# Whole Cancer Genome Sequencing by Next-Generation Methods

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**Key Words:** Cancer; Next-generation sequencing; Massively parallel sequencing; Personalized oncology; Molecular diagnostics; Colorectal cancer; Non–small cell lung cancer; Melanoma

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Upon completion of this activity you will be able to:
- contrast the advantages of next-generation gene sequencing technologies with the traditional Sanger, pyrosequencing, and allele-specific polymerase chain reaction approaches.
- list the current uses of cancer cell gene sequencing for the management of patients with solid tumors.

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Questions appear on p 653. Exam is located at www.ascp.org/ajcpcme.

## Abstract

*Traditional approaches to sequence analysis are widely used to guide therapy for patients with lung and colorectal cancer and for patients with melanoma, sarcomas (eg, gastrointestinal stromal tumor), and subtypes of leukemia and lymphoma. The next-generation sequencing (NGS) approach holds a number of potential advantages over traditional methods, including the ability to fully sequence large numbers of genes (hundreds to thousands) in a single test and simultaneously detect deletions, insertions, copy number alterations, translocations, and exome-wide base substitutions (including known "hot-spot mutations") in all known cancer-related genes. Adoption of clinical NGS testing will place significant demands on laboratory infrastructure and will require extensive computational expertise and a deep knowledge of cancer medicine and biology to generate truly useful "clinically actionable" reports. It is anticipated that continuing advances in NGS technology will lower the overall cost, speed the turnaround time, increase the breadth of genome sequencing, detect epigenetic markers and other important genomic parameters, and become applicable to smaller and smaller specimens, including circulating tumor cells and circulating free DNA in plasma.*

During the past 13 years since the launch of the targeted therapy era, there has been a keen interest in determining the cancer cell gene sequence to predict the response to anticancer drugs ▮**Table 1**▮.[1-4] In late 1998, the era of personalized oncology began with the simultaneous regulatory approval of the anti–HER2-targeted monoclonal antibody therapeutic, trastuzumab, for the treatment of HER2-overexpressing breast cancer and the immunohistochemical-based diagnostic HercepTest (DAKO, Carpinteria, CA) for the identification of patients who are eligible for treatment.[5] During the ensuing 12 years, interest in these "drug-test" combinations, also referred to as "companion diagnostics," has increased exponentially as more anticancer agents have been approved and more new drugs have entered clinical trials based on biomarker profiles.[4] The selection of anti–epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non–small cell lung cancer (NSCLC) and the use of anti-EGFR monoclonal antibody therapeutics in colorectal cancer (CRC) are now firmly based on pretreatment sequence determinations for hot spots in the *EGFR* (NSCLC) and *KRAS* (CRC) genes.[1-3]

Recently, 2 novel targeted small-molecule drugs have advanced into late-stage clinical trials that feature a genetic sequence detection–based companion diagnostic. PLX-4032 targets papillary thyroid cancers and metastatic malignant melanomas that feature the V600E mutation of the *BRAF* gene.[6] The second drug, crizotinib, has shown impressive efficacy for the treatment of NSCLCs that feature an *EML4-ALK* translocation.[7] The recent introduction of the more thorough and in-depth sequencing technologies (next-generation sequencing [NGS] or massively parallel sequencing) has

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**Table 1**
**Treatment Selection and Cancer Genomics**

| Genetic Event | Disease | Drug | Current Test Platform | Regulatory Status |
|---|---|---|---|---|
| *HER2* gene amplification | Breast and upper GI cancer | Trastuzumab and lapatinib | IHC; FISH | FDA approved |
| *BCR-ABL* translocation | CML | Imatinib and dasatinib | Cytogenetics; FISH; PCR | FDA approved |
| *RARA-PML* translocation t(15;17) | APL | ATRA | Cytogenetics; FISH; PCR | FDA approved |
| *KRAS* mutation | CRC | Cetuximab and panitumumab | Traditional sequencing | FDA approved |
| *BRAF* mutation | CRC | Cetuximab and panitumumab | Traditional sequencing | Clinical trials |
| *EGFR* mutation | NSCLC | Gefitinib and erlotinib | Traditional sequencing | FDA approved |
| *BRAF* mutation | Melanoma and thyroid cancer | PLX-4032 | Traditional sequencing | Clinical trials |
| *EML-ALK* translocation | NSCLC | Crizotinib | FISH | Clinical trials |
| *CKIT* mutation | GIST and melanoma | Imatinib and sunitinib | IHC | FDA approved |

APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; CML, chronic myelogenous leukemia; CRC, colorectal cancer; FDA, US Food and Drug Administration; FISH, fluorescence in situ hybridization; GI, gastrointestinal; GIST, gastrointestinal stromal tumor; IHC, immunohistochemical analysis; NSCLC, non–small cell lung cancer; PCR, polymerase chain reaction.

heightened concerns that the traditional so-called Sanger-type sequencing method used to sequence the human genome 10 years ago will prove incapable of scaling sufficiently to support the breadth of information required to manage an ever-growing arsenal of targeted anticancer drugs. Thus, as PLX-4032 and crizotinib approach regulatory approval in 2011, a significant debate has arisen as to which technology approach is best for detecting gene sequence abnormalities in clinical samples of solid tumors and hematologic malignancies.

## Traditional Gene Sequencing Approaches in Clinical Oncology

Traditional DNA sequencing for cancer patient management **Table 2** was initially focused on the detection of cancer predisposition in the patient's germline.[8,9] Currently, traditional sequencing approaches remain the cornerstones for detecting *BRCA1* and *BRCA2* mutations, hereditary nonpolyposis coli (*hMLH1* and *hMSH2*), familial adenomatous polyposis coli, and other familial cancer syndromes[8,9] and have also been used to genotype germline DNA to determine polymorphisms associated with anticancer drug metabolism and toxic effects.[10-12] In addition, DNA sequence determination has expanded in routine oncology practice to include tumor cell assessments that predict the efficacy of drugs on the market and in various stages of clinical development.[13-15]

### Sanger Sequencing

The original sequencing of the human genome used the chain-termination sequencing method developed by Sanger and colleagues.[16-18] The modern Sanger method uses automated sequencing instruments that detect fluorescently labeled

**Table 2**
**Traditional DNA Sequencing Methods**

| Method | Basic Technique | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|
| Sanger "chain-termination method" | Fluorescent dye–labeled bases; DNA fragments separated by capillary electrophoresis | High | "Gold standard"; complete sequence | Time-consuming; cannot detect deletions, translocations, copy number changes |
| Pyrosequencing "sequencing by synthesis method" | Chemiluminescent detection; DNA polymerase synthesizes DNA complementary to a target template; pyrophosphate release detected at each base addition | Higher | More sensitive than Sanger; provides percentage of mutated vs wild-type DNA; works well with fragmented DNA from FFPE samples | Short length reads limit technique to hot spots; limited accuracy detecting changes in homopolymer runs; scalability limited compared with other NGS methods |
| Mass spectroscopy–based mutation analysis (MALDI-TOF) | Primer extension with chain termination using PCR amplicon templates identifies variant alleles | Higher | High sensitivity; high resolution of DNA fragments; detects frame-shift mutations; readily identifies germline SNPs | Mass spectrometry resolution window balanced with PCR amplicon design requirements combine to limit scalability |
| Allele-specific RT-PCR | Primers span DNA sites of interest; probes detect specific mutations | Higher | Very high sensitivity; widely used for clinical testing for oncogene mutations in CRC, NSCLC | Scalability constraints limit application to hot spots |
| RT-PCR melting curve analysis | Heterogeneous DNA PCR products melt at temperatures different from those for homogeneous DNA PCR products | High | High sensitivity; provides percentage of mutated vs wild-type DNA | Often difficult to resolve differences in melt curves; difficult to standardize; multiplex capability limited |

CRC, colorectal cancer; FFPE, formalin-fixed, paraffin-embedded; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NGS, next-generation sequencing; NSCLC, non–small cell lung cancer; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; SNPs, single nucleotide polymorphisms.

