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Quality Control Probes for Spot-Uniformity and Quantitative Analysis of Oligonucleotide Array

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Quality control QC for spot-uniformity is a critical point in fabricating an oligonucleotide array, and quantification of targets is very important in array analysis. We developed two new types of QC probes as a means of confirming the quality of the uniformity of attached probes and the quantification of targets. We compared the signal intensities and fluorescent images of the QC and target-specific probes of arrays containing only target-specific probes and those containing both QC and target-specific probes. In a comparison of quality control methods, it was found that the arrays containing QC probes could check spot-uniformity or spot defects during all processes of array fabrication, including after spotting, after washing, and after hybridization. In a comparison of quantification results, the array fabricated by the method using QC probes showed linear and regular results because it was possible to normalize variations in spot size and morphology and amount of attached probe. This method could avoid errors originating in probe concentration and spot morphology because it could be normalized by QC probes. There were significant differences in the signal intensities of all mixtures ($P<0.05$). This result indicates that the method using QC probes is more useful than the ordinary method for quantification of mixed target. In the quantification of mixed targets, this method could determine a range for mixed targets of various amounts. Our results suggest that methods using QC probes for array fabrication are very useful to the quality control of spots in the fabrication processes of quantitative oligonucleotide arrays.

Keywords: Quality control probe, oligonucleotide array, spot-uniformity, quantitative analysis

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Oligonucleotide array technology is an ideal tool for expression profiling, genotyping, DNA sequencing, diagnostics, immunology, drug discovery, and drug development [6]. The array process flow is highly variable and is still evolving [19]. Moreover, the large number of array elements makes it impossible to examine each array process manually. Because of these reasons, quality control is an important factor in array fabrication [7]. Quality control QC of spots on a glass surface is important in order to assess the surface quality, integrity, and homogeneity of each spot [14, 15]. There are several features that affect the quality of a spot on an array: signal intensity, spot size, and morphology. There are several experimental factors that may cause low signal intensities: a low amount of DNA (or oligonucleotide) in the spot, the molecular and physical compositions of the spot (the purity of the DNA or oligonucleotide, attachment to the glass, and availability to hybridization, *etc.*), uneven or incomplete hybridization, among others [11]. The spot size defects include spot coalescence, small spots, large spots, and missing spots. Various-sized spots can be caused by debris in the spotting solution, a spotting pin that does not make sufficient contact with the glass surface, damaged or dirty pins, and high humidity during spotting, among other causes [11, 16, 19]. In spot morphology, defects include ringing or doughnuts and bulls-eyes effects, noncircular spots, and other irregular shapes [16, 20].

In order to obtain accurate and reproducible array results, the quality of the array in various respects, such as printing, hybridization condition, and image analysis, must be evaluated [2, 11, 20]. Several methods of evaluating the quality of array spots have been reported [12, 19]. Staining methods using dye, such as ethidium bromide, OliGreen, SYBR Green II, and SYTO 61, can assess spot quality after hybridization. However, these methods require the time-consuming steps of incubation, staining, and dye-removing. Furthermore, the assayed slides are not available for subsequent

hybridization, because fluorescent dye can remain on the slide surface. Therefore, only one or a few slides (mainly the first and last slides of a printed batch) can be quality assessed [12, 13, 19]. Recently, several reports have introduced QC methods that can confirm spot-uniformity before hybridization. There is a method using a spotting solution containing a fluorescence dye [19], and another method using hybridization by spotted QC probe and fluorescently labeled QC product [7, 18, 21]. The former method provides information on probe retention on the slide prior to hybridization, but the latter method can provide information on a probe after hybridization. However, neither of these methods simultaneously provides spot-uniformity before and after hybridization and quantitative assessment of a bound probe on the slide. Quantification for mixed targets is important to determine which one is dominant (or that they are of equal amounts), especially in clinical diagnosis [10]. The purpose of this study was to develop new quality control methods that enable an effective evaluation of the quality of oligonucleotide arrays and quantification of the mixed target in the sample.

In this study, we developed new types of QC probes as a means of visualizing probe morphology and quantifying probe retention on all slides before and after hybridization. We compared the results of evaluation for spot-uniformity and quantification for mixed target with the ordinary method and the methods using QC probes.

