Digital quantification of mutant DNA in cancer patients

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Purpose of review
The accumulation of somatic mutations is the major driving force for tumorigenesis. These mutations uniquely differentiate tumor cells from their normal counterparts. Mutations within tumor cells and mutant DNA released by tumor cells into blood, lymph, stool, tissues and other bodily compartments can thereby be used for cancer detection. Here we discuss technologies available for the detection and quantification of mutant DNA in clinical samples and the value of such measurements for patient management.
Recent findings
Conventional mutation detection technologies are either qualitative or only roughly estimate the abundance of mutant DNA molecules. Recently-developed approaches, however, use single molecule counting to determine the genotype of each individual member of a DNA population, providing a more accurate and precise digital output.
Summary
In this review, we discuss the clinical utility of mutant DNA quantification in cancer patients in the context of recent technical advances made in digital mutation detection.

Keywords
biomarker, molecular diagnostics, mutation, quantification, single molecule detection

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Abbreviations

APC	adenomatosis polyposis coli
ATP	adenosine triphosphate
BCR-ABL	breakpoint cluster region-Abelson
EBV	Epstein-Barr virus
dHPLC	denaturing high performance liquid chromatography
EGFR	epidermal growth factor receptor
FRET	fluorescence resonance energy transfer
PCR	polymerase chain reaction
PSA	prostate specific antigen
RCA	rolling-circle amplification
SBE	single base extension
SMD	single molecule detection

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Introduction

The molecular basis of cancer is rapidly being deciphered and recent studies suggest that a complex array of genetic alterations exist in most human cancers [1]. Genetic alterations including gene deletions, gene amplifications, point mutations, and chromosomal rearrangements play a major role in the development and progression of cancers and are therefore unique identifiers that distinguish tumor cells from their normal counterparts.

In order to best use gene alterations as a biomarker in clinical oncology, it must be possible to detect the tumor-specific genetic changes in a background of DNA from normal cells. Gene deletions and amplifications consist of copy number alterations, instead of changes in the primary DNA sequence and thus are not easily distinguishable from DNA of normal cells. Detection and enumeration of gene copy number changes therefore require tumor cell isolation or direct cellular visualization. For example, in-situ hybridization or laser capture microdissection followed by real-time polymerase chain reaction (PCR) is used for the quantification of *v-erb-b2* erythroblastic leukemia viral oncogene homolog 2 amplifications in breast cancer tissue [2].

Unlike deletions and amplifications, point mutations and chromosomal rearrangements represent changes of the primary DNA sequence that are substantially different from normal DNA and thus can be detected within clinical samples without the need for prior tumor cell isolation or visualization. Mutation detection has often been performed qualitatively without the ability to quantify the amount of mutant and wild-type DNA present in the sample. Qualitative mutation detection assays can have two potential problems. First, an assay could yield a false-negative result because the amount of starting DNA is too low to detect minority mutations. Second, an assay could yield a stochastic false-positive result because rare random mutations are present in a sample. Quantitative technologies could overcome these problems. They have the ability to directly measure the number of DNA molecules tested per assay and therefore ensure that the amount of starting material is sufficient to detect minority mutations in the predicted frequency range. Quantification also allows one to distinguish between random and pathogenic mutations by establishing a baseline mutant-to-wild-type ratio (e.g. the background mutation frequency of human cells), where mutations found at a ratio below the baseline are considered random. Quantitative assays also allow standard

quality control monitoring which is a necessary requirement for routine diagnostic testing [3•].

Several quantitative mutation detection technologies are currently in development, and translational studies are needed to demonstrate whether quantification of mutant DNA is beneficial for early detection and as a biomarker of malignancy. Technologies attempting to quantify cancer mutations and their potential clinical application are the focus of this review.

Mutation detection and quantification principles

Over the past decade, many techniques have been developed for the analysis of mutated DNA. The impetus for this effort has been the desire to increase sensitivity, specificity and efficiency while decreasing cost. Analytical sensitivity and specificity are parameters that refer to the lowest amount of mutant DNA that can be detected in a high background of normal DNA and the ability to exclusively detect actual mutations, respectively. Assay efficiency and cost are linked and refer to the time, labor and cost required for each analysis. The performance of the currently available technologies varies considerably. To better understand the variability, we grouped them based on (i) how many mutations can be analyzed per assay, (ii) the order of amplification and allele discrimination, and (iii) the quantitative nature of the assay signals.

