Environmental and Molecular Mutagenesis 51:851–870 (2010)

Review Article

Selected Technologies for Measuring Acquired Genetic Damage in Humans

Joe Shuga,[1,2] Yong Zeng,[2,3] Richard Novak,[2,4] Richard A. Mathies,[2,3] Pierre Hainaut,[5] and Martyn T. Smith[1,2]*

[1]*School of Public Health, University of California, Berkeley, California 94720*
[2]*Center for Exposure Biology, University of California, Berkeley, California 94720*
[3]*Department of Chemistry, University of California, Berkeley, California 94720*
[4]*UC San Francisco/UC Berkeley Joint Graduate Group in Bioengineering, University of California, Berkeley, California 94720*
[5]*Section of Mechanisms of Carcinogenesis, International Agency for Research on Cancer, Lyon, France*

Technical advances have improved the capacity to detect and quantify genetic variants, providing novel methods for the detection of rare mutations and for better understanding the underlying environmental factors and biological mechanisms contributing to mutagenesis. The polymerase chain reaction (PCR) has revolutionized genetic testing and remains central to many of these new techniques for mutation detection. Millions of genetic variations have been discovered across the genome. These variations include germline mutations and polymorphisms, which are inherited in a Mendelian manner and present in all cells, as well as acquired, somatic mutations that differ widely by type and size [from single-base mutations to whole chromosome rearrangements, and including submicroscopic copy number variations (CNVs)]. This review focuses on current methods for assessing acquired somatic mutations in the genome, and it examines their application in molecular epidemiology and sensitive detection and analysis of disease. Although older technologies have been exploited for detecting acquired mutations in cancer and other disease, the high-throughput and high-sensitivity offered by next-generation sequencing (NGS) systems are transforming the discovery of disease-associated acquired mutations by enabling comparative whole-genome sequencing of diseased and healthy tissues from the same individual. Emerging microfluidic technologies are beginning to facilitate single-cell genetic analysis of target variable regions for investigating cell heterogeneity within tumors as well as preclinical detection of disease. The technologies discussed in this review will significantly expand our knowledge of acquired genetic mutations and causative mechanisms. Environ. Mol. Mutagen. 51:851–870, 2010.

Key words: molecular epidemiology; microfluidics; digital PCR; BEAMing; single-cell genetic analysis

INTRODUCTION

Intense study of the human genome has been underway for decades in an attempt to uncover genetic factors that contribute to common human disease; however, the evolving human genome contains much more variation than first anticipated, and researchers are still discovering new variants. Approximately 1.4 million single nucleotide polymorphisms (SNPs) were identified in the draft sequence of the Human Genome Project, and, in the past decade, the International HapMap Project and other efforts have increased the number of entries in NCBIs dbSNP database to more than 10 million [Lander et al., 2001; Venter et al., 2001; Frazer et al., 2007]. As both haploid and diploid versions of individual genomes are finished, we now know that the draft reports of the human

Grant sponsor: Trans-NIH Genes, Environment and Health Initiative, Biological Response Indicators of Environmental Systems Center; Grant Number U54 ES016115-01; Grant sponsor: Superfund Basic Research Program NIEHS; Grant Number P42 ES004705; Grant sponsor: Canary Foundation and ACS Early Detection Postdoctoral Fellowship; Grant sponsor: NSF Graduate Research Fellowship to RN.

*Correspondence to: Martyn T. Smith, Center for Exposure Biology, University of California, Berkeley, CA, 94720.
E-mail: martynts@berkeley.edu

Received 2 February 2010; provisionally accepted 2 August 2010; and in final form 3 August 2010

DOI 10.1002/em.20630

Published online 24 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

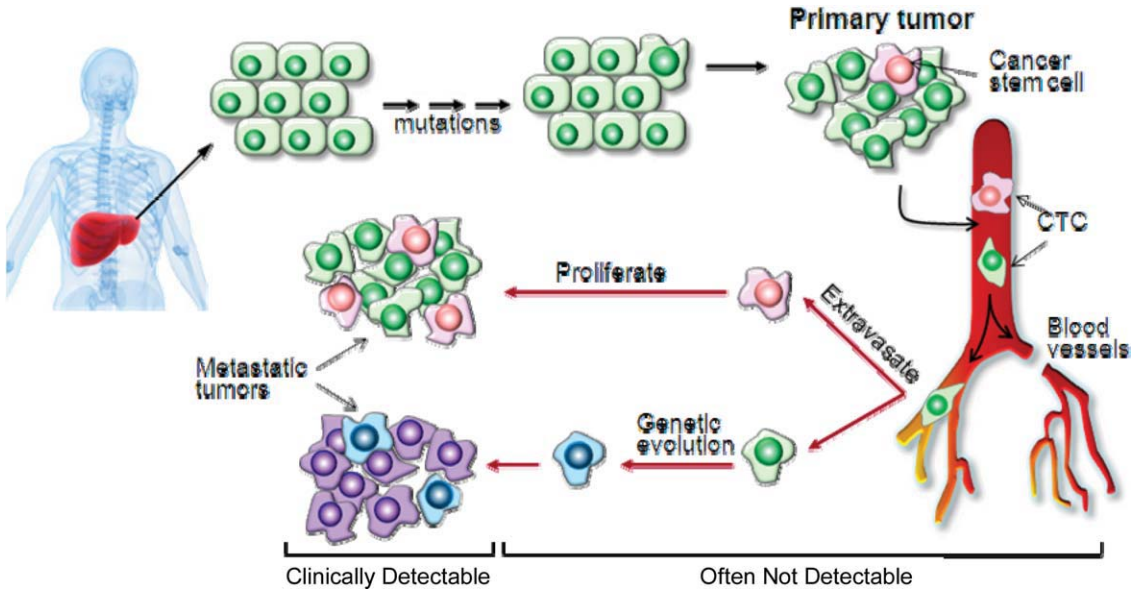

Fig. 1. Carcinogenic somatic mutations occur at the single-cell level and then persist in tumor clones that differ genetically from the germline sequence and sometimes from each other. DNA, along with other cellular components, is under constant attack by reactive metabolites generated by physiological processes as well as by chemical, physical, or infectious agents present in the human environment. It is estimated that each individual human cell can undergo damage to its DNA at a rate of up to 10^6 molecular lesions per day [Lodish, 2003]. DNA repair systems (primarily protein networks) detect and compensate for this extensive damage, and failure of these repair networks to detect, process, or repair DNA damage correctly leads to mutations. Here, a group of normal cells (green cells with regular morphology) gradually acquire mutations before one (top-right) undergoes carcinogenic transformation (becoming a green cell with irregular morphology). This cell gives rise to a primary tumor clone composed of cells that share its acquired mutation pattern, and part of the tumor undergoes metastatic transformation to become a cancer stem cell (pink) that is capable of generating secondary tumors after entering circulation. Both the cancer stem cells and other parts of the primary tumor can enter the circulation and become circulating tumor cells (CTCs), and these CTCs might be detectable in whole blood samples, but will be present only at very low levels. Eventually, secondary tumors are formed, and if other parts of the primary tumor cells undergo separate transformation and become metastatic through distinct mutations (generating the blue cell), they too may go on to form secondary tumors that will differ in their acquired mutational spectrum from the original cancer stem cell (pink). Eventually, the cancer presents clinically and the tumors may be biopsied and analyzed to determine the pattern of mutation that is present; however, these changes are often undetectable early in carcinogenesis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

genome greatly underestimated the number of SNPs in the human population, and we have learned that non-SNP changes, such as block substitutions and indels, can account for ~20% of the variants in individuals [Levy et al., 2007; Ng et al., 2008; Wang et al., 2008; Wheeler et al., 2008; McKernan et al., 2009; Pushkarev et al., 2009; Venter, 2010].

Laboratories that study the genetic basis for human disease are now equipped with the consensus human genome, knowledge regarding millions of common variants in the human population, and a variety of computational and analytical postgenomic tools; however, these tools do not always allow extremely rare genetic variation to be identified in a population or detected in an individual. Elusive mutations that contribute to common human disease can be rare in two senses: (1) the variant can be rare within the human population, so that the variant might not be discovered even after the genome of 10s or even 100s of individuals have been sequenced and (2) the variant can be rare in an individual, because it results from an acquired somatic mutation. If a variant is rare in either sense, it will evade detection in many typical genotyping assays. For example, a feature for the variant will not be present on a DNA microarray if it has not been discovered among the general human population. Similarly, without prior knowledge of the variant, specific polymerase chain reaction (PCR) assays cannot be designed for its detection. Even if the variant is known, it might evade detection in genotyping assays if the variant arose as an acquired somatic mutation in an individual, because the clone harboring the variant will be masked by the pool of "normal" tissue that surrounds it in any routine biological specimen.

Most cancers are the result of acquired mutations in somatic cells that transform them into cells with unregulated growth. The acquired mutations are transmitted to all cells descended from the original cell that underwent the mutation, giving rise to a clone of cells carrying the mutation as a marker and possibly as a phenotypic trait (Fig. 1). This is particularly spectacular in the case of cancer, which often results from the proliferation of a single or a small number of clone(s) having acquired a selective growth advantage as the result of mutation. Cancer involves extensive modifications of the cell genome through multiple steps of somatic mutation [Vogelstein and Kinzler, 1993; Lengauer et al., 1998;

Greenman et al., 2007; Yeang et al., 2008]. These acquired mutations only exist in a subset of cells, and they cannot be detected by analyzing the germline sequence of individuals, but instead must be detected at the level of single cells or clones. Discovering "driver" mutations that lead to carcinogenesis in a rare subset of cells is one key approach to the early detection and treatment of cancer [Carter et al., 2009]. However, the detection of clonally expanding "driver" mutations in tumors is further complicated by recent reports (and reinterpretations of old data) that suggest a polyclonal origin for many tumors in a variety of tissues (Parsons, 2008). The technologies discussed in this review can generally be used to detect acquired mutations of both polyclonal and monoclonal origin, as long as the overall mutation frequency in a specimen is above the limit of detection for a given methodology. However, the identification of key "driver" mutations within a polyclonal tumor is a complex task, since there may be a different "driver" or "drivers" for each clone. Furthermore, even after a "driver" mutation is identified, the mutation will only be present in a fraction of polyclonal tumor tissue and will thus have a lower overall frequency in a specimen.

This review focuses on some of the established technologies for detecting and quantifying rare somatic mutations in human samples before turning to the application of next-generation sequencing (NGS) and digital PCR in microdroplets to discover and detect rare genetic markers that are associated with disease at the level of single cells or clones. The use of NGS to completely sequence the diploid genome in normal and diseased tissue from the same individual is particularly powerful, as this approach facilitates the discovery of new variants among the population at large while also identifying key mutations that have occurred on the pathway to a particular disease. To constrain the review to a reasonable scope, we will not review decade-old approaches in any detail, although many of these approaches are still applied to achieve highly sensitive detection of somatic mutations. Furthermore, we will constrain our focus to studies that analyze genomic DNA (gDNA) directly for acquired mutations and have thus excluded all discussion of epigenetic analyses, mitochondrial DNA analyses, and transcript-based methodologies, although many of the methods discussed here can easily be adapted to study these analytes.

ESTABLISHED METHODS

Many well-established methods are available for detecting somatic mutations. These methods differ in their technical approach, sensitivity, scope (one or multiple genes), and resolution within the DNA sequence. Methods that attempt to detect or quantify rare somatic mutations rely on physical separation or enrichment of mutant copies to increase their effective concentration relative to the background of germline DNA. This enrichment for mutant DNA can be achieved at the level of tissue or cells with laser capture microdissection and cell sorting, or it can occur at the molecular level through allele-specific primer design and other strategies that bias PCR amplification in favor of mutant alleles. In this section, we review a selection of current technologies that are suitable for the detection of low levels of point mutations in a large background of wild-type DNA. Many of these highly sensitive methods have the same limitation: they require prior knowledge of the exact position and type of the mutation and are therefore limited to the detection of known variants. In this section, we review the following: (1) traditional methods with high sensitivity; (2) detection of translocations by PCR and fluorescence in situ hybridization (FISH); (3) allele-specific PCR (AS-PCR) and related techniques; (4) DNA microarrays as cytogenetic tools; (5) arrayed primer extension (APEX); and (6) short oligonucleotide mass analysis (SOMA) and related techniques.

Traditional Methods with High Sensitivity

Mutant-enriched PCR (ME-PCR) is the most widely used procedure for genotypic selection of mutant DNA. It is based on enzymatic digestion of wild-type gDNA at known mutation sites. This method selectively cleaves wild-type sequences, thus providing enrichment in mutant sequences. Two versions of this type of assay have been commonly used. Restriction site mutation is based on digestion before PCR amplification [Jenkins et al., 2003], and restriction fragment length polymorphism (RFLP) is based on restriction digestion after PCR [Kirk et al., 2000]. Comprehensive review of these techniques is beyond the scope of this manuscript, but excellent reviews are available in the literature [Pourzand and Cerutti, 1993; Jenkins, 2004]. With both these techniques, mutations are confirmed by sequencing of digestion-resistant PCR products. Recently, a modified version of this assay has been developed to detect mutations at DNA positions that do not fall within restriction sites. This assay uses two consecutive rounds of PCR to introduce a synthetic restriction site in the wild-type allele, thus generating a PCR product amenable to restriction. Variations of this method have recently been applied to detect infrequent ($<0.1\%$) KRAS mutations both in circulating free DNA present in the plasma of healthy subjects before diagnosis of cancer (Fig. 2A) and in tumors and serum from nonsmall cell lung cancer cases [Gormally et al., 2006; Cortot et al., 2010; Wu et al., 2010].

