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Ratio quantification of gene dosage by Agilent 2100 Bioanalyzer for detection of somatic gene deletions

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ABSTRACT

We applied the Agilent 2100 Bioanalyzer, a microfluidics-based electrophoresis instrument, and introduced an accurate and consistent parameter, the relative product yield ratio (ROY), to detect somatic gene deletions in tumor cells. Briefly for such purpose, the Agilent 2100 Bioanalyzer quantified the ROY of a target gene to an endogenous internal control, both of which were initially co-amplified by Robust-Dosage PCR (RD-PCR). Herein, we extensively validated this approach. We first tested the effect of well positions on the Agilent DNA chip. We found that no matter which wells the samples were loaded in, the ROY was consistent with coefficient of variation (CV) < 2%. Then we tested the effect of product concentrations that varied 8-fold, and the ROY was also consistent with CV < 3.5%. Furthermore, we applied this approach to identify six somatic *KRAS* deletions in non-small cell lung cancer patients, confirming our previous findings. Thus, the Agilent 2100 Bioanalyzer is simple, accurate, quick, and ultimately able to replace conventional gel electrophoresis for the detection of somatic gene deletions.

Keywords: Electrophoresis; Gene Deletion Detection; Agilent 2100 Bioanalyzer; RD-PCR; NSCLC

1. INTRODUCTION

Chromosomal deletions and amplifications often occur in malignancies such as lung cancer [1] (http://cgap.nci.nih.gov/Chromosomes/Mitelman Database of Aberrations in cancer). However, early detection of deletions and amplifications is difficult largely because of the low frequency of aberrations, multiploidy in many cancer genome regions, and the low portion of tumor cells in tissue specimens.

We applied the Agilent 2100 Bioanalyzer, a microfluidics-based electrophoresis instrument which takes nearly 40 minutes to analyze a set of DNA samples. In brief, the Agilent 2100 Bioanalyzer quantified the relative product yield ratio (ROY) of a target gene to an endogenous internal control, both which were initially co-amplified by Robust-Dosage PCR (RD-PCR), so that somatic gene deletions could be detected accurately.

RD-PCR is a highly quantitative duplex PCR which co-amplifies a target locus on an autosome and an endogenous internal control on a X-chromosome [2,3]. Through measuring the ROY of the two products, the template copy number (ROT) can be accurately deduced. Previously, we developed and validated 110 RD-PCR assays for detection of heterozygous deletions and duplications with high linearity and consistency [4,5]. However, the PCR products needed to be electrophoresed on agarose gel and measured by Typhoon imager, a labor intensive, expensive, and time consuming procedure.

The Agilent 2100 Bioanalyzer can quantify the absolute product yields of DNA products [6,7]. However, the ROY, exemplified by a target of the *KRAS* gene on CH.12 and an endogenous internal control of the *F9* gene on CH.X, had not been extensively explored, particularly for detection of somatic gene deletions. Thus, we extensively validated the consistency and accuracy of the Agilent bioanalyzer in obtaining the ROY. We first tested the effect of well positions on the chip and found the ROY consistent in all individual wells. Then we tested the effect of product concentrations and found that the ROY was consistent throughout 8-fold product concentrations. Finally, we applied this approach to identify six somatic *KRAS* deletions in non-small cell lung cancer (NSCLC) patients, which confirmed our previous experiments. Thus, the Agilent 2100 Bioanalyzer is easy to use, accurate, quick, relatively cheap, and able to replace conventional gel electrophoresis for the detection of somatic gene deletions.

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2. MATERIALS AND METHODS

2.1. NSCLC Patients and Tissue Specimens

NSCLC patients from Taiwan were diagnosed at an early stage (IA, IB, IIA, or IIB) and treated with standard adjuvant therapy. Cancer tissue and their paired marginal normal tissues were sectioned in surgery and immediately frozen at –70˚C. Tumor tissues contained sufficient portion of tumor cells, typically ≥40%, and normal tissues had no tumor cells, as judged by a pathologist.

