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KRAS, BRAF, and EGFR Mutational Analysis in Ovarian, Colon, and Lung Cancers by Highly Multiplex PCR/Barcoded Magnetic Bead (BMB) Suspension-Array Assays

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ABSTRACT

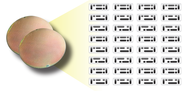
Anti-epithelial growth factor receptor (EGFR) monoclonal antibodies and small molecule EGFR inhibitors have been used for treating metastatic colorectal cancer (mCRC) and non-small cell lung cancer (NSCLC), respectively. However, since a relatively large proportion of the mCRC and NSCLC patients carry the activating KRAS/BRAF mutations that can render these EGFR-targeted therapies ineffective, the KRAS/BRAF mutation testing is now required for such prescription. In contrast, epithelial ovarian cancer (EOC) thus far has been treated by surgery, radiotherapy, and/or chemotherapy depending on the tumor stages. However, it has been shown that a high percentage of tumors of the mucinous, endometrioid, low-grade serous, and other types of EOC patients also contain KRAS or BRAF somatic mutations, suggesting that drugs targeting the mediators of the EGFR pathway may have implications on treating EOC of certain types. The conventional analysis of the KRAS, BRAF, and EGFR mutations has been done by multiple reactions with one mutation target per reaction, thus requiring a large amount of precious patient sample for complete testing. Here we present three highly multiplex molecular diagnostic assays, utilizing amplification by allele-specific PCR and automatic detection by a platform designed around the barcoded-magnetic-bead (BMB) suspension-array technology, for detection of 12 KRAS (in codons 12 and 13), six BRAF (in codon 600), and 22 EGFR (in exons 18-21) mutations in mCRC, NSCLC, and EOC patient samples. The results indicate that, albeit all the reaction components are in single wells, these highly multiplex PCR/BMB assays, by comparing with the sequencing results, not only are sensitive and specific but also can conserve precious tissue specimens, save operating time and labor, reduce turnaround time, and increase assay throughput.

Technology Overview

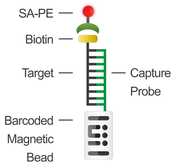
Barcoded Magnetic Bead (BMB) Technology

BMBs Made from Wafers

- Made from 6-in. silicon wafers
- 12 binary digits = 2^{12} (4,096) potential barcodes
- 3.5 million beads per wafer
- Bead dimension = 68 x 40 x 6 micron

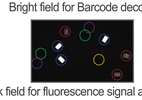


Probe Coating & Detection



Barcode Decoding & Signal Acquisition by BMB Analyzer

Bright field for Barcode decoding



Dark field for fluorescence signal acquisition

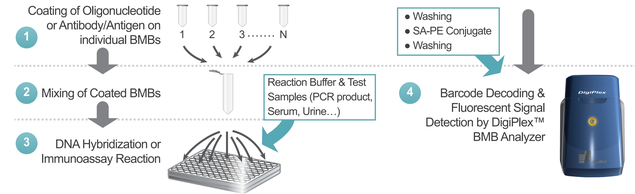
BMB Analyzer - DigiPlex™ Multiplex Analyzer

1 96-well plate holder

2 Optical module

3 DeXipher controlling software

Development and Performing the IntelliPlex™ BMB Assays



Assays

The IntelliPlex™ KRAS, BRAF, and EGFR Mutation Assays are based on target amplification by allele-specific PCR and detection by hybridization to BMBs coated with amplicon-specific probes.

All the three multiplex mutation assays are performed in a single-tube format both for the PCR amplification and BMB hybridization, and can not only detect but also differentiate all of the mutation types covered by the assays:

KRAS Mutation Assay

- G12A, G12D, G12V, G12C, G12R, and G12S on codon 12
- G13A, G13D, G13V, G13C, G13R, and G13S on codon 13

BRAF Mutation Assay

- V600E (V600E1 & V600E2), V600D, V600K, V600G, V600R, and V600M on codon 600

EGFR Mutation Assay

- G719S, G719C, G719A in exon 18
- 14 Deletion types, including the most frequently occurred deletions-Cosmic IDs 6223 & 6225, in exon 19
- Insertion, S768I, and T790M in exon 20
- L858R and L861Q in exon 21

Assay Performance

Analytical Sensitivity and Specificity of the IntelliPlex™ KRAS, BRAF, and EGFR Assays