Screening and locus specific mutation detection

Screening or scanning methods can detect a range of possible mutations in a target region. They are particularly important for the discovery of new cancer genes [4]. For this application, the sensitivity of standard Sanger sequencing is sufficient. The identification of minority mutations, however, requires higher sensitivities and specificities. Current scanning technologies are in general not sensitive enough, due to larger numbers of positions analyzed. Once a new mutation is associated with a certain cancer, it is usually sufficient to detect it by locus-specific methods. Locus-specific assays have the advantage of being more sensitive, cheaper and easier to perform. Several assays that can be used for the quantification of known and unknown mutations are described below.

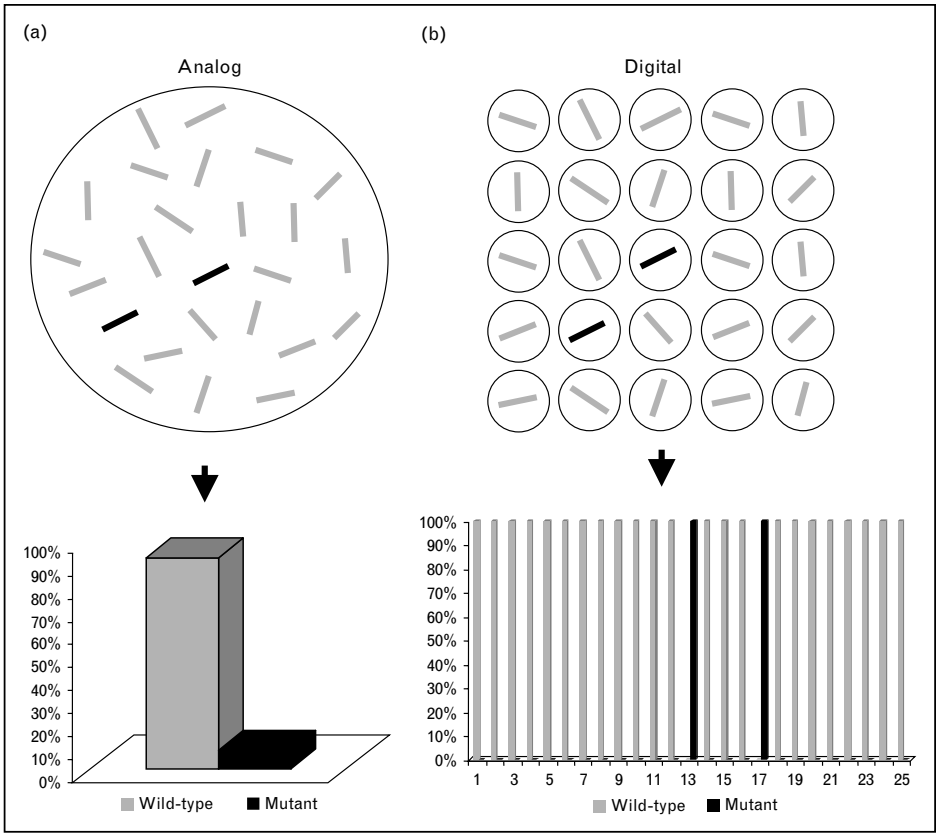
Direct and indirect allele discrimination

Most techniques rely on the following four components: (i) amplification of the target sequence, (ii) discrimination of the mutant and wild-type DNA sequences, (iii) separation, and (iv) detection. Approaches that amplify the DNA before genotype discrimination are named indirect detection methods. The amplification is most commonly accomplished by the use of PCR and therefore suffers from typical PCR-associated complications, such as the generation of random errors by the DNA polymerase and allele bias that results in poor quantitative accuracy. Direct methods, on the other hand, discriminate the alleles prior to the amplification or detection step. The presence or absence of the mutation is determined by allele-specific methods. DNA ligases, DNA polymerases and DNA nucleases, as well as thermodynamic differences between matched and mismatched DNA duplexes are used to discriminate between alleles. The initial allele discrimination step greatly influences the specificity and sensitivity of any direct mutation detection test. For some assay platforms, the DNA molecules need to be separated before detection by binding to a solid phase or by gel electrophoresis. Unfortunately, the separation step introduces the risk of cross-contamination and requires additional handling of the samples. An assay format that avoids these problems is a homogeneous test that does not require separation and can combine the amplification, discrimination and detection step essentially in a single tube [5].