2.2. Extraction of Genomic DNA from Frozen Tissues

The genomic DNA was extracted from frozen tissue using DNeasy Mini Kit according to the manufacturer's protocol (Qiagen). Concentration was measured by NanoDrop-1000 spectrophotometer at 260 nm (Nano-Drop).

2.3. RD-PCR for Chromosomal Deletions

Each assay co-amplified a target of the *KRAS* gene on CH.12 and an endogenous internal control of the *F*9 gene on CH.X. Primers were designed according to Liu *et al*. 2003 [3] (**Table 1**).

Before RD-PCR, genomic DNA samples were incubated at 95˚C in 1× Expand High Fidelity buffer #3 without $MgCl_2$ (Roche) for 10 minutes in order to completely denature the genomic DNA and minimize RD-PCR bias.

A RD-PCR reaction contained a total volume of 25 µl: 1× Expand High Fidelity buffer #3 (Roche), 4.5 mM $MgCl_2$, 200 µM each dNTP, 0.1 µM each primer of the *F*9 gene (internal control), 0.2 µM each primer for exon 2 (target), 1U of Platinum *Taq* DNA polymerase and 1U of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen), and 60 ng of genomic DNA. Samples were heated to 94˚C for 2 minutes before cycling. Each of 23 cycles consisted of denaturation at 94˚C for 15 seconds, annealing at 55˚C for 30 seconds, and elongation at 72˚C for 90 seconds.

2.4. Agilent 2100 Bioanalyzer and DNA 1000 Chip

We prepared the DNA 1000 Chip, suitable for separation and detection of DNA segments from 15 bp to 15,000 bp, according to the manufacturer's instruction. DNA gel matrix containing DNA dye and buffer was spread through microchannels on the DNA chip. 5 µl of marker and 1 µl of RD-PCR product were added into each well, followed by electrophoresis and measurement.

The absolute peak areas of the *KRAS* and *F*9 products, which were used to represent the amount of RD-PCR product yield, were obtained by subtracting local background signal from total signal in arbitrary fluorescence units (FU) using Expert software version B.02.08.SI648.

The relative product yield ratio (ROY) of the *KRAS* gene to the *F*9 gene of a sample is the net signal of the target segment divided by the net signal of the control segment.

For normal product, a linear equation of ROY = m·ROT + b was regressed when ROY was correlated with the template copy number ratio (ROT) per cell (normal male ROT = 2 and female ROT = 1). Then for an unknown sample, the linear equation was used to convert the ROY to its ROT. 85% ROT ratio of tumor tissues to their paired normal tissues was set as detection limit for deletions, an equivalent when 30% of diploid cells delete one copy of the *KRAS* gene.

3. RESULTS AND DISCUSSION

3.1. The Effect of Well Positions on Measured Peak Areas

In order to test the effect of well positions, we added two RD-PCR products, one amplified from a female and the other from a male, in the same amount into 6 wells each, placed in alternating order across the DNA chip.

With well positions noted, we found that the absolute peak areas of the *KRAS* and *F*9 products, representatives of the *KRAS* and *F*9 product yields, were heavily affected by well positions on the chip (**Figure 1**), even though the same amount of RD-PCR products was applied to each well.

However, the relative peak area ratios, representing the relative product yield ratios (ROY), were much more consistent (**Table 2(a)**). This was also supported by the analysis of average, standard deviation, and coefficient of variation (CV) (**Table 2(b)**). For example, the CV was 0.015 for the female sample, and was 0.014 for the male sample.

3.2. The Effect of Product Concentrations on Measured Peak Areas

In order to test the effect of product concentrations, each of three PCR samples was prepared over 8-fold concentrations. Again, the ROY was highly consistent, regardless of product concentrations or well positions (**Table 3(a)**, **Figure 2**). The average, standard deviation, and CV were calculated (**Table 3(b)**). For example, the CV was 0.016 for Female 2, 0.018 for Male 2, and 0.035 for Male 3.