MATERIALS AND METHODS

Design of Target-Specific and Quality Control Probes

The target DNA used for hybridization of an HBV polymerase gene was generated by a nested PCR. A total of 5 target-specific probes (wild type of rtM204 and mutants of rtM204V, rtM204I1, rtM204I2, and rtM204I3), which were based on the HBV polymerase gene, were designed to detect lamivudine-resistant HBV. The target-specific probes have been described earlier [14]. We prepared two kinds of QC probes. Fig. 1 describes the designs and working principles of QC probes. The array fabricated by the ordinary method contained only target-specific probes (Fig. 1A). The T20-F probe was composed of 20-mer polyT labeled by TAMRA at the 3' end. This was mixed with target-specific probes. The target-specific probes and the T20-F probe were spotted on the slide at the same time (Fig. 1B). Then, END-F probes were labeled by TAMRA at the 3' end of target-specific probes. These probes were used simultaneously as QC and target-specific probes (Fig. 1B). Two kinds of QC probes were labeled at the 3' end by TAMRA and were modified by an aminolink group at the 5' end. Five kinds of END-F probes were designed with reference to Jang *et al.* [14]: END-rtM204, 5'-spacer-CAGTTA TATGGATGA-TMARA-3'; END-rtM204V, 5'-spacer-CAGTTATGTGGATGA-TAMRA-3'; END-rtM204I1, 5'-spacer-CAGTTATATAGATGATG-TAMRA-3'; END-rtM204I2, 5'-spacer-CAGTTATATCGATGATG-TAMRA-3'; and END-rtM204I3, 5'-spacer-CAGTTATATTGATGATG-TAMRA-3'.

Target DNA Preparation

We used the YMDD motif (codon 204) of the reverse transcriptase region of the polymerase gene connected with lamivudine-resistant

