

## SIMULTANEOUS DETECTION OF COLORECTAL CANCER MUTATIONS IN STOOL SAMPLES WITH BIOCHIP ARRAYS

### SIMULTANA DETEKCIJA MUTACIJA KOLOREKTALNOG KANCERA U UZORCIMA STOLICE POMOĆU BIOČIP SKUPOVA

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**Summary:** Colorectal cancer (CRC) is the second main cause of cancer-related death in the Western world and like many other tumours is curable if detected at an early stage. Current detection options include faecal occult blood testing and invasive direct visualisation techniques such as flexible sigmoidoscopy, colonoscopy and barium enema. The availability of a more simple, non-invasive test that detects tumour specific products with optimal analytical performance might overcome barriers among patients who are not willing to undergo more sensitive but invasive tests. One such emerging technology, which has shown promise in recent years, is the analysis of DNA alterations exfoliated from tumour cells into stool. Here we report an analytical platform for non-invasive detection of 28 common mutations within CRC-related genes *APC*, *TP53*, *K-ras* and *BRAF* in stool samples based on biochip array technology and applied to the semi-automated Evidence Investigator analyser. Mutation detection was possible in 1000-fold excess of wildtype DNA and analysis of 10 CRC-positive patient samples showed presence of targeted mutations with equivalent mutations also identified by an alternative method. This application represents an excellent tool for the multiplex detection of CRC-specific mutations using a single platform.

**Keywords:** biochip array technology, colorectal cancer, mutation, non-invasive, stool

### Introduction

Colorectal cancer (CRC) has a long pre-malignant phase and relatively slow progression from organ defined invasive disease to local and distant metastatic disease and as such there is ample

**Kratak sadržaj:** Kolorektalni kancer je drugi glavni uzrok smrtnosti usled raka u zapadnom svetu i poput mnogih drugih tumora izlečiv je ukoliko se otkrije u ranoj fazi. Trenutno dostupne mogućnosti detekcije uključuju testiranje okultnog krvarenja u stolici i invazivne tehnike direktne vizualizacije kao što su fleksibilna sigmoidoskopija, kolonoskopija i barijum enema. Dostupnost jednostavnijeg, neinvazivnog testa za otkrivanje produkata specifičnih za tumor uz optimalne analitičke performanse doprinela bi prevazilaženju barijera kod pacijenata koji se nerado podvrgavaju senzitivnijim ali invazivnim testovima. Takva tehnologija koja se poslednjih godina razvija i daje ohrabrujuće rezultate je analiza alteracija DNK koje iz ćelija tumora dospevaju u stolicu. U radu će biti predstavljena analitička platforma za neinvazivnu detekciju 28 uobičajenih mutacija u okviru gena povezanih sa kolorektalnim kancerom, *APC*, *TP53*, *K-ras* i *BRAF* u uzorcima stolice, zasnovana na tehnologiji biočip skupova primenjenoj na poluautomatskom analizatoru Evidence Investigator. Detekciju mutacija bilo je moguće uraditi u 1000-strukom višku DNK a analiza 10 uzoraka pacijenata pozitivnih na kolorektalni kancer je otkrila prisustvo ciljanih mutacija sa ekvivalentnim mutacijama takođe identifikovanim alternativnom metodom. Takva primena predstavlja sjajnu alatku za detekciju mutacija specifičnih za kolorektalni kancer pomoću jedinstvene platforme.

**Ključne reči:** tehnologija biočip skupova, kolorektalni kancer, mutacija, neinvazivni, stolica

opportunity to identify patients at a curable stage (1). Current tests are either non-specific as with faecal occult blood testing (2) or invasive. Analysis of DNA markers in stool represents an attractive alternative basis for the molecular detection of CRC (3).

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Abbreviations: CIN – Chromosomal Instability Pathway; CRC – Colorectal Cancer; MCR – Mutation Cluster Region; ME-PCR – Mutant-enriched Polymerase Chain Reaction; MPCR – Multiplex Polymerase Chain Reaction; PCR-RFLP – Polymerase Chain Reaction – Restriction Fragment Length Polymorphism; RLU – Relative Light Unit; WT – Wildtype.

