Past, current and future approaches to querying MAPK pathway activation: status and clinical implications

MAPK pathway activation related to cancer development has drawn a great deal of attention in the field of personalized medicine in recent years. Many different approaches and assays have been developed to query the activation of this pathway and to develop life-saving treatments. The goal of this review article is threefold. First, to provide a brief overview of the many mutation assays that have been used to detect MAPK pathway activation, and to compare pros and cons of these assay platforms. Second, to focus on one custom-designed multiplexing mutation assay that is currently used to support an ongoing clinical trial and to show the novel features of this assay and its relevance in addressing unmet clinical needs. Third, to provide future perspectives of these MAPK pathway gene mutation detection efforts and to suggest how what we have learned from past and current approaches should guide future developments.

Keywords: BRAF • FFPE • KBN-SNPE • KRAS • MAPK • MEK • NGS • NRAS • SNaPshot

Background

Defining the mutational status of solid tumors is becoming one of the most important personalized medicine approaches in guiding treatment options for cancer patients [1]. Treatments developed against targets within a specific signaling pathway may not be effective when activating mutations are downstream of the pathway. One well-known example is that tumors carrying activating mutations in RAS and RAF do not respond well to anti-EGFR therapy [2]. Inhibitors against proteins further downstream in the pathway, such as ERK, may be more effective [3]. Therefore, proteins that lie downstream of RAS, RAF and MEK in the RAS/MAPK pathway (Figure 1) are likely potential targets for anti-cancer agent development [4–6]. Approved and investigational treatments selective for MAPK pathway targets are listed in Table 1 [3,7–27].

RAS, RAF mutation distributions among different cancers

Approximately 25% of all cancers carry RAS-activating mutations [28]. In pancreatic cancer

and colorectal cancer, these mutations, especially in KRAS, are present at even higher rates [4,28]. Roughly 10–25% of melanomas also carry NRAS mutations [4,6,29] and KRAS mutations were detected in nearly 30% of non-small cell lung cancers [4,30]. For thyroid cancers, RAS mutations (HRAS, KRAS, or NRAS) represent 55–60% of cases, and BRAF mutations were found in nearly 60% of malignant melanomas [6,31,32]. These BRAF mutations are mainly located in the kinase domain, and a single base substitution (V600E) accounts for ~80% of mutations [6,31,32]. Not all activating BRAF mutations are found in melanoma. Approximately 10% of colorectal cancer tumors harbor BRAF mutations and approximately 50% of thyroid cancers carry BRAF mutations [29], as does several percent of non-small-cell lung cancer cases [33]. The high frequency of RAS or RAF mutations in these cancers makes targeting this pathway an attractive strategy for new anti-cancer agent development for those individuals most likely to benefit from MAPK inhibitors [34]. The percentage of selected

Ken CN Chang^{*1}
& Matthew J Marton¹

¹Molecular Biomarkers & Diagnostics,
Merck & Co, Inc., Rahway, NJ, USA

^{*}Author for correspondence:

ken.chang@merck.com

Figure 1. MAPK pathway.

GF: Growth factor.

tumors with mutations in KRAS, BRAF or NRAS as reported in the COSMIC database is summarized in Table 2. Other MAPK pathway genes such as ERK and MEK are also targets for pathway intervention; however, they are not oncogenes, and therefore strategies to target these proteins must rely on pathway activation assays other than those in ERK or MEK, since mutations in these genes cannot be used as a diagnostic tool to identify a responsive patient population.

Mutations & clinical outcomes

Hotspot mutations in these MAPK oncogenes, such as those located in KRAS codons 12, 13 and 61, and BRAF 600, have long been the primary scientific and clinical focus. Increasingly, however, recent evidence indicates mutations in other locations can also lead to the tumorigenic phenotype [35,36] and that it is important to detect mutations beyond those in well-known hotspots. For example, a recent clinical trial revealed

panitumumab (Vectibix), an anti-EGFR monoclonal antibody (mAb), was beneficial in patients with wild-type KRAS or NRAS but potentially harmful in patients with KRAS or NRAS mutations that are not routinely covered in regular tests [37]. Using KRAS as an example, not all mutations have the same clinical outcome, and often rarer mutations detected outside of the frequently encountered codons 12 and 13 are mutually exclusive making them an important category of mutations with diagnostic value [38]. Furthermore, approximately 1–10% of KRAS mutated tumors responded to anti-EGFR mAb therapy [39]. Retrospective analysis suggested that patients who received mAb therapy with KRAS G13D mutation had better outcomes than those with other KRAS mutations [39]. *In vitro* analysis also supported the hypothesis that not all KRAS mutations have the same biological impact [40]. Therefore, it may be necessary to include a more extensive list of known MAPK pathway gene

MAPK target	Tumor	Treatment options or investigational drugs	Ref.
KRAS, HRAS	Melanoma, NSCLC	BMS-214662, L-778123	[7–11]
MEK	Breast, lung	CI-1040	[12]
MEK	NSCLC, melanoma	PD-0325901	[13,14]
MEK	Pancreatic, thyroid	BAY 86–9766	[15]
MEK	Advanced malignancies	AZD8330	[16]
BRAF	Melanoma	Dabrafenib	[17]
MEK/BRAF	Melanoma	Trametinib	[18]
BRAF	Melanoma	Vemurafenib	[19,20]
BRAF	NSCLC, leukemia, and so on	BAY 43–9006	[21,22]
BRAF	Melanoma	PLX4720	[23,24]
RAF	NSCLC, CRC, and so on	XL281	[25]
BRAF	Melanoma	GSK2118436	[26,27]
ERK	Melanoma, CRC	MK-8353	[3]