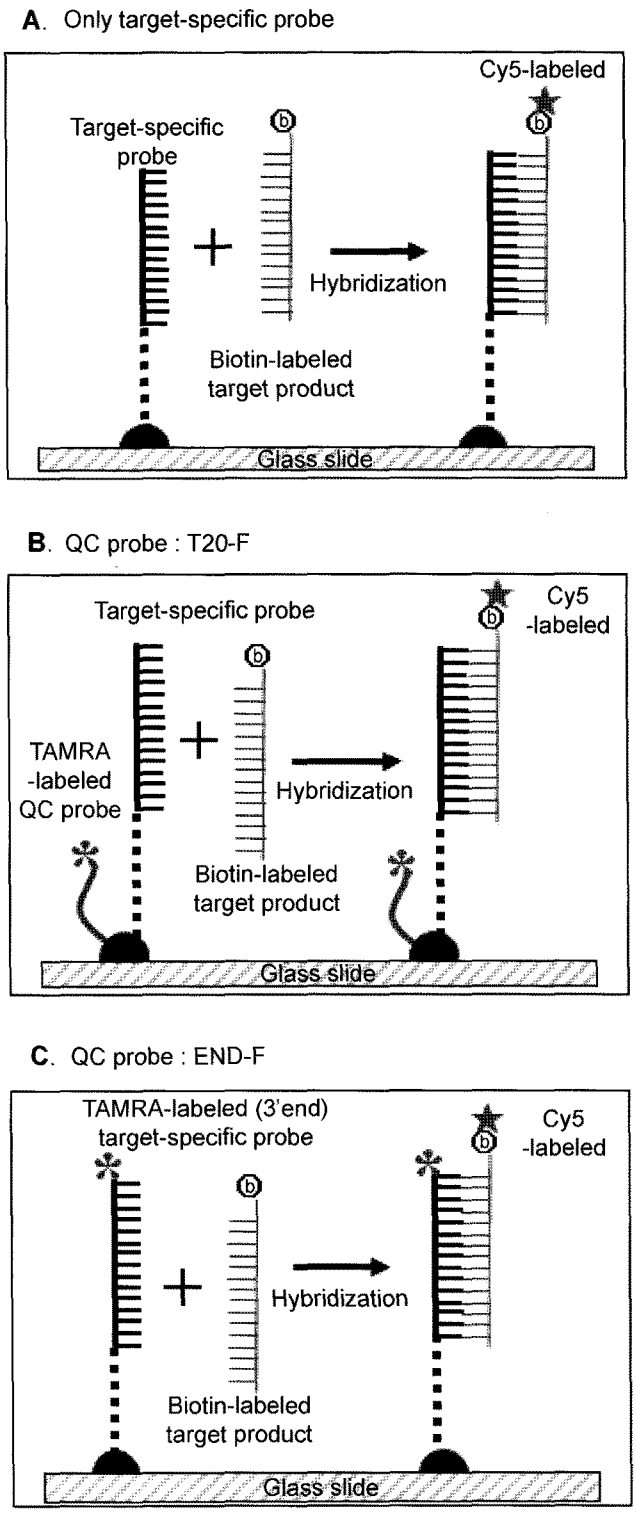

Fig. 1. Designs and working principles of QC probes.
A. The ordinary method was spotted only target-specific probe without QC probes. **B** and **C**, Two kinds of QC probes were labeled at the 3' end by TAMRA and were modified by an aminolink group at the 5' end. Two QC probes could be detected during all processes of array after spotting. **B.** The T20-F probe was mixed with target-specific probe. **C.** The END-F probes were performed as QC probes and target-specific probes. Asterisk, TAMRA; b, biotin; star-shape, Cy5 fluorescence.

HBV as the target for the array test. The rtM204 indicated the wild type (methionine, ATG) at codon 204 and the rtM204V indicated the mutant type (valine, GTG) at codon 204. The target DNA of clones rtM204 and rtM204V used for hybridization was generated by mutagenesis. Target DNA amplification was accomplished using the biotin-labeled primers (bio-HBF2, 5'-biotin-CCATCATCTTGGG CTTTCGC-3', and bio-HBR2, 5'-biotin-CAAAAGAAAATTGGTA ACAGCGGTA-3') according to Jang *et al.* [14]. The site-directed mutagenesis method and the PCR condition have already been described [14].

Fabrication and Quality Control of Arrays

Fabrication of arrays. To evaluate the QC probes in the array, we fabricated three kinds of arrays. The array was fabricated with 50 μM of target-specific probe (without QC probes) in 3× SSC (450 mM NaCl, 45 mM Na-citrate, pH 7.0). The array using the T20-F probe was fabricated by spotting a mixture containing 50 μM of a target-specific probe and a TAMRA-labeled QC probe in 3× SSC. The array using the END-F probe was fabricated with 50 μM of END-F probe in 3× SSC. The probes were printed in duplicate, as shown in Fig. 3. The hybridization and scanning processes of the oligonucleotide array have been described previously [14, 15].

Quality control of arrays. The array images were taken by the nonconfocal fluorescent scanner GenePix 4000A (Axon Instruments, U.S.A.) at wavelengths of 532 nm and 635 nm for TAMRA and Cy5, respectively. As shown in Fig. 3A, fluorescent images from a QC probe were obtained at 532 nm and those from specific probes were obtained at 635 nm.

Quantification by Signal Intensity Ratio

The quantification of the mixed samples was based on the normalization of the QC probes as follows. First, we normalized the signal intensity of the QC probes. By using the QC probe, it was possible to normalize variations in slide-to-slide, spot-to-spot, and hybridization efficiencies. Second, we multiplied the signal intensity of the target-specific probes by the normalized ratios of the QC probe for each spot. Subsequently, the ratio values (rtM204V/rtM204) were calculated from the wild-type and mutant probes of each case. The standard deviations reflected slide-to-slide and spot-to-spot variations. To compare the more precise results of quantification and to test the reproducibility of our method, experiments were performed 16 times for each mixed target. Since each array contained two replicate spots for each target-specific probe, the average values for the hybridization signal ratios were obtained from 32 spots.

Statistical Analysis

Statistical analysis was done with the SPSS ver.11.0 for Windows software (SPSS Inc., Chicago, IL, U.S.A.). The significance of

