Clinical validation of qPCR Target Selector™ assays using highly specific switch-blockers for rare mutation detection

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ABSTRACT

Aims The identification of actionable DNA mutations associated with a patient's tumour is critical for devising a targeted, personalised cancer treatment strategy. However, these molecular analyses are typically performed using tissue obtained via biopsy, which involves substantial risk and is often not feasible. In addition, biopsied tissue does not always reflect tumour heterogeneity, and sequential biopsies to track disease progression (eg, emergence of drug resistance mutations) are not well tolerated. To overcome these and other biopsy-associated limitations, we have developed non-invasive 'liquid biopsy' technologies to enable the molecular characterisation of a patient's cancer using peripheral blood samples.

Methods The Target Selector ctDNA platform uses a real-time PCR-based approach, coupled with DNA sequencing, to identify cancer-associated genetic mutations within circulating tumour DNA. This is accomplished via a patented blocking approach suppressing wild-type DNA amplification, while allowing specific amplification of mutant alleles.

Results To promote the clinical uptake of liquid biopsy technologies, it is first critical to demonstrate concordance between results obtained via liquid and traditional biopsy procedures. Here, we focused on three genes frequently mutated in cancer: *EGFR* (Del19, L858, and T790), *BRAF* (V600) and *KRAS* (G12/G13). For each Target Selector assay, we demonstrated extremely high accuracy, sensitivity and specificity compared with results obtained from tissue biopsies. Overall, we found between 93% and 96% concordance to blinded tissue samples across 127 clinical assays.

Conclusions The switch-blocker technology reported here offers a highly effective method for non-invasively determining the molecular signatures of patients with cancer.

INTRODUCTION

Large-scale efforts to obtain genome sequencing data from patient tumours have revealed an array of DNA mutations associated with specific cancer types. This information has led to the development of targeted therapies that counteract a subset of these molecular alterations, and patients harbouring such mutations benefit dramatically. For example, patients with non-small cell lung cancer (NSCLC) generally do not respond to chemotherapy, resulting in an extremely poor prognosis. However, 14%–38% of NSCLC tumours are associated with mutations in the epidermal growth factor receptor (EGFR),[1] and treating these patients with EGFR tyrosine kinase inhibitors (TKIs) significantly extends overall survival from 10.8 to 24.3 months.[2]

A number of practical constraints can prevent the identification of treatable genetic alterations in patients with cancer. Fine-needle biopsies, common in lung cancer, frequently lack sufficient tissue for molecular testing. Performing a second biopsy is one way of remedying this situation, but these procedures are associated with significant risk and cost, and some patients are either unable or unwilling to tolerate such an invasive solution. In addition, because tumours are heterogeneous, biopsied tissue often does not reflect the complete genomic landscape of a patient's disease. Targetable molecular aberrations may be present in regions of the tumour not sampled, or metastatic tumours may be molecularly distinct from the primary tumour. Finally, because resistance to targeted therapies eventually emerges, postprogression biopsies are needed to select a secondary course of treatment, exposing patients to additional discomfort and risk. Alternatives to traditional tissue biopsies are therefore needed to ensure that all patients receive the benefits of molecular diagnostic tests, both at diagnosis and following disease progression.

Scientific advances now enable the capture of tumour content for molecular analyses via a simple peripheral blood draw, a technique generally referred to as a liquid biopsy. Liquid biopsies offer a complementary approach to traditional biopsies, in many cases alleviating the tissue challenges noted above. Biocept has developed liquid biopsy assays for detecting tumour-associated genetic mutations within circulating tumour DNA (ctDNA), which is released into the peripheral blood by apoptotic or necrotic tumour cells. Biocept's Target Selector ctDNA platform uses a real-time PCR-based approach to detect low frequency mutant alleles. This system uses a patented blocking approach to suppress amplification of wild-type DNA, while allowing the specific amplification of mutant alleles. Assays have been developed to detect mutations in three genes frequently mutated in cancer: *EGFR*, *BRAF* and *KRAS*.

Mutations in the *EGFR* gene are associated with numerous cancers, including colorectal, anal, head and neck, breast, ovarian, brain, prostate and lung, with two of these hotspots accounting for a vast majority of cases including >90% of these cases: a

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point mutation in exon 21 (L858R) and a deletion within exon 19 (Del19).[3] These mutations function through constitutive activation of the kinase domain in EGFR, and EGFR-TKIs are generally effective in these patients. However, resistance inevitably emerges, often via the T790M secondary mutation, and therapies targeting this alteration have now been developed. *KRAS* is a member of the Ras family of small GTPases and is frequently mutated during tumour progression. Activating *KRAS* mutations are found in >90% of pancreatic cancers,[4–6] 35%–45% of colon cancers[7] and ~25% of lung cancers.[8] In patients with advanced colorectal cancer or NSCLC, *KRAS* mutations are associated with resistance to anti-EGFR antibody therapy[9] and poor survival.[10] As a result, it is important to test for mutations in the *KRAS* gene to determine eligibility for EGFR-targeting therapies.

