Introduction

With an ever expanding armamentarium of molecularly targeted therapies that provide clinical benefit in a small subset of patients, the use of predictive biomarkers to appropriately select those who will benefit is crucial. Though the characterization of some predictive markers entails simple testing that can be completed in most labs, such as immunohistochemistry (IHC), other testing can be much more complex (1). The United States Food and Drug Administration (FDA) has recognized the importance of companion diagnostic testing and in 2014 released a position statement highlighting the need for the development and approval of companion in vitro diagnostic tests contemporaneously in order to ensure FDA approval of novel agents (2).

While the pairing of targeted therapies with biomarkers provides an opportunity for personalized care, it also presents significant challenges. As more complex biomarkers emerge, significant variation in assay methodology leads to difficulties in standardizing diagnostic tests and their results. Institutions and funding agencies often struggle with choosing the most cost efficient but clinically useful test from a myriad of competing platforms. The rapid advancement in molecular diagnostics has also resulted in many assays becoming obsolete shortly after adoption as they are replaced with more sensitive or comprehensive tests. Despite these challenges, recent advances have resulted in companion diagnostics with improved clinical performance. Here we review currently available and investigational molecular pathology assays of importance to the treatment of metastatic colorectal cancer (mCRC) and

Current companion diagnostics in advanced colorectal cancer; getting a bigger and better piece of the pie

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Abstract: While the treatment of colorectal cancer continues to rely heavily on conventional cytotoxic therapy, an increasing number of targeted agents are under development. Many of these treatments require companion diagnostic tests in order to define an appropriate population that will derive benefit. In addition, a growing number of biomarkers provide prognostic information about a patient’s malignancy. As we learn more about these biomarkers and their assays, selecting the appropriate companion diagnostic becomes increasingly important. In the case of many biomarkers, there are numerous assays which could provide the same information to a treating physician, however each assay has strengths and weaknesses. Institutions must balance cost, assay sensitivity, turn-around time, and labor resources when selecting which assay to offer. In this review we will discuss the current state of companion diagnostics available in metastatic colorectal cancer and explore emerging biomarkers and their assays. We will focus on KRAS, BRAF, HER2, and PIK3CA testing, as well as microsatellite stability assessment and multigene panels.

Keywords: Colorectal cancer; biomarker; next generation sequencing (NGS); companion diagnostic

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provide insights into future opportunities. We will focus on biomarkers with current or anticipated future actionability (see Figure 1).

**Current standard of care companion diagnostic tests**

**KRAS/NRAS**

**Overview and clinical significance**

In mCRC, the use of monoclonal antibodies (cetuximab and panitumumab) to target the epidermal growth factor receptor (EGFR) has proven to be an effective treatment strategy (3-9). These agents bind the extra-cellular domain of EGFR and prevent downstream signaling. Correlative studies identified *Kirsten rat sarcoma viral oncogene (KRAS)* mutations as an important driver of tumor resistance (9). Although initially only *KRAS* exon 2 (codons 12 and 13) mutations were evaluated as a biomarker for anti-EGFR resistance, recent data has shown that mutations in *KRAS* exon 3 (codons 59 and 61), exon 4 (codons 117 and 146) or *neuroblastoma rat sarcoma viral oncogene (NRAS)* exon 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146) are also associated with anti-EGFR resistance (10,11). Together these extended *RAS* mutations are present in up to 56% of mCRC (12). Due to the strength of *RAS* testing as a predictive biomarker, guidelines suggest that all patients with mCRC should have extended *RAS* mutation testing and must not have mutations in *RAS* if receiving EGFR directed therapy (13-15).

Given the importance to *RAS* testing, optimizing molecular detection of mutations is of utmost importance. At present, no one methodology is preferred and all assays appear to have similar cost implications in large health care systems (16). Laboratories must either validate their own independent test or adopt a commercially available kit. Concordance between primary tumors and metastatic lesions is high, ranging between 90% and 100% in most studies, suggesting that testing can be completed on whichever lesion is easiest to biopsy, or on archival tissue (17-22). While highly conserved between liver metastasis and primary, some studies have suggested a higher level of discordance (up to 32%) between primary and lung or lymph node metastasis, which may complicate mutation analysis in patients with metastases beyond the liver (23,24).

