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Original Article

Development and validation of a TaqMan Array for cancer mutation analysis

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ABSTRACT

Introduction: Optimal cancer treatment with targeted agents requires rapid, comprehensive and accurate molecular assays to analyse actionable oncogenic mutations across multiple tumour types.
Materials and Methods: We describe a PCR panel based on the 384 well TaqMan Array® (Thermo Fisher Scientific). This allows measurement of common RAS (NRAS and KRAS), EGFR and BRAF mutations in a single assay (the REB Array), analysing 44 mutations in 7 samples per plate. This retrospective study includes 96 patients with NSCLC (n = 42), colorectal cancer (n = 26), and melanoma (n = 28) with previous mutational analysis. Samples with discrepant results were sequenced to confirm the result.
Results: The REB achieved 93% concordance with the Therascreen EGFR assay (Qiagen), 95% concordance with the KRAS castPCR assay (Thermo Fisher), and 100% concordance with the cobas BRAF assay (Roche). There were 2 true discrepancies, most likely a result of sample quality or differences in sensitivity between the assays that depend on set thresholds to determine the presence of mutations. Analysis of the performance of the REB Array gave an overall sensitivity of 92%, with a positive predictive value of 100% and negative predictive value of 84.24%.
Conclusion: The REB array is comparable to competing PCR methods with the additional advantages of a broader range of mutations, simplified manual handling, and reduced overall cost per sample.

1. Introduction

The identification of mutations within an expanding number of human cancers is important to guide choice of treatment, and pathology departments are increasingly expected to provide rapid mutation detection.

Activating mutations in EGFR allow patients to be treated with EGFR inhibitors such as gefitinib (Iressa) or erlotinib (Tarceva). In the same patients, identifying those with RAS mutations suggests that further testing would not be beneficial as these are driver mutations and mutually exclude other targetable mutations. In colorectal cancer, KRAS, NRAS and probably BRAF indicate a lack of response to anti-EGFR antibody therapy [1]. BRAF is measured in all melanoma patients with metastatic disease to identify those that may benefit from vemurafenib and other BRAF inhibitors [2,3]. While other mutations in melanoma are of importance, around 40% of patients will have BRAF mutations and do not require further testing for other mutations.

While larger centres are turning to next-generation sequencing (NGS) to assess mutations within cancers, most of the information obtained is not currently actionable, and is not required routinely by clinical teams. As more targeted agents come onto the market, there will be a need to identify mutations in a large number of genes, but this is not yet the case, and single gene PCR methods for the detection of common mutations still have an important role. Many PCR based assays for cancer markers exist; however, they typically suffer

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Table 1
Complete list of castPCR assays incorporated within the TLDA card.