Finally, the BRAF serine/threonine kinase is mutated in a range of cancers that include colorectal, thyroid, lung, ovarian and melanoma.[11] Detection of *BRAF* V600 mutations can predict response to anti-EGFR antibody agents in colon cancer,[12 13] determine prognosis in thyroid and colon cancers[14] and inform the selection of targeted BRAF inhibitors.[15]

Thus, determining whether the *EGFR, KRAS* and *BRAF* genes are mutated in a patient's tumour, both at diagnosis and progression, can indicate which therapeutic intervention may provide the most benefit.

In this study to evaluate the clinical usefulness of the Target Selector liquid biopsy ctDNA platform as a complementary approach to traditional tissue biopsies, we analysed blood samples taken from patients who had previously received a traditional biopsy.

The Target Selector ctDNA assays are real-time PCR-based enrichment assays and are based on selective blocking of wild-type amplification with a switch-blocker that contains a switch and an anchor portion. In contrast to blocked wild-type amplification, mutant templates are readily amplified in the presence of the same switch-blocker allowing for selective enrichment. A mismatch in the mutant template leads to a significant reduction in the melting temperature of the switch portion of the blocker allowing the forward primer to extend with the mutant template. Under the same conditions with wild-type, the forward primer is blocked, due to the stability of the switch region which is fully complementary to wild-type. When the temperature is raised above the melting temperature of the forward primer, as shown in figure 1, the non-extended forward primer on the wild-type template falls off before the switch-blocker dissociates. However, when a mutant template is present, the extended forward primer has a high enough melting temperature allowing it to remain bound to the template beyond the point that the switch begins to open, thus extending further and supporting amplification. Detection of the amplification product is accomplished by unquenching of the fluorophore (F) signal when the blocker is bound to the template. The location of the quencher (Q) is at the 5' end of the switch region. For wild-type amplification, primers and a blocker for a region on *EGFR* exon 20 were used. Since any mutation of the DNA template in the region covered by the switch portion of the blocker can potentially lead to amplification of this template, Sanger sequencing was used to confirm the presence and identity of the specific mutation. The amplification products were purified after the qPCR Target Selector assay and used for templates in Sanger sequencing reactions to confirm the presence of the specific mutation.

Switch-blockers were designed as described previously.[16] Briefly, the anchor portion contains modifications to prevent it

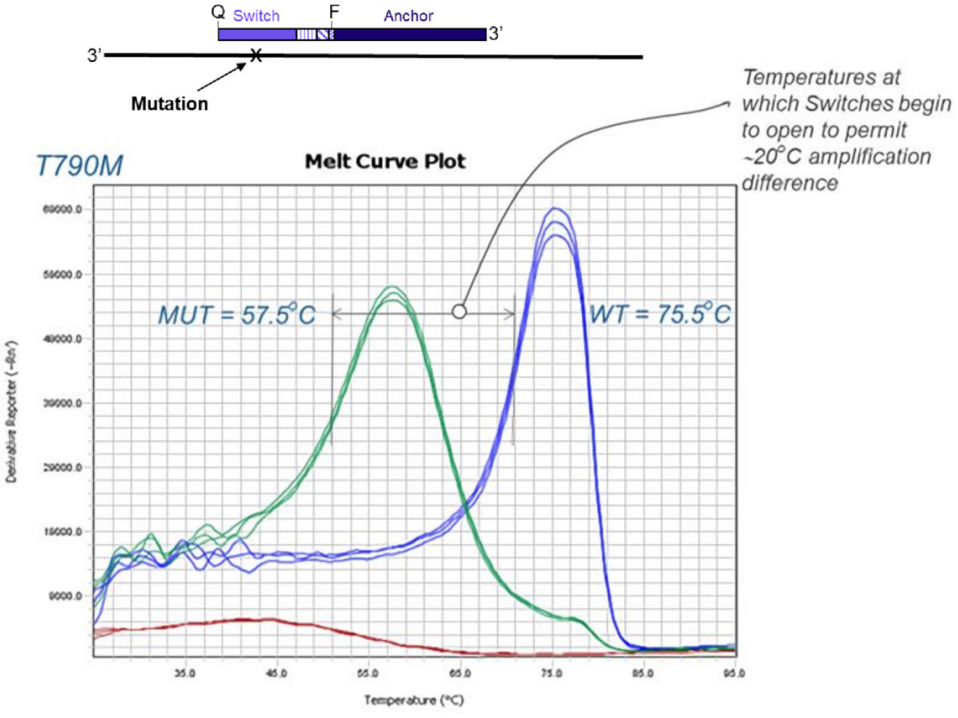

Figure 1 A melt profile illustrating the thermodynamic properties of a switch-blocker when hybridised to wild-type compared with mutant. Shown here is a switch-blocker targeting T790M where the switch is CATCACGCAG. This shows the vast differences for switch opening due to a single C/A mismatch wherein A is the mutation in the target strand associated with T790M. The C highlighted in red is the wild-type sequence, which shows switch closed to about 75°C. The fluorophore (F) is quenched by the quencher (Q) as the switch opens. The melt profiles shown here are plotted as derivative curves such that peaks become the T_ms for the corresponding mutant and wild-type targets. Adapted from our previously published analytical validation study using this same technology.[21]

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from serving as a primer on its 3' end and to block digestion by 3' exonuclease repair enzymes. The bridging portion of the switch-blocker comprises nucleotide or non-nucleotide moieties that do not have Watson-Crick base pair and they span four or more bases relative to the target sequence. The switch portion comprises 7 to about 13 nucleotides and optionally contain modifications to increase duplex affinity thus enabling switches on the shorter size to be used, which increases specificity. Additionally, the switch-blockers contain a quencher and fluorophore separated by about 15–18 nucleotides to allow them to report during real-time PCR amplification.