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nucleotide sequences. DNA strands of increasing length are resolved by capillary electrophoresis after DNA elongation along single-stranded amplified DNA templates is randomly terminated by incorporating fluorescent dideoxynucleotide chain terminators.[18] Sanger sequencing has been used to identify many clinically significant DNA variants. Sanger and other traditional sequencing platforms (Table 1) have been used for the following: (1) *EGFR* sequencing in NSCLC to select patients for treatment with EGFR tyrosine kinase inhibitors[19-21]; (2) *KRAS* mutation testing of CRC specimens to predict resistance to the anti-EGFR antibody therapeutics, cetuximab and panitumumab[22-24]; (3) *BRAF* mutation testing to predict anti-EGFR antibody resistance in CRC[25-29]; (4) *BRAF* mutation testing to select patients with metastatic thyroid cancers and melanoma for treatment with the BRAF inhibitor PLX-4032[30]; (5) sequence-based detection of the *EML4-ALK* translocation in NSCLC to select patients for treatment with crizotinib[31-33]; and (6) *CKIT* sequencing for treatment of gastrointestinal stromal tumors, a subset of malignant melanomas that feature activating *CKIT* mutations,[34-38] and a number of hematologic malignancies, including the *BCR-ABL* translocation in chronic myelogenous leukemia and the *RARA-PML* translocation in acute promyelocytic leukemia. *HER2* gene amplification is not readily detected by traditional Sanger sequencing methods but is detected by NGS technologies.

### Pyrosequencing

Pyrosequencing differs from the chain-termination method by relying on an integrated biochemical reaction to detect pyrophosphate released by DNA polymerase after each nucleotide addition during the synthesis of the DNA strand complementary to the DNA strand being sequenced.[39] This method is also referred to as a "sequencing by synthesis" method. As each new base is added to the complementary DNA strand by DNA polymerase, the pyrophosphate released from the added nucleotide is combined by a sulfurylase to adenosine 5' phosphosulfate to form adenosine triphosphate. The adenosine triphosphate is hydrolyzed by luciferase as it converts luciferin to oxyluciferin, releasing photons as visible light detected with a high-sensitivity charge-coupled device (CCD) camera.

Pyrosequencing has been shown to be more sensitive than Sanger sequencing and also provides a measure of the percentage of DNA that harbors detected mutations. However, pyrosequencing is limited in the length of the template DNA strand that can be sequenced, which is significantly shorter than that for Sanger chain-termination sequencing and is prone to errors in reading through homopolymer sequences. Thus, pyrosequencing is most often applied in clinical settings focused on hot-spot sequencing of short-length DNA segments or specific exons where mutations are commonly found to determine mutation status of oncogenes.[40-42] Finally, pyrosequencing is better suited for sequencing the heavily fragmented, short-strand-length DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections than is the Sanger method.

### MALDI-TOF Sequencing

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has been applied to clinical samples for DNA sequence analysis with very high resolution and sensitivity, especially for the detection of single nucleotide polymorphisms (SNPs) in germline DNA.[43-45] MALDI-TOF has also been used to find disease susceptibility–associated genetic markers and biomarkers of drug response.[44,45] MALDI-TOF is particularly adept for finding frameshift and heterozygous mutations. Although MALDI-TOF has been used to evaluate the products of Sanger sequencing reactions, the short read lengths that can be detected in the mass spectrometry detection window preclude using this technique for large-scale sequencing projects.

### Allele-Specific RT-PCR

Allele-specific real-time polymerase chain reaction (RT-PCR) is a method of determining the sequence of targeted hot spots in the cancer cell genome, known to be commonly mutated in cancer, that are important to guide therapy decisions for such tumors as CRC and NSCLC. A variety of commercial PCR-based systems have been developed to perform DNA sequencing and have shown high sensitivity for mutation detection in closed-tube systems designed to reduce the risk of sample contamination.[46,47] Primer and probe sets of oligonucleotides are designed to detect specific mutations of clinical interest when run in the closed RT-PCR system. The probes are designed for specific mutations and will not detect other mutations, deletions, or translocations involving related genes. In NSCLC, this method is reported to be sensitive enough for detecting a *KRAS* mutation at a frequency as low as 1% of the total DNA extracted from an FFPE specimen.[48]

### Quantitative PCR Melting Curve Analysis

This approach is based on the principle that homogeneous DNA amplicons will melt at a characteristic temperature and that the melting curves of DNA products produced by PCR amplification can be analyzed to determine the presence of heterogeneous amplicon products. In addition, any double-stranded amplicon containing a mismatch along its length will dissociate ("melt") at a lower temperature than expected. When a mixture of DNA amplicons is present, each has a characteristic melting temperature based on its length and sequence composition that can then identify the specific DNA mutations of interest.[49] When mutated DNA and wild-type DNA amplicons are present in a PCR mixture and they are

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**Table 3**
**Commercial Next-Generation Sequencing Platforms***

| | Basic Technique | Major Uses | Time Needed | Instrument Cost ($, thousands) |
|---|---|---|---|---|
| Illumina | Flow cell-based, reversible dye termination, and 4-color optical imaging | Whole exome sequencing; whole genome sequencing; SNP detection | Intermediate, 4 d fragment; 9 d paired end | 500-900 |
| 454 Pyrosequencing | Emulsion PCR with bead-based pyrosequencing and CCD light imaging | Targeted exon sequencing; confirmatory sequencing; SNP detection | Fast, <1 d | 500-700 |
| Helicos | Oligo-dT captured PolyA-tailed DNA fragments; flow cell 4-color dNTP optical imaging | Single molecule sequencing; whole genome sequencing | Slowest, 8 d | 999 |
| SOLiD | Sequential dinucleotide ligation; flow cell–based 4-color optical imaging | Whole exome sequencing; whole genome sequencing; SNP detection | Slow, 7 d fragment run; 14 d paired end | 600-700 |
| Ion Torrent | Semiconductor-based nonoptical detection; standard dNTP sequencing chemistry | Targeted sequencing projects not demanding deep sequencing | Fast, <1 d | 50 |

CCD, charge-coupled device; dNTP, deoxynucleoside triphosphate; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.
* Adapted from Metzker.[55]

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denatured and reannealed, melt curves characteristic of the homozygous and heterozygous products can be generated, indicating the sequence composition of the mixture.

A variation of this method is the PCR clamp method, which uses a peptide nucleic acid probe to block the amplification of the wild-type sequence in a DNA sample while permitting amplification of a specific mutation.[50] Although this method has high sensitivity, it cannot be used to calculate the relative percentage of mutated DNA, nor can it discriminate the exact nature or location of a sequence alteration.

## NGS Sequencing Technologies

The initial near-complete human genome sequence assembly, at the time of its publication in 2001, required more than 12 years of sequencing at multiple laboratories at a cost of more than $3 billion.[17,18] Today, the continuous demand for more rapid and low-cost sequencing has driven the development of novel approaches designed to parallel the sequencing process.[51-54] In comparison with traditional Sanger sequencing and other sequence analysis methods, these new massively parallel or next-generation strategies **Table 3**[55] have increased sequencing rates by orders of magnitude and driven down the per base sequencing cost significantly.[56,57] It is now widely anticipated that NGS will enable the in-depth characterization of the cancer cell genome and further advance the fields of molecular pathology and personalized medicine for patients with cancer.