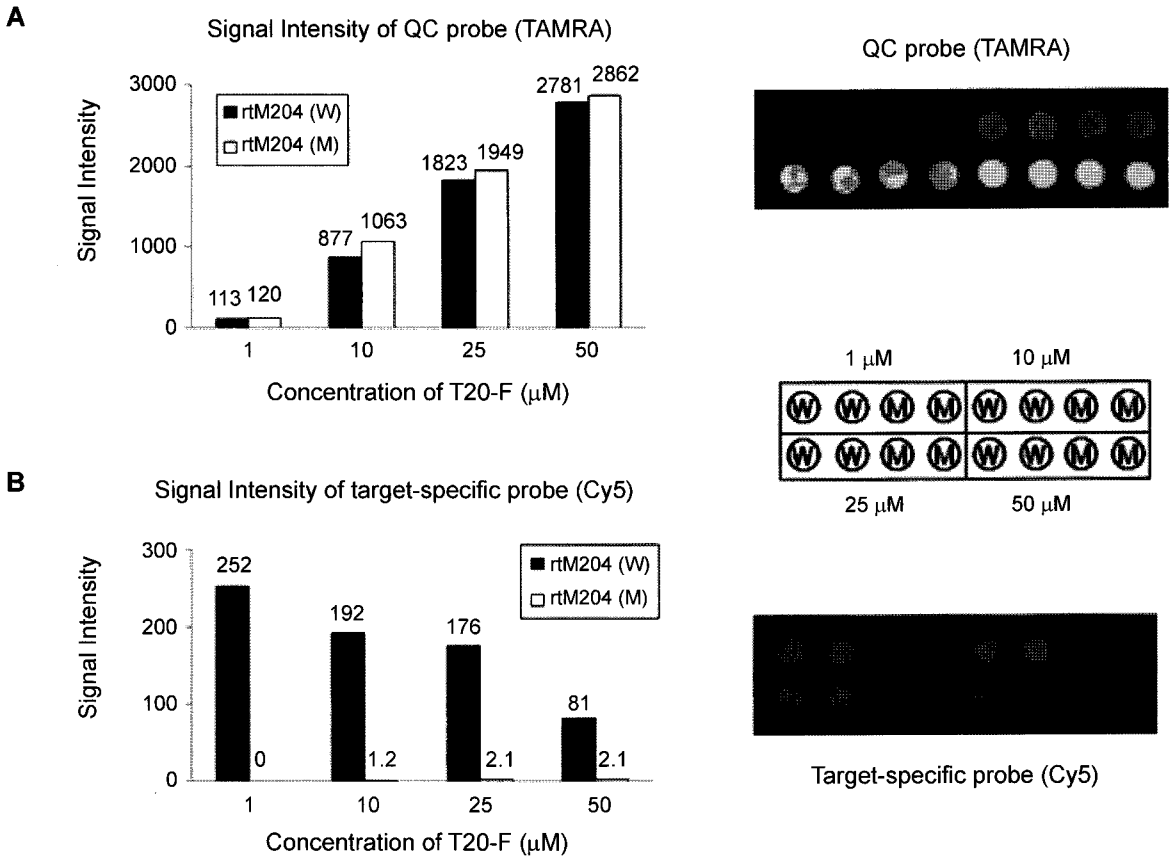

Fig. 2. Determination of optimal concentration of T20-F probe.
Four concentrations of T20-F probes (1, 10, 25, and 50 μM) were mixed with 50 μM of target-specific probe. **A.** The graph of the signal intensity and the fluorescent image were obtained by T20-F probe for wild type and mutant after hybridization. **B.** The graph of the signal intensity and the fluorescent image were obtained by Cy5-labeled target-specific probe for wild type and mutant after hybridization.

differences among three kinds of arrays for the hybridization signal intensity of each mixed type was analyzed using the Kruskal-Wallis test. The significance of differences between two kinds of arrays for the hybridization signal intensity of each mixed type was analyzed using the Mann-Whitney U test. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Optimization of T20-F Probe Concentration

To evaluate the influence of the T20-F probe on target-specific hybridization, we tested various concentrations (1, 10, 25, and 50 μM) of T20-F probe (Fig. 2). To confirm the reproducibility of our method, the experiments for each array were performed three times. The signal intensity of the T20-F probe was detectable at concentrations of 10, 25, and 50 μM, but was not detected at 1 μM after hybridization. The signal intensity for target-specific probes was detectable at all concentrations. Increasing concentrations of the T20-F probe showed a reduced intensity of the Cy5 signal according to target-specific hybridization. In the case of the rtM204 probe, increasing the T20-F probe concentration increased the T20-F probe intensity but decreased the Cy5 intensity of the target-specific hybridization. We optimized the QC probe concentration for target-specific probes. For 50 μM of the target-specific probe, the optimal concentration of the T20-F probe was confirmed to be 10 μM. For the array containing 10 μM of the T20-F probe, the signal intensity for the target-specific probe was high and the signal of the T20-F probe was detectable. Based on a previous array assay [4], the END-F probe was used at the optimal concentration of 50 μM, because it was labeled by TAMRA at the 3' end of the target-specific probe.