MATERIALS AND METHODS

Extraction of circulating cell-free DNA from plasma

Blood was collected in CEE-Sure tubes (Biocept, San Diego, California, USA). The plasma fraction was isolated by two centrifugations (3000 g, 5 min, 25°C followed by 16 000 g, 10 min, 4°C) and stored at −80°C as appropriate, prior to further processing. ctDNA was isolated from 3 mL of plasma using the QIAamp circulating nucleic acid kit by manual extraction with vacuum manifold. Purified ctDNA was eluted in 50 µL, and 3 µL aliquots were used in individual Target Selector assays. Target Selector assays were then run as described previously.

Sequencing of Target Selector assay products

After qPCR, Target Selector assay products were purified using the MinElute PCR purification kit (QIAGEN) and purified dsDNA PCR products quantified on a NanoDrop. Thirty nanograms of purified PCR product were used in Sanger cycle sequencing reactions using BigDye Terminator 1.1 mix and the corresponding sequencing primer for each of the assays. After purification on Centri-Sep spin columns, the fragments were analysed on a 3500Dx Genetic Analyzer using POP-7 polymer.

For clinical samples, blood or plasma samples were obtained from patients with breast, colorectal or lung cancer who had previously been subjected to a tissue biopsy where molecular analyses included *EGFR*, *BRAF* and *KRAS*.

RESULTS

The Target Selector platform for ctDNA is enabled using a unique switch-blocker construct that is a combination of a probe and steric blocker. As previously discussed, switch-blockers are tripartite oligonucleotides that contain an anchor portion, a bridging portion and a switch region (figure 2). In addition to consisting of three parts, a quencher and fluorophore are positioned to span the switch portion of the switch-blocker. When the entire switch-blocker hybridises to amplification products at lower temperatures (50°C range), the quencher and fluorophore are separated, leading to a fluorescent signal. When the switch opens, a significant portion of the fluorescence is quenched as the quencher comes into closer contact with the fluorophore. When the entire switch-blocker fully separates from the target, additional quenching of the fluorophore occurs (figure 2).

Overall, switch-blockers are used to block amplification of targets that are perfect complements to the switch region, while targets that contain even a single nucleotide variant are fully amplified. This is made possible by using the unique thermodynamic properties of oligonucleotides.

In the tripartite structure of switch-blockers, the bridging portion is sufficiently long, spanning four nucleotides or more, such that the anchor and switch regions hybridise as essentially independent elements. At the same time, the anchor portion is approximately 30 nucleotides in length, which brings the entire switch-blocker construct, with high specificity, to the exact position where the genetic alteration is to be selectively amplified. Most significantly from a thermodynamics standpoint, hybridisation of the anchor sequence massively increases the local concentration of the switch in the regions where blocking of perfectly matched target sequence is to occur. For purposes of illustration, once the anchor has hybridised, the tether length between the 5' end of the anchor and the switch is approximately 45 Å. This confines the switch to a spherical volume element defined by $4/3\pi r^3$, where r=45 Å. Mathematically, this translates to a local concentration of the switch at approximately 4 mM. Since approximately 0.25 µM probes concentrations are generally used in hybridisation reactions, this represents a 16 000-fold

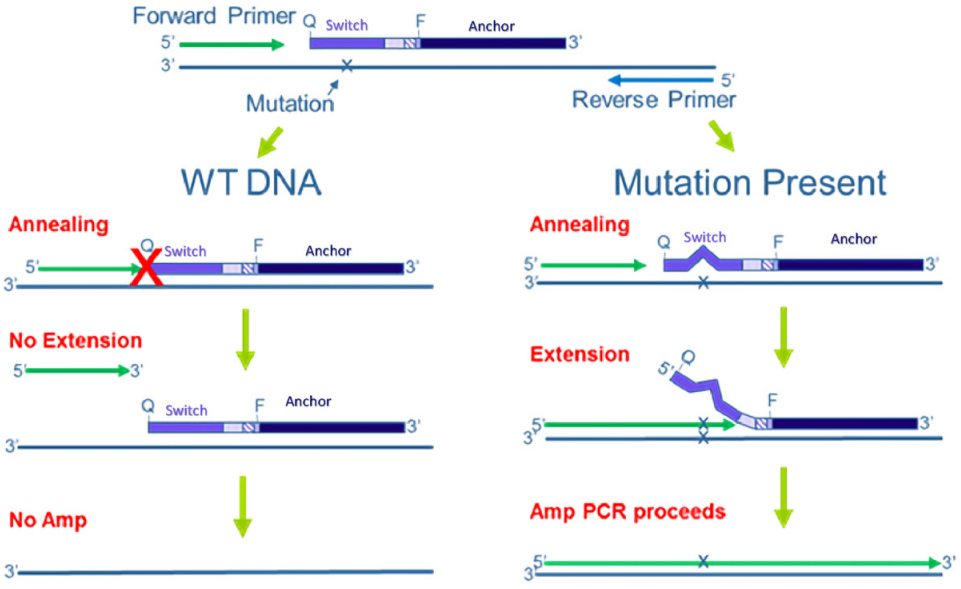

Figure 2 A diagram showing the principle of a switch-blocker to block wild-type (WT) amplification, while not blocking mutant amplification. When the target is WT, the switch is fully complementary, resulting in the arrest of amplification. When a mutation occurs within the switch, the switch opens during the extension step in PCR amplification, allowing mutant sequences to be fully amplified.