### Illumina HiSeq and Genome Analyzer Systems

The massively parallel signature sequencing developed by Lynx Therapeutics was the second or next-generation approach to DNA sequencing. The original Lynx Therapeutics platform was a microsphere (bead)-based system that read nucleotides in groups of 4 via an adapter ligation and adapter decoding strategy using reversible dye terminators.[58] Lynx Therapeutics (Hayward, CA) merged into Solexa, which was subsequently acquired by Illumina. This short read sequencing approach is now incorporated into a fluidic flow cell design (HiSeq and Genome Analyzer systems, Illumina, San Diego, CA) with 8 individual lanes. The flow cell surface is populated with capture oligonucleotide anchors, which hybridize the appropriately modified DNA segments of a sequencing library generated from a genomic DNA sample **Image 1**, **Image 2**, and **Image 3**. By a process called "bridge amplification," captured DNA templates are amplified in the flow cell by "arching" over and hybridizing to an adjacent anchor oligonucleotide primer.[58] Actual sequencing is performed by hybridizing a primer complementary to the adapter sequence, then cyclically adding DNA polymerase and a mixture of 4 differently colored fluorescent reversible dye terminators to the captured DNA in the flow cell.[58] By using this approach, nonmodified DNA fragments and unincorporated nucleotides are washed away, while captured DNA fragments are extended 1 nucleotide at a time. After each nucleotide-coupling cycle takes place, the flow cell is scanned, and digital images are acquired to record the locations of fluorescently labeled nucleotide incorporations. Following imaging, the fluorescent dye and the terminal 3' blocker are chemically removed from the DNA before the next nucleotide coupling cycle **Figure 1**.[58]

The Illumina technique is the most widely used NGS platform, but it is limited by a relatively low multiplexing capability.[59] A number of technical issues, particularly due to


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| Length of Reads (bases) | Advantages | Disadvantages |
|---|---|---|
| Intermediate, 75-100 | Widest use; most experienced users | Low multiplex capability |
| Longest >500 | Fast; high accuracy; long reads useful for mapping and confirming | Low parallelism; errors in homo-polymer runs |
| Intermediate, 35 | Unbiased sequence reads | High error rates |
| Intermediate, 50-75 | Error reduction via 2-base encoding system | Slow speed with long run times |
| Intermediate, 50 | Lowest cost, fastest process | Lowest parallelism; high error rates |

aberrant nucleotide incorporation rates driven by polymerase error incorporating the modified deoxynucleotides, place major responsibility on the roles of the bioinformaticists and computational biologists to interpret the sequencing results that are produced by the Illumina systems.[60,61] The Illumina system has been applied in programs for gene discovery, whole exome analysis, and SNP detection by resequencing.[62] Examples of use of the Illumina HiSeq technique on clinical surgical pathology–processed tissue samples are shown in Image 1.

### 454 Pyrosequencing

First introduced in 2005, the 454 Genome Sequencer FLX Titanium System (Roche, Branford, CT) NGS platform relies on highly parallel PCR reactions taking place in tiny emulsions composed of a primer-coated bead with a single captured DNA template encased with the DNA polymerase,

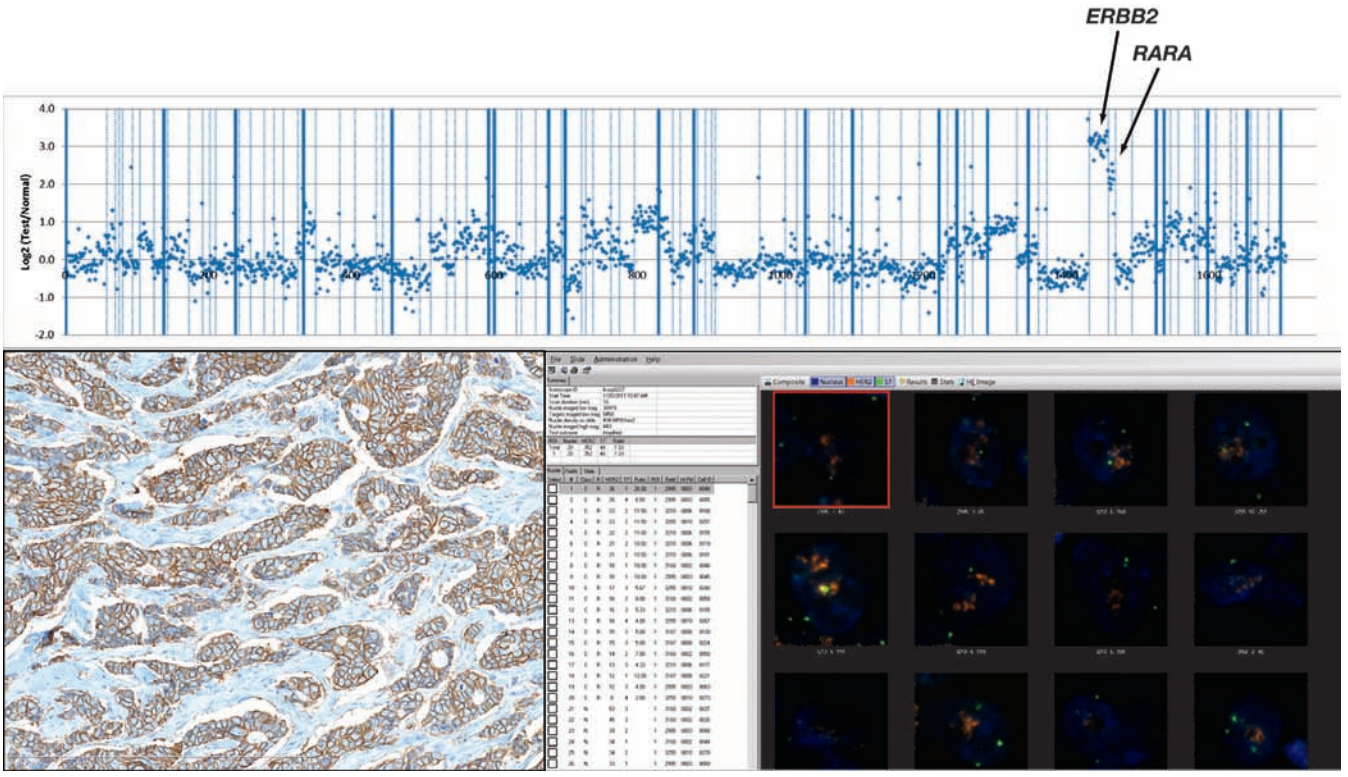


**Image 1** *HER2* gene amplification in breast cancer detected by next-generation sequencing (NGS). In this surgically resected recurrent invasive duct carcinoma of the breast, NGS found an 8.6-fold amplification of the *HER2* gene and a 4.6-fold amplification of the *RARA* gene. Fluorescence in situ hybridization (FISH) testing of this specimen (not shown) revealed an average *HER2* copy number of 11.6. Immunohistochemical staining of this tumor was strongly 3+, as seen at the lower left (HercepTest [DAKO, Carpinteria, CA], ×100), and the *HER2* gene amplification for this case detected by FISH (Abbott-Vysis PathVysion [Abbott Molecular, Downers Grove, IL], ×400) using the Ikoniscope automated fluorescence digital imaging system (Ikonisys, New Haven, CT) is seen at the lower right. Sanger and other types of traditional DNA sequencing do not detect alterations in gene copy number. NGS was performed using the Illumina HiSeq2000 System (Illumina, San Diego, CA) for 182 oncogenes, tumor suppressor genes, and drug-metabolizing enzymes from formalin-fixed paraffin embedded (FFPE) specimens. FFPE sections of 20-μm total thickness were obtained, and extracted DNA was subjected to hybrid capture and paired-end sequencing featuring high-coverage sequence (median, 300-fold coverage) of 2,574 exons encompassing the 182 genes. bp, base pairs.