Comparison of Quality Control Methods in Array Fabrication Using QC Probes

The spot quality of the array by the ordinary method containing only target-specific probes without QC probes and the arrays containing two kinds of QC probes were analyzed. The arrays were evaluated for spot-uniformity immediately after spotting, after washing, and after hybridization (Fig. 3). Before the hybridization experiment, we could evaluate the spot-uniformity of the target-specific probes with T20-F and END-F probes. However, for the array containing only target-specific probes, no images could be shown until hybridization. After hybridization, the array containing only target-specific probes could not be evaluated

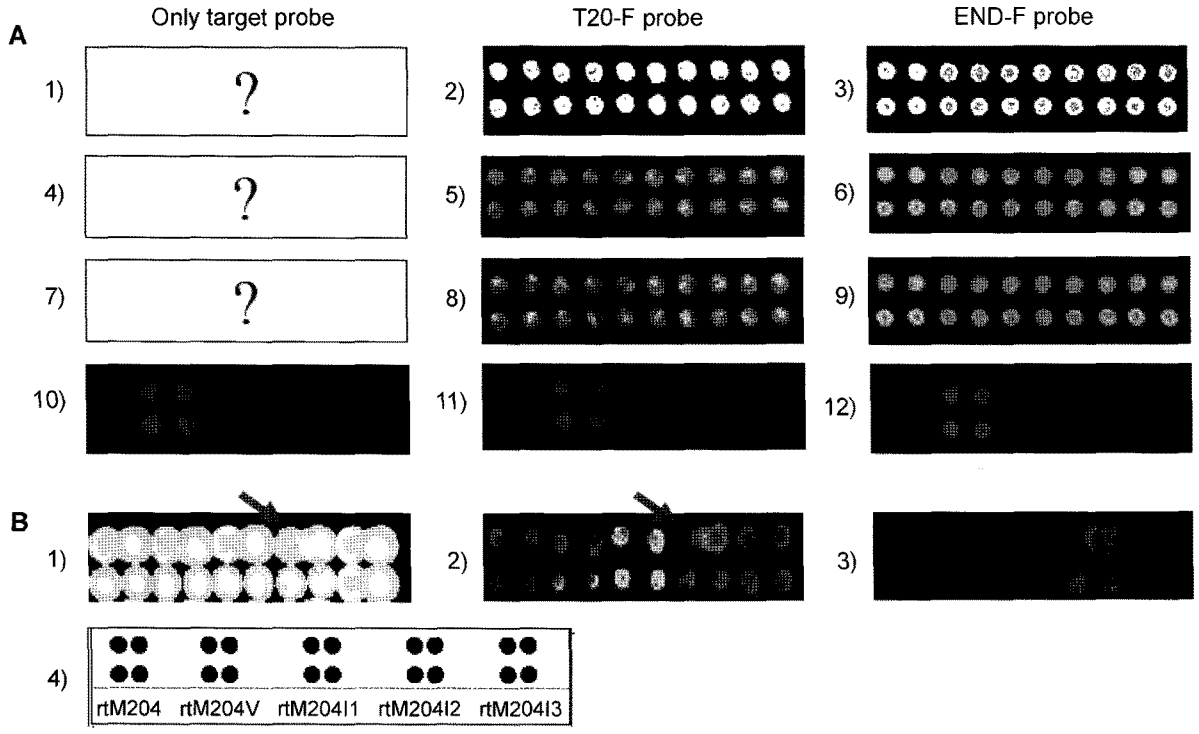

Fig. 3. Comparison of quality control methods during all processes of array fabrication.
A. Images obtained by QC probes and target-specific hybridization using the three quality control methods. Images obtained by QC probe after spotting (A1 to A3), after washing (A4 to A6), and after hybridization (A7 to A9). Images obtained by target-specific hybridization of fluorescently labeled product and target-specific probe (A10 to A12). **B.** Effect of QC probes on spot-uniformity assessment. Images obtained by T20-F probe after spotting (B1) and after washing (B2). Image obtained by target-specific hybridization of fluorescently labeled product and target-specific probe (B3). The defect spots of various morphologies were identified by QC probe. The arrow indicates a lost spot after washing. **B4.** Scheme of probes layout in array. The question mark indicates that it is impossible to get the result of the quality evaluation for an array not containing QC probes.