CRC is a disease in which many DNA mutations associated with the process of carcinogenesis have been characterised (4). This characterisation makes it potentially valuable to examine stool for the presence of DNA with different mutations as indicators of both pre-clinical and clinical disease (5). This is important since no single mutation has been identified which is expressed across all colorectal cancers. DNA is seen as a good marker as it is stable in stool and can be assayed with sensitive techniques (6). DNA is also a consistent marker as it is shed continuously from colorectal cancer and its precursor polyps meaning only a single stool sample is required for analysis. Bleeding from cancers or polyps usually occurs only intermittently, requiring the collection of multiple samples for occult blood testing (5). Analysis also relies on functional, not spatial detection of polyps and cancers. By using a molecular profile rather than a physical shape, location or size, a reduction in false positives is thought to occur when compared to using direct visualisation techniques (5). Overall, assaying stool DNA is a much more patient-friendly option, as it is non-invasive, requires no unpleasant cathartic preparation and allows for off-site collection of samples (6).

The most common pathway of CRC development is the chromosomal instability (CIN) pathway, which includes point mutations that occur within *K-ras*/*BRAF*, *APC* and *TP53* genes (4, 7). The CIN pathway leads to about 85% of all CRCs that are primarily sporadic. While there are thousands of possible mutations a relatively small number of mutations are actually associated with the vast majority of lesions. More than 50% of colorectal cancers display specific mutations in the *K-ras* gene, with an increasing frequency in larger and more advanced lesions (8). In contrast to other genes involved in tumourigenesis, mutations in the *K-ras* gene occur almost exclusively in codons 12, 13 and 61 (>90% in codons 12 and 13) (2). *BRAF* somatic mutation presents in 15% of sporadic CRCs (9) with a single hotspot at codon 600 accounting for 80% of *BRAF* mutations in CRC (10). Mutations tend to occur in a mutually exclusive relationship with *K-ras* mutations (7, 9, 11). Mutations in *K-ras* predominantly occur during the transformation of small to intermediate adenomas and it has been demonstrated that *BRAF* mutations arise within a similar phase of CRC development (7, 11) albeit in a much small percentage of cases. Approximately half of all CRCs display *TP53* mutations, with higher frequencies observed in distal colon and rectal tumours and lower frequencies in proximal tumours (12). *TP53* mutations tend to occur in the late adenoma stage (13). Mutations in five hotspot codons (175, 245, 248, 273 and 282) account for approximately 43% of all *TP53* mutations in CRC (14–16). Somatic mutations in *APC* occur in about 75% of sporadic CRCs (17) and transpire early during tumourigenesis. Over 60%

of somatic mutations present within <10% of the coding sequence of the *APC* gene between codons 1286 and 1513 known as the mutation cluster region (MCR) (18). Within the MCR, there are also two hotspots at codons 1309 and 1450 (19).

Here we therefore describe a platform for the simultaneous detection of multiple DNA mutations within *K-ras*, *BRAF*, *TP53* and *APC* genes from stool samples by a combination of multiplex PCR, probe hybridisation, ligation, PCR amplification and biochip hybridisation. The latter stage is based on biochip array technology. This technology permits the simultaneous detection of multiple analytes within a single patient sample. This has implications with regards a reduction in sample/reagent consumption and as such the overall cost-effectiveness of assays. Applications of this methodology to protein and drug analysis have been previously described (20–24). The core of this technology is the biochip (9mm x 9mm), which represents not only the solid support, but also the vessel where hybridisation occurs. Chemiluminescent signal detection of array hybridisation and corresponding results are then processed on the Evidence Investigator semi-automated analyser.

## Materials and Methods

### *Patient samples and extraction of stool DNA*

CRC-positive human stool samples (n=10) were obtained from Medical Solutions (Nottingham, UK). Samples from apparently healthy volunteers (n=10) were collected in-house and frozen at -80 °C within 24 hours of defaecation. Long-term storage of all samples was maintained at -80 °C.

DNA was extracted from each specimen using the QIAamp DNA Stool Mini Kit (51504, Qiagen, Germany) following the manufacturer's protocol for the isolation of DNA from stool for human DNA analysis. 220 mg sections of stool were analysed per specimen in three different locations. Purified DNA was eluted in 200 µL of supplied elution buffer. DNA yield was quantified by ultraviolet spectrometry (260/280 nm). Long-term storage of extracted DNA was maintained at -20 °C. Triplicate aliquots of extracted stool DNA per patient sample were therefore available for downstream analysis.