Most samples assessed for *RAS* status will be formalin-fixed paraffin embedded archival tissue, however in patients without sufficient tissue, cytological samples have been shown to be sufficient for determining *RAS* and *BRAF*...
(\textit{v-raf murine sarcoma viral oncogene B1}) status with high concordance to primary lesions (17,19,25-27).

While conventional assays identify patients as \textit{RAS} mutant when a mutation is detected in >10\% of reads sequenced, sensitivities approaching 0.1\% are now possible (28). This heightened sensitivity allows for greater characterization of sub-clonal populations that may result in early treatment resistance. Multiple studies have described early treatment failure and lack of benefit from \textit{EGFR} directed therapy in patients with low frequency \textit{RAS} mutations that would not be detected using standard clinical assays (29-32). It remains to be seen, however, whether these higher sensitivity assays will result in improved patient selection and outcomes. Recently drafted guidelines recommend that assay sensitivity should be at least 5\%, although the outcome data supporting this recommendation is limited. Important considerations during assay selection include workload, turnaround time, equipment costs, assay costs, sensitivity and comprehensiveness of assays. \textit{Table 1} summarizes some key differences between available platforms. While some assays may allow for detection of only targeted “hot-spot” mutations, others are much more comprehensive and may detect mutations of unclear significance. Sanger sequencing is the gold standard and most techniques are compared to this, however, there are many options available.

**Direct sequencing techniques**

Sanger sequencing and pyrosequencing are methods of direct DNA sequencing which use detection of fluorescent nucleotides or photon emission during nucleotide incorporation to elicit DNA sequence information and provide information about an entire sequenced region (46,47). Direct sequencing is able to determine mutations at all base-pairs throughout an entire gene, but Sanger sequencing has difficulty assessing low frequency mutations or samples with tumor content below 20–30\% (48-50). Unlike Sanger sequencing, pyrosequencing allows detection of low level mutations because of its ability to sequence numerous templates concurrently, however it can be limited by the ability to only sequence short templates, which can make detection of uncharacterized mutations more challenging (47,51). Pyrosequencing is more sensitive than Sanger sequencing and comparable in sensitivity to Therascreen, which has been established as a regulatory approved companion biomarker for \textit{anti-EGFR} inhibitors (33,52). Pyrosequencing is available via independently developed assays or commercially available kits such as PyroMark (Qiagen, Hilden, Germany).

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{Assay} & \textbf{Mutant allele frequency threshold required for detection} & \textbf{Full gene or targeted} & \textbf{Estimated turnaround time (28)} & \textbf{Cost} \\
\hline
Sanger/direct sequencing & 10–20\% (30) & Full gene & Days to weeks & Low cost per sample but high equipment cost to start \\
\hline
Pyrosequencing & 2.5–5\% (33,34) & Full gene & Few days & Low cost per sample but high equipment cost to start \\
\hline
Allele specific PCR & 1–5\% (30,35-38) & Targeted, Cobas: 18 mutations in codons 12, 13, and 61 of \textit{KRAS}. Therascreen: 7 mutations in codons 12 and 13 (G12A, G12D, G12R, G12C, G12S, G12V, G13D) & Rapid & Low cost per sample \\
\hline
High resolution melting & 2.5–10\% (37,39,40) & Full gene or targeted & Days to weeks & Low cost per sample but high equipment cost to start \\
\hline
NGS & 1\% (28,41-43) & Full gene or targeted & Days to weeks & Varied-moderate to high \\
\hline
Multiplex mutation assays & 3–5\% (44,45) & Targeted & Days to weeks & Low cost per sample but high equipment and assay validation cost to start \\
\hline
\end{tabular}
\caption{Comparison of select tissue sequencing platforms for \textit{RAS/BRAF} status}
\end{table}

PCR, polymerase chain reaction; NGS, next generation sequencing.
Mutant allele specific polymerase chain reaction (PCR)