KRAS Assay

BMB Probe	Genomic DNA								Mutant plasmid DNA						Neg Ctrl
	WT (50ng)	CCL155 (G12A) 1%	CRL2558 (G12D) 1%	CCL228 (G12V) 1%	CRL5807 (G12C) 1%	CCL255 (G12S) 1%	HCT116 (G13D) 1%	CRL5891 (G13C) 1%	G12R 1%	G13A 1%	G13V 1%	G13R 1%	G13S 5%		
G12A	0.5	5.0	0	0.7	0.5	0.2	0	0.2	0.8	0.7	0.6	0	0		0
G12D	0.4	1.0	1.6	0.7	0.6	0.7	0.5	0.3	1.2	0.6	1.3	1.3	0.4		0
G12V	0.1	0.7	0.1	2.8	0.3	0	1.1	0	0.1	0	0	0	0		0
G12C	0.1	0.8	0.6	0	6.0	0	0	0.6	0.9	0	0.8	0.5	0.1		0
G12S	0.6	0.9	1.4	0.8	0.8	2.0	0.8	0.6	0.9	0.7	0.6	0.7	0.8		0
G13D	0	1.4	0.4	0.9	0	0.9	1.4	0.4	0.7	0	0.1	0.8	0.6		0
G13C	0	0	0	0	0	0	0	8.6	0	0	0	0	0		0
G12R	0.6	0.8	1.0	0.8	0.8	0.6	1.1	0.4	2.7	0.3	0.2	0.6	1		0
G13A	0.4	0.9	0.3	0.3	1.1	0.7	0.1	0.4	1.0	3.5	0.7	0.2	0.2		0
G13V	0.1	0.3	0.3	0.2	0.1	0	0	0	0.2	0.3	2.4	0	0		0
G13R	0.1	0	0.5	0	0.6	0.3	0.4	0.5	0.8	0	0.6	7.5	0		0
G13S	0.2	0.2	0.6	0.5	0.3	0.4	0.2	0	0.3	1.0	0.1	0.4	4.2		0
Ref Gene Ctrl	11.3	2.0	11.1	11.0	11.0	11.1	12.0	10.0	2.5	10.9	6.2	4.4	10.8		0
Int Ctrl	0.8	2.5	2.2	2.5	2.0	2.6	2.6	0.2	0	0	0.3	3.6	2.1		3.1

BRAF Assay

BMB Probe	Genomic DNA		Mutant plasmid DNA							Neg Ctrl
	WT (50ng)	WiDr (V600E1) 1%	V600E1 1%	V600E2 1%	V600D 1%	V600G 1%	V600K 1%	V600R 1%	V600M 5%	
V600E/D	0	12.0	11.2	6.8	10.7	0	0.3	0	0	0
V600D	0	0.7	0.6	0.4	9.7	0	0.2	0	0.3	0
V600G	0	0.1	0.3	0.2	0.4	2.8	0.4	0.4	0.6	0
V600K	0	0.3	0.2	0	0.2	0	3.7	0	0	0
V600R	0	0	0	0	0	0	0	6.0	0	0
V600M	0	2.0	1.9	1.3	3.5	0	1.4	0.1	1.4	0
Ref Gene Ctrl	8.9	9.6	9.6	8.7	9.0	9.2	9.6	9.2	9.3	0
Int Ctrl	2.0	2.7	3.0	2.0	2.6	1.8	3.0	2.5	2.7	2.0

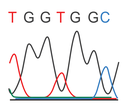
EGFR Assay

BMB Probe	Genomic DNA				Neg Ctrl
	WT (50ng)	SW48 (G719S/R252) 1%	CRL5883 (Exon19 Del/ 6223) 1%	CRL5908 (T790M/E240 & L858R/E224) 1%	
Cosmic ID 6252	0.6	2.7	0	0.4	0
6253	0.4	0.2	0.4	0.3	0
6239	0.2	0.3	0.2	0.3	0
6223	0.2	0.3	1.8	0.4	0
6225	0.1	0.1	0.2	0.2	0
26038	0.7	0.6	0.8	0.9	0
12678	0.4	0.2	0.4	0.5	0
12383	0	0	0	0	0
6218	0	0	0	0.1	0
6255	0.2	0.2	0.2	0.7	0
12370	0	0	0	0.1	0
12384	0.3	0.3	0.1	0.2	0
12382	0.1	0	0	0	0
12387	0.3	0.2	0.2	0.4	0
6210	0	0	0	0	0
6254/ 12369	0.4	0.4	0.3	0.2	0
6240	0.4	0.3	0.3	1.6	0
6241	0.2	0.3	0.5	0.5	0
12376	0	0.1	0.4	0.4	0
6224	0.1	0.3	0.5	1.6	0
6213	0	0.1	0	0	0
Ref Gene Ctrl	5.5	5.7	5.5	5.1	0
Int Ctrl	4.5	4.2	4.4	4.6	7.7

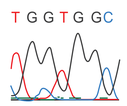
Wild-type genomic DNA alone or mutant cell-line genomic/plasmid DNA mixed in the wild-type genomic DNA at the specified percentages were first amplified by PCR and the resultant reaction products were then mixed with BMBs coated with different amplicon-specific probes for hybridization. After reaction with streptavidin-phycoerithrin (SA-PE) conjugate, the barcodes were decoded and hybridization signals acquired with the DigiPlex BMB analyzer. The S/CO ratios shown in the tables are derived by dividing sample median fluorescence intensity (MFI) signals over the predetermined cutoff MFI values for each different BMBs.

