Implications of Key Differences Across 12 *KRAS* Mutation Detection Technologies and Their Relevance in Clinical Practice

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Background

- *KRAS* mutations define one of the largest genomic subsets of NSCLC,[1] and various clinical trials have incorporated targeted therapies for patients with advanced NSCLC containing these mutations.[2,3]
- Testing for actionable mutations in NSCLC, such as *EGFR* exon 19 deletions, is well established in routine clinical practice,[4] however *KRAS* testing is not part of the clinical guidelines.
- *KRAS* mutation testing is commonly employed to inform treatment decisions in colorectal cancer[5], and a number of *KRAS* testing technologies exist that can aid clinical practice.
- There is a need for high performing, easy-to-use, affordable tests that provide robust results and can guide clinical decisions.
- We assessed a 'snap-shot' of the state-of-the-art landscape of *KRAS* mutation detection technologies that are commonly used in today's molecular clinical diagnostic setting.

Methods

Cell line models

- Five distinct *KRAS* mutant cell lines reflecting the most common variant alleles in NSCLC, including all codons relevant to NSCLC were obtained from American Type Culture Collection (Teddington, UK) and used in the analyses.
- The cell lines (and corresponding *KRAS* mutations) used were: MIA PACA-2 (p.G12C),[6] PANC-1 (p.G12D),[7] MDA-MB231 (p.G13D),[8] SW620 (p.G12V),[9] and NCI-H460 (p.Q61H).[10]
- Cells were grown according to recommended culturing conditions.

DNA extraction and creation of admixtures

- Fifty-six admixtures were prepared at very low copy numbers and concentrations that can be typically encountered from clinical NSCLC biopsies.[11]
- Cell line DNA was extracted from frozen cell pellets containing ~5x10^6 cells using the DNeasy Blood & Tissue kit (Qiagen).
- 1000 copies/µL DNA mutant standards were made from each cell line, and diluted with known *KRAS* wild-type Human Genomic Reference DNA (Roche Diagnostics).
- Two admixtures of 100 copies/µL and 50 copies/µL mutant alleles were created for each concentration (20%, 10%, 5%, 1%, and 0.5%), to create 50 admixtures in total.
- Six wild-type control samples were also prepared with total input DNA copy numbers equivalent to those in cell line admixtures.

KRAS mutation detection technologies

- All admixtures were analysed across 12 distinct technologies and assays in a blinded manner: three real-time quantitative PCR (RT-qPCR), two matrix-assisted laser desorption/ionisation time-of-flight spectrometry (MALDI-TOF), five next-generation sequencing (NGS), one droplet digital PCR (ddPCR), and one Sanger capillary sequencing method.
- *therascreen*® and **cobas**® *KRAS* mutation testing was performed at AstraZeneca laboratories; remaining methods were performed at external partner laboratories where *KRAS* testing is routinely performed to assist clinical practice or conducted for research purposes.
- In each technology, the admixtures were used directly as input and sample pre-processing was allowed; each technology was used according to the established manufacturer's protocol or following the laboratory's standard operating procedures.
- Characteristics of each technology were compared using a questionnaire completed by clinical labs or technology manufacturers.

Results

Relative sensitivity of technologies

- Analysis of admixture DNA demonstrated a range of performance between individual technologies (**Figure 1**).
  - Overall, the greatest levels of detection were achieved with the '100 copy' DNA samples.
  - Only 384/672 data points were correctly identified across all 12 technologies and assays.
  - Successful genotyping ranged from 0% using Sanger sequencing to 100% using NGS.

Real-time quantitative PCR

- The **cobas**® KRAS Mutation Test (Roche Molecular Systems) detected *KRAS* mutations below the specified 5% limit of detection for p.G12D, p.G13D and p.G12V in the 100 copy and 50 copy samples; the lowest level of detection for p.G12C was at the 10% level.
- The *therascreen*® KRAS RGQ PCR kit (Qiagen) was able to detect all codon 12 and codon 13 mutations at the 10% and 20% level in the 100 copy sample, but does not cover *KRAS* p.Q61 codon mutations which account for ~5% of *KRAS* mutations in NSCLC.[12]
- The Idylla™ *KRAS* mutation test (Biocartis) accurately identified 96% of samples, including all 100 copy mutant and 23/25 of the 50 copy mutant samples.

