

Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients

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ABSTRACT

Aims To evaluate the accuracy, consumable cost and time around testing (TAT) of a next-generation sequencing (NGS) assay, the Ion Torrent AmpliSeq Colon and Lung Cancer Panel, as an alternative to Sanger sequencing to genotype KRAS, NRAS and BRAF in colorectal cancer patients.

Methods The Ion Torrent panel was first verified on cell lines and on control samples and then prospectively applied to routine specimens (n=114), with Sanger sequencing as reference.

Results The Ion Torrent panel detected mutant alleles at the 5% level on cell lines and correctly classified all control tissues. The Ion Torrent assay was successfully carried out on most (95.6%) routine diagnostic samples. Of these, 12 (11%) harboured mutations in the BRAF gene and 47 (43%) in either of the two RAS genes, in two cases with a low abundance of RAS mutant allele which was missed by Sanger sequencing. The mean TAT, from sample receipt to reporting, was 10.4 (Sanger) and 13.0 (Ion Torrent) working days. The consumable cost for genotyping KRAS, NRAS and BRAF was €196 (Sanger) and €187 (Ion Torrent).

Conclusions Ion Torrent AmpliSeq Colon and Lung Cancer Panel sequencing is as robust as Sanger sequencing in routine diagnostics to select patients for anti-epidermal growth factor receptor (EGFR) therapy for metastatic colorectal cancer.

INTRODUCTION

In metastatic colorectal cancer (mCRC), the epidermal growth factor receptor (EGFR) is targeted by cetuximab and panitumumab monoclonal antibodies.¹ However, several clinical trials have demonstrated that patients whose tumour has KRAS mutations in codon 12 or 13 do not benefit from these drugs,^{2,3} although there is significant evidence supporting a partial response for codon 13 mutants.⁴ The College of American Pathologists (CAP)⁵ and the European Society of Pathology (ESP)⁶ have not recommended any single KRAS testing method, but suggested the use of kits approved for in vitro diagnostic (IVD) use by the Food and Drug Administration (FDA) or by the European Community (EC), or validation using a laboratory-based assay.¹ Our laboratory, a large volume reference centre for South Italy, adopted Sanger sequencing; this method, although labour-intensive, was accurate and efficiently predicted response to cetuximab treatment.⁷

A paradigm shift in our testing strategy, however, may be driven by the recent requirement of the European Medicine Agency to analyse codons 12

and 13 in exon 2, codons 59 and 61 in exon 3, and codons 117 and 146 in exon 4 for both KRAS and NRAS.⁸ In addition, testing may also include assays for the emerging genomic determinant of prognosis and/or resistance, BRAF.¹ The increase in gene targets makes Sanger sequencing even more labour intensive and IVD use-approved kits very expensive, while the required amount of genomic DNA (gDNA) is not always available.⁹ Multiplexing assays based on single nucleotide primer extension analysed by capillary electrophoresis or by mass spectrometry can provide a solution.¹⁰ However, these assays can interrogate only a limited number of common variants and are not easily scalable to accommodate additional biomarkers for future testing.¹⁰

Multiple genes of multiple mCRC patients can simultaneously be analysed by next-generation sequencing (NGS); thus, this technology may soon replace Sanger sequencing to select patients for anti-EGFR treatment.¹¹ Among the most popular NGS benchtop platforms is the Ion Torrent Personal Genome Machine (PGM; Life Technologies, Carlsbad, California, USA) which requires only a small amount of gDNA (10 ng).¹² Until recently, this NGS platform has employed very broad panels containing around 50 cancer driver gene targets.^{10, 13–15} To encourage implementation of the NGS in predictive molecular diagnostics, Ion Torrent recently released a 22 gene target panel called the Ion AmpliSeq Colon and Lung Cancer Panel. The performance of this panel has previously been evaluated for clinical cancer testing by other groups retrospectively on archival¹⁶ and clinical trial specimens.¹⁷ The current study was conducted to verify the AmpliSeq Colon and Lung Cancer assay in the different setting of routine diagnostics. Prospectively, a large number of consecutive and unselected samples were simultaneously processed by Sanger sequencing and by the AmpliSeq Colon and Lung Cancer assay. Diagnostic accuracy, cost, time around testing (TAT) and the overall practicality of the two methodologies were evaluated. The aim was to assess whether the AmpliSeq Colon and Lung Cancer Panel on the Ion Torrent PGM could be an alternative to Sanger sequencing for genotyping KRAS, NRAS and BRAF genes in our laboratory.

