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Clinical Validation of Newly Developed Multiplex Kit Using Luminex xMAP Technology for Detecting Simultaneous *RAS* and *BRAF* Mutations in Colorectal Cancer: Results of the RASKET-B Study[1]

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Abstract

Detection of *RAS* and *BRAF* mutations is essential to determine the optimal treatment strategy for metastatic colorectal cancer (CRC). We prospectively evaluated the MEBGEN RASKET-B KIT (RASKET-B), a novel multiplex kit, simultaneously detecting 48 types of *RAS* mutations and the *BRAF* V600E mutation using Luminex xMAP technology. The aim was to obtain market approval for RASKET-B as an *in vitro* diagnostic (IVD) option in Japan. Genomic DNA was extracted from 302 formalin-fixed paraffin-embedded tissues obtained from CRC patients. The

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primary endpoints were the concordance rate (CR) between the results from RASKET-B and the previously approved IVD kit (RASKET) for *RAS* mutations, and CR between the results from RASKET-B and direct sequencing (DS) for *BRAF* mutations. The secondary endpoints included the CR between RASKET-B and DS for *RAS* mutations and between RASKET-B and the pyrosequencing (PYRO) for the *BRAF* V600E mutation. Among the 302 samples, 142 *RAS* mutations (47%) and 18 *BRAF* V600E mutations (6.0%) were detected by RASKET-B. All mutations detected in the recruited patients were mutually exclusive. Both *RAS* and *BRAF* mutation rates were statistically higher in right-sided than left-sided CRC. The CR between RASKET-B and RASKET for *RAS* gene and RASKET-B and DS for *BRAF* V600E mutation was 100% for both (95% CI: 99%-100%). The results from RASKET-B were also highly concordant with DS for *RAS* (97.4%) and with PYRO for the *BRAF* (V600E) gene (99.7%). RASKET-B thus provides rapid, precise, and simultaneous detection of *RAS* and *BRAF* mutations in CRC.

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Introduction

RAS (*KRAS* and *NRAS*) mutations are present in approximately 50% to 55% of colorectal cancer (CRC) cases. The clinical significance of the detection of *RAS* mutations has been previously established as a required test prior to the initiation of anti–epidermal growth factor receptor (EGFR) antibody therapy to predict the efficacy in metastatic CRC [1–5]. Prospective-retrospective biomarker analyses in randomized clinical trials have consistently demonstrated that anti-EGFR antibodies, cetuximab and panitumumab, are unlikely to benefit patients with *KRAS* exon 3 and 4 and *NRAS* exons 2, 3, and 4 mutations, in addition to those with a *KRAS* exon 2 mutation [6–8]. Moreover, recent results from clinical trials revealed that overall survival is possibly better when patients are treated with anti-EGFR therapy as a first-line treatment than when treated with bevacizumab in the *RAS* wild-type population [9,10]. This suggests that *RAS* mutation status has a large impact on the treatment decision in patients with metastatic CRC.

Many studies have reported that the *BRAF* V600E mutation is detected in approximately 5%-12% of metastatic CRC patients. *RAS* and *BRAF* V600E mutations are almost mutually exclusive [11]. Unlike *RAS* mutations, the predictive value of *BRAF* mutations for anti-EGFR mAb efficacy is less certain. On the other hand, the *BRAF* V600E mutation leads to a poor prognosis or rapid progression, regardless of treatment in metastatic CRC [12,13]. Recently, the possibility was reported that triplet chemotherapy combining 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI) with bevacizumab is more effective than other chemotherapies for patients with the *BRAF* V600E mutation [14,15], and both European Society for Medical Oncology (ESMO) consensus guidelines and pan-Asian adapted ESMO consensus guidelines recommend FOLFOXIRI plus bevacizumab as the preferred choice for these patients [16,17]. Therefore, the *BRAF* mutation status should be assessed before starting the first-line chemotherapy. The latest edition of the Japanese Society of Medical Oncology Clinical Guidelines: Molecular Testing for Colorectal Cancer Treatment states that proper testing for *BRAF* V600E mutation and mismatch repair deficiency is necessary in addition to testing for *RAS* mutation [18].