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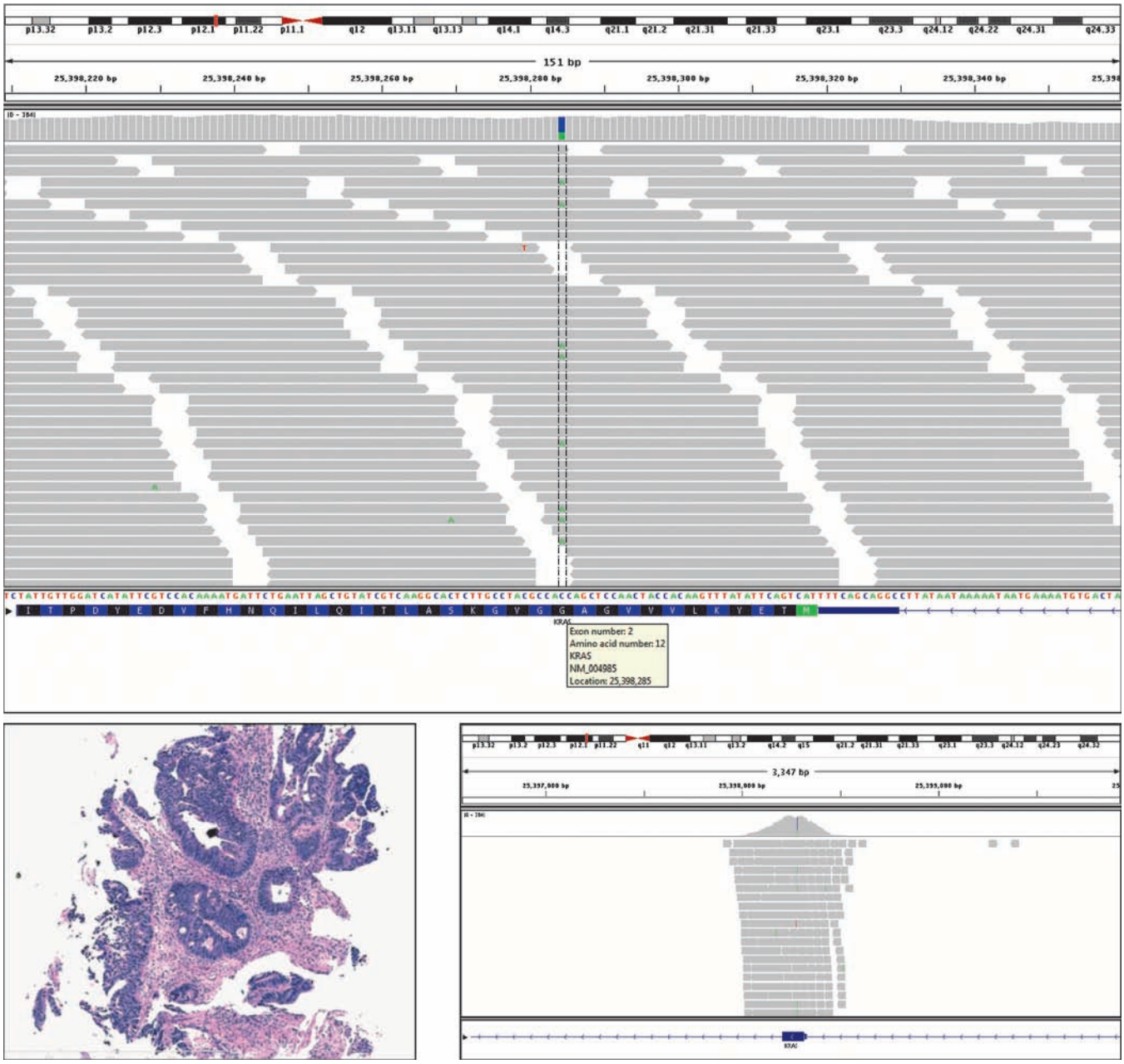


**Image 2** *KRAS* mutation in colorectal cancer (CRC) detected by next-generation sequencing (NGS). With NGS, this endoscopic biopsy of a deeply invasive CRC is shown to have a *KRAS* point mutation at codon 12 of the second exon of the *KRAS* gene located on chromosome 12 where a thymidine (T) has substituted for a G (glycine). This is known as the *KRAS* G12V mutation and has been linked to deregulation of cell cycle, apoptosis, and nitrogen metabolism. CRC cases with a G12V *KRAS* mutation have been associated with resistance to treatment with anti–epidermal growth factor receptor antibodies (cetuximab and panitumumab).[22-24] The biopsy of this deeply invasive CRC in a 52-year-old man, pathologic stage pT2pN2pMx, is shown in the lower left (H&E, ×200). Detail of the NGS detection of the mutation is seen at the lower right. NGS was performed using the Illumina HiSeq2000 System (Illumina, San Diego, CA) for 182 oncogenes, tumor suppressor genes, and drug-metabolizing enzymes from formalin-fixed, paraffin-embedded (FFPE) specimens. FFPE sections of 20-μm total thickness were obtained, and extracted DNA was subjected to hybrid capture and paired-end sequencing featuring high-coverage sequence (median, 300-fold coverage) of 2,574 exons encompassing the 182 genes. bp, base pairs.

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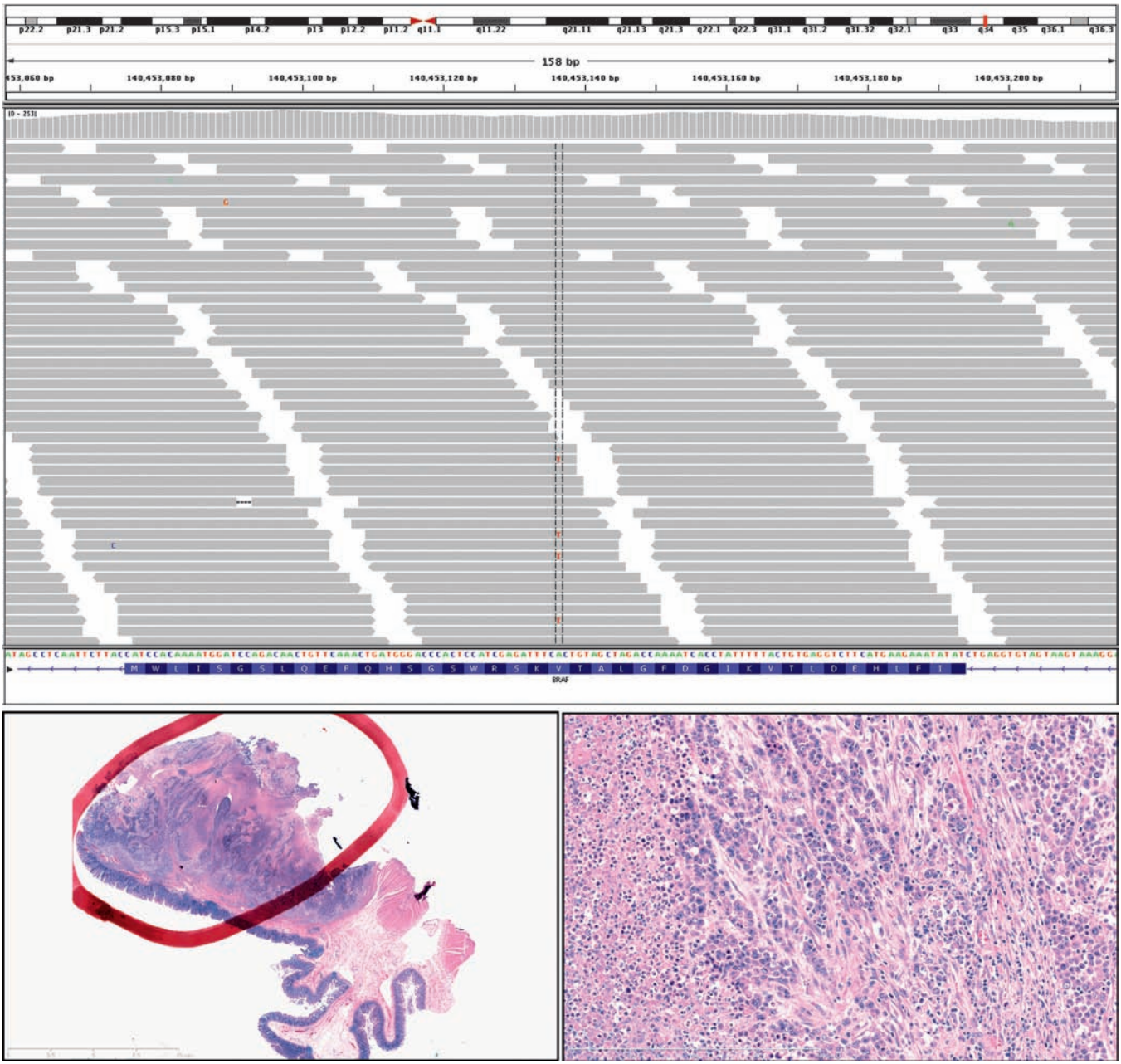