Mutant allele specific PCR uses probes for mutated and non-mutated alleles and allows for enrichment of mutant transcripts in samples with low frequency mutations (35). Probes for each allele are labeled with fluorescent reporter dyes that allow detection and quantification. There are a number of modifications to the principle of mutant allele specific PCR that can enhance assay performance, such as peptide-nucleic acid linking or locked nucleic acid incorporation (53). These assays are able to detect mutant allele frequencies that are much lower than Sanger sequencing (as low as 1%) but do not detect mutations outside of those selected for inclusion as primers in the assay (35,36,54). This enhanced sensitivity can result in the re-classification of up to 20% of patients compared to Sanger sequencing or Therascreen and is clinically relevant (30,55). Patients defined as wild-type by PCR based assays have been shown to have improved response rates, progression free, and overall survival (OS) compared to Sanger sequencing defined wild type populations (30,56).

Patented mutant allele specific PCR detection kits, such as the Roche Cobas KRAS mutation kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA) or the Qiagen Therascreen kit (Qiagen, Hilden, Germany), are available and perform well. Cobas uses TaqMelt real-time PCR. Following amplification, samples are heated and mutations are detected by discrepancies between wild type and mutant amplicon melting temperatures. Compared to Sanger sequencing and Therascreen, the Cobas assay has demonstrated superior detection rates, which were in line with pyrosequencing and high resolution melting (HRM) assays (34,37,57). A limitation of the Cobas assay is the inability to confirm which specific mutation is present in a sample. Therascreen uses an allele specific amplification refractory mutation system (ARMS) and incorporates a fluorescent probe (Scorpion) during real-time PCR when a mutation is present in template material. It detects the seven most common mutations in KRAS exon 2 (codon 12 and 13) and has been shown to result in equivalent, if not better sensitivity compared to direct sequencing and HRM, with the ability to detect mutant allele frequencies of 1% (38,49,58,59). The assay identifies the specific mutation which is present in a sample but does not offer extended RAS coverage outside of exon 2 (59).

HRM analysis

HRM analysis uses PCR amplification followed by monitoring for fluorescence changes during heated denaturation to detect mutant alleles. The inclusion of a fluorescent dye or probe that emits more strongly when bound to dsDNA than ssDNA allows detection of variants. Minimum mutant allele frequency required for detection ranges between 2.5% and 10%, making the assay more sensitive than direct sequencing (37,39,40,60). Some reports suggest that HRM RAS detection may result in excessive false positive results but this has not been universally noted and validity was comparable to pyrosequencing and standard sequencing in other studies (59,61). An advantage to HRM is the ability to detect all mutations within multiple genes concurrently (i.e., RAS and BRAF), however the assay is unable to report the specific mutation present in a sample (61).

Gel electrophoresis methods

Single strand conformation polymorphism analysis and denaturing gradient gel electrophoresis both use fluorescent labeling PCR reactions followed by gel electrophoresis with amplified wild-type RAS co-run across gel matrices. Templates with mutations will assume different structures and migrate at different speeds. Mutations are detected by noting when additional bands are present in the gel beyond the wild type band (47,62). Single strand conformation polymorphism analysis, HRM PCR, and Therascreen show similar sensitivities, and appear more sensitive for low frequency mutations than pyrosequencing (62). Gel electrophoresis methods can detect an array of mutations across entire genes but do not identify the specific gene mutation present (63). The Ampli-set-K-RAS commercial kit (Bird, Siena, Italy) uses restriction fragment length polymorphism PCR and has been shown to have similar sensitivity and specificity to Therascreen and multiplex assays (64).