CRC: Colorectal cancer; NSCLC: Non-small-cell lung cancer.

mutations in future routine testing. The goals of this review are: to provide a brief overview of many mutation assays that could be or have been used to detect MAPK pathway gene activation and also compare pros and cons of these assay platforms; to focus on one custom-designed multiplexing mutation assay that is currently used to support an ongoing clinical trial and to show the novel features of this assay and its relevance in addressing unmet clinical needs; and to provide our perspective on future MAPK pathway gene mutation detection efforts and to suggest how best to apply what we have learned from past and current approaches to guide future developments.

Brief overview of mutation assays that detect MAPK pathway gene activation

In general, one can classify the type of MAPK pathway activation mutation assays into two broad categories: those that detect mutations in specific, limited nucleotide locations, and those that take a general sequencing approach such as Sanger sequencing or pyrosequencing. If the location of the mutations is known, there are many more assay platforms from which to select, each having its own advantages and disadvantages.

Sanger sequencing, or dideoxy sequencing, is still considered the ‘gold standard’ for mutation detection, even though next-generation sequencing (NGS) may someday replace it as the new standard [41]. The dideoxy-sequencing methodology became widespread in the 1990s when Applied Biosystems (ABI)’s capillary electrophoresis DNA sequencers were used ubiquitously to first sequence the human genome. It is well accepted and understood, in part because base-calling

is automated and because no complex bioinformatics are needed to analyze the raw data, as the electropherograms, with color-coordinated trace peaks corresponding to each base, can be unambiguously visualized. However the amount of input DNA needed is usually relatively large, especially if longer sequences need to be analyzed. The use of clinical formalin-fixed paraffin embedded (FFPE) tissue samples, which normally are highly fragmented, also adds to the requirement of higher input DNA to generate clear-cut data. Another challenge is that all different alleles within the same amplicon are sequenced at the same time using the same set of sequencing primers, therefore it is very difficult to distinguish if two mutations are present in one allele in the same amplicon region or one mutation is present in two alleles.

Pyrosequencing can be considered the original ‘sequencing-by-synthesis’ technology. As DNA is synthesized from an immobilized template, the released pyrophosphate triggers the sulfurylase–luciferase cascade

Table 2. Percentage of selected tumors with mutations in KRAS, BRAF or NRAS as reported in the COSMIC Database v68.

Tumor	Cosmic		
	KRAS	BRAF	NRAS
Colorectal	35	13	4
Melanoma	2	42	15
Ovarian	12	8	1
Pancreatic	57	2	1
Thyroid	2	39	6
Lung	17	2	1

resulting in the emission of light [42]. Nucleotides are added one at a time in order, and the emission of light and the amount of emission allow the determination of which nucleotide base and the number of nucleotides that is incorporated. Since the amount of light emitted is proportional to the number of nucleotides incorporated it is possible to identify different alleles and to determine the variant frequencies.

Many technologies have been developed to detect mutations of known locations, but they differ by degree of sensitivity, sample requirements and the suitability of multiplexing. The SNaPshot multiplex kit is designed to interrogate SNPs at known locations on DNA templates [43–45]. Genomic DNA is isolated from FFPE samples and specific exons from different target genes are amplified in one or more multiplexed PCR reactions. These PCR products then act as templates in the SNaPshot reaction. This reaction consists of oligonucleotide probes designed to hybridize immediately adjacent to the 3' side of the nucleotides of interest. The probe is then extended by the addition of a single fluorescently-labeled dideoxynucleotide. Detection of mutations is made by a combination of the identification of the fluorescently tagged nucleotide added and the migration of the probe, based on its size as determined by capillary electrophoresis.

The design strategy of shifted termination assay (e.g., ABI, TrimGen) is similar to that of the SNaPshot assay in that it utilizes a primer with complementary sequence up to the base 3' to the target nucleotide site. This is followed by supplying the mutant nucleotide with respective fluorescently-labeled ddNTP to allow early termination of chain extension if a variant or mutation is present [46]. The resulting wild-type fragment will be larger than the mutant fragment, therefore allowing separation using electrophoresis.

The StripAssay™ [47] from ViennaLab (Vienna, Austria) is a mutation assay technology in which biotin-labeled PCR amplicons of target genes are hybridized to a solid matrix with up to 48 immobilized probes complementary to the target DNA. Since the probes are labeled with biotin, streptavidin–alkaline phosphatase incubation allows color to be observed and the assay result to be observed and scored. Although assays for several genes are available, adding assays for additional mutations may be cumbersome because each would have to be designed separately, that is, the assay does not appear to be easily scalable. In addition, the assay does not permit quantification of the mutant allele frequency, in other words, percent mutant for each mutation in the corresponding samples can only be estimated, as only 'present' or 'absent' results are obtained. This type of mutation detection has a very specific purpose and may not be applicable for broad applications

since treatment responses may be directly related to the variant frequency of a particular mutation, and retrospective analysis of such information may lead to hypotheses to better predict patient treatment response.