**Image 3** *BRAF* V600E mutation in colorectal cancer (CRC) detected by next-generation sequencing (NGS). In this surgically resected undifferentiated carcinoma of the colon, sequence analysis by NGS revealed a wild-type *KRAS* sequence and a V600E point mutation in the *BRAF* gene. The *BRAF* mutation was detected in 13% of the extracted DNA at a sequencing depth of 184 (sequencing depth equals the average number of nucleotides contributing to a portion of NGS-based DNA sequence assembly). At the lower left, the scanning power view shows that a significant amount on nonneoplastic colon tissue was present in the sample submitted for NGS. To guard against the possibility of a dilutional effect on the total DNA extracted, the specimen was enriched for tumoral DNA by removing the tissue within the paraffin block not within the red circle before thick sectioning and subsequent DNA extraction. At the lower right, the highly necrotic, poorly differentiated histologic features of this resected pT3pN1pMx primary CRC are shown (H&E, ×10). Although *BRAF* mutation status has also been used as a negative selector for anti–epidermal growth factor receptor antibody therapy in CRC, recent evidence suggests that, rather than being a specific marker of resistance to these drugs, the *BRAF* mutation status imparts an overall negative prognosis for these tumors, which are generally resistant to all types of therapy.[25-29] NGS was performed using the Illumina HiSeq2000 System (Illumina, San Diego, CA) for 182 oncogenes, tumor suppressor genes, and drug-metabolizing enzymes from formalin-fixed, paraffin-embedded (FFPE) specimens. FFPE sections of 20-μm total thickness were obtained, and extracted DNA was subjected to hybrid capture and paired-end sequencing featuring high-coverage sequence (median, 300-fold coverage) of 2,574 exons encompassing the 182 genes. bp, base pairs.

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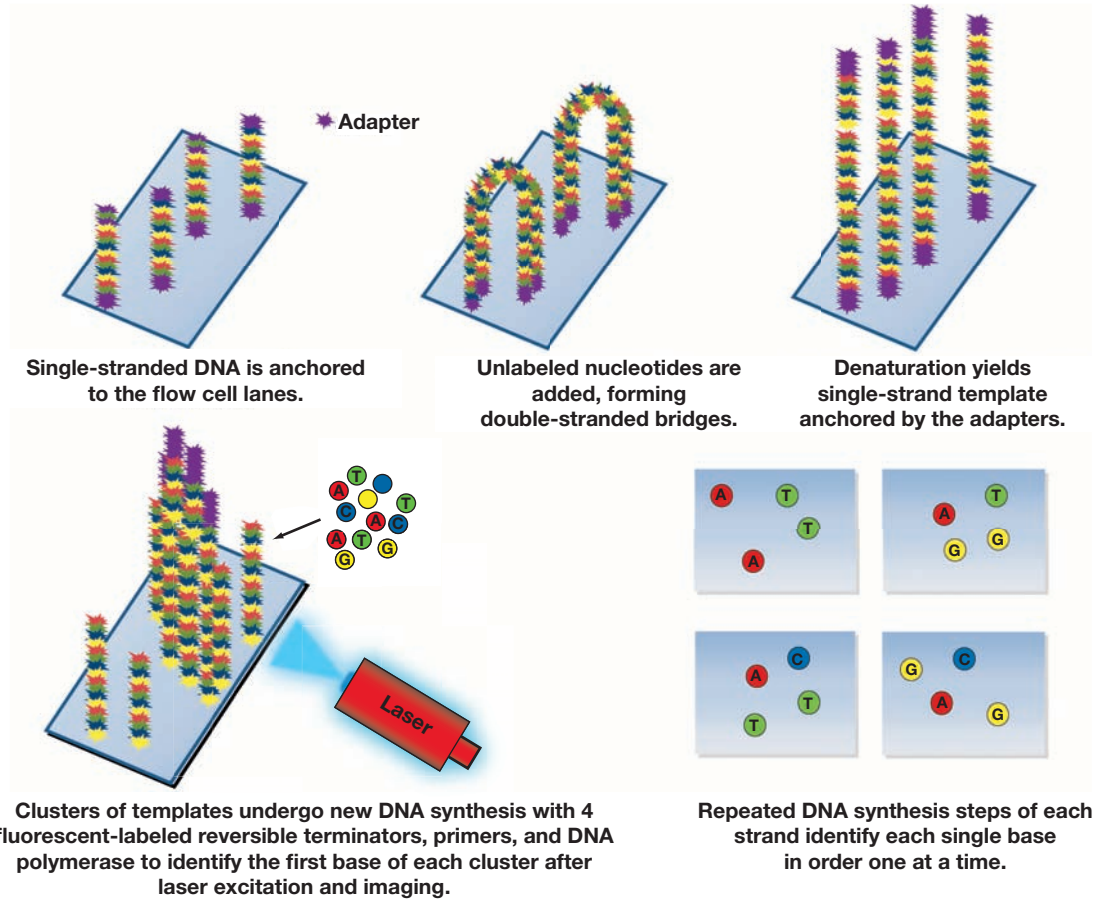


**Figure 1** Schematic diagram of the Illumina sequence by synthesis next-generation sequencing platform.

primers, and nucleoside triphosphates (NTPs) necessary for PCR in an oil droplet. PCR amplification results in each bead becoming coated with a single DNA amplicon. The emulsions are broken, and the DNA-coated beads are loaded onto an array of picoliter wells for the sequencing reaction.[55,63] Pyrosequencing is performed over the picoliter well array, and the nucleotide additions are visualized and located by a fiberoptic-coupled imaging camera.

The system provides longer read lengths than other NGS technologies, a strength of this system.[62] The 454 NGS platform features the strengths of relatively fast instrument run times but is limited by the contamination risks associated with emulsion PCR and problems with error rates in genetic regions rich in homopolymer repeats.[62] 454 sequencing has mainly been applied to targeted capture sequencing, high-resolution confirmatory sequencing for other NGS platforms, and plant and insect whole genome mapping.[5,63,64]

### Helicos Heliscope

The Heliscope platform involves fragmenting the sample DNA and performing polyadenylation at the 3' ends of the fragments. Denatured polyadenylated strands are captured by hybridization to poly(dT) oligonucleotides immobilized on a flow-cell surface. This approach was the first method to successfully perform single molecule sequencing.[65] The flow cell is cyclically flooded with fluorescently labeled deoxynucleoside triphosphates (dNTPs) in the presence of DNA polymerase, which incorporates nucleotides from the oligo-dT primer. The flow cell is imaged at each cycle using a CCD camera, allowing the identification of the location of each nucleotide incorporation event. As in other systems, the fluorescent label is cleaved and washed away before each subsequent sequencing cycle.[62,65] The Heliscope system is considered to provide the most nonbiased DNA sequence, which is its strength, although relative to competing NGS platforms, it has relatively high NTP incorporation error rates.[62]

### SOLiD Sequencing

SOLiD sequencing (Supported Oligonucleotide Ligation and Detection) is based on DNA ligase–mediated oligonucleotide ligation after PCR amplification in an emulsion


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format.[64] The primers in SOLiD NGS are progressively offset to allow the adapter bases to be sequenced when used in conjunction with the color-space coding for determining the template sequence by deconvolution.[58] Fluorescent signals are captured by CCD camera imaging before enzymatic cleavage of the ligated probes and, after washing, repeating the sequencing process. The SOLiD approach has been used in applications similar to the Illumina NGS, including whole genome sequencing, whole exome capture, and sequencing and SNP discovery.[65] Strengths of the SOLiD approach include reduction in sequencing error rates relative to the Illumina NGS by using 2-base encoding. A drawback of the SOLiD system has been its relatively long run times and complex analysis requirements.[62]

### Ion Torrent Sequencing

This platform was acquired by Life Technologies, which claims that this PostLight sequencing technology has the major advantage of being the first platform to eliminate the cost and complexity associated with the 4-color optical detection currently used in all other NGS platforms. The Ion Torrent method relies on standard DNA polymerase sequencing with unmodified dNTPs but uses semiconductor-based detection of hydrogen ions released during every cycle of DNA polymerization.[66] Each nucleotide incorporation into the growing complementary DNA strand causes the release of a hydrogen ion that is sensed by a hypersensitive ion sensor.[66] The initial Ion Torrent system has relatively low parallelism, so it tends to be focused on short sequence determination of mutation hot spots throughout the genome.