We previously reported that the MEBGEN RASKET KIT (RASKET) is useful for rapid detection of 48 types of mutations in codons 12, 13, 59, 61, 117, and 146 of *KRAS* and *NRAS* using PCR-reverse sequence specific oligonucleotide (PCR-rSSO) and xMAP technology [19]. The RASKET clinical validation study confirmed the precise detection of *RAS* mutations, with a concordance rate (CR) of 98.4% between the RASKET KIT and direct sequencing in *RAS* mutations (UMIN000011781). The RASKET KIT was approved in Japan as an *in vitro* diagnostic (IVD) and has become widely used in daily practice and is recognized as an *RAS* testing platform in Japan.

As mentioned above, the detection of *RAS* and *BRAF* mutations is an essential step for decision-making regarding therapeutic approaches and predicting resistance to EGFR-targeted therapy. The PCR-rSSO and xMAP technologies allow multiplex molecular testing in a single well. It would be clinically beneficial to develop a new kit for the simultaneous detection of *BRAF* V600E mutations and *RAS* gene mutations. In this study, we evaluated the newly developed MEBGEN RASKET-B KIT (RASKET-B) to detect 48 different *RAS* amino acid mutations and the *BRAF* V600E mutation in CRC patients. This study was performed as a registration trial for regulatory approval of the kit in Japan.

Material and Methods

Patients and Tumor Samples

The RASKET-B study used the identical cohort and the DNA sample sets that were used in the RASKET study (Study ID: UMIN 000011784) [19]. Briefly, the eligibility criteria for patients were 1) histologically confirmed adenocarcinoma of colorectal origin, 2) age ≥20 years at the time of informed consent, and 3) patients' written consent for participation in the study. Patients with insufficient amounts of formalin-fixed paraffin-embedded (FFPE) tissues, those with an undetermined *RAS* status by the RASKET kit in the previous RASKET study, and those who withdrew consent were excluded from the RASKET-B study. One central pathologist assigned for the study microscopically confirmed cancer in each patient, classified the tumor into the appropriate histologic type, calculated the tumor area ratio and tumor cell ratio, and then marked the tumor area on the prepared hematoxylin and eosin–stained slides for manual microdissection (MMD).

Study Design

All specimens were anonymized. Only the participating affiliations were able to access patients' information using a correspondence table, which was only available at each study site to eliminate any

disclosure to outsiders. Sample anonymity was the task of one employee of G&G Science Co., Ltd., who was not involved in the study.

The set of extracted DNA from the FFPE specimens was sent to three different reference laboratories (G&G Science Co., Ltd.; Health Sciences Research Institute, Inc.; and SRL Inc.), where independent assays were performed with RASKET-B, direct sequencing (DS), and pyrosequencing (PYRO), respectively. All samples were deidentified and blinded to the tissue genotype and clinical characteristics of each patient.

The primary endpoints in this study were the CR between results from RASKET-B and RASKET for *RAS* mutations, and the CR between RASKET-B and DS for *BRAF* mutations. The results of *RAS* mutations by the RASKET study [19] were used for comparison with those obtained by RASKET-B. As the secondary endpoints, we determined the CR between results from RASKET-B and DS for the *RAS* gene, and results from RASKET-B and PYRO for the *BRAF* gene. In addition, the accuracy of genotyping was evaluated by comparing data between RASKET-B and DS. The original and revised protocols were approved by the ethical committees in each of the participating affiliations. The study was conducted in accordance with the Declaration of Helsinki and ethical guidelines for clinical research.

Direct Sequencing

After the pathological confirmation of cancer in each patient, 10-μm–thick sections were processed by MMD. DNA extraction was performed with QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and as previously reported [19,20]. Briefly, each extracted DNA was amplified using six sets of primers to amplify exon 2, exon 3, and exon 4 in *KRAS* and *NRAS*, and exon 15 in *BRAF*. The mutations in these regions were detected using the BigDye Terminator Cycle Sequencing Kit (Thermo Fischer Scientific, Waltham, MA).

Pyrosequencing

DNA samples were analyzed for codon 600 in exon 15 of *BRAF* with the Therascreen *BRAF* Pyro Kit (Qiagen) as described by the manufacturer's protocol. The PYRO method was performed without MMD. For one patient with a discrepancy between RASKET-B and PYRO, MMD was additionally performed for PYRO to carefully confirm the existence of *BRAF*-mutated tumor cells.