Using a PCR-RFLP-based methodology, Harris and collaborators adapted an assay initially developed by Cerrutti and collaborators [Chiocca et al., 1992] to measure TP53 mutations in noncancerous liver, colon, and lung [Hussain et al., 2000a,b, 2001]. This highly sensitive method is based on the cloning of PCR products of the mutant allele into phage lambda followed by plaque assay and oligonucleotide hybridization to quantify mutant PCR products. Its sensitivity is an impressive one mutant DNA copy in about 10^7

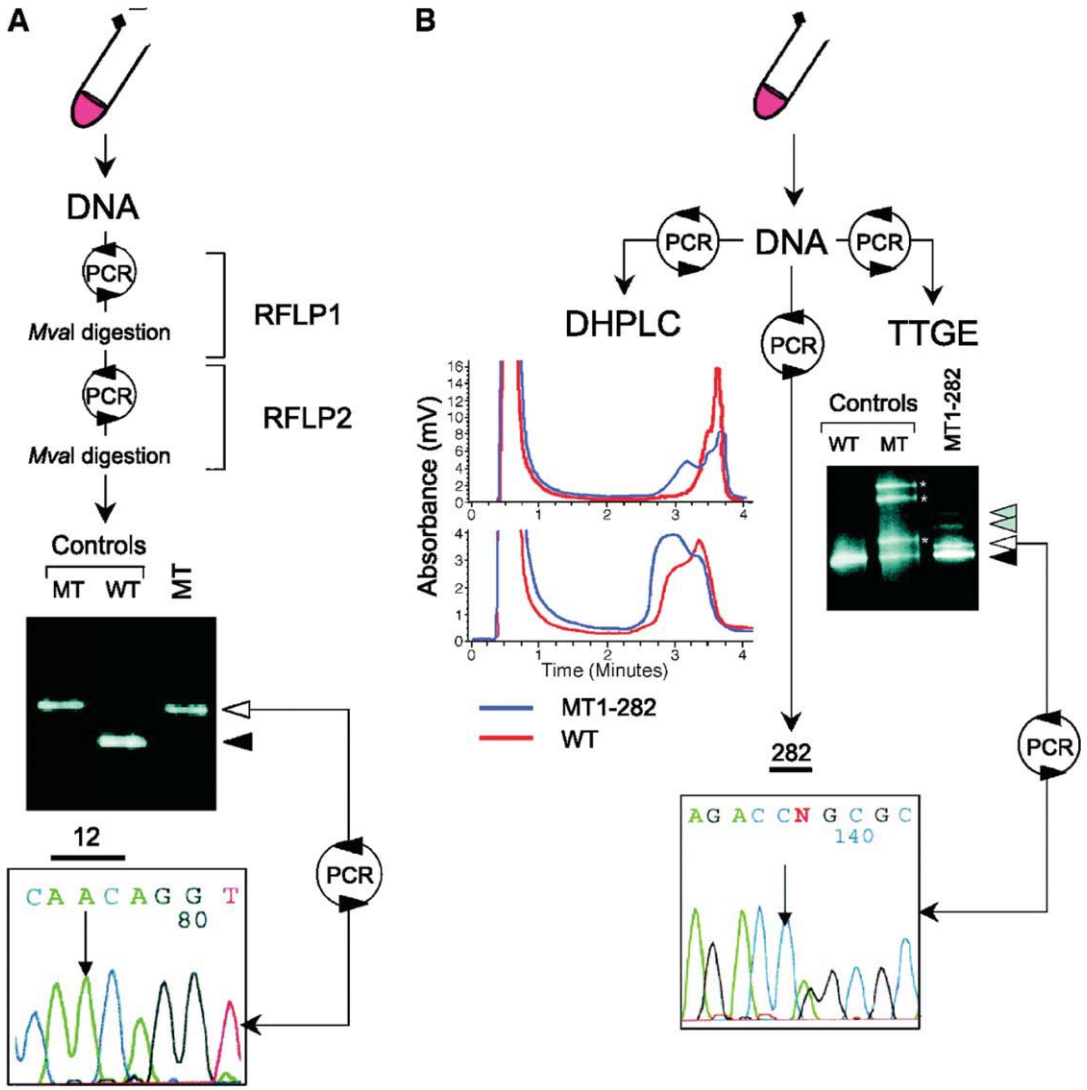

Fig. 2. Detection of low levels of KRAS (codon 12) mutation or of TP53 mutation in circulating free plasma DNA (cfDNA) of healthy subjects. In cancer patients, it is known that mutant DNA is released by cancer cells. The significance of plasma DNA mutations for subsequent cancer development in healthy subjects was assessed in a large longitudinal prospective study by Gormally and collaborators. **A**: KRAS2 mutations were detected by ME-PCR and sequencing (n = 1,098), and (**B**) TP53 mutations were detected by DHPLC, TTGE, and sequencing (n = 550). Specifically, detection of mutations in codon 12 of KRAS2 was achieved by two consecutive PCR-RFLP analyses, followed by sequencing of the mutant PCR product (*white arrow*). The DNA-binding domain of TP53 was analyzed by DHPLC, and samples with abnormal chromatograms were sequenced from an independent PCR product. If the mutation was not detected by sequencing, a new PCR product was analyzed by TTGE. Homoduplex products were excised from the TTGE gel, reamplified, and sequenced (*gray arrow*, mutant-wild-type heteroduplexes; *white arrow*, mutant homoduplexes; *black arrow*, wild-type homoduplexes; *white star*, mutant control heteroduplexes [*top two bands*] and homoduplexes). This is the first report of TP53 or KRAS2 mutations in the plasma of healthy subjects in a prospective study, suggesting that KRAS2 mutation is detectable ahead of bladder cancer diagnosis. Because other studies have shown that TP53 mutation may be associated with environmental exposures, these observations have implications for monitoring early steps of carcinogenesis. Figure from Gormally et al. [2006]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells. Results have demonstrated increased mutation loads in liver of patients with Wilson disease and in colon of patients with ulcerative colitis (two oxyradical overload cancer precursor diseases) [Hussain et al., 2000a,b] and in the normal lung of heavy smokers without clinical evidence of cancer [Hussain et al., 2001; Langerod et al., 2007]. However, these methods are labor-intensive and expensive, limiting their application in molecular epidemiology.

Other common techniques include single-strand conformation polymorphism (SSCP), denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis, or related methods [Welsh et al., 1997; Borresen-Dale et al., 2001; Bazan et al., 2005; Keohavong et al., 2005; Yamanoshita et al., 2005; Janne et al., 2006; Olivier et al., 2006; Young et al., 2008; Szymanska et al., 2010]. Several protocols are

available to retrieve and reamplify mutant DNA after prescreening, either by excision of shifted bands detected by temporal temperature gradient electrophoresis (TTGE) or SSCP or by collection of shifted peaks in DHPLC [Bazan et al., 2005; Langerod et al., 2007]. This reamplified, mutant-enriched material can then be analyzed by direct sequencing. In one recent study, DHPLC and TTGE were applied to detect mutant TP53 in the plasma of healthy subjects (Fig. 2B) [Gormally et al., 2006].

Finally, in unique situations where mutant cells can be identified by immunohistochemistry of morphology, microdissection can be used to analyze single cells or clones for mutations. In normal skin, the epithelium is made of juxtaposed patches of cells originating from single progenitors. Because many missense TP53 mutations induce protein stabilization, immunohistochemistry can be used to detect patches of cells with TP53 accumulation, which are then microdissected and analyzed by PCR/sequencing at the level of single cells or clones [Williams et al., 1998; Ling et al., 2001].

Detection of Translocations by PCR and FISH

Translocations were the first class of mutations definitively linked to cancers, and some of the earliest cancer screens were based on the cytogenetic detection of translocations. The association between the Philadelphia chromosome, which is produced by a translocation between chromosomes 9 and 22 [t(9;22)], and chronic myeloid leukemia (CML) was first reported in 1960, and the association between t(8;21) and acute myeloid leukemia (AML) was reported not long after [Rowley, 1973; Rowley and Potter, 1976; Nowell, 2007]. Several hematological malignancies have a remarkably strong association with translocations; for example, t(14;18) is detectable in ~90% of follicular lymphoma cases, and t(9;22) occurs in virtually all CML patients [Bende et al., 2007; Vardiman, 2009]. It has now become evident that chromosomal translocations are associated with several other blood cancers and that they also play a role in prostate, breast, and lung cancers among others [Nambiar et al., 2008]. Furthermore, t(9;22) and t(14;18) are detectable in healthy individuals, making these mutations promising biomarkers of early effect [Liu et al., 1994; Biernaux et al., 1995; Fuscoe et al., 1996; Schmitt et al., 2006; Nambiar and Raghavan, in press; Schuler et al., 2009; Bayraktar and Goodman, 2010]. Both these translocations have been found to increase with age, and the frequency of t(14;18) was increased in cigarette smokers [Bell et al., 1995]. More recent studies report an association between pesticide exposure and both $t(14;18)^{+}$ and $t(14;18)^{-}$ non-Hodgkin lymphoma [Roulland et al., 2004; Chiu et al., 2006; Agopian et al., 2009].

Highly sensitive and specific PCR assays to detect translocations in gDNA can be easily designed if the chromosomal breakpoints are well-defined by designing forward and reverse primers that target different chromosomes. The use of a Taqman probe between the primers enhances the specificity of the assay and also provides a quantitative readout in real-time PCR (RT-PCR). Nested primers and a second round of PCR can boost the sensitivity of a translocation assay dramatically, and quantitative detection has been achieved at frequencies as low as 10^{-7} for some mutations [Roulland et al., 2006; Agopian et al., 2009; Nambiar and Raghavan, in press]. Accordingly, PCR testing for these mutations has become common practice both when first diagnosing many hematologic malignancies and when monitoring for minimal residual disease [Bonassi et al., 1995; Schuler and Dolken, 2006; Jolkowska et al., 2007; Gu et al., 2008].

However, even if the breakpoint locations are well known, as is the case with t(14;18), many reports maintain that FISH is superior to PCR in sensitivity and comparable to PCR in specificity [Einerson et al., 2005; Belaud-Rotureau et al., 2007; Gu et al., 2008]. FISH-based detection of translocations relies on fluorescent DNA probes that bind specifically to selected regions on one or both of the involved chromosomes. Probe sets may be designed in a variety of manners, including break-apart, single-fusion, and dual-fusion designs. Dual-color, dual-fusion FISH uses two different colored DNA probes, one of which targets one chromosome, whereas the other targets its translocation partner. The dual-color, dual-fusion technique yields one normal signal for each chromosome involved as well as a two fusion signals (one from each of the derivative chromosomes) when the targeted translocation is present. Multicolor karyotyping and spectral karyotyping have expanded the capacity of FISH techniques, facilitating broad cytogenetic analysis without the need for specifically designed probes [Schrock et al., 1996; Speicher et al., 1996; Bayani and Squire, 2004]. Recently, a microfluidic device was described for FISH analyses that can reduce reagent costs by 20-fold and technician labor by 10-fold [Sieben et al., 2007, 2008]. A comprehensive review of FISH-based techniques for assessing acquired translocations and other cytogenetic abnormalities is a challenging task and is beyond the scope of this review; instead, we refer the interested reader to several recent reviews available in the literature [Najfeld, 2003; Ventura et al., 2006; Sreekantaiah, 2007; Halling and Kipp, 2007].

Allele-Specific PCR

Allele-specific PCR (AS-PCR) is based on the use of mutant-specific primers (MSPs) primers that preferentially anneal with mutant sequence. The PCR products are then analyzed using conventional methods (e.g., SSCP plus sequencing). AS-PCR analysis of TP53 mutations resulted in the detection of mutated cells accounting for 0.01–1% of cells, providing enough sensitivity to detect rare TP53 mutations as early biomarkers of relapse in AML and acute

lymphocytic leukemia [Wada et al., 1994; Zhu et al., 1999]. AS-PCR may be combined with Taqman probes to detect rare mutations in a quantitative manner. Behn and Schuermann [1998] developed a variant of AS-PCR to target mutational hotspots in the TP53 gene. This method combines PCR-SSCP with sequence specific-clamping by peptide nucleic acids (PNAs). PNAs are designed to preferentially bind to wild-type DNA, and not extend, thereby blocking amplification of wild-type DNA to yield a mutant-enriched sample. A recent study combined PNA-clamping with asymmetric primers and melting curve analysis using an unlabeled detection probe to develop a simple and economical assay for the detection of mutations in codon 12 of KRAS [Oh et al., in press]. These authors were able to detect 0.1% mutant DNA in colon cancer tissue. Another recent variation on AS-PCR involved shortening the MSP at its 5′-end to reduce its T_m and then use a blocking oligonucleotide, complementary to the wild-type sequence but phosphorylated at the 3′-end to prevent extension to suppress nonspecific amplification of the wild-type allele by the MSP and achieve detection at mutant frequencies $<0.1\%$ [Morlan et al., 2009]. In another recent variation on this theme, aberrant plasma cells were first enriched using fluorescence-activated cell sorting in cases of hematologic malignancies, and then wild-type primer extension was suppressed using primers that were modified at the 3′ position with a C7 amino linker to block polymerase elongation [Rasmussen et al., 2005; Rasmussen et al., in press]. Finally, bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification assays, which use two opposing 3′-terminal-blocked oligonucleotides, have recently been developed for the human TP53 gene [Shi et al., 2007]. These assays are extremely sensitive and specific, allowing for single mutant molecule detection in a large background ($>3 \times 10^9$ copies) of wild-type DNA [Liu and Sommer, 2004].

DNA Microarrays as Cytogenetic Tools

Single nucleotide polymorphism arrays (SNP-A) and comparative genomic hybridization arrays (CGH-A) have been adapted for karyotypic analysis, and these technologies enable precise scanning of the genome for copy number variations (CNVs) with a sensitivity between 2 and 30% [Maciejewski et al., 2009]. Although these array-based methods are less sensitive than FISH, their resolution is much higher, and they do not rely on cell division. Furthermore, SNP-A can be used to detect copy number neutral loss of heterozygosity (CN-LOH), which is not detected using conventional metaphase cytogenetics. CGH-A measures the difference between test and control DNA, and the use of paired germline and tumor DNAs from the same individual allows for definitive identification of somatic lesions. Similarly, SNP-A analysis can be rerun on paired, unaffected tissue (such as $CD3^+$ cells in cases of myeloid malignancy) to confirm the somatic origin of CNVs [Huh et al., 2010; Jasek et al., 2010]. Unlike conventional cytogenetics, these techniques rely on completely automated data analysis and generate results that can be objectively and systematically analyzed using computational algorithms [Price et al., 2005; Nannya et al., 2005; Shah et al., 2006; Yamamoto et al., 2007]. A comprehensive review of these technologies is beyond the scope of this review, but excellent reviews on both the technology and its application are available in the literature [Carter, 2007; Maciejewski and Mufti, 2008; Maciejewski et al., 2009; Brenner and Rosenberg, in press].

Genomic CNVs have long been associated with specific chromosomal rearrangements and genomic disorders, and CGH-A and SNP-A have recently been applied to study human disease [McCarroll and Altshuler, 2007]. Many recent studies have used array-based methods to detect CN-LOH (also known as uniparental disomy) of the NF1 allele, which is strongly associated with neurofibromatosis type I and juvenile myelomonocytic leukemia [Flotho et al., 2007; Pasmant et al., 2009; Steinemann et al., 2010]. CGH-A and SNP-A methods have also been applied recently to detect somatic mutation of TP53, JAK2 (V617F), BRCA1, and BRCA2 in both tumor and peripheral blood in cases of hematalogic malignancy and ovarian cancer [Gondek et al., 2007; Dunbar et al., 2008; Walsh et al., 2008; Bea et al., 2009].

