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Implementation of next generation sequencing technology for somatic mutation detection in routine laboratory practice

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Summary

The introduction of next generation sequencing (NGS) in the routine diagnostic setting is still in the development phase and has been limited by its complexity. Targeted NGS offers an attractive alternative to performing multiple single target assays and is very useful in meeting the increasing clinical demand for testing of multiple genetic aberrations in cancer specimens.

To this end, we carried out a blinded validation study on 113 tumours in a diagnostic laboratory and compared mutation results from targeted NGS with those from Sanger sequencing, pyrosequencing, competitive allele specific TaqMan polymerase chain reaction (CAST PCR) and Cobas assays. DNA was extracted from formalin fixed, paraffin embedded (FFPE) tissue samples that included core biopsies, resections and cytology samples from three common and one rare cancer types [non-small cell lung cancer (NSCLC), colorectal cancer (CRC), malignant melanoma (MM) and gastrointestinal stromal tumour (GIST)]. Libraries were prepared using the TruSight Tumour 26 gene panel and NGS was carried out on the MiSeq instrument.

Results from NGS were concordant with the mutational status determined by other platforms in 107 of the 113 cases tested (94.7%). The sequencing quality for NGS failed in four of the six false negative cases, while a further two samples gave false negative results because the c-*KIT* mutations were located outside the range of the NGS panel. One NSCLC sample contained an *EGFR* mutation previously detected by the Cobas assay. Reanalysis of the NGS data for this sample using a cut-off allele frequency of 1% revealed the mutation had an allele frequency of 2%, which was below the recommended software-determined threshold of 3%. NGS detected 113 additional mutations that were not previously known from analysis by the conventional methods. Twenty-six of these have known clinical importance, 37 have potential clinical significance, while 50 were novel mutations with unknown clinical significance. NGS detected variants using inputs of 10–20 ng of FFPE extracted DNA and from specimens with a tumour cell content less than 50%, for which when possible we recommend microdissection.

We conclude that results from targeted NGS are highly concordant with those from other mutation testing platforms. Targeted NGS is suitable for a range of sample types received in the diagnostic pathology laboratory, including those with limited material or with low tumour cell content (TCC). This work has allowed us to determine the quality parameter settings required in order to obtain robust mutation data by NGS.

Key words: Next generation sequencing; somatic mutation; gene panels; molecular diagnostic testing laboratory.

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INTRODUCTION

Next generation sequencing (NGS) technology has revolutionised the ability to analyse genomic aberrations in cancer tissues.[1] In recent years this technology has become more affordable, leading to large, collaborative whole genome studies of cancers.[2–6] The resulting data have improved our understanding of the genes and pathways that drive tumour development, provided a rational basis for drug development and treatment strategies, and identified potential markers for cancer stratification, diagnosis and prognostication.[7] As a consequence, the number of specific gene aberrations now being requested for testing is rapidly increasing. There is an urgent need for validation of NGS technology in the clinical setting. Studies that address the entire workflow starting from sample acquisition through to reporting are critical for its clinical implementation.[8,9]

With the rapidly increasing number of clinical biomarkers, multiple tests are frequently requested for a single patient sample and hence there is a greater need for multiplexing with a suite of tests that are often common to several cancer types. In this context, NGS is increasingly being recognised as highly suitable to meet these demands.[10]

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NGS has a number of distinct advantages in this regard, notably its capacity to screen multiple targets in multiple samples in a single assay, with high sensitivity and low DNA input requirement. It is highly suited for reflex testing and has a faster turn-around time, is more cost effective and requires less sample DNA input than conventional platforms. NGS is versatile because it can readily identify single-nucleotide variants, deletions and insertions. Furthermore, it can provide definitive variant allele frequencies and also detect novel sequence variants.

Several laboratories, mainly from the USA, have published their findings with NGS for a range of different cancer types using several different gene panels and platforms.[11–15] Guidelines for the use of NGS in the clinical diagnostic setting have been published recently by regulatory bodies including the European Society of Genetics[16] and the College of American Pathologists.[17] However, the use of NGS in the clinical setting is still in the early developmental stage and there is a need for further validation studies conducted by diagnostic laboratories.[18] Clearer policies are also required with regard to the reporting of incidental or novel aberrations.[19]

In the blinded validation study reported here, we evaluated NGS in the diagnostic setting for mutational analysis of 113 samples from non-small cell lung cancer (NSCLC), colorectal cancer (CRC), malignant melanoma (MM) and gastrointestinal stromal tumour (GIST) and two normal tissues. Results from NGS were compared against the mutation status previously determined by four other techniques. The study involved scientists, pathologists and clinicians, reflecting their essential core contributions in our workflow algorithms. The MiSeq platform in combination with the TruSight Tumour panel was determined at the start of the study to best fit the diagnostic testing criteria of our laboratory. The assay offers paired-end reading and delivers highly accurate and reproducible sequencing data in a single, integrated bench-top instrument.[20,21] This study was aimed at addressing the specific challenges encountered during routine implementation of NGS.[7] These include the suitability of low quantities of DNA extracted from a variety of formalin fixed, paraffin embedded (FFPE) sample types, the optimal laboratory workflow, the ease of use of available variant analysis software, and finally the reporting of results.