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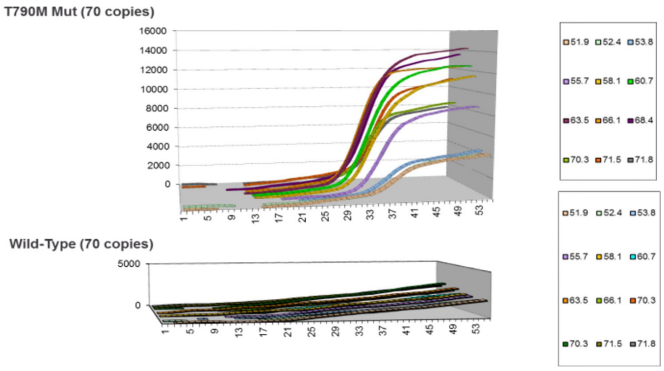

Figure 3 An illustration of the amplification discrimination when switch-blockers are used to suppress wild-type targets, while allowing mutant targets to amplify fully. Displayed here are real-time PCR amplification curves with mutant (T790M) and wild-type targets. The fluorescent read-outs occur on amplification with either mutant or wild-type targets and are detected using the switch-blockers at lower temperatures (~50°C). Shown here is a slight background drift of fluorescence with wild-type target, but no amplification from 52°C to 72°C. Over this same temperature range, there is good to excellent amplification of the mutant G/T associated with T790M.

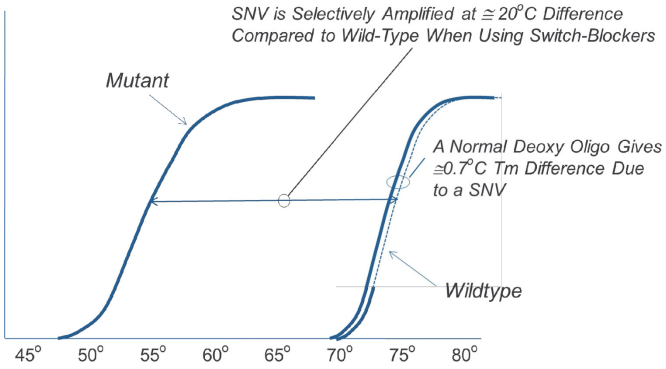

Figure 4 A diagram of the observed amplification profile for mutant and wild-type targets from the results shown in figure 3. For comparison, the expected discrimination that would be expected for a single nucleotide variant (SNV) when using a normal deoxy oligonucleotide that had a T_m of 73°C would be approximately 0.7°C . Clearly, the switch-blocker approach has far higher discrimination capability compared with conventional deoxy oligonucleotides.

increase in local concentration of the switch relative to its corresponding target sequence. This increase in switch concentration relative to the target concentration is equivalent to an increase in T_m of 30°C or greater (Oligo Calc., Northwestern), from 28°C to 58°C or more. When combined with other switch modifications to increase affinity, the T_m of the 10mer switch is increased even more to approximately 75°C for a perfectly matched wild-type target to give a huge temperature discrimination of wild-type versus mismatch targets (figure 2). Key, however, is that the specificity of the short 10mer switch is maintained, wherein a single mismatch is highly destabilising, such that a single G/T mismatch causes a T_m drop of 18°C. At the same time, switch-blockers are used with enzymes that are easily sterically blocked, such that switch-closed, blocks amplification, and switch-open allows amplification to occur (figure 1.) This effect is shown using real-time PCR reaction in figure 3, where the permissive temperatures for a mismatch (G/T) amplification reaction (switch-open) ranges from about 52°C to 72°C, while wild-type (perfect match/switch-closed) is fully blocked to amplification over the same temperature range. This vast temperature difference permits the simple selection of temperatures where the mismatch targets amplify fully (switch-open during extension) and wild-type target are essentially completely blocked to amplification (switch-closed during extension). This unprecedented mismatch discrimination difference is illustrated in figure 4. Additionally, figure 4 shows the temperature discrimination that would be expected, based on nearest neighbour calculation with a median of about 0.7°C (range 0.4°C–1.4°C) for a normal deoxy oligonucleotide that would have a T_m around 73°C.

The ability of the switch-blocker methodology to detect rare, minor alleles is further shown in figure 5. Figure 5 shows a dilution series of a T790M target, using real-time PCR detection in a switch-blocker amplification assay. The dilution series gives a perfectly linear response relative to target T790M copies from 7000 to 7 copies. Also, shown in this figure are theoretical plots of what would have been expected if there had been 1% (420 copies), 0.1% (42 copies), 0.01% (4.2 copies) as copy equivalents of wild-type target breakthrough, if wild-type targets were not essentially completely suppressed. This figure alone demonstrates that wild-type targets are suppressed >10 000-fold relative to amplification of a single G/T mutations. It also demonstrates that the linear dilution series of mutant, all the way down to 7 mutant copies is not impacted by the presence of 42 000 copies of wild-type. In essence, the wild-type target is so completely suppressed that it has no effect on the mutant dilution series.