## Comparison of NGS Platforms

The relative strengths and weaknesses of the NGS platforms as they are applied to the sequencing of the somatic cancer cell genome are summarized in Table 3. All NGS platforms have high entry costs, but all also have the potential to dramatically reduce the cost of comprehensive genomic profiling of cancer cells in the near future. Some techniques offer speed, such as the 454 Pyrosequencing and Ion Torrent platforms, but, compared with the Illumina and SOLiD platforms, may not be as well suited for clinical somatic tumor DNA sequencing owing to their relatively limited capacities for supporting highly parallel, deep sequencing. Some NGS platforms are better adapted to wide genome scans because their massively parallel capacity can support the depth of sequencing that somatic mutation detection requires, whereas others are more adept at the sequencing of targeted genomic regions. It is also important to note that all NGS technologies generate unparalleled quantities of data and are heavily reliant on the data processing, storage, and analysis tools that accompany the instruments and on downstream data analysis pipelines and storage strategies.[62,67-70]

There is great interest in simplifying the sample and data analysis workflow and improving the accuracy of mutation detection (also known as "mutation calling") and identification of other genetic abnormalities obtained from NGS data sets. Similarly, cost reduction is being addressed by improving how efficiently each sequencing run is loaded. One approach relies on applying bar-code sequence tags or radio-frequency identification tags to sequencing libraries before pooled hybrid capture of the sequencing libraries.[71]

Despite recent advances in the computational biology required to align the genome and differentiate the significant findings among the massive amounts of sequencing data generated by NGS, these emerging technologies will continue to face analytic challenges for some time. Clinical applications, particularly in oncology, demand high levels of accuracy, sensitivity, and specificity for sequence-based tests focused on calling out specific genetic alterations.

## Comparison of Traditional and NGS Strategies for Cancer Cell Genomic Analysis

A comparison between traditional Sanger DNA sequencing and NGS for somatic cancer cell analysis is given in **Table 4**. The relative costs of the 2 approaches are of great importance to current and future test providers, consumers, and payers. Without question, the expertise, especially in computational biology, required to perform clinical NGS testing for patients with cancer is significantly higher for this NGS approach than for traditional Sanger sequencing. Although the cost per base sequenced for the traditional approach is higher on a per test basis, this "one gene, hot spots only" approach is less expensive than the cost for an NGS test incorporating hundreds or thousands of genes or a whole exome.

The predominant cell cycle stage of the sample to be sequenced can influence the final result, especially when gene copy number is being assessed by NGS. In regard to daily clinical pathology practice, traditional and NGS sequencing approaches are challenged by defining the best sample to test (eg, primary vs metastatic tumor tissue or tumor tissue vs circulating tumor cells), small samples such as from fine-needle aspiration biopsies, extensive necrotic rather than viable tumor tissue, tumor heterogeneity for mutations and other genetic abnormalities, and samples that feature a very low percentage of tumor DNA.

The relatively restricted sequencing capacity of traditional Sanger sequencing limits this approach to single-gene or hot-spot sequence analysis (eg, codons 12 and 13 of exon 2 in the *KRAS* gene), and the difficulties and high cost of

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**Table 4**
**Comparison of Sanger and NGS for the Cancer Cell Genome**

| Parameter | Traditional | NGS | Advantage |
|---|---|---|---|
| Cost | | | |
| per Base | High | Low | NGS |
| per Single clinical "test" ($) | Low to moderate, 300-500 | Moderate to high, 800-2,000 (estimated) | Traditional |
| per 10-gene multiplex multigene test ($) | High, 3,000-5,000 | Moderate, 800-2,000 | NGS |
| Equipment | Moderate | High | Traditional |
| Expertise required for sequencing and data analysis | Moderate | High | Traditional |
| Can be performed using FFPE samples | Yes | Yes | — |
| Challenged by small samples, necrotic tumor, tumor heterogeneity, and low percentage of tumor sample DNA | Yes | Yes | — |
| Generally restricted to 1 gene at a time | Yes | No | NGS |
| Can easily sequence hundreds of cancer-related genes in 1 sample | No | Yes | NGS |
| Generally restricted to hot-spot analysis | Yes | No | NGS |
| Can detect deletions | No | Yes | NGS |
| Can detect translocations | No | Yes | NGS |
| Can detect gene copy number alterations | No | Yes | NGS |
| Sensitivity | Low | High | NGS |
| Accuracy | High | High | — |
| Regulatory status | "Gold standard" | Pending | Traditional |
| Turnaround time | | | |
| Single gene | Shorter | Longer | Traditional |
| per Multiplex multigene test | Longer | Longer | — |

FFPE, formalin-fixed, paraffin-embedded; NGS, next-generation sequencing.

multiplexing this technology for multiple gene analysis are significant drawbacks to this approach for cancer genomics. NGS platforms accommodate large-scale gene sequencing, which can determine the status of "expected" potential mutations in a given clinical situation and also discover "unexpected" sequence abnormalities, which, in turn, may significantly alter treatment planning based on genomic analysis. NGS sequencing can provide gene copy information such as homozygous and heterozygous deletions and gene amplifications, whereas traditional sequencing approaches cannot. NGS can also detect translocations, which also drive therapy selection, as in the case of the *EML4-ALK* translocation and the selection of crizotinib in NSCLC. Although the turnaround time for NGS of a multiplex (eg, >100-gene) cancer genome sequence is longer (eg, 7-14 days) than traditional single-gene hot-spot sequencing, it is anticipated that this difference will rapidly narrow as NGS technology continues to evolve.

Although published NGS data describing clinical cancer samples are limited, it seems that NGS will exceed traditional Sanger sequencing in sensitivity for mutation detection in samples in which a mutation is present in only a small percentage of the total DNA extracted from a specimen. Although traditional Sanger sequencing and NGS sequencing results are most easily interpreted for a tumor cell sequence when a matched reference germline sequence is available, improving algorithms and public databases of germline and somatic mutations that can be used to filter results are making direct tumor sequence assessment increasingly reliable. Despite rapidly advancing sequencing technology in an era of growing demand for a more personalized approach to oncology practice, it remains likely that traditional and emerging cancer cell diagnostics, including histopathologic slide–based results (immunohistochemical studies and fluorescence in situ hybridization), epigenetic testing such as methylation-specific RT-PCR profiling, full transcriptional analysis, and microRNA profiling, will be combined with tumor cell DNA sequencing in some form of integrated laboratory report.

## Sample Procurement and Preanalytic Issues

By using their morphologic skills to identify appropriate tumor tissues, anatomic pathologists will have major roles in the introduction of whole genome sequencing into routine clinical practice. Although for the present the benefits of using FFPE specimens for clinical cancer cell gene sequencing make it the preferred source of DNA to be sequenced, the growth in molecular diagnostics may, in time, support a transition to freshly collected, buffer-stabilized tissue specimens.

In either case, a number of preanalytic factors are known to potentially affect the results of molecular diagnostic tests on solid tumors and hematologic malignancies. Among the factors affecting all FFPE surgical and biopsy specimens are specimen age, warm ischemia (time within the patient devoid of blood supply), and cold ischemia (time outside the patient before stabilization in buffer or formalin). For fixed specimens, important factors include characteristics of the

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formalin fixative (eg, concentration, zinc content, and pH), duration of fixation, type of tissue processing (routine or microwave-based), temperature of paraffin during embedding, and storage conditions of paraffin blocks.[72-74]

In general, DNA sequencing has been successful using FFPE material of relatively recent vintage, although as the age of FFPE specimens increases, they generally demonstrate increasingly significant DNA degradation. It is also well known that nucleic acids degenerate more quickly when they are stored as unstained microscopic slides vs intact paraffin blocks, most likely a result of enhanced tissue oxidation. Specimens with fragmented DNA are more difficult to sequence owing to the relative reduction in long DNA segments and the accumulation of short segments that are less likely to result in successful sequencing library generation.[75]

## Summary

NGS sequencing can produce upwards of 1 billion sequences per instrument in a 4-day run, and the cost per base sequenced is far cheaper than for the traditional dye-terminator methods. NGS is highly dependent on sophisticated bioinformatic analysis programs and faces significant data management and interpretation challenges for confirming whether a specific locus in a single cancer-related gene is wild type, an SNP, or a somatic mutation. A great strength of NGS approaches is that, they not only can detect base substitutions but also can simultaneously find insertions, deletions, copy number alterations, and translocations.