3.3. Detection of Somatic Gene Deletions in Non-Small Cell Lung Patients

RD-PCR first co-amplified a target of exon 2 of the *KRAS* gene and an endogenous internal control of exon 1 of the

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Table 1. List of primers for RD-PCR.

Gene and exon	Name[a]	3' gene specific region of the primer[b]		Core segment		
		Sequence (5' - 3')	T_m (˚C)[c]	Size (bp)	T_m (˚C)[d]	GC (%)
*F*9 exon 1 on CH.X (Control)	F9(2724)D	ATGTAGCCACTATGCCTATC	64.4	486	83.0	40.7
	F9(3210)U	CTGGCTGTTAGACTCTTCAA	61.7			
KRAS exon 2 on autosome 12p12.1	RAS(5445)D	CTGGTGGAGTATTTGATAGTGT	61.9	413	80.4	34.9
	RAS(5857)U	GAACATCATGGACCCTGACA	67.9			
	RAS(23768)U	GCATGGCATTAGCAAAGACT	66.4			

[a]For example, F9(2724)D represents: *F*9, the human coagulation factor IX gene; (2724), 5' end of the 3' gene-specific region of the primer begins at nucleotide 2724 according to GenBank accession K02402. D, downstream (*i.e.*, in the direction of transcription). The precise sizes and locations of the PCR fragment can be obtained from the informative names. The *KRAS* sequence is from Genbank accession NG_007524; [b]The sequence of the 3' gene-specific region is shown. A 10-nucleotide universal tail of 5'ggccaagtga3' is attached to the 5' end of each primer; Oligo 5 calculates the melting temperature of a primer by the nearest neighbor method at 50 mM KCl, 0.7 mM free Mg, and 200 nm DNA. The melting temperature of a PCR product, the T_m value, of each PCR segment is estimated under the above salt conditions by the formula: $T_m^{product} = 31.5 + 16.6 \log\{[K^+]/(1 + 0.7[K^+])\} + 0.41(\%G + \%C) - 500/length$.