METHODS

AmpliSeq Colon and Lung Cancer Panel: preliminary verification

Like all laboratory-developed tests in molecular diagnostics, the analytical performance of NGS



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procedures must be internally confirmed before clinical implementation. Since the performance of the Ion AmpliSeq Colon and Lung Cancer Panel has previously been evaluated,¹⁶ verification (rather than formal validation) was carried out for three clinically actionable genes: KRAS, NRAS and BRAF.

The HT29 (BRAF V600E), the SW480 (KRAS G12V) and the H1299 (NRAS Q61K) cell lines were obtained from CNR/IEOS (Naples, Italy). Each DNA cell line was serially diluted in wild-type DNA (derived from the SW48 cell line) to produce mixtures with 50%, 20% and 5% mutant alleles, the last percentage being the threshold usually adopted for clinical decision making.^{13–14} The ability of the Ion AmpliSeq Colon and Lung Cancer Panel to detect the most relevant mutational hotspots was also verified on a set of 15 mutation-positive (Sanger sequencing) formalin-fixed paraffin-embedded (FFPE) samples with known mutations in the BRAF (n=1; V600E), KRAS (n=9; G12R, G12S, G12C, G12D, G12V, G12A, G13D, Q61H, A146T) and NRAS (n=5; G12D, G13S, G13R, Q61K, Q61R) genes.

Routine clinical samples

Following approval from the institutional review board, the relative performance of the Ion Torrent assay and of Sanger sequencing on prospectively collected and processed routine diagnostic samples was assessed. Routine samples were simultaneously processed by our current technology, based on Sanger sequencing, and by Ion Torrent PGM sequencing. To this end, 114 unselected consecutive (including 99 surgical samples and 15 endoscopic biopsies) FFPE samples, referred from 18 different institutions, were prospectively processed by both methods. Only one single tumour sample from a given location (primary tumour n=105; metastases n=9) was tested for each patient.

Our molecular laboratory is an accredited Italian Society of Pathology reference centre for RAS testing and the organiser in Italy for the ESP Colon External Quality Assessment Scheme. After obtaining the patient's consent, oncologists and the primary pathologists from outside institutions record the clinical and pathological data (including the original pathology report) on a dedicated website. Then, the corresponding tissue sample is express-mailed to our central laboratory. Upon receipt of each sample, a representative H&E stained slide is reviewed by a pathologist and the area with the highest density of neoplastic cells is marked, annotating the percentage of neoplastic cells. Depending on the complexity of histology and on the density of the tumour, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) from two (resection specimens) or three (biopsy specimens) 10 µm-thick serial sections. An additional section (biopsy specimens only) was stained by H&E to confirm tumour cell percentage.

Sanger sequencing

Sanger sequencing was performed as previously described. Briefly, DNA targets for exons 2, 3 and 4 of KRAS and NRAS, and exon 15 of BRAF were amplified using laboratory-developed primer pairs, as previously reported.^{18–19} The concentration of reagents was optimised using 80 ng of DNA, 0.4 mM of each primer and 0.5 U of 5 PRIME Taq DNA Polymerase (Eppendorf, Milan, Italy) in a total volume of 25 µL. PCR conditions were as follows: initial denaturation for 5 min at 95°C, cyclic denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation at 65°C for 30 s for 35 cycles, and final extension at 72°C for 2 min. Following PCR, the fragments were purified using the QIAquick DNA purification kit (Qiagen) according to the manufacturer's instructions.