Several qualitative allele-specific PCR technologies, such as the Amplification Refractory Mutation System (ARMS, Qiagen), achieve high sensitivity by blocking amplification of the wild-type sequence [48]. ARMS is a mutation assay that employs so-called Scorpion™ probes that, under ambient temperature, will form a hair-pin loop with a fluorescent tag that is quenched at one end and a target primer sequence attached at the other end [48]. After target amplification through the primer end, heat denaturation allows the newly synthesized strand to be opened up together with the hair-pin loop. During the cooling step, this hair-pin loop with a pre-designed sequence complement hybridizes to the newly synthesized target sequence. Since the hybridization between the newly synthesized sequence and the complementary sequence within the loop will keep the quencher separated from the dye, fluorescent signal will be emitted. Kinetic analysis of the fluorescent signals and delta Ct calculation allow the determination of the presence of potential mutations.

TaqMan™ SNP Genotyping Assay (ABI) is one of the most well-established variant detection (or allelic discrimination) assays [49]. Thousands of known single nucleotide variant assays can be ordered directly from ABI, many of which have been prevalidated by the company. Assays for novel mutations can be easily custom designed and ordered. The design principle is basically a Taqman™ assay with fluorescently-quenched probe designed to hybridize to a region including nucleotide of the mutation of interest. If the mutation is present, the probe is degraded by the 5' exonuclease activity of the Taq polymerase, which releases the quencher from the probe and allows its fluorescence to be detected. Using different primer-probe sets and different fluorophores for mutant and wild-type probes allows both alleles to be amplified in the same reaction and permits quantification of the relative abundance of the mutant and wild-type alleles.

OncoCarta Mass Array (Sequenom) is a mass spectrometry-based (MALDI-TOF) mutation assay based on a single-nucleotide primer extension (SNPE) strategy [50]. A panel of pre-designed mutations (up to several hundred mutations) can be assembled; however, the bigger the panel the more DNA is required. Detection sensitivity for this assay is not very high, and some of the pros and cons of these assays can be found in Table 3.

Other mutation detection assays are also available if qualitative analysis of presence or absence of mutations is a sufficient result. These assays include a melting

Platform	Pros	Cons (or limitations)
Sanger sequencing	<ul style="list-style-type: none"> • Foreknowledge of mutation not needed • Current gold standard assay • Able to detect any mutation including in/dels 	<ul style="list-style-type: none"> • Low sensitivity (10–20%)
Pyro-sequencing (Qiagen)	<ul style="list-style-type: none"> • High sensitivity (1–5%) • Able to detect any mutation including in/dels • Commercial kit for KRAS codons 12, 13 and 61 	<ul style="list-style-type: none"> • Potential false positives • Higher costs than Sanger sequencing
SNaPshot (ABI)	<ul style="list-style-type: none"> • High sensitivity (1%) • Easily configurable to add mutations of interest 	<ul style="list-style-type: none"> • Detects user-defined mutations
STA (ABI)	<ul style="list-style-type: none"> • High sensitivity (1%) • Commercial kit available for KRAS codons 12 and 13 	<ul style="list-style-type: none"> • Detects KRAS: Gly12/13 (Ser, Arg, Cys, Asp, Ala, Val) BRAF: Val600 (Glu, Ala, Gly)
STA (TrimGen)	<ul style="list-style-type: none"> • High sensitivity (1%) • Commercial kit available for KRAS codons 12 and 13 	<ul style="list-style-type: none"> • Detects KRAS: Gly12Ser, Gly12/13 (Arg, Cys, Asp, Ala, Val)
StripAssay (Vienna Labs)	<ul style="list-style-type: none"> • High sensitivity (1%) • Commercial kit available for codons 12 and 13 	<ul style="list-style-type: none"> • Detects KRAS: Gly12Ala, Arg, Ile, Leu, Ser, Val, Gly12/13 (Asp, Cys) • BRAF: Val600Glu
ARMS & Scorpions (Qiagen)	<ul style="list-style-type: none"> • EU approved; US FDA approved as IVD (Qiagen); • RUO kit available in the USA • High sensitivity (1%) 	<ul style="list-style-type: none"> • Detects KRAS Gly12Ala, Arg, Cys, Ser, Val, Gly12/13Asp
TaqMan (ABI)	<ul style="list-style-type: none"> • Many off-the-shelf assays available 	<ul style="list-style-type: none"> • Detects user-defined mutations
MALDI-TO (Sequenom)	<ul style="list-style-type: none"> • Large number of hotspot mutations in many genes are analyzed in a single assay 	<ul style="list-style-type: none"> • Low sensitivity (10%)
MeltingCurve (Roche)	<ul style="list-style-type: none"> • EU approved; FDA approved as IVD (cobas) 	<ul style="list-style-type: none"> • Assay output is ‘mutant’ or ‘mutation not detected’
Surveyor (Transgenomic)	<ul style="list-style-type: none"> • High sensitivity (1%) • Commercial kit available for KRAS codons 12, 13 and 61 	<ul style="list-style-type: none"> • Assay output is ‘mutant’ or ‘mutation not detected’

ABI: Applied Biosystems; IVD: *In vitro* diagnostics; RUO: Research use only.

curve assay like those Roche cobas™ assays [51,52] and the hybridization nuclease digestion assays such as Surveyor® KRAS Mutation Kit from Transgenomic [53].

