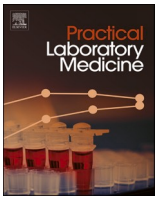
Practical Laboratory Medicine 33 (2023) e00306

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm



Development of a clinical microarray system for genetic analysis screening

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ARTICLE INFO

Keywords:
Genetic analysis
Microarray
Medical care
Screening test
Surface treatment

ABSTRACT

Objectives: Research on the relationship between diseases and genes and the advancement of genetic analysis technologies have made genetic testing in medical care possible. There are various methods for genetic testing, including PCR-based methods and next-generation sequencing; however, screening tests in clinical laboratories are becoming more diverse; therefore, novel measurement systems and equipment are required to meet the needs of each situation. In this study, we aimed to develop a novel microarray-based genetic analysis system that uses a Peltier element to overcome the issues of conventional microarrays, such as the long measurement time and high cost.
Methods: We constructed a microarray system to detect the UDP-glucuronosyltransferase gene polymorphisms *UGT1A1*6* and *UGT1A1*28* in patients eligible for irinotecan hydrochloride treatment for use in clinical laboratories. To evaluate the performance of the system, the hybridization temperature and reaction time were determined, and the results were compared with those obtained using a conventional hybridization oven.
Results: The hybridization temperature reached its target in 1/27th of the time required by the conventional system. We assessed 111 human clinical samples and found that our results agreed with those obtained using existing methods. The total time for the newly developed device was reduced by 85 min compared to that for existing methods, as the automated DNA microarray eliminates the time that existing methods spend on manual operation.
Conclusions: The surface treatment technology used in our system enables high-density and strong DNA fixation, allowing the construction of a measurement system suitable for clinical applications.

1. Introduction

Owing to research on the relationship between diseases and genes and the advancement of genetic analysis technologies, genetic testing has become an essential part of current medical care. In the USA, President Obama announced the Precision Medicine Initiative

Abbreviations: PCR, Polymerase chain reaction; UGT, UDP-glucuronosyltransferase; DLC, diamond-like carbon; CCD, charge coupled device; ctDNA, circulating tumor DNA; cfDNA, cell-free DNA.

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https://doi.org/10.1016/j.plabm.2022.e00306
Received 7 June 2022; Received in revised form 14 October 2022; Accepted 16 December 2022
Available online 23 December 2022

in 2015, and as a result, precision medicine has advanced. In precision medicine, preoperative or postoperative genetic testing can be used to stratify patients according to disease states and select appropriate drugs, enabling efficient delivery of personalized treatment. Postoperative monitoring can also be used to predict the possibility of recurrence and other prognostic factors. With the advancement of liquid biopsy genetic testing, preventive medicine is expected to have an impact in the future in terms of estimating the risk of morbidity and preventing diseases.

Various methods for genetic testing are available, such as PCR-based methods and next-generation sequencing; however, as screening tests in clinical laboratories are becoming more diverse, novel measurement systems and equipment are needed to meet their requirements. Microarrays are systems wherein multiple probes are immobilized on a chip for gene analysis [1–8], genetic testing [10–22], or protein analysis [9,37,42]. The use of microarrays in clinical applications has been extensively investigated [24–35]. However, it is difficult to take measurements rapidly and at a low cost using conventional devices. To solve this problem, we developed a novel genetic analysis system based on surface treatment technology. Surface treatment is a major factor determining microarray performance as it allows for immobilizing DNA at a high density without peeling. Various surface treatment technologies for immobilizing DNA and proteins have been developed and applied to DNA chips and other reagents in various life science fields.

When microarray technology was developed in the 1990s, substrates were coated with positively charged poly-L-lysine, and negatively charged DNA was electrostatically immobilized [1,3,36]. Currently, two major methods exist for immobilizing DNA: electrostatic binding and covalent binding [23,37–41]. In the electrostatic immobilization method, long strands of DNA with high charge density are easily immobilized, however, the efficiency of forming complementary target DNA strands is low as all of the genetic materials are adsorbed in the substrate surface. Because electrostatically fixed DNA is easily detached, substrate DNA is cross-linked to the polymer coated on the surface by ultraviolet irradiation. In the covalent binding method, which has been employed to coat substrate surfaces with aldehyde, amine, or carboxyl groups, immobilizing DNA at a high density is important, and the immobilized substrate DNA and target DNA in the liquid must be in close contact to enable the formation of double-stranded bonds. To increase the reaction efficiency, coating the substrate with dendritic polymers modified with functional groups has been investigated to achieve freedom of both oscillation and functional group density in solution [42].