### *Tumour cell lines and extraction of DNA*

Two colorectal adenocarcinoma cell lines were assessed (SW-480 ATCC No. CL-228 and HT-29 ATCC No. HTB-38). Both were purchased from ATCC and grown according to the supplier's instructions. DNA was extracted using the DNeasy Blood and Tissue kit (69504, Qiagen, Germany) and DNA yield quantified by ultraviolet spectrometry (260/280 nm). Long-term storage of extracted cell line DNA was maintained at -20 °C.

### RanplexCRC Array Analysis

Analysis of extracted DNA was performed using RanplexCRC Array Kit (EV3536A/B Randox Laboratories Ltd, Crumlin, UK) according to the manufacturer's instructions and as summarised below.

**Pre-enrichment.** Pre-enrichment of each replicate of extracted DNA per stool specimen was performed via a multiplex PCR (MPCR) reaction. Two MPCR reactions were carried out per DNA replicate. Reaction 1 (MPCR1) permits simultaneous amplification of *K-ras*, *BRAF* and *TP53* gene regions of interest with reaction 2 (MPCR2) amplifying the *APC* gene regions of interest. Amplification was achieved using Phusion High-Fidelity DNA Polymerase (F-530S, FINNZYMES, Finland). Products were visualised using agarose gel electrophoresis with ethidium bromide staining. Each positive MPCR reaction was purified using QIAquick PCR purification kit (28104, Qiagen, Germany). Purified DNA was eluted in 30 µL of 10 mmol/L Tris.Cl pH 8.5. Two MPCR purified reactions (MPCR1 and MPCR2) are therefore available per replicate of extracted stool DNA assessed.

**Hybridisation-ligation-PCR amplification.** Two hybridisation reactions were carried out per MPCR reaction available. Hybridisation of MPCR1 reactions (*K-ras/BRAF/TP53*) was carried out with RanplexCRC mutant probe mix 1 and RanplexCRC wildtype probe mix 1. MPCR2 (*APC*) reactions were hybridised with RanplexCRC mutant probe mix 2 and wildtype probe mix 2. After hybridisation for up to 16 hours at 60°C in a thermal cycler, a ligation step at 54°C followed. Aliquots of each ligation reaction were PCR amplified using RanplexCRC primers and AmpliTaq Gold (N808-0240, Applied Biosystems, USA). Products were visualised using agarose gel electrophoresis with ethidium bromide staining. A total of four hybridised-ligated-PCR amplified products are available per replicate of extracted stool DNA assessed.

**Biochip hybridisation.** Array hybridisation of hybridised-ligated-PCR amplified products is performed on two biochips. Biochip 1 corresponds to *K-ras/BRAF/TP53* targets (16 mutations; 3 WT controls) and biochip 2 to *APC* (12 mutations; 2 WT controls) as shown in Table I. Hybridisation was carried out for 1 hour at 60 °C in the thermoshaker provided with the system. Post-hybridisation stringency washes followed. Conjugation with streptavidin-HRP was then performed at 37 °C for 1 hour before chemiluminescence detection within Evidence Investigator semi-automated analyser (EV3602, Randox Laboratories Ltd., Crumlin, UK). Signal was expressed as relative light units (RLUs). Results were processed automatically using dedicated software.

### Mutant-enriched PCR

Mutant-enriched PCR (ME-PCR) analysis was performed on 10 CRC-positive patient samples to

**Table I** Targets detectable via RanplexCRC Array.

Gene	Mutation	WT control
<i>K-ras</i>	<i>K-ras</i> 12.2AL, <i>K-ras</i> 12.1AR, <i>K-ras</i> 12.2AS, <i>K-ras</i> 12.1CY, <i>K-ras</i> 12.1SE, <i>K-ras</i> 12.2VA, <i>K-ras</i> 13.2AS	<i>K-ras</i> 12.1WT
<i>BRAF</i>	BRAF600.2	BRAF600.2WT
<i>TP53</i>	<i>TP53</i> 175.2, <i>TP53</i> 245.1, <i>TP53</i> 245.2, <i>TP53</i> 248.1, <i>TP53</i> 248.2, <i>TP53</i> 273.1, <i>TP53</i> 273.2, <i>TP53</i> 282.1	<i>TP53</i> 175.2WT
<i>APC</i>	<i>APC</i> 876.1, <i>APC</i> 1306.1, <i>APC</i> 1309.1, <i>APC</i> 1309.5del, <i>APC</i> 1312.1, <i>APC</i> 1338.1, <i>APC</i> 1367.1, <i>APC</i> 1378.1, <i>APC</i> 1379.1, <i>APC</i> 1450.1, <i>APC</i> 1465.2del, <i>APC</i> 1554.1ins	<i>APC</i> 876.1WT, <i>APC</i> 1450.1WT

assess agreement with RanplexCRC Array generated results.