Arrayed Primer Extension

Arrayed primer extension is a genotyping and resequencing technology that allows the scanning of mutations over large regions of DNA and that can detect "unknown" mutations within a given sequence [Kurg et al., 2000; Tonisson et al., 2002; Le Calvez et al., 2005]. It combines the advantages of Sanger dideoxy sequencing with the high-throughput potential of the microarray format (Fig. 3). A DNA sample is amplified, fragmented enzymatically, and annealed to arrayed 25-mer oligonuclotides that cover the sequence of interest. Each oligonucleotide hybridizes one base downstream of the preceding one, with their 3′ ends one base upstream of the base to be identified. Once hybridized, they serve as primers for template-dependent DNA polymerase extension reactions by using four fluorescently labeled dideoxynucleotides. Each base is probed with two primers, one for the sense and another for the antisense strand. Image analysis and interpretation of fluorescence signals at each position then provide a sequence read. Recently, this concept has been extended to a second generation APEX device that allows multiplex (640-plex) DNA amplification and detection of SNPs and mutations [Krjutskov et al., 2008]. This robust genetic test has minimal requirements per variant target: two primers, two spots on the microarray, and a low-cost four-color detection system for each targeted base pair (Fig. 3).

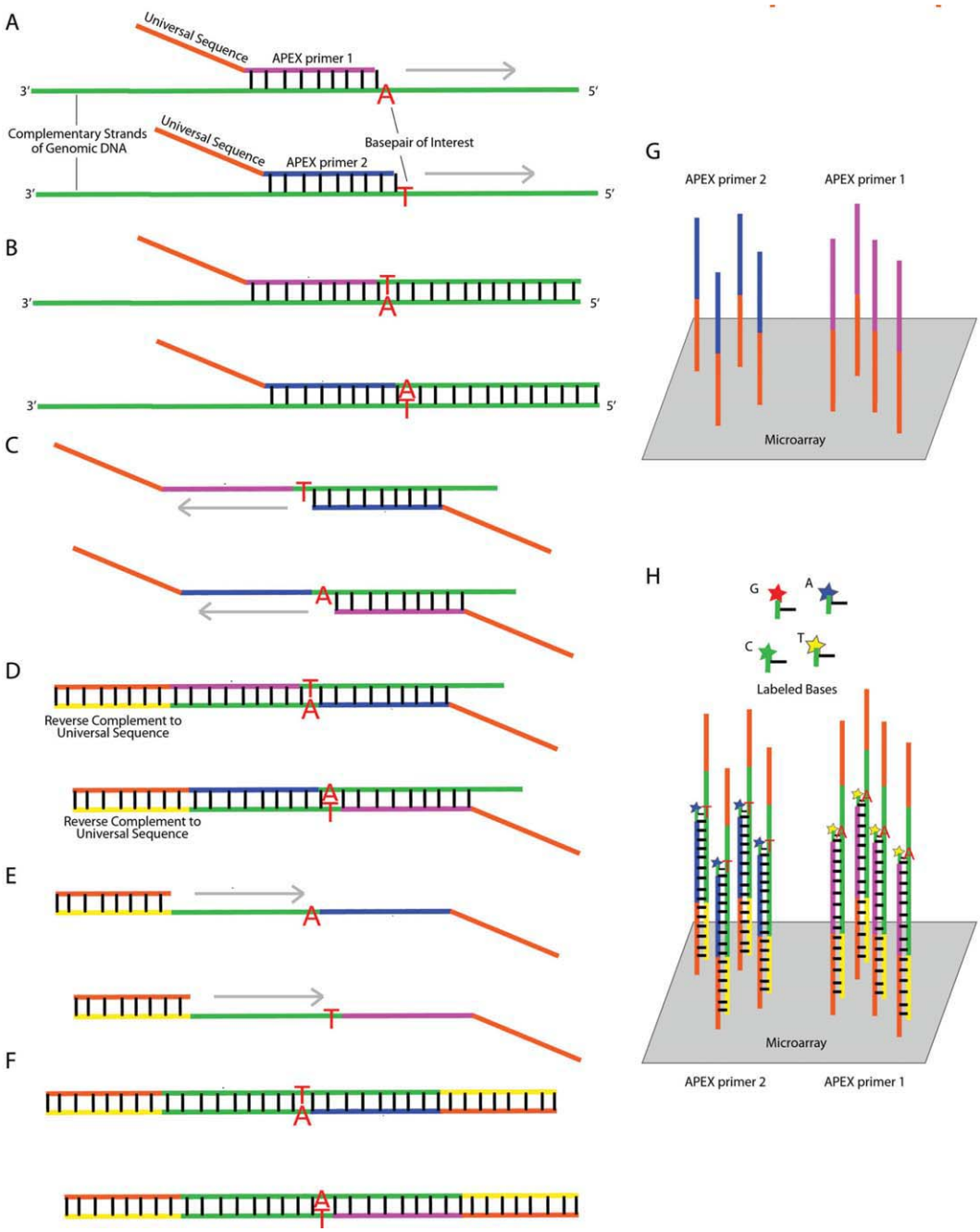

Fig. 3. The APEX-2 Method for a Basepair of Interest. **A**: The first phase of PCR is driven by APEX-2 primers, which contain both context-specific sequence (purple and blue) and universal sequence (red); the specific portion of an APEX-2 primer pair bind gDNA to frame a basepair of interest. **B**: Polymerase then extends the APEX-2 primers, using the gDNA as template, to form products that contain the nucleotide of interest as well as a complement for the alternate APEX-2 primer in the pair. **C**: As the APEX-2 primer-driven phase of PCR continues, the products formed in part B serve as template, and the complementary specific portions of the APEX-2 primers once again anneal and extend via polymerase activity. **D**: Note that the reverse complement to the universal sequence is found in the PCR products formed after this second APEX-2 primer-driven extension. **E**: Universal primers now bind template sequences and ensure balanced amplification for all base pairs to be genotyped. **F**: The products formed by Universal primer-driven amplification. **G**: APEX-2 primers with a 5′-amino modification are immobilized on a microarray to facilitate detection. **H**: The purified products from Universal primer amplification are hybridized to the immobilized APEX-2 primers; genotyping is then performed via four-color single-base extension reaction and analysis of fluorescence data at each position of the array.

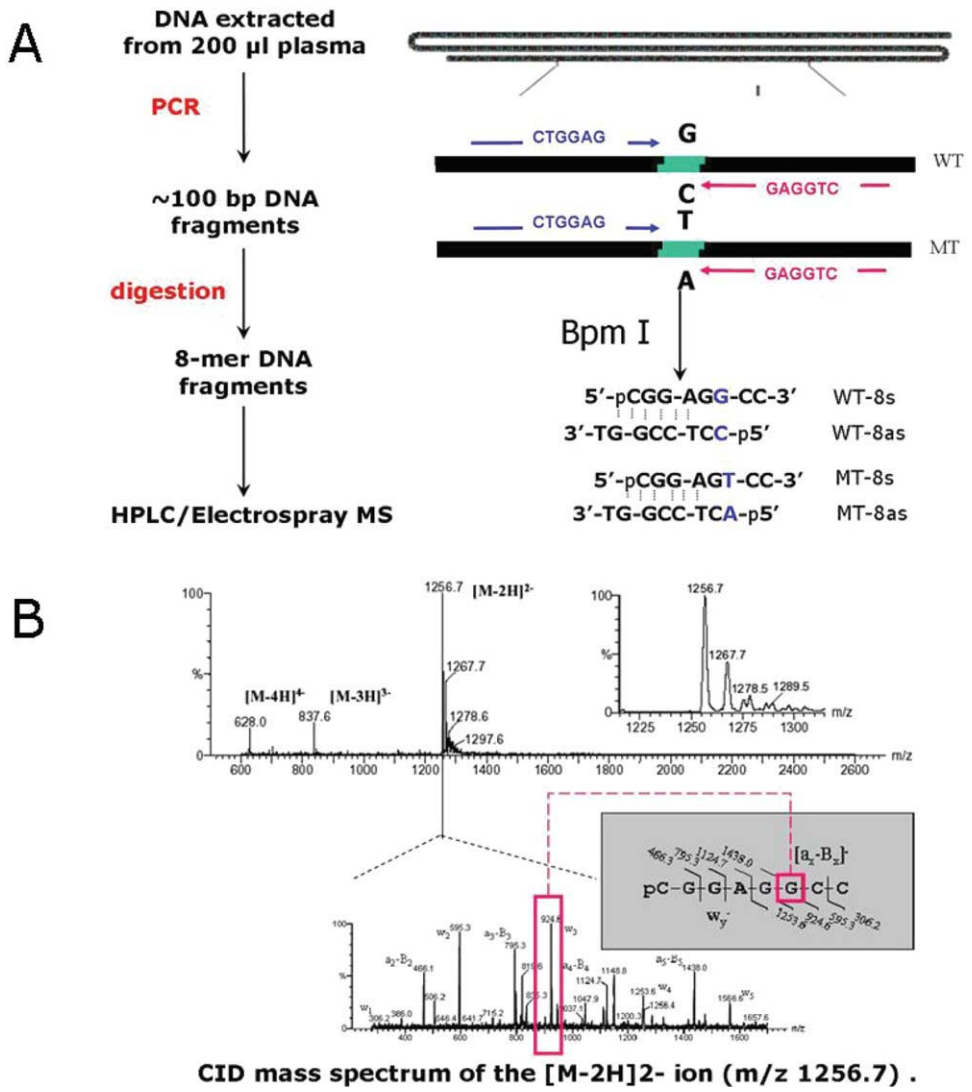

Fig. 4. Short oligonucleotide mass analysis (SOMA) of TP53 R249S mutations. **A**: Principle of SOMA. DNA is amplified by PCR using primers that introduce a site for BpmI, a restriction enzyme that cleaves DNA away from its recognition site. Short oligonucleotides (8-mers) are generated by digestion, purified by HPLC and analyzed by electrospray mass spectrometry. **B**: Mass spectrum of the sense strand of the wild-type 8-mer (top spectrum) and of its breakdown products (bottom spectrum). Inset: expected mass of breakdown products. Presence of a specific species (framed) identifies the wild-type sequence (with G at third position of codon 249). Figure adapted from Laken et al. [1998] and Lleonart et al. [2005]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

APEX has been adapted for the detection of TP53 mutations in DNA isolated from plasma or from solid tumors, with a sensitivity of 0.1–5%, depending upon the sequence context and the nature of the mutation [Tonisson et al., 2002; Le Calvez et al., 2005]. The method has been applied to ovarian and breast cancer samples and dilutions of cell human cancer cell lines and proved to be comparable in sensitivity to DHPLC and TTGE at some variant sites in the TP53 gene [Kringen et al., 2005; Le Calvez et al., 2005]. These results suggest that the platform is a robust, high-throughput, and comprehensive tool for genetic analysis. However, this array platform is relatively new and has only recently been commercialized; as such, it is currently unknown whether this method will prove robust enough for large scale studies using nondiseased tissues or surrogate samples.

Short-Oligonucleotide Mass Analysis and Related Techniques

Short-oligonucleotide mass analysis (SOMA) is a technique developed by Groopman and collaborators in which small sequences of mutated and wild-type DNA, produced by PCR amplification and restriction digestion, are characterized by HPLC-electrospray ionization tandem mass spectrometry (ESI-MS/MS) [Laken et al., 1998; Jackson et al., 2001; Qian et al., 2002]. DNA is amplified using primers that introduce restriction sites for enzymes, like BpmI,

TABLE I. Resequencing Tumors with NGS

Reference	Diseased tissue	Control tissue
Mardis et al. (2009)	AML-M1 (bone marrow)	Skin
Ding et al. (2010)	Breast, brain metastasis, and tumor xenograft	Peripheral blood
Ley et al. (2008)	AML-M1	Skin
Ding et al. (2008)	Lung adenocarcinoma (623 genes only)	Normal lung or skin
Castle et al. (2010)	UMC-11 adenocarcinoid-derived cell line	Pooled male and female DNA
Pleasance et al. (2010b)	NCI-H209 small-cell lung cancer bone marrow metastasis cell line	NCI-BL209 lymphoblastoid cell line (EBV transformed)
Pleasance et al. (2010a)	COLO-829 malignant melanoma cell line	COLO-BL829 EBV transformed lymphoblastoid cell line
Stephens et al. (2009)	Twent-four different breast cancer cell lines	NCBI genome assembly
Lee et al. (2010)	Lung adenocarcinoma	Lung
Beck et al. (2010)	Plasma circulating DNA from ductal breast carcinoma	Healthy volunteer circulating DNA

which cleave DNA several base pairs away from the restriction enzyme-binding site. Short DNA fragments spanning the mutation site (7–15 base pair oligomers) are then produced by restriction digestion and separated by HPLC before ESI-MS/MS. The first MS analysis distinguishes the four single-stranded oligonucleotides corresponding to sense and antisense, wild-type, and mutant DNA. The second MS analyzes oligonucleotide fragmentation products and detects mass fragments characterizing the mutated base (Fig. 4). The use of an internal standard plasmid alongside test DNA allows the precise quantification of mutant and wild-type sequences, which can be expressed in absolute copy numbers.

This method has been applied to the detection of KRAS and TP53 mutations in the plasma DNA and tissues of healthy subjects and cancer patients [Szymanska et al., 2004, 2009; Lleonart et al., 2005]. Quantification of mutant circulating free DNA by SOMA in a case–control study on liver cancer in The Gambia (West Africa) has shown that TP53 gene serine 249 mutation median levels were higher in hepatocellular carcinoma cases (2.8×10^3 copies/mL plasma, range: $5 \times 10^2 - 1.1 \times 10^4$) compared to median levels in cirrhotic patients and healthy controls (5×10^2 copies/mL plasma; range, $5 \times 10^2 - 2.6 \times 10^3$ and 5×10^2 copies/mL plasma; range, $5 \times 10^2 - 2 \times 10^3$, respectively) [Lleonart et al., 2005]. When SOMA was directly compared to RFLP for analysis in liver-derived gDNA, it was found that SOMA was 2.5–15 fold more sensitive for the detection of specific mutations in TP53 [Qian et al., 2002]. This powerful method is rapid and amenable to scaling-up, making it one of the most powerful approaches for mutation detection in a large series of specimens.