Column	Row	HGVS	Row	HGVS
1	**A**	EGFR_rf	**B**	KRAS c.35G>C p.Gly12Ala
2	**A**	EGFR c.2238_2252del15 p.L747_T751delLREAT	**B**	KRAS c.34G > C p.Gly12Arg
3	**A**	EGFR c.2573T>G p.Leu858Arg; EGFR c.2572_2573CT>AG p.Leu858Arg	**B**	KRAS c.436G>A p.Ala146Thr
4	**A**	EGFR c.2369C>T p.Thr790Met	**B**	KRAS c.37G>T p.Gly13Cys
5	**A**	EGFR c.2582T>A p.Leu861Gln	**B**	KRAS c.183A>C p.Gln61H
6	**A**	c.2303G > T p.Ser768Ile	**B**	KRAS c.182A>T p.Gln61Leu
7	**A**	EGFR c.2156G>C Gly719Ala	**B**	KRAS c.37G>C p.Gly13Arg
8	**A**	EGFR c.2155G>A p.Gly719Ser	**B**	NRAS_rf
9	**A**	EGFR c.2155G>T P.Gly719Cys	**B**	NRAS c.37G>C p.Gly13Arg
10	**A**	EGFR c.2125G>A p.Glu709Lys	**B**	NRAS c.34G>T p.Gly12Cys
11	**A**	EGFR c.2126A>C p.Glu709Ala	**B**	NRAS c.34G>A p.Gly12Ser
12	**A**	EGFR c.2311_2312insGCGTGGACA	**B**	NRAS c.38G > A p.Gly13Asp
13	**A**	EGFR c.2319_2320ins9	**B**	NRAS c.182A>T p.Gln61Leu; NRAS c.181_182CA>TT p.Gln61Leu
14	**A**	BRAF_rf	**B**	NRAS c.35G>A p.Gly12Asp
15	**A**	BRAF c.1799T>A p.Val600Glu	**B**	NRAS c.181C>A p.Gln61Lys
16	**A**	BRAF c.1798_1799GT > AA p.Val600Glu	**B**	NRAS c.182A>G p.Gln61Arg; NRAS c.181_182CA>AG p.Gln61Arg
17	**A**	BRAF c.1798_1799GT>AG p.Val600Arg	**B**	NRAS c.38G>T p.Gly13Val
18	**A**	BRAF c.1799_1800TG>AT p.Val600Asp	**B**	NRAS c.35G>T p.Gly12Val
19	**A**	KRAS_rf	**B**	NRAS c.183A>T p.Gln61His
20	**A**	KRAS c.35G>A p.Gly12Asp	**B**	NRAS c.183A>C p.Gln61His
21	**A**	KRAS c.35G>T p.Gly12Val	**B**	NRAS c.35G>C p.Gly12Ala
22	**A**	KRAS c.38G > A p.Gly13Asp	**B**	NRAS c.37G>T p.Gly13Cys
23	**A**	KRAS c.34G>T p.Gly12Cys	**B**	NRAS c.37G>A p.Gly13Ser
24	**A**	KRAS c.34G>A p.Gly12Ser	**B**	NRAS c.38G>C p.Gly13Ala

from either low sensitivity or are complicated to set up. Many only analyse a limited range of mutations in one gene, so that several tests must be performed to cover several genes, adding significant expense and time to the diagnostic pathway. We have therefore developed a simple PCR-based array system to cover all of the common mutations in four actionable genes for colorectal cancer, lung cancer, and melanoma, using a small amount of DNA that would be readily obtainable from biopsy as well as surgical material. The immediate need for these patients is to identify mutations in EGFR, KRAS, NRAS, and BRAF. We have previously used 384 well TaqMan arrays® (Thermo Fisher Scientific, Paisley, UK) to study gene expression in cancer [4–6] and they have been used for miRNA research for many years [7,8]. We realised that these had the potential to detect common mutations in several genes using a single array. This would not only streamline workflow in the laboratory but could also identify patient samples that may benefit from next-generation sequencing and other molecular methods to identify less common actionable mutations or gene amplification.

The TaqMan Array (Thermo Fisher Scientific, Paisley, UK) was developed some 10 years ago by Applied Biosystems Inc., now part of Thermo Fisher Scientific. It consists of a microfluidic card with 384 wells fed by eight ports. Each well is manufactured with primers and probes lyophilised within it. The volume of each well is 1 µL, and the cards can be run on a variety of machines available from the manufacturer. The REB Array (RAS-EGFR-BRAF) custom TaqMan Array card is intended for the detection of 44 somatic mutations in RAS (KRAS and NRAS), EGFR and BRAF oncogenes (Table 1). TaqMan® Mutation Detection Assays (incorporated into the TaqMan Array card) were designed based on competitive allele specific TaqMan® PCR (castPCR™) technology, which combines allele specific TaqMan® qPCR with allele-specific MGB blocker oligonucleotides that effectively suppress nonspecific amplification from the off-target allele. This gives enhanced specificity and sensitivity. The KRAS and BRAF assays we chose for the plate were the same as those used in our previous castPCR plates [9], using a series of patient samples with known mutational status.

In this study, we compared the newly designed REB array to commercial (Therascreen and cobas) and in house PCR assays currently in use to provide mutation analysis for patients with colorectal cancer, non-small cell lung cancer (NSCLC) and melanoma.