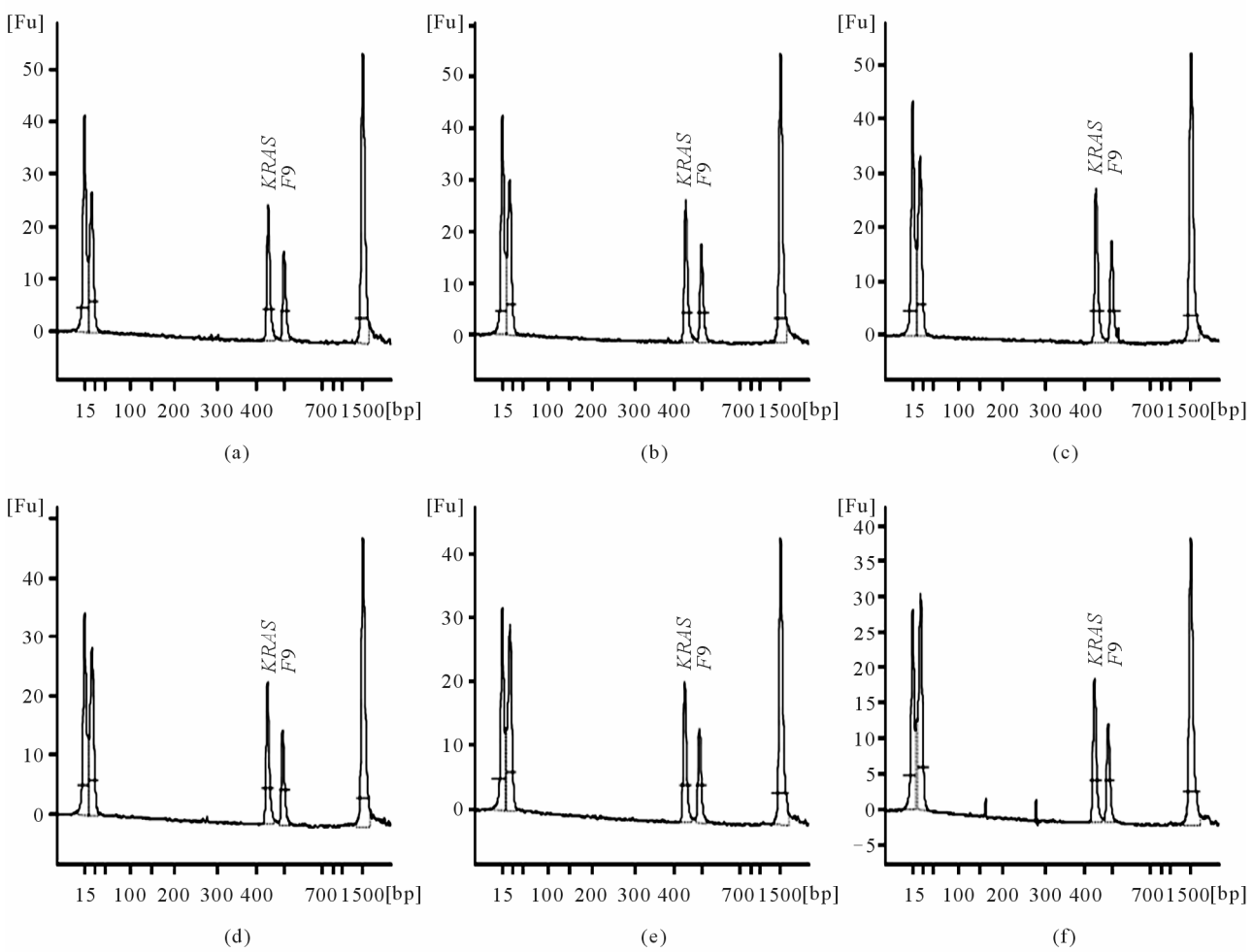

Figure 1. The effect of well positions. The X-axis represents the product size in bp and the Y-axis is the arbitrary fluorescence intensity. The *KRAS* and *F*9 signals are labeled. Panels (a) to (f) were conducted on the same chip from the same RD-PCR DNA sample Male 1 with the same volume of product added to each well. (a) Examples are from Well 2; (b) Well 4; (c) Well 6; (d) Well 8; (e) Well 10; (f) Well 12. The DNA markers that are at 15 bp and 15,000 bp are present.

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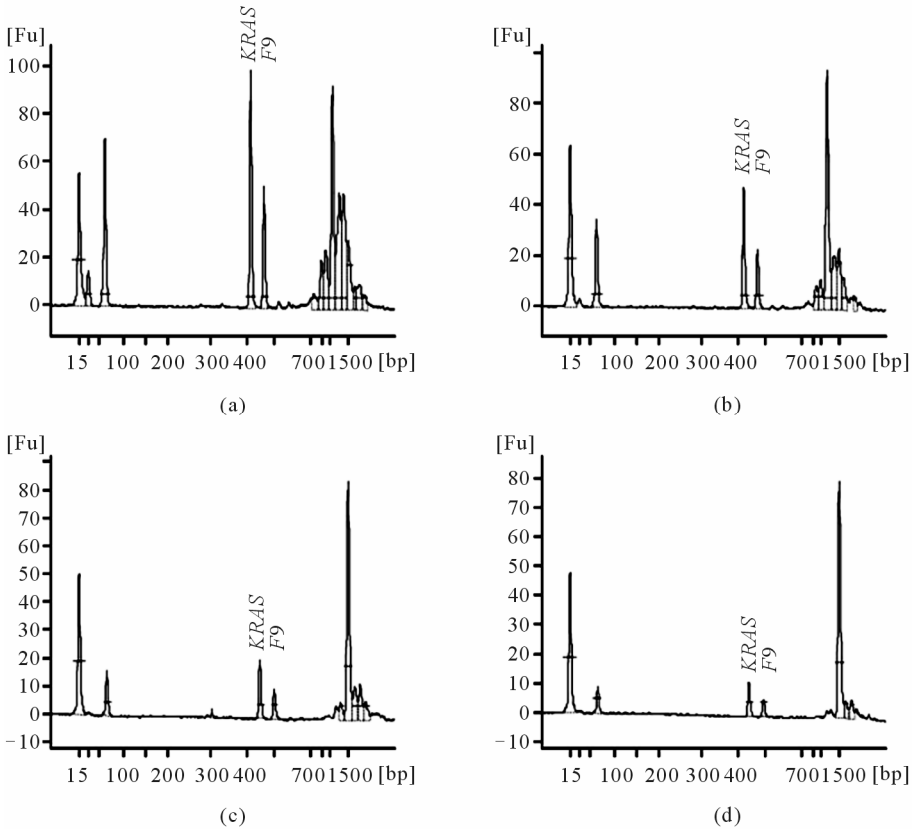