Additionally, the ability to essentially completely block wild-type amplification, while not blocking mutant amplification, allows the use of a variety of mismatch discrimination methods that have very poor sensitivity to be used downstream of switch-blocker amplification reactions to vastly increase the sensitivity of these generally poorly sensitive discrimination methods. These include Sanger sequencing, microarrays, mass-spectrometry

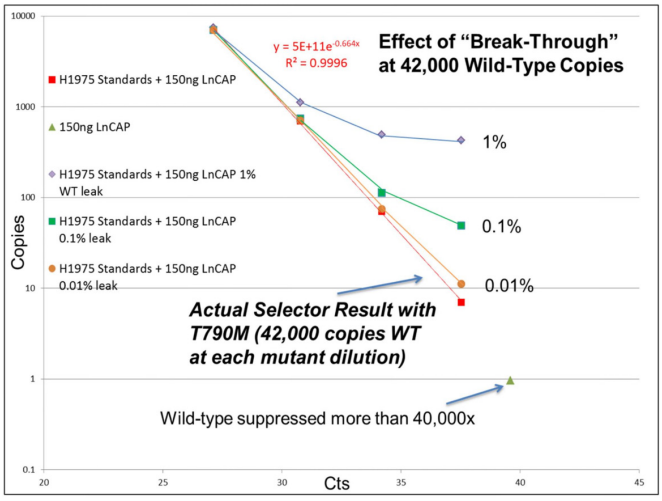

Figure 5 Demonstration of the ability to precisely detect mutant targets in a vast excess of wild-type (WT) target. The dilution for the mutation shown here in red is the average of triplicate data points and it shows essentially perfect linearity when plotted against CTs obtained from real-time PCR assays. The blue, green and orange lines are depictions of what would have been expected if there had been 1%, 0.1% and 0.01% 'breakthrough' of the 42 000 copies of WT present in the assay. WT was suppressed here to less than a single copy, or >40 000-fold.

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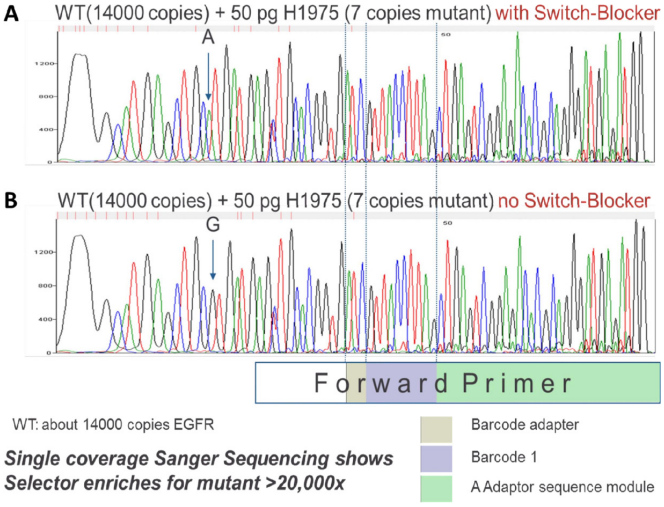

Figure 6 Switch-blocker amplification. (A) When a switch-blocker is present that spans the T790M region, only the mutant (A) is detected. In fact, the wild-type (WT) sequence is so completely suppressed that there is no hint of it at the 790 position, even though it was initially present at 99.95% allele prevalence. This suppression of WT represents >20 000-fold selective enrichment of the mutation allele using the switch-blocker methodology. (B) In contrast, the lower panel shows the amplification result using Sanger sequencing in the absence of a switch-blocker when 14 000 copies of WT (99.95%) and 7 copies of mutant T790M (0.05%) are present in an assay. Since the sensitivity of Sanger for detecting minor alleles is approximately 20%, there is no chance that a 0.05% minor allele frequency (MAF) will be detected, and only the WT (G) allele is detected. At the bottom of panel B are shown sequences embedded during amplification to facilitate next-generation sequencing, if desired. EGFR, epidermal growth factor receptor.

(mass-spec), capillary methods and even next-generation sequencing (NGS) at lower levels of sequence coverage.

The ability of the switch-blocker technology to increase the sensitivity of detection of downstream analysis methods is illustrated in figure 6. This figure illustrates visually the sensitivity of Sanger sequencing alone and in combination with switch-blocker amplification. In the lower panel of figure 6B are the Sanger outputs in the absence of the switch-blocker when interrogating 7 copies (0.05%) of mutant T790M in the presence of 14 000 copies (99.95%) of wild-type. As mentioned above, Sanger sequencing can typically detect only about 20% of a minor allele at any nucleotide location. As a result, Sanger sequencing cannot detect a mutant at the 790 amino acid position, when it is present at only 0.05% minor allele frequency (MAF). In contrast, as can be seen in figure 6A, when amplification is carried out in the presence of a switch-blocker targeting the region of 790, only a mutant nucleotide is seen, which is correctly identified as T790M. Overall, in figure 6A, under the influence of a switch-blocker, the T790M mutation has been so completely selectively amplified that there is now no hint of wild-type at the 790 position, even though it was originally present at 99.95%. Taking into consideration the 20% sensitivity of Sanger sequencing for detecting minor alleles, this result demonstrates >20 000-fold selective amplification of the mutant relative to wild-type.