2. Materials and Methods

2.1. Design of REB Arrays

Plates were designed as shown in Table 1. The COSMIC database was interrogated to identify mutations that were most commonly reported. CastPCR assays for each of these were identified from the Life Technologies website (http://www.lifetechnologies.com/it/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-mutation-detection-assays/somatic-mutation-real-time-pcr.html) and used to populate a spreadsheet, which was used to manufacture the array cards. TaqMan® Mutation Detection Assays for the detection of mutant alleles must be run in parallel with corresponding wild type allele assays. This acts as a positive control for the wild-type DNA. For each gene, a reference assay was therefore required. Each well on the 384 well REB Array contains a separate assay (Fig. 1), allowing detection of a large number of targets without multiplexing. The card is divided along the rows into 8 separate channels, each containing 48 wells housing separate castPCR assays (four reference wild-type assays, 44 mutant assays) into which a single sample is loaded with a single pipetting step. This elimination of individual well pipetting dramatically reduces the risk of contamination or operator error. As each port uses 48 wells, and the individual assays are very reliable, a single well is sufficient for each assay. It is, however, good practice to run control plates at regular intervals, particularly with new plate or reagent batches. If duplicates or triplicates for individual samples or controls are required, two or three ports respectively must be used.

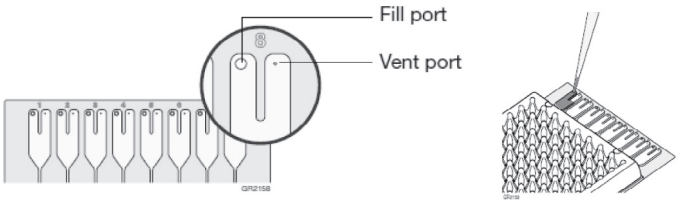

Fig. 1. Diagram of the TaqMan Array fill port and filling procedure (with permission from Thermo Fisher).

2.2. Patients and samples

We retrospectively identified a series of 96 patients with known mutational status and either extracted DNA from remaining formalin-fixed, paraffin-embedded FFPE blocks, or used stored DNA from the previous diagnostic PCR assays to validate the REB array, the characteristics of which are summarised in Table 2 according to the BRISQ (Biospecimen reporting for improved study quality) guidance [10]. Our study conforms to the tenets of the Declaration of Helsinki. Due to its retrospective nature for laboratory developed test validation, UK Health Research Authority Ethics Committee approval was not required. Consent for publication is not required as long as the patients are not identifiable.

FFPE blocks were identified for a series of patients with NSCLC (n = 42), colorectal cancer (n = 26), and melanoma (n = 28) for which mutational analysis had previously been performed using Therascreen or castPCR. It should be noted that these were not consecutive or randomly selected patients, but were instead chosen to reflect the range of mutations seen in our laboratory, for the purpose of clinical validation of the REB array from samples with sufficient remaining tissue surplus to diagnostic requirements. Where it was necessary to obtain further DNA, the same block was used when this could be identified from the pathology report.

2.3. DNA extraction

Areas of high cellularity cancer were identified by a histopathologist and marked on a slide. These were matched with the corresponding block and case. Samples from the areas marked were punched out using a 1 mm diameter skin punch, as previously described [9]. The samples were then placed in the vial of a Maxwell DNA extraction robot (Promega, Southampton, UK) and DNA extracted according to the manufacturer's instructions. Stored or newly extracted DNA was checked for content and quality using a Nanodrop or Qubit instrument (both from Thermo Fisher Scientific) according to the manufacturer's instructions.

2.4. REB arrays

Individual samples were diluted with MM and dH_2O to give a concentration of 500 ng/mL in a volume of 100 μL (total DNA load 50 ng). After mixing, 100 μL of each sample extract was added to each port of a REB array. The array plates were sealed, spun and placed in a previously calibrated ViiA7 PCR machine (Thermo Fisher Scientific). Standard PCR conditions were used as follows: 50 °C for 2 min, followed by 95 °C for 1 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each run took 118 minutes to complete.

For efficient use of the REB arrays, samples must be grouped into batches of 7 (to fill the 8 channel card, 7 samples + 1 negative control). Smaller batch sizes require empty channels to be loaded with master mix before running, to avoid inaccurate data analysis by the ViiA7 software.