Irinotecan hydrochloride is a prodrug hydrolyzed by carboxylesterases expressed *in vivo* to form the potent anticancer activator SN-38. SN-38G, a non-active metabolite with no anticancer activity, is released from the body [43,44]. SN-38 is known to cause leukopenia, neutropenia, and diarrhea as side effects. Homozygous or heterozygous genotypes of UDP-glucuronosyltransferase gene (*UGT1A1*) polymorphisms *UGT1A1*28* or *UGT1A1*6* result in reduced enzyme level and activity of *UGT1A1*, rendering it unable to inactivate SN-38. This is known to increase the incidence of adverse effects. *UGT1A1* is associated with the metabolism of anticancer drugs, and the clinical significance of measuring *UGT1A1*28* and *UGT1A1*6*, in particular, has been recognized. Therefore, in the present study, we aimed to construct a system to detect *UGT1A1*28* and *UGT1A1*6* in patients eligible for irinotecan hydrochloride treatment for use in clinical laboratories.

2. Materials and methods

2.1. Development of a novel microarray system

We developed a DNA chip of 3×3 mm^2 in size. In this DNA chip, one sample can be measured per DNA chip, and up to 64 different probes can be immobilized on a single DNA chip. To detect target genes, probes for the detection of wild-type and variants must be immobilized. Since at least two probes are required for each target gene (for detecting the wild type and variant DNA), a single DNA chip can detect up to 32 target genes. For the hybridization reaction between the DNA probe immobilized on the glass slide and the target DNA, an oven set at a uniform temperature is generally used; however, we used a Peltier device to enable hybridization using 10 μL of the sample. For detection, the measurement must be completed rapidly using a simple mechanism. To this end, we used a confocal laser system with a semiconductor laser for excitation and an optical amplifier tube for detection. This setup negatively affects the system by increasing its size; however, by using a DNA chip of only 3×3 mm^2 in size, detection could be performed using a charge-coupled device (BU-60, BITRAN Corp., Saitama, Japan). To evaluate the performance of the developed system, the hybridization temperature and reaction time were varied and compared with those in a conventional oven (Hybridization Incubator HB-100, TAITEC

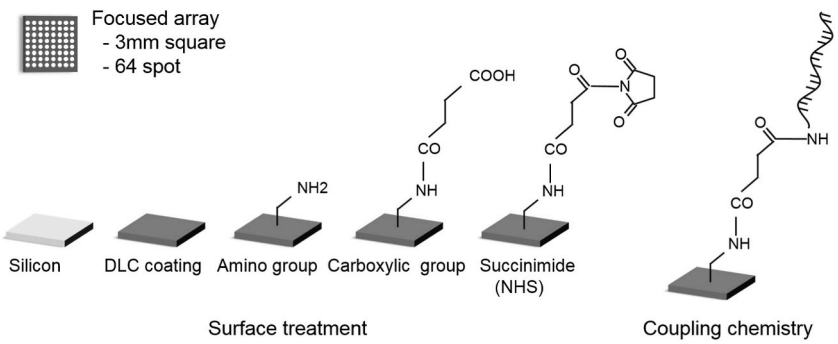

Fig. 1. Surface treatment of microarrays used for clinical diagnosis.

Corp., Koshigaya, Japan). The reaction time varied up to 60 min for the oven and up to 5 min for the Peltier device.

2.2. Microarray for UGT1A1 genotyping

Fig. 1 shows the microarray surface treatment used in systems for clinical diagnosis. We used a covalent surface treatment method that enables strong, high-density DNA fixation and efficient hybridization reactions [45]. High detection sensitivity is expected as oligonucleotides immobilized at a high density hybridize with the target DNA. In the method we used, diamond-like carbon (DLC) is deposited on a silicon substrate via ionization deposition, followed by coating with a polymer with amino groups and, subsequently, a polymer with carboxyl groups. The high-density functional groups are *N*-hydroxysuccinimide (NHS)-activated and covalently bound to the amino groups modified on the ends of the oligonucleotides for fixation.

