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TECHNICAL ADVANCE

Multiplex SuperSelective PCR Assays for the Detection and Quantitation of Rare Somatic Mutations in Liquid Biopsies

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SuperSelective primers, by virtue of their unique design, enable the simultaneous identification and quantitation of inherited reference genes and rare somatic mutations in routine multiplex PCR assays, while virtually eliminating signals from abundant wild-type sequences closely related to the target mutations. These assays are sensitive, specific, rapid, and low cost, and can be performed in widely available spectrofluorometric thermal cyclers. Herein, we provide examples of SuperSelective PCR assays that target eight different somatic *EGFR* mutations, irrespective of whether they occur in the same codon, occur at separate sites within the same exon, or involve deletions. In addition, we provide examples of SuperSelective PCR assays that detect specific *EGFR* mutations in circulating tumor DNA present in the plasma of liquid biopsies obtained from patients with non—small-cell lung cancer. The results suggest that multiplex SuperSelective PCR assays may enable the choice, and subsequent modification, of effective targeted therapies for the treatment of an individual's cancer, utilizing frequent noninvasive liquid biopsies. *(J Mol Diagn 2022, 24: 189—204; https://doi.org/10.1016/j.jmoldx.2021.11.006)*

The identification of particular somatic mutations in a patient's cancer cells enables targeted therapies to selectively destroy specific cancer cells, without significantly harming normal cells that do not possess those mutations.[1,2] Moreover, new somatic mutations arise over time in cancer cells, often necessitating a change in the choice of targeted therapy for treatment to remain effective.[3] These relevant somatic mutations can be identified by an analysis of DNA fragments that transiently appear in a patient's blood plasma as the result of the death of that patient's cancer cells by apoptosis and by necrosis.[4] Significantly, these noninvasive liquid biopsies can be obtained frequently over time, enabling the therapy to be adjusted as needed.[5,6] However, the identification of these relatively rare DNA fragments that possess somatic mutations is difficult because of the presence in the blood sample of abundant closely related

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DNA fragments from normal cells, whose sequences often differ from the target mutant DNA fragments by only a single nucleotide.[7]

Currently, the main means of analyzing cell-free DNA fragments in liquid biopsy samples is the use of next-generation sequencing analysis.[8] This technique is expensive, time-consuming, and requires specialized equipment and experienced personnel. And most significantly, the exponential amplification process occasionally misincorporates nucleotides, generating mutant sequences that were not present in the original sample; and this undesired consequence limits the sensitivity of the resulting assays. Pretreatment of the DNA with a nuclease that selectively destroys the normal fragments improves the situation,[9] but next-generation sequencing analysis is still required.

Conventional PCR assays for the detection of somatic mutations utilize outside primers that amplify both the mutant target sequences and their closely related wild-type sequences. These multiplex assays employ differently colored TaqMan probes or molecular beacon probes to distinguish the mutants from the wild types. However, when the mutant sequence is rare and the closely related wild-type sequence is abundant, as often occurs in liquid biopsy samples from cancer patients, the relatively weak signal from the amplified rare mutant sequence is obscured by the strong signal from the amplified abundant closely related wild-type sequence, limiting the ability to detect the rare mutant sequence. In addition, digital PCR assays, which also amplify both the mutant and the wild type, are currently limited in their ability to be multiplexed.

In the hopes of overcoming these limitations, PCR assays have been developed that are designed to selectively amplify the mutant fragments, while ignoring the closely related wild-type fragments. These selective PCR techniques include the amplification refractory mutation system,[10] which depends on the enzymatic discrimination of perfectly complementary hybrids formed by the PCR primers with the mutant templates, while ignoring the mismatched hybrids that are formed with the closely related wild-type templates. However, the sensitivity of these PCR assays is still limited, because of the effects of keto-enol tautomerism that occasionally enables the wild-type templates to be amplified. A promising, extremely sensitive, alternative approach utilizes switch blockers that selectively bind to the wild-type sequences, preventing their amplification, while enabling the related mutant sequences to be amplified.[11] However, subsequent next-generation sequencing analysis is needed to distinguish the rare somatic mutations present in the sample. Consequently, there is a need to develop simple, multiplex, real-time PCR assays that are rapid, low cost, and extraordinarily sensitive, and that do not require subsequent next-generation sequencing analysis to provide meaningful results.