2.5. Therascreen EGFR RGQ PCR

The Therascreen assay (Qiagen Ltd, Manchester, UK) requires the preparation of eight different master mixes for the detection of the full panel of mutations the assay covers, and was performed according to the manufacturer's instructions. The Therascreen assay

Table 2
BRISQ summary of sample characteristics [10].

Data Elements	Examples
Biospecimen type	Solid tissue
Anatomical site	Variable – colorectal cancer, melanoma primary or lymph node, lung cancer primary or lymph node metastasis
Disease status of patients	Patients with colorectal cancer, melanoma, or NSCLC
Clinical characteristics of patients	No previous chemotherapy or targeted therapy
Vital state of patients	Alive – biopsy material
Clinical diagnosis of patients	Colorectal cancer, melanoma, or NSCLC
Pathology diagnosis	Colorectal cancer, melanoma, or NSCLC
Collection mechanism	Surgical, endoscopic or needle biopsy
Type of stabilisation	10% neutral buffered formalin
Type of long-term preservation	Formalin fixation and Paraffin embedding
Constitution of preservative	10% neutral-buffered formalin
Storage temperature	20 to 25 °C
Storage duration	Up to 3 years
Shipping temperature	Not applicable
Composition assessment and selection	Minimum 10% neoplastic cells, material in block or extracted DNA surplus to diagnostic requirements

requires the preparation of eight master mixes (seven mutation detection assays and one control assay), giving a total DNA requirement of 80 ng. The assays were run in a Qiagen RotorGene QPCR instrument, and analysed according to manufacturer's instructions.

2.6. Life Technologies KRAS castPCR

The castPCR assays were performed as recently reported [9], using standard operating procedures. Fifty nanograms of gDNA is required per reaction and up to 6 samples can be run per plate. The castPCR assay format is a predesigned 96-well plate in which the wells are preloaded with the primers and probes for mutation and control assays (16 in total); therefore, the only reagents that need to be prepared are PCR master mix and gDNA from each of the samples to be analysed. Every sample and control must be analysed by all 16 assays (20 μL per reaction), and therefore the PCR master mix and gDNA are combined and diluted with nuclease free dH_2O to achieve the correct concentration and volume. The plates were run in a Life Technologies ViiA7 instrument (Thermo Fisher Scientific) according to manufacturer's instructions.

2.7. IonTorrent next generation sequencing

Sequencing was performed as previously described [11], with the following changes to accommodate IonChef loading of the IonTorrent 316 or 314 chips. Stored or newly extracted DNA was checked for content and quality using a Qubit 2.0 Fluorometer. Ten nanograms of gDNA from each of the samples chosen for NGS analysis was combined with the Ampliseq™ reagents and primer pool for the Onconetwork 22 gene panel [11] and amplified for 22 cycles. After initial amplification, the amplified products are partially digested before IonExpress Adapters and Barcode sequences are ligated to the library fragments. Following barcoding, the libraries are cleaned up using a magnetic bead method. The cleaned up products are then quantified by the Ampliseq Q-PCR method. Once the libraries have been successfully quantified they are combined and diluted to 50 pM. For IonTorrent 314 chips, 3 libraries were combined per chip. These library pools were then loaded into the IonChef instrument for further library preparation and chip loading. The loaded 314 chips were then run on the IonTorrent PGM instrument according to manufacturer's instructions. The Variant Caller plugin (included in the provided Ion Suite software) was used to analyse the aligned sequence data for the identification of hotspot mutations and novel variants.

2.8. Data analysis

The ViiA7 software produces a spreadsheet of Ct values for each well, clearly identified by sample number. The results from the spreadsheets were then collected into a further summary spreadsheet in Excel (Microsoft). The following exclusions were applied:

- Samples with insufficient DNA content (<50 ng total card input) were excluded from the analysis.
- Samples where one or more controls failed were excluded from the analysis as failed samples.
- Samples giving positive Ct values for targets on genes not present in the comparator assays were excluded from analysis as the significance of these results could not be determined.