Matrix-assisted laser desorption/ionisation time-of-flight spectrometry

- UltraSEEK™ (Agena Bioscience) accurately identified 92% of samples, detecting all codon 12 and codon 13 mutations down to 0.5% in the 100 and 50 copy admixtures. Detection down to the 5% level was achieved for p.Q61H in both the 100 and 50 copy samples.

Next-generation sequencing

- The Oncomine™ Focus Assay (Thermo Fisher Scientific) was the only technology to correctly identify all 56 samples in the 100 and 50 copy admixtures, and did so at all levels of allele frequency down to 0.5%.
- The Illumina Nextera® Rapid Capture Custom Lung Panel did not detect any *KRAS* mutations and therefore was excluded from the analysis; however, only three samples met the manufacturer's recommended concentration of ≥50 ng per 10 µL.

Droplet digital PCR

- The *KRAS* PrimePCR™ ddPCR™ assay (Bio-Rad Laboratories) identified 60% of samples (100 copies input) and 52% (50 copies input) correctly, highlighting the need for further optimisation.

Sanger capillary sequencing

- Sanger capillary sequencing (Applied Biosystems) produced weak PCR products and only very low mutant levels were detected in a few samples, as expected due to its low sensitivity.

Comparison of technology characteristics

- **Table 1** provides an overview of the characteristics and advantages of each technology and assay.
- Overall, the fastest turnaround time achieved from sample to result was 2 hours (Idylla™ CE-IVD; Biocartis). NGS methods took between 2 days (Sentosa® SQ NSCLC Panel; Vela Diagnostics) to 2–3 weeks for the NGS Illumina Nextera® Rapid Capture Custom Lung Panel.
- Required lab expertise ranged from minimal (Idylla™ CE-IVD) to high (several other technologies).

Conclusions

- *KRAS* mutant detection technologies and assays varied greatly in terms of technical requirements and performance when using samples with DNA concentrations representative of clinical NSCLC biopsies.
- This highlights the need to select the most appropriate test for the sample type and quality and quantity of DNA.
- The NGS hybridisation based approach is more sensitive to suboptimal DNA input than amplicon based NGS assays.
- The admixtures created employed high molecular weights and are not necessarily reflective of technology performance in fragmented DNA, or DNA containing sequence artefacts, such as might be observed with FFPE samples.[13]
- Appropriate optimisation and validation of tests, representative of the intended clinical sample, is essential in every laboratory.
- Most of the technologies used are also available for other molecular biomarkers; our findings may be applicable to other solid tumours where mutation status can inform treatment decisions.
- The *therascreen*®, **cobas**® and Idylla™ tests are CE-IVD approved, and the Sentosa® SQ NSCLC Panel was the only fully automated NGS CE-IVD approved technology assessed.

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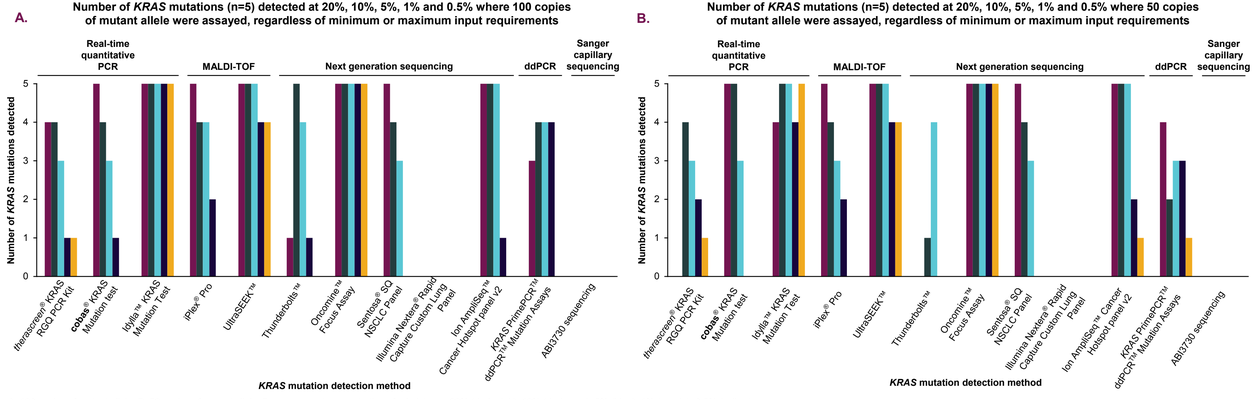
Figure 1: Relative sensitivity of technologies