To this end, we assessed the sensitivity, specificity, reproducibility and limit of detection of NGS in comparison to other validated testing platforms used in our laboratory. Through this study we have been able to set quality parameters for NGS analysis in the diagnostic setting that allows us to provide robust and accredited reporting to clinicians. We believe these results will contribute to the development of standardised approaches to NGS testing for clinical implementation.

MATERIAL AND METHODS

Tissue samples

FFPE tumour samples (n = 111, see Table 1 for details) and two normal tissues dating from 2010 to 2015 were selected from the archives of PathWest Laboratory Medicine, Western Australia. Twenty-eight of these were referred from external pathology laboratories. Samples were chosen according to their mutation status for the *BRAF*, *KRAS*, *NRAS*, *KIT*, *EGFR*, *PIK3CA* and *PDGFRA* genes as previously determined by either Cobas 4800, competitive allele specific TaqMan polymerase chain reaction (CAST PCR), Sanger bi-directional sequencing or pyrosequencing platforms. The samples comprised 21 NSCLC, 52 CRC, 32 MM and 6 GIST. They consisted of cytology (fine needle aspiration, pleural fluid) and non-cytology (excisions, core biopsies, resections) samples from 111 individual patients.

A total of 87 unique mutations (74 single base substitutions and 13 deletions) were previously detected by conventional methods in these samples (Supplementary Table 1, Appendix A). All cases had been previously tested for mutations in at least one gene using conventional platforms. Cases were arranged in order of internal diagnostic case number identifiers and then the sample identity and molecular status were blinded by re-numbering the cases 1–111. Samples from the pool of 111 patients were selected for each run by a scientist not performing the assay and then tested using the Trusight Tumour 26 gene panel (Illumina, USA) on the MiSeq instrument (Illumina). The scientist performing the test and reviewers of the data had no knowledge of each case background and corresponding molecular status.

Eight cell lines with well characterised *EGFR*, *BRAF*, *KRAS* and *NRAS* mutations (Horizon Diagnostics, UK; Table 2) were used to evaluate the sensitivity of the Miseq Illumina Platform. The two normal tissue samples were included to test specificity of the assay.

Three commercial control DNA multiplex reference standards (Horizon Diagnostics) that contain characterised mutations in commonly tested genes (*BRAF, EGFR, KRAS, NRAS, KIT, PDGFRA, PIK3CA*) at 5%, 2.5% and 1% variant allele frequencies were also used to evaluate the sensitivity and specificity of the TruSight tumour kit.

DNA extraction

Haematoxylin and eosin (H&E) stained sections were assessed by a pathologist to estimate the percentage of tumour cells within cell blocks or biopsy specimens. FFPE tissue blocks with the highest tumour cell content were selected. Where applicable, for cases with <50% tumour cell content, manual microdissection under a dissecting microscope was carried out after preparing 15×5 μm unstained sections from FFPE tissue. The majority of cytology cell block cases with <50% tumour cell content were not suitable for microdissection due to the mixed population of tumour infiltrate amongst a background of inflammatory cells. The tissue fragments were transferred to Eppendorf tubes and DNA was extracted with the QIAamp DNA mini kit (Qiagen, Australia) using the Qiacube automated method. Samples for analysis with the Cobas were isolated with Cobas DNA sample preparation kit (Roche Diagnostics, USA). DNA quantification was performed using the Nanodrop (Thermo Scientific, USA) or Qubit 2.0 Fluorometer (Life Technologies, USA) instruments. See Supplementary Materials and Methods (Appendix A) for further details.

For most cases the same DNA aliquot was used for somatic mutation testing by all platforms. This ensured that the TCC was consistent in both conventional methods and NGS. In instances where an additional DNA sample was required for further testing, the same DNA extraction procedure was performed, including microdissection or whole section DNA isolation.

Table 1 Tissue characteristics

Tumour type	Total cases	Resection	Cytology	Biopsy
Lung	21	2	14	5
Colorectal	52	26	1	25
Melanoma	32	1	5	26
GIST	6			6

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