Cases with discrepant results were sequenced on the IonTorrent PGM using the Onconetwork 22 gene panel [11]. Descriptive statistics, including sensitivity and specificity, were produced using Excel.

3. Results

A total of 96 cases were included in the study, of which 26 had colorectal cancer, 42 had lung cancer, and 28 had melanoma. A summary of the results for all three tumour types is shown in Table 3 and the accompanying STARD diagram (Fig. 2). Analysis of the performance of the REB Array gave an overall sensitivity of 92%, with a positive predictive value of 100% and negative predictive value of 84.24%. Discrepant cases were identified and subjected to further analysis as described below.

3.1. Lung cancer

A total of 42 samples were tested with 50 ng input DNA. Of these, 39/42 (92.9%) matched the results of previous Therascreen (Qiagen) testing for EGFR. The three discordant samples were submitted to sequencing using the IonTorrent 22 gene panel with 10 ng input DNA as previously described [11].

In one sample, the T790M mutation detected by Therascreen was not identified by REB array or IonTorrent NGS. The REB array did detect a KRAS mutation (c.34G > C) with Ct < 36.5, confirmed by IonTorrent NGS (COS518). This is regarded as a true discrepancy, incorrectly allocated by Therascreen, but correctly by REB array.

A second sample was reported as wild-type by the REB array, but had an Exon 20 mutation by Therascreen. IonTorrent sequencing detected the Exon 20 mutation (c.2307_2308insGCCAGCGTG), matching the Therascreen result with 57% of 1520 reads. This is a rare insertion, with just 10 entries on COSMIC, and was not included in the REB arrays assays, and does not represent a true discrepancy. The Therascreen kit manual does not specify exactly which insertions Therascreen can detect, but says "3 insertions in exon 20 (detects the presence of any of 3 insertions, but does not distinguish between them)".

A third sample was reported as wild-type by the REB array, but had a S768I mutation (c.2303G > T) by Therascreen. The sample was wild-type by Ion Torrent for EGFR. This is regarded as a true discrepancy, incorrectly allocated by Therascreen, but correctly by REB array.

After incorporating the findings of NGS analysis, the final concordance of the REB Array for EGFR was 40/42 (95.2%). Sensitivity and specificity for EGFR are therefore 92% and 100% respectively, with a negative and positive predictive value of 93% and 100% respectively.

Table 3
Summary of mutations found by tumour type and gene. One lung cancer had two EGFR mutations, L861Q and G719X, so that a total of 19 mutations were reported from 42 cases, with 24 wild-type (WT) results. In colorectal cancer, of 19 samples with good quality DNA, 7 were WT with 10 KRAS and 2 NRAS mutations.