Assay with MEBGEN RASKET-B KIT

Extracted DNA samples were diluted to a concentration of 10-20 ng/μl with sterile TE buffer (1 mmol/l Tris–HCL [pH = 8.0], 0.1 mmol/L EDTA). Assays with the RASKET-B kit (MBL, Nagoya, Japan) were performed according to the manufacturer's protocol. Briefly, a 5-μl template of each sample was mixed with 20 μl master mix, including primers, Taq DNA polymerase, and uracil-DNA-glycosylase. Reactions were heated for 5 minutes at 40°C and 2 minutes at 95°C; 10 repeating cycles of 94°C for 20 seconds and 62°C for 30 seconds; 45 repeating cycles of 90°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and finally 72°C for 1 minute and 94°C for 10 minutes. Each amplification product was then hybridized to mutation detection probes and immobilized with color-coded beads. Five microliters of PCR products and 45 μl of hybridization solution containing probe-coupled beads were hybridized at 95°C for 2 minutes followed by 55°C for 30 minutes. After washing, the PCR amplification-bead complexes were reacted with streptavidin-phycoerythrin at 52°C for 15 minutes. Using the Luminex 100/200 system (Luminex, Austin, TX), the median fluorescence intensity was determined for the color-coded beads and PE, which represented the types of *RAS* mutations and their signal intensities, respectively. UniMAG (MBL, Nagoya Japan) data analysis software was used to analyze the raw data from Luminex 100/200. Thus, using the RASKET-B KIT, we simultaneously examined 12 types of *RAS* exon 2 (G12S, G12C, G12R, G12D, G12V, G12A, G13S, G13C, G13R, G13D, G13V, and G13A), 8 types of *RAS* exon 3 (A59T, A59G, Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H), 4 types of *RAS* exon 4 (K117N, A146T, A146P, and A146V) mutations, and *BRAF* exon 15 (V600E). The evaluation criterion for the performance of the RASKET-B was CR ≥90% with the DS, PYRO, and RASKET reference assays in the primary and secondary endpoint analyses.

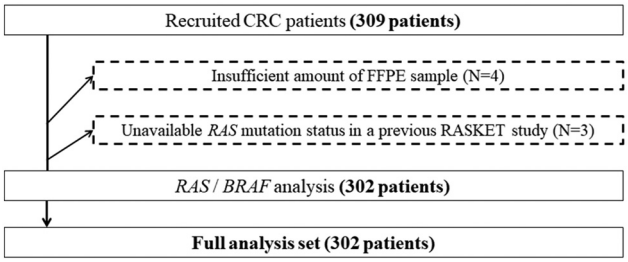

Figure 1. Study design and patient eligibility for primary and secondary endpoint analyses. Among 309 assessable samples, 4 were excluded because of an insufficient amounts of FFPE samples, and 3 were excluded because of unavailability of *RAS* mutation status in the previous study.

TaqMan Assays

In the case of any controversial data between the RASKET-B and the reference assays, we confirmed the results with TaqMan Mutation Detection Assays (Thermo Fisher Scientific) [21].

Statistical Analysis

The number of specimens required for the RASKET-B study was estimated as the acceptable number of specimens to satisfy that the CR between the RASKET and the reference assays would be >90%,

Table 1. Patient Characterization in the Blinded Clinical Evaluation Study for the MEBGEN RASKET-B KIT

		Total (*N* = 302)
Age, years	Median (minimum-maximum)	64 (26-89)
Sex	Male	178
	Female	124
Primary tumor location	Right-sided colon *	62
	Left-sided colon †	98
	Rectum ‡	142
Stage	Stage 0-I	33
	Stage II	48
	Stage III	125
	Stage IV	96
Histologic type	Well to moderately differentiated adenocarcinoma	273
	Poorly differentiated or mucinous	29
Tumor area ratio (%)	Median (minimum-maximum)	50 (5-100)
Tumor cell ratio (%)	Median (minimum-maximum)	50 (5-90)

* Cecum, ascending colon, and transverse colon.
† Descending colon and sigmoid colon.
‡ Rectum (Ra, and Rb). Ra: upper rectum (above peritoneal reflection), Rb: lower rectum (below peritoneal reflection).