*K-ras* codon 12 together with *TP53* codons 175, 245, 248 and 273 were assessed using primers and corresponding restriction enzymes as previously detailed (25) with one adaptation in the initial assay pre-amplification step to include the addition of Phusion High-Fidelity DNA Polymerase (F-530S, FINNZYMES, Finland). Up to three subsequent PCR-RFLP enrichments were performed.

*K-ras* codon 13 and *BRAF* codon 600 analysis was carried out using the previously described method of (10).

*TP53* codon 282 and *APC* codons 876, 1306, 1309, 1312, 1338, 1367, 1378, 1379 and 1450 were assessed using in-house designed mutant-enriched primers and corresponding restriction enzymes. Enrichment was performed for these codons using the adapted method of Behn et al. (25).

Final restriction reactions were analysed on a 3% Nusieve gel (50081, Lonza, Rockland, USA) using ethidium bromide staining. Mutant-harboring products were easily distinguished from the remaining wildtype alleles given their different base pair (bp) lengths. Positive products were excised and forwarded for gel purification using MinElute Gel Extraction kit (28606, Qiagen, Germany).

### Sequencing

External automated sequencing of 1 ng/µL per 100bp of gel purified ME-PCR product was performed using relevant primers at 3 pmol and v3.1 Cycle Sequencing RR-100 (4336917, Applied Biosystems, USA) according to the manufacturer's protocol.

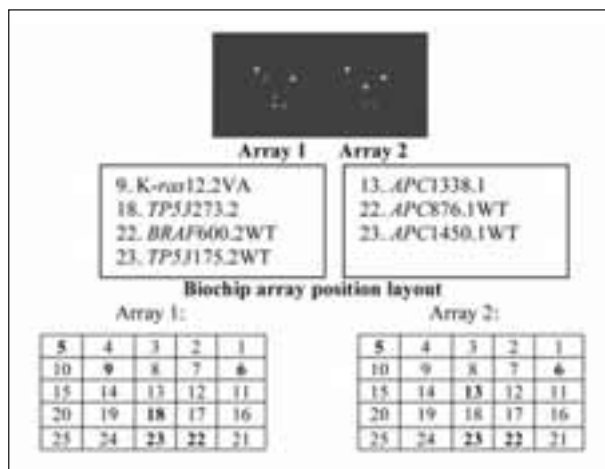
## Results

### Simultaneous detection of CRC-related mutations

Figure 1 illustrates an example of the simultaneous detection of the targeted mutations and wild-type controls with a single sample and Table II shows the corresponding RLU values for the CRC biochip arrays.

### Probe/Array Specificity

Specificity of each target probe combination was confirmed via the use of target-specific complementary single-stranded synthetic oligonucleotides. Each of them was assessed under multiplex probe conditions through hybridisation-ligation-PCR amplification and biochip hybridisation confirming detection of each specific target. Each relevant target specific hybridisation-ligation-PCR product ( $\geq 100$ bp) was generated and no misligation or biochip cross-hybridisation was observed. Specificity of the unique tag array was confirmed through hybridisation of each target specific hybridised-ligated PCR product and also via hybridisation of complementary biotin-labelled tag sequences. Each individual array tag position was detected accordingly with no cross-hybridisation noted. Figure 2 illustrates some examples.



**Figure 1** Example of an image of CRC biochip arrays from a sample.

### Sensitivity

Two colorectal adenocarcinoma cell lines SW-480 and HT-29 covering 5 assay targets (*K-ras*12.2VA, *BRAF*600.2, *TP53*273.2, *APC*1338.1, *APC*1554.1ins) were used to confirm assay sensitivity. DNA from these cell lines was separately spiked into wildtype DNA (WT DNA) at various ratios from 1:1 to 1:1000 using a total volume of 100 ng of DNA. Mutant targets were detected in the presence of 1000-fold excess WT DNA.

### Patient Samples Analysis

No mutations were identified within the control samples assessed ( $n=10$ ). Mutant targets were detected in eight out of ten of the CRC-positive samples analysed, as shown in Table III. Mutations were present in three out of the four genes assessed *APC*, *TP53* and *K-ras*. A patient sample (sample number 4) also produced multiple mutations within *TP53* plus a single *APC* mutation.

