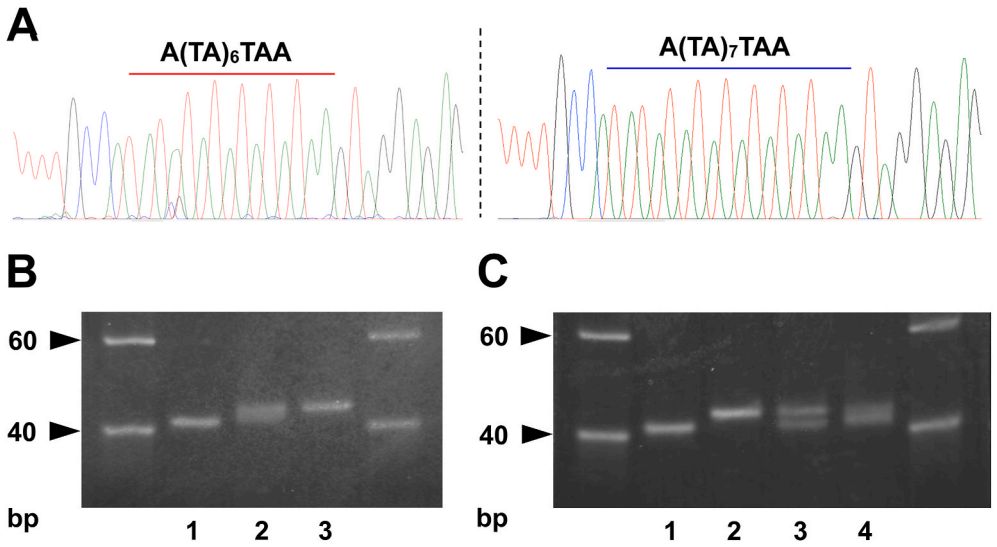

Fig. 4. DNA sequencing of control plasmids (A), agarose gel electrophoresis of amplicons obtained by PCR with control plasmids (B), and effect of temperature cycle on heterozygous-derived amplicon gel image (C).
(**A**) TA repeats of control plasmids were confirmed by DNA sequencing. The left is pA(TA)$_6$TAA and the right is pA(TA)$_7$TAA plasmid. (**B**) Lane **1** and **3** are PCR amplicons using pA(TA)$_6$TAA and pA(TA)$_7$TAA as templates. Both lanes are homozygote simulations. Lane **2** is the amplicon obtained by PCR using a mixture of pA(TA)$_6$TAA and pA(TA)$_7$TAA as template. This lane is a heterozygote simulation. Electrophoresis conditions are described above (*see materials and methods, section 5)*. (**C**) Lane **1** and **2** contain amplicons derived from pA(T)$_6$TAA and pA(TA)$_7$TAA, respectively. Lane **3** and **4** contain amplicons mixture of pA(TA)6TAA and pA(TA)7TAA. Lane 4 was loaded with an amplicon mixture that was heated at 95 °C for 30 s and then cooled before electrophoresis.

electrophoresis time (for 30 min) on a 5 % agarose gel, clearer amplicon separation was observed (Fig. 3B, lane **2**). This distinct separation was attributed not only to the high-resolution of agarose, but also buffer and voltage. The low concentration of 0.5 × TB buffer prevented diffusion of short DNA fragments within the gel by maintaining the temperature of the buffer and gel. Furthermore, the high voltage of 135 V shortened electrophoresis duration, completing the electrophoresis before the gel temperature increase [15]. In subsequent experiments, the following electrophoresis conditions were used: 5 % agarose concentration, buffer of 0.5 × TB, voltage of 135 V, and electrophoresis duration of 1.5 h (until xylene cyanol pigment in the DNA ladder marker reached the end of positive side). The pA(TA)$_6$TAA amplicon was observed slightly above the 40 bp marker. It was attributed to the action of Taq DNA polymerase. Taq polymerase adds an A residue to the 3′ end of newly synthesized DNA strands during PCR. Additionally, the DNA ladder manufacturer indicated that even between DNAs of identical base numbers, varying ratios of the four constituent bases may lead to slight band position variations. For these reasons, the pA(TA)$_7$TAA amplicon may also have been observed slightly higher than expected.

3.3. Separation of amplicons

Amplicons amplified by PCR using pA(TA)$_6$TAA and pA(TA)$_7$TAA were resolved on 5 % agarose gel (Fig. 4A, 4B and 4C). Furthermore, along with successful identification of each pA(TA)$_6$TAA and pA(TA)$_7$TAA amplicons, the heterozygous model amplicon, an equal mixture of pA(TA)$_6$TAA and pA(TA)$_7$TAA, was identified. This amplicon was observed between 40 bp and 42 bp (Fig. 4B). That homozygous model amplicons were observed as a broadened band, rather than two bands of 40 bp and 42 bp, is attributed to hybridization between the 40 base and 42 base strands during PCR temperature cycling. To examine this hypothesis, an amplicon mixture of pA(TA)$_6$TAA and pA(TA)$_7$TAA was subjected to one cycle of temperature cycling and analyzed by 5 % agarose gel electrophoresis. As a result, a broadened band was observed between the pA(TA)$_6$TAA and pA(TA)$_7$TAA amplicons (Fig. 4C). These results suggest that A(TA)$_6$TAA/A(TA)$_6$TAA homozygotes yield a band slightly above the 40 bp base pairs, A(TA)$_7$TAA/A(TA)$_7$TAA homozygotes yield a higher band, and A(TA)$_6$TAA/A(TA)$_7$TAA heterozygotes yield a broadened band between the A(TA)$_6$TAA and A (TA)$_7$TAA amplicons. To ensure consistency, the pA(TA)$_6$TAA and pA(TA)$_7$TAA amplicons must always be electrophoresed simultaneously as standards in adjacent lanes.