The use of mass spectrometry for mutation detection is not limited to SOMA, and the recent commercialization of the Sequenom MassArray provides another powerful technology for identifying genetic variants. The MassArray system combines proprietary primer extension technology with matrix-assisted laser desorption/ionization-time of flight MS analysis for flexible high-throughput detection of somatic mutations. This platform was recently applied to conduct high-throughput genotyping and to test for 238 previously described oncogenic mutations in 1,000 human tumor samples [Thomas et al., 2007]. The authors of this study found robust mutation distributions spanning 17 cancer types. Furthermore, they found 14 of 17 oncogenes analyzed to be mutated at least once, and that 298 of 1,000 samples carried at least one mutation. In another recent study, the OncoCarta platform from Sequenom was used to profile 239 colon cancers and 39 metastatic lymph nodes [Fumagalli et al., 2010]. The authors interrogated 238 hotspot mutations in 19 genes and found mutations in seven different genes at 26 different nucleotide positions in colon cancer samples. To validate the analytical platform, the authors conducted a subset of the assays a second time and obtained identical results. Furthermore, the mutation frequencies of the most common colon cancer mutations were similar to the Catalog of Somatic Mutations in Cancer database. Specifically, the frequencies were 43.5% for KRAS, 20.1% for PIK3CA, and 12.1% for BRAF along with infrequent mutations in NRAS, AKT1, ABL1, and MET.

EMERGING METHODS

Given the complexity of biological mechanisms involved in acquired somatic mutations, there has been a push for faster, more sensitive, and high-throughput technologies for mutation analysis. Technology parallelization in the form of next-generation sequencing methods combined with powerful bioinformatics has created an explosion of extremely high-resolution data. Large-scale genome sequencing projects have begun to focus on comparing tumors with normal tissue from the same individuals (Table I). These comparative genome-sequencing studies are currently the most informative in comprehensively revealing true acquired somatic DNA mutations in a cancer by removing germline variation that would otherwise obscure the relatively few acquired mutations. In a genome of 3×10^9 bases, one would expect to find $\sim 3 \times 10^6$ SNP as well as $\sim 1 \times 10^6$ other variations based on comparison to the reference sequence of the genome [Levy et al., 2007].

This is in stark contrast to the 8–500 acquired mutations of potential pathological relevance identified by comparative sequencing studies and subsequent validation [Ding et al., 2008; Ley et al., 2008; Lee et al., 2010]. In addition to a revolution in sequencing hardware, the software and databases required to process the massive data sets and give meaning to the raw sequence information have undergone drastic expansion and many resources now exist for interpreting the role of specific mutation sites [Carter et al., 2009; Barrett, 2010; Robison, in press]. So-called third-generation sequencers are poised to further accelerate the discovery of novel mutations and disease association with their even greater throughput, lower cost, and lower sample quantity requirements.

Although rapid genome sequencing is already greatly increasing the amount of information available about mutations in entire tumors, the fundamental biological mechanisms behind carcinogenesis reside inherently at the level of the single cell. Some of the third-generation sequencing technologies may eventually offer single-cell resolution to some degree, but alternative (primarily microfluidic) platforms exist and may soon be extended to achieve high-throughput analysis of cellular heterogeneity in targeted loci at the digital level [Tewhey et al., 2009]. With standard technologies, the underlying technical barriers to single-cell analysis are primarily: (1) cell/DNA manipulation; (2) analytical reaction rates hindered by extremely low relative analyte concentrations; and (3) the ability to scale analytical throughput to the level required for statistically significant detection of rare mutation events. Microfluidic approaches have been demonstrated to address all points effectively and will be instrumental for single-cell resolution studies of carcinogenesis mechanisms stemming from acquired genetic mutations [El-Ali et al., 2006; Sims and Allbritton, 2007; Schmid et al., 2010]. A combination of next-generation sequencing technology for target mutation discovery and focused single-cell analysis of tumor heterogeneity will likely trigger a revolutionary understanding of the role of acquired genetic mutations in carcinogenesis.

Next-Generation Sequencing

The term "next-generation sequencing" has been applied to all sequencing technologies developed to succeed automated Sanger sequencing, but it is more commonly used for second generation methods that are currently on the market and being applied with increasing frequency in diverse research fields. A third generation of technologies is in varying stages of development and application. There is a vast diversity to next-generation technologies, but these sequencing approaches generally use massively parallel amplification and detection strategies. A sheared DNA sample is amplified evenly through the use of emulsions (Roche 454, Life SOLiD, Polonator G.007) and substrates (Illumina Genome Analyzer) to segregate amplicons from each other. The amplified DNA is then arrayed in the sequencing device to enable parallel optical detection of the fluorescence-based sequencing process. The Helicos Biosciences system is an exception in that it does not call for DNA amplification and instead uses the original single fragments of sample DNA in the sequencing reaction. In all second-generation sequencing approaches, an enzyme such as polymerase or ligase is used to replicate the separated clusters of amplicons and provide nucleotide specificity. As a result of the sequential addition of nucleotides or dinucleotides, a fluorescence signal is generated and recorded at each amplicon location. The large-scale parallelization results in billions of sequence reads that are then computationally assembled. A detailed technical overview of each technology is available in several excellent reviews [Voelkerding et al., 2009; Metzker, 2010].

In all large-scale sequencing efforts, the underlying computational algorithms for base calling, assembly, and analysis of raw sequence reads have undergone parallel innovation. A primary goal of the algorithms is to remove polymerase and other errors, and each sequencing platform contains software to produce relatively clean sequence. Additional accuracy is provided with increased sequence reads, so that a desired level of accuracy can be reached with any sequencing technology with sufficient data [Metzker, 2010]. Notably, a variety of algorithms to detect CNVs have been developed, and these aberrations can be assessed with reads as low as 0.3× of the genome [Alkan et al., 2009; Medvedev et al., 2009; Robison, in press]. Once a clean sequence is produced, the acquired mutations discovered by subtracting out the germline sequence of the same individual still need to be differentiated into "driver" and "passenger" mutations. Only a small subset (~10%) of all acquired mutations are thought to be responsible for cancer progression, and this premise is providing clues to the mechanistic origins of specific cancer types [Carter et al., 2009; Robison, in press]. These "driver" mutations are parsed from the neutral "passenger" mutations by various bioinformatics approaches involving algorithms such as CHASM or the use of databases, followed by experimental validation [Carter et al., 2009; Barrett, 2010; Robison, in press]. However, it is possible that mutations in noncoding stretches of the genome that have been excluded from comparative mutation analyses play at least some role in disease progression, and future work will likely address this point [Calin and Croce, 2009; Garzon et al., 2009].

Although the recent sequencing technology advances offer unprecedented ability to analyze genomes without a priori assumptions, relatively few studies with a goal of identifying acquired genetic mutations have been published to date. The first full comparative genome-sequencing effort by Ley et al. [2008] of cytogenetically normal AML subtype M1 (AML-M1) compared to skin tissue revealed eight genes with novel mutations thought to drive the cancer with another 500–1,000 additional mutations specific to the tumor found across noncoding regions

of the genome. With broader use of next-generation sequencers, other tumor types have been sequenced in a similar manner, including malignant melanoma, small-cell lung cancer bone metastasis, lung adenocarcinoma, and a second AML-M1 (Table I). Recently, Ding et al. [2010] presented the resequencing results of a basal-like breast cancer tumor, a brain metastasis, and a tumor xenograft compared to peripheral blood indicating ~50 point mutation and small indel sites in genes and splice sites in all three tumors not shared with germline sequence, 20 sites enriched in metastasis and xenograft, and 2 de novo mutations in the metastatic tumor [Ding et al., 2010]. An additional 41 large-scale genomic rearrangements or deletions were observed as a result of the chromosomal instability characteristic of this cancer type. The findings suggest that a few cells from the original tumor disseminate throughout the body with only several additional mutations but with large CNVs of existing mutations. The results demonstrate the power of the comparative genome-sequencing approach in parsing the complex mutation landscape present in carcinogenesis. Further applications of next-generation sequencing to the study of mutations in noncoding regions as well as correlation with germline SNPs will drastically enhance our understanding of the biological mechanisms and clinical outcomes associated with acquired genetic mutations.

Third-generation sequencing platforms are beginning to emerge in the marketplace and will offer greater throughput, lower cost, and, in general, will obviate the need for sample amplification. Helicos has already demonstrated whole genome sequencing of an individual as well as in-depth analysis of germline mutations potentially associated with increased risk of sudden cardiac arrest, but the platform has yet to be applied to comparative sequencing of acquired mutations [Pushkarev et al., 2009; Ashley et al., 2010]. Somewhat more distant technologies include the Pacific Biosciences platform that may enable direct epigenetic analysis of the genome in addition to the basic sequence and scanning and nanopore systems that avoid fluorescent chemistries altogether and will bring the cost of comparative genome analysis within reach of most laboratories [Lund and Parviz, 2009; Flusberg et al., 2010].

Digital Genetic Analysis in Emulsions and Microfluidic Devices

To quantitatively measure tumor heterogeneity and detect extremely low-level variants, advanced technologies are required that provide high-throughput, quantitative analysis at the level of single cells or molecules. Digital PCR was first applied to detect low concentrations of mutations in alleles associated with colorectal cancer more than a decade ago through limiting dilution of template in 7-μL reaction volumes in 96-well plates [Vogelstein and Kinzler, 1999]. This powerful technique facilitates the detection of a single copy of target nucleic acid in a complex background. Digital PCR methods now include emulsion PCR techniques, such as BEAMing, which significantly improve the throughput, efficiency, and detection sensitivity by conducting single-molecule amplification in isolated nanoliter or picoliter droplets [Dressman et al., 2003; Kojima et al., 2005; Griffiths and Tawfik, 2006]. These methods have been implemented recently to detect and quantify genetic variation in DNA samples [Dressman et al., 2003; Diehl et al., 2005, 2008; Li et al., 2006] and have been applied to detect extremely rare mutational variants in colorectal tumors and in circulating tumor DNA [Diehl et al., 2005, 2008; Li et al., 2006].

BEAMing is an original method aimed at one-to-one conversion of a population of DNA fragments into a population of beads that can be counted [Dressman et al., 2003; Diehl et al., 2006]. It derives its name from its principal components: beads, emulsion, amplification, and magnetics. First, PCR is used to amplify target DNA using primers that contain a sequence tag. Second, PCR products are mixed with oligonucleotide-labeled beads that anneal with the tag, and this mixture is emulsified to enable the amplification of individual PCR products on individual beads. Third, the DNA immobilized on the beads is denatured, hybridized with primers that anneal just upstream of the mutation site, and then a single nucleotide primer extension reaction is carried out using four fluorescently labeled nucleotide terminators. Flow cytometry is then used to rapidly measure the fluorescence of individual beads. The nature of the base changes is given by the fluorescence of the incorporated nucleotide. Counting fluorescent beads provides a precise estimate of the number of wild-type or mutant DNA copies and allows quantification of mutant and wild-type frequencies even when they are present at ratios less than 10^{-4}. BEAMing has recently been used to quantify mutant APC in the circulating plasma DNA of patients with colorectal cancer [Diehl et al., 2005, 2008]. In plasma from cases with advanced colorectal cancer, the method revealed elevated levels of both total and mutant APC relative to early stage cancer and normal individuals (median 4.78×10^4 copies APC/mL plasma with 8% mutant APC vs. $\sim 4 \times 10^3$ copies APC/mL plasma with 0.01–1.7% mutant APC) [Diehl et al., 2005].

One drawback of the BEAMing method is that mechanical agitation is used to generate highly polydisperse picoliter emulsion droplets, which impose inherent limitations in statistical quantification and PCR amplification efficiency. The implementation of microfluidics for droplet generation overcomes these limitations by enabling production of practically monodisperse droplets with precise control over the droplet size, while maintaining high-throughput [Beer et al., 2007, 2008; Kiss et al., 2008; Tewhey et al., 2009]. Such capability not only enables uniform efficiency of enzymatic reactions in the droplets [Beer et al., 2007], but also permits digital quantification of the absolute number of targets present in the initial sample through Poisson statistical analysis [Kiss et al., 2008; Mazutis et al., 2009]. In addition, inte-

grated microfluidic circuits allow programmable in situ manipulation of droplets, such as droplet steering, trapping, and fusion, leading to a much broader spectrum of applications, such as real-time droplet PCR [Beer et al., 2007].

The term "microfluidics" typically refers to networks of channels with dimensions ranging from 5 to 500 μm that are used to manipulate small volumes ($10^{-6} - 10^{-15}$ L) of fluid in a precisely controlled manner [Weibel and Whitesides, 2006]. These channels are often etched in glass and silicon substrates, but they can also be embossed in the surface of a polymer, such as poly(dimethylsiloxane) (PDMS). Their small scale is exploited to control reaction volume, manipulate single cells and molecules, and increase throughput with massive parallelization. The key advantages to the use of microfluidic technologies include minimal reagent and sample consumption, low waste generation, rapid reaction and analysis times, and minimal space requirements in the laboratory [Weibel and Whitesides, 2006]. Furthermore, microfluidic platforms generate volumes with similar length scale to the intrinsic volumes of eukaryotic cells and organelles, making them particularly well-suited for single-cell analyses. Finally, microfluidic technology is highly amenable to automation, integration, and parallelization, enabling the development of robust bioanalytical systems with unprecedented throughput and sensitivity. Many comprehensive reviews of the large field of microfluidic devices and their application in genetic analysis exist in the literature [El-Ali et al., 2006; Weibel and Whitesides, 2006; Liu and Mathies, 2009; Schmid et al., 2010].

Several recent research reports have successfully conducted analysis of gDNA in microfluidic devices. In one recent report, digital PCR amplification of chromosome 21 and GAPDH (control) was conducted in a PDMS microfluidic chip composed of 12 panels that each contained 765 discrete reaction volumes defined by microvalves [Fan and Quake, 2007]. The gDNA of a cell line with trisomy 21 was then compared to the gDNA from a normal cell line, and the authors found that the expected 1:1 ratio of positive wells was observed following PCR for GAPDH, whereas the expected 3:2 ratio of chromosome 21 positive wells was observed. These authors then diluted the trisomy 21^{+} cell line in the wild-type control and found that they needed to use 4,000 compartments to be 95% confident in assessing trisomy 21 in a sample that contained 10% mutant gDNA within a pool of normal gDNA. By continuing to increase the number of compartments analyzed, the authors theorize that one could achieve nearly arbitrary sensitivity by continuing to increase the scale of the assay [Fan and Quake, 2007]. Another recent report used lab-on-a-chip-level integration of thermal cycling, sample purification, and capillary electrophoresis to enable complete Sanger sequencing from only 1 fmol of DNA template [Blazej et al., 2006]. As many as 556 continuous bases were sequenced with 99% accuracy, demonstrating read lengths required for de novo sequencing of human and other complex genomes. This achievement opens the possibility of direct sequencing of genetic targets following amplification from single cells.