Next generation sequencing (NGS)

NGS uses massively paralleled sequencing technology to perform sequencing with significant depth and covering many genes with similar time and resource costs compared to sequencing a single mutation with Sanger sequencing (65). It can utilize highly selective hot-spot panels with significant depth at each mutation site or more comprehensive sequencing such as whole-exome or whole-genome analysis. With more comprehensive sequencing, data management and informatics pipelines becomes increasingly important to deal with the massive amounts of information generated. The costs of NGS are falling significantly and costs to sequence a single genome are now on the scale of a few thousand dollars, with targeted panels available for several hundred dollars a sample. Both Haley et al. and Altimari et al. demonstrated that NGS had
better sensitivity and specificity than Sanger sequencing, pyrosequencing and ARMS-Scorpion PCR assays for the detection of known KRAS mutations (41,42). NGS has a sensitivity as low as 1% mutant allele frequency and had 100% concordance with a panel of KRAS mutant patients compared to 98% with Therascreen (43). Other major benefits of NGS will be discussed later when we address the role of multiplex panels in mCRC.

**Emerging biomarkers in colorectal cancer**

**BRAF**

**Overview and clinical significance**

*BRAF**V600E mutations are found in 8–10% of mCRC and are strongly associated with *RAS* wild-type and microsatellite instable (MSI) tumors (66). BRAF mutant tumors associated with MSI result from high level CpG island hypermethylation [CpG island hypermethylation phenotype (CIMP)+] (67). This CIMP+ phenotype accounts for almost all cases of *BRAF*V600E mutant metastatic CRC. Testing for *BRAF*V600E mutation is recommended for its prognostic and potentially predictive role (68). Results of biomarker analysis from the PRIME randomized trial comparing the efficacy of panitumumab plus oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) with FOLFOX4 alone, revealed that the presence of *BRAF*V600E mutation in *RAS* wild-type tumors portends poor prognosis, regardless of treatment (4). The median progression-free survival (PFS) and OS in *BRAF* mutant patients compared to wild type patients was 6.1 vs. 10.8 months and 10.5 vs. 28.3 months, respectively (69). Due to their poor prognosis, these patients need to be identified early and considered for clinical trials assessing BRAF inhibitors (70). Additionally, *BRAF*V600E mutation is strongly associated with MLH1 promoter methylation and can therefore distinguish between sporadic and hereditary (Lynch-related) MLH1-deficient MSI CRC (71).

**BRAF**V600E mutation assays

*BRAF*V600E mutation testing is performed commonly using PCR based methods. In a study comparing PCR-Sequencing (Sanger sequencing) with a real time PCR (allelic discrimination) assay, real time PCR was found to be more sensitive for detection of BRAF mutations in CRC, especially in cases with an allelic frequency of less than 20% (72). NGS platforms can also be used for detecting *BRAF*V600E mutations and offers the advantage of testing multiple genes for mutations of interest. In a prospective comparison, the performance of the Ion Torrent NGS assay was found comparable to Sanger sequencing and detected similar proportions of *BRAF* mutant cases (73). Since these methods require DNA extraction, they are resource intensive, and often inaccessible. Notably, *BRAF*V600E mutation testing can facilitate screening of Lynch syndrome related MSI CRCs (74,75). Emerging data has shown that *BRAF*V600E mutation in plasma cfDNA can be tested using PCR based tests, has moderate concordance (concordance rate of 74%) with standard tumor tissue testing and may have prognostic and predictive significance (76).

**Human epidermal growth factor receptor 2 (HER2)**

**Overview and clinical significance**

HER2 amplifications are found in 3–4% of all CRC and are strongly associated with *RAS* and *BRAF* wild-type tumors. In patients harboring wild-type *RAS/BRAF* genes, HER2 amplifications are seen in 5–6% cases (66,77). Presence of HER2 amplification has been implicated in resistance to anti-EGFR monoclonal antibodies (78). In the clinical setting, HER2 gene copy number status significantly correlated with differential response rate (RR), PFS and OS. Patients with HER2 amplification have shorter PFS on treatment with anti-EGFR based therapies compared to HER2 non-amplified cases (79). HER2 amplification has been validated as a negative predictive biomarker for anti-EGFR antibody therapy in metastatic CRC. Patients with HER2 amplification had significantly shorter PFS (median: 2.9 vs. 8.1 months) compared to HER2 non-amplified patients on anti-EGFR based therapy (80). Furthermore, these patients can derive benefit from dual-anti-HER2 inhibition using trastuzumab in combination with lapatinib (HERACLES study) or trastuzumab and pertuzumab (My Pathway study) and should be referred for clinical trials assessing HER-2 directed therapy (81-83).