Now that NGS is being widely applied to FFPE specimens, data defining its sensitivity and specificity limits for detecting cancer gene abnormalities will soon be known. This information will be critical for interpreting the many somatic genetic aberrations that occur in cancer with very low frequencies, many far less than 1%. Experts in the cancer sequencing field hold that as many as 75% or more of cancer gene sequence variations may be missed by a hot-spot-only analysis. However, it is equally clear that tens or hundreds of individual traditional mutation analysis assays to test for all of the possible important mutations in a given tumor are not practical or feasible given the amount of tumor sample, time, and money it would take to perform so many individual tests. Given the potential generation of "actionable" gene sequence abnormalities and abnormalities of unknown clinical importance, the successful NGS-based clinical service will have a result report that is focused on the abnormalities that can lead directly to the best available therapy, direct a patient to an ongoing clinical trial, or result in some other actionable response from the treating oncologist.

Cancer-specific NGS will rapidly expand from a focus on mutations and gene copy number alterations to include translocations followed by assessment of epigenetic abnormalities. Moreover, the starting sample may well change as understanding of cancer biology progresses. Currently, surgical biopsy and resection specimens are the most widely analyzed samples; however, medical practice suggests that a transition to testing ever-smaller amounts of DNA is likely to be required as fine-needle aspiration biopsy specimens, circulating tumor cells, and even DNA from apoptotic cancer cells circulating in plasma become clinically relevant patient specimens. The technologic advantages offered by NGS will eventually reduce the cost of sequencing and decrease the turnaround times that will support unprecedented analytic depths of understanding about tumor biology, enabling highly individualized patient care. Thus, as NGS enters clinical testing in 2011 **Figure 2**, the impact on physician decision making and cancer outcomes will be widely followed by oncologists, pathologists, payers, and, most of all, patients with the disease.

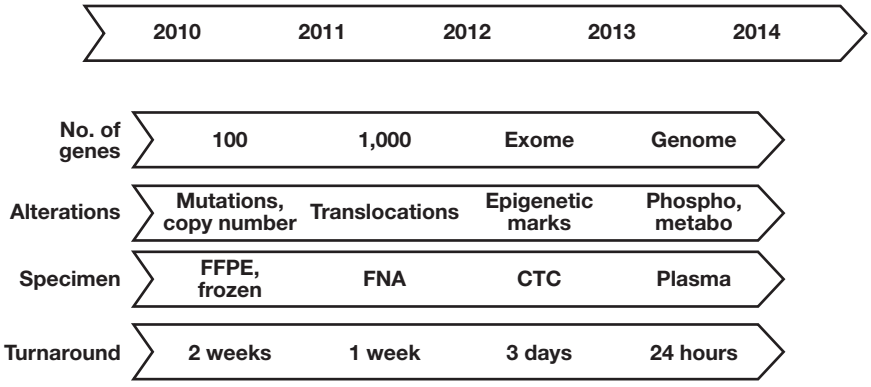


**Figure 2** Anticipated future development of next-generation sequencing. CTC, circulating tumor cells; FFPE, formalin-fixed, paraffin-embedded; FNA, fine-needle aspiration; metabo, functional status of drug metabolism pathways; phospho, phosphorylation or activation of cell surface receptors.

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## References

1. Bronte G, Rizzo S, La Paglia L, et al. Driver mutations and differential sensitivity to targeted therapies: a new approach to the treatment of lung adenocarcinoma. *Cancer Treat Rev*. 2010;36(suppl 3):S21-S29.
2. Saif MW, Shah M. K-*ras* mutations in colorectal cancer: a practice changing discovery. *Clin Adv Hematol Oncol*. 2009;7:45-53, 64.
3. Tejpar S, Bertagnolli M, Bosman F, et al. Prognostic and predictive biomarkers in resected colon cancer: current status and future perspectives for integrating genomics into biomarker discovery. *Oncologist*. 2010;15:390-404.
4. Papadopoulos N, Kinzler KW, Vogelstein B. The role of companion diagnostics in the development and use of mutation-targeted cancer therapies. *Nat Biotechnol*. 2006;24:985-995.
5. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
6. Smalley KS. PLX-4032, a small-molecule B-Raf inhibitor for the potential treatment of malignant melanoma. *Curr Opin Investig Drugs*. 2010;11:699-706.
7. Hallberg B, Palmer RH. Crizotinib: latest champion in the cancer wars [editorial]? *N Engl J Med*. 2010;363:1760-1762.
8. Robson M, Offit K. Inherited predisposition to cancer: introduction and overview. *Hematol Oncol Clin North Am*. 2010;24:793-797.
9. Weinstein JL, Ayyanar K, Watral MA. Cancer predisposition syndromes. *Cancer Treat Res*. 2009;150:223-238.
10. Deeken JF, Figg WD, Bates SE, et al. Toward individualized treatment: prediction of anticancer drug disposition and toxicity with pharmacogenetics. *Anticancer Drugs*. 2007;18:111-126.
11. Bernig T, Chanock SJ. Challenges of SNP genotyping and genetic variation: its future role in diagnosis and treatment of cancer. *Expert Rev Mol Diagn*. 2006;6:319-331.
12. Algeciras-Schimnich A, O'Kane DJ, Snozek CL. Pharmacogenomics of tamoxifen and irinotecan therapies. *Clin Lab Med*. 2008;28:553-567.
13. Mardis ER, Wilson RK. Cancer genome sequencing: a review. *Hum Mol Genet*. 2009;18:R163-R168.
14. Swanton C, Caldas C. From genomic landscapes to personalized cancer management: is there a roadmap? *Ann N Y Acad Sci*. 2010;1210:34-44.
15. Taylor BS, Ladanyi M. Clinical cancer genomics: how soon is now? *J Pathol*. 2011;223:318-326.
16. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74:5463-5467.
17. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860-921.
18. International Human Genome Consortium. Finishing the euchromatic sequence of the human genome. *Nature*. 2004;431:931-945.
19. Kobayashi S, Boggon TJ, Dayaram T, et al. *EGFR* mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2005;352:786-792.
20. Sequist LV, Joshi VA, Jänne PA, et al. Response to treatment and survival of patients with non–small cell lung cancer undergoing somatic *EGFR* mutation testing. *Oncologist*. 2007;12:90-98.
21. Cooper WA, O'toole S, Boyer M, et al. What's new in non–small cell lung cancer for pathologists: the importance of accurate subtyping, *EGFR* mutations and *ALK* rearrangements. *Pathology*. 2011;43:103-115.
22. Monzon FA, Ogino S, Hammond ME, et al. The role of *KRAS* mutation testing in the management of patients with metastatic colorectal cancer. *Arch Pathol Lab Med*. 2009;133:1600-1606.
23. Ross JS, Torres-Mora J, Wagle N, et al. Biomarker-based prediction of response to therapy for colorectal cancer: current perspective. *Am J Clin Pathol*. 2010;134:478-490.
24. Weichert W, Schewe C, Lehmann A, et al. *KRAS* genotyping of paraffin-embedded colorectal cancer tissue in routine diagnostics: comparison of methods and impact of histology. *J Mol Diagn*. 2010;12:35-42.
25. Loupakis F, Ruzzo A, Cremolini C, et al. *KRAS* codon 61, 146 and *BRAF* mutations predict resistance to cetuximab plus irinotecan in *KRAS* codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer*. 2009;101:715-721.
26. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type *BRAF* is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol*. 2008;26:5705-5712.
27. Souglakos J, Philips J, Wang R, et al. Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. *Br J Cancer*. 2009;101:465-472.
28. Sartore-Bianchi A, Di Nicolantonio F, Nichelatti M, et al. Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. *PLoS One*. 2009;4:e7287. doi:10.1371/journal.pone.0007287.
29. Tejpar S, Popovici V, Delorenzi M, et al. Mutant KRAS and BRAF gene expression profiles in colorectal cancer: results of the translational study on the PETACC 3-EORTC 40993-SAKK 60-00 trial [abstract]. *J Clin Oncol*. 2010;28(suppl 15):7s. Abstract A-3505.
30. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364:2507-2516.
31. Shaw AT, Solomon B. Targeting anaplastic lymphoma kinase in lung cancer [published online ahead of print February 2, 2011]. *Clin Cancer Res*. 2011;17:2081-2086. doi:10.1158/1078-0432.CCR-10-1591.
32. Gerber DE, Minna JD. ALK inhibition for non–small cell lung cancer: from discovery to therapy in record time. *Cancer Cell*. 2010;18:548-551.
33. Rodig SJ, Shapiro GI. Crizotinib, a small-molecule dual inhibitor of the c-Met and ALK receptor tyrosine kinases. *Curr Opin Investig Drugs*. 2010;11:1477-1490.
34. Martín-Broto J, Rubio L, Alemany R, et al. Clinical implications of *KIT* and *PDGFRA* genotyping in GIST. *Clin Transl Oncol*. 2010;12:670-676.