Significant interest has been invested in microfluidic droplet technologies and their application in high-throughput genetic analysis. Recent applications of microfluidics in genetic analysis have used model systems to demonstrate proof-of-concept that may soon be extended to clinical samples. In one study, a T-junction channel in a silicon device was used to generate a stream of monodisperse picoliter droplets that were isolated from both the microfluidic channel surfaces and each other by the oil carrier phase [Beer et al., 2007]. A system of valves was used to stop the droplets on-chip, and then the droplets were thermally cycled without droplet motion. Using this system, 10-pL droplets, encapsulating single copies of viral gDNA, showed highly efficient RT-PCR amplification with a cycle threshold that was ~18, which is 20 cycles earlier than when the same sample was assayed in bulk using commercial instruments [Beer et al., 2007]. This application of an established RT-PCR assay in a microfluidic device demonstrates the power of this technology for isolating single-copy nucleic acids in a complex environment and thus conducting digital PCR analyses. In another recent study, adenovirus gDNA was quantified using a high-throughput microfluidic chip that encapsulates PCR reagents in millions of picoliter droplets in a continuous oil flow [Kiss et al., 2008]. The oil stream carries the aqueous droplets through alternating zones of temperature to achieve thermal cycling, and inclusion of fluorescent probes in the PCR reaction mix allows the amplification to be tracked in individual droplets within the microfluidic channels [Kiss et al., 2008]. Ultimately, these authors found that an adenovirus-specific product could be detected and quantified in 35 min at template concentrations as low as one template molecule per 167 droplets. The observed frequencies of positive reactions over a range of template concentrations agreed closely with the frequencies expected from Poisson statistics, demonstrating the quantitative power of the method [Kiss et al., 2008]. Another recent application of microfluidic technology achieved quantitative isothermal amplification of digitally encapsulated plasmid via hyperbranched rolling circle amplification of a plasmid containing the lacZ gene [Mazutis et al., 2009]. The authors then went on to fuse the amplified plasmid droplets with droplets containing all necessary reagents for in vitro translation in a highly controlled and pairwise manner and thus demonstrated that active β-galactosidase was only produced from droplets containing amplified plasmid [Mazutis et al., 2009]. Although these recent reports used model systems (plasmid or viral genomes) rather than truly relevant targets in the human genome, they demonstrate state-of-the-art technology that may soon be applied to the study of rare human genetic variants.

Single-cell sensitivity and resolution is crucial to in-depth understanding of tumor heterogeneity and detection of rare events for the early diagnosis of diseases including cancer. Single-cell genetic analysis (SCGA) methods have

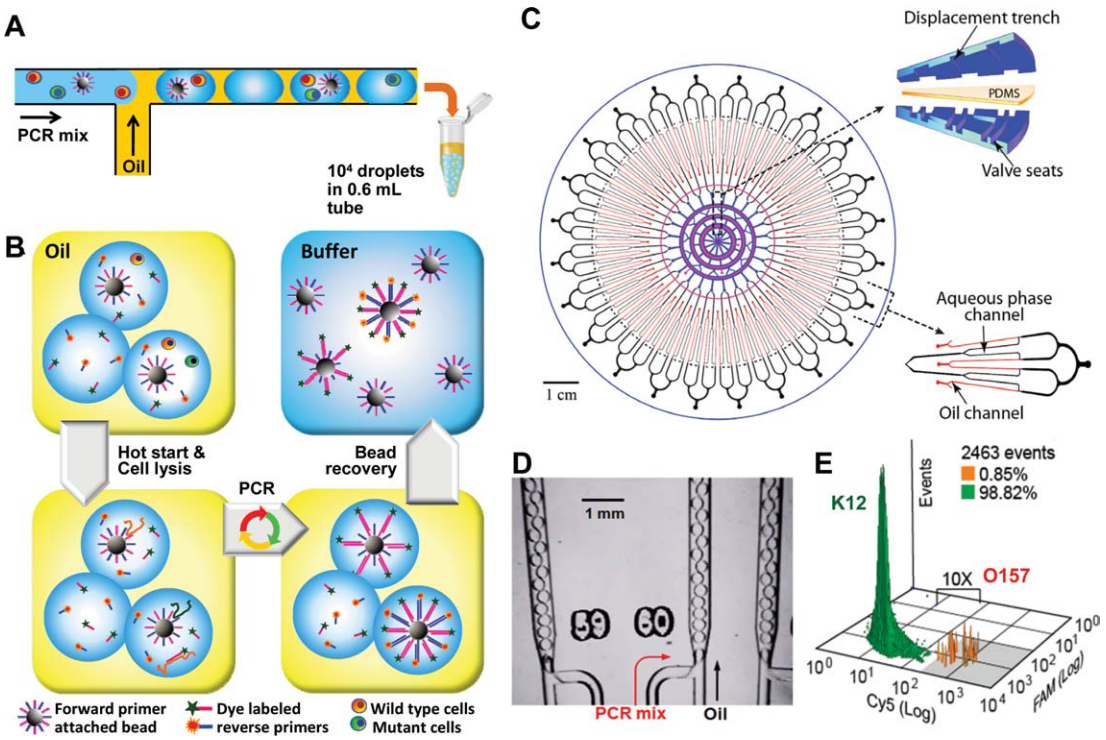

Fig. 5. Microfluidic device and methods for single-copy DNA template amplification. **A**: target DNA (or cells) and beads are mixed with the PCR reagent (blue) at very dilute concentrations and pumped through a microfabricated droplet generator. Monodisperse nanoliter volume droplets of the PCR reagent are formed in carrier oil (yellow) at the T-injector and routed into a tube for thermal cycling. The number of droplets containing a single bead and a single template DNA/cell is controlled by varying their concentrations in the PCR solution and by controlling the droplet volume. **B**: Each functional PCR mix droplet contains a bead covalently labeled with the reverse primers, dye-labeled forward primers, and a single target copy. Subsequent steps of PCR generate dye-labeled double-stranded product on the bead surface. Following emulsion PCR, the droplets are broken, and the beads are analyzed by flow cytometry to quantify the signal for each clonal amplification product. **C**: Microdroplet generation array for controlled formation of nanoliter PCR droplets: layout of device, showing the aqueous phase channels (black), the oil phase channels (red), and the detail of the pumping structure. A three layer (glass-PDMS-glass) pneumatically controlled micropump is integrated on-chip to deliver PCR reagent containing dilute 34-μm beads and template. **D**: Optical micrograph of droplet generation at three T-injectors in the array. Droplets are typically generated at each T-injector at a frequency of ~6 Hz, meaning that 2–3 × 10^6 microdroplets can be generated each hour. **E**: Two-color flow cytometry data showing the expected frequency of mutant signal in a large wild-type background (total input of 100 total copies per droplet with mutant frequency of 10^{-4}). Figure adapted from Zeng et al. [2010].

been reported combining bead-bound PCR methods and flow cytometric quantification, similar to BEAMing methods, with microfluidic droplet generation to achieve highly controlled reaction volumes and precise quantitation of genetic targets in a high-throughput manner (Fig. 5) [Kumaresan et al., 2008; Zeng et al., 2010]. A hybrid microsystem used integrated, on-chip micropumps to coencapsulate primer-functionalized microbeads and single cells in monodipserse, nanoliter-scale PCR reaction microdroplets to achieve quantitative single-cell detection of the GAPDH gene in human lymphocytes, and the gyrB gene in bacteria [Kumaresan et al., 2008]. The SCGA method has been improved to detect multiple genetic targets by multiplexing the bead-bound primers (Fig. 5). The microdroplet generator has been scaled up to a 96-channel array, which is capable of producing 3.4 × 10^6-nL volume PCR droplets per hour, and the multiplexed SCGA method was then applied to achieve quantitative measurement of single pathogenic bacteria cells in a large background of >10^5 wild-type bacteria (Fig. 5) [Zeng et al., 2010]. The results indicate the promising potential of the microfluidic technique for high-throughput SCGA and quantitative detection of rare mutations, such as CTCs in whole blood.

Another recent study used a 672-microwell chip to conduct genetic analysis of two adherent human cell lines following on-chip culture [Lindstrom et al., 2009]. Following culture in 500-nL microwells, the authors lysed the cells and conducted PCR using biotinylated and fluorescently labeled primers to capture and detect amplicon on streptavidin-coated magnetic beads. The authors then denatured the double-stranded product, trapped the microbeads with a magnetic field for washing, and conducted a minisequencing reaction to detect the two expected genotypes (wild-type p53 and a p53 point mutation) simultaneously in the expected ratio using an array scanner [Lindstrom et al., 2009].

Finally, it should be noted that microfluidics has also provided promising platforms for developing low-cost,

high-performance sequencing technologies. Examples include pyrosequencing methods [Margulies et al., 2005; Russom et al., 2005], digital PCR enrichment using a droplet primer library for large-scale target sequencing [Tewhey et al., 2009], and microscale PCR that is promising for single molecule or single-cell sequencing [Blazej et al., 2006]. Furthermore, solid-phase emulsion PCR technologies enable low-cost and high-throughput preparation of template DNA for new genome-sequencing techniques [Margulies et al., 2005; Shendure et al., 2005]. Microfluidic droplet technologies generate uniform droplets, which may improve the quality of template DNA to improve the performance of next-generation sequencing, such as the read length [Kumaresan et al., 2008].

CONCLUSIONS

Thanks to resequencing and genome-wide association studies, thousands of genetic biomarkers of early effect in carcinogenesis have been discovered. Their validation in translation studies is currently a major focus in molecular epidemiology, and several of these markers are now available for application in large-scale studies on human populations. However, for these genetic markers to have an impact in early detection and treatment of cancer, highly sensitive and specific technologies must be used to detect rare mutations in human specimens. Despite decades of research, some of the most sensitive methods that are currently being applied to detect acquired mutations in human specimens are highly optimized and specifically targeted versions of ME-PCR, AS-PCR, and nested PCR. These methods are often applied to homogenized gDNA from tumor, whole blood, plasma, or other routinely available tissues. However, these types of analyses may fail to analyze variants contributing to human disease, because unknown variants cannot be discovered with such targeted, though highly sensitive, techniques. Furthermore, when homogenized gDNA is analyzed, information regarding the co-occurrence of mutations in single cells or clones is lost, although these data might be important for understanding disease progression.

For these reasons, more studies using NGS and high-throughput digital PCR are needed to further elucidate the genetic basis of human disease. Studies that resequence the genome of diseased tissue (e.g., tumor) and compare to the germline sequence in the same individual (e.g., adjacent normal tissue) are particularly powerful, as this approach compensates for the individual's background germline variants and reveal only acquired mutations that "drove" disease progression or that were acquired as "passengers" in the process. Furthermore, high-throughput, digital PCR techniques are powerful methods that will soon enable single-cell analyses within tumors and other diseased tissues. These approaches will soon allow researchers to study the cellular population genetics within diseased tissue and to reveal heterogeneity that is masked in bulk analyses of homogenized gDNA. The use of digital methods will inevitably reveal unprecedented levels of heterogeneity or internal variation within an individual that is intrinsic to the complexity of cancer initiation and development. Measuring, interpreting, and mastering this ensemble complexity is an important challenge, which will be critical for fully understanding cancer and for the cost-effective implementation of molecular biomarkers and treatments.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for their expert comments regarding this manuscript; their comments lead to major revisions and greatly improved this review.

REFERENCES

Agopian J, Navarro JM, Gac AC, Lecluse Y, Briand M, Grenot P, Gauduchon P, Ruminy P, Lebailly P, Nadel B, Roulland S. 2009. Agricultural pesticide exposure and the molecular connection to lymphomagenesis. J Exp Med 206:1473–1483.

Alkan C, Kidd JM, Marques-Bonet T, Aksay G, Antonacci F, Hormozdiari F, Kitzman JO, Baker C, Malig M, Mutlu O, Sahinalp SC, Gibbs RA, Eichler EE. 2009. Personalized copy number and segmental duplication maps using next-generation sequencing. Nat Genet 41:1061–1067.

Ashley EA, Butte AJ, Wheeler MT, Chen R, Klein TE, Dewey FE, Dudley JT, Ormond KE, Pavlovic A, Morgan AA, Pushkarev D, Neff NF, Hudgins L, Gong L, Hodges LM, Berlin DS, Thorn CF, Sangkuhl K, Hebert JM, Woon M, Sagreiya H, Whaley R, Knowles JW, Chou MF, Thakuria JV, Rosenbaum AM, Zaranek AW, Church GM, Greely HT, Quake SR, Altman RB. 2010. Clinical assessment incorporating a personal genome. Lancet 375: 1525–1535.

Barrett IP. 2010. Cancer genome analysis informatics. Methods Mol Biol 628:75–102.

Bayani J, Squire J. 2004. Multi-color FISH techniques. Curr Protoc Cell Biol Chapter 22:Unit 22.5.

Bayraktar S, Goodman M. 2010. Detection of BCR-ABL positive cells in an asymptomatic patient: A case report and literature review. Case Report Med 939706.

Bazan V, Agnese V, Corsale S, Calo V, Valerio MR, Latteri MA, Vieni S, Grassi N, Cicero G, Dardanoni G, Tomasino RM, Colucci G, Gebbia N, Russo A. 2005. Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: Results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study. Ann Oncol 16 (Suppl 4):iv50–iv55.

Bea S, Salaverria I, Armengol L, Pinyol M, Fernandez V, Hartmann EM, Jares P, Amador V, Hernandez L, Navarro A, Ott G, Rosenwald A, Estivill X, Campo E. 2009. Uniparental disomies, homozygous deletions, amplifications, and target genes in mantle cell lymphoma revealed by integrative high-resolution whole-genome profiling. Blood 113:3059–3069.

Beck J, Urnovitz HB, Mitchell WM, Schutz E. Next generation sequencing of serum circulating nucleic acids from patients with invasive ductal breast cancer reveals differences to healthy and nonmalignant controls. Mol Cancer Res 8:335–342.

Beer NR, Hindson BJ, Wheeler EK, Hall SB, Rose KA, Kennedy IM, Colston BW. 2007. On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets. Anal Chem 79:8471–8475.

Beer NR, Wheeler EK, Lee-Houghton L, Watkins N, Nasarabadi S, Hebert N, Leung P, Arnold DW, Bailey CG, Colston BW. 2008. On-chip single-copy real-time reverse-transcription PCR in isolated picoliter droplets. Anal Chem 80:1854–1858.

Behn M, Schuermann M. 1998. Sensitive detection of p53 gene mutations by a 'mutant enriched' PCR-SSCP technique. Nucleic Acids Res 26:1356–1358.

Belaud-Rotureau MA, Parrens M, Carrere N, Turmo M, Ferrer J, de Mascarel A, Dubus P, Merlio JP. 2007. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in detecting IGH-BCL2 rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol 38:365–372.