Commercially available US-FDA-reviewed KRAS and BRAF kits, such as the Qiagen Therascreen® KRAS RGQ kit and the cobas KRAS and BRAF mutation tests, detect mutations only at limited locations and do not include an extended set of mutations that might be critical to treatment response. The Therascreen KRAS assay is based on Scorpions and ARMS technologies and detects seven frequently encountered mutations in codon 12 and 13 [54]. The cobas KRAS mutation kit is

a real-time PCR assay which detects 21 mutations in codons 12, 13 and 61 [51]. However, specific mutations are not reported, which is important for exploratory data analysis. The cobas BRAF assay only detects BRAF V600E mutation [52]. In the bioMerieux THxID™-BRAF kit, which uses the ARMS methodology, two different probes labeled with two different dyes allow the simultaneous detection of the BRAF internal control and a BRAF mutation [55]. In the THxID assay, primers specific for the BRAF gene allow the amplification of a non-polymorphic gene area, which is used as an internal control. Primers specific for the mutations

V600E and V600K allow the amplification of mutated fragments, and hence, *BRAF* mutation detection. Although many mutation assays are considered semi-quantitative assays, no FDA-regulated clinical assays currently report the mutation variant frequency (VF), partially due to the difficulty of interpreting the clinical meaning of VF, as well as the reproducibility of these semi-quantitative measures.

Clinical consideration, clinical objectives & assay platform requirements

Based on the clinical objectives, many clinical considerations are important to the selection of a specific assay for the clinical application. Table 3 summarizes the major pros and cons of each platform mentioned above and can be used as a reference when considering platform selection options. Other approaches for querying MAPK pathway include identification of MAPK gene expression signature [56], however it is not the intention to review this strategy here.

Additional assay development considerations include tissue types, sample collection and preservation methods, clinical objectives, turn-around time (TAT), and acceptable false positive/false negative rates [57]. For tissue types, FFPE tissue is one of the most unique and challenging since nucleic acids isolated from there are typically highly fragmented. Many assay designs involve PCR amplification of target amplicons and if the length of target amplicon is too long (such as greater than 150 bp) there will be a very limited number of copies of the target sequences available for PCR amplification, which can introduce large variation simply due to the sampling variation. Sample collection and preservation methods will also likely impact the quality of nucleic acids, since fragmentation and degradation can affect data variation and reproducibility. In the clinical setting, including clinical trials, a fast TAT is always a goal as patients may not be able to wait for 2 weeks for data to be available, and a clinical decision can be made. In our experience, the normal expectation of TAT is 7 calendar days or 5 business days. Finally, a good balance of low false-positive and low false-negative rates is also the key to making an assay 'the platform of choice'. The following custom-designed multiplexing mutation assay, which was developed and analytically validated in our lab, took most of the above-mentioned key factors into consideration and is currently being used with great success to support an ongoing clinical trial.

Novel features of a custom-designed multiplexed mutation assay (*KRAS*, *BRAF*, *NRAS* SNPE mutation detection assay)

The *KRAS*, *BRAF*, *NRAS* SNPE (KBN-SNPE) mutation detection assay was designed to support an

ongoing clinical trial for patient prospective enrollment [58]. The assay plan and design started with identifying 35 mutations in the *KRAS/BRAF/NRAS* genes that are thought to activate the MAPK pathway or increase activity of one of the kinases [59–64]. A custom-designed SNPE called the KBN-SNPE assay, was then developed using SNaPshot mutation assay kits from ABI. The SNaPshot methodology takes advantage of PCR-based amplification of the target sequence followed by specific primers/probes to interrogate the mutation sites of interest through single nucleotide extension reactions using fluorescently labeled ddNTP and capillary gel electrophoresis to separate the resulting products. Similar assays were published in several different research studies and some were for clinical sample analysis [60,61,65]; however, none of these assays were designed and validated to support a clinical trial for patient prospective enrollment.

This assay begins with the PCR amplification of regions of the *KRAS*, *BRAF* and *NRAS* genes from gDNA isolated from one or two 5 µm FFPE tissue slides. A set of custom-designed probes with different lengths of tails with repeating sequences is hybridized to the PCR amplicons, followed by a single base extension reaction of the probes. The final products are then separated by the capillary electrophoresis, and GeneMapper software (ABI) is used to make automated base calls based on size and incorporated fluorescent label. An example of a KBN-SNPE assay output is shown in Figure 1.

This KBN-SNPE assay was designed to detect the mutations of interest in *KRAS*, *BRAF* and *NRAS* (Table 4) in support of patient stratification in clinical trials. The design and validation of KBN-SNPE assay took into the consideration of the challenge of FFPE samples, which generally yield fragmented, low quality genomic DNA. Based on our clinical experience, the average fragment size of FFPE tissue gDNA is around 200 base pair [45]. Therefore, it is necessary to design PCR primers that amplify short stretches (100–150 bp) of DNA and to balance the primer concentrations so that all target regions within same reaction can be equally amplified.

The KBN-SNPE assay concurrently interrogates nine and six nucleotide positions within *KRAS* and *NRAS*, respectively, and twelve within *BRAF* (Table 4). Hot spot mutation regions of *KRAS* exons 2, 3 and 4, *NRAS* exons 2 and 3, and *BRAF* exons 11 and 15 were amplified in three separate PCR reactions. Due to the number of SNPE probes used for *BRAF* and sequence similarity between *KRAS* and *NRAS* plus a potential need for accommodating newly discovered critical mutations while the clinical trial progresses, it was necessary to perform the primer extension portion

Table 4. Mutations identified by the KBN-SNPE assay and their resulting amino acid change.