We developed a chip microarray for the analysis of *UGT1A1* polymorphisms. The probes used are listed in Table 1. Human genomic DNA was amplified by PCR with a reaction volume of 32 μL (in a reaction tube; SARSTEDT, Nümbrecht, Germany) comprising 8 μL (1.25 ng/μL) human DNA as a template, 3.2 μL amplification buffer, 1.6 μL nucleotide mix, 1.6 μL (18 pmol/μL) *UGT1A1* primer mix, 0.32 μL amplification reagent, and 17.28 μL purified water. The primers used are listed in Table 1. The amplification was performed on a Mastercycler Pro S PCR system (Eppendorf, Hamburg, Germany) using the following thermal cycling condition: hold at 95 °C for 5 min, followed by 37 cycles at 95 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 30 s.

Next, 15 μL hybridization buffer was added to 30 μL of the PCR solution and gently mixed with a pipette. The mixture was spun down and placed in a genetic analyzer. The microarray was fixed using the instrument's needles, washing and rinse solutions were set in the instrument, and hybridization and detection were carried out. The system parameters were as follows: hybridization temperature, 54 °C; hybridization time, 60 min; and heat lid temperature, 80 °C. For washing, 0.01 × saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate was used, and the washing solution was shaken 200 times. For rinsing, 1 × SSC was used, and the rinsing solution was shaken 80 times. For detection, the exposure time was set to 2 and 5 s, and the output exposure time was set to 7 s.

The numerical data obtained from the measurements were saved as CSV files and analyzed using Excel (Microsoft, Washington, USA). The background was taken as the average value of the top two center points (without the probe). The signal-to-noise (S/N) ratio, fluorescence intensity value, and genotype determination value were calculated by substituting the fluorescence intensity and background into the following equations:

$$\mathrm{S}\Big/\mathrm{N\ ratio} = \frac{Fluorescence\ intensity}{Background},$$

$$\mathrm{Fluorescence\ intensity\ value} = \mathrm{Fluorescence\ intensity} - \mathrm{Background},$$

$$\mathrm{Genotype\ determination\ value} = \frac{(Average\ of\ variant\ probe\ fluorescence\ intensity\ values) \times 2}{(Average\ of\ wild\ type\ probe\ fluorescence\ intensity\ values) + (Average\ of\ variant\ probe\ fluorescence\ intensity\ values)}.$$

2.3. Temperature settings for UGT1A1 genotyping

To set an optimal hybridization temperature, eight human samples were assessed at hybridization temperatures of 52–56 °C, and the genotype determination values and S/N ratios were calculated. Appropriate hybridization temperatures were set based on the genotype determination values and S/N ratios. Human DNA samples were obtained with informed consent from the patients in the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine [46].

Table 1
Sequences of the primers and probes used to detect the *UGT1A1*28* and *UGT1A1*6* polymorphisms using the DNA microarray.

Name	Sequence (5' to 3')
Primers	
*UGT1A1*28*-S	TAGTCGTCCTTCTTCCTCTCTGGT
*UGT1A1*28*-AS	IC5-ATGGCGCCTTTGCTCCT
*UGT1A1*6*-S	IC5-CCATGCTGGGAAGATACTGTTGA
*UGT1A1*6*-AS	GATCACACGCTGCAGGAAAG
Probes	
*UGT1A1*28*-TA5	CCCCCCCCTTTTTGCCATATATATATATAAGTAGGA
*UGT1A1*28*-TA6	CCCCCCCCTTTTTGCCATATATATATATATAAGTAGGA
*UGT1A1*28*-TA7	CCCCCCCCGTTTTTGCCATATATATATATATATAAGTAGG
*UGT1A1*28*-TA8	CCCCCCCCGTTTTTGCCATATATATATATATATATAAGTAGGA
*UGT1A1*6*-211G	TAAAATGCTCCGTCTCTGATG
*UGT1A1*6*–211A	TAAAATGCTCTGTCTCTGATGT

2.4. Threshold settings and comparison with an existing method for UGT1A1 genotyping

Threshold values were set based on the mean and standard deviation of genotype determination values for each genotype calculated from 22 human specimens. Then, genotype determination values were calculated using the developed genetic analyzer for 111 human specimens that had already been examined using the existing Invader *UGT1A1* Molecular Assay (Sekisui Medical, Tokyo, Japan), and the genotypes were determined based on the genotype determination thresholds that were set. The results were compared with those obtained using the Invader assay.