While other related blocker strategies have been employed in the past, such as PNA blockers(ref) and minor groove binder (MGB) conjugates they do not have the ability to block wild-type as effectively as switch-blockers. Due to the high degree of wild-type blocking, we are able to use both allele-specific, as well as regional blocking of wild-type sequences. The challenge with allele-specific assays is that a specific assay is required to interrogate each allele of interest. In the case of KRAS, for example, this could require up to 40 different assays to test for the mutations present in just codons 12 and 13 of exon 2. In the case of KRAS, when using regional or 'foot-print' blocking with switch-blockers, only a single blocker assay is required. This is the mode of the assay we prefer to use, and in this mode, the forward primer lies outside of the region where the mutations. In this way, the amplification reactions are 'agnostic' to the mutations that are present. That said, the switch-blocker constructs can also be used to carry out allele-specific amplification reactions where the forward primer is a perfect match on its 3' end for the allele of interest that is being detected. Even in the allele-specific mode, we found that switch-blockers increased the sensitivity of detection >30-fold, even though these reactions were purportedly allele specific, due to the allele-specific primer (data not shown). Separately, we found that we could carry out allele-specific reactions under the same cycling conditions as our 'foot-print' mode assays such that both assay formats could be run on the same plate, at the same time. For the reasons stated above, however, we routinely use 'foot-print' mode assays due to their agnostic performance relative to the mutations being interrogated, and due to the fact that for a series of possible mutation in a region of interest, only a single assay is needed. This greatly increases the cost-effectiveness and ease of development of these assays.

Other factors that reduce the sensitivity of previously used steric blocking assays that use, for example, PNA oligos or minor groove binder (MGB) conjugates, are issues related to amplification errors and cytosine deamination. In the case of amplification errors, we employ high fidelity enzymes to reduce amplification errors >100-fold compared with conventional TagMan amplification reactions. Our ability to completely block wild-type also helps reduce amplification errors, since if the wild-type is highly blocked to amplification, there is little opportunity for amplification errors to occur in wild-type targets.

At the same time, cytosine deamination poses a significant obstacle to reducing the performance of other steric blocking assays. It has gone largely unappreciated that even relatively short periods of heating of nucleic acids can deaminate cytosine.[17] Once deaminated, cytosine residues become deoxyuridine that are 'read' by most polymerases as thymidine, and, as a result, create cytosine to thymine transversions that were not present in the original biological material. These tranversions then appear as mutations that, in fact, are really not there. To overcome this challenge, we have engineered the switch-blocker assays to reject templates whenever cytosine deamination has occurred. By combining all these features, we are able to develop analogue-type assays with extremely high sensitivity. Related to this, we have recently reported on the ability to detect single copy mutations in a background of 10 000–15 000 copies of wild-type molecules.[18]

In summary, these improvements include the following. First, almost complete suppression of wild-type amplification (this is due to the large mismatch discrimination window of about 20 degrees that are unique to switch-blockers). Second, suppression of amplification errors. Finally, prevention of cytosine deamination products serving as templates for amplification.

While a rigorous side-by-side comparison to other methodologies using the same clinical samples is not practical, we can approximate these comparisons using performance data that have been published by others. In table 1 are comparison data for a number of other assays including digital PCR, Beaming,

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Table 1 ctDNA platform comparison

Methodology	Limit of detection	Diagnostic offering
Allele specific		
Droplet digital PCR (ddPCR)[22]	0.01%–0.02%	
BEAMing[23]	0.01%	OncoBEAM (Sysmex)
ARMS-PCR[24]	0.05%–0.1% 25–100 copies per mL 1.42% median MAF	Cobas EGFR V.2 (Roche)[25 26] Therascreen EGFR (QIAGEN)[27 28]
TaqMan[29]	0.01% 1–10 copies	
Non-allele specific/'foot-print'		
NGS, targeted	≥0.04% MAF for SNVs ≥0.125% MAF for SNVs	Guardant360[30] FoundationOneLiquid[31] *Note: In a recent publication comparing 4 NGS platforms they become discordant below 1% MAF.*[32]
Switch-blocker (Biocept/Aegea)	0.01%–0.02%	
PNA clamp PCR[33]	0.1%–1%	
LNA clamp PCR[34]	0.1%–1%	

ctDNA, circulating tumour DNA ; MAF, minor allele frequency; NGS, next-generation sequencing; SNV, single nucleotide variant.

Cobas assays (ARMS), TagMan assays, as well as NGS platforms. These can be used to compare assay performance with our switch-blocker assays.

Switch-blocker assays are as sensitive as allele-specific assays that depend on a combination of a blocker as well as allele-specific forward primers to achieve this same performance. Switch-blockers achieve this performance in both allele-specific and 'foot-print' modes. As a result, the switch-blocker assays are more sensitive than far more expensive 'foot-print' mode assays, like NGS, and as sensitive as allele-specific assays that require single assays for single alleles.