Sequencing reactions were performed for both DNA strands by the Big Dye Terminator V1.1 (Applied Biosystems, Monza, Italy) on a total of 10 ng of purified PCR products. Dye purification was carried out by alcohol/sodium acetate precipitation. Sequence analysis was performed on an Applied Biosystems 310 genetic analyser. The files obtained were aligned to the reference sequence and examined for mutations by the CodonCode software.

Ion Torrent sequencing

DNA was extracted from cell lines and clinical tissue samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was suspended in 30 µL of molecular biology water. DNA quantity and quality were assessed using the Qubit photometer (Life Technologies) and the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

According to the manufacturer's protocols, 10 ng of DNA for each sample was used for library preparation with the Ion AmpliSeq Library 96LV Kit 2.0 (Life Technologies) and the Colon and Lung Cancer Panel (Life Technologies). This panel gives 90 amplicons covering 504 mutational hotspot regions in 22 genes (AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53), with performance of at least 500× sequence coverage for eight samples on one Ion 316 chip. For samples yielding less than 10 ng DNA input, additional cycling conditions were used for library preparation as recommended by the manufacturer. Each library was barcoded with the Ion Xpress Barcode Adapters 1–16 Kit (Life Technologies). Barcoded libraries were combined to a final concentration of 100 pM. Template preparation by emulsion PCR (emPCR) was performed on the Ion OneTouch 2 system (Life Technologies). Library quality control was performed using the Ion Sphere Quality Control Kit according to the manufacturer's instructions, ensuring that 10–30% of template positive Ion Sphere particles (ISP) were targeted in the emPCR reaction. Sequencing primer and polymerase were added to the final enriched ISPs prior to loading onto 316 (100 Mb output) chips. Sequencing was carried out on the PGM (Life Technologies). Data analysis was carried out with Torrent Suite Software V3.2 (Life Technologies), considering only KRAS, NRAS and BRAF, while all other genes were masked. After alignment to the hg19 human reference genome, the Variant Caller plug-in was applied using the Colon and Lung hotspot file as a reference (downloaded from Ion Community, <http://www.ioncommunity.lifetechnologies.com>, last accessed 15 September 2014). The Ion Reporter suite (Life Technologies) was used to filter polymorphic variants. In addition, all nucleotide variations with less than a 5% variant frequency were masked. All detected variants were manually reviewed with the Integrative Genomics Viewer (IGV V2.1, Broad Institute, Cambridge, Massachusetts, USA).

Evaluation of TAT and consumable costs

In order to evaluate the overall practicality of performing Ion Torrent sequencing in our referral centre, TAT and consumable costs were taken into account. The TAT (the period from sample receipt to interpretation of the results) was recorded for every sample for both Sanger and Ion Torrent sequencing. The first step of our routine testing algorithm is evaluation of KRAS exon 2, where most resistance mutations are clustered. Only KRAS exon 2 wild-type cases undergo direct sequencing of PCR products of the remaining RAS and BRAF exons. For Sanger

sequencing, consumable cost was evaluated for a single exon analysis and multiplied for the number of reactions needed for any given gene. For Ion Torrent sequencing, each patient analysis cost was estimated considering the fact that eight barcoded samples were loaded for each 316 chip.

RESULTS

AmpliSeq Colon and Lung Cancer Panel: preliminary verification

Serial dilution of the HT29, SW480 and H1299 cell lines to 20%, 10%, 5% of mutant alleles demonstrated that the Ion Torrent platform consistently detected mutations at the 5% level of mutant alleles. All of the 15 verification tissue specimens were correctly genotyped for KRAS, BRAF and NRAS by the Ion Torrent NGS.

Ion Torrent sequencing in routine settings

Most of the routine samples (109/114; 95.6%) processed on the PGM yielded an adequate library for subsequent sequencing, although library preparation failed in five cases. Three of the failed cases did not yield adequate results by Sanger sequencing either. In most of the adequate cases (85/109), amplification for library generation was carried out without major technical problems; in a minority of cases (24/109), the low level of library concentration (<100 pM) required DNA re-amplification.

