94 Defining Best Practices for Determining KRAS Mutations in Colon Cancer

Wilbur Franklin MD

2011 Annual Meeting – Las Vegas, NV

AMERICAN SOCIETY FOR CLINICAL PATHOLOGY
33 W. Monroe, Ste. 1600
Chicago, IL 60603
94 Defining Best Practices for Determining KRAS Mutations in Colon Cancer

Treatment of colon carcinoma with the anti-epidermal growth factor receptor antibody Cetuximab is reported to be ineffective in KRAS-mutant tumors and, therefore, mutation testing techniques have become an urgent concern. A variety of methods are currently in use for tissue preparation and mutation detection. This session will review the evidence supporting the different approaches for defining best practices.

- Familiarity with discovery and biological significance of KRAS and clinical significance of KRAS mutations of
- Understanding of preanalytic requirements for KRAS mutation testing
- Survey of currently available KRAS testing platforms, their sensitivity (levels of detection), specificity, gene coverage, specimen requirements, turnaround time, technical simplicity, startup cost, cost per exon and clinical validation.

FACULTY:

Wilbur Franklin MD
Entire Pathology Team
Molecular Pathology
Molecular Pathology
1.0 CME/CMLE Credit

Accreditation Statement: The American Society for Clinical Pathology (ASCP) is accredited by the Accreditation Council for Continuing Medical Education to provide continuing medical education (CME) for physicians. This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education (ACCME).

Credit Designation: The ASCP designates this enduring material for a maximum of 1 AMA PRA Category 1 Credits™. Physicians should only claim credit commensurate with the extent of their participation in the activity. ASCP continuing education activities are accepted by California, Florida, and many other states for relicensure of clinical laboratory personnel. ASCP designates these activities for the indicated number of Continuing Medical Laboratory Education (CMLE) credit hours. ASCP CMLE credit hours are acceptable to meet the continuing education requirements for the ASCP Board of Registry Certification Maintenance Program. All ASCP CMLE programs are conducted at intermediate to advanced levels of learning. Continuing medical education (CME) activities offered by ASCP are acceptable for the American Board of Pathology’s Maintenance of Certification Program.
Defining Best Practices for Determining KRAS Mutation in Colon Cancer
Wilbur A. Franklin, MD
University of Colorado

**Historical Introduction**

The first steps that ultimately lead to the discovery of KRAS were taken in the 1960’s when Werner Kirsten, a pathologist at the University of Chicago was able to isolate a murine leukemia virus containing the KRAS oncogene by serial passage of murine leukemia in rats. In the early 1980’s the nucleotide sequence of the virus, the transforming capability the activated oncogene and the identification of the cellular homologue of the viral oncogene were all accomplished within a span of a few months in the laboratories of Weinberg and Barbacid among others (reviewed in 1).

We now know that KRAS is a member of a superfamily of guanosine-5-triphosphatase (GTPase) proteins that also includes NRAS and HRAS and a number of less well known proteins. The role of these proteins is to transduce stimuli from surface growth factor receptors, but as multiple simultaneous sources of signals from different receptors and ligands have been identified, these proteins are increasingly recognized as integrators and processors of signals from the cell surface and not just transducers. In addition, the existence of negative and positive feedback loops adds to this complexity. On activation, RAS undergoes prenylation (addition of a 15-carbon chain) of a CAAX (C, cystein; A, aliphatic amino acid; X, serine or methionine) motif by a farnesyl transferase. This makes RAS more hydrophobic and adherent to the inner aspect of the cytoplasmic membrane, where it activates subsequent transducers in an intracellular signaling cascade that involves numerous effector molecules including PI3K and MAPK.

Consistent mutations in several components of this signaling pathway (starred in adjacent figure 1) occur in human solid tumors. Mutations in KRAS are particularly common. They permit stimulus-independent activation and perpetuate activation because they occur in the region of the molecule that regulates its level of enzymatic activity. Hydrolysis of GTP bound to RAS must occur in order for RAS to return to an inactive state. Mutations in the codon normally
encoding glycine in position 12, which does not contain a side chain, may result in substitution with an amino acid possessing a side chain that will sterically interfere with GTP hydrolysis. Thus, acquisition of certain mutations in KRAS leads to a permanently active state that permits cells to evade apoptosis and acquire a growth advantage.

KRAS is one of the most frequently mutated genes in human solid tumors. The attached figure 1 shows the frequency of mutation entered into the Sanger center Cosmic database as of September, 2011. The highest frequency is found in GI tumors with pancreas, colon and biliary tumors together accounting for nearly 77% of the nearly 20,000 KRAS mutations in the database and lung cancer accounts for an additional 14%. In colon carcinoma multiple series have indicated that KRAS is mutated in 40-45% of invasive cancers. KRAS mutations that occur in colon tumors are largely limited to three codons, 12, 13, and 61. That the number of specific mutations in human tumors is limited and that the mutations are stable and readily detectable make KRAS mutations an important pharmacodiagnostic marker.

**KRAS as a Biomarker**

**KRAS as a Prognostic Marker in Colorectal Carcinoma (CRC)**

The independent effect of KRAS mutation on outcome in colon cancer is still unclear and remains controversial. In a series of 3,439 of CRC patients it was found that of the 12 possible mutations on codons 12 and 13, only the glycine to valine mutation on codon 12 (8.6%) had a statistically significant impact on outcome. Smaller series have shown similar results, but retrospective data from other large randomized studies has failed to consistently demonstrate a meaningful effect of KRAS mutation on outcome in CRC. In a study of a large number of untreated patients, there was no difference in progression free survival (PFS, 7.3 weeks) between mutant and non-mutant tumors. A National Cancer Institute of Canada reported only in poster format indicated that among patients treated with best supportive care there was no significant difference in survival between KRAS WT and mutant tumors [4.8 and 4.6 months(P=0.97), respectively].
KRAS as A Predictor of Treatment Response in CRC

In 2007, a study published in the New England Journal of Medicine\(^6\) reported that administration of the monoclonal antibody, Cetuximab, to patients with advanced CRC expressing immunohistochemically detectable EGFR resulted in improved survival in comparison to untreated controls. Both treatment and control arms had been treated with a fluoropyrimidine, irinotecan, and oxaliplatin or had contraindications to treatment with these drugs. The difference in survival, while not large suggested that this specific treatment could be useful in managing CRC and prompted a closer look at who responds and who does not. Several subsequent studies indicated that KRAS mutation status dictates response to anti-EGFR monoclonal antibody treatment in CRC. In the randomized studies summarized in the attached table, wild type tumors treated with anti-EGFR antibody (Cetuximab or Panatumumab) have a progression free survival advantage of several months and significantly reduced hazard ratio in comparison with mutated tumors in first line and third line settings.

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment*</th>
<th>Pts</th>
<th>KRAS MT**</th>
<th>HR</th>
<th>KRAS WT**</th>
<th>HR</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amado</td>
<td>P versus BSC (3rd line)</td>
<td>427</td>
<td>7.4 wks</td>
<td>0.99</td>
<td>12.3 wks</td>
<td>0.45</td>
<td>JCO, 2008</td>
</tr>
<tr>
<td>Van Cutsem (CYRSTAL)</td>
<td>FOLFIRI + C (1st line)</td>
<td>540</td>
<td>7.6 mos</td>
<td>1.07</td>
<td>9.9 mos</td>
<td>0.68</td>
<td>NEJM, 2009</td>
</tr>
<tr>
<td>Bokemeyer (OPUS)</td>
<td>FOLFOX + C (1st line)</td>
<td>233</td>
<td>5.5 mos</td>
<td>1.83</td>
<td>7.7 mos</td>
<td>0.57</td>
<td>JCO, 2009</td>
</tr>
<tr>
<td>Karapetis</td>
<td>C versus BSC (3rd line)</td>
<td>572</td>
<td>1.8 mos</td>
<td>0.99</td>
<td>3.7 mos</td>
<td>0.40</td>
<td>NEJM, 2008</td>
</tr>
</tbody>
</table>