3. Results

3.1. Development of the novel microarray system

The microarray system used for genetic analysis consisted of a 3×3 mm^2 DNA chip with 64 probes immobilized on its surface that was fixed to special needles for analysis. Eight needles capable of fixing four chips can be mounted simultaneously in the automated detection system. Therefore, the developed device can be set with 32 DNA chips and can measure 32 samples simultaneously. The device is approximately 70 cm wide, 30 cm high, and 30 cm deep and is connected to a PC for control. The output data were analyzed using the "BIOSHOT" software (Toyo Kohan, Tokyo, Japan) dedicated to the developed device. After PCR of the template DNA sample, the hybridization solution is added to the amplification product to prepare the reaction solution for hybridization. The hybridization reaction is performed by immersing the DNA chip in the reaction solution, and fluorescence images are acquired using a CCD camera after the DNA chip is washed. The fluorescence intensities of all the reacted probes are calculated from the obtained fluorescence images, and the presence or absence of mutations is judged from the genotype determination value.

Fig. 2 compares the heating temperatures for the conventional oven and analyzer developed in this study. As shown in the figure, when using the conventional oven, it took approximately 40 min for the temperature to reach a plateau and approximately 40 min for the solution to reach the set temperature. In contrast, in the system developed in this study, it took approximately 1.5 min for the Peltier element to reach a constant temperature and approximately 1.5 min for the solution to reach the set temperature. Thus, the hybridization temperature reached the target temperature in 1/27th of the time required by the conventional system.

3.2. Temperature settings for UGT1A1 genotyping

The results of the optimal hybridization temperature determination are shown in Fig. 3. The genotype determination value of *UGT1A1*28* increased with increasing hybridization temperature for the homo- and heterozygous genotypes and increased slightly for the wild type. The genotype determination values for the homo- and heterozygous *UGT1A1*6* were similar, whereas the wild type genotype determination value decreased as the temperature increased. The values were sufficiently separated at all temperatures. S/N ratio measurements indicated that the optimum hybridization temperature was 54 °C as genotype determination values for both *UGT1A1*28* and *UGT1A1*6* tended to decrease at temperatures below 52 °C or above 56 °C.

3.3. Threshold settings and comparison with an existing method for UGT1A1 genotyping

The mean and standard deviation of the genotype determination values for homo- and heterozygous genotypes of *UGT1A1*28* and *UGT1A1*6* were calculated. The mean values ± 3σ (error) for each genotype are shown in Fig. 4. We considered that it was possible to set the threshold values in the range where the genotype determination values did not overlap, considering that they were sufficiently separated. The threshold values set based on the measurement results are listed in Table 2.

Fluorescence images representative of the 111 human specimens analyzed using the system developed in this study are shown in Fig. 5, and the genotype determination values are shown in Fig. 6. All genotype determination values were sufficiently separated, and it was possible to identify the genotypes from the obtained values. The results for the 111 specimens obtained using the set threshold values are shown in Table 3. Two specimens that did not meet the determination threshold were re-assessed using PCR. The results

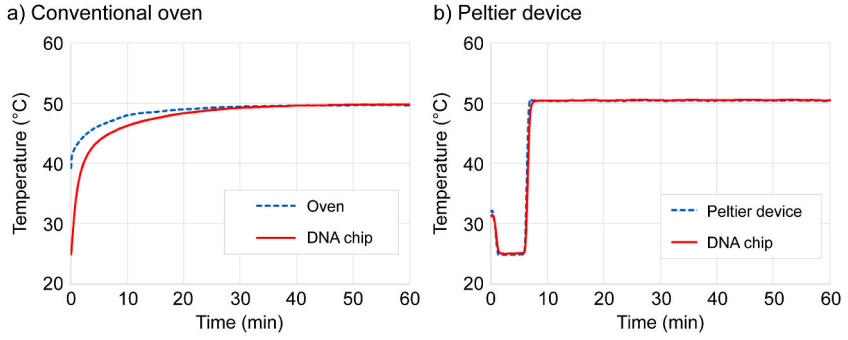

Fig. 2. Comparison of heating time between (A) conventional oven and (B) our DNA microarray system using a Peltier device.