Equivalent mutations as generated by CRC biochip arrays were identified by mutant-enriched PCR (refer to Table III). Three out of the twenty-eight array mutational targets could not be assessed via ME-PCR, *APC*1309.5del, *APC*1465.2del and *APC*1554.1ins. An *APC* 1309 mutation, which is not present on the CRC biochip arrays, was also identified via ME-PCR for a single patient sample (sample number 5).

Sequencing data confirmed base change and examples of ME-PCR sequencing data are shown in Figure 3.

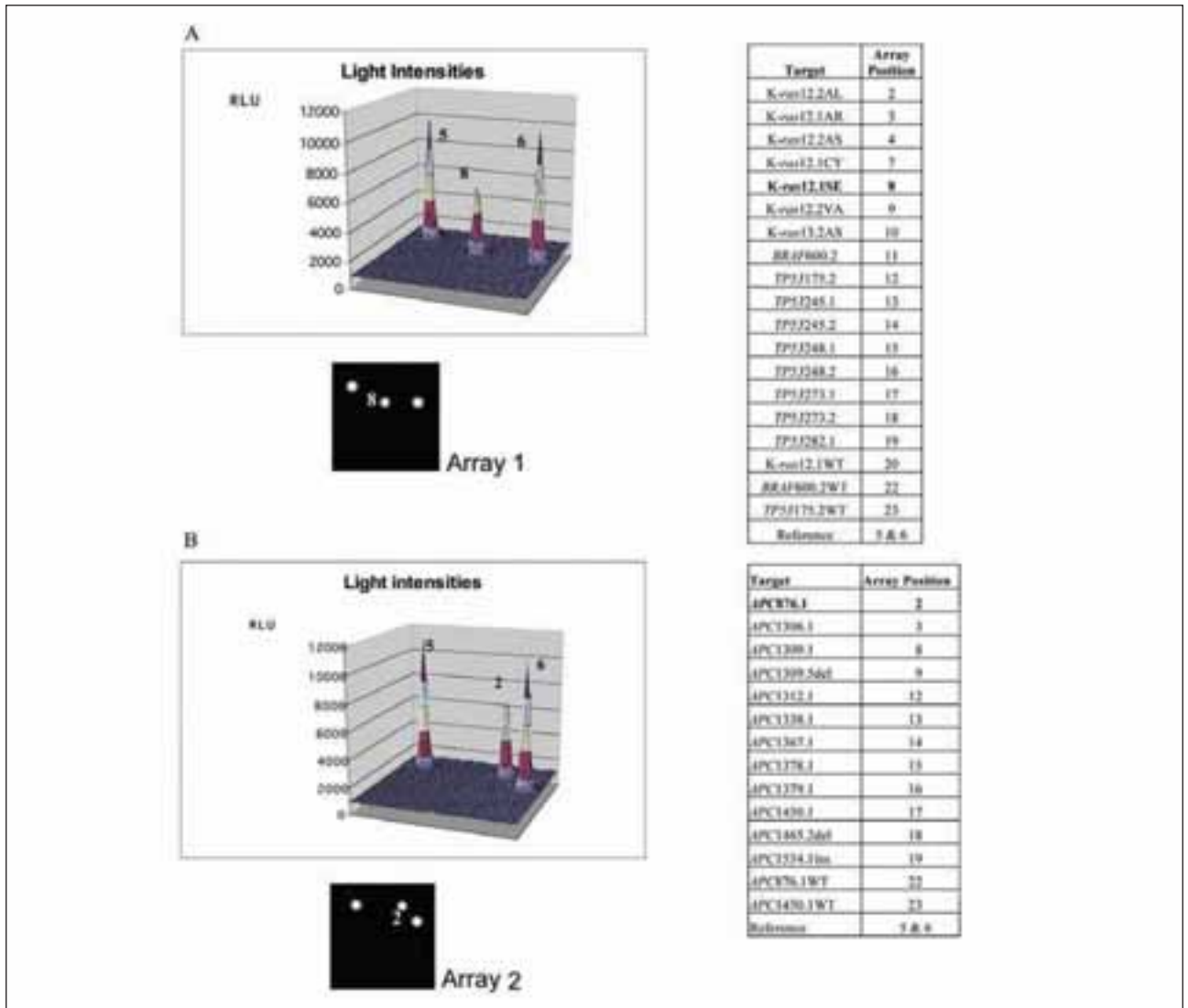
## Discussion

To our knowledge, this is the first analytical evaluation of the application of biochip array technology to DNA analysis on the semi-automated Evidence Investigator analyser. The technology enables simultaneous specific detection of up to 28 CRC-specific mutations within a single patient stool sample in less than 48 hours. This is due to the unique combination of probe design, sensitivity of ligase for a mismatch next to the ligation site, single PCR primer