3.4. Implementation in healthy volunteers

To validate this *UGT1A1*28* detection method, 15 volunteers participated. From the collected saliva, genomic DNA was purified using a kit from Norgen. Despite possible storage of the collected saliva for long periods (≥3 months), purified genomic DNA only survives storage for 2–3 days under refrigeration. Therefore, genomic DNA was subjected to PCR immediately after purification. The obtained genomic DNA concentration was approximately 50–200 ng/μL. However, PCR using this genome DNA as a template yielded

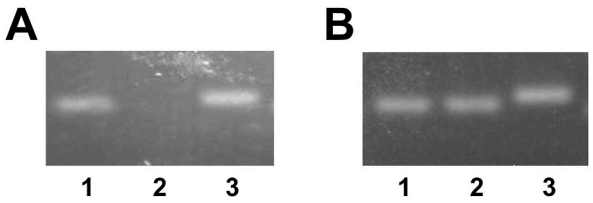

Fig. 5. Amplification using genomic DNA extracted from saliva as template.
(**A**) Lane **1** and **3** indicate amplicons derived from pA(TA)$_6$TAA and pA(TA)$_7$TAA, respectively. Lane **2** indicates PCR mixture using genomic DNA as template. (**B**) Lane **1** and **3** indicate amplicons derived from pA(TA)$_6$TAA and pA(TA)$_7$TAA, respectively. Lane **2** indicates PCR mixture using the PCR product for DNA sequencing as template (*see materials and methods, section **6***).

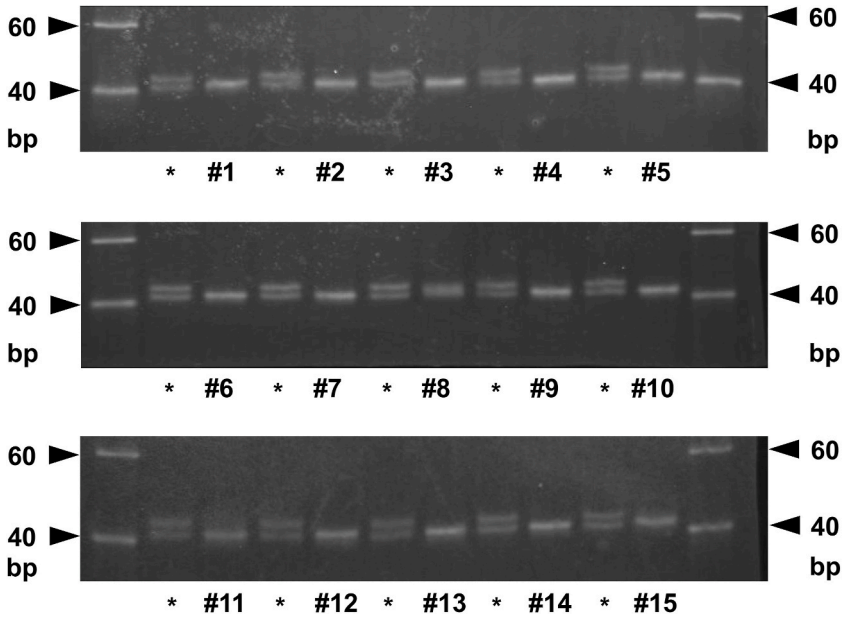

Fig. 6. Photo images of agarose gel electrophoresis on all subjects.
For convenience, subject numbers are shown (**#1**–**15**). PCR and electrophoresis conditions are described in *materials and methods, section **6***. To facilitate band size discrimination, controls were electrophoresed together. Control lanes are indicated with asterisk (*). The upper band in the control lane corresponds to the pA(TA)$_7$TAA amplicon, while the lower band corresponds to the pA(TA)$_6$TAA amplicon.

no visual confirmation of amplicons by agarose gel electrophoresis. In addition to the small amount of genomic DNA used in the PCR, it is thought amplification was inhibited due to poor primer hybridization with the template. Therefore, after long DNA strand amplification, short DNA fragment of amplification followed. This method is commonly referred to as "nested PCR". As described above (*see materials and methods section **6***), a DNA fragment approximately 540 bp was first amplified (*see materials and methods, section **7***) by PCR. This long DNA fragment contains the TA repeat sequence. Then, the second PCR uses the long DNA fragment as a template to amplify the short chain DNA. The reaction mixture was then purified and used as a template for internal TA repeat sequence amplification. The amplified short chain DNA was visually confirmed using agarose gel electrophoresis (Fig. 5).