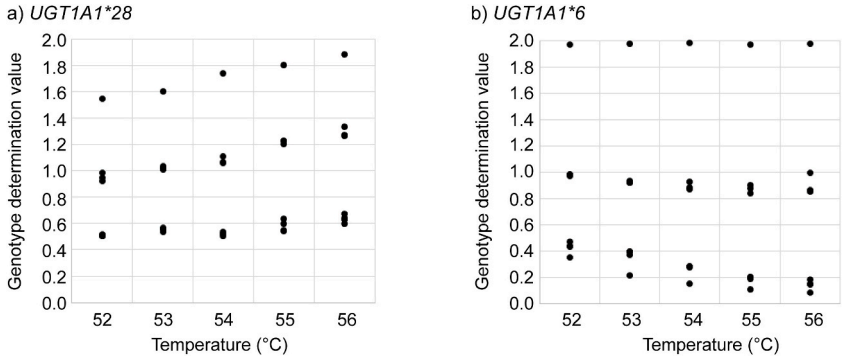

Fig. 3. Relationship between hybridization temperature and genotype determination value.

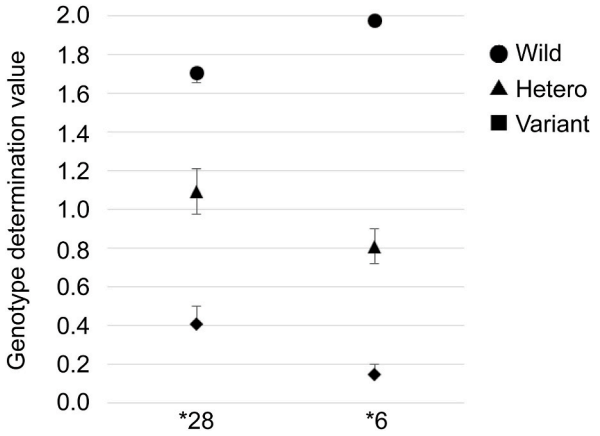

Fig. 4. Mean genotype determination value.

Table 2
Threshold values for genotyping.

UGT1A1*28		UGT1A1*6	
TA6/TA6	0.000–0.706	G/G	0.000–0.327
TA6/TA7	0.801–1.478	G/A	0.437–1.157
TA7/TA7	1.579–2.000	A/A	1.792–2.000

Wild type *UGT1A1*28* contains six TA repeats (TA6), and variants contain seven TA repeats (TA7).
Wild type *UGT1A1*6* contains a G allele (G), and variants contain an A allele (A).

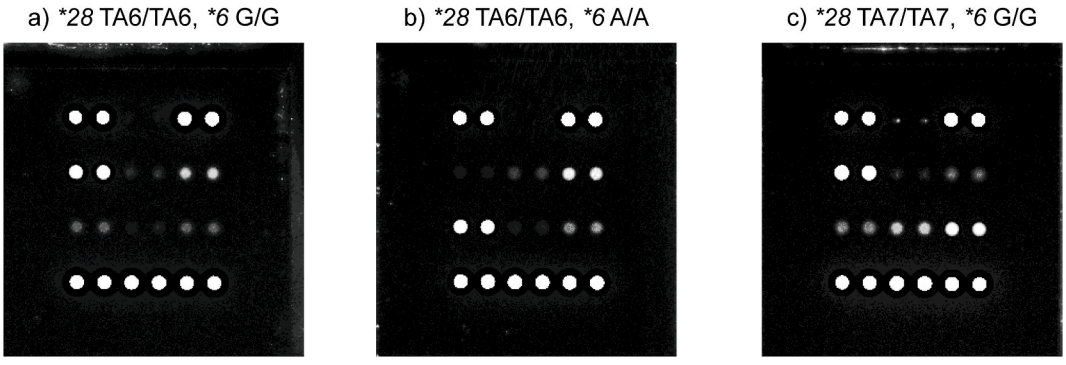

Fig. 5. Representative fluorescence images.

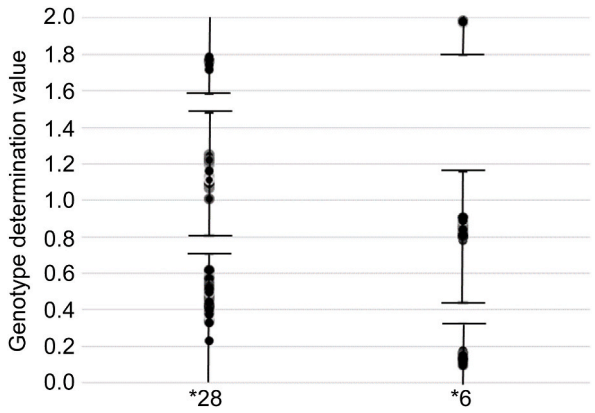

Fig. 6. Genotype determination values of measurements using human DNA samples (111 samples, error bars indicate threshold values).