for spot quality. The array containing the T20-F and END-F probes could be evaluated for spot quality by the TAMRA. The specific hybridization signal for the rtM204V probe was showed in all three kinds of arrays. Fig. 3B shows the images obtained from the array containing QC probes. The images were obtained by the QC probes after spotting and after washing. We checked for a high degree of variation in the spot morphology and a lost spot after washing. Spot morphology defects, such as donut spots and noncircular spots by probe diffusion, were detected and discarded. This spot existed on the array before washing, but was lost after washing. This was likely due to the weak attachment of the spot to the array.

The fluorescent image of each spot could be detected by the two types of QC probes during all of the processes of array fabrication. Therefore, all of the slides of the printed batch could be quantitatively assessed.

Quantification Comparison Between the Three Kinds of Arrays

To quantify mixed targets using QC probes, an experiment was carried out with mixed targets including the wild type and the mutant from the mutagenesis clones. Two biotin-labeled PCR products were mixed at 100:0, 90:10, 50:50, 10:90, and 0:100 wt-to-mutant ratios. The arrays were hybridized with biotin-labeled target product. The mixed targets were quantified based on the normalization of the QC probes.

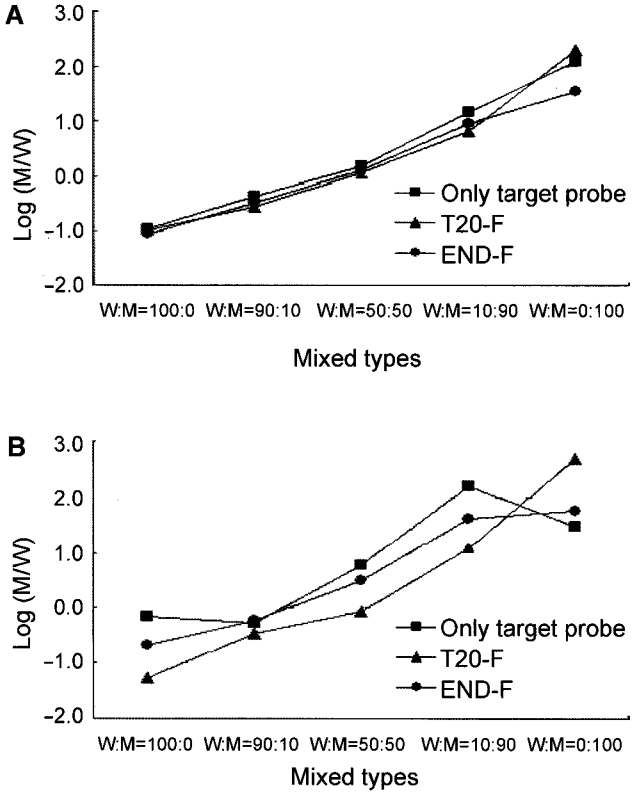

Fig. 4. Comparison of quantification results.
A. Linearity between log hybridization signal ratios and mixed types of target products. The graph shows a representative result for linearity at 16 experiments. **B.** In some cases, the array fabricated by the ordinary method containing only target-specific probes showed a nonlinear graph. Two kinds of array containing QC probes showed a linear graph.

Fig. 4 shows the results of the quantification comparison for 16 independent experiments. The data obtained by the array containing the T20-F and END-F probes were normalized based on the signal intensity of the QC probes after hybridization. However, the array containing only target-specific probes could not be normalized. The graph shows the log signal ratios (rtM204V/rtM204) of the mutant. Linearity between the log signal ratios and the mixed targets (rtM204V) was observed in most of the arrays (Fig. 4A). In some cases of an array containing spots of low quality, those containing QC probes showed linear results. The arrays containing only target-specific probes showed nonlinear results. We observed a nonlinear result two times (Fig. 4B).