Our laboratory has developed an alternative multiplex real-time PCR design that is able to detect extremely rare DNA fragments containing somatic mutations in the presence of abundant closely related nonmutant DNA fragments. These unique assays utilize SuperSelective PCR primers that initiate exponential amplification on targeted mutant DNA fragments, but do not initiate amplification on closely related nonmutant DNA fragments.[12] The sensitivity of PCR assays that utilize SuperSelective primers to quantitate rare mutant sequences in the presence of abundant closely related wild-type sequences is far greater than the sensitivity of the approaches described above. Moreover, this increased sensitivity occurs for all rare target sequences amplified with SuperSelective primers in multiplex PCR assays.

Conventional PCR primers are relatively long oligodeoxyribonucleotides that are designed to be complementary to a target sequence, and whose length and sequence are chosen so that, under the relatively high annealing temperature of a PCR assay, they bind to their target sequence, but do not bind to the other DNA sequences in the sample. Unfortunately, conventional PCR primers bind to both the rare mutant sequences and to their closely related wild-type sequences in a liquid biopsy sample. Shorter PCR primers, although thermodynamically much more likely to bind to a perfectly complementary mutant sequence than to form a mismatched hybrid with a closely related target sequence, cannot be used to selectively bind to the mutant target sequences because, due to their shortness, they also bind to many other nontarget genes in the sample.

SuperSelective primers, on the other hand, are designed to satisfy two desired properties of highly discriminatory primers that selectively initiate synthesis only on mutant target sequences. These primers bind only to gene sequences of interest; but by virtue of their unique design, they only initiate exponential synthesis on the mutant target sequences.[12] Their structural design is illustrated in Figure 1. SuperSelective primers are single-stranded DNA oligonucleotides that possess three functional segments. At their 5′ end is an anchor sequence that is virtually identical to the sequence of a conventional PCR primer, in that its length and sequence are chosen to selectively bind only to DNA templates of interest under the relatively high annealing temperature of the PCR assay. At their 3′ end is a relatively short foot sequence that forms a perfectly complementary hybrid with an intended mutant target sequence, but that forms a thermodynamically less stable hybrid with a mismatched closely related sequence. These two separate sequence segments of SuperSelective primers are joined by a bridge sequence that is chosen by the primer designer to not be complementary to the intervening sequence in the DNA template that is located between the anchor target sequence and the foot target sequence. Moreover, the sequence of the bridge is chosen to not be complementary to any other sequence that may occur in the sample. Therefore, under PCR annealing conditions, the bridge sequence in the SuperSelective primer and the intervening sequence, which is located in the template strand to which the SuperSelective primer binds, form a single-stranded bubble that effectively