As reported in the Methods section, mutations detected by Ion Torrent with at least a 5% variant frequency were annotated (figure 1). Ten BRAF mutant cases (V600E, n=7; G596R, n=1; K601E, n=1; D594G, n=1) were detected by both Sanger and Ion Torrent sequencing. In addition, two BRAF mutations

(G466E, n=1; G469A, n=1) not covered by our Sanger sequencing-based assay were only detected by Ion Torrent. A total of 38 KRAS mutations (exon 2, n=32; exon 3, n=2; exon 4, n=4) were detected by both techniques (figure 1). One KRAS Q22K mutation detected by Ion Torrent at a 5.5% mutant allele level was missed by Sanger sequencing (figure 1). Eight cases harbouring NRAS mutations (exon 2, n=2; exon 3, n=6) were detected by both sequencing methods. One G13R mutation with a 5.2% mutant allele frequency was only detected by Ion Torrent.

Evaluation of TAT and consumable costs

The Ion Torrent mean TAT was 13.0 working days (range 7–14). The mean TAT for Sanger sequencing evaluation of KRAS exon 2, was 4.2 working days (range 3–6); in the cases also evaluated for the remaining KRAS, NRAS and BRAF exons, the entire process had a mean TAT of 10.4 working days.

The cost of consumables for any single exon analysis by Sanger sequencing was €28. Consequently, the consumable cost of testing KRAS, NRAS and BRAF, including seven exons, was €196. For Ion Torrent sequencing, as eight barcoded samples were loaded for each 316 chip, the cost for each patient analysis was €187.23. This amount was slightly higher (€262.20) in a minority of cases (24/109) where the low level of library concentration required DNA re-amplification. Similarly, initialisation failures, occurring twice for a total of 16 samples, led to an increase of €7.80 per sample.

DISCUSSION

Ion Torrent NGS assays have been retrospectively evaluated on previously characterised positive and negative archival control

