High Sensitivity Detection of Tumor Gene Mutations


DiaCarta, Inc. 3535 Breakwater Avenue, Hayward, California, USA

Gene Mutations and Cancer

It is widely accepted that cancer develops as a result of mutations in cell growth pathway related genes particularly those mutations that occur in growth factor receptor pathways such as the cell surface Epidermal Growth Factor Receptor (EGFR) and Fibroblast Growth Factor Receptor (FGFR) pathways and in tumor suppressor genes such as p53, APC and PTEN. Detection of these mutations is critical not only for early detection of cancerous cells but also for decisions on what therapy should be applied to treat the patient. For example, mutations in the intracellular downstream signaling proteins rat sarcoma viral oncopenes KRAS, NRAS and HRS can produce resistance to a particular therapeutic agent. In this context of ‘Precision Medicine’ tumor gene mutations have classically been measured in the DNA isolated from formalin-fixed paraffin embedded (FFPE) tumor biopsy tissue. However, surgical tumor biopsies are not only invasive and risky, but are extremely difficult for inaccessible and fragile organs such as the lungs. A recent study confirmed that this standard prognostic procedure is woefully inadequate [1]. A localized tumor biopsy could miss mutations in a distal region of the tumor that might radically change a person’s chances for survival. And although biopsies can provide data about specific mutations that might make a tumor vulnerable to targeted therapies, that information is static and bound to become inaccurate as the cancer evolves. Minimally invasive procedures such as taking blood is simple in comparison, urine sampling is even simpler. Several groups have reported that the mutational landscape of a patient’s tumor can be measured simply by monitoring the mutational status of the circulating cell-free tumor DNA (ctDNA) in the patient’s blood. However, this requires a highly sensitive technique and to date most large oncology research centers have resorted to BEAMing PCR2 to achieve this. This is partly because tumor DNA is much harder to detect in the circulation due to the large excess of wild-type DNA. There is typically less of it in the blood. In people with very advanced cancers, tumors might be the source of most of the circulating DNA in the blood, but more commonly, ctDNA makes up barely 1% of the total and possibly as little as 0.01%. Several groups in recent years have reported using ctDNA to study patients who were being treated with EGFR inhibitors. Looking for example for known KRAS mutations that confer resistance; or for mutations that prevent drugs from binding to their target [3-5].

The classical “gold standard” for tumor mutational analysis; DNA sequencing is not entirely satisfactory for detecting low frequency mutations in ctDNA. In fact it is one of the least sensitive methods for characterizing mutation. For DNA sequencing, a mutation must be present in 10-20% of the sample to be readily detected. Below this threshold, tumorigenic mutations go undetected. Such is the case with colon tumors, most of which are polyclonal and heterogeneous. In one study of colon tumors, investigators affiliated with the FDA concluded that tumorigenic mutations may be undetectable using standard DNA sequencing methods [6].

To improve sensitivity, several alternative approaches have been developed. These include developments in real-time PCR-based detection methods, such as allele-specific PCR (ASPCR) and hydrolysis-based probes [7]. Although these techniques show better sensitivity, lowering the detection threshold to at least 5%, they still fall short of a crucial goal—detecting a mutation that is present in less than 1% of tissue samples. All these techniques are inadequate because they cannot eliminate the large excess of wild-type genomic DNA present in the samples which leads to a high background signal. Droplet digital PCR (ddPCR) [8] has been developed in an effort to improve the sensitivity of PCR so that it can reach below a detection limit of 0.1% mutated DNA. The technology makes millions of droplets to separate the mutant DNA from wild-type genomic DNA. However, some droplets generated can contain both wild-type and mutated DNA. The presence of such droplets poses a problem when one is working with clinical samples. Wild-type DNA continues to form a large background, so sensitivity does not quite reach below 0.1% sensitivity on a routine basis and is not without some issues. Some of the key performance features of the various methods are summarized in (Table 1) [7 to 17].

A New Molecular Paradigm: QClamp Xeno-Nucleic Acid Clamped PCR

To reduce the wild-type background and improve sensitivity, a molecular clamp has been designed to hybridize selectively to wild-type template DNA and block its amplification. This molecular clamp consists of a synthetic, sequence-specific Xeno-nucleic acid (XNA) probe. It is called QClamp”. In the presence of a mutation such as a single nucleotide polymorphism (SNP) gene deletion,

*Corresponding author: Michael Powell J, DiaCarta, Inc. 3535 Breakwater Avenue, Hayward, California, USA, Tel: 510-314-8859; E-mail: mpowell@diacarta.com

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In addition, it can detect low frequency genetic mutations (<0.05%) in DNA samples obtained from patient tumor biopsy or whole blood samples. This level of sensitivity enables detection of gene mutations in the oncology therapeutic clinical setting utilizing patient biopsy, surgical tissue, or formalin fixed, paraffin-embedded (FFPE) tissue.