**HER2 amplification testing**

No consensus exists regarding methodology for HER2 amplification testing in CRC. The HERACLES study used HER2 IHC for patient selection (82). Using this cohort, the investigators proposed a criterion for HER2-IHC positivity to identify HER2 amplification in CRC. IHC was performed using both HercepTest antibody and Bench Mark Ultrasystem using the VENTANA 4B5 antibody. In this cohort, none of the IHC 0 or 1+ cases were amplified. Furthermore, there was complete concordance between silver in situ hybridization (SISH) and fluorescent in situ
hybridization (FISH) (84). However, in the absence of prospective validation, the application of this algorithmic interpretation of IHC and ISH in CRC is incomplete. In another systematic analysis of 2,573 CRC cases using HER2 IHC and in situ hybridization (ISH), the diagnostic sensitivity and specificity of HER2 IHC was 71% and 96%, respectively. HER2 IHC scores of 0 or 1+ exhibited good agreement with ISH (concordance rate of 97%). However, the concordance rates of HER2 IHC 2+ and 3+ were (38% and 78%, respectively) low, necessitating the additional need for ISH analysis to confirm HER2 status in these cases (85). Although, not validated in CRC, NGS assay can accurately identify HER2 amplifications in breast cancer samples (86). HER2 amplifications in plasma cfDNA can be identified using digital PCR. In mCRC patients who developed resistance to anti-EGFR antibodies, 22% (4/18) of patients were found to have HER2 amplifications (87), although this methodology requires tissue concordance validation studies before it is ready for clinical implementation.

**PIK3CA**

Overview and clinical significance
Phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA) is a key enzyme in the MTOR signaling pathway. Mutations in exon 9 and 20 have been reported in 14–32%, and had been reported to associate with resistance to EGFR directed therapy (88-91). However, these early studies may have been confounded by the frequent co-mutation of both PIK3CA and KRAS in CRC tumors, and subsequent studies have not demonstrated a role for PIK3CA as an independent predictive biomarker for EGFR antibodies. Exon 20 mutations appear to be more strongly associated with EGFR resistance, however a recent meta-analysis suggests the prognostic significance of PIK3CA mutations is unclear at this point (92,93). PIK3CA mutations have been noted to be a potential biomarker for benefit from ASA or COX-2 inhibition in the adjuvant setting after several studies demonstrated improved outcomes in patients with PIK3CA mutations taking ASA after a colorectal cancer diagnosis (94,95). At this point in time there is no proven therapeutic intervention directed at PIK3CA mutations in mCRC. Due to this, testing for these mutations is not indicated outside of a clinical trial.

**Mutation detection**
Similar to KRAS and BRAF, PIK3CA mutations are highly conserved between primary and metastasis, with a concordance of over 90% (19). There is no preferred assay to detect PIK3CA mutations and individual reports suggest NGS platforms, gel electrophoresis, and PCR sequencing all have excellent sensitivities, while Sanger sequencing is less sensitive. HRM has been shown to have similar sensitivity to Sanger sequencing, while pyrosequencing appears to be more sensitive than Sanger sequencing (96-100).