*P=Panatumumab, BSC=Best Supportive Care, C=Cetuximab

**Progression Free Survival

The CRYSTAL trial was a randomized phase III trial assessing cetuximab with bolus and infusional FU-leucovorin-irinotecan as first line treatment in patients with EGFR-expressing metastatic CRC. A statistically significant improvement in the overall response rate and median PFS in the patients enrolled on the Cetuximab-containing arm was found. However, in the subset of patients that could be analyzed for KRAS mutational status, the benefit of cetuximab appeared to be restricted to patients without mutations in the KRAS gene. This was a retrospective study conducted on a subset of the intent-to-treat population, but the KRAS mutant and KRAS WT patients were similar in terms of demographics and disease characteristics. Similarly, in the OPUS phase II study that evaluated FOLFOX-cetuximab in first-line, KRAS WT patients obtained benefit from receiving cetuximab in addition to FOLFOX compared to those receiving FOLFOX alone as measured by response rate (61% v 37%; P=0.01) and PFS (7.7 v 7.2 months; P=0.02). The figure above shows Kaplan-Meier curves for overall survival for a trial in which Cetuximab is compared with...
best supportive care alone. Cetuximab treatment was associated with improved overall survival among patients with KRAS wild-type tumors but not among those with mutated KRAS tumors. Panel A plots results for patients with mutated KRAS tumors, and Panel B results for patients with wild-type KRAS tumors. The difference in treatment effect according to mutation status was significant (test for interaction, P=0.01). On the basis of these results the European Medicines Agency (EMEA) has approved Panitumumab only for patients with tumors that are WT KRAS. This was the first approval of an agent for CRC based on the presence/absence of a gene mutation, and opens a new era in biomarker-driven therapy in this disease.

Finally, it has recently been suggested that not all mutations are equally predictive of poor response to Cetuximab. Patients with codon 13 mutation (p.G13D) appear to respond to Cetuximab treatment with a significantly longer overall survival and reduced hazard ratio in comparison to patients with other KRAS mutations7.

Best Practices

In view of the strong data indicating that KRAS mutation predicts unresponseness to anti-EGFR treatment, the American Society of Clinical Oncology together with the College of American Pathologists published recommendations for KRAS testing in patients with metastatic carcinoma8 as follows:

Based on systematic reviews of the relevant literature, all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a CLIA-accredited laboratory. If KRAS mutation in codon 12 or 13 is detected, then patients with metastatic colorectal carcinoma should not receive anti-EGFR antibody therapy as part of their treatment.

This recommendation presents Oncology groups and clinical laboratories with several challenges that must be addressed to implement these recommendations.

- Which patients should be tested and when should they be tested?

The recommendation is for all patients with metastatic tumor who are candidates for Cetuximab treatment to be tested. Ideally testing should be conducted by mutual agreement between oncologist and laboratory when patients are considered for anti EGFR treatment. However, many factors can prevent optimal communication between busy oncologists and pathologists. Also, in many cases, changes in clinical status may engender urgent need for testing results and delay may create anxiety that could be avoided in both patients and clinical staff. Reflex testing of all resected metastatic carcinomas can eliminate these problems and allows KRAS status to be prospectively factored into the management plan of patients with metastatic tumors. For these reasons we believe that reflex testing should be implemented for all patients with metastatic colon cancer.

- What samples are suitable for mutational testing?

The ASCO recommendation is that test samples ... “should be specifically chosen by a pathologist to include predominantly tumor cells without significant necrosis or inflammation.” To implement this recommendation, several aspects of preanalytical processing must be taken into account.

**Tissue Collection and Processing:** Tissues obtained for molecular testing may come from surgical resection or biopsies (fine needle aspirates and cores). Most often, the tissue needed for molecular testing is derived from diagnostic material, unless special arrangements are made to specifically collect tissues for molecular studies. Issues regarding specific specimen types are discussed below.
Critical issues for resection specimens

- **Gross dissection**: Identification of tumor tissue in resected specimens requires a high level of morphological and dissection skill. For this reason, it is essential that a trained pathologist be involved in establishing protocols for the collection of specimens for molecular studies. For DNA-based assays including mutation analysis, timing of collection procedure can be somewhat flexible since DNA is a particularly robust analyte. However, DNA is not indestructible and prolonged delay in processing should be avoided. For all specimens, it is helpful to document the interval between vascular clamping and processing of the remnant tissue specimen (warm ischemia time).

- **Resection specimen preservation**: Fresh tissue, tissue preserved in transport media such as RNAlater, or frozen samples are acceptable starting materials for KRAS mutational testing, according to ASCO/CAP guidelines. However, these samples may be inconvenient to process in clinical laboratories and have the disadvantage that tumor cell purification by macro- or microdissection may be more difficult and complicated that with paraffin sections cut from fixed tissue. Moreover, testing protocols have been well adapted to fixed tissue. Formalin-fixed paraffin embedded specimens are preferred by our laboratory and many others for molecular testing. The most cost effective fixative for molecular testing of solid tumors laboratories is 3.7% buffered neutral (pH 7.4-7.6) formalin. Buffering is needed to eliminate possible DNA depurination which may occur at acid pH. Phosphate is the preferred buffering salt. Formalin works by crosslinking amino groups in proteins, introducing a -CH2- linkage. This linkage is stable and permits long term storage of tissues in paraffin with minimal degradation. Fixatives such as acetone and alcohol work by rapidly dehydrating tissues. These fixatives may not completely inactivate autolysis and may require modification of testing platforms. These types of specimens should be accessioned only after consultation with the molecular pathologist. Metal-based (zinc, mercury, etc.) fixatives, which are favored by anatomical pathologists in some clinical contexts because of the excellent histological detail they provide fixatives may inactivate polymerase used in molecular analyses. For this reason, metal-based fixatives are contraindicated for molecular biomarker studies.

- **Fixation technique**: Most clinical samples are fixed by immersion in a solution that inactivates autolytic enzymes, typically formalin. The adequacy of fixation depends on the size of the tissue sample to be fixed and the amount and concentration of fixative. For formalin fixation, tissue samples should be no more than 2 cm in maximum lateral diameter by 0.5 cm thickness.

Fixed tissue may be accepted in the molecular laboratory as wet tissue, paraffin blocks, or cut sections either stained or unstained. Blocks are evaluated for quantity by gross inspection on receipt in the laboratory. A microscopic slide cut from the block should be evaluated to confirm presence and adequacy of tumor.

Surgical pathology and histology laboratories at submitting institutions may possibly have varying procedures for fixation and processing times for FFPE tissues. Variances can contribute to significant differences in the testing and evaluation of specific biomarkers in the tissues. Common deviations from standard operating procedures that are detrimental to testing outcome include:

- Excessive fixation: We recommend no more than 72 hours.
- Inadequate fixation: Biopsy specimens should be fixed at least 6 hours. Resection specimens should be fixed overnight with a change in formalin to offset the effect of
absorption of fixative by blood. Tissue should be sliced at 5 mm thickness during fixation.
✓ Decalcification causes DNA deterioration and may be a cause amplification failure. Decalcification status should be kept in mind in interpretation of testing results.

2. Critical issues for biopsy tissue:
- **Sample collection:** Diagnostic biopsy material, obtained by endoscopy, needle core, and fine needle aspiration biopsy, is suitable for molecular testing. Desiccation occurs rapidly in small specimens, so they must be processed immediately after removal. However, the limited size of biopsy material has the advantage that processing usually begins immediately following removal from the patient, penetration of fixative is usually excellent and quality may be excellent.

- **Biopsy yield:** Diagnostic biopsy procedures are often designed to obtain only enough tissue for diagnosis and few cells may remain for molecular testing. The most frequent cause of failure of molecular testing on biopsies is inadequate tumor sampling. An estimate of the number of cells present a biopsy or cell block by examination of a stained section is a prerequisite for beginning to process a specimen for molecular analysis.

- **Quantification of tumor cells in a block:** Cell numbers that are required for specific molecular procedures can be roughly estimated on the assumption that each eukaryotic cell contains 10 picograms of DNA. This means that approximately 100 cells would be required to generate a nanogram of DNA and 100,000 cells would be required for a microgram. This estimate is complicated by the fact that DNA is truncated in tissue sections so that a large proportion of the cells examined under the microscope may not contain the full DNA complement. As a general rule, we have found that a minimum of 100 tumor cells in a 10 µm section is required for consistent adequacy of PCR-based assays.