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35. Antonescu CR. The GIST paradigm: lessons for other kinase-driven cancers. *J Pathol*. 2011;223:251-261.
36. Woodman SE, Davies MA. Targeting *KIT* in melanoma: a paradigm of molecular medicine and targeted therapeutics. *Biochem Pharmacol*. 2010;80:568-574.
37. Macconaill LE, Garraway LA. Clinical implications of the cancer genome. *J Clin Oncol*. 2010;28:5219-5228.
38. Berger MF, Garraway LA. Applications of genomics in melanoma oncogene discovery. *Hematol Oncol Clin North Am*. 2009;23:397-414.
39. Ronaghi M, Uhlén M, Nyrén P. A sequencing method based on real-time pyrophosphate. *Science*. 1998;281:363-365.
40. Ahmadian A, Ehn M, Hober S. Pyrosequencing: history, biochemistry and future. *Clin Chim Acta*. 2006;363:83-94.
41. Marsh S. Pyrosequencing applications. *Methods Mol Biol*. 2007;373:15-24.
42. Ronaghi M, Shokralla S, Gharizadeh B. Pyrosequencing for discovery and analysis of DNA sequence variations. *Pharmacogenomics*. 2007;8:1437-1441.
43. Gut IG. DNA analysis by MALDI-TOF mass spectrometry. *Hum Mutat*. 2004;23:437-441.
44. Edwards JR, Ruparel H, Ju J. Mass-spectrometry DNA sequencing. *Mutat Res*. 2005;573:3-12.
45. Tost J, Gut IG. Genotyping single nucleotide polymorphisms by MALDI mass spectrometry in clinical applications. *Clin Biochem*. 2005;38:335-350.
46. Higuchi R, Fockler C, Dollinger G. Kinetic PCR: real-time monitoring of DNA amplification reactions. *Biotechnology*. 1993;11:1026-1030.
47. Heid CA, Stevens J, Livak KJ, et al. Real time quantitative PCR. *Genome Res*. 1996;6:986-994.
48. Clayton SJ, Scott FM, Walker J, et al. K-*ras* point mutation detection in lung cancer: comparison of two approaches to somatic mutation detection using ARMS allele-specific amplification. *Clin Chem*. 2000;46:1929-1938.
49. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*. 1997;245:154-160.
50. Dabritz J, Hanfler J, Preston R, et al. Detection of Ki-*ras* mutations in tissue and plasma samples of patients with pancreatic cancer using PNA-mediated PCR clamping and hybridisation probes. *Br J Cancer*. 2005;92:405-412.
51. Schuster SC. Next-generation sequencing transforms today's biology. *Nat Methods*. 2008;5:16-18.
52. Voelkerding KV, Dames SA, Durtschi JD. Next-generation sequencing: from basic research to diagnostics. *Clin Chem*. 2009;55:641-658.
53. Meyerson M, Gabriel S, Getz G. Advances in understanding cancer genomes through second-generation sequencing. *Nat Rev Genet*. 2010;11:685-696.
54. Schweiger MR, Kerick M, Timmermann B, et al. The power of NGS technologies to delineate the genome organization in cancer: from mutations to structural variations and epigenetic alterations. *Cancer Metastasis Rev*. 2011;30:199-210. doi:10.1007/s10555-011-9278-z.
55. Metzker ML. Sequencing technologies: the next generation. *Nat Rev Genet*. 2010;11:31-46.
56. Roukos DH. Trastuzumab and beyond: sequencing cancer genomes and predicting molecular networks [published online ahead of print October 26, 2010]. *Pharmacogenomics J*. 2011;11:81-92. doi:10.1038/tpj.2010.81.
57. Church GM. Genomes for all. *Sci Am*. 2006;294:46-54.
58. Mardis ER. Next-generation DNA sequencing methods. *Annu Rev Genomics Hum Genet*. 2008;9:387-402.
59. Erlich Y, Mitra PP, delaBastide M, et al. Alta-Cyclic: a self-optimizing base caller for next-generation sequencing. *Nat Methods*. 2008;5:679-682.
60. Quail MA, Kozarewa I, Smith F, et al. A large genome center's improvements to the Illumina sequencing system. *Nat Methods*. 2008;5:1005-1010.
61. Ding L, Wendl MC, Koboldt DC, et al. Analysis of next-generation genomic data in cancer: accomplishments and challenges. *Hum Mol Genet*. 2010;19:R188-R196.
62. Margulies M, Egholm M, Altman WE, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005;437:376-380.
63. Tawfik DS, Griffiths AD. Man-made cell-like compartments for molecular evolution. *Nat Biotechnol*. 1998;16:652-656.
64. Shendure J, Porreca GJ, Reppas NB, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*. 2005;309:1728-1732.
65. Braslavsky I, Hebert B, Kartalov E, et al. Sequence information can be obtained from single DNA molecules. *Proc Natl Acad Sci U S A*. 2003;100:3960-3964.
66. Rusk N. Torrents of sequence. *Nat Methods*. 2011;8:44. doi:10.1038/nmeth.f.330.
67. Schwartz S, Oren R, Ast G. Detection and removal of biases in the analysis of next-generation sequencing reads. *PLoS One*. 2011;6:e16685. doi:10.1371/journal.pone.0016685.
68. Cantacessi C, Jex AR, Hall RS, et al. A practical, bioinformatic workflow system for large data sets generated by next-generation sequencing. *Nucleic Acids Res*. 2010;38:e171. doi:10.1093/nar/gkq667.
69. Koboldt DC, Ding L, Mardis ER, et al. Challenges of sequencing human genomes. *Brief Bioinform*. 2010;11:484-498.
70. Buehler B, Hogrefe HH, Scott G, et al. Rapid quantification of DNA libraries for next-generation sequencing. *Methods*. 2010;50:S15-S18.
71. Teer JK, Bonnycastle LL, Chines PS, et al. Systematic comparison of three genomic enrichment methods for massively parallel DNA sequencing. *Genome Res*. 2010;20:1420-1431.
72. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*. 2007;25:118-145.
73. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol*. 2002;161:1961-1971.
74. Hewitt SM, Lewis FA, Cao Y, et al. Tissue handling and specimen preparation in surgical pathology. *Arch Pathol Lab Med*. 2008;132:1929-1935.
75. Wang F, Wang L, Briggs C, et al. DNA degradation test predicts success in whole-genome amplification from diverse clinical samples. *J Mol Diagn*. 2007;9:441-451.


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