Fifteen samples were analyzed using the developed method. Of these 13 were A(TA)$_6$TAA/A(TA)$_6$TAA homozygote (87 %) and 2 were A(TA)$_6$TAA/A(TA)$_7$TAA heterozygote (13 %, #8 and #15 in Fig. 6), while the A(TA)$_7$TAA/A(TA)$_7$TAA homozygote was not observed. These results are consistent with those obtained from DNA sequencing by Sanger's method. For example, the DNA sequences identified as A(TA)$_6$TAA/A(TA)$_6$TAA homozygote, #7 and #14, as well as those identified as A(TA)6TAA/A(TA)7TAA heterozygote, #8 and #15, are shown in Fig. 7. The genotype frequencies detected also appear consistent with the genotype frequency in healthy Japanese [16].

Thus, a new, inexpensive, and simple *UGT1A1*28* detection method requiring no special regents or equipment was successfully developed. As this detection method distinguishes a difference of two-bp, it is applicable not only to the *UGT1A1*28* detection but also potentially to other microsatellite mutations and insertion/deletion mutations.

4. Conclusion

A simple and straightforward *UGT1A1*28* detection method was developed. This method combines the nested PCR of short chain

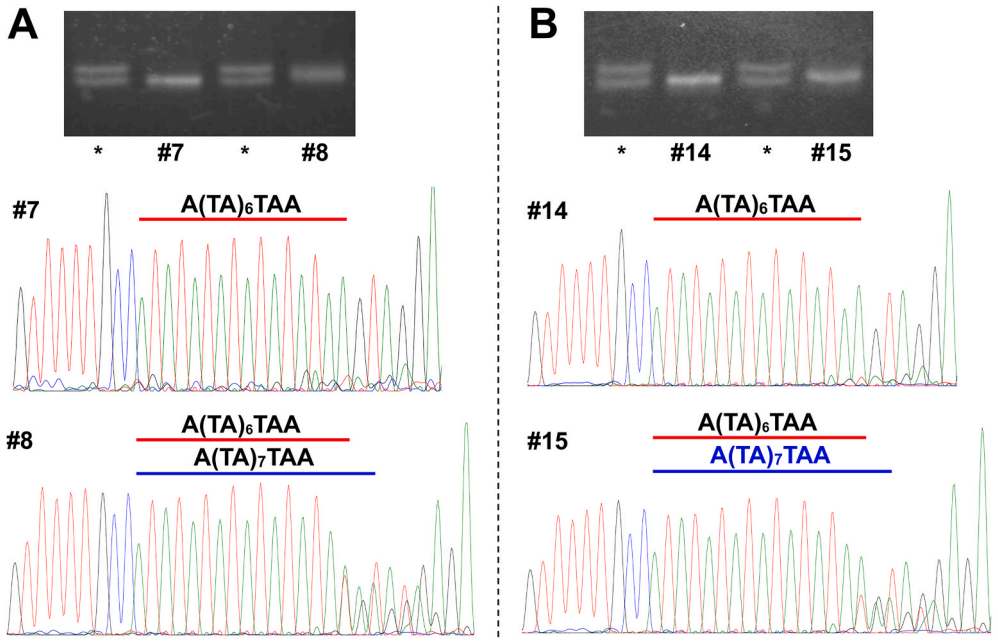

Fig. 7. Agarose gel electrophoresis profiles and DNA sequences.
Subject **#7** and **#8** were identified as $A(TA)_6AA/A(TA)_6TAA$ homozygous and $A(TA)_6TAA/A(TA)_7TAA$ heterozygous, respectively by DNA sequencing (**A**). And subject **#14** and **#15** were identified as $A(TA)_6AA/A(TA)_6TAA$ homozygous and $A(TA)_6TAA/A(TA)_7TAA$ heterozygous, respectively, by DNA sequencing (**B**). Agarose gel images are enlargements of Fig. 6. In agarose gel images, asterisk (*) indicates control lane. The upper band is $pA(TA)_7TAA$ amplicon and lower band is $pA(TA)_6TAA$ amplicon.

DNA with high-resolution agarose gel electrophoresis. Fifteen healthy volunteers were tested using this method, and the results were consistent with DNA sequencing. Overall, the method represents a significant improvement in terms of its applicability, ease of use, and cost-effectiveness.

5. Declaration

This study was reviewed and approved by the Ethics Committee of the Health Sciences University of Hokkaido, with the approval number: 23P002. All participants provided informed consent to participate in the study.

Data availability statement

Data will be made available on request from the corresponding author.

CRediT authorship contribution statement

Shirou Tsuchida: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Takaaki Hirayama:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Hayato Nunose:** Validation, Investigation, Formal analysis, Data curation. **Hinako Suzuki:** Validation, Data curation. **Ryo Hakota:** Validation. **Tsugumi Shindo:** Validation. **Koji Nakagawa:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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