Table 2. Frequency of Breakdown of *RAS* and *BRAF* Mutations Detected in Colorectal Cancer Patients

Mutation Status	No. of Cases	Proportion Among 302 Cases
WT *RAS* or *BRAF*	142	47.0%
KRAS exon 2 mutant	113	37.4%
p.G12S	5	1.7%
p.G12C	8	2.6%
p.G12R	4	1.3%
p.G12D	44	14.6%
p.G12 V	23	7.6%
p.G12A	6	2.0%
p.G12A, p.G12R	1	0.3%
p.G13D	20	6.6%
p.G12D, p.G13D	2	0.7%
Other *KRAS* exon 2 mutant*	0	0.0%
KRAS exon 3 mutant	6	2.0%
p.A59E	1	0.3%
p.Q61H	5	1.7%
Other KRAS exon 3 mutant*	0	0.0%
KRAS exon 4 mutant	10	3.3%
p.K117N	2	0.7%
p.A146T	6	2.0%
p.A146P	1	0.3%
p.A146V	1	0.3%
NRAS exon 2 mutant	6	2.0%
p.G12D	4	1.3%
p.G12V	2	0.7%
Other NRAS exon 2 mutants	0	0.0%
NRAS exon 3 mutant	8	2.6%
p.Q61K	2	0.7%
p.Q61L	5	1.7%
p.Q61R	1	0.3%
Other NRAS exon 3 mutant†	0	0.0%
NRAS exon 4 mutant‡	0	0.0%
BRAF V600E mutant	18	6.0%

*KRAS p.G13S, p.G13R, p.G13V, and p.G13A.
**KRAS p.A59T, p.A59G, p.Q61K, p.Q61E, p.Q61L, p.Q61P, and p.Q61R.
§RAS p.G12S, pG12C, p.G12R, and p.G12A.
†NRAS p.A59T, p.A59G, pQ61E, p.Q61P, and Q61H.
‡NRAS p.K117N, p.A146T, p.A146P, and p.A146V.

at the lower limit of the 95% confidence interval. We then determined the number of specimens for the RASKET-B study needed to exceed 278. Patients' demographic and disease characteristics were reported as standard statistics. Statistical analysis was carried out using StatFlex (Artech Co. Ltd., Osaka, Japan). The RASKET-B study is registered with UMIN22742.

Results

Patients

Tissues were obtained from 309 consenting patients with histologically confirmed CRC. Insufficient amounts of FFPE were available in four patients, and *RAS* gene status in three patients was not reportable using RASKET in the previous RASKET study. Therefore, 302 patients were eligible for the primary endpoint analysis (Figure 1) in the RASKET-B study. Patient data are provided in Table 1.

Frequency of RAS and BRAF Mutations in MEBGEN RASKET-B KIT

Among the 302 samples, RASKET-B detected 142 *RAS* mutations [113 (37.4%) *KRAS* exon 2, 5 (1.7%) *KRAS* exon 3, 10 (3.3%) *KRAS* exon 4, 6 (2.0%) *NRAS* exon 2, 8 (2.6%) *NRAS* exon 3] and 18 (6.0%) *BRAF* exon 15 mutations (Table 2). All mutations detected from the recruited patients were mutually exclusive. Both *RAS* and *BRAF* mutation rates were statistically higher in colon cancer on the right side than on the left side. Especially, the frequency of *BRAF* mutations among patients with *RAS* wild type was six-fold higher on the right side than on the left side (Table 3). Among patients with ascending colon cancer, 25% (9/36) had *BRAF* V600E mutations, a higher frequency than the other tumor locations (Figure 2). In terms of histologic types, the frequency of *BRAF* V600E mutations was significantly higher in patients with poorly differentiated or mucinous colon cancer. Also, the frequency of *BRAF* V600E mutations in females was not significant but tended to be higher than in males (P = .0745, Table 3).

Concordance of RAS and BRAF Status

In the primary endpoint analysis, the CR between results concerning *RAS* mutation status obtained from RASKET-B and RASKET was 100% (302/302) (95% CI: 98.8%-100%) (Table 4A). Concerning the *BRAF* mutation status, the CR between results obtained from RASKET-B and DS was also 100% (302/302) (95% CI: 98.8%-100%) (Table 4B).

In the secondary endpoint analyses of all samples, the CR between RASKET-B and DS was 97.4% (294/302) (95% CI: 94.9%-98.9%) in *RAS* (Table 5A). Among the eight samples with conflicting results between RASKET-B and DS, six samples were positive with *RAS* mutations (two cases with *KRAS* G12D, two cases with *KRAS* G13D, one case with *KRAS* G12R, and one case with *KRAS* Q61H) in RASKET-B but negative in DS. Genotypic results of these samples in RASKET-B were consistent with RASKET and with results from the TaqMan Mutation Detection Assay, which is a more sensitive method (cutoff levels 0.1%-1%). The other two samples were negative with *RAS* mutations in RASKET-B and positive with DS. One of the samples had two mutations in *KRAS* codon 11 and codon 12 (G12C). The other sample had a *KRAS* mutation in A59E, which was not covered by RASKET-B (Table 6).