Comparison of the IntelliPlex™ Mutation Assays with Sanger Sequencing by Testing FFPE Tissue Samples from Non-Small Cell Lung Carcinoma (NSCLC), Colorectal Carcinoma (CRC), Melanoma, and Ovarian Carcinoma

Example Results



G (WT/WT)
FFPE CRC Sample #2



G>C (KRAS G12A/WT)
FFPE CRC Sample #7

BMB Probe	FFPE CRC Sample #2	FFPE CRC Sample #7
G12A	0.3	9.0
G12D	0.7	0.2
G12V	0.8	0.4
G12C	0.4	0.4
G12S	0.1	0.2
G13D	0.4	0.4
G13C	0.4	0.9
G12R	0.3	0.4
G13A	0.2	0.5
G13V	0	0
G13R	0	0.1
G13S	0.3	0.9
Ref Gene Ctrl	13.7	14.2
Int Ctrl	7.7	7.3

DNA extracted from FFPE samples by using QIAamp FFPE DNA kit was tested with the IntelliPlex™ KRAS, BRAF, or EGFR Mutation Kit. The determined mutation types were then compared against the types determined by Sanger sequencing.

Method Comparison Summary

Colorectal Carcinoma

IntelliPlex™ Mutation Kit		Sanger Sequencing: Dectected	Sanger Sequencing: Not Dectected
KRAS	Dectected	15	0
	Not Dectected	1	19
BRAF	Dectected	3	0
	Not Dectected	0	32

Ovarian Carcinoma

IntelliPlex™ Mutation Kit		Sanger Sequencing: Dectected	Sanger Sequencing: Not Dectected
KRAS	Dectected	7	0
	Not Dectected	0	5
BRAF	Dectected	0	0
	Not Dectected	0	12

Non-Small Cell Lung Carcinoma

IntelliPlex™ Mutation Kit		Sanger Sequencing: Dectected	Sanger Sequencing: Not Dectected
KRAS	Dectected	2	0
	Not Dectected	0	19
EGFR	Dectected	11	0
	Not Dectected	0	10

Melanoma

IntelliPlex™ Mutation Kit		Sanger Sequencing: Dectected	Sanger Sequencing: Not Dectected
BRAF	Dectected	2	0
	Not Dectected	0	4

Overall Clinical Performance

	IntelliPlex™ Mutation Assay		
	EGFR	KRAS	BRAF
Sensitivity	11/11 (100%)	24/25 (96%)	5/5 (100%)
Specificity	10/10 (100%)	43/43 (100%)	48/48 (100%)

Frequency of Mutations Determined by the IntelliPlex™ Mutation Kits

Colorectal Carcinoma		
KRAS	Frequency	%
G12D	4	12
G12A	3	9
G12V	2	6
G13D	6	24
Others	0	0
Total	15	43
WT	20	57
BRAF	Frequency	%
V600E	3	9
Others	0	0
Total	3	9
WT	32	91

Ovarian Carcinoma		
KRAS	Frequency	%
G12D	4	33
G12V	2	17
G12R	1	8
Others	0	0
Total	7	58
WT	5	42
BRAF	Frequency	%
V600E	0	0
Others	0	0
Total	0	0
WT	12	100

Non-Small Cell Lung Carcinoma		
KRAS	Frequency	%
G12D	1	5
G12C	1	5
Others	0	0
Total	2	10
WT	19	90
EGFR	Frequency	%
Exon18	0	0
Exon19	8 4 ID6223 2 ID12370 1 ID12383 1 ID12387	38
Exon20	0	0
Exon21	3 L858R/ ID6224	14
Others	0	0
Total	11	52
WT	10	48

Melanoma		
BRAF	Frequency	%
V600E	2	33
Others	0	0
Total	2	33
WT	4	67

Summary

We present here three highly multiplex assays, based on PCR amplification and barcoded magnetic bead (BMB) suspension array technologies, for detection and genotyping of mutations occurred in the hot spots of three oncogenic biomarkers (KRAS, BRAF, and EGFR) for lung, colorectal, melanoma, and ovarian cancers:

- **Single-tube multiplex format:**
  - KRAS assay: a total of 12 mutations on codon 12 and codon 13
  - BRAF assay: a total of 6 mutations on codon 600
  - EGFR assay: a total of 22 mutations in exons 18, 19, 20, and 21
- **Highly specific, highly sensitive, and no post-PCR purification required before hybridization detection**
- **QC controls (internal and reference gene controls) embedded and assayed together with the targets in the same reaction**
- **Highly concordant results on FFPE sample testing when using Sanger sequencing as the Gold Standard method for comparison**

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Intelligent Multiplexing Solutions for Better Health