Relationship Between the Quantification Results of the QC Probe Methods

To compare the more precise results of quantification and to test the reproducibility of our method, the experiments for each mixed target were performed 16 times. The relationship between the results of quantification for the three kinds of arrays is shown in Table 1. The mean ±standard deviation is shown for the quantification result for the mutant of each mixture. The data obtained for the T20-F and END-F probes were normalized based on the signal intensity of the QC probes after hybridization. The array not containing QC probes could not be normalized. We compared the following cases. First, in the comparison of the three kinds of arrays, we found significant differences in the hybridization signal intensities of the mixtures containing 50%, 90%, and 100% mutant ($P<0.05$). Second, in the comparison of the two kinds of arrays containing only target-specific probe and T20-F probes, we found no significant differences in the hybridization signal intensities of the mixtures containing 0%, 10%, and 100% mutant ($P>0.05$). However, we found significant difference in the hybridization signal intensities of the mixtures containing 50% and 90% mutant ($P=0.011$ and $P=0.003$, respectively). Third, in the comparison of arrays containing only target-specific probe and END-F probes, we found significant differences in the hybridization signal intensities of all the mixtures (10%, 50%, 90%, and 100% mutant) ($P<0.05$). However, there was no significant difference in hybridization signal intensity of 0% mutant (100% wild-type, $P=0.838$). Fourth, in the comparison of the two kinds of QC probes, we found no significant differences in the hybridization signal intensities of all the mixtures except 100% mutant. For the quantification of mixed targets in the samples, the methods using QC probe was very useful, especially the method using the END-F probe.

Table 1. Quantitative analysis of mixed targets using the three kinds of arrays. The *P* value was analyzed using the Kruskal-Wallis test.

Mixed types	Mean±SD of log signal ratios			
	Target only	T20-F	END-F	*P* value
W:M=100:0	-0.882±0.424	-0.889±0.401	-0.887±0.146	0.904
W:M=90:10	-0.391±0.504	-0.393±0.161	-0.484±0.155	0.081
W:M=50:50	0.317±0.261	0.141±0.139	0.162±0.189	0.022
W:M=90:10	1.291±0.382	0.855±0.458	1.015±0.328	0.006
W:M=0:100	2.132±0.547	2.041±0.760	1.722±0.266	0.017

DISCUSSION

Arrays have been reported to be a powerful tool for the high-throughput detection and quantification of many nucleic acid molecules [3]. A high quality of array is a prerequisite for the reliability and reproducibility of results. However, there are some difficulties in obtaining reliable and reproducible results. These include slide heterogeneity, spotting pin variation, and spot size differences caused by technical reasons [5]. Various methods have been reported for the quality control of spots. First, the method reported by Schena *et al.* [17] is used to inspect spot-uniformity and glass surface damage by laser scanning using salts present in the spots immobilized on a glass surface. This method can be applied only after spotting. It cannot be applied after washing and after hybridization because the salts are removed from the spots through immobilization and the washing processes after spotting. Second, another QC method applied a fluorescent staining dye, such as SYBR Green, after spotting and post-processing [1]. Only one or a few slides (mainly the first and last slides of a printed batch) can be quality assessed, and not all slides of a printed batch can be quality assessed [12, 13, 19]. This method requires a number of washing processes for staining and dye-removing, and the fluorescent dye residue affects the hybridization. This method is unlikely to be acceptable for diagnostic use because of the increase in noise and the loss of signal by reuse [9]. Recently, a method using probes, each spiked with a common reference gene fragment, has been developed [3, 8]. Quantification of this method is based on the ratios of Cy3 to Cy5. It cannot be applied to the quantification of oligonucleotide arrays because of the differences in hybridization efficiencies between oligonucleotide probes [14]. Finally, Bodrossy *et al.* [4] have developed a quantification method using two-color hybridization. For the quantitative analysis, the hybridization results for a mixture of Cy5-labeled reference target and Cy3-labeled sample target were used. This method can be applied to the quantification of oligonucleotide arrays but cannot be applied to the quality control of spots.

In this study, we developed methods for quality control and quantification of fabricated arrays. This was achieved using two types of QC probes. We used TAMRA as a fluorescent dye for all of the QC probes. This avoids the potential spectral interference of the Cy5 dye of hybridization with the target-specific probe. Therefore, the dye indicating different wavelengths from the hybridization signal with a target-specific probe can be applied to the fluorescent labeling of QC probes. These QC probes are important in order to assess the surface quality, integrity, and homogeneity of each probe spot.