**Table 1.** Currently available methods for determining cancer gene mutation status.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%) (mutant/wild type)</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dideoxy sequencing</td>
<td>10–30</td>
<td>Currently considered the ‘gold-standard’ method for genotypic analysis</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>≤5</td>
<td>Provides short reads of sequence (40–50 nucleotides), but better analytical sensitivity than dideoxy-sequencing methods</td>
</tr>
<tr>
<td>PCR/PCR + allele-specific probes</td>
<td>10</td>
<td>Use of selective probes to distinguish mutations from wild-type sequence</td>
</tr>
<tr>
<td>Amplification refractory mutation system (ARMS)</td>
<td>1–5</td>
<td>Good sensitivity, requires specifically engineered primer and probe combinations</td>
</tr>
<tr>
<td>PCR amplification with high-resolution melt–curve analysis</td>
<td>10–20</td>
<td>Fast turnaround time, open and/or closed system with regard to reagent usage</td>
</tr>
<tr>
<td>Multiplex PCR and array hybridization</td>
<td>1–10</td>
<td>Bead or linear array detection</td>
</tr>
<tr>
<td>COLD-PCR (Coamplification at lower denaturation temperature)</td>
<td>~1</td>
<td>COLD-PCR to selectively amplify mutant alleles in a background of wild-type sequences.</td>
</tr>
<tr>
<td>Allele-specific PCR with peptide nucleic acid probes</td>
<td>1–5</td>
<td>Use of peptide nucleic acid-clamping probes to enhance sensitivity of mutation detection</td>
</tr>
<tr>
<td>Digital PCR and digital droplet PCR</td>
<td>~0.1</td>
<td>Requires specialized chemistry and instrumentation and highly skilled operators</td>
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As discussed earlier, mutations in growth factor receptor pathways can guide a physician's choice for a patient's precision therapy for a particular cancer. Lung cancer is the leading cause of cancer-related deaths worldwide. There are two main types, of which non-small-cell-lung-cancer (NSCLC) constitutes approximately 80% of cases. Patients with NSCLC usually present with advanced, metastatic disease. If left untreated, they have a median survival of less than 5 months. Even with chemotherapy, which is often associated with significant toxicity, survival is usually prolonged by less than 6 months. The introduction of targeted therapy such as tyrosine kinase inhibitors (TKIs) has improved survival in a subset of NSCLC patients [18,19]. It is known that patients who respond to TKI harbor activating mutations, notably in the kinase domain of (Exons 18-21) of the epidermal growth factor receptor (EGFR) gene [20-22]. Given that exon 19 deletions and the exon 21 L858R mutations account for approximately 85% of clinically important EGFR mutations, the need to detect these mutations present in minute quantities in plasma or serum against a background of wild-type (wt) sequences is imperative. Employing xenonucleic acid (XNA) clamp probes designed to inhibit amplification of wild-type EGFR Exon 18-21 templates around the clinically relevant mutation sites Exon 19 del and L858R a typical QClamp real-time PCR assay profile result is shown (Figure 3) which demonstrates the high sensitivity and specificity of the assay. The QClamp XNA clamp probes can span from 14 to 25 nucleotides of target sequence and the QClamp EGFR assay detects all clinically relevant deletions seen in exon 19. The QClamp method is highly sensitive and can detect below 0.1% Exon 19del and L858R in a background of wild-type. In comparison scorpion-amplification refractory mutation system (scorpion-ARMS) can detect only 3.7-4.8% L858R in plasma samples [23].

The high sensitivity of QClamp and the ability to be performed on regular widely available real-time PCR instrumentation platforms such as ABI 7500, Roche LC480, Rotorgene Q etc. makes it highly attractive for clinical applications were a rapid Sample Answer is needed for precision medicine applications.

**QClamp PCR Enrichment for DNA Sequencing**

QClamp can also be employed to enrich samples for mutant templates prior to Sanger Sequencing or Next Generation Sequencing (NGS) applications. Detection sensitivities can easily and efficiently be reduced to <0.1% simply by the addition of wild-type XNA clamp probes to the pre-sequencing PCR reaction. As an illustrative example Sanger DNA sequencing currently has a sensitivity of 20-24% with addition of an XNA clamp probe to the pre-sequencing PCR reaction that spans the wild-type sequence in KRAS exon 2 codon 12 and codon 13 allows the sensitivity of detection of c12 and c13 mutations to be increased to <0.1%. A typical sequencing trace obtained by Sanger sequencing of the PCR amplicon,
generated from DNA extracted from an FFPE sample that was originally called wild-type by Sanger DNA sequencing before QClamp PCR but was in fact G12D mutant is shown (Figure 4).

Recent publications highlight the utility of this approach for detection of KRAS mutations in ctDNA in pancreatic cancer patients [24], discovery of a rare BRAF mutation (V600_K601 delinsE) in a fine-needle aspirate biopsy from thyroid cancer patient [25] and detection of low frequency KRAS mutations in CRC patient tumor biopsies [26].

**Figure 3.** QClamp EGFR assay performed with template DNA containing Exon 19del or L858R mutations at the allelic frequencies shown. Real-time PCR profiles A = EGFR Exon 19del amplification plots, B = EGFR Exon 19del melting profiles, C = EGFR L858R amplification plots, D = EGFR L858R melting profiles, clearly demonstrating detection sensitivity <0.1%.

QClamp PCR based sample enrichment will have wide utility in improving clinical studies utilizing DNA sequencing or Next Generation Sequencing (NGS) for patient stratification or for targeted therapy.
References


7. Glaab WE, Skopek TR (1999) A novel assay for allelic discrimination that combines the fluorescent 5’ nuclease polymerase chain reaction (TaqMan®) and mismatch amplification mutation assay. Mutation Research430 (1); 1-12.