BRAF				KRAS				NRAS			
Codon number	WT codon	Mutant codon	Protein description	Codon number	WT codon	Mutant codon	Protein description	Codon number	WT codon	Mutant codon	Protein description
466	GGA	GAA	G466E	12	GGT	AGT	G12S	12	GGT	GAT	G12D
466	GGA	GTA	G466V	12	GGT	TGT	G12C	12	GGT	GTT	G12V
592	ATA	GTA	I592V	12	GGT	GAT	G12D	13	GGT	CGT	G13R
594	GAT	GTT	D594V	12	GGT	GCT	G12A	13	GGT	GAT	G13D
594	GAT	GAA	D594E	12	GGT	GTT	G12V	61	CAA	AAA	Q61K
594	GAT	GAG	D594E	13	GGC	CGC	G13R	61	CAA	CGA	Q61R
596	GGT	CGT	G596R	13	GGC	GAC	G13D	61	CAA	CTA	Q61L
597	CTA	TCA	L597S	61	CAA	AAA	Q61K	61	CAA	CAC	Q61H
600	GTG	GAG	V600E	61	CAA	CTA	Q61L	61	CAA	CAT	Q61H
600	GTG	AGG	V600R	61	CAA	CAC	Q61H				
600	GTG	AAG	V600K	61	CAA	CAT	Q61H				
600	GTG	GAT	V600D	146	GCA	ACA	A146T				
601	AAA	GAA	K601E	146	GCA	GTA	A146T				

WT: Wild-type.

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of the assay in three separate reactions [45]. Collectively, this assay can detect 35 specific mutations (81 potential mutations for all possible A/T/G/C changes at each nucleotide location excluding wild-type) at 27 nucleotide positions in three genes spread over seven exons.

The analytical validation process of KBN-SNPE assay involved a triplicate strategy and relied on several novel approaches to redefine and calculate sensitivity, specificity and accuracy. Some highlights of this novel assay validation strategy are captured in the following sections.

Calculation of specificity, sensitivity & concordance

For accuracy study, concordance analysis was done at the sample (patient) level. Each sample was subjected to mutation analysis by both KBN-SNPE assay and Sanger sequencing. If the two methods agreed, the result was assumed to be accurate. If only one method detected a mutation, the sample was analyzed by Ion Torrent Ion PGM sequencing. If the Ion PGM result matched either the KBN-SNPE or Sanger result, the matched result was considered accurate.

This concordance approach using next-generation sequencing to confirm the Sanger sequencing result (considered as a gold-standard sequencing assay) is somewhat unconventional since NGS is a more sensitive sequencing assay and some mutations detected by the KBN-SNPE assay may not be detected using the

Sanger sequencing. The following terms were defined in advance in order to carry out the analytical validation for this multi-gene/multi-mutation site validation and to set up the acceptance criteria:

Specificity or sample-level negative agreement:

- Specificity = # of True negatives / (# of True negatives + # of False positives), where the truth is defined as the consensus result obtained by two or more assay methods (KBN-SNPE, Sanger sequencing, NGS)

Sensitivity or sample-level positive agreement:

- Sensitivity = # of True positives / (# of True positives + # of False negatives)

Overall concordance (accuracy)

The overall ability of the KBN-SNPE assay to make the same wild-type or mutation call as the consensus call is defined as follows:

- Overall concordance = (# of True positives + # of True negatives) / (# of True positives + # of False positives + # of False negatives + # True negatives)

Establishing analytical accuracy (using NGS as a tie-breaker assay)

To establish the analytical accuracy of the assay, three sets of samples were analyzed by both the KBN-SNPE assay and Sanger sequencing: cell lines with previously documented mutations in *BRAF* or *KRAS*; synthetic oligonucleotides containing all mutations detected by

the KBN-SNPE assay; and a set of 60 FFPE tumor samples (from six different tissues). In summary, the assay was able to make correct calls from all three sample types and the details of the experimental data can be found in the original publication [45].

For FFPE tissue samples, gDNA was isolated from the corresponding 60 FFPE tissue slides, ten samples each of colorectal, ovarian, melanoma, lung (non-small cell carcinomas), pancreatic and thyroid cancers. Each sample was subjected to mutation analysis by the KBN-SNPE assay and Sanger sequencing.

Designing SNPE primers to detect certain mutations involving two consecutive nucleotide changes

The detection of BRAF V600K and V600R requires special probe design since there are two mutations present consecutively in the same codon. In addition to designing oligonucleotide primers/probes, one from sense and one from anti-sense directions, it was necessary to design additional primer/probe in order to cover potential mutant populations. A probe was designed to cover any mutants with A at 1798 nucleotide position to allow detecting mutants with mutations at both 1798 and 1799 nucleotide positions (double-mutation) such as V600R (GTG to AGG) or V600K (GTG to AAG) (see Figure 2 and the original publication). This design was necessary in order to detect the double mutations including the more aggressive V600K BRAF mutation, which has drawn a lot of attention recently for its possible link to tumor metastasis [66,67].