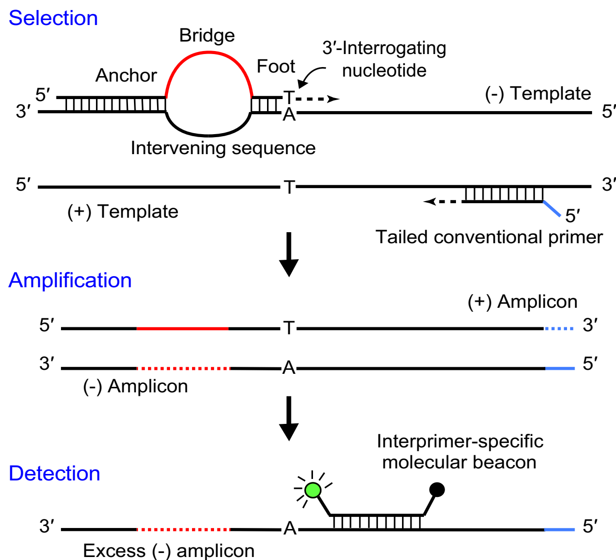

Figure 1 Three aspects of SuperSelective PCR assays. Selection occurs repeatedly during each thermal cycle. SuperSelective primers, by virtue of their unique design, bind to all (−) templates, irrespective of whether those templates are mutant or wild type, but they only initiate (+) amplicon synthesis on the mutant (−) templates. The tailed conventional primers initiate synthesis on all (+) templates and on all (+) amplicons. Exponential amplification of the resulting (+) and (−) amplicons is highly efficient, as the entire SuperSelective primer sequence is complementary to the (−) amplicons, and the entire conventional primer sequence is complementary to the (+) amplicons. Detection is efficient, because the concentration of the SuperSelective primers is limited, and the synthesis of (+) amplicons comes to a halt as thermal cycling proceeds. **Solid red lines** identify the SuperSelective primer's bridge sequence, and **dotted red lines** identify the complement of the bridge sequence. **Solid blue lines** identify the conventional primer's 5′-tail sequence, and **dotted blue lines** identify the complement of the conventional primer's tail sequence.

separates the target-specific binding function of the relatively long anchor sequence from the mutation-selective function of the relatively short foot sequence.

PCR assays that utilize SuperSelective primers are able to detect 10 mutant DNA fragments in a sample containing 10,000 closely related wild-type DNA fragments.[12] There are at least three different elements that enable this inherent degree of selectivity: i) thermodynamically, short perfectly complementary foot hybrids are much more stable than short foot hybrids that possess a mismatched base pair, and are therefore considerably more likely to be present at any given moment; ii) the Brownian motion of the water molecules in the PCR buffer exerts a force on the single-stranded bubble in the primer-template hybrids that tends to pull the foot hybrids apart, and mismatched foot hybrids are more easily pulled apart than perfectly complementary foot hybrids; and iii) although the mechanism is not well understood, the presence of the selectivity enhancing agent tetramethylammonium chloride in the PCR buffer tends to stabilize the perfectly complementary foot hybrids, while destabilizing the mismatched foot hybrids.[13,14]

The net effect of these factors is that the perfectly complementary foot hybrids tend to persist for hundreds of milliseconds before they come apart, which is a sufficient period of time for DNA polymerase molecules to kinetically interact with these foot hybrids, leading to the initiation of amplification; while mismatched foot hybrids tend to persist for only a few milliseconds, which is kinetically an insufficient time period for these hybrids to encounter a polymerase and initiate amplification.

Figure 1 also illustrates the overall design of SuperSelective PCR assays. When bound to a mutant (−) template, an interrogating nucleotide at the 3′ end of the SuperSelective primer is complementary to the single-nucleotide polymorphism present in that mutant (−) template, resulting in a perfectly complementary foot hybrid, which is then extended by DNA polymerase, generating a (+) amplicon. In addition, conventional reverse primers that bind to (+) templates initiate the synthesis of (−) amplicons. Moreover, the conventional reverse primers possess a noncomplementary, G:C-rich 5′-tail sequence that is incorporated into the resulting (+) amplicons, enabling robust synthesis in subsequent rounds of amplification.

After selective initiation has occurred, subsequent rounds of amplification are highly efficient. This is due to the incorporation of the entire SuperSelective primer sequence into the resulting (+) amplicons that occurs when the SuperSelective primer is bound to a mutant (−) template. When that (+) amplicon is copied, the resulting (−) amplicon contains the complement of the entire SuperSelective primer sequence. Because those (−) amplicons possess the complement of the SuperSelective primer's bridge sequence in place of the intervening sequence that was present in the (−) template, subsequent rounds of amplification are primed by the entire SuperSelective primer sequence, as well as by the entire tailed conventional primer sequence.