Table 3. Correlation Between Tumor Location Side and Mutation Status, Sex, or Tissue Type

		n	*RAS*			*BRAF*			*Braf* mutant/*ras* Wild Type
			Wild Type	Mutant	*P* Value	Wild Type	Mutant	*P* Value	
			160	142 (47.0%)		284	18 (6.0%)		18/160 (11.3%)
Age, median (minimum-maximum)			64 (33-89)	63 (26-84)	P = .275	63 (26-85)	70 (46-89)	**P = .0077**	
Sex (%)	Male	178	101	77 (43.3%)	P = .117	171	7 (3.9%)	P = .0745	7/77 (9.1%)
	Female	124	59	65 (52.4%)		113	11 (8.9%)		11/59 (18.6%)
Histologic types (%)	Well to moderately differentiated adenocarcinoma	273	144	129 (47.3%)	P = .804	263	10 (3.7%)	**P < .0001**	10/144 (6.9%)
	Poorly differentiated or Mucinous	29	16	13 (44.8%)		21	8 (38.1%)		8/16 (50%)
Primary tumor location (%)	Right-sided colon	62	26	36 (58.1%)	P = .0506	52	10 (16.1%)	**P < .0001**	10/26 (38.5%)
	Left-sided colon and Rectum	240	134	106 (44.2%)		232	8 (3.3%)		8/134 (6.0%)

Each parameter was analyzed via the Fisher's test, except age, which was analyzed via the Student's t test.

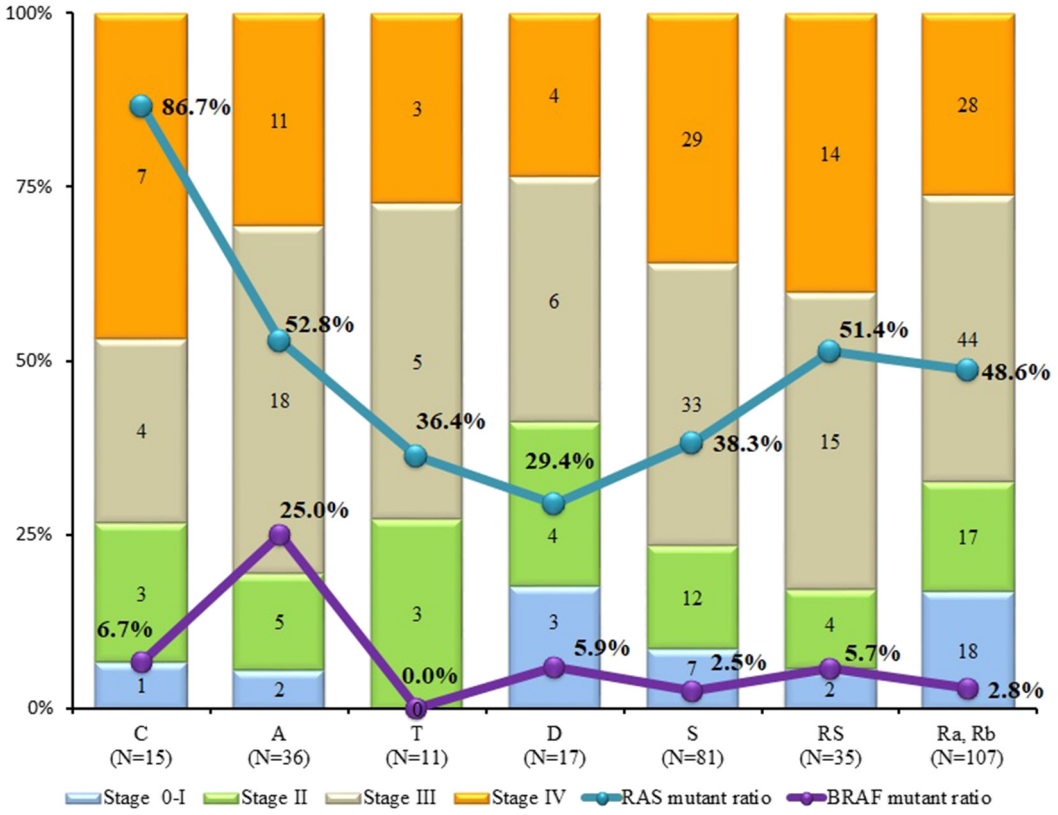

Figure 2. Frequency of *RAS* and *BRAF* mutations for each tissue location and stage.