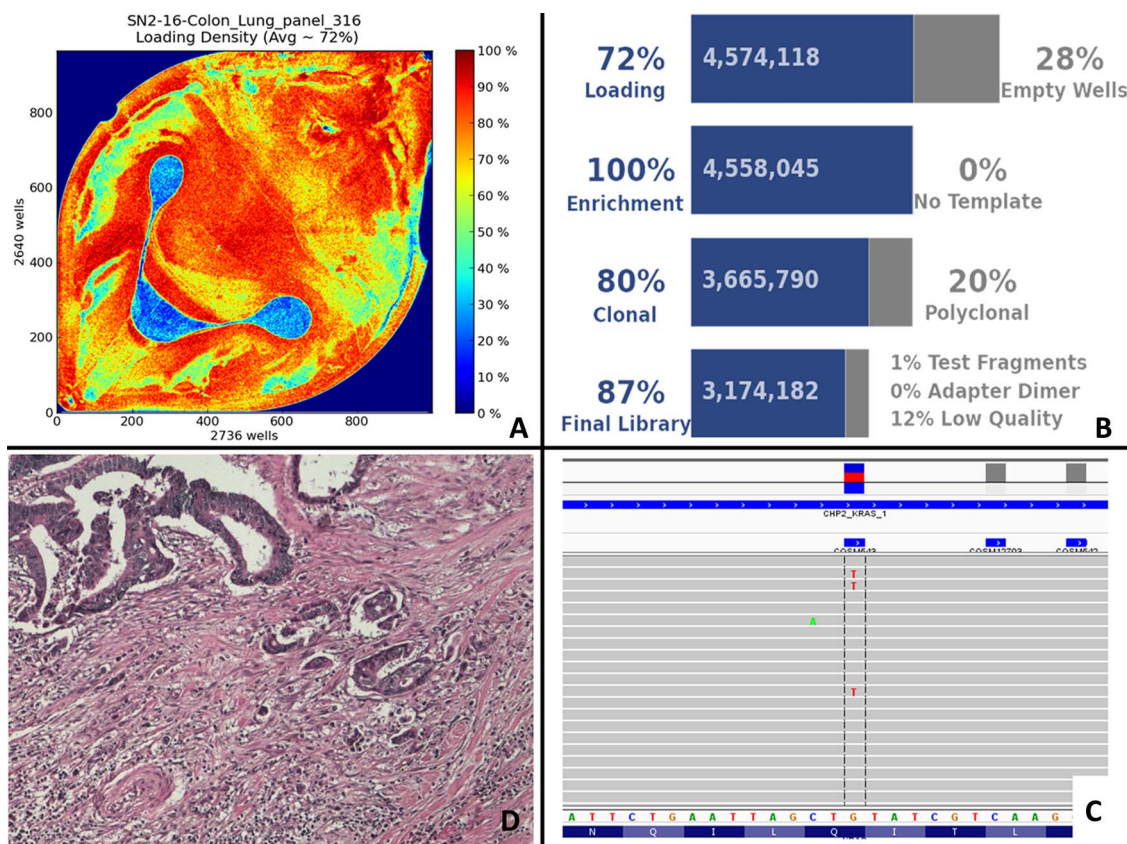


Figure 1 Loading density (A) and performance parameters (B) of an Ion Torrent sequencing run, carried out using a 316 chip are shown. A low frequency of a KRAS Q22K mutant allele (C) was observed with an integrated genomics viewer in a case of colorectal cancer (D) with 30% neoplastic cells.

samples.^{10 13 14 20} However, in our study, routine clinical samples have been prospectively received by our central laboratory from several local pathology laboratories. Sanger sequencing and Ion Torrent NGS were performed simultaneously, unlike in previous reports where these techniques were performed at different times in different laboratories and on different histological sections.^{17 21} In the diagnostic setting, challenges include the less than optimal DNA quality of some samples due to formalin over-fixation, the low tumour cell content in tumour tissues with abundant inflammatory cells, and insufficient starting material, for example, minimal biopsy fragments. The Ion AmpliSeq Colon and Lung Cancer Panel failed in a small minority of cases (4.4%), in contrast to the 100% success rate of a recent clinical trial whose design included preliminary sample selection.¹⁷

All of the 56 point mutations detected by Sanger sequencing were also correctly identified by Ion Torrent NGS, confirming the high level of specificity of the Ion AmpliSeq Colon and Lung Cancer Panel.¹⁶ In addition, the NGS assay detected two BRAF mutations in gene regions not covered by Sanger sequencing. The differential sensitivity of methods can differ; the NGS technique is able to detect mutations with low variant frequencies.¹⁷ To avoid false positive results, the 5% variant frequency threshold is generally recommended for AmpliSeq.^{13 14} In our series, two mutations (KRAS Q22K and NRAS G13R) with variant frequency just above the 5% threshold were missed by Sanger sequencing. These discordant results have a number of technical and clinical implications. From a technical point of view, a laboratory that adopts NGS in clinical practice should consider having an in-house validated single gene assay with at least 5% sensitivity to confirm mutations occurring at a low level, in particular for the most clinically relevant hotspots. In this study, however, one of the discordant mutations occurred in codon 22, which would also be missed by high-sensitive assays targeted at codons 12 and 13.²² The occurrence of less common mutations is expected to increase, as referral laboratories adopt NGS as a screening tool. From a biological point of view, further investigation is required to confirm our previous results suggesting that low abundance of RAS mutant alleles might lead to resistance to anti-EGFR mAbs.²³