Moreover, when SuperSelective primers are present in a multiplex PCR assay, the unique bridge sequence of each primer plays a special role. When a particular target is present in an assay and is amplified by a SuperSelective primer, resulting in the complement of that primer's bridge sequence becoming incorporated into the (−) amplicons, none of the other SuperSelective primers that are present in the assay mixture is able to initiate synthesis by hybridizing to those (−) amplicons, because they possess a completely different bridge sequence.

We purposely utilize a limited concentration of the SuperSelective primer and an excess concentration of the conventional reverse primer.[15,16] As a consequence of this nonsymmetrical arrangement, exponential amplification comes to a halt when the SuperSelective primers are depleted, and the synthesis of (+) amplicons ceases. However, because of the excess concentration of the conventional reverse primers, linear synthesis of the (−) amplicons continues, resulting in the presence of more (−) amplicons than (+) amplicons. This arrangement enables a fluorescently labeled molecular beacon probe[17,18] to bind to the excess (−) amplicons with little interference from

the complementary (+) amplicons. The target sequence for the molecular beacons in these (−) amplicons is purposely located in the interprimer sequence that lies between the regions whose sequences are related to the primers. This arrangement assures that the resulting fluorescent signals are indicative of the presence of (−) amplicons generated from the mutant target sequences in the original sample.

This report provides an example of a sensitive, multiplex PCR assay that simultaneously utilizes nine different SuperSelective primers, five different tailed conventional reverse primers, and five differently colored interprimer-specific molecular beacons to detect and quantitate eight different somatic mutations that can occur in four different exons of the human epidermal growth factor receptor (*EGFR*) gene of patients who have non−small-cell lung cancer[19,20]; as well as quantitating a β-actin reference gene that enables the relative abundance of each somatic mutation to be determined. These particular target mutations in the *EGFR* gene were chosen because the presence or absence of each of them can be used to determine which targeted therapy (gefitinib or erlotinib,[21] osimertinib,[22] or dacomitinib[23]) is likely to be effective. These particular mutations were also chosen as targets because two of them occur in each of the four exons, enabling us to demonstrate the ability of SuperSelective primers to distinguish which mutation is present in an exon, despite differences in how each pair of mutations is located within the exon. The results of the experiments described in this report demonstrate the ease with which multiplex real-time SuperSelective PCR assays can be designed to obtain highly specific and sensitive information concerning the presence and abundance of relevant somatic mutations present in noninvasive liquid biopsies.

In addition, this report demonstrates the specificity, selectivity, and consequent excellent sensitivity of multiplex SuperSelective PCR assays that analyze DNA fragments isolated from the plasma in liquid biopsies obtained from non−small-cell lung cancer patients. The results demonstrate that the presence of a small number of target mutant DNA fragments provides a true-positive signal, and the absence of target mutant DNA fragments does not produce false-positive signals.

Materials and Methods

PCR Primers and Molecular Beacons

Nine different SuperSelective primer sequences and seven different tailed conventional primer sequences were designed. All of these primers were purchased from Integrated DNA Technologies (Coralville, IA). Six differently colored interprimer-specific molecular beacon probes for detecting the resulting amplicons were designed in house, and purchased from LGC Biosearch Technologies (Petaluma, CA). The concentration of each primer, and the concentration of each molecular beacon, was determined in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Human Genomic DNA

Wild-type human DNA (from multiple anonymous donors), catalog number G1521, was purchased from the Promega Corp. (Madison, WI). Approximately 9 μg of this DNA was digested for 120 minutes at 37°C in 50 μL containing 10 units of restriction endonuclease DraI (New England Biolabs, Ipswich, MA) in a buffer provided by New England Biolabs that contained 100 μg/mL bovine serum albumin, 10 mmol/L magnesium acetate, 50 mmol/L potassium acetate, and 20 mmol/L Tris-acetate (pH 7.9); followed by incubation for 20 minutes at 65°C to inactivate the enzyme. The resulting mixture of genomic DNA restriction fragments was analyzed by conventional PCR to determine the concentration of *EGFR* wild-type DNA target fragments, after which that mixture was diluted with water to generate two stock solutions (of either 2000 or 600 *EGFR* target fragments/μL). The lengths of these *EGFR*-containing DraI fragments, all of which contained intact *EGFR* target sequences, varied from 1000 to 3000 bp.