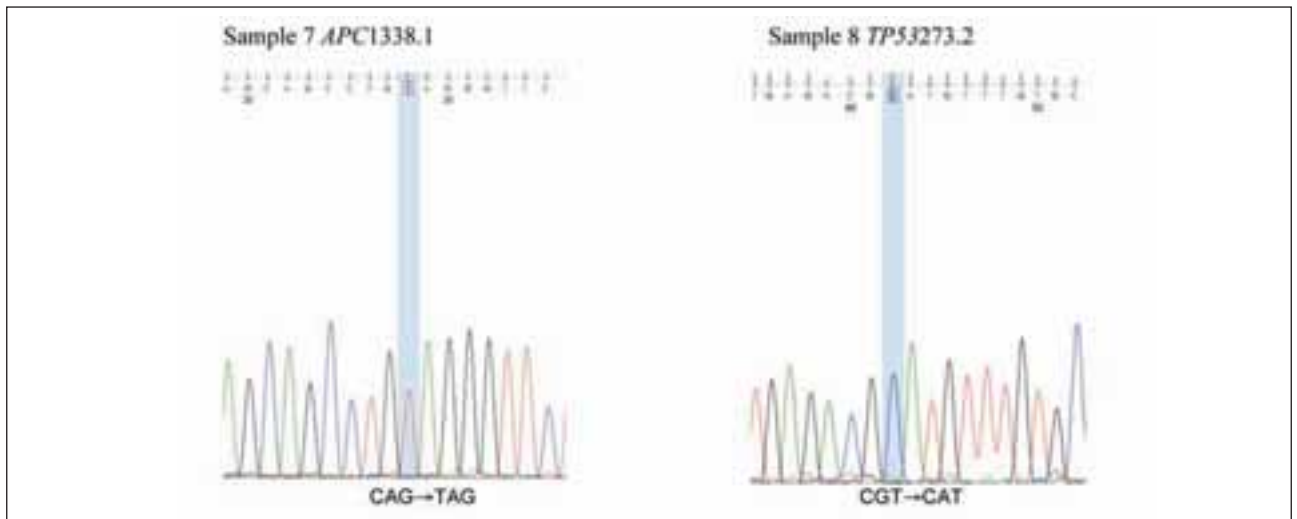
**Table II** Corresponding RLU values for the positive targets on the CRC biochip arrays from a sample.

Target	Array 1 Position	RLU	Target	Array 2 Position	RLU
<i>K-ras</i> 12.2VA	9	1816	<i>APC</i> 1338.1	13	3616
<i>TP53</i> 273.2	18	1985	<i>APC</i> 876.1WT	22	1132
<i>BRAF</i> 600.2WT	22	2207	<i>APC</i> 1450.1WT	23	1167
<i>TP53</i> 175.2WT	23	2500	Reference	5&6	
Reference	5&6				





**Figure 2** Examples of array/probe specificity. A: Array 1 (K-ras/BRAF/TP53) specific detection of K-ras 12.1SE. B: Array 2 (APC) specific detection of APC876.1.