A. 100 copies of mutant allele; B. 50 copies of mutant allele. Bars represent concentration of admixtures: 20% (dark pink); 10% (dark grey); 5% (blue); 1% (purple); 0.5% (yellow)
ddPCR, droplet digital polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption/ionisation time-of-flight spectrometry; PCR, polymerase chain reaction.

Table 1: Comparison of technology characteristics

Parameter	Real-time quantitative PCR			MALDI-TOF		Next generation sequencing					ddPCR	Sanger capillary sequencing
	therascreen® KRAS RGQ PCR Kit	cobas® KRAS Mutation Test	Idylla™ KRAS Mutation Test	iPlex® Pro	UltraSEEK™	Thunderbolts™	Oncomine™ Focus Assay	Sentosa® SQ NSCLC Panel	Illumina Nextera® Rapid Capture Custom Lung Panel	Ion AmpliSeq™ Cancer Hotspot panel v2	*KRAS* PrimePCR™ ddPCR™ Mutation Assays	ABI3730 sequencing
Ease of use												
No. handling steps (wet work)	6–8	3–5	1–2	3–5	3–5	11–20	6–8	6–8	>20	11–20	6–8	11–20
No. handling steps (analysis to report)	6–8	3–5	1–2	1–2	1–2	6–8	3–5	1–2	9–10	1–2	3–5	3–5
Level of expertise required (1–4 [low to high])	3	2	1	3	3	4	4	2	4	3	4	4
Turnaround time												
Hands on wet-lab work time (exc. DNA extraction)	2–5 hrs	31–60 mins	0–30 mins	31–60 mins	31–60 mins	5–10 hrs	5–10 hrs	2–5 hrs	>20 hrs	10–20 hrs	2–5 hrs	2–5 hrs
Hands on analysis	31–60 mins	0–30 mins	0–30 mins	0–30 mins	0–30 mins	1–2 hrs	31–60 mins	0–30 mins	2–5 hrs	1–2 hrs	31–60 mins	2–5 hrs
Total turnaround from DNA to clinical reporting (minimum)	1–2 days	3–4 days	2–4 hrs	1–2 days	1–2 days	5–10 days	3–4 days	2–3 days	2–3 weeks	5 days	1–2 days	1–2 days
Multiplexing level												
No. of reactions per sample	8+	2	3–5	1–24	1–4	2	1	1	1	1	3–5	2
Max. no. of samples per run	9	25–48	1	16–384	24–96	25–48	2–10	2–10	2–10	2–10	49–96	11–24
No. of genes covered	1	1	1	11+	11+	11+	11+	11+	11+	11+	1	1
No. of codons covered	2	3	6–10	11+	11+	Hotspots	Hotspots	Hotspots	Complete coverage	Complete coverage	3–5	Complete coverage
No. of individual mutations	6–10	All possible	11+	11+	11+	All possible	All possible	All possible	All possible	All possible	3–5	All possible
Tissue/DNA requirements												
Minimum required DNA input	20 ng	50 ng	N/A	80 ng	10 ng	20–30 ng	10 ng	5 ng	50–75 ng	10 ng	1 ng	10 ng
Amount of FFPE tissue required*	20 µm	5 µm	5–10 µm	5 µm	5 µm	15–20 µm	5 µm	10 µm	15–20 µm	5 µm	5 µm	10 µm
Limit of detection												
Claimed sensitivity (%)	5%	5%	5%	10%	0.1%	5%	5%	5%	10%	5%	0.001%	20–30%

ddPCR, droplet digital polymerase chain reaction; FFPE, formalin-fixed, paraffin-embedded; NGS, next-generation sequencing; MALDI-TOF, matrix-assisted laser desorption/ionisation time-of-flight spectrometry; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction
*Slide requirements may vary according to the amount of tumour tissue on the slide.

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