Bell DA, Liu Y, Cortopassi GA. 1995. Occurrence of bcl-2 oncogene translocation with increased frequency in the peripheral blood of heavy smokers. J Natl Cancer Inst 87:223–224.

Bende RJ, Smit LA, van Noesel CJ. 2007. Molecular pathways in follicular lymphoma. Leukemia 21:18–29.

Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. 1995. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. Blood 86:3118–3122.

Blazej RG, Kumaresan P, Mathies RA. 2006. Microfabricated bioprocessor for integrated nanoliter-scale Sanger DNA sequencing. Proc Natl Acad Sci USA 103:7240–7245.

Bonassi S, Abbondandolo A, Camurri L, Dal Pra L, De Ferrari M, Degrassi F, Forni A, Lamberti L, Lando C, Padovani P, Sbrana I, Vecchio D, Puntoni R. 1995. Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. Cancer Genet Cytogenet 79:133–135.

Borresen-Dale AL, Hovig E, Smith-Sorensen B. 2001. Detection of mutations by denaturing gradient gel electrophoresis. Curr Protoc Hum Genet Chapter 7:Unit 7.5.

Brenner BM, Rosenberg D. 2010. High-throughput SNP/CGH approaches for the analysis of genomic instability in colorectal cancer. Mutat Res (in press).

Calin GA, Croce CM. 2009. Chronic lymphocytic leukemia: Interplay between noncoding RNAs and protein-coding genes. Blood 114: 4761–4770.

Carter H, Chen S, Isik L, Tyekucheva S, Velculescu VE, Kinzler KW, Vogelstein B, Karchin R. 2009. Cancer-specific high-throughput annotation of somatic mutations: Computational prediction of driver missense mutations. Cancer Res 69:6660–6667.

Carter NP. 2007. Methods and strategies for analyzing copy number variation using DNA microarrays. Nat Genet 39(7 Suppl):S16–S21.

Castle JC, Biery M, Bouzek H, Xie T, Chen R, Misura K, Jackson S, Armour CD, Johnson JM, Rohl CA, Raymond CK. DNA copy number, including telomeres and mitochondria, assayed using next-generation sequencing. BMC Genom 11:244.

Chiocca SM, Sandy MS, Cerutti PA. 1992. Genotypic analysis of *N*-ethyl-*N*-nitrosourea-induced mutations by Taq I restriction fragment length polymorphism/polymerase chain reaction in the c-H-ras1 gene. Proc Natl Acad Sci USA 89:5331–5335.

Chiu BC, Dave BJ, Blair A, Gapstur SM, Zahm SH, Weisenburger DD. 2006. Agricultural pesticide use and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Blood 108:1363–1369.

Cortot AB, Italiano A, Burel-Vandenbos F, Martel-Planche G, Hainaut P. 2010. KRAS mutation status in primary nonsmall cell lung cancer and matched metastases. Cancer 116:2682–2687.

Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, Diaz LA Jr, Goodman SN, David KA, Juhl H, Kinzler KW, Vogelstein B. 2005. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci USA 102:16368–16373.

Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. 2006. BEAMing: Single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 3:551–559.

Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA, Kinzler KW, Vogelstein B, Diaz LA Jr. 2008. Circulating mutant DNA to assess tumor dynamics. Nat Med 14:985–990.

Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, Sougnez C, Greulich H, Muzny DM, Morgan MB, Fulton L, Fulton RS, Zhang Q, Wendl MC, Lawrence MS, Larson DE, Chen K, Dooling DJ, Sabo A, Hawes AC, Shen H, Jhangiani SN, Lewis LR, Hall O, Zhu Y, Mathew T, Ren Y, Yao J, Scherer SE, Clerc K, Metcalf GA, Ng B, Milosavljevic A, Gonzalez-Garay ML, Osborne JR, Meyer R, Shi X, Tang Y, Koboldt DC, Lin L, Abbott R, Miner TL, Pohl C, Fewell G, Haipek C, Schmidt H, Dunford-Shore BH, Kraja A, Crosby SD, Sawyer CS, Vickery T, Sander S, Robinson J, Winckler W, Baldwin J, Chirieac LR, Dutt A, Fennell T, Hanna M, Johnson BE, Onofrio RC, Thomas RK, Tonon G, Weir BA, Zhao X, Ziaugra L, Zody MC, Giordano T, Orringer MB, Roth JA, Spitz MR, Wistuba II, Ozenberger B, Good PJ, Chang AC, Beer DG, Watson MA, Ladanyi M, Broderick S, Yoshizawa A, Travis WD, Pao W, Province MA, Weinstock GM, Varmus HE, Gabriel SB, Lander ES, Gibbs RA, Meyerson M, Wilson RK. 2008. Somatic mutations affect key pathways in lung adenocarcinoma. Nature 455:1069–1075.

Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL, Abbott RM, Hoog J, Dooling DJ, Koboldt DC, Schmidt H, Kalicki J, Zhang Q, Chen L, Lin L, Wendl MC, McMichael JF, Magrini VJ, Cook L, McGrath SD, Vickery TL, Appelbaum E, Deschryver K, Davies S, Guintoli T, Crowder R, Tao Y, Snider JE, Smith SM, Dukes AF, Sanderson GE, Pohl CS, Delehaunty KD, Fronick CC, Pape KA, Reed JS, Robinson JS, Hodges JS, Schierding W, Dees ND, Shen D, Locke DP, Wiechert ME, Eldred JM, Peck JB, Oberkfell BJ, Lolofie JT, Du F, Hawkins AE, O'Laughlin MD, Bernard KE, Cunningham M, Elliott G, Mason MD, Thompson DM Jr, Ivanovich JL, Goodfellow PJ, Perou CM, Weinstock GM, Aft R, Watson M, Ley TJ, Wilson RK, Mardis ER. 2010. Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature 464:999–1005.

Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. 2003. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci USA 100:8817–8822.

Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, Sekeres MA, Wang XF, McDevitt MA, Maciejewski JP. 2008. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 68:10349–10357.

Einerson RR, Kurtin PJ, Dayharsh GA, Kimlinger TK, Remstein ED. 2005. FISH is superior to PCR in detecting t(14;18)(q32;q21)-IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. Am J Clin Pathol 124:421–429.

El-Ali J, Sorger PK, Jensen KF. 2006. Cells on chips. Nature 442:403–411.

Fan HC, Quake SR. 2007. Detection of aneuploidy with digital polymerase chain reaction. Anal Chem 79:7576–7579.

Flotho C, Steinemann D, Mullighan CG, Neale G, Mayer K, Kratz CP, Schlegelberger B, Downing JR, Niemeyer CM. 2007. Genome-wide single-nucleotide polymorphism analysis in juvenile myelomonocytic leukemia identifies uniparental disomy surrounding the NF1 locus in cases associated with neurofibromatosis but not in cases with mutant RAS or PTPN11. Oncogene 26:5816–5821.

Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW. 2010. Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7:461–465.

Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, Belmont JW, Boudreau A, Hardenbol P, Leal SM, Pasternak S, Wheeler DA, Willis TD, Yu F, Yang H, Zeng C, Gao Y, Hu H, Hu W, Li C, Lin W, Liu S, Pan H, Tang X, Wang J, Wang W, Yu J, Zhang B, Zhang Q, Zhao H, Zhou J, Gabriel SB, Barry R, Blumenstiel B, Camargo A, Defelice M, Faggart M, Goyette M, Gupta S, Moore J, Nguyen H, Onofrio RC, Parkin M, Roy J, Stahl E, Winchester E, Ziaugra L, Altshuler D, Shen Y, Yao Z, Huang W, Chu X, He Y, Jin L, Liu Y, Sun W, Wang H, Wang Y, Xiong X, Xu L, Waye MM, Tsui SK, Xue H, Wong JT, Galver LM, Fan JB, Gunderson K, Murray SS, Oliphant AR, Chee MS, Montpetit A, Chagnon F, Ferretti V, Leboeuf M, Olivier JF, Phillips MS, Roumy S, Sallee C, Verner A, Hudson TJ, Kwok PY, Cai D, Koboldt DC, Miller RD, Pawlikowska L, Taillon-Miller P, Xiao M, Tsui LC, Mak W, Song YQ, Tam PK, Nakamura Y, Kawaguchi T, Kitamoto T, Morizono T, Nagashima A, Ohnishi Y, Sekine A, Tanaka T, Tsunoda T, Deloukas P, Bird CP, Delgado M, Dermitzakis ET, Gwilliam R, Hunt S, Morrison J, Powell D, Stranger BE, Whittaker P, Bentley DR, Daly MJ, de Bakker PI, Barrett J, Chretien YR, Maller J, McCarroll S, Patterson N, Pe'er I, Price A, Purcell S, Richter DJ, Sabeti P, Saxena R, Schaffner SF, Sham PC, Varilly P, Stein LD, Krishnan L, Smith AV, Tello-Ruiz MK, Thorisson GA, Chakravarti A, Chen PE, Cutler DJ, Kashuk CS, Lin S, Abecasis GR, Guan W, Li Y, Munro HM, Qin ZS, Thomas DJ, McVean G, Auton A, Bottolo L, Cardin N, Eyheramendy S, Freeman C, Marchini J, Myers S, Spencer C, Stephens M, Donnelly P, Cardon LR, Clarke G, Evans DM, Morris AP, Weir BS, Mullikin JC, Sherry ST, Feolo M, Skol A, Zhang H, Matsuda I, Fukushima Y, Macer DR, Suda E, Rotimi CN, Adebamowo CA, Ajayi I, Aniagwu T, Marshall PA, Nkwodimmah C, Royal CD, Leppert MF, Dixon M, Peiffer A, Qiu R, Kent A, Kato K, Niikawa N, Adewole IF, Knoppers BM, Foster MW, Clayton EW, Watkin J, Muzny D, Nazareth L, Sodergren E, Weinstock GM, Yakub I, Birren BW, Wilson RK, Fulton LL, Rogers J, Burton J, Carter NP, Clee CM, Griffiths M, Jones MC, McLay K, Plumb RW, Ross MT, Sims SK, Willey DL, Chen Z, Han H, Kang L, Godbout M, Wallenburg JC, L'Archeveque P, Bellemare G, Saeki K, An D, Fu H, Li Q, Wang Z, Wang R, Holden AL, Brooks LD, McEwen JE, Guyer MS, Wang VO, Peterson JL, Shi M, Spiegel J, Sung LM, Zacharia LF, Collins FS, Kennedy K, Jamieson R, Stewart J. 2007. A second generation human haplotype map of over 3.1 million SNPs. Nature 449:851–861.

Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, Pogue-Geile KL. 2010. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer 10:101.

Fuscoe JC, Setzer RW, Collard DD, Moore MM. 1996. Quantification of t(14;18) in the lymphocytes of healthy adult humans as a possible biomarker for environmental exposures to carcinogens. Carcinogenesis 17:1013–1020.

Garzon R, Calin GA, Croce CM. 2009. MicroRNAs in Cancer. Annu Rev Med 60:167–179.

Gondek LP, Dunbar AJ, Szpurka H, McDevitt MA, Maciejewski JP. 2007. SNP array karyotyping allows for the detection of uniparental disomy and cryptic chromosomal abnormalities in MDS/MPD-U and MPD. PLoS One 2:e1225.

Gormally E, Vineis P, Matullo G, Veglia F, Caboux E, Le Roux E, Peluso M, Garte S, Guarrera S, Munnia A, Airoldi L, Autrup H, Malaveille C, Dunning A, Overvad K, Tjonneland A, Lund E, Clavel-Chapelon F, Boeing H, Trichopoulou A, Palli D, Krogh V, Tumino R, Panico S, Bueno-de-Mesquita HB, Peeters PH, Pera G, Martinez C, Dorronsoro M, Barricarte A, Navarro C, Quiros JR, Hallmans G, Day NE, Key TJ, Saracci R, Kaaks R, Riboli E, Hainaut P. 2006. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: A prospective study. Cancer Res 66:6871–6876.

Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR. 2007. Patterns of somatic mutation in human cancer genomes. Nature 446:153–158.

Griffiths AD, Tawfik DS. 2006. Miniaturising the laboratory in emulsion droplets. Trends Biotechnol 24:395–402.

Gu K, Chan WC, Hawley RC. 2008. Practical detection of t(14;18)(IgH/BCL2) in follicular lymphoma. Arch Pathol Lab Med 132:1355–1361.

Halling KC, Kipp BR. 2007. Fluorescence in situ hybridization in diagnostic cytology. Hum Pathol 38:1137–1144.

Huh J, Tiu RV, Gondek LP, O'Keefe CL, Jasek M, Makishima H, Jankowska AM, Jiang Y, Verma A, Theil KS, McDevitt MA, Maciejewski JP. 2010. Characterization of chromosome arm 20q abnormalities in myeloid malignancies using genome-wide single nucleotide polymorphism array analysis. Genes Chromosomes Cancer 49:390–399.

Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, Shields PG, Ham AJ, Swenberg JA, Marrogi AJ, Harris CC. 2000a. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: A cancer-prone chronic inflammatory disease. Cancer Res 60:3333–3337.

Hussain SP, Raja K, Amstad PA, Sawyer M, Trudel LJ, Wogan GN, Hofseth LJ, Shields PG, Billiar TR, Trautwein C, Hohler T, Galle PR, Phillips DH, Markin R, Marrogi AJ, Harris CC. 2000b. Increased p53 mutation load in nontumorous human liver of wilson disease and hemochromatosis: Oxyradical overload diseases. Proc Natl Acad Sci USA 97:12770–12775.

Hussain SP, Amstad P, Raja K, Sawyer M, Hofseth L, Shields PG, Hewer A, Phillips DH, Ryberg D, Haugen A, Harris CC. 2001. Mutability of p53 hotspot codons to benzo(a)pyrene diol epoxide (BPDE) and the frequency of p53 mutations in nontumorous human lung. Cancer Res 61:6350–6355.

Jackson PE, Qian GS, Friesen MD, Zhu YR, Lu P, Wang JB, Wu Y, Kensler TW, Vogelstein B, Groopman JD. 2001. Specific p53 mutations detected in plasma and tumors of hepatocellular carcinoma patients by electrospray ionization mass spectrometry. Cancer Res 61:33–35.

Janne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, Distel RJ, Meyerson M, Johnson BE. 2006. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin Cancer Res 12(3, Pt 1):751–758.

Jasek M, Gondek LP, Bejanyan N, Tiu R, Huh J, Theil KS, O'Keefe C, McDevitt MA, Maciejewski JP. 2010. TP53 mutations in myeloid malignancies are either homozygous or hemizygous due to copy number-neutral loss of heterozygosity or deletion of 17p. Leukemia 24:216–219.