Gene	HGVS	Protein	Lung	Colorectal	Melanoma
EGFR	WT	WT	24	7	8
	EGFR c.2238_2252del15 p.L747_T751delLREAT	Deletion	9		
	EGFR c.2573T>G p.Leu858Arg; EGFR c.2572_2573CT>AG p.Leu858Arg	L858R	1		
	EGFR c.2369C>T p.Thr790Met	T790M			
	EGFR c.2582T>A p.Leu861Gln	L861Q	2		
	c.2303G > T p.Ser768Ile	S768I	1		
	EGFR c.2156G>C Gly719Ala	G719A			
	EGFR c.2155G>A p.Gly719Ser	G719S	1		
	EGFR c.2155G>T P.Gly719Cys	G719C			
	EGFR c.2125G>A p.Glu709Lys	E709K			
	EGFR c.2126A>C p.Glu709Ala	E709A			
	EGFR c.2311_2312insGCGTGGACA	D770_N771insSVD			
	EGFR c.2319_2320ins9	H773_V774insNPH			
BRAF	BRAF c.1799T>A p.Val600Glu	V600E			11
	BRAF c.1798_1799GT > AA p.Val600Glu	V600E			1
	BRAF c.1798_1799GT>AG p.Val600Arg	V600R			
	BRAF c.1799_1800TG>AT p.Val600Asp	V600K			
KRAS	KRAS c.35G>A p.Gly12Asp	G12D	1	4	
	KRAS c.35G>T p.Gly12Val	G12V		1	
	KRAS c.38G > A p.Gly13Asp	G13D		2	
	KRAS c.34G>T p.Gly12Cys	G12C	2	1	
	KRAS c.34G>A p.Gly12Ser	G12S			
	KRAS c.35G>C p.Gly12Ala	G12A		2	
	KRAS c.34G > C p.Gly12Arg	G12R	1		
	KRAS c.436G>A p.Ala146Thr	A146T			
	KRAS c.37G>T p.Gly13Cys	G13C			
	KRAS c.183A>C p.Gln61H	Q61H			
	KRAS c.182A>T p.Gln61Leu	Q61L			
	KRAS c.37G>C p.Gly13Arg	G13R	1		
NRAS	NRAS c.37G>C p.Gly13Arg	G13R			1
	NRAS c.34G>T p.Gly12Cys	G12C			
	NRAS c.34G>A p.Gly12Ser	G12S			
	NRAS c.38G > A p.Gly13Asp	G13D			
	NRAS c.182A>T p.Gln61Leu; NRAS c.181_182CA>TT p.Gln61Leu	Q61L			
	NRAS c.35G>A p.Gly12Asp	G12D		1	
	NRAS c.181C>A p.Gln61Lys	Q61K		1	1
	NRAS c.182A>G p.Gln61Arg; NRAS c.181_182CA>AG p.Gln61Arg	Q61R			6
	NRAS c.38G>T p.Gly13Val	G13V			
	NRAS c.35G>T p.Gly12Val	G12V			
	NRAS c.183A>T p.Gln61His	Q61H			
	NRAS c.183A>C p.Gln61His	Q61H			
	NRAS c.35G>C p.Gly12Ala	G12A			
	NRAS c.37G>T p.Gly13Cys	G13C			
	NRAS c.37G>A p.Gly13Ser	G13S			
	NRAS c.38G>C p.Gly13Ala	G13A			

3.2. Colorectal cancer

For colorectal cancer, 26 samples were tested, but 7 samples were later found to have less than 50 ng input DNA or had failed controls suggesting poor DNA quality. Initial analysis showed that 20/26 (77%) samples matched the results from previously used assays, but when samples with insufficient DNA content (<50 ng total card channel input) were excluded from the analysis, 18/19 (95%) were concordant.

The single discordant sample H09-322 was found to be KRAS mutant by castPCR (p.Gly13Asp, c38G > A) but was wild type by REB array. IonTorrent sequencing detected a KRAS mutation at low level, c.38G > A (COSM532) with 12% of 1437 reads. The low number of reads suggests that this may be a sensitivity issue.

Sensitivity and specificity for KRAS are therefore 92% and 100% respectively, with a negative and positive predictive value of 85.71% and 100% respectively.

3.3. Melanoma

A total of 28 melanoma samples were tested, all of which had previously been sequenced for BRAF and NRAS mutations, and 10 of which had previously been tested for BRAF mutations alone by cobas PCR (Roche). BRAF V600E mutation was identified in 12 cases, and NRAS Q61K in 6 cases, NRAS Q61R in 1 case and NRAS G13R in 1 case. The remaining 8 cases were WT for NRAS and BRAF. Of the ten cases tested by cobas, 4 had BRAF mutations at V600E: all were found by REB array and IonTorrent sequencing.

There was one possible discrepancy with sequencing in a case not previously tested by cobas: a rare BRAF V600E variant (BRAF c.1798_1799GT > AA p.Val600Glu) was detected by REB array, which was found as an unexpected deletion by sequencing using a laboratory developed Ampliseq panel. This probably reflected a difference in PCR amplicons between Ampliseq and castPCR, and is not regarded as a true discrepancy.