For secondary endpoint analysis of *BRAF* V600E mutation detection, the result of RASKET-B was compared with PYRO. The CR was 99.7% (301/302) (95% CI: 98.2%-100%) (Table 5B). One sample was positive with *BRAF* V600E by RASKET-B and negative by PYRO without MMD. We also performed PYRO assays with MMD and then confirmed the detection of a mutation in *BRAF*. The percentage of *BRAF* mutant alleles in the sample transcript was 11.2%.

Table 4A. Consistency of *RAS* Mutation Status (Primary Endpoint Analysis)

		RASKET		
		Positive	Negative	Total
RASKET-B	Positive	142	0	142
	Negative	0	160	160
	Total	142	160	302
Overall agreement percentage		100% (95% CI, 98.8%-100%)		
Positive agreement percentage		100% (95% CI, 98.8%-100%)		
Negative agreement percentage		100% (95% CI, 98.8%-100%)		

Table 4B. Consistency of *BRAF* V600E Mutation Status (Primary Endpoint Analysis)

		DS		
		Positive	Negative	Total
RASKET-B	Positive	18	0	18
	Negative	0	284	284
	Total	18	284	302
Overall agreement percentage		100% (95% CI, 98.8%-100%)		
Positive agreement percentage		100% (95% CI, 98.8%-100%)		
Negative agreement percentage		100% (95% CI, 98.8%-100%)		

Genotyping Performance in RASKET-B

One hundred and fifty-seven specimens with positive *RAS* or *BRAF* mutation results by both the RASKET-B and DS were included. The concordance of each genotype for the overall population assessed by RASKET-B and DS was 100% (157/157) (95% CI: 98.3%-100%) (data not shown).

Table 5A. Consistency Between RASKET-B and DS in *RAS* Gene Mutations (Secondary Endpoint-1 Analysis)

		DS		
		Positive	Negative	Total
RASKET-B	Positive	136	6	142
	Negative	2	158	160
	Total	138	164	302
Overall agreement percentage		97.4% (95% CI, 94.9%-98.9%)		
Positive agreement percentage		95.8% (95% CI, 92.1%-99.5%)		
Negative agreement percentage		98.8% (95% CI, 96.4%-100%)		

Table 5B. Consistency Between RASKET-B and PYRO in *BRAF* Gene Mutations (Secondary Endpoint-1 Analysis)

		PYRO		
		Positive	Negative	Total
RASKET-B	Positive	17	1	18
	Negative	0	284	284
	Total	18	284	302
Overall agreement percentage		99.7% (95% CI, 98.2%-100%)		
Positive agreement percentage		94.4% (95% CI, 80.2%-100%)		
Negative agreement percentage		100% (95% CI, 98.8%-100%)		

Table 6. Discrepancy Samples in *RAS* Gene Mutation Detection Between the RASKET-B KIT and DS

RASKET-B	DS	Percentage of Mutant DNA in TaqMan Detection Assays
KRAS p.G12D	WT	2.81% (*KRAS* p.G12D)
KRAS p.G12D	WT	1.78% (*KRAS* p.G12D)
KRAS p.G12R	WT	0.31% (*KRAS* p.G12R)
KRAS p.G13D	WT	0.41% (*KRAS* p.G13D)
KRAS p.G13D	WT	2.03% (*KRAS* p.G13D)
KRAS p.Q61H	WT	38.9% (*KRAS* p.Q61H)
WT	*KRAS* p.A11A *KRAS* p.G12C	Not tested
WT	*KRAS* p.A59E	Not tested

Discussion

This study is the first to demonstrate the clinical usefulness of the RASKET-B, which can simultaneously detect *RAS* and *BRAF* mutations using the PCR-rSSO and xMAP technologies. For *RAS* genes, we compared the clinical significance of the RASKET-B to RASKET (previously confirmed and approved in Japan [19]) and DS with MMD. The overall CRs of *RAS* gene detections were 100% and 97.4%, respectively. For the *BRAF* gene, the results from the RASKET-B were compared to DS with MMD and PYRO; CRs were 100% and 99.7%, respectively. The CRs satisfied the predefined criteria. Based on these results, the RASKET-B was approved by the Ministry of Health, Labour, and Welfare of Japan as an IVD kit for simultaneous determination of both *RAS* and *BRAF* mutation status in FFPE and fresh frozen tissues of CRC patients on 05 December 2017.