Jenkins GJ. 2004. The restriction site mutation (RSM) method: Clinical applications. Mutagen 19:3–11.

Jenkins GJ, Doak SH, Griffiths AP, Tofazzal N, Shah V, Baxter JN, Parry JM. 2003. Early p53 mutations in nondysplastic Barrett's tissue detected by the restriction site mutation (RSM) methodology. Br J Cancer 88:1271–1276.

Jolkowska J, Derwich K, Dawidowska M. 2007. Methods of minimal residual disease (MRD) detection in childhood haematological malignancies. J Appl Genet 48:77–83.

Keohavong P, Lan Q, Gao WM, Zheng KC, Mady HH, Melhem MF, Mumford JL. 2005. Detection of p53 and K-ras mutations in sputum of individuals exposed to smoky coal emissions in Xuan Wei County, China. Carcinogenesis 26:303–308.

Kirk GD, Camus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, Brechot C, Hainaut P, Montesano R. 2000. Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from The Gambia. J Natl Cancer Inst 92:148–153.

Kiss MM, Ortoleva-Donnelly L, Beer NR, Warner J, Bailey CG, Colston BW, Rothberg JM, Link DR, Leamon JH. 2008. High-throughput quantitative polymerase chain reaction in picoliter droplets. Anal Chem 80:8975–8981.

Kojima T, Takei Y, Ohtsuka M, Kawarasaki Y, Yamane T, Nakano H. 2005. PCR amplification from single DNA molecules on magnetic beads in emulsion: Application for high-throughput screening of transcription factor targets. Nucleic Acids Res 33:e150.

Kringen P, Bergamaschi A, Due EU, Wang Y, Tagliabue E, Nesland JM, Nehman A, Tonisson N, Borresen-Dale AL. 2005. Evaluation of arrayed primer extension for TP53 mutation detection in breast and ovarian carcinomas. Biotechniques 39:755–761.

Krjutskov K, Andreson R, Magi R, Nikopensius T, Khrunin A, Mihailov E, Tammekivi V, Sork H, Remm M, Metspalu A. 2008. Development of a single tube 640-plex genotyping method for detection of nucleic acid variations on microarrays. Nucleic Acids Res 36:e75.

Kumaresan P, Yang CJ, Cronier SA, Blazej RG, Mathies RA. 2008. High-throughput single copy DNA amplification and cell analysis in engineered nanoliter droplets. Anal Chem 80:3522–3529.

Kurg A, Tonisson N, Georgiou I, Shumaker J, Tollett J, Metspalu A. 2000. Arrayed primer extension: Solid-phase four-color DNA resequencing and mutation detection technology. Genet Test 4:1–7.

Laken SJ, Jackson PE, Kinzler KW, Vogelstein B, Strickland PT, Groopman JD, Friesen MD. 1998. Genotyping by mass spectrometric analysis of short DNA fragments. Nat Biotechnol 16:1352–1356.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, Gibbs RA, Muzny DM, Scherer SE, Bouck JB, Sodergren EJ, Worley KC, Rives CM, Gorrell JH, Metzker ML, Naylor SL, Kucherlapati RS, Nelson DL, Weinstock GM, Sakaki Y, Fujiyama A, Hattori M, Yada T, Toyoda A, Itoh T, Kawagoe C, Watanabe H, Totoki Y, Taylor T, Weissenbach J, Heilig R, Saurin W, Artiguenave F, Brottier P, Bruls T, Pelletier E, Robert C, Wincker P, Smith DR, Doucette-Stamm L, Rubenfield M, Weinstock K, Lee HM, Dubois J, Rosenthal A, Platzer M, Nyakatura G, Taudien S, Rump A, Yang H, Yu J, Wang J, Huang G, Gu J, Hood L, Rowen L, Madan A, Qin S, Davis RW, Federspiel NA, Abola AP, Proctor MJ, Myers RM, Schmutz J, Dickson M, Grimwood J, Cox DR, Olson MV, Kaul R, Shimizu N, Kawasaki K, Minoshima S, Evans GA, Athanasiou M, Schultz R, Roe BA, Chen F, Pan H, Ramser J, Lehrach H, Reinhardt R, McCombie WR, de la Bastide M, Dedhia N, Blocker H, Hornischer K, Nordsiek G, Agarwala R, Aravind L, Bailey JA, Bateman A, Batzoglou S, Birney E, Bork P, Brown DG, Burge CB, Cerutti L, Chen HC, Church D, Clamp M, Copley RR, Doerks T, Eddy SR, Eichler EE, Furey TS, Galagan J, Gilbert JG, Harmon C, Hayashizaki Y, Haussler D, Hermjakob H, Hokamp K, Jang W, Johnson LS, Jones TA, Kasif S, Kaspryzk A, Kennedy S, Kent WJ, Kitts P, Koonin EV, Korf I, Kulp D, Lancet D, Lowe TM, McLysaght A, Mikkelsen T, Moran JV, Mulder N, Pollara VJ, Ponting CP, Schuler G, Schultz J, Slater G, Smit AF, Stupka E, Szustakowski J, Thierry-Mieg D, Thierry-Mieg J, Wagner L, Wallis J, Wheeler R, Williams A, Wolf YI, Wolfe KH, Yang SP, Yeh RF, Collins F, Guyer MS, Peterson J, Felsenfeld A, Wetterstrand KA, Patrinos A, Morgan MJ, de Jong P, Catanese JJ, Osoegawa K, Shizuya H, Choi S, Chen YJ. 2001. Initial sequencing and analysis of the human genome. Nature 409:860–921.

Langerod A, Zhao H, Borgan O, Nesland JM, Bukholm IR, Ikdahl T, Karesen R, Borresen-Dale AL, Jeffrey SS. 2007. TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res 9:R30.

Le Calvez F, Ahman A, Tonisson N, Lambert J, Temam S, Brennan P, Zaridze DG, Metspalu A, Hainaut P. 2005. Arrayed primer extension resequencing of mutations in the TP53 tumor suppressor gene: Comparison with denaturing HPLC and direct sequencing. Clin Chem 51:1284–1287.

Lee W, Jiang Z, Liu J, Haverty PM, Guan Y, Stinson J, Yue P, Zhang Y, Pant KP, Bhatt D, Ha C, Johnson S, Kennemer MI, Mohan S, Nazarenko I, Watanabe C, Sparks AB, Shames DS, Gentleman R, de Sauvage FJ, Stern H, Pandita A, Ballinger DG, Drmanac R, Modrusan Z, Seshagiri S, Zhang Z. 2010. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature 465:473–477.

Lengauer C, Kinzler KW, Vogelstein B. 1998. Genetic instabilities in human cancers. Nature 396:643–649.

Levy S, Sutton G, Ng PC, Feuk L, Halpern AL, Walenz BP, Axelrod N, Huang J, Kirkness EF, Denisov G, Lin Y, MacDonald JR, Pang AW, Shago M, Stockwell TB, Tsiamouri A, Bafha V, Bansal V, Kravitz SA, Busam DA, Beeson KY, McIntosh TC, Remington KA, Abril JF, Gill J, Borman J, Rogers YH, Frazier ME, Scherer SW, Strausberg RL, Venter JC. 2007. The diploid genome sequence of an individual human. PLoS Biol 5:e254.

Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, Dooling D, Dunford-Shore BH, McGrath S, Hickenbotham M, Cook L, Abbott R, Larson DE, Koboldt DC, Pohl C, Smith S, Hawkins A, Abbott S, Locke D, Hillier LW, Miner T, Fulton L, Magrini V, Wylie T, Glasscock J, Conyers J, Sander N, Shi X, Osborne JR, Minx P, Gordon D, Chinwalla A, Zhao Y, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson M, Baty J, Ivanovich J, Heath S, Shannon WD, Nagarajan R, Walter MJ, Link DC, Graubert TA, DiPersio JF, Wilson RK. 2008. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature 456:66–72.

Li M, Diehl F, Dressman D, Vogelstein B, Kinzler KW. 2006. BEAMing up for detection and quantification of rare sequence variants. Nat Methods 3:95–97.

Lindstrom S, Hammond M, Brismar H, Andersson-Svahn H, Ahmadian A. 2009. PCR amplification and genetic analysis in a microwell cell culturing chip. Lab Chip 9:3465–3471.

Ling G, Persson A, Berne B, Uhlen M, Lundeberg J, Ponten F. 2001. Persistent p53 mutations in single cells from normal human skin. Am J Pathol 159:1247–1253.

Liu P, Mathies RA. 2009. Integrated microfluidic systems for high-performance genetic analysis. Trends Biotechnol 27:572–581.

Liu Q, Sommer SS. 2004. Detection of extremely rare alleles by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification (Bi-PAP-A): Measurement of mutation load in mammalian tissues. Biotechniques 36:156–166.

Liu Y, Hernandez AM, Shibata D, Cortopassi GA. 1994. BCL2 translocation frequency rises with age in humans. Proc Natl Acad Sci USA 91:8910–8914.

Lleonart ME, Kirk GD, Villar S, Lesi OA, Dasgupta A, Goedert JJ, Mendy M, Hollstein MC, Montesano R, Groopman JD, Hainaut P, Friesen MD. 2005. Quantitative analysis of plasma TP53 249Ser-mutated DNA by electrospray ionization mass spectrometry. Cancer Epidemiol Biomarkers Prev 14:2956–2962.

Lodish HF. 2003. Molecular Cell Biology. New York: W.H. Freeman and Company. xxxiii, 973, [79] p.

Lund J, Parviz BA. 2009. Scanning probe and nanopore DNA sequencing: Core techniques and possibilities. Methods Mol Biol 578:113–122.

Maciejewski JP, Mufti GJ. 2008. Whole genome scanning as a cytogenetic tool in hematologic malignancies. Blood 112:965–974.

Maciejewski JP, Tiu RV, O'Keefe C. 2009. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. Br J Haematol 146:479–488.

Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, Fulton LA, Locke DP, Magrini VJ, Abbott RM, Vickery TL, Reed JS, Robinson JS, Wylie T, Smith SM, Carmichael L, Eldred JM, Harris CC, Walker J, Peck JB, Du F, Dukes AF, Sanderson GE, Brummett AM, Clark E, McMichael JF, Meyer RJ, Schindler JK, Pohl CS, Wallis JW, Shi X, Lin L, Schmidt H, Tang Y, Haipek C, Wiechert ME, Ivy JV, Kalicki J, Elliott G, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson MA, Baty J, Heath S, Shannon WD, Nagarajan R, Link DC, Walter MJ, Graubert TA, DiPersio JF, Wilson RK, Ley TJ. 2009. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med 361:1058–1066.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380.

Mazutis L, Araghi AF, Miller OJ, Baret JC, Frenz L, Janoshazi A, Taly V, Miller BJ, Hutchison JB, Link D, Griffiths AD, Ryckelynck M. 2009. Droplet-based microfluidic systems for high-throughput single DNA molecule isothermal amplification and analysis. Anal Chem 81:4813–4821.

McCarroll SA, Altshuler DM. 2007. Copy-number variation and association studies of human disease. Nat Genet 39(7 Suppl):S37–S42.

McKernan KJ, Peckham HE, Costa GL, McLaughlin SF, Fu Y, Tsung EF, Clouser CR, Duncan C, Ichikawa JK, Lee CC, Zhang Z, Ranade SS, Dimalanta ET, Hyland FC, Sokolsky TD, Zhang L, Sheridan A, Fu H, Hendrickson CL, Li B, Kotler L, Stuart JR, Malek JA, Manning JM, Antipova AA, Perez DS, Moore MP, Hayashibara KC, Lyons MR, Beaudoin RE, Coleman BE, Laptewicz MW, Sannicandro AE, Rhodes MD, Gottimukkala RK, Yang S, Bafna V, Bashir A, MacBride A, Alkan C, Kidd JM, Eichler EE, Reese MG, De La Vega FM, Blanchard AP. 2009. Sequence and structural variation in a human genome uncovered by short-read, massively parallel ligation sequencing using two-base encoding. Genome Res 19:1527–1541.

Medvedev P, Stanciu M, Brudno M. 2009. Computational methods for discovering structural variation with next-generation sequencing. Nat Methods 6(11 Suppl):S13–S20.

Metzker ML. 2010. Sequencing technologies—The next generation. Nat Rev Genet 11:31–46.

Morlan J, Baker J, Sinicropi D. 2009. Mutation detection by real-time PCR: A simple, robust and highly selective method. PLoS One 4:e4584.

Najfeld V. 2003. Diagnostic application of FISH to hematological malignancies. Cancer Invest 21:807–814.

Nambiar M, Raghavan SC. 2009. Prevalence and analysis of t(14;18) and t(11;14) chromosomal translocations in healthy Indian population. Ann Hematol (in press).

Nambiar M, Kari V, Raghavan SC. 2008. Chromosomal translocations in cancer. Biochim Biophys Acta 1786:139–152.

Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S. 2005. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 65:6071–6079.

Ng PC, Levy S, Huang J, Stockwell TB, Walenz BP, Li K, Axelrod N, Busam DA, Strausberg RL, Venter JC. 2008. Genetic variation in an individual human exome. PLoS Genet 4:e1000160.

Nowell PC. 2007. Discovery of the Philadelphia chromosome: A personal perspective. J Clin Invest 117:2033–2035.

Oh JE, Lim HS, An CH, Jeong EG, Han JY, Lee SH, Yoo NJ. 2010. Detection of low-level KRAS mutations using PNA-mediated asymmetric PCR clamping and melting curve analysis with unlabeled probes. J Mol Diagn 12:418–424.

Olivier M, Langerod A, Carrieri P, Bergh J, Klaar S, Eyfjord J, Theillet C, Rodriguez C, Lidereau R, Bieche I, Varley J, Bignon Y, Uhrhammer N, Winqvist R, Jukkola-Vuorinen A, Niederacher D, Kato S, Ishioka C, Hainaut P, Borresen-Dale AL. 2006. The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clin Cancer Res 12:1157–1167.

Parsons BL. 2008. Many different tumor types have polyclonal tumor origin: Evidence and implications. Mutat Res 659:232–247.

Pasmant E, Sabbagh A, Masliah-Planchon J, Haddad V, Hamel MJ, Laurendeau I, Soulier J, Parfait B, Wolkenstein P, Bieche I, Vidaud M, Vidaud D. 2009. Detection and characterization of NF1 microdeletions by custom high resolution array CGH. J Mol Diagn 11:524–529.

Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordonez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR. 2010a. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 463:191–196.