**Figure 3** Examples of ME-PCR sequencing chromatograms.

**Table III** CRC biochip arrays and ME-PCR data from CRC-positive patient samples.

Sample No.	Disease State	RanplexCRC Array Mutations				ME-PCR Mutations			
		<i>K-ras</i>	<i>BRAF</i>	<i>TP53</i>	<i>APC</i>	<i>K-ras</i>	<i>BRAF</i>	<i>TP53</i>	<i>APC</i>
1	Cancer	<i>K-ras</i> 12.1AR	–	–	–	<i>K-ras</i> 12.1AR	–	–	–
2	Cancer	–	–	–	<i>APC</i> 1338.1	–	–	–	<i>APC</i> 1338.1
3	Cancer	–	–	–	<i>APC</i> 1338.1	–	–	–	<i>APC</i> 1338.1
4	Cancer	–	–	<i>TP53</i> 175.2 <i>TP53</i> 245.1 <i>TP53</i> 273.1 <i>TP53</i> 273.2	<i>APC</i> 1338.1	–	–	<i>TP53</i> 175.2 <i>TP53</i> 245.1 <i>TP53</i> 273.1 <i>TP53</i> 273.2	<i>APC</i> 1338.1
5	Cancer	–	–	<i>TP53</i> 273.2	–	–	–	<i>TP53</i> 273.2	<i>APC</i> 1309.2*
6	Cancer	–	–	–	<i>APC</i> 1306.1	–	–	–	<i>APC</i> 1306.1
7	Cancer	–	–	–	<i>APC</i> 1338.1	–	–	–	<i>APC</i> 1338.1
8	Cancer	–	–	<i>TP53</i> 273.2	–	–	–	<i>TP53</i> 273.2	–
9	Cancer	–	–	–	–	–	–	–	–
10	Cancer	–	–	–	–	–	–	–	–

\*not present on RanplexCRC panel

pair usage and tag array. Furthermore, the addition of an initial pre-enrichment stage enhances the sensitive detection of tumour-specific products against the high background of non-specific DNA present in stool, enabling mutation detection in a 1000-fold excess of WT DNA presence.

Mutations were detected in 8 out of 10 CRC-positive patient samples assessed and were predominantly within the *APC* gene. A single patient sample produced multiple mutations within *TP53* plus a single *APC* mutation (sample number 4). Detection of multiple mutations within a single gene and across several genes has been observed previously (26–28). Array mutations were not detected for two CRC-positive patient samples. This result does not rule out the possibility that a mutation may be present within these samples as the CRC biochip arrays assess for the 28 common ‘hotspot’ mutations for CRC. No mutations were identified within the control specimens.

The equivalent CRC-positive samples were subsequently analysed via the conventional ME-PCR method. A total of 17 codons were assessed for each patient sample confirming presence of mutant and/or WT targets. ME-PCR analysis resulted in the detection of equivalent targets as generated by the CRC biochip arrays. Furthermore an *APC* codon 1309 mutation (GAA→GCA) was identified within a single patient sample (sample number 5), which is not present on the CRC biochip arrays panel. This patient sample also produced a *TP53* codon 273 mutation which was confirmed with CRC biochip arrays.

Current systems for the detection of CRC-related mutations include the lengthy mutant-enriched PCR method (10, 25). Such procedures

require numerous PCR-RFLP stages in a bid to ‘enrich’ for mutant targets against the background of WT alleles. Confirmation of mutations obtained is then performed via automated sequencing further increasing the time required to complete the assay. ME-PCR is also known to be prone to false-positive results due to the numerous stages of PCR and inherent error rate of *Taq* polymerase (29–30).

Several faecal DNA tests are also currently available on the market (31–35) and most are based on the detection of multiple molecular markers for CRC including point mutations within *K-ras*, *BRAF*, *TP53* and *APC*. Analysis includes numerous different technologies such as real-time PCR, single-base extension, variation scanning technology and automated sequencing (31–34). Interpretation of results must therefore be performed individually for each technique rather than on a single platform as with the RanplexCRC Array panel. Moreover, with the biochip arrays reported here, full assessment is completed in the individual laboratory.

Additionally, genotyping of tumours has proven valuable in identifying genes whose alterations are associated with primary or acquired resistance to targeted therapies (36). Recent studies indicate that patients with metastatic CRC carrying tumours with *K-ras* mutations do not respond to monoclonal antibody treatment (cetuximab and panitumumab) (37, 38). The presence of *K-ras* mutations could therefore potentially be used routinely to select patients eligible for cetuximab and panitumumab treatment (36). In the future, identification of a patient specific mutational profile through analysis of stool and/or tumour could therefore be used to provide a personalised CRC treatment program.

In conclusion, data obtained clearly demonstrated the detection of mutations within CRC-related genes from CRC-positive single stool specimens using biochip array technology. The availability of such a non-invasive test that can detect tumour specific products with optimal analytical performance may be beneficial among patients who are unwilling to undergo more invasive procedures. Furthermore this

type of assay may improve the overall cost-effectiveness of screening for CRC by limiting the need for colonoscopy strictly to individuals with adenomatous polyps or cancer identified through this method (5). Further long term testing of altered DNA in stool samples may also decrease the number of surveillance colonoscopies needed after therapy for colonic neoplasia (5).

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