While most reports have addressed Ion Torrent sequencing technological issues in detail,^{10 13 14 20} few data are available to show what is a reasonable clinical turnaround time and when Ion Torrent sequencing becomes cost-effective.⁹ Since experience in this new and complicated technology is still limited, our data may show that NGS assays are not overly time consuming and expensive. We found that the Ion Torrent mean TAT for all clinically relevant analysis was only slightly longer than for Sanger sequencing (13.0 vs 10.4 working days), reflecting the long learning curve and more hands-on technical time required for library preparation, chip loading and data analysis. It is likely that the TAT will soon be greatly reduced by adopting the Ion 318 chip, whose higher number of fluidic addressable wells (11 million) will allow simultaneous processing of 16 samples with at least 500× sequencing coverage. In addition, the NGS assay TAT will continue to improve with the implementation of a fully integrated robotic station. Consequently, more efficient sample batching will improve the cost effectiveness of the whole procedure. To date, our data have shown that the consumable cost for testing KRAS, NRAS and BRAF using Ion Torrent sequencing (€187.23) is comparable to that for Sanger sequencing (€196) and much cheaper than the total for individual FDA/CE IVD-approved single-gene tests. However, while the most expensive tests are the easiest to interpret, NGS data

analysis requires more expertise than usually available in academic institutions or in large clinical hospitals.²⁴

In conclusion, AmpliSeq-based procedures are feasible in a central laboratory setting and represent a powerful tool to refine even further personalised treatment regimens for patients with mCRC.

Take home messages

- ▶ A paradigm shift in colorectal cancer predictive testing is being driven by the recent requirement of the European Medicine Agency to analyse codons 12 and 13 in exon 2, codons 59 and 61 in exon 3, and codons 117 and 146 in exon 4 for both KRAS and NRAS.
- ▶ As multiple genes of multiple metastatic colorectal cancer (mCRC) patients can simultaneously be analysed by next-generation sequencing, this technology may soon replace Sanger sequencing to select patients for anti-epidermal growth factor receptor (EGFR) treatment.
- ▶ Ion Torrent AmpliSeq Colon and Lung Cancer Panel sequencing is as robust as Sanger sequencing in routine diagnostics to select patients for anti-EGFR therapy for mCRC.

Contributors UM, GT: conceived and designed the experiments; UM, RS, DV: performed the experiments; UM, GT, EV, PP, CB: analysed the data; UM, EV, CB, PP, GT; contributed to the writing of the manuscript.

Competing interests None.

Ethics approval The Institutional Review Board 'Carlo Romano' approved the study.

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Scopo. Valutare l'accuratezza, il costo dei reagenti ed il *time around testing* (TAT) di un saggio di next-generation sequencing (NGS), *Ion Torrent AmpliSeq Colon and Lung Cancer Panel*, come alternativa al sequenziamento secondo Sanger per genotipizzare KRAS, NRAS and BRAF in pazienti con carcinoma metastatico coloretale.

Metodi. Il pannello *Ion Torrent AmpliSeq Colon and Lung Cancer* è stato dapprima verificato su linee cellulari e su campioni controllo e poi applicato prospetticamente a campioni di routine (n=114), avendo come riferimento il sequenziamento secondo Sanger.

Risultati. Il pannello Ion Torrent ha rilevato la presenza di alleli mutati fino ad una diluizione pari al 5% su DNA estratto da linee cellulari ed ha correttamente classificato tutti i campioni del set di controllo. L'analisi condotta su campioni routinari ha generato risultati valutabili nel 95.6% dei casi. Di questi 12 (11%) presentavano mutazioni a carico del gene BRAF e 47 (43%) in uno dei due geni RAS. In 2 casi, data la bassa percentuale di allele mutato in un gene RAS, il sequenziamento sec. Sanger non ha rilevato la mutazione. Il TAT medio è risultato essere 10.4 giorni, per il Sanger e 13 giorni per l'Ion Torrent. Il costo dei reagenti per genotipizzare KRAS, NRAS e BRAF è stato €196 per il Sanger e €187 per l'Ion Torrent.

Conclusioni. Il pannello *Ion Torrent AmpliSeq Colon and Lung Cancer* si è dimostrato essere affidabile e sostenibile nella diagnostica di routine, rappresentando una valida alternativa al sequenziamento secondo Sanger nella selezione di pazienti per il trattamento personalizzato del carcinoma coloretale metastatico.

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