Table 3
Results for the 111 specimens using the DNA microarray and Invader systems.

DNA microarray system						Invader system					
*UGT1A1*28*			*UGT1A1*6*			*UGT1A1*28*			*UGT1A1*6*		
TA6/TA6	TA6/TA7	TA7/TA7	G/G	G/A	A/A	TA6/TA6	TA6/TA7	TA7/TA7	G/G	G/A	A/A
85	19	7	72	33	6	85	19	7	72	33	6

Wild type UGT1A1*28 contains six TA repeats (TA6), and variants contain seven TA repeats (TA7).
Wild type UGT1A1*6 contains a G allele (G), and variants contain an A allele (A).

indicated that for all 111 specimens, the results agreed with those obtained using the Invader *UGT1A1* Molecular Assay. The 111 specimens showed good agreement, indicating that the automated DNA microarray system achieved satisfactory genotype determination performance. Furthermore, the time requirements for the newly developed device was lower (165 min) than that for the Invader assay (250 min).

4. Discussion

Surface treatment of the microarray substrate affects the final detection efficiency. In this study, we developed a microarray system by modifying the surface treatment, which will be useful for clinical genetic analyses. Our surface treatment involved modifying the high-density functional groups on the DLC coating. The high-density functional groups were NHS-activated and covalently bonded to the amino groups modified on the ends of the oligonucleotides for fixation. The use of this surface treatment in clinical genetic analyses is novel, and using this novel system, high detection sensitivity is expected as the oligonucleotides immobilized at high density hybridize with the target DNA [47,48]. The electric double layer on the immobilized surface forms an obstacle to hybridization to a solid surface, and diffusion by convection is an important factor for efficient hybridization [49–52]. Convection currents generated in a closed container break down this barrier layer, which is thought to enable short reaction times. In addition, using a limited number of reaction vessels to ensure a short and stable reaction time is advantageous in microarray hybridization.

The developed device eliminates the need for conventional manual operation related to washing and detection after hybridization. In addition, the reaction can be completed rapidly as a Peltier device is used for hybridization. Peltier devices can heat up more rapidly than conventional ovens, in which the temperature of the air inside the oven increases gradually during heating. Therefore, the container holding the chips requires a long time to increase its temperature. In our system, the tube containing the solution is in direct contact with the Peltier device, and we speculate that the direct contact with the heat source caused the solution to heat rapidly. To our knowledge, a Peltier device has never been used in microarray hybridization and is considered an effective tool for completing the reaction rapidly.

The Tm of the fixed probes and target amplified material affected the genotype determination values when using our device. The best separation was achieved at 54 °C. The optimal temperature depends on the type of probe to be fixed and should be considered on a case-by-case basis [53]. Achieving sufficient separation (of 3σ or more) is necessary for accurate genotype determinations. We believe our system achieved sufficient separation because the reaction was carried out stably, and the variation in the genotype determination value was small. The use of an automated system also contributed to the stability of the reaction conditions.

In fluorescence imaging, the background should be sufficiently low, and the fluorescence intensity of each spot should be sufficient to allow for accurate measurements [54–56]. In our system, stray light does not enter the system, and autofluorescence of the microarray substrate is low; therefore, we can say that our system can accurately detect weak but almost pure fluorescence of each spot.

The genotype determination values obtained using our method were assessed for correlation and were confirmed to be within the set thresholds; this demonstrates that the genetic analysis is reliable. Although there may be interfering factors in actual samples, such as fragmentation and contamination by foreign substances, which lead to uncertain measurements, the system developed in this study could effectively genotype samples without being affected by such factors [57].

The developed device showed similar detection performance to an existing method, one of the reasons being that a large number of oligonucleotide probes immobilized on the microarray substrate resulted in sufficient fluorescence intensity and S/N ratio. This allowed us to obtain a separable signal with sufficient dynamic range. Furthermore, the automated system stabilized the hybridization temperature, enabling a short reaction time.