Figure 2. The effect of product concentrations. Panels A to D are of RD-PCR DNA sample Male 2, which were conducted on the same chip, but with differing concentrations. (a) Concentration of 1× in Well 2; (b) 1/2× in Well 5; (c) 1/4× in Well 8; (d) 1/8× in Well 11.

Table 2(a). The effect of well positions on ROY of the *KRAS* and *F*9 genes.

Well[a]	Sample[b]	Copy number ratio of *KRAS* to *F*9 per cell[b]	*KRAS* peak area[c]	*F*9 peak area[c]	*KRAS*/*F*9 peak area ratio
1	Female 1	2:2	23.1	34.9	0.662
3			23.2	34.8	0.667
5			24.4	36.8	0.663
7			19.8	30.3	0.653
9			19.9	29.9	0.666
11			17.9	26.2	0.683
2	Male 1	2:1	21.9	14.2	1.542
4			23.8	15.8	1.506
6			24.2	15.8	1.532
8			19.4	12.5	1.552
10			17.7	11.8	1.500
12			16.5	10.9	1.514

[a]1 µl of RD-PCR products was added to each well; [b]Normal Female 1 has two copies of the *KRAS* gene and two copies of the *F*9 gene in each cell, and normal Male 1 has two copies of the *KRAS* gene and one copy of the *F*9 gene in each cell; [c]Peak area, the integral of fluorescence intensity, is assigned in arbitrary fluorescence units (FU).

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Table 2(b). Statistical analysis of ROY of the KRAS and F9 genes[a].

Sample	Average	Standard deviation	Coefficient of variation (CV)
Female 1	0.666	0.010	0.015
Male 1	1.524	0.021	0.014

[a]ROY of the *KRAS* and *F*9 genes from **Table 2(a)** was analyzed.

Table 3(a). The effect of product concentrations on ROY of the *KRAS* and *F*9 genes.

Well[a]	Sample	Concentration[b]	*KRAS* peak area[c]	*F*9 peak area	*KRAS*/*F*9 peak ratio
1	Female 2	1×	34	38.9	0.874
4		1/2×	18.6	20.6	0.903
7		1/4×	7.6	8.7	0.874
10		1/8×	3.3	3.7	0.892
2	Male 2	1×	60	32.2	1.863
5		1/2×	29	15.3	1.895
8		1/4×	12.1	6.3	1.921
11		1/8×	6.8	3.5	1.943
3	Male 3	1×	59	29	2.034
6		1/2×	32.8	16.1	2.037
9		1/4×	12.9	5.9	2.186
12		1/8×	7.2	3.4	2.118

[a]1 µl of RD-PCR products at a concentration was added to each well; [b]RD-PCR products were serially diluted to their 1/2, 1/4, and 1/8 concentrations; [c]Peak area assigned in arbitrary FU.

Table 3(b). Statistical analysis of ROY of the *KRAS* and *F*9 genes[a].

Sample	Average	Standard deviation	Coefficient of variation (CV)
Female 2	0.886	0.014	0.016
Male 2	1.906	0.034	0.018
Male 3	2.094	0.073	0.035

[a]ROY of the *KRAS* and *F*9 genes from **Table 3(a)** were analyzed.