- **Biopsies obtained specifically for molecular testing:** The low tumor cell numbers and marginal adequacy of remnant tissue from FNA procedures coupled with the compelling data supporting the predictive value of molecular markers have prompted an interest in obtaining biopsy specimens specifically for molecular testing. It is increasingly accepted by patients and clinicians that obtaining additional tissue strictly for molecular purposes is worth the effort and is likely to generate successful retrieval of DNA.

Our preferred method is to obtain two core biopsies and an aspirate from an FNA procedure. A cell block is prepared from the aspirate by fixation of pelleted cells in 37% paraformaldehyde solution. The cohesive fixed pellet is wrapped in tissue paper, processed in an automated tissue processor and embedded in paraffin. It is important that solutions used in the automated tissue processing system be clean and uncontaminated by particulate of soluble contaminating materials.

**Enriching for Tumor Cells:** Because non-malignant cells invariably accompany ("contaminate") all clinical samples, specimens must be enriched for tumors to a level that is compliant with the analytical sensitivity of the platform that will be used for the testing. Three different procedures may be used for tumor cell enrichment depending on the amount of contaminating normal tissue that is present in the specimen.

1. **Enface sectioning**
Paraffin blocks may be sectioned enface if the tumor cell concentration is high (>50%). In this procedure sections are cut from the surface of the paraffin block. These sections have
a tendency to roll up into tight scrolls. These scrolls are easily transferred to a test tube for
deparaffinization and DNA extraction. The thickness of the section may be adjusted
according to DNA yield desired. As mentioned above thicker sections contain few truncated
nuclei.

2. Coring of paraffin blocks
Coring of paraffin blocks is a rapid and inexpensive way to enrich for tumor cells. In this
procedure, glass slide is annotated by dotting with a cytology marking pen over a region
containing a high concentration of tumor cells with minimal normal cell contamination. A
region containing 50% or more tumor cells can usually be identified is solid tumors. In
specimens that are uniformly admixed with large numbers of stromal/inflammatory cells,
microdissection may be appropriate (see below). A needle is used to sample at the site
indicated on the annotated slide. A 1 mm needle core routinely yields over a µg good
quality DNA. Core are thoroughly deparaffinized with xylenes and DNA extracted.

3. Microdissection
Many methods of microdissection for molecular analysis have been described and the
choice of methods will depend on local capabilities and preferences. Whatever the method,
it is important that microdissection be overseen by an experienced pathologist. Routine
molecular analysis does not require extreme levels of tumor cell purity. Tumor cell
concentrations of 50% will usually suffice even for the least sensitive of testing methods.
Most methods involve scraping of tumor cells from a glass slide with a scalpel or glass
dissecting needle. We prefer to microdissect hematoxylin-stained cells cut at 10 µm using a
dissecting microscope. Hematoxylin-stained cells are covered with a thin layer of glycerol
and tumor cells specifically scraped from a histological section with a glass Pasteur pipette
that has been drawn out using a pipette puller. Specific cells are then aspirated into the
pipette using “pneumatic cell collector” and then expelled into a microcentrifuge tube. The
cells can then be washed and processed using conventional DNA extraction methods.

Specimen quality: DNA quality can be measured using the Bioanalyzer technique and with
paraffin cores, we have generally been able to harvest DNA that is somewhat sheared but has
an approximate average length of 6,000 base pairs. Sectioning and microdissection may
increase shearing considerably and in our experience fragment length in these specimens is
approximately 500 bp. Nevertheless, adequate DNA for PCR based procedures is usually
retrievable from ordinary stained sections.
In summary, most but not all cases submitted to the molecular laboratory are of acceptable
quality. Reasons specimens may be rejected for cellular or molecular testing may be specific to
the particular testing protocol and most frequently include:
✓ Insufficient viable tumor in block
✓ Poor fixation
✓ Incomplete dehydration (processing error)
✓ Decalcification

Documentation: A record of histological examination for specimen adequacy is retained by the
testing laboratory, and includes:
✓ Time from removal to fixation (warm ischemia time, if available)
✓ Length of fixation time (if available)
✓ Presence of tumor
✓ Histological tumor type
✓ Percentage of viable tumor present in the specimen
✓ Final determination of specimen adequacy
Specimens determined to be inadequate should not be processed further for molecular or cellular studies.

- What testing method should be used for KRAS testing?

Once DNA has been extracted and is of sufficient quality that it can go forward for molecular testing, a choice must be made among the several methods that may be used to detect KRAS mutation. This includes conventional Sanger sequencing, which remains the gold standard for mutation detection, high resolution melting, allele specific PCR, amplification refractory system (ARMS), often coupled with a bifunctional self-probing primer (Scorpions), pyrosequencing, and allele specific tests that may involve placement of a lock on mutant DNA and digestion of wild type. Each of these platforms must be independently evaluated and validated in the specific setting of the testing laboratory. Among the parameters that must be assessed are 1) amount and quality of starting material (DNA), 2) assay sensitivity, 3) specificity, 4) coverage of genome, 5) turnaround time, 6) simplicity, 7) start up costs, 8) cost per test, and 9) clinical validation.

Several methods currently employed for the identification of KRAS mutation in clinical samples are listed in the table and discussed below.

**Recent KRAS Testing Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR + Sanger sequencing (dideoxy chemistry)</td>
<td>Non-mutation-specific sequence comparison with normal sequence</td>
</tr>
<tr>
<td>High resolution melting (HRM) analysis (Roche Lightcycler)</td>
<td>Sequences with mutations hybridize at different, fixed temperatures</td>
</tr>
<tr>
<td>Bifunctional primer/probe (ARMS/Scorpion, DxS)</td>
<td>Mutation specific PCR/detection</td>
</tr>
<tr>
<td>Maldi-tof mass spectrometry array (Sequenom iPLEX)</td>
<td>Single base extension with mass bias nucleotide probes</td>
</tr>
<tr>
<td>Capillary electrophoresis, single base extension (SNaPshot, ABI)</td>
<td>Single base extension with fluorescent nucleotide probes</td>
</tr>
<tr>
<td>Pyrosequencing sequencing</td>
<td>PCR with phosphorescent probes</td>
</tr>
</tbody>
</table>

**Sanger Sequencing:**

The first, Sanger sequencing, has been the gold standard for many years and is based on, as are all other assays, the dideoxy-chemistry illustrated in Figure 2.
Sanger Sequencing

Dideoxy nucleotide inhibition of DNA synthesis due to nonreactivity of 3' hydrogen

In the precursor deoxynucleoside triphosphates (dNTPs) that are the building blocks of replicating DNA, the 3' hydroxyl group in the deoxyribose can be replaced with a hydrogen forming dideoxynucleosides (ddNTP). This substitution prevents binding of incoming dNTPs, preventing the formation of phosphodiester bonds and halting elongation of the replicating nucleotide chain. Replication is precisely truncated at the position where addition of the ddNTP occurs. In the Sanger sequencing reaction, labeled ddNTPs are added to the transcription reaction of a nucleotide chain. This results in the truncation of the transcription reaction, leaving behind nucleotides of variable length, each of which can be identified by separating the transcription product using a variety of methods including autoradiography, gel chromatography, and, most commonly, capillary electrophoresis (Figure 3).
Figure 3

The sequencing reaction is read as a color code that distinguishes oligonucleotides of variable length with four specific color labels. A ladder of nucleotides is created that can be identified by their electrophoretic mobility and color or fluorescence (Big Dye) terminal nucleotide. The identification of abnormal (mutant) peaks in chromatograms of this ladder can be facilitated by computer programs that not only create the chromatogram but compare them with reference sequences (refseq), and identify abnormalities that represent mutations and polymorphisms, as shown in the adjacent Mutation Surveyor result (Figure 4).