The *UGT1A1* gene test kit using the DNA chip method reported here has been approved as *an in vitro* diagnostic reagent in Japan. The *UGT1A1* gene test kit using the Invader method, which is being compared, is also available in Japan as an *in vitro* diagnostic test reagent. Below, we discussed a comparative account of these two methods, particularly the operation time required and throughput.

The time required to perform the DNA chip method is 85 min shorter than that required using the invader method. Briefly, the DNA chip method requires a total of 165 min: 90 min for PCR and 75 min for hybridization and washing. On the contrary, the Invader method requires a total of 250 min: 5 min for denaturing, 240 min for incubation, and 5 min for detection. Furthermore, the actual measurement requires time for the manual preparation of reagents in addition to the time described above. In clinical practice, it is assumed that 96 samples, which is equivalent to one PCR plate, are measured in a day. Assuming that work is performed for 96 samples, 90 min is required for the Invader method and 55 min for the DNA chip method; therefore, the time required for reagent preparation is 35 min shorter for the DNA chip method than that for the Invader method.

To compare throughput, we compared the time required to measure 96 samples, which is the equivalent of one PCR plate, assuming actual operation. Since the Invader method can measure 48 samples simultaneously, two runs are required to measure 96 samples, which would require 500 min in total. In contrast, the DNA chip method can perform PCR for 96 samples simultaneously, so PCR measurements for 96 samples can be completed in 90 min. After PCR, it requires three rounds of hybridization (60 min each) and washing (15 min each); therefore, the total time required for 96 samples is 315 min, which is 185 min shorter than the time required by the Invader method for the same number of samples. Furthermore, 96 specimens can be assayed by the DNA chip method within 8 h, the standard length of a workday, making it advantageous in actual clinical practice.

Although there is no difference in clinical outcomes by reducing the testing time to 85 min, as discussed above, it is advantageous for use in a clinical setting because it reduces work time and increases throughput. For laboratories that perform genetic testing, the DNA chip method is more suitable for clinical laboratory than the Invader method because shortening work time and improving throughput are the key points that can lead to improved testing efficiency and lower costs. In addition, in the DNA chips, it is easy to mount additional probes that may be clinically necessary when research on clinical significance is advanced. The measurement process and time remain the same even when new probes are added, so the DNA chip method will be even more advantageous when the number of test targets increases. If future research reveals the clinical significance of other genetic polymorphisms, we plan to incorporate them into the DNA chip.

The DNA chip method described in this report is effective as a platform for *in vitro* diagnostics for clinical testing when the target gene is determined and has been approved as an *in vitro* diagnostic in Japan.

We believe the features of the DNA chip, combined with additional new technologies, will offer significant advantages to improve the currently available platforms. Currently, we are developing a new platform that combines DNA chips with microfluidic technology and examining systems that can detect circulating tumor DNA (ctDNA) and cell-free DNA (cfDNA). If it becomes possible to easily measure multiple targets such as ctDNA and cfDNAin blood, it will contribute to early cancer detection by liquid biopsy.

5. Conclusion

A microarray system suitable for clinical use was developed using a surface treatment that enables high-density and strong DNA fixation. Using the assay system for the *UGT1A1*28* and *UGT1A1*6* polymorphisms, the optimal hybridization temperature and thresholds for genotype determination were set. In a performance evaluation using 111 samples, the results of the present device were comparable to those of an existing assay. Therefore, we propose that the system developed in this study is suitable for genetic testing in clinical laboratories.

Author contribution

Hiroshi Okamura: Conceptualization, Methodology, Formal analysis, Writing - Original Draft. Hirofumi Yamano: Conceptualization, Methodology. Toshiya Tsuda: Methodology. Junichi Morihiro: Methodology. Koichi Hirayama: Methodology, Formal analysis, Validation. Hiroaki Nagano: Conceptualization, Methodology.

Funding

The research was partially funded by Yamaguchi Prefecture's "Grant-in-Aid for Yamaguchi Industrial and Strategic Research and Development" project.

Ethical approval

Not applicable.

Informed consent

Human DNA samples were obtained with informed consent from the patients in the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine.

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.

Data availability

Data will be made available on request.

Acknowledgments

We are deeply grateful to Dr. Akiyoshi Fukamizu, Dr. Shoichi Hazama, and Dr. Ryouichi Tsunedomi for their technical advice. We would like to thank Editage (www.editage.com) for English language editing.

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