**Microsatellite instability (MSI)**
While MSI is a long standing prognostic biomarker in early stage CRC, interest in this biomarker in the metastatic setting has been renewed with the advent of immune checkpoint inhibitors (101-103). The use of pembrolizumab in MSI-high CRCs has been associated with significant response rates (104). A plausible hypothesis is that deficient DNA repair results in higher mutational burden in MSI-high tumors, and the resultant increase in neo-antigens provides an immunogenic environment. Notably, level of mutational burden correlates with response to immune checkpoint inhibitors in other tumor sites (105,106). MSI is present in 15–20% of all CRCs and about 4% of mCRC and arises from either germline mutations in mismatch repair (MMR) proteins (MSH2, MSH6, MLH1, PMS2) or from somatic hypermethylation of the MLH1 promoter (107,108). Loss of these MMR proteins results in expansions of short repetitive sequences throughout the genome called microsatellites that can be detected via PCR-based assays which target standard DNA sequences containing these repeats. A simpler way to infer MMR function is IHC to detect loss of expression in the four key MMR proteins. IHC has been shown to have similar sensitivity to PCR based MSI testing, however is not able to distinguish germline mutations from somatic hypermethylation of MLH1 (1). While this deficit has implications for hereditary screening programs, it does not impact the phenotypic behavior of tumors. Besides MSI status, other markers such as tumor infiltrating lymphocytes and mutational burden have not been clinically validated in colorectal cancer.

**Novel assay methodologies**

**Cell free DNA (cfDNA), circulating tumor DNA (ctDNA), and circulating tumor cells**
There are a number of novel assays capable of assessing RAS and BRAF status from plasma or serum. The ability to ascertain this information from a “liquid biopsy” reduces complications associated with biopsy procedures and
provides another means to assess response to therapy and detect emergence of resistance. ctDNA genotyping of \textit{RAS} and \textit{BRAF} has been shown to have similar, if not better, ability to detect low frequency mutations compared to tissue based assays in known \textit{RAS/BRAF} mutant patients and allows monitoring of clonal dynamics (109,110). While many studies report rates of discordance between 20\% and 30\% in \textit{RAS} and nearing 10\% in \textit{BRAF} for ctDNA, ctDNA, and CTC based assays, it is difficult to interpret whether this demonstrates inadequacies in an assay or temporal clonal dynamics (111-115). A recent phase II trial of irinotecan and cetuximab demonstrated that ctDNA \textit{KRAS} mutation status was often discordant compared to archival tissue, but was actually more predictive of patient response and survival than archival status. This work highlights how plasma assays may actually provide a better representation of the current mutational burden of a patient (116). The ability to follow these clonal dynamics has also been shown to offer potential re-treatment options. Siravegna \textit{et al.} demonstrated emergence of a \textit{RAS} resistant clone after treatment that subsequently diminished upon cessation of EGFR directed therapy. These patients were able to be re-challenged with cetuximab/panitumumab and achieved responses prior to the re-emergence of the prior clone (109).

\textbf{Multigene panels}

As we move towards the increasing use of molecularly targeted agents, it is becoming crucial to assess samples for multiple biomarkers concurrently. Utilizing multiple different assays to test for individual targets is challenging due to time constraints, cost, and pathologic specimen exhaustion. At this time, whole genome and whole exome sequencing are still out of reach for clinical utilization, however the use of NGS targeted gene panels allows a focused assessment of key genes and have been adopted at many institutions. By inserting a barcode into sample template material, a multiplexed platform that can sequence multiple samples concurrently is possible, facilitating increased throughput. Once barcoded, oligonucleotides are hybridized to a solid matrix with mutation specific sequences followed by PCR amplification to create clonal clusters. DNA synthesis of sequences complementary to the clonal clusters is detected by a marker such as pH change or fluorescence emission, depending on the platform used, and this information is computationally merged across all of the concurrent reactions to create the full output of the sequencing.

Multiplex assays allow assessment of numerous genes within one reaction and have similar or better sensitivity to direct sequencing methods (58,117,118). They are cheaper than direct sequencing and are better able to deal with degraded or poor quality DNA. Typically, a panel is designed to detect “hot-spot” mutations that have been previously described with clinical significance, however mutation coverage can differ based on the specific assay. The decision concerning how much of a gene or the number of genes to include on a panel must be balanced with the depth of coverage desired. By limiting the size of a panel, increasing depth at each mutation is possible with similar cost. Mutant allele frequency required for detection is reported in the range of 3-5\% and with significant depth of coverage or paired reads that span breakpoints, copy number variation (CNV) can be detected with sensitivities nearing 100\% (44,45,86,119,120). This does require that the break point and adjacent territory both have adequate coverage which may not always be present depending on the particular panel. Detection of gene fusions is difficult unless the panel is designed to include targets for a known fusion. Most novel fusions are detected using WGS or RNA-Seq technology (121,122).