Analog and digital quantification of mutations

As discussed above, the assay strategy determines the specificity and sensitivity as well as the precision and accuracy of allele quantification. These parameters also depend on the nature of the assay signal generated. Traditional genotyping assays determine the identity of a particular base as an average contribution to a heterogeneous population of DNA molecules. Thus, such methods only convey an 'analog' signal for the individual members of the DNA pool. If more than one genotype is queried at a time, a ratio can be calculated between the different alleles present in the reaction (Fig. 1a). Most assays available today are analog and have been reviewed elsewhere [5–7]. Here, we present examples of assays that have recently been used for quantification of mutations in clinical samples. Pyrosequencing, for example, is a nucleotide extension sequencing approach where pyrophosphate is generated when a particular nucleotide anneals to the template and is incorporated by DNA polymerase. Subsequently, pyrophosphate is converted to adenosine triphosphate (ATP) by ATP sulfurylase, which provides the energy for luciferase to oxidize luciferin and generate light. The intensity of the light is proportional to the amounts of annealed and extended nucleotide molecules. The peak sizes of the pyrogram are used to quantify the relative amount of each allele down to a mutant to wild-type ratio of 5% [8]. Another example of analog mutation detection is a DNA endonuclease-based assay followed by denaturing high performance liquid chromatography (dHPLC). The quantitative output signals are the peak sizes on the dHPLC chromatograms. The assay can be used to quantify unknown mutations if present above 1% [9]. Two final examples are the LigAmp assay and the Scorpion primer-based quantitative PCR assay which can

Figure 1 Principle of analog and digital mutation analysis



(a) In analog assays, an average signal is acquired from the mutant and wild-type DNA molecules present in the sample. The ratio between the mutant and wild-type signal is an estimate of the mutation frequency. (b) In digital assays, the genotype of the individual DNA molecules is determined separately. Counting is used to quantify the mutant and wild-type DNA molecules present in the sample.

quantify mutations down to 0.01% [10,11••]. As homogeneous assays, these measure in real-time the accumulation of mutant and wild-type PCR product during each cycle. In the log-linear phase of the amplification the amount of the target DNA correlates with the initial copy numbers.

A more precise and accurate approach to mutation quantification is based on discrete counting of the mutant and wild-type alleles present in a sample. Techniques incorporating this methodology are termed 'digital' as they are able to generate binary results (mutation present or absent) on each individual member of a DNA pool (Fig. 1b) [12,13••]. The quantitative precision and accuracy of digital assays is limited only by the number of molecules being analyzed. Statistics become important for counting rare events, which is the case when rare mutant alleles are present in a high background of wild-type molecules. Based on the Poisson distribution, the standard deviation of the number of rare events equals the square root of the number of detected events. Only the measured background events need to be subtracted from the positives to get the net counts. This phenomenon has been called Poisson noise, and it limits the precision of single molecule detection methods [14]. The precision can be improved only by increasing the number of molecules analyzed. For example, a precision of 10% would require the detection of 100 mutant molecules per measurement. Considering a sensitivity limit of 0.01% for the detection of mutant DNA in plasma, this would require the analysis of a total of 1×10^6 DNA molecules. This translates into 3 μg of human genomic DNA, which exceeds the amount of DNA present in most plasma samples [15••].

Digital mutation quantification methods

Various approaches have been described that allow the confined digital analysis of single molecules. We will review direct and indirect methods that can be used for mutation quantification in DNA fragments at known or unknown base positions (Table 1).