For the QC probes, we could confirm the following results. First, we could evaluate the spots quality for all processes of array fabrication including after spotting, after washing, and after hybridization. In the experiments using QC probes, we successfully identified spots morphology defects such as donut spots and noncircular spots. Moreover, we detected a lost spot after washing from the light attachment of the spot to the array (Fig. 3). Second, for the cases with the QC probes and without the QC probes, we compared the signal intensity of target-specific probes after hybridization. The signal intensity of the target-specific probes of the array containing T20-F probes was an average 1,508 unit, the array fabricated by END-F probes was an average 1,908 unit, and the array not containing QC probes was an average 1,682 unit. In the results of the comparison of Cy5 signal intensities, we could confirm that the application of QC probes had no effect on either the spot morphology or the signal intensity of hybridization with target-specific probes. Third, we compared the quantification results for mixed targets of various amounts (Fig. 4 and Table 1). In almost all of the arrays containing spots of high quality, linearity between the log signal ratios and the mixed targets was shown. However, for some cases of arrays containing spots of low quality, those containing QC probes showed linear results. The arrays containing only target-specific probes showed nonlinear results (Fig. 4B). The array fabricated by spotting pin can cause various spot sizes, spot morphology, and attached probe amount [11, 16, 19]. Therefore, the arrays containing only target-specific probes showed nonlinear and irregular results because arrays fabricated by the ordinary method cannot be normalized. However, the arrays fabricated by this method using QC probes showed linear and regular results because it was possible to normalize variations in spot size and morphology and attached probe amount.

For the quantification results for each mixed type obtained by the 16 experiments, we compared the average

values and standard deviation for the log signal ratios (Table 1). In the analysis results, the mixed types containing equal amounts of the two products showed log signal ratios >0. Because of the sequence properties of the oligonucleotide probe, the inherent signal intensity value of each probe mixture containing equal amounts (50:50) showed log signal ratios >0. It was observed that the signal intensity of the 100% wild type was relatively low and that of the 100% mutant was relatively high. The signal intensity difference between these two types of targets was due to the probe properties. The center position of the probe is more important in stabilizing hybridization; thus, a probe with most of its GC content in the center binds to its target more strongly than another one with homogenous GC distribution [4]. The mutant probe showed a high signal intensity, because the center position of the mutant probe is located G (the center position of the wild-type probe was located A). The cases of 0% wild type and 0% mutant did not show zero signal intensity, because the results of hybridization with two probes by single base substitution has arisen a background signal from a low rate of nonspecific hybridization. The results from the signal intensity of Cy5 dye (hybridization with target-specific probes) were used to gain a rough estimate. The signal intensity of hybridization with target-specific probes obtained after normalization of the QC probes was used to refine the quantitative assessment of single target or mixed targets.

In this study, we compared quantification results of the three kinds of arrays. For the mixture containing only wild type, we found no significant differences between the results of each assay. For the mixture containing 100% mutant, we found significant differences in the results of only target-specific and END-F probes and T20-F and END-F probes (P=0.012 and P=0.015). In the comparison of the ordinary method and QC probe methods, the arrays containing two kinds of QC probes could evaluate spot-uniformity for all processes of array fabrication. The QC probe methods can avoid errors originating from the probe concentration and spot morphology because it can be normalized by QC probes. Therefore, the arrays using QC probes were very useful to the quantification of the mixed targets. Quantification using the T20-F probe was useful for the mixture containing 50% and 90% mutant. For the quantification using the END-F probe, the signal ratio of target-specific probe increased in proportion to the target DNA ratio of the mixture. The relationship between signal ratio and target DNA ratio is directly proportional. The quantification using the END-F probe was particularly useful for all the mixed types (P<0.05).

In summary, we developed the methods of evaluating the spot-uniformity of fabricated arrays and of quantifying mixed targets in samples. The T20-F and END-F probes were readily available and very useful as QC probes for spot-uniformity and probe attachment. The arrays using QC probes were very useful to the quantification of the mixed targets. The oligonucleotide array fabricated with END-F probes can be used simultaneously in the detection and quantification of mixed targets in samples. In our previous study [4, 5], we limited our experiments to low-density arrays of short oligonucleotides (<30 nt for target sequences) used for oligonucleotide arrays for clinical diagnosis. However, these developed QC probes discussed here are applicable to various oligonucleotide arrays. The methods using QC probes are very useful for the quality control of spots on fabricated arrays and the quantification of mixed targets.

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