Sanger sequencing is readily accomplished in DNA extracted from formalin-fixed paraffin-embedded tissue that has been amplified by PCR provided that the amplified sequence is short (<400 bp). This method has the advantage that it provides unbiased sequence results that will detect virtually any mutation in the amplified template. However, the sensitivity is limited with tumor cell concentration of approximately 20% required for accurate results. Because of the PCR amplification, the amount of DNA starting material required is usually small, depending on
the number of DNA segments to be amplified with 5 ng of DNA usually sufficient for a single amplification.

**PCR-based Mutation Specific Assays**

Mutation specific techniques depend largely on the observation that mismatch of a 3’ terminal nucleotide in PCR primer results in failure of PCR amplification so that primers can be created that are highly selective for a single nucleotide. This is the basis for the Amplification Refractory Amplification System (ARMS) that is used to detect mutations in KRAS codons 12 and 13.

Allele specific amplification has been coupled with quantitative fluorescent probe detection methods to create efficient real-time platforms for the rapid analysis of specific mutations at sites of frequent mutation such as codon 12 and 13 of KRAS. In one variation of this, ARMS/Scorpions, primer and probe are combined in a single molecule. The figure below illustrates how a mutation specific probe provides a high level of specific amplification that is further enhanced by the Scorpion probes capacity to fold back on target sites, releasing a fluorescent signal. This methodology simplifies the preparation of reagents for the test and has a high level of sensitivity.

A typical quantitative reaction is shown below where the mismatch at the 3’ prime end of the primer results in minimal amplification of the normal base but successful amplification of the sequence containing mutant base. Elongation of the amplified segment displaces a fluorophore from its quencher resulting in quantitative increase in signal that can be read on either thermal block systems such as the Roche LiteCycler 480 and ABI 7900 or the recently introduced Qiagen Rotor-Gene Q that is based on centrifugal rotation of reaction tubes in a chamber of heated moving air. The number of PCR cycles needed to detect signal above a background referred to as Cycle threshold (Ct) is used to quantify fluorescence. Differences in Ct between exogenous control DNA and test DNA (∆Ct) reflect the quantity of mutant DNA in each sample.

A computer algorithm determines what level ∆Ct can be considered positive (significantly elevated over background). Typically, this assay can detect 1% mutant copy in a 2-3 ng DNA sample.
Clinical assays deploying this technology have the advantages of simplicity, high sensitivity (<6% in our hands) and rapid turnaround. Below is a schema showing the time line for testing clinical samples in a typical clinical laboratory. Results can be available in less than two days and this can be a major advantage in a clinical context.

**Performance of ARMS/Scorpions under field conditions**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 am</td>
<td>8 am</td>
</tr>
<tr>
<td>FEDEX Delivery</td>
<td>DNA Extraction (Qiacube)</td>
</tr>
<tr>
<td>11 am</td>
<td>10 am</td>
</tr>
<tr>
<td>Block Evaluation</td>
<td>Scorpion Assay</td>
</tr>
<tr>
<td>12 pm</td>
<td>1 pm</td>
</tr>
<tr>
<td>5 pm</td>
<td>2 pm</td>
</tr>
<tr>
<td>1 mm Punch taken from Block</td>
<td>Results assessed and signed off</td>
</tr>
<tr>
<td>Deparaffinization</td>
<td>Results Reported</td>
</tr>
<tr>
<td>Proteinase K Digestion</td>
<td></td>
</tr>
</tbody>
</table>

*A multicenter phase III Trial of Irinotecan/S-FU/Leucovorin or Oxaliplatin/S-FU/Leucovorin with Bevacizumab, or Cetuximab (C225), or with the combination of Bevacizumab and Cetuximab for patients with untreated metastatic adenocarcinoma of the colon or rectum*
On the down side however, only a single mutation per amplification reaction is detected resulting in the need for a considerable amount of DNA and increasing the cost of the overall analysis. In currently available kits for KRAS analysis, 7 primer/probe sets are needed for testing each target allele at codon 12 and 13. Thus, although only a small amount of DNA is needed for each reaction, the amount of DNA required for the whole analysis can escalate quickly and an upper limit of clinical feasibility is quickly reached. In the adjacent table are some representative results for a recently tested cooperative group cohort using a kit format showing the number of specific alleles tested and representative results.

In summary, the advantages of simplicity and speed of turnaround make this platform desirable for performing large numbers of tests for a small number of mutations.

**Performance of ARMS/Scorpions**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Codon</th>
<th># Specimens</th>
<th>Mutation Rate (%)</th>
<th>% of Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 GLY</td>
<td>GGT</td>
<td>684</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 ALA</td>
<td>GGT→GCT</td>
<td>23</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>12 ASP</td>
<td>GGT→GAT</td>
<td>153</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>12 ARG</td>
<td>GGT→CGT</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12 CYS</td>
<td>GGT→TGT</td>
<td>49</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>12 SER</td>
<td>GGT→AGT</td>
<td>28</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>12 VAL</td>
<td>GGT→GTT</td>
<td>99</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>13 ASP</td>
<td>GGC→GAC</td>
<td>89</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

Subtotal (mutant) 447 100 100
Total 1131 40 *

High Resolution Melting Analysis

High resolution melting analysis measures differences in melting point temperatures between matched and mismatched DNA doublets. Mismatching causes a reduction in melting point temperatures that can be quantified using software that is readily available in quantitative PCR instrumentation. An example of melting point difference plot is shown in the adjacent figure. In the top frame (A) are normalized high resolution melt curves. PCR products are labeled with a fluorescent dye and fluorescent signal is plotted as the temperature increases. Strand melting results in a decrease in fluorescent signal.
Below, in frame (B) a difference plot displays the melting curve of each sample subtracted from the reference curve to visually accentuate the melt curve differences and aid in genotype grouping. Products of three mutant templates are shown in red and pink. Products of wild type templates are shown in blue. The upper (pink curve) represents codon 12 mutation G12S, while the red curves represent amplification products for a single mutation at codon 12 (G12V) at two different concentrations. The latter two curves are similar in shapes but have different in heights due to variation in the quantity of PCR amplicon.

The advantage of the melting point analysis is that it is sensitive and requires relatively small amounts of DNA (< 5ng). It also has excellent coverage, detecting any mutational abnormality that may be present in the amplified segment. However, any perturbation of DNA structure including untargeted SNPs and adducts may produce aberrant melting point curves and nonspecific background for this method is relatively high. The specificity of this reaction is therefore relatively low without confirmation by a second technology, typically direct sequencing. This can negate the rapid turnaround and relative low cost of the assay. We regard this test as a screening assay that requires confirmation of abnormal results by a second assay.

**Pyrosequencing**

Pyrosequencing unlike Sanger sequencing does not rely on truncation of the transcription reaction but on the release of the pyrophosphate during chain elongation, which initiates a chemoluminescent response that can be detected with appropriate instrumentation. For this technology, DNA is immobilized and reagents needed for addition of successive nucleotides added to the template. The result of addition of each NTP is recorded. Additions of nucleotide that result in addition of nucleotide to the growing oligonucleotide chain are registered by the release of a pyrophosphate which is turned into ATP that induces a fluorescent signal in a reaction catalyzed by luciferase.

Pyrosequencing is relatively inexpensive and sensitive, and largely unbiased in that it detects all possible mutations in a target sequence. Sensitivity is higher than Sanger sequencing and approximates the sensitivity of allele specific and multiplex methods (~5%). However, the technology is somewhat complicated and it must be carefully validated and monitored. Turnaround times are relatively short so that this is a popular technology.
Multiple Assays

MASSarray (Sequenom) and SNaPshot (ABI) are based on similar chemistry but use different probes to measure the outcome of sequencing reactions. For Sequenom, a mass biased probe is used and this is detected by mass spectrometry. For SNaPshot, a fluorescent probe is used and standard sequencing capillary gel electrophoresis is used to detect mutant or nonmutant peaks. PCR reactions are performed with multiplex primers and panels of primer sets are constructed to detect mutations at multiple sites. The chemistry is based on single base extension from a tagged primer. The primers are positioned immediately adjacent to the mutated base the addition of a ddNTP at the mutation site results in the formation of a labeled nucleotide that can then be measured by the appropriate instrumentation, either mass spectroscopy (Sequenom) or capillary gel electrophoresis (SNaPshot chromatogram illustrated above). The primer sets can be designed at lengths for specific alleles so that they can be multiplexed into panel of up to 8 or 9 panels. The amplification and PCR of this technology results in a high level of sensitivity so that only 5% concentration of tumor cells is required for detection of mutation in this assay. An example of the readout for such a panel (Sequenom Oncarta) that has been provided by Dr. Chris Corless is shown below.