Table 1 Digital assays applied to mutation detection

Technique	Assay principle	Amplification	Discrimination	Separation	Detection	Sensitivity[b]	Gene	References
Ligation and FRET	Direct, locus-specific	NA	Ligation of molecular beacons	NA	Microfluidic device	1/1000	*K-ras*	[22]
Ligation and RCA	Direct, locus-specific	RCA	Ligation of oligonucleotides	NA	Fluorescence Microscope	1/500–1500	*CFTR*	[16]
Invasive cleavage and gold nanoparticles	Direct, locus-specific	NA	Invasive cleave	NA	Scanning electron microscope	ND	*CFTR*	[23•]
Restriction digest and digital PCR	Direct, locus-specific	PCR in solution	Restriction digest	Magnetic separation	Real-time PCR machine	1/100 000[a]	*p53*	[19•]
Digital PCR and molecular beacon hybridization	Indirect, locus-specific	PCR in solution	Molecular beacons	NA	Fluorescence plate reader	1/100–1000	*K-ras*	[29–32]
Digital PCR and molecular beacon hybridization	Indirect, locus-specific	Emulsion, PCR on magnetic beads	Molecular beacons	Magnetic separation	Flow cytometry	1/1000	*K-ras*	[36]
Digital PCR and single base extension	Indirect, locus-specific	Emulsion, PCR on magnetic beads	Single base extension	Magnetic separation	Flow cytometry	1/10 000	*APC*	[15••]
Digital PCR, RCA and single base extension	Indirect, locus-specific	Emulsion, PCR on magnetic beads	Single base extension	Magnetic separation	Flow cytometry	1/10 000	*K-ras, PIK3CA, p53*	[38•]
Digital PCR and protein truncation test	Indirect, scanning for stop codons	PCR in solution	Protein truncation test	PAGE	Gel scanner	1/500	*APC*	[33,34]
Digital PCR and pyrosequencing	Indirect, scanning	Emulsion, PCR on nonmagnetic beads	Pyrosequencing	NA	GS20, 454 Corp.	1/1000	*EGFR*	[40••]

NA, not applicable; ND, not determined.
[a] Quantification of mutant population only;
[b] Mutant/wild-type.

Direct digital mutation detection

Direct digital quantification methods are based on the discrimination of the genotype of single DNA molecules followed by single molecule amplification and/or detection. As described above, the enzymatic or physical methods used to distinguish mutant from wild-type sequences limit the sensitivity and specificity of these assays. For example, the initial allele discrimination of DNA molecules immobilized on glass slides can be done using a ligation assay [16] or a surface-invasion cleavage assay [17]. These strategies can at best detect one mutant in 1500 wild-type molecules. After the allele discrimination reaction, allele-specific circularized single-stranded DNA molecules are hybridized to the ligation or cleavage products and used as a template for subsequent rolling-circle amplification (RCA). RCA produces a long single-stranded concatemerized molecule containing multiple copies of the complementary circular starting sequence. The amplified DNA sequences are then labeled by hybridizing allele-specific fluorescent oligonucleotides to the RCA products. The image of the slide taken after the labeling is used to count the individual molecules. Recently, a similar solution-based approach was used for the digital analysis of DNA from pathogens [18••]. This homogeneous test has the potential to be adapted to mutation detection and quantification. Briefly, the assay uses ligation for the circularization of padlock probes, subsequent RCA, and a microfluidic device attached to a fluorescence microscope to count the molecules. Another type of assay allows the absolute quantification of unknown mutations in DNA fragments, but not the number of wild-type DNA molecules [19•]. The test is based on capturing the target sequences on beads coated with complementary probes, selective digestion of wild-type molecules, and the subsequent quantification of intact mutant molecules by digital PCR (see below). The high sensitivity of this test (1/100 000) can only be accomplished by performing several rounds of mutant enrichment, which unfortunately makes the assay difficult to use for clinical applications.

Several years ago, fluorescence-based single molecule detection (SMD) approaches were introduced. These are based on the direct visualization of individual fluorescently labeled DNA molecules without the need for enzymatic amplification [20,21••] So far only one of these approaches has been used for the enumeration of point mutations in solution [22]. The assay is based on the allele-specific ligation of fluorescence resonance energy transfer (FRET) probes generating molecular beacons upon successful ligation. A laser-based fluorescence system attached to a heatable microfluidic device is used to detect photon bursts generated by the FRET reaction. SMD technologies have also been developed for the analysis of single DNA molecules immobilized on solid

supports. Currently, there is only one base-specific approach described in the literature that could readily be applied to the enumeration of mutations. The test is based on a surface-invasion cleavage assay followed by the detection of mutant alleles using gold nanoparticles [23•]. Recently, technologies for the sequencing of single DNA molecules on solid support have also been introduced. These are intended for the low-cost resequencing of complete genomes, but could eventually be adapted for the quantification of genetic variations in clinical samples [24–26]. Most notable are two approaches that use cyclic array sequencing. These rely on the extension of a DNA template hybridized to immobilized primers by a polymerase in the presence of fluorescently-labeled nucleotides [25,27]. Unfortunately, the sensitivity of these methods is limited by the error rates of the DNA polymerases currently available for sequencing. These error rates are at least one log higher compared with DNA polymerases conventionally used for PCR.