We routinely validate the assay performance of switch-blocker assays at 0.05% MAF. This is routinely done at 7 mutant copies in 14 000 wild-type copies. This, of course, is already at MAF levels that are more sensitive than the LOD for many other assays designed for sensitive detection, yet our assays are showing >98% sensitivity and specificity at these MAF levels.

Once the analytical performance of these assays was established, liquid-biopsy results were then compared with data obtained by analysing biopsied tissue. In total, samples from 91 unique patients were used for clinical validation of hotspot mutation assays for *EGFR* (Del19, L858 and T790), *BRAF* (V600) and *KRAS* (G12 or G13). For this analysis, when discordant results were found, we attempted to obtain additional tissue to resolve the discordance. This was successful in one T790M patient who was believed to be negative by tissue, but was found to be positive by our Target-Selector assay using blood as the sample type. On obtaining additional biopsy material, the patient was indeed found to be T790M positive. In other cases, we used digital PCR from an outside vendor, as an orthogonal method to help resolve discrepancies. If the digital PCR using ctDNA confirmed our ctDNA results, we considered our results to be correct. If, however, digital PCR confirmed the tissue results, our ctDNA results were considered as discordant. In a number of cases, due either to the lack of a digital PCR assay, such as BRAF, or due to inadequate amounts of ctDNA, we were not able to resolve contrary results. Even taken these limitations into consideration, we obtain excellent clinical correlations to tissue.

Patients with positive tissue biomarker status had a confirmed diagnosis of lung cancer, melanoma or colorectal cancer. Repeating these molecular analyses using the Target Selector liquid biopsy platform revealed the following: EGFR (del19, L858R, T790M) concordance to tissue results were 93%, sensitivity 83%, specificity 98%, positive predictive value (PPV) 95%, negative predictive value (NPV) 93%, BRAF concordance to tissue results were 93%, sensitivity 75%, specificity 100%, PPV 100%, NPV 91% and KRAS concordance to tissue results were 96%, sensitivity 92%, specificity 100%, PPV 100%, NPV 92% (table 2).

DISCUSSION

Target Selector liquid biopsy assays for detecting *EGFR, KRAS* and *BRAF* mutations yielded results that were highly concordant with those obtained via tissue-based molecular analyses. This indicates that the Target Selector liquid biopsy platform is an effective tool for characterising the molecular drivers of a patient's disease, and can be used to complement traditional tissue biopsy procedures. Incorporating liquid biopsies into the diagnostic procedures will better enable physicians to devise personalised treatment strategies, and to help reduce adverse health events associated with biopsy procedures.

Although obtaining tissue via traditional biopsy remains the gold standard for performing molecular analyses, there are many instances in which biopsy procedures fail to yield enough tissue to perform the relevant diagnostic tests. For example, initial diagnostic biopsy procedures for patients with lung cancer often involve minimally invasive procedures, such as fine-needle aspirations, bronchial washing and bronchial brushing. However, these procedures frequently do not yield enough tissue for molecular testing, so more invasive procedures are then recommended. Many patients cannot tolerate these invasive alternatives because of their poor health, while others are unwilling to subject themselves to the discomfort, risk and cost (in 2012, a thoracic biopsy cost on average US$15 000, and increased to US$60 000 for the 19% of patients who experienced an adverse event.[19] Furthermore, metastatic tumours can arise in regions that preclude traditional biopsy procedures, such as in the brain or bone. For patients in which tissue biopsy procedures have failed or are impracticable, liquid biopsies represent a viable alternative for performing molecular analyses.

Characterisation of multiple tumour biopsies from the same patient have revealed both intratumour and intertumour heterogeneity. This means that a single tumour biopsy may not reveal the entire genomic landscape of a patient's disease. A targetable molecular aberration may only be present in an expanding region of the tumour that was not sampled, resulting in a false negative. In addition, metastatic tumours can have a distinct molecular signature from the primary tumour. It has even been shown that novel resistance mutations can arise in separate metastases, resulting in heterogeneous responses to secondary therapies. Liquid biopsies can capture this spatial heterogeneity, as all tumours within the body release ctDNA. This feature eliminates the guesswork from the biopsy procedure (which tumour to sample, which region of the selected tumour to biopsy), providing a more comprehensive view of the patient's disease.

Once a targeted therapy is initiated, resistance to these therapies inevitably emerges and the disease progresses. Thus, patients undergoing these types of treatments are subjected to frequent CT scans to monitor tumour burden. When progression is detected, subsequent biopsies and molecular analyses can help determine the next course of treatment. For patients initially diagnosed with an *EGFR* activating mutation and placed on a first-generation or second-generation EGFR-TKI, a secondary EGFR mutation, T790M, often drives this progression.