The presently detected frequency of *RAS* mutations obtained using the RASKET-B agrees with those reported in several previous studies [2,3,6,7,22], while the frequency of *BRAF* mutations was slightly lower than that in Western countries [23–26]. The detection limit of RASKET-B was approximately 1%-5% (Supplementary Table 1, *A*, *B*, and *C*), which is identical toRASKET [19]. This detection sensitivity is similar to that of other allele-specific PCR-based technologies, which suggests that the discrepancy of the *BRAF* mutation rate was not due to sensitivity differences. The frequency of *BRAF* mutations in Asian countries is approximately 5% [12,27–31], which is consistent with the present study. The *BRAF* frequency was higher in the right-sided CRC and in females, which is consistent with a previous report [32].

In this study, we observed several inconsistencies between the results of the RASKET-B and the reference assays. Six specimens with positive results in the RASKET-B and negative results in DS were confirmed to be *RAS* mutation positive via the TaqMan method. The discrepancy may be mainly caused by the sensitivity difference: the detection limit of RASKET-B is higher than the DS (> 10%). In fact, five of six cases included a smaller amount of mutant DNA. For the *BRAF* gene, one sample showed discrepant results between RASKET-B and PYRO, possibly due to its small tumor ratio (tumor cell ratio 70%, tumor area ratio 15%). MMD additionally performed with PYRO showed a positive *BRAF* mutation result.

Conversely, among the two specimens with a negative result in RASKET-B and a positive result in DS, one sample had a KRAS A59E mutation that was not reported in the PRIME study [3]. The other false-negative sample had a double mutation in *KRAS* codon 11 and codon 12 (G12C). Based on the assay principle, PCR amplifications including codon 11 mutations cannot hybridize to the detection beads for codons 12 and 13 because the codons are adjacent. However, this would have little impact in clinical practice due to the very rare frequency [1,3,7,8]. Thus, this kit can provide clinically appropriate detection of *RAS* and *BRAF* mutations.

The Luminex xMAP technology is widely applied for multiplex molecular testing, such as tissue and virus genotyping, which requires differential detections from a number of similar sequences [33,34]. Additionally, for the amplification of multiple genes or their regions, any possible cross-reactions should be minimized to provide appropriate assays. The RASKET-B allows the simultaneous PCR in the same well of nine regions (four regions in each of *KRAS* and *NRAS* genes and one region in the *BRAF* gene) with few cross-reactions. The turnaround time for detection of both *RAS* and *BRAF* gene mutations is approximately 4.5 hours, regardless of the number of samples (<96). Thus, the RASKET-B can potentially solve unmet medical needs of clinicians and reference laboratories, as it can be designed for the rapid, high-throughput, and multiplex detection of all *RAS* and *BRAF* mutations.

There were some limitations in this study. First, all samples were obtained through surgery and not biopsies. Also, there may be a bias in that only samples with available *RAS* mutation data were recruited in this study. Poor quality DNA extracted from FFPE tissues could possibly lead to an inaccurate result. However, a sensitivity of 1%-5% in the RASKET-B would be enough to provide *RAS*/*BRAF* mutation status in biopsy samples and in clinical practice. Another limitation was that the RASKET-B was designed to detect only V600E mutations in the *BRAF* gene. This is because the clinical significance of other *BRAF* mutations still remains unclear in CRC. Even so, the RASKET-B could provide results of *RAS* and *BRAF* (V600E) mutations simultaneously with lower cost and a shorter turnaround time compared to other methods, such as next-generation sequencing.

In conclusion, clinical evaluation of the MEBGEN RASKET-B KIT met the predefined primary and secondary endpoints and displayed a high CR with existing *RAS* and *BRAF* assays. The RASKET-B provides rapid and precise detection of *RAS* and *BRAF* mutations from FFPE tissue from CRC patients.

Conflict of Interest

The study was designed under the responsibility of MBL and was funded by MBL, Japan. The RASKET-B and RASKET were provided by MBL. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.10.004.

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