Assay precision & the limit of detection (analytical sensitivity)

Assay precision including intra-run and inter-run variation, was done in a traditional way and the detail data can be found in the original publication. As described in the publication, the limit of detection was significantly different for different mutations, ranging from 1.6 to 12%, but since this was determined using cultured cell lines, the behavior and detection sensitivity of these mutations could be quite different from those samples obtained from the FFPE tissue slides. Artifactual mutations such as C to T and C to A from FFPE tissue samples have been reported in many publications [68–70]. These high background artifactual variant calls interfere with mutation detection calls and will likely affect the detection sensitivity of true mutations. A more practical and perhaps meaningful analytical validation might be to determine the limit of quantification using the triplicate approach, to define the minimum VF for each mutation that could achieve greater than 95% reproducibility of

concordant variant calls. This approach could be very valuable for mutation assay validation in NGS as multiple mutations, including rare and unknown mutations, are impossible to validate individually simply due to the large total number of potential mutations.

Sample analysis in triplicate improves assay performance

In the experiments described above it was noted that miscalls could occur from time to time depending on the sample quality and the mutant variant frequency. For clinical applications, it would be preferable to reduce these errors, which represent either false positives or false negatives. After careful consideration it was concluded that the error rate of the assay could be reduced by assaying samples in triplicate and taking a 'majority rules' approach. The rules developed for calling KBN-SNPE results are given in Table 5. When samples were assayed in triplicate there was 100% (42/42) concordance between the KBN-SNPE result and the consensus result (defined by agreement with Sanger or NGS). Samples assayed in triplicate where two or more calls were indeterminate were excluded from calculations for concordance.

The KBN-SNPE assay is a clinical trial assay custom-designed to select patients whose tumors display an activated MAPK pathway. Many analytical validation approaches for this multiplexing mutation assay were modified from the traditional single mutation assay validation strategy, such as gene-level analysis versus sample- or patient-level analysis, however the validation principles remain the same. A triplicate strategy clearly improved the overall performance of this assay where the most challenging clinical FFPE tissue samples were involved and low variant frequency of certain mutations could be encountered.

In general the custom-designed KBN-SNPE assay performance can be summarized below:

- Assay accuracy, as measured by overall concordance to the consensus call for the validation samples, was greater than 94% at both the patient- and gene-levels for *KRAS*, *NRAS* and *BRAF*. The validation samples consisted of ten FFPE sections from six tissue tumor types for a total of 60 samples;
- Intra- and inter-run precision was 97% at the sample level and greater than 97% at the gene level;
- Samples assayed in triplicate agreed with the consensus result 100% of the time at patient and gene levels;
- Concordance was 100% using gDNA isolated from cell lines with previously defined mutations in *KRAS* and *BRAF*;

Figure 2. Example of KBN-SNPE results for BRAF probe pools. (A) BRAF probe Pool 1, wild-type control; **(B & C)** Examples of V600E and V600D mutations; **(D)** BRAF probe Pool 2 wild-type control. Positions of codons and nucleotides are indicated at the bottom of the figure. Nucleotide labels for each peak have been converted to the sense strand.

MT: Mutant; SNPE: Single-nucleotide primer extension; WT: Wild-type.

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- The assay correctly identified all mutations when synthetic oligonucleotides replaced gDNA in the assay;
- The analytical sensitivity of each assay was dependent on the mutation detected and ranged from 1.6 to 12% mutant DNA against a wild-type background;

Table 5. Rules for calling KBN-SNPE results at the mutation level when assaying samples in triplicate.			
Replicate/call			Consensus
1	2	3	
Wild-type	Wild-type	Wild-type	Wild-type
Wild-type	Wild-type	Mutation-1	Wild-type
Wild-type	Mutation-1	Mutation-1	Mutation-1
Mutation-1	Mutation-1	Mutation-1	Mutation-1
Wild-type	Wild-type	ND	Wild-type
Mutation-1	Mutation-1	ND	Mutation-1
Wild-type	ND	ND	Indeterminate-redo
ND	ND	ND	Indeterminate-redo
Mutation-1	ND	ND	Indeterminate-redo
Wild-type	Mutation-1	ND	Indeterminate-redo
Mutation-1	Mutation-1	Mutation-2	Mutation-1
ND: Not determined. Reproduced with permission from [45].			

- The assay efficiency (call rate) for the assay was >94% when the claim to have detection sensitivity assay was performed as a single point determination and 100% when performed in triplicate.

Assay detection sensitivity percentage reproducibility is crucial to accurate & consistent diagnosis

Both the Therascreen *KRAS* and the cobas *KRAS* and *BRAF* assays claim to have detection sensitivity of less than 5% using 100 ng gDNA isolated from FFPE tissue samples with at least 10% tumor cells on the FFPE tissue slide. For average quality FFPE tissue samples, this translates into approximately 200–400 DNA copies with intact target sequences available for PCR amplification. Therefore, 5% of a mutant population equals to approximately 10–20 copies, and hence if an aliquot of gDNA isolated from such FFPE tissue slides is used to perform mutation tests, the chance of accurately detecting such a low percentage mutant population is very low [45]. Efforts are being made to establish PCR-based methods of quality control (e.g., determining the number of amplifiable copies of target DNA) to identify samples that meet minimum quality and quantity requirements [71,72]. However, the reality is that if many clinical samples fail to meet these requirements during a clinical trial, it will be essential to find a way to profile these samples otherwise the result may be a failed trial.