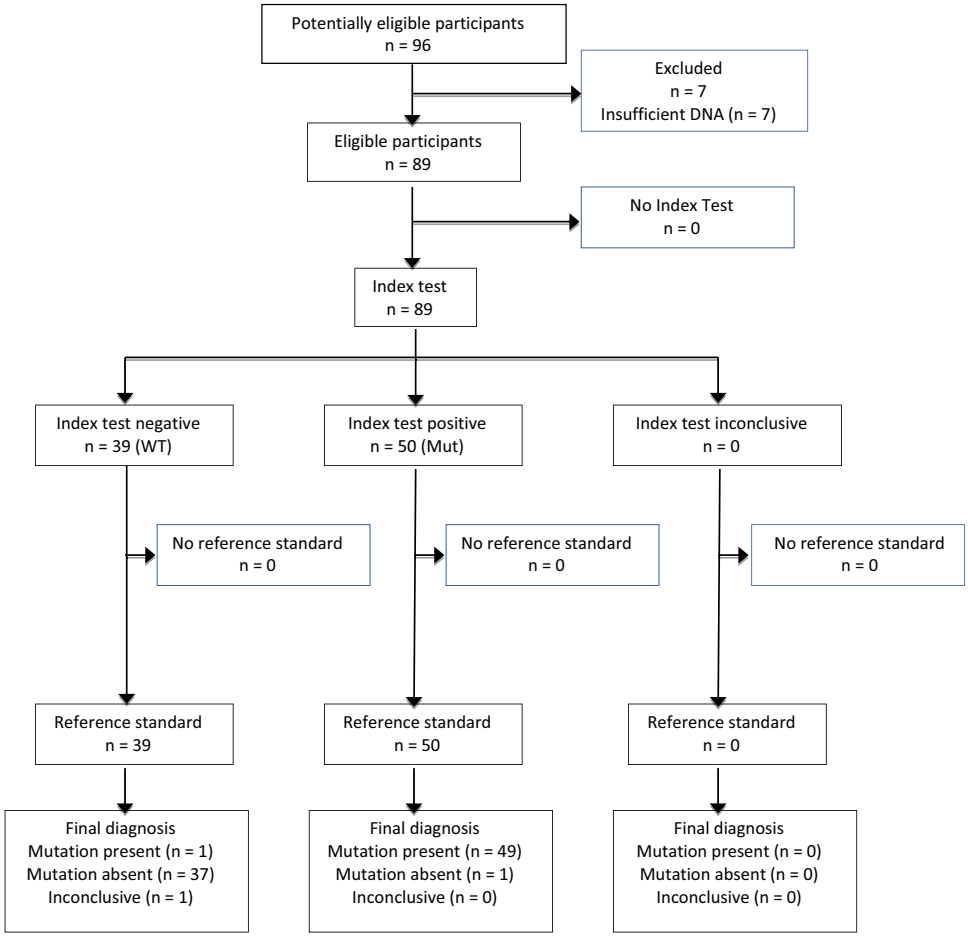

Fig. 2. STARD diagram. The REB array is the Index test, and the reference test is taken as EGFR for lung cancer, KRAS for colorectal cancer, and BRAF for melanoma. One NSCLC case found WT by REB array was regarded as mutation inconclusive, as this test was not present on the array but was detected by Therascreen.

Final concordance was therefore 28/28 (100%). Sensitivity and specificity for BRAF were both 100%, with negative and positive predictive values of 100%.

4. Discussion

There was generally good concordance between REB array and previous mutational analysis by either PCR or sequencing, though four true discrepant results were identified: two in EGFR, one in BRAF, and one in KRAS. Following sequencing, only one of these cases (the KRAS mutation in a colorectal cancer) was found to be a REB array miss, and this showed low reads suggesting low allelic frequency. It should be noted that for many of the cases (45%) stored DNA was used. The quantity of DNA remaining may therefore have been suboptimal and in several cases re-extraction of DNA was successful in finding the mutation previously identified. This may explain the KRAS case missed by REB array. Some loss of sensitivity is expected by performing castPCR within TaqMan arrays due to dilution in the plate.

Two EGFR mutations called WT by Therascreen were picked up by the REB array. The EGFR mutant case missed by REB array had a rare Exon 20 mutation, which is not present on the REB array, though it is found by Therascreen. This is therefore not regarded as a true discrepancy.