Plasmids Containing *EGFR* Target Sequences

Eight different target plasmids, each consisting of a pIDTSmart Amp vector containing a synthetic 200-bp gene fragment encompassing a different *EGFR* mutant target sequence, were purchased from Integrated DNA Technologies, which also verified the inserted nucleotide sequence. Each plasmid was approximately 3000 bp long and contained a single endonuclease ScaI restriction site located away from the inserted sequence. Approximately 4 μg of each plasmid was digested for 120 minutes at 37°C in 20 μL containing 10 units of ScaI (New England Biolabs) in a buffer provided by New England Biolabs that contained 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L dithiothreitol, and 50 mmol/L Tris-HCl (pH 7.9); followed by incubation for 20 minutes at 80°C to inactivate the enzyme. The concentration of each linearized plasmid was determined in a NanoDrop spectrophotometer, after which the plasmids were substantially diluted with water to generate three stocks of 200, 20, or 2 DNA target fragments/μL.

Multiplex PCR Assay Design

Figure 2 summarizes the design of the demonstration assays that utilize plasmid DNA targets containing mutant *EGFR* sequences. There are nine different SuperSelective primers present in these multiplex real-time PCR assays, each designed to selectively amplify a different DNA target sequence. One of these nine SuperSelective primers was

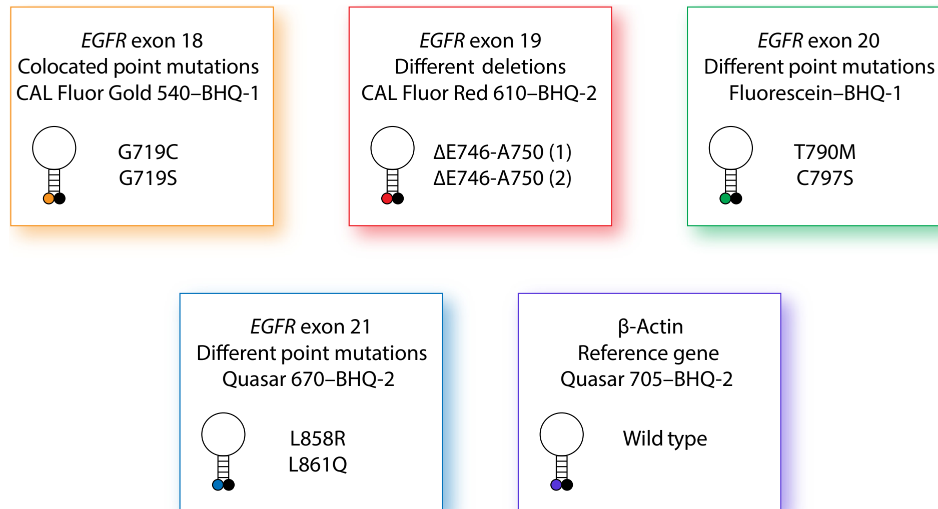

Figure 2 Design of the demonstration multiplex PCR assay. Eight different somatic mutations that can occur in the *EGFR* gene were chosen as targets, because the presence of any one of these mutations enables a targeted therapy to be used for the treatment of patients who have non—small-cell lung cancer. A ninth target gene, β-actin, serves as an internal reference that reflects the amount of DNA in the sample, enabling the relative abundance of each somatic mutation that is present to be estimated. Five differently colored molecular beacon probes are present in the assay; four are each specific for the amplicons generated by the presence of a somatic mutation in a different *EGFR* exon, and one is specific for the β-actin reference gene.