For the custom-designed KBN-SNPE multiplexing mutation assay, the detection sensitivity is similar to the FDA-approved *KRAS/BRAF* assays mentioned above. In our study, to determine assay accuracy, triplicate

samples were run using 15 ng of gDNA as starting material for each replicate. Since the KBN-SNPE assay involves fragment separation by capillary electrophoresis and since base calls are based on both migration and color-labeling differences, false positives and false negatives are rare. For samples with low VF mutations, the likely major reason for a discordant call is sampling variation that falls below the detection sensitivity stochastically. Therefore, if only a small number of amplifiable gDNA copies are available as the starting material, a triplicate sample preparation strategy starting from the same gDNA might be necessary to ensure accurate mutation status of clinical samples, especially when low level of mutation detection is critical.

Since most of clinical mutation assays are used for patient treatment guidance and different mutation detection assays have different detection sensitivity, it is very important to recognize that a designation of ‘mutation not detected’ is very dependent on the assay being used. A mutation status determined to be wild-type for any given samples may not obtain the same mutation status from a different assay. This can be very important clinically, since certain low VF mutations detected in leukemia samples are highly significant in terms of impacting clinical outcome [73].

Furthermore, a recent retrospective analysis of samples from an anti-EGFR clinical study showed that 17% of the patients called ‘mutation not detected’ actually had a *KRAS* mutation detectable by a more sensitive assay [68], some of whom responded to anti-EGFR therapy. Thus, although the correlation between mutation VF and the treatment outcome is yet to be established, these data suggest that some

patients whose samples contain as much as 10% mutation may be as responsive as those called wild-type [74]. More and more recent reports have shown that clinical studies using Sanger sequencing for patient enrollment have experienced false-negative calls, in which mutation status was called wild-type, but after retesting the samples were found to harbor mutations using more sensitive FDA-approved tests [35,75]. Many mutations previously believed to be rare, such as BRAF V600K, are now being detected at higher rates due to the improvements of detection sensitivity of new qPCR-based mutation detection assays and very sensitive and widely used NGS assays, which are reported to permit detection of mutant allele frequency at low single-digit percentage levels [76,77]. These examples illustrate that the definition of wild-type ('mutation not detected') is assay-dependent. While this has always been true, this has important implications for the development of future mutation detection assays, as discussed in the following sections.

NGS assay design & special considerations for querying MAPK pathway activation

As mentioned above, if the location of a possible mutation is unknown, then a general sequencing approach is necessary; this includes next generation sequencing. NGS is also known as massively parallel sequencing, as millions of sequencing reads are done in the same chip or flow cell in each sequencing run. Reviews of this exciting new technology and its application in clinical research can be found in numerous journals, and a selective set of articles is included here [78–82]. The recent FDA clearance of the Illumina MiSeqDx platform [83] is likely to encourage clinical application of this novel technology. If NGS is to be used in querying MAPK pathway activation, one can easily design a MAPK pathway-focused gene panel, and all targeted sequence regions of each gene could be fully covered with both known and unknown mutations, profiled in detail. Previous assay design strategies querying MAPK pathway activation were focused on the interrogation of hotspot mutations of each gene. However, as mentioned above, recent data suggest that the omission of rare or unknown mutations from the common mutation assays may cause the misdiagnosis of many patients and the underestimation of the efficacy of some investigational drugs, by including patients that should have been excluded according to the study inclusion criteria. Therefore, it may be beneficial to cover the entire gene of interest in a focused NGS gene panel. Recently, Amgen and Illumina announced a companion diagnostics agreement in which Illumina will develop a multi-gene, NGS-based test as a companion diagnostic for Vectibix

(panitumumab), a metastatic colorectal cancer drug, to more comprehensively detect the MAPK pathway mutations [84]. Analytical validation of such an NGS panel for clinical application may be very challenging since many performance metrics for more traditional mutation assays will need to be modified. First, many traditional mutation assays are designed to detect one mutation at a time. The degree of complexity for detecting well-defined mutations is much smaller than that for an NGS mutation detection assay where hundreds, if not thousands, of variant calls could be generated from one sample. How to calculate this type of multivariant repeatability or reproducibility is a challenge, not to mention that the assessment of accuracy for hundreds of variant calls may render the task extremely difficult and time consuming, if not entirely impossible. Therefore, novel approaches may need to be devised in order to optimally utilize this powerful technology. For example, using a triplicate approach and a concordance calculator to sort out concordant and discordant variant calls could be very valuable [85]. Furthermore, using a triplicate strategy to optimize quality control filters, including VF, coverage, quality scores, and other bioinformatics filters, to reduce false positive and false negative rates could potentially be beneficial for determining/defining the assay specificity, precision and limit of detection [85]. Another similar approach used quality metrics to rank order the target variants and plot the accumulation rate for both concordant and discordant variants with decreasing score stringency [86]. A more detailed discussion is outside of the scope of this review.

Discussion & future perspective

In this article, many genomics assays that could be employed to probe MAPK pathway activation were reviewed. A recent example of developing and analytically validating a custom-designed mutation assay to detect 35 mutations of interest in the *KRAS*, *BRAF* and *NRAS* genes in FFPE tumor tissue for patient stratification in clinical trials, was provided. In this multiplexed mutation assay, several novel analytical validation approaches were taken to reduce the likelihood of false-positive calls, which are an issue associated with clinical FFPE tissue [63]. Recently, several publications showed that many mutations in COSMIC were reported only once, suggesting a strong possibility of a false-positive call [69,87]. This will likely trace back to the validity of many analytically validated clinical mutation assays. Whether the mutation assays used in the clinical sample testing were properly validated or not is debatable [88,89]. Nonetheless, the reproducibility of rare or unknown mutations detected from these assays should always be addressed.