Between 29-72\% of patients had potentially actionable targets after sequencing with multiplex panels in results from several large cohorts (123-126). The number of actionable targets is highly variable depending on the available compounds for treatment at a center and the number of mutations assessed in each panel. At this point in time, these panels are most effective in centers with large early drug development programs that can provide access to numerous investigational compounds. The Assessment of Targeted Therapies Against Colorectal Cancer (ATTACC) umbrella trial protocol at MD Anderson Cancer Center recently reported the results of the first 484 patients screened. Ninety-five percent of patients had a biomarker identified, of which 31.2\% were enrolled onto one of the 18 companion trials available during the study (127). Other ongoing umbrella and basket studies, such as NCI-MATCH, will hopefully demonstrate the utility of multigene panels to complete accrual to trials of uncommon molecular subtypes (128).

With advances in NGS technology and reduction in cost, panels with a larger number of genes and/or hotspots are possible. Given the lack of targeted agents that have demonstrated clinical effectiveness in metastatic colorectal cancer at this point in time, there is no clinical indication to utilize larger panels beyond those that would include \textit{RAS} and potentially \textit{BRAF} mutations outside of a clinical
One of the major reasons to include a larger number of genes within a panel is to create a platform that can be used across numerous tumor sites to save resources and allow batching of different histologies together.

**Conclusions**

Despite a lack of new agents available for the treatment of mCRC over the past decade, the refinement of current biomarkers to better select patients who will benefit from EGFR inhibition has been an important step towards improving outcomes. The current drug development pipeline includes exciting new targeted agents that may be available in the next few years, however these therapies are likely to provide benefit in only a subset of patients. In order for these agents to move into the clinic and receive regulatory approval, they will require tandem development of companion diagnostic tests. Current commonly assessed biomarkers are outlined in Table 2. Selection of the appropriate companion diagnostic in some settings may be dictated by regulatory authorities during drug approval, however in many situations institutions will need to select one of a variety of assays that may provide similar information. These decisions will be driven by assay performance, cost, and labor intensity. With the rapid improvement in NGS technology, significant reduction in sequencing cost, and ability to test specimens from many patients on the same assay, we expect future companion diagnostics will rely heavily on multiplex panels that can effectively screen patients for numerous agents concurrently. These assays not only benefit patients by providing information about multiple mutational targets concurrently, but better utilize pathologic specimens.

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**Footnote**

*Conflicts of Interest: The authors have no conflicts of interest to declare.*

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**Table 2 Commonly tested biomarkers in advanced colorectal cancer**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Commonly used assays</th>
<th>Current status</th>
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<tbody>
<tr>
<td>KRAS (codon 12, 13, 59, 61, 117, 146) and NRAS (codon 12, 13, 59, 61, 117, 146) mutations</td>
<td>Direct sequencing, allele specific PCR, high resolution melting, multiplex panels</td>
<td>Prognostic and predictive Predicts resistance to EGFR targeted therapy—standard of care</td>
</tr>
<tr>
<td>BRAF<strong>V600E</strong> mutation</td>
<td>PCR</td>
<td>Prognostic and predictive (investigational) Suggests potential response to BRAF targeted agents—experimental</td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>Microsatellite expansion PCR or IHC</td>
<td>Prognostic and predictive (investigational) Suggests response to immunotherapy—experimental Hereditary screening implications</td>
</tr>
<tr>
<td>Her2 amplification</td>
<td>Combination of IHC and FISH</td>
<td>Predictive (investigational) Suggests response to HER2 targeted therapy—experimental</td>
</tr>
<tr>
<td>PIK3CA mutation (exon 9 and 20)</td>
<td>No standard assay</td>
<td>Unclear role May suggest resistance to EGFR targeted therapy May suggest sensitivity to ASA/COX-2 inhibitors in adjuvant setting</td>
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PCR, polymerase chain reaction; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; FISH, fluorescent in situ hybridization.
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