Using this technology, it is possible not only to measure all of the mutations in a specific locus but to measure a large number of additional mutations to provide a better profile for individual
lungs cancers. In the table below, we show results for a test set of 32 colon carcinomas.

<table>
<thead>
<tr>
<th>Gene Method</th>
<th>KRAS</th>
<th>cMET</th>
<th>PIK3CA</th>
<th>EGFR</th>
<th>BRAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DxS</td>
<td>HRM</td>
<td>Dseq</td>
<td>Sqom</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C001</td>
<td>G13D</td>
<td>MT</td>
<td>G13D</td>
<td>G13D</td>
<td>WT</td>
</tr>
<tr>
<td>C002</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C003</td>
<td>G12C</td>
<td>MT</td>
<td>WT</td>
<td>G12C</td>
<td>WT</td>
</tr>
<tr>
<td>C004</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C005</td>
<td>G13D</td>
<td>MT</td>
<td>WT</td>
<td>G13D</td>
<td>WT</td>
</tr>
<tr>
<td>C006</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C007</td>
<td>G12D</td>
<td>MT</td>
<td>G12D</td>
<td>G12D</td>
<td>WT</td>
</tr>
<tr>
<td>C008</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C009</td>
<td>G12C</td>
<td>?</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C010</td>
<td>WT</td>
<td>MT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C011</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C012</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C013</td>
<td>G12C</td>
<td>MT</td>
<td>G12C</td>
<td>G12C</td>
<td>WT</td>
</tr>
<tr>
<td>C014</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C015</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C016</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C017</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C018</td>
<td>WT</td>
<td>MT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C019</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C020</td>
<td>G12C</td>
<td>MT</td>
<td>G12C</td>
<td>G12C</td>
<td>R970C</td>
</tr>
<tr>
<td>C021</td>
<td>G12D</td>
<td>MT</td>
<td>G12D</td>
<td>G12D</td>
<td>WT</td>
</tr>
<tr>
<td>C022</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C023</td>
<td>G12D</td>
<td>MT</td>
<td>G12D</td>
<td>G12D</td>
<td>WT</td>
</tr>
<tr>
<td>C024</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C025</td>
<td>G13D</td>
<td>MT</td>
<td>G13D</td>
<td>G13D</td>
<td>WT</td>
</tr>
<tr>
<td>C026</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C027</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C028</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C029</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C030</td>
<td>G12V</td>
<td>MT</td>
<td>G12V</td>
<td>G12V</td>
<td>WT</td>
</tr>
<tr>
<td>C031</td>
<td>WT</td>
<td>?</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>C032</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

*Cloned sequences positive

Multiple mutations were found in nearly a third of the tumors and most cases overlapped with mutation in KRAS. However, BRAF mutations of which we identified 4, did not overlap with KRAS and this may be of considerable clinical importance since BRAF mutation confer a particularly poor prognosis in colon cancer.

Choosing a Testing Platform: Strengths and Weakesses

At the present time, there is no preferred platform for KRAS testing and there are few examples of direct comparison of multiple methodologies. One study that has directly compared three different platforms on a single sample set is attached as an appendix. Detection rates of these assays are similar with some minor exceptions. First, it is clear that Sanger sequencing is relatively insensitive, requiring at least 20% tumor cell concentration. For this reason, a small number of mutations may be missed in tumors that are highly contaminated with stromal background. Second, many of the remaining less specific tests are more sensitive, reaching a
level of 5% or less, so that the frequency of positive results this assay may be increased for tumor specimens in which contamination with normal cells is expected.

Direct comparison reveals the strengths and weaknesses of the various platforms and these summarized in the KRAS scorecard below.

<table>
<thead>
<tr>
<th>Testing Method</th>
<th>Analytical Sensitivity</th>
<th>Specificity</th>
<th>Coverage of gene</th>
<th>Turnaround time</th>
<th>Simplicity</th>
<th>Startup Costs</th>
<th>Cost per exon</th>
<th>Clinical Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger Seq</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>High</td>
<td>Mod</td>
<td>+</td>
</tr>
<tr>
<td>HRM</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>Mod</td>
<td>Low</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRM + Clamp</td>
<td>++++++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>Mod</td>
<td>Mod</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ARMS/Scorpion</td>
<td>++++++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>Mod</td>
<td>High</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iPLEX mass spectrometry</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>High</td>
<td>Low</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>Mod</td>
<td>Mod</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In general, differences among platforms are small and can be accommodated by adjustments to preanalytical processing protocols and other modifications of technique. For example, the coverage of Sanger sequence is an advantage of this method but analytical sensitivity of the method is less robust than that of other methods. The expense of some assays may preclude their adoption by laboratories on a tight budget. Clinical mandates may dictate which method is adopted, particularly in regard to FDA approved platforms that may be required for establishing eligibility for a clinical trial. All of these considerations will weigh into which platform is locally adopted.

It is imperative that the laboratory developed tests for clinical application be vigorously validated locally prior to be offered for clinical decision making. This includes establishing analytical sensitivity by dilution studies employing engineered oligonucleotides and cell lines and clinical samples with known mutations. Clinical sensitivity must also be assessed in a locally assembled cohort to test application of a particular platform in a local setting. This process may be less onerous for FDA approved tests that have been prevalidated but proficiency testing for platforms of all types are essential in a well-run laboratory.

**Conclusions:**

In conclusion, any of the methods discussed above when combined with the appropriate preanalytical processing steps may be appropriate for clinical testing. Sanger sequencing may be a perfectly acceptable technology or platform if samples are purified to a concentration of > 20% tumor cells and usually in the range of 50-70%. Sanger sequencing, however, may not be appropriate for high volume, rapid turnover laboratories and would be certainly inappropriate without assessment of tumor cell content and enrichment of test samples for tumor cells by appropriate technology. Alternatively, allele specific testing may be the appropriate in high throughput situations where enrichment methods less rigorous than direct microdissection such as block coring or whole sectioning may be employed. The choice of which technology to use
for KRAS testing will be depend on the expertise and resources of the individual laboratory doing the testing. In large clinical trials where rapid turnover is required and volume discounts on kit reagents may be obtained, platforms with high sensitivity and rapid turnaround may be most appropriate. Lower volume operations may be drive more by scheduling constraints and reagent costs. In all settings validation of method is paramount. In the near future FDA approval for specific platforms may standardize methods and limit the diversity of methodologies that are currently available and deployed for this important biomarker.