One frequently encountered issue during clinical sample testing is the high sample failure rate resulting from the samples' inability to meet minimum quality or quantity requirements. Typically, a biomarker strategy starts with acquiring high quality preclinical and clinical biosamples in order to facilitate biomarker discovery. However, from a clinical assay development and validation point of view, this approach may have unintended consequences, since it may lead to assays that are difficult to validate clinically with actual clinical samples, which normally have lower quality. Without improving sample collection and storage issues at the clinical sites, the use of high quality samples for biomarker discovery and development may make it difficult to reproduce a preclinically observed biomarker effect in a clinical setting. Therefore, in the biomarker discovery-development stage, preclinical samples with quality that mimics that of poorer quality clinical samples should be evaluated. This could be done by using preclinical samples that went through a controlled degradation/fragmentation process, for the preclinical biomarker validation. The analytical validation plan should also consider including a section to address the limitation of the assay when lower quality samples are encountered, therefore allowing the clinical team to more accurately calculate or predict the enrollment rate. It would be a disastrous situation if a clinical trial were terminated not because the lack of drug efficacy, but instead due to a poor enrollment rate that was not anticipated by the clinical team.

Validating the reproducibility of any specific mutation does not guarantee the validity of different

mutations. The KBN-SNPE mutation assay described in this review article showed that different mutations have different detection sensitivity and any mutations detected close to their detection sensitivity will be unlikely to be reproducible. Therefore, the KBN-SNPE assay employed a triplicate strategy to address this issue which resulted in the improvement of assay performance and reduced false positives and false negatives to almost non-existence. The fundamental issue for FFPE tissue samples is the degree of fragmentation of the nucleic acids and the limited quantity available from each FFPE tissue slide. In other words, when the mutation assay uses only a small amount of gDNA, the limited number of copies of DNA available for PCR amplification shifts the focus of the question to sampling variation and the fundamental principle of probability. If a limited copy number of DNA is used, simple sampling variation can easily cause a two to threefold variation in variants with low VF, and further variation will be introduced if additional manipulations throughout the library preparation or entire protocol, including PCR amplification, are needed. With a small number of DNA copies available, improper or insufficient sample and reagent mixing during each step of the assay could also introduce large variation. Additional considerations from a thermodynamics and kinetics point of view, such as proper hybridization to the specific target sequence, and the competition among minor and major alleles for the primers/probes or even ligation adaptors, could also play critical roles. Many analytical validation strategies for mutation assays do not use the true clinically mimetic samples,

Executive summary

Brief overview of mutation assays that detect MAPK pathway gene activation

- Type of MAPK pathway activation mutation assays can be classified into two broad categories: those that detect mutations in specific, limited nucleotide locations, and those that take a general sequencing approach such as Sanger sequencing or pyrosequencing.
- Many clinical considerations based on clinical objectives are important to the selection of a specific assay for the clinical application.
- Additional assay development considerations include tissue types, sample collection and preservation methods, clinical objectives, turn-around time, and acceptable false-positive/false-negative rates.

Novel features of a custom-designed multiplexed mutation assay

- The *KRAS*, *BRAF*, *NRAS* SNPE (KBN-SNPE) mutation detection assay was designed to support an ongoing clinical trial for prospective patient enrollment.
- The analytical validation process of KBN-SNPE assay involved a triplicate strategy and relied on several novel approaches to redefine and calculate sensitivity, specificity and accuracy.
- The KBN-SNPE mutation assay described in this review article showed different mutations have different detection sensitivity and any mutations detected close to their detection sensitivity are unlikely to be reproducible.

Future perspective

- In the biomarker discovery-development stage, preclinical samples with quality that mimics that of poorer quality clinical samples should be evaluated.
- The triplicate strategy should be considered to improve assay reproducibility for any mutation assays including next generation sequencing, especially for those variant calls around detection sensitivity.

which usually have poorer average quality than those procured from the commercial tissue source. Even if the clinically representative average-quality samples are used for the analytical validation, those clinical samples with below-average quality might have irrational results that were not covered by the validation strategy. Hence, accepting those results based on a successful analytical validation will still lead to the identification of non-reproducible mutations which could be mostly eliminated by implementing a triplicate strategy. Of course, cost is always a concern for running singlet mutation assay versus triplicate mutation assay. However, based on clinical experience, samples usually arrive in batches and due to TAT requirements they are likely to be processed a few samples at a time. Either using a 96-well plate assay for PCR-based mutation detection or a chip or flow cell for NGS assay, running triplicate (starting from the same gDNA stock solution) will be unlikely to increase the cost three-fold. Nonetheless, cost should not be the reason for compromising the clinical assay reproducibility.

As discussed in the previous NGS assay design and considerations section, it is likely that NGS will be used more widely in querying MAPK pathway activation

in the future. Since NGS can cover both known and unknown mutations, and use of the less sensitive mutation assays may cause the misdiagnosis of many patients, it may be beneficial to cover the entire gene of interest using a focused NGS gene panel. The above mentioned Amgen and Illumina companion diagnostics agreement should pave the way for querying the MAPK pathway activation in a more comprehensive manner.

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