The melanoma cases show excellent concordance between all three assays (REB array, cobas, and Ampliseq panel). It should be noted that no V600K cases were included in the series of samples used for validation: this mutation was initially omitted from this version of the REB array, but has now been included as our sequencing and other published data [12,13] suggest that such cases may account for up to 10% of BRAF mutated melanomas, which we would otherwise miss unless sequencing was undertaken.

Discrepancies between mutation assays are to be expected, since calling mutations by PCR is based on PCR efficiency and thresholds, which differ between technologies, and primer pairs used for PCR differ between assays, including targeted sequencing panels. Equally, particularly for EGFR in this instance, amplicon size varies with the technology used, and may be a further source of difference. The KRAS technology was castPCR in both the REB array and the standard assay, and the performance of these assays in TaqMan array plates versus 96 well plates was comparable, apart from a single discrepancy, which probably reflects slightly lower sensitivity in the TaqMan array. Use of a third technology is often necessary to determine the true status of the sample for accuracy estimation. In this instance, the IonTorrent Colon and Lung Cancer panel proved very helpful in deciding the true status of discrepant samples, as it has the ability to use very small amounts of DNA (10 ng) in comparison with castPCR (50 ng) and Therascreen (80 ng).

In our view, the advantages of REB array outweigh its any minor loss of sensitivity, since it allows coverage of a greater number of genes and mutations than its comparators in this study for either lung or colorectal cancer. There is certainly a need for multiple gene

assessment without the expense of NGS, and this is one solution. No PCR assay is capable of finding complete coverage of all the mutations that may be present, but this approach does allow common mutations to be detected accurately, and in patients with these mutations, the effect of treatment is known.

The other advantage of the REB Array is its ease of use. Loading of DNA is simple and rapid, and less prone to pipetting errors than Therascreen or castPCR. The assay takes 2 hours to perform, and even allowing a further 90 min for DNA extraction; results can be reported in a morning, compared with three days for IonTorrent NGS using current protocols. There is no question that this is an advantage to clinical laboratories, which are often under considerable pressure to produce rapid results for oncologists, particularly in lung cancer. We have used the CMD-ImPACT tool (http://www.rcpath.org/cmd-impact) to cost the tests involved in this pathway. In our laboratory, use of the REB array to triage patients for ALK immunohistochemistry and IonTorrent NGS is the most cost-effective option, partly because savings in staff time outweigh the cost of the plates.

Our intended use of the REB array is as a simple, rapid triage test allowing efficient use of expensive NGS facilities, while meeting guidance for molecular pathology testing [14]. This is particularly important in lung cancer, where biopsies are often small and difficult to repeat. Other laboratories may wish to adopt different approaches, in consultation with their clinical team, but reflex testing of samples can be very cost-effective [14], and in our practice, the use of the REB array will make it affordable to use NGS for mutation detection [11,15] in patients without common actionable mutations. There is a wide range of competing technologies for PCR-based detection of cancer mutations. Therascreen and cobas are both commonly used, and the new cassette-based Idylla™ system (Biocartis), which automates extraction and measurement, is also gaining market share as it avoids the need for molecular laboratory expertise [16–18]. Finally, antibodies to common mutations are now available and could be used as screening methods [19,20]. The choice of method is an individual decision for those providing cancer pathology services, but can be informed by comparative studies [9,14,21–24]. Most newer methods have subjected to such assessments [16–18].

In conclusion, we have developed a rapid assay system for common actionable mutations in colorectal cancer, lung cancer and melanoma. This newly validated assay has the potential to personalise patient treatment and direct further investigation in those without actionable mutations.

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Contributorship

IC developed the REB array, HK did the testing with assistance from JN and AR, supervised by LB, DS, and TW. All authors contributed to the analysis and write up of the data. HK is funded by a University of Warwick PhD studentship.

Conflict of interest

IC has received unrestricted research grants and has acted as a consultant for Life Technologies, now part of Thermo Fisher. IC has shares in CanTech Ltd. IC and AR are part of the Onconetwork led consortium developing NGS solutions on the IonTorrent PGM.

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