*F*9 gene. Then the ROY was quantified and converted to its template copy number ratio (ROT) due to high linearity and high correlation. In detail, from analyzing the results of 6 male and 4 female NSCLC patient samples, the linear equation is set as ROY = 1.094·ROT + 0.163 with R = 0.961. Thus, for an unknown patient sample, the linear equation was used to convert the ROY to its ROT. 85% ROT ratio of tumor tissues to their paired normal tissues (T/N ROT ratio) was set as detection limit for deletions, an equivalent when 30% of diploid cells delete one copy of the *KRAS* gene.

Thus, we identified six *KRAS* deletions in tumor tissues, but not in their paired normal tissues (**Table 4**), confirming our previous finding. In addition, our results were similar to those by gel electrophoresis and Typhoon™ 9410 Variable Mode Imager except for one patient with a boundary value (T/N ROT ratio = 0.96 by Agilent 2100 Bioanalyzer vs 0.85 by gel electrophoresis). The *KRAS* deletions were also verified by fluorescence *in situ* hybridization (FISH) in individual tumor cells (data not shown).

4. SUMMARY

We applied the Agilent 2100 Bioanalyzer, a microfluidics-based electrophoresis instrument, rather than conventional gel electrophoresis, to detect somatic gene deletions.

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Table 4. Comparison of Agilent 2100 Bioanalyzer with conventional gel electrophoresis.

NSCLC Patient	Tissue	Gel electrophoresis and Typhoon		Agilent 2100 Bioanalyzer		% tumor cells[d]	FISH[e]		Copy number of *KRAS* deletion to overall genome[f]
		KRAS/*F*9[a]	T/N ROT[b]	*KRAS*/*F*9	T/N ROT[b]		*KRAS*	CH.12 Centromere	
Male 4	Tumor	1.73	0.808	2.05	0.748	60% - 70%	2	3	1/6
	Normal	2.14		2.74					
Male 5	Tumor	1.61	0.809	2.08	0.849	80%	2	3	1/4
	Normal	1.99		2.45					
Female 3	Tumor	0.82	0.796	0.96	0.774	60%	3	3	2/6
	Normal	1.03		1.24					
Female 4	Tumor	0.80	0.748	0.90	0.744	80%	2	3	2/6
	Normal	1.07		1.21					
Male 6	Tumor	0.96	0.478[c]	1.06	0.482[c]	50-60%	3	3	3/6
	Normal	2.01		2.20					
Male 7	Tumor	1.18	0.584	1.42	0.648	60%	2	3	2/4
	Normal	2.02		2.19					

[a]ROT is the copy number ratio of the *KRAS* gene to the *F*9 gene; [b]T/N ROT is the ROT ratio of the tumor tissue to its paired normal tissue. 85% ROT ratio of tumor tissues to their paired normal tissues was set as detection limit for *KRAS* deletions; [c]In this male patient, a tumor cell is expected to have three copies of the *F*9 gene but one copy was found deleted. This was confirmed by RD-PCR targeting four other loci of EGFR and MET genes; [d]% tumor means the ratio of tumor area to the total area in tissue specimen; [e]The copy numbers of the *KRAS* gene and CH.12 centromere per tumor cell were averaged from 60 cells through FISH. The nearest integer for the copy number is rounded; [f]The copy number of KRAS deletion per cell was estimated from PD-PCR quantification and % tumor in tissue specimen with FISH scoring in individual tumor cells. The copy number of overall genome per cell is estimated from FISH scoring in individual tumor cells. The nearest integer for the copy number is rounded.

In this study, we extensively validated the consistency and accuracy of the approach. Furthermore, we applied this approach to identify six somatic KRAS deletions in NSCLC patients, confirming our previous finding. Thus, the Agilent 2100 Bioanalyzer is simple, reliable, quick, and ultimately able to replace conventional gel electrophoresis for the detection of somatic gene deletions.

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