References

KRAS Mutation

Comparison of Testing Methods and Tissue Sampling Techniques in Colon Cancer

Wilbur A. Franklin,* Jerry Haney,* Michio Sugita,* Lynne Bemis,† Antonio Jimeno,† and Wells A. Messersmith†

From the Departments of Pathology* and Medical Oncology,†
University of Colorado Denver, Aurora, Colorado

Treatment of colon carcinoma with the anti-epidermal growth factor receptor antibody Cetuximab is reported to be ineffective in KRAS-mutant tumors. Mutation testing techniques have therefore become an urgent concern. We have compared three methods for detecting KRAS mutations in 59 cases of colon carcinoma: 1) high resolution melting, 2) the amplification refractory mutation system using a bifunctional self-probing primer (ARMS/Scorpion, ARMS/S), and 3) direct sequencing. We also evaluated the effects of the methods of sectioning and coring of paraffin blocks to obtain tumor DNA on assay sensitivity and specificity. The most sensitive and specific combination of block sampling and mutational analysis was ARMS/S performed on DNA derived from 1-mm paraffin cores. This combination of tissue sampling and testing method detected KRAS mutations in 46% of colon tumors. Four samples were positive by ARMS/S, but initially negative by direct sequencing. Cloned DNA samples were retested by direct sequencing, and in all four cases KRAS mutations were identified in the DNA. In six cases, high resolution melting abnormalities could not be confirmed as specific mutations either by ARMS/S or direct sequencing. We conclude that coring of the paraffin blocks and testing by ARMS/S is a sensitive, specific, and efficient method for KRAS testing. (J Mol Diagn 2010, 12:43–50; DOI: 10.2353/jmoldx.2010.080131)

The KRAS gene was recognized more than 25 years ago as the component of Kirsten sarcoma virus responsible for oncogenesis.1 Since that time, mutations of KRAS conferring constitutive activity on KRAS protein have been described in a large proportion of solid tumors ranging from >90% of pancreatic carcinomas2 to 20% to 30% of pulmonary adenocarcinomas.3,4 KRAS is a component of the tyrosine kinase signaling pathway mediated through ErbB, insulin-like growth factor, and met recep-
tors, among others. The presence of KRAS mutations has recently taken on increased clinical significance, as response of colon tumors to the epidermal growth factor receptor-targeted agent Cetuximab has been linked to absence of such mutations.5–8 KRAS mutation testing currently is being incorporated into patient selection protocols for anti-epidermal growth factor receptor colon cancer trials and is increasingly important for effective management of other solid tumors with targeted agents.9–12

Still unresolved however, are the specific protocols that are most sensitive, specific, and efficient for detection of KRAS mutation in clinical samples. Methodologies used to test for KRAS mutations were reviewed more than a decade ago.13 Typically, the specimens available for mutational analysis are formalin-fixed, paraffin-embedded tissue blocks. Formalin is a convenient fixative with a large installed user base, and formalin-fixed tissue embedded in paraffin blocks provides outstanding histological detail for pathological diagnosis. However, formalin fixation may introduce sequence alterations in DNA, and the frequency of such artificial mutations is inversely correlated with the number of cells used in PCR.14 Which paraffin block sampling method can yield an optimal quantity of DNA is yet to be determined.

Also unclear is which of the currently available methods of testing can be most effectively be applied to clinical material while meeting turnaround time requirements of current targeted treatment protocols. The current gold standard for KRAS testing remains direct sequencing of PCR amplification products.13 Two recently described methods, high resolution melting (HRM) analysis15–18 and amplification refractory mutation system (ARMS),5,19–21 which incorporates a unique bifunctional fluorescent primer/probe molecule (Scorpion),5,22,23 have high

Supported by NIH NCI Cancer Center grant P30-CA046934 and NIH NCI Early Detection Research Network Grant U01-CA85070.
DxX Limited provided ARMS/S test kits for this study free of charge. No other financial support for this study was received other than the federal grants indicated.

Accepted for publication July 2, 2009.
Address reprint requests to Wilbur A. Franklin, M.D., Department of Pathology, University of Colorado Denver, Fitzsimons Campus, 12801 E. 17th Ave., Rm L18–5104, MS F1804, Aurora, CO 80045. E-mail: wilbur.franklin@uchsc.edu.
sensitivity and specificity for detection of KRAS mutations with low turnaround time, and could be applied in a clinical setting.

The objective of the study described in this report was to test two methods of sampling paraffin-embedded tissue blocks for KRAS testing and to cross compare the two new testing methodologies, HRM and ARMS/S, with direct sequencing in regard to sensitivity, specificity, and efficiency of testing.

Materials and Methods

The design of the study is depicted in Figure 1. Paraffin blocks from 59 patients with invasive adenocarcinoma of colon were tested under a Colorado Multiple Institutional Review Board-approved protocol. The proportion of the slide that was composed of tumor was variable and was separately recorded in 10% increments for each tumor. All blocks were processed by two rapid methods: 1.) sectioning at 5 microns and 2.) coring using a Beecher Instruments tissue arrayer (Beecher Instruments, Inc., Sun Prairie, WI) that can precisely sample specific regions of the paraffin block. Sectioned tissue tended to form a tight spiral that could be readily placed directly in a microcentrifuge tube for DNA extraction. To prepare tissue cores, a stained slide made from a tumor-containing paraffin block was marked with a cytology marking pen to indicate a tumor rich focus. The block was then placed in the arrayer and the tumor-rich focus was sampled with a 1 mm needle. DNA was also extracted from tumor cells scraped from glass slides with a scalpel under a dissecting microscope in a subset of samples to determine whether microdissection might improve the sensitivity of the various detection assays (see below).

For deparaffinization, both sections and cores were incubated at room temperature in several volumes of xylene for 6 to 12 hours. Complete removal of paraffin was critical to obtain maximum DNA yield. For DNA extraction, deparaffinized tissue was digested with protease K (Qiagen, Inc., Valencia, CA) overnight at 37°C. If a pellet remained following overnight digestion, protease K was refreshed and the specimen reincubated at 90°C for 1 hour, which resulted in the dissolution of all residual pellet observed in this trial. DNA was then isolated from the incubation mixture using a QIAcube robotic workstation (Qiagen Inc., Valencia, CA) extraction protocol. DNA yields were then quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA).

DNA was also isolated from non-small cell lung cancer lines to evaluate the sensitivity and specificity of the assays in unfixed snap frozen tissue. To obtain cell line DNA, cells were grown to confluence, centrifuged at 1500 x g and frozen until use. DNA was extracted from the thawed cell pellet by protease K digest and purified by the QIAcube fresh tissue protocol. All purified DNA samples were then tested by the three methods described below.

ARMS/S

Template DNA was analyzed for a set of seven known KRAS point mutations using the Therascreen KRAS Mutation Detection kit (DxS Ltd., Manchester, UK). Reactions and analysis were performed on a Lightcycler 480 real-time PCR instrument (LC480) that was calibrated using a dye calibration kit provided by the kit manufacturer. Reactions were performed on a 96-well plate in

![Figure 2](image-url) Raw amplification curves for KRAS mutant tumor sample tested by the ARMS/S method. Each curve represents an amplification product of the same DNA template for one of eight specific primer sets. The curve on the farthest left is for wild-type control primers. The next curve to right represents amplification products for mutant template (GGT→GAT, G12D). The remaining curve is for the wild-type template. A positive result is determined by the cross point, the point on each curve where the slope of the curve becomes linear. There is a clear separation between mutant product (CP 29.6) and non-mutant product (CPs 38.5). The difference between CP for the wild-type control DNA and the mutant DNA (ΔCP) is small (2.4 cycles), indicating a mutation.

Table 1. KRAS Exon 1 Mutations Detected by DxS ARMS/Scorpion Kit

<table>
<thead>
<tr>
<th>Codon</th>
<th>BP Substitution</th>
<th>AA Change (Abbrev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>GGT→GCT</td>
<td>Gly→Ala (G12A)</td>
</tr>
<tr>
<td>12</td>
<td>GGT→GAT</td>
<td>Gly→Asp (G12D)</td>
</tr>
<tr>
<td>12</td>
<td>GGT→CCT</td>
<td>Gly→Arg (G12R)</td>
</tr>
<tr>
<td>12</td>
<td>GGT→TGT</td>
<td>Gly→Cys (G12C)</td>
</tr>
<tr>
<td>12</td>
<td>GGT→AGT</td>
<td>Gly→Ser (G12S)</td>
</tr>
<tr>
<td>12</td>
<td>GGT→GTT</td>
<td>Gly→Val (G12V)</td>
</tr>
<tr>
<td>13</td>
<td>GGC→GAC</td>
<td>Gly→Asp (G12D)</td>
</tr>
</tbody>
</table>
20 μl reactions using approximately 60 ng of each DNA template. Sample DNA was amplified with eight separate primer sets (one for the wild-type sequence and one for each of seven different point mutations) with an internal Scorpion reporter probe (Table 1). Cycle cross point (Cp) values were calculated using the LC480 Fit-point software suite, and the control Cp was subtracted from the Cp of each mutation specific primer set (Figure 2). Because there may be spuriously low level amplification in the absence of mutant template, amplification products are often visible at later cycle numbers for most of the primer sets. To avoid false-positive results due to background amplification, the assay is considered valid only if the control Cp value is less than or equal to 35 cycles. Cp thresholds are calculated to compensate for this background amplification. Mutations are called when the Cp is less than the statistically-set 5% confidence-value threshold.