Pleasance ED, Stephens PJ, O'Meara S, McBride DJ, Meynert A, Jones D, Lin ML, Beare D, Lau KW, Greenman C, Varela I, Nik-Zainal S, Davies HR, Ordonez GR, Mudie LJ, Latimer C, Edkins S, Stebbings L, Chen L, Jia M, Leroy C, Marshall J, Menzies A, Butler A, Teague JW, Mangion J, Sun YA, McLaughlin SF, Peckham HE, Tsung EF, Costa GL, Lee CC, Minna JD, Gazdar A, Birney E, Rhodes MD, McKernan KJ, Stratton MR, Futreal PA, Campbell PJ. 2010b. A small-cell lung cancer genome with complex signatures of tobacco exposure. Nature 463:184–190.

Pourzand C, Cerutti P. 1993. Genotypic mutation analysis by RFLP/PCR. Mutat Res 288:113–121.

Price TS, Regan R, Mott R, Hedman A, Honey B, Daniels RJ, Smith L, Greenfield A, Tiganescu A, Buckle V, Ventress N, Ayyub H, Salhan A, Pedraza-Diaz S, Broxholme J, Ragoussis J, Higgs DR,

Flint J, Knight SJ. 2005. SW-ARRAY: A dynamic programming solution for the identification of copy-number changes in genomic DNA using array comparative genome hybridization data. Nucleic Acids Res 33:3455–3464.

Pushkarev D, Neff NF, Quake SR. 2009. Single-molecule sequencing of an individual human genome. Nat Biotechnol 27:847–852.

Qian GS, Kuang SY, He X, Groopman JD, Jackson PE. 2002. Sensitivity of electrospray ionization mass spectrometry detection of codon 249 mutations in the p53 gene compared with RFLP. Cancer Epidemiol Biomarkers Prev 11(10, Pt 1):1126–1129.

Rasmussen T, Kuehl M, Lodahl M, Johnsen HE, Dahl IM. 2005. Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors. Blood 105:317–323.

Rasmussen T, Haaber J, Dahl IM, Knudsen LM, Kerndrup G, Lodahl M, Johnsen HE, Kuehl M. 2010. Identification of translocation products but not K-RAS mutations in memory B cells from multiple myeloma patients. Haematologica (in press).

Robison K. 2010. Application of second-generation sequencing to cancer genomics. Brief Bioinform (in press).

Roulland S, Lebailly P, Lecluse Y, Briand M, Pottier D, Gauduchon P. 2004. Characterization of the t(14;18) BCL2-IGH translocation in farmers occupationally exposed to pesticides. Cancer Res 64:2264–2269.

Roulland S, Navarro JM, Grenot P, Milili M, Agopian J, Montpellier B, Gauduchon P, Lebailly P, Schiff C, Nadel B. 2006. Follicular lymphoma-like B cells in healthy individuals: A novel intermediate step in early lymphomagenesis. J Exp Med 203:2425–2431.

Rowley JD. 1973. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293.

Rowley JD, Potter D. 1976. Chromosomal banding patterns in acute nonlymphocytic leukemia. Blood 47:705–721.

Russom A, Tooke N, Andersson H, Stemme G. 2005. Pyrosequencing in a microfluidic flow-through device. Anal Chem 77:7505–7511.

Schmid A, Kortmann H, Dittrich PS, Blank LM. 2010. Chemical and biological single cell analysis. Curr Opin Biotechnol 21:12–20.

Schmitt C, Balogh B, Grundt A, Buchholtz C, Leo A, Benner A, Hensel M, Ho AD, Leo E. 2006. The bcl-2/IgH rearrangement in a population of 204 healthy individuals: Occurrence, age and gender distribution, breakpoints, and detection method validity. Leuk Res 30:745–750.

Schrock E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. 1996. Multicolor spectral karyotyping of human chromosomes. Science 273:494–497.

Schuler F, Dolken G. 2006. Detection and monitoring of minimal residual disease by quantitative real-time PCR. Clin Chim Acta 363: 147–156.

Schuler F, Dolken L, Hirt C, Kiefer T, Berg T, Fusch G, Weitmann K, Hoffmann W, Fusch C, Janz S, Rabkin CS, Dolken G. 2009. Prevalence and frequency of circulating t(14;18)-MBR translocation carrying cells in healthy individuals. Int J Cancer 124:958–963.

Shah SP, Xuan X, DeLeeuw RJ, Khojasteh M, Lam WL, Ng R, Murphy KP. 2006. Integrating copy number polymorphisms into array CGH analysis using a robust HMM. Bioinformatics 22:e431–e439.

Shendure J, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, Wang MD, Zhang K, Mitra RD, Church GM. 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. Science 309:1728–1732.

Shi J, Liu Q, Sommer SS. 2007. Detection of ultrarare somatic mutation in the human TP53 gene by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification. Hum Mutat 28:131–6.

Sieben VJ, Debes Marun CS, Pilarski PM, Kaigala GV, Pilarski LM, Backhouse CJ. 2007. FISH and chips: Chromosomal analysis on microfluidic platforms. IET Nanobiotechnol 1:27–35.

Sieben VJ, Debes-Marun CS, Pilarski LM, Backhouse CJ. 2008. An integrated microfluidic chip for chromosome enumeration using fluorescence in situ hybridization. Lab Chip 8:2151–2156.

Sims CE, Allbritton NL. 2007. Analysis of single mammalian cells on-chip. Lab Chip 7:423–440.

Speicher MR, Gwyn Ballard S, Ward DC. 1996. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet 12:368–375.

Sreekantaiah C. 2007. FISH panels for hematologic malignancies. Cytogenet Genome Res 118:284–296.

Steinemann D, Arning L, Praulich I, Stuhrmann M, Hasle H, Stary J, Schlegelberger B, Niemeyer CM, Flotho C. 2010. Mitotic recombination and compound-heterozygous mutations are predominant NF1-inactivating mechanisms in children with juvenile myelomonocytic leukemia and neurofibromatosis type 1. Haematologica 95:320–323.

Stephens PJ, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ, Greenman CD, Jia M, Latimer C, Teague JW, Lau KW, Burton J, Quail MA, Swerdlow H, Churcher C, Natrajan R, Sieuwerts AM, Martens JW, Silver DP, Langerod A, Russnes HE, Foekens JA, Reis-Filho JS, van't Veer L, Richardson AL, Borresen-Dale AL, Campbell PJ, Futreal PA, Stratton MR. 2009. Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462:1005–1010.

Szymanska K, Lesi OA, Kirk GD, Sam O, Taniere P, Scoazec JY, Mendy M, Friesen MD, Whittle H, Montesano R, Hainaut P. 2004. Ser-249TP53 mutation in tumour and plasma DNA of hepatocellular carcinoma patients from a high incidence area in the Gambia, West Africa. Int J Cancer 110:374–379.

Szymanska K, Chen JG, Cui Y, Gong YY, Turner PC, Villar S, Wild CP, Parkin DM, Hainaut P. 2009. TP53 R249S mutations, exposure to aflatoxin, and occurrence of hepatocellular carcinoma in a cohort of chronic hepatitis B virus carriers from Qidong, China. Cancer Epidemiol Biomarkers Prev 18:1638–1643.

Szymanska K, Moore LE, Rothman N, Chow WH, Waldman F, Jaeger E, Waterboer T, Foretova L, Navratilova M, Janout V, Kollarova H, Zaridze D, Matveev V, Mates D, Szeszenia-Dabrowska N, Holcatova I, Bencko V, Le Calvez-Kelm F, Villar S, Pawlita M, Boffetta P, Hainaut P, Brennan P. 2010. TP53, EGFR, and KRAS mutations in relation to VHL inactivation and lifestyle risk factors in renal-cell carcinoma from central and eastern Europe. Cancer Lett 293:92–98.

Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, Kotsopoulos SK, Samuels ML, Hutchison JB, Larson JW, Topol EJ, Weiner MP, Harismendy O, Olson J, Link DR, Frazer KA. 2009. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol 27:1025–1031.

Thomas RK, Baker AC, Debiasi RM, Winckler W, Laframboise T, Lin WM, Wang M, Feng W, Zander T, MacConaill L, Lee JC, Nicoletti R, Hatton C, Goyette M, Girard L, Majmudar K, Ziaugra L, Wong KK, Gabriel S, Beroukhim R, Peyton M, Barretina J, Dutt A, Emery C, Greulich H, Shah K, Sasaki H, Gazdar A, Minna J, Armstrong SA, Mellinghoff IK, Hodi FS, Dranoff G, Mischel PS, Cloughesy TF, Nelson SF, Liau LM, Mertz K, Rubin MA, Moch H, Loda M, Catalona W, Fletcher J, Signoretti S, Kaye F, Anderson KC, Demetri GD, Dummer R, Wagner S, Herlyn M, Sellers WR, Meyerson M, Garraway LA. 2007. High-throughput oncogene mutation profiling in human cancer. Nat Genet 39:347–351.

Tonisson N, Zernant J, Kurg A, Pavel H, Slavin G, Roomere H, Meiel A, Hainaut P, Metspalu A. 2002. Evaluating the arrayed primer extension resequencing assay of TP53 tumor suppressor gene. Proc Natl Acad Sci USA 99:5503–5508.

Vardiman JW. 2009. Chronic myelogenous leukemia, BCR-ABL1+. Am J Clin Pathol 132:250–260.

Venter JC. 2010. Multiple personal genomes await. Nature 464:676–677.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafha V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A, Zhu X. 2001. The sequence of the human genome. Science 291:1304–1351.

Ventura RA, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason DY, Siebert R. 2006. FISH analysis for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded tissue. J Mol Diagn 8:141–151.

Voelkerding KV, Dames SA, Durtschi JD. 2009. Next-generation sequencing: From basic research to diagnostics. Clin Chem 55:641–658.

Vogelstein B, Kinzler KW. 1993. The multistep nature of cancer. Trends Genet 9:138–141.

Vogelstein B, Kinzler KW. 1999. Digital PCR. Proc Natl Acad Sci USA 96:9236–9241.

Wada H, Asada M, Nakazawa S, Itoh H, Kobayashi Y, Inoue T, Fukumuro K, Chan LC, Sugita K, Hanada R, Akuta N, Kobayashi N, Mizutani S. 1994. Clonal expansion of p53 mutant cells in leukemia progression in vitro. Leukemia 8:53–59.

Walsh CS, Ogawa S, Scoles DR, Miller CW, Kawamata N, Narod SA, Koeffler HP, Karlan BY. 2008. Genome-wide loss of heterozygosity and uniparental disomy in BRCA1/2-associated ovarian carcinomas. Clin Cancer Res 14:7645–7651.

Wang J, Wang W, Li R, Li Y, Tian G, Goodman L, Fan W, Zhang J, Li J, Guo Y, Feng B, Li H, Lu Y, Fang X, Liang H, Du Z, Li D, Zhao Y, Hu Y, Yang Z, Zheng H, Hellmann I, Inouye M, Pool J, Yi X, Zhao J, Duan J, Zhou Y, Qin J, Ma L, Li G, Zhang G, Yang B, Yu C, Liang F, Li W, Li S, Ni P, Ruan J, Li Q, Zhu H, Liu D, Lu Z, Li N, Guo G, Ye J, Fang L, Hao Q, Chen Q, Liang Y, Su Y, San A, Ping C, Yang S, Chen F, Li L, Zhou K, Ren Y, Yang L, Gao Y, Yang G, Li Z, Feng X, Kristiansen K, Wong GK, Nielsen R, Durbin R, Bolund L, Zhang X, Yang H. 2008. The diploid genome sequence of an Asian individual. Nature 456:60–65.

Weibel DB, Whitesides GM. 2006. Applications of microfluidics in chemical biology. Curr Opin Chem Biol 10:584–591.

Welsh JA, Castren K, Vahakangas KH. 1997. Single-strand conformation polymorphism analysis to detect p53 mutations: Characterization and development of controls. Clin Chem 43:2251–2255.

Wheeler DA, Srinivasan M, Egholm M, Shen Y, Chen L, McGuire A, He W, Chen YJ, Makhijani V, Roth GT, Gomes X, Tartaro K, Niazi F, Turcotte CL, Irzyk GP, Lupski JR, Chinault C, Song XZ, Liu Y, Yuan Y, Nazareth L, Qin X, Muzny DM, Margulies M, Weinstock GM, Gibbs RA, Rothberg JM. 2008. The complete genome of an individual by massively parallel DNA sequencing. Nature 452:872–876.

Williams C, Ponten F, Ahmadian A, Ren ZP, Ling G, Rollman O, Ljung A, Jaspers NG, Uhlen M, Lundeberg J, Ponten J. 1998. Clones of normal keratinocytes and a variety of simultaneously present epidermal neoplastic lesions contain a multitude of p53 gene mutations in a xeroderma pigmentosum patient. Cancer Res 58:2449–2455.

Wu S, Zhu Z, He J, Luo X, Xu J, Ren-Heidenreich L. 2010. A novel mutant-enriched liquidchip technology for the qualitative detection of somatic mutations in KRAS gene from both serum and tissue samples. Clin Chem Lab Med 48:1103–1106.

Yamamoto G, Nannya Y, Kato M, Sanada M, Levine RL, Kawamata N, Hangaishi A, Kurokawa M, Chiba S, Gilliland DG, Koeffler HP, Ogawa S. 2007. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. Am J Hum Genet 81:114–126.

Yamanoshita O, Kubota T, Hou J, Ping YM, Zhang XL, Li XP, Li SS, Li XX, Zhu DC, Fukushima Y, Nakajima T. 2005. DHPLC is superior to SSCP in screening p53 mutations in esophageal cancer tissues. Int J Cancer 114:74–79.

Yeang CH, McCormick F, Levine A. 2008. Combinatorial patterns of somatic gene mutations in cancer. FASEB J 22:2605–2622.

Young KH, Leroy K, Moller MB, Colleoni GW, Sanchez-Beato M, Kerbauy FR, Haioun C, Eickhoff JC, Young AH, Gaulard P, Piris MA, Oberley TD, Rehrauer WM, Kahl BS, Malter JS, Campo E, Delabie J, Gascoyne RD, Rosenwald A, Rimsza L, Huang J, Braziel RM, Jaffe ES, Wilson WH, Staudt LM, Vose JM, Chan WC, Weisenburger DD, Greiner TC. 2008. Structural profiles of TP53 gene mutations predict clinical outcome in diffuse large B-cell lymphoma: An international collaborative study. Blood 112:3088–3098.

Zeng Y, Novak R, Shuga J, Smith MT, Mathies RA. 2010. High-performance single cell genetic analysis using microfluidic emulsion generator arrays. Anal Chem 82:3183–3190.

Zhu YM, Foroni L, McQuaker IG, Papaioannou M, Haynes A, Russell HH. 1999. Mechanisms of relapse in acute leukaemia: Involvement of p53 mutated subclones in disease progression in acute lymphoblastic leukaemia. Br J Cancer 79:1151–1157.