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Table 2

Clinical validation		T790M	
Total population	Condition positive	Condition negative	Prevalence
31	11	20	35%
Test outcome positive	True positive 10	False positive 1	PPV 91%
Test outcome negative	False negative 1	True negative 19	NPV 95%
Accuracy 94%	Sensitivity 91%		
		Specificity 95%	
Clinical validation		**L858R**	
Total population	Condition positive	Condition negative	Prevalence
22	6	16	27%
Test outcome positive	True positive 5	False positive 0	PPV 100%
Test outcome negative	False negative 1	True negative 16	NPV 94%
Accuracy 95%	Sensitivity 83%		
		Specificity 100%	
Clinical validation		**Del19**	
Total population	Condition positive	Condition negative	Prevalence
21	6	15	29%
Test outcome positive	True positive 4	False positive 0	PPV 100%
Test outcome negative	False negative 2	True negative 15	NPV 88%
Accuracy 90%	Sensitivity 67%		
		Specificity 100%	
Clinical validation		**EGFR combined**	
Total population	Condition positive	Condition negative	Prevalence
74	23	51	31%
Test outcome positive	True positive 19	False positive 1	PPV 95%
Test outcome negative	False negative 4	True negative 50	NPV 93%
Accuracy 93%	Sensitivity 83%		
		Specificity 98%	
Clinical validation		**KRAS**	
Total population	Condition positive	Condition negative	Prevalence
25	13	12	52%
Test outcome positive	True positive 12	False positive 0	PPV 100%
Test outcome negative	False negative 1	True negative 12	NPV 92%
Accuracy 96%	Sensitivity 92%		
		Specificity 100%	
Clinical validation		**BRAF**	
Total population	Condition positive	Condition negative	Prevalence
28	6	22	21%
Test outcome positive	True positive 6	False positive 0	PPV 100%
Test outcome negative	False negative 2	True negative 20	NPV 91%
Accuracy 93%	Sensitivity 75%		
		Specificity 100%	

EGFR, epidermal growth factor receptor; NPV, negative predictive value; PPV, positive predictive value.

Thankfully, therapies against this mutation have now been developed,[20] and switching to this new course of treatment can further extend survival. However, patients are often unable or unwilling to subject themselves to multiple rounds of tissue biopsies to guide therapeutic adjustments. Liquid biopsies offer a safe, inexpensive, non-invasive and effective alternative to the CT/biopsy procedure normally used to monitor disease progression. In fact, tracking the molecular evolution of a tumour via liquid biopsy can help predict progression (the T790M mutation will likely be detected before an increase in tumour size is detected via CT scan), enabling rapid adjustment of the therapeutic strategy.

Take home messages

- We demonstrate here the ability to detect cancer-associated mutations with extremely high sensitivity of 0.1%–0.01% or better.
- A compelling question remains, however, as to the need for assays with extremely high sensitivity in clinical practice to inform medical decision making.
- In a recent paper by Mehrotra *et al*, a general conclusion was that assays ranging between 0.01% and 0.1% are needed to adequately monitor patients with solid tumour using circulating tumour DNA (ctDNA).[35]
- Thus, the ultra-high sensitivity reactions, such as switch-blocker assays, are needed to provide the best level of patient management.
- A vast majority of assays for identifying rare cancer-associated mutations using ctDNA fall far short of this requirement.
- Routine next-generation sequencing has sensitivity of about 1%,[36–38] mass-spectrometry about 0.1%–5% and Sanger sequencing about 20%.
- Using the switch-blocker technology described here, we are able to achieve a sensitivity in the range of 0.1%–0.01% at very nominal costs, since the switch-blocker methodology is based on routine PCR amplification.
- At the same time, it is possible to combine multiple switch-blocker assays into single multiplex reactions, to further lower assay costs.

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In addition to combining switch-blocker technology with Sanger sequencing as illustrated here, it can also be combined with mass-spec, NGS, arrays and various gel-based systems, as well as droplet-digital PCR. We routinely use switch-blocker in combination with NGS, and have demonstrated greater than a million-fold selective mutant amplification in combination with a MiSeq (Illumina, San Diego, California, USA) (data not shown). This shows that NGS can be combined with switch-blocker technology to significantly increase sensitivity, while using very low fold coverage. This corresponds to an increase of 100-fold to 1000-fold or more in the efficiency of NGS.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. The accurate clinical detection of actionable mutations in patients with cancer is essential for treatment decisions related to Food and Drug Administration-approved targeted therapies and other precision medicine applications. While traditional tissue biopsies have been considered as the gold standard for molecular profiling, insufficient tissue and tumour heterogeneity often pose barriers towards correct biomarker status determination. Liquid biopsy methodologies address these challenges and provide a complementary approach to tissue testing. However, assays with extremely high sensitivity are key for being able to inform on patients that present with low circulating tumour DNA mutant copy levels. The clinical validation studies presented in this paper demonstrate the utility of a highly sensitive PCR assay that enriches mutants of interest in 'hot-spot' regions of the genome. This assay has sensitivities in the range of 0.01%–0.02% minor allele frequency. In earlier studies, we demonstrated analytically the ability to detect down to single copy mutations. In this study, we report on the clinical validation of this assay, using tissue results as the 'gold standard' when carried out with blinded clinical samples. These assays are more sensitive than most previously reported assays, and do not require that they be allele specific. At the same time, these assays are more sensitive than a vast majority, if not all next-generation sequencing assays, and are comparable or more sensitive than allele-specific digital assays. The availability of these assays for informing on rare, cancer-associated, actionable alterations should be of interest to the broader medical community.

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