HRM

Template DNA was tested by HRM analysis using a Lightcycler 480 real-time PCR instrument (Roche Applied Science, Indianapolis, IN). Approximately 60 ng of tumor template DNA, wild-type control DNA and mutant control DNA were amplified on the Lightcycler 480 instrument using HRM master mix (Roche cat# 04909631001), with the RASO1 and RASA2 primers and 1.75 mmol/L MgCl₂ in a 96 well plate using a 2-step cycling program (95° melting, 72° annealing and extension) for 45 cycles. PCR products were analyzed by HRM with 25 data acquisitions per degree of temperature increase, from 40° to 90°C. Lightcycler 480 Gene Scanning software using the known wild-type control samples for baseline calculation (Figure 3, A and B) was used for these analyses.

Direct Sequencing

Approximately 60 ng of template DNA were PCR amplified using 10 pmol each of forward and reverse KRAS primers (forward: RASO1 5'-AAGGGCTGCTGAAAATGAC-3' reverse:RASA2 5'-TGTCCTGCACCAGTAATAG3') and Taq polymerase PCR master mix (Promega cat# M750) in a 25 μl reaction. PCR was performed on an ABI 9700 thermocycler with 20 cycles of touchdown PCR (starting annealing temperature of 65°C, decremented 0.5°C per cycle) and 15 cycles at 55°C annealing temp.
perature. The resultant PCR products were purified with the QiAmp PCR cleanup kit (Qiagen cat# 28106) run through the appropriate protocol on the QiAcube robotic workstation. The purified PCR products were sequenced in forward and reverse directions using an ABI 3730 automated sequencer (Applied Biosystems, Inc., Foster City, CA). Each chromatogram was visually inspected for any abnormalities with particular attention directed to codons 12 and 13. Sequences were also evaluated using Mutation Surveyor software (Soft Genetics, State College, PA) against known wild-type control sequences. Mutations were determined to be present when peaks reached a threshold value above baseline calculated from background level. Visual reads confirmed mutation peaks that fell just below the threshold of detection of the Mutation Surveyor software and identified false-positive calls where the software misinterpreted band compression and other anomalies as sequence changes (Figure 4).

**Statistical Analysis**

χ² analysis, Student t-test and Pearson correlation were used to compare test results. Analyses were conducted with the SPSS v.16 statistical package (SPSS, Inc., Chicago, IL).

**Results**

**Cell Line DNA**

To validate testing methods in unfixed control DNA, a total of 14 non-small cell lung carcinoma cell line DNA samples were tested by all three methods. Three (H157, H358, and HCC44) have been previously reported to be KRAS mutant while 11 (H157, H226, H322, H358, H520, H820, H1435, H1650, H1703, H1975, H2126, HCC44, HCC2279, and HCC4006) are considered to be wild-type. All three mutations were confirmed (H157, G12R; H358, G12S; and HCC44, G12S) by all three methods—ARMS/S, HRM, and direct sequencing.

**Comparison of Sections with Cores in Paraffin Blocks**

Estimated tumor cellularity of tumors in paraffin blocks varied from 1% or less to approximately 90% of the cross-sectional area of the block. No relationship was noted between the detection of mutation and the percentage of tumor in sections (P = 0.638, t-test). All specimens (sections and cores) yielded sufficient DNA for successful amplification. The average quantity obtained from sections was 6.0 micrograms while the average obtained from 1 to 2 cores was 10.3 micrograms. Both sections and cores from a total of 59 surgically resected invasive adenocarcinomas of colon were evaluated by the three methods—ARMS/S, HRM, and direct sequencing.

The two methods of specimen preparation resulted in similar but not identical results. Results from total of 177 samples processed by both methods were compared. There was agreement between the sampling methods in 158 of the 177 samples with 14 of the cores and five of the sections mutation-positive while the corresponding samples were wild-type as shown in the cross tabulation (Table 2). When these results were broken down by testing method it was found that all but one of the samples positive in section DNA but negative in core DNA were tested by HRM.

Finally, the question remains whether microdissection may further increase the sensitivity of the assay. To address this question we microdissected the all of the specimens that were negative in whole sections but positive in core specimens and all but one specimen that were mutation-negative in both sections and core samples. (Insufficient cellular material was available for retesting of one case.) Two of the three cases that were negative in whole tissue sections were positive in the microdissected material but none that were negative in both whole sections and cores were positive in microdissected samples.

**Comparison of Molecular Testing Methods**

A total of 118 specimens (59 sections and 59 cores) were tested by each of the three mutation detection methods. Results for the various methods are shown in Table 3.

The most frequently positive method was HRM. Pearson correlation indicated a high degree of agreement among the various methods, but with the highest between ARMS/S and direct sequencing and the lowest between direct sequencing and HRM (Table 4). Cross tabulation tables (Table 5) for two-way comparisons are shown below. All 10 discrepancies between ARMS/S and direct sequencing were due to the higher frequency of MUT by ARMS/S than directed sequencing. Discrepancies between HRM and the other two methods were due exclusively to the higher frequency of MUT in the HRM groups.

**Table 2. Mutations in DNA Extracted from Tissue Sections and Cores**

<table>
<thead>
<tr>
<th></th>
<th>Sections</th>
<th>Cores</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>89</td>
<td>14</td>
</tr>
<tr>
<td>MUT</td>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>83</td>
</tr>
</tbody>
</table>

*WT = wild-type.
*MUT = mutant.

**Table 3. Frequency of Mutations Detected by Separate Testing Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Frequency of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMS/S</td>
<td>43%</td>
</tr>
<tr>
<td>HRM</td>
<td>54%</td>
</tr>
<tr>
<td>Seq</td>
<td>36%</td>
</tr>
</tbody>
</table>

*ARMS/S = amplification resistant mutation system/Scorpions.
*HRM = high resolution melting analysis.
*Seq = direct sequencing.
Combined Results

Combined results are shown in Table 6. The method with the most frequent positive results was HRM, which detected melting point abnormalities in 53% of sections and 56% of cores. However, HRM does not provide the identity of mutations that are potentially detected and requires direct sequencing to confirm the mutation.\(^{15}\)

Mutations were confirmed by direct sequencing in only 19 of 31 (specificity 61%) of HRM-positive section specimens and 23 of 33 (specificity 70%) of HRM-positive cores. All cases that were MUT by ARMS/S or direct sequencing were positive by HRM, indicating a sensitivity of 100%.

The highest level of agreement among any of the subgroups was the association between ARMS/S and direct sequencing in core specimens. The cross tabulation table for this comparison is shown below (Table 7). There was 93% concordance in these data. All of the discrepancies in results were in the direction of greater sensitivity for ARMS/S.

The high sensitivity of ARMS/S raises the question of the specificity of this test. On resequencing the test samples, one sample was found to have a mutation that had previously been overlooked and that matched the mutation identified by ARMS/S (Figure 5). Close examination of the sequence chromatogram shows a very tiny peak corresponding to the mutation detected by ARMS/S, but its height is below the level of background and cannot be reasonably called. To further explore this question with the remaining three samples and to estimate the concentration of mutant DNA in the starting DNA sample, we cloned the DNA of these samples and sequenced 12 to 24 of the resulting clones from each sample. In two samples we found one mutant clone per 12 wild type, and in one sample we found 1 mutant clone per 24 wild-type, suggesting a concentration of mutant DNA at 4 to 8% in these samples.

### Discussion

We evaluated sample preparation and compared three current methods for the determination of KRAS mutation in formalin-fixed, paraffin tissue samples. We find that methods of sample preparation and testing result in small, but possibly significant differences in results. ARMS/S proved to be 100% accurate with a single round of testing, which could translate into economical and clinical advantage.