Indirect digital mutation detection

Indirect digital mutation quantification involves an initial compartmentalized amplification of single DNA molecules followed by allele discrimination and detection. Cloning of DNA fragments followed by the sequencing of individual bacterial colonies is the most basic form of digital analysis. Unfortunately, this approach is time-consuming and labor-intensive. In 1988, it was demonstrated that PCR can be efficiently performed on single DNA templates [28]. This opened the way for several applications, reviewed elsewhere [12,13••]. One such technique, which came to be known as digital PCR, proved to be a powerful tool for single molecule counting and quantification of somatic mutations in clinical samples [29]. In digital PCR assays, multiple PCR reactions are performed in parallel at DNA concentrations so low that most reactions contain zero or one template molecule and thus can be amplified clonally. Each resulting DNA pool is then analyzed individually for the presence of mutant and wild-type sequences by using fluorescent allele-specific molecular beacons. The digital PCR approach has been used for detection of *K-ras* mutations in various clinical samples [29–32]. Protein truncation tests which can be used to scan for stop codons within a target sequence have also been combined with digital PCR [33,34]. In contrast to more conventional mutation detection methods, the sensitivity of digital PCR approaches is not limited by the DNA polymerase error rate. In the worst-case scenario, an error occurs in the first cycle of a double-stranded single molecule PCR reaction which will result in a mutant to wild-type ratio of one to four. This would translate to a mixed mutant/wild-type signal in the subsequent genotyping assay and thus be excluded from the calculation. The sensitivity is instead limited by the number of molecules that can be analyzed and the false-positive rate of the mutation detection assay. In principle, the latter has the least influence on the detection limit as the analysis only needs to distinguish analytes that are exclusively wild-type or exclusively mutant. The main limitation of the originally described digital PCR techniques was the cost and labor involved in performing a large number of individual PCR reactions. To address these issues, several methods allowing millions of single-molecule PCR reactions to be performed in a single assay have been developed. One way to achieve this is by performing single-molecule PCRs in a thin polyacrylamide film poured on a glass microscope slide [35]. The amplification results in discrete DNA colonies in the polymer matrix (polonies). Another approach involves BEAMing (beads, emulsions, amplification and magnetics) which allows single-molecule PCR reactions to be performed on magnetic beads in water-in-oil emulsions [36]. An alternative way to generate beads coated with clonally amplified PCR products is by adding single beads into wells of a picotiter plate that can be used for digital PCR [37]. The bead suspension obtained after solid-phase PCR accurately reflects the DNA diversity present in the template population and can therefore be used for mutation quantification. Recently, BEAMing followed by single base extension (SBE) and flow cytometry was used to quantify the level of mutated DNA circulating in the plasma of colorectal tumor patients [15••]. The detection limit of this assay was one mutant DNA molecule in a background of 10 000 wild-type DNA molecules. This threshold was determined by the error rate of the DNA polymerase used for the preamplification of the limited amounts of plasma DNA. The sensitivity of BEAMing-based assays can also be constrained by the signal-to-noise ratio of the bead-based SBE assay. Therefore, RCA was used to increase the DNA copy number on the beads, making the SBE assay more specific [38•]. Besides analyzing a mutation at a specific location, the DNA on beads can also be used as a template for sequencing. 454 Life Sciences developed an approach to sequence DNA on individual beads in parallel [39••]. This system is commercially available and has been used for the identification and quantification of *epidermal growth factor receptor* (*EGFR*) gene mutations in lung cancer biopsies [40••]. Another strategy used DNA coated beads immobilized in a polyacrylamide gel as a template for cycle sequencing by ligation [41••].

The clinical application of counting mutations

Cancers are currently managed by a variety of clinical markers, which generally include patient symptoms, radiographic evaluation, routine laboratory tests and pathologic evaluation. These markers are used not only for diagnosis, but also as prognostic and predictive markers, for tumor staging, and as markers for tumor response and detection of residual disease. More sensitive and specific biomarkers could aid cancer diagnosis and man-

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