In sample preparation, there are several potential problems that could affect testing results. Tumor samples may vary in the size of the overall sample that is available for testing. This is dependent in part on the procedure that was used to obtain the sample. Surgical resection yields many more tumor cells for testing than more limited diagnostic procedures such as snare or fine needle aspiration biopsy. For this reason testing of resection specimens is favored whenever these are available. However, when resection specimens are not available, adequate results may be obtained from smaller biopsies provided that well preserved tumor cells are present in the block.

Paraffin blocks are highly heterogeneous with respect to the quantity and distribution of tumor within the blocks and microscopic verification that sufficient tumor cells are present in the block is critical for accurate testing. After microscopic inspection, blocks may be directly sectioned to obtain tumor cells for DNA extraction. However, sectioning without further tumor cell enrichment may result in dilution of tumor DNA template with non-tumor stromal DNA that may compete for primers used in PCR reactions used to amplify potentially mutant loci. To address this problem, microdissection of the histological sections has been used to enrich for tumor cell DNA. The coring procedure used in this study proved to be an adequate and less time consuming method to enrich for tumor DNA than microdissection. In addition, the procedure yielded generous amounts of DNA, important in minimizing the chance of detecting artificial mutations.

### Table 4. Correlation among Results Detected by Different Testing Methods

<table>
<thead>
<tr>
<th>Pearson correlation</th>
<th>DxS</th>
<th>HRM</th>
<th>Seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>DxS</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRM</td>
<td>0.801</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Seq</td>
<td>0.816</td>
<td>0.683</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table 5. 2 × 2 Comparison of Mutation Frequencies Detected by the Three Testing Methods

<table>
<thead>
<tr>
<th></th>
<th>ARMS/S v. Seq</th>
<th>HRM v. Seq</th>
<th>ARMS/S v. HRM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT* MUT† Total</td>
<td>WT MUT Total</td>
<td>WT MUT Total</td>
</tr>
<tr>
<td>ARMS/S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>66 6 66</td>
<td>54 0 54</td>
<td>54 13 67</td>
</tr>
<tr>
<td>MUT</td>
<td>10 42 52</td>
<td>22 42 64</td>
<td>0 51 51</td>
</tr>
<tr>
<td>Total</td>
<td>76 42 118</td>
<td>76 42 118</td>
<td>54 64 118</td>
</tr>
</tbody>
</table>

*WT = wild-type.
†MUT = mutant.
which is greater when small amounts of starting material are available.24 For these reasons we have used a simple and widely accessible tissue microarray instrument for coring paraffin blocks. This instrument is equipped with set screws to stabilize the paraffin block for further manipulation. The 1 mm coring needle, with which the instrument may be equipped, can be superimposed on areas of the block that contain tumor and the tumor-enriched region can be cored for DNA extraction. Cores are only partially deparaffinized after up to 6 hours of incubation with xylenes at room temperature so that for highest DNA yields overnight deparaffinization is necessary. We have found this procedure is efficient, inexpensive and accurate with no false positives and >20% increase in the frequency of mutations detected by direct sequencing and 12% by ARMS/S. Moreover, microdissection failed to increase the sensitivity of detection in cases selected for their negative results in whole section but positive results in core samples. The yield of DNA averaged >10 micrograms from one or two cores so the ample template was available for retesting and archiving.

Beyond specimen processing is the choice of mutation detection method. Until recently the gold standard and most widely available method for mutation detection was direct sequencing13 of PCR products resulting from the amplification of tumor DNA with KRAS specific primers. This method detects all mutations in amplified DNA sequences, but mutant copies must have a concentration that is at least 20% to 50% of any accompanying wild-type sequences, a sensitivity that may not be optimal for clinical testing.25,26 We found a mutation frequency of 39% for direct sequencing, 7 percentage points lower than the frequency of mutation detected by the more sensitive ARMS/S method.

ARMS is based on the observation that oligonucleotides with a single base mismatch at the 3' base will not function as primers.19 With proper primer design at the 3' base, mutant alleles can be preferentially amplified in specimens where mutant copies are at a low concentration. ARMS was used to document KRAS mutation in colon carcinoma a decade ago.21 More recently ARMS has been used in a quantitative PCR platform20 and linked with a bifunctional fluorescent primer/probe molecule23 (“Scorpions”).22 The combined assay uses 7 primer/probes for 7 different mutations KRAS in a single kit that directly detects the presence of KRAS mutation in heterogeneous specimens at a low allelic concentration (1%) without the need for confirmation by direct sequencing. This assay has been successfully used in phase III clinical trials for metastatic colon carcinoma.5,7

In the present study, ARMS/S detected four (17%) more mutations than were initially detected through direct sequencing of DNA-derived from paraffin cores. One of the specimens that was negative by direct sequencing proved positive by repeat testing. The remaining three samples were negative on duplicate testing. That these were not ARMS/S false positives was proven by cloning the DNA and sequencing the resulting clones. Mutant sequence was detected in all samples that were initially negative, indicating the cause of the negative results was low sensitivity (<10% mutant/wild-type ratio). The combination of core sampling of tumor tissue and ARMS/S

### Table 7. Comparison of ARMS/Scorpions with Direct Sequencing in DNA Prepared for Tissue Cores

<table>
<thead>
<tr>
<th></th>
<th>cARMS/S</th>
<th>cSeq</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>MUT</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Totals</td>
<td>36</td>
<td>23</td>
</tr>
</tbody>
</table>

*WT = wild-type.
†MUT = mutant.

![Figure 5. Example of discrepancy between ARMS/S and direct sequencing.](image)
technique thus provides a particularly sensitive and rapid method for KRAS mutation test. However, ARMS/S is not without limitation since it covers only the common codon 12 and 13 mutations but misses uncommon mutations in codons 12 and 61, which together account for approximately 1% of reported KRAS mutations in colon carcinoma (Sanger Institute, http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action = gene&ln = KRAS).

A second new method for mutation testing, HRM, directly measures differences in melting point temperatures between matched and mismatched double stranded DNA, either polymorphisms or somatic mutations. HRM of KRAS PCR products is inexpensive and rapid. The reported sensitivity of the assay is high with carcinoma and non-small cell lung carcinoma sug-

either direct sequencing or ARMS/S, suggesting a false HRM was found in six cases they were not confirmed by sequencing. In the present study, while all sequencing confirmed mutations were positive by HRM, abnormal curves must be confirmed by sequencing. In the present study, while all sequencing confirmed mutations were positive by HRM, abnormal HRM was found in six cases they were not confirmed by either direct sequencing or ARMS/S, suggesting a false positive frequency of >20%. Although HRM may prove to be a helpful screening tool, the requirement for confirmation by a second method increases turn around time and expense and decreases the value of the high sensitivity of the method.

Conclusions

Several methodological variables can affect the outcome of testing for KRAS mutations in formalin-fixed, paraffin-embedded clinical samples. These include the method of sampling the paraffin block and the testing platform used for mutation detection. This study shows that selective sampling of microscopically recognizable tumor cells by full thickness coring of paraffin blocks yields more DNA and results in a higher rate of mutation detection than testing a surface cross section of tumor bearing paraffin block. In comparison with direct sequencing of PCR products, ARMS/S and HRM both detect KRAS mutations at higher frequency but ARMS/S could be verified in all cases by either direct sequencing or a cloning assay, while HRM abnormalities frequently could be verified by either direct sequencing nor by ARMS/S. ARMS/S offers higher sensitivity and specificity compared with the other two methods tested in this study. The high accuracy of ARMS/S is clinically relevant, as the toxicity and economic burden of a false negative result for a negative predictor is significant. Finally, it should be noted that results for the mutation detection systems reported here apply to KRAS only. Validation of these detection systems for other genes of interest such as epidermal growth factor receptor will require the same rigorous cross comparisons used in this KRAS study.

Acknowledgment

We thank Christopher Korch, PhD, for expert technical assistance.

References

15. Do H, Krypuy M, Mitchell PL, Fox SB, Dobrovic A: High resolution
melting analysis for rapid and sensitive EGFR and KRAS mutation detection in formalin-fixed paraffin-embedded biopsies. BMC Cancer 2008, 8:142


50 Franklin et al

JMD January 2010, Vol. 12, No. 1