designed to amplify a segment of the functional β-actin genes that occur in sister chromosomes of human wild-type DNA, to provide a reference that reflects the amount of DNA in the sample. The β-actin target sequence was distinguished from the sequence of β-actin pseudogenes by the inclusion of unique intron sequences as targets for the foot of the SuperSelective primer and as targets for the conventional reverse primer.[24] Each of the other eight SuperSelective primers was designed to selectively amplify a different *EGFR* target somatic mutation if that target sequence is present in an assay sample. *EGFR* mutations G719C and G719S in exon 18 are single-nucleotide variations that occur at the same position in the same codon; mutations ΔE746-A750 (1) and ΔE746-A750 (2) are slightly different deletions that occur at the same position in exon 19; mutations T790M and C797S are single-nucleotide variations that occur at relatively distant positions in exon 20; and mutations L858R and L861Q are single-nucleotide variations that occur in different codons close to each other in exon 21. Every assay also contained five different tailed conventional reverse primers, one for each of the two particular mutations present in each of the four *EGFR* exons, and one for the β-actin reference gene. And, finally, each assay contained five differently colored molecular beacon probes, one for the amplicons generated from each of the four *EGFR* exons, and one for the amplicons generated from the β-actin reference gene. Figure 2 also identifies the differently colored fluorophores and quencher moieties that were used to label each of the five molecular beacon probes. All of the assay samples used in these demonstration assays contained a relatively large amount of human wild-type genomic DNA that had been digested with a restriction endonuclease to generate wild-type DNA target fragments.

Multiplex PCR Assay Components and Procedures

All of the PCR demonstration assays were performed in 30-μL volumes that contained: 50 mmol/L KCl; 2.5 mmol/L $MgCl_2$; 20 mmol/L Tris-HCl (pH 8.4); 250 μmol/L deoxy-ATP; 250 μmol/L deoxy-CTP; 250 μmol/L deoxy-GTP; 250 μmol/L deoxy-TTP; 0.5% of the nonionic surfactant Tween 20 (Sigma Aldrich, St. Louis, MO); 70 mmol/L tetramethylammonium chloride (Sigma Aldrich); 0.05 units/μL Platinum *Taq* DNA polymerase (Thermo Fisher Scientific); 60 nmol/L of each of the nine different SuperSelective forward primers; 500 nmol/L of each of the five different tailed conventional reverse primers; 50 nmol/L of the molecular beacon for the detection of the β-actin amplicons; and 150 nmol/L of each of the four differently colored molecular beacons for the detection of the *EGFR* mutant amplicons. The PCR assays were performed in 0.2 mL white polypropylene tubes (USA Scientific, Ocala, FL). For each assay, 20 μL containing all of the components listed above was added to each reaction tube cooled in an ice bath. Then, 10 μL containing the target DNAs was added to each tube and mixed, after which the PCR amplifications were performed simultaneously in a CFX96 Touch spectrofluorometric thermal cycler (Bio-Rad Laboratories, Hercules, CA).

The thermal cycling program was 2 minutes at 95°C to activate the DNA polymerase, followed by 50 or 60 thermal cycles that consisted of 95°C denaturation for 20 seconds, 60°C annealing for 20 seconds, and 72°C chain elongation

for 20 seconds. Molecular beacon fluorescence intensity was monitored in five different color channels at the end of the 60°C annealing stage of each thermal cycle. The number of thermal cycles that occurred before the intensity of each colored signal reached a predetermined level (the C_T) was determined automatically by the spectrofluorometric thermal cycler. The C_T value is inversely linearly proportional to the logarithm of the number of target DNA fragments originally present in the sample.[25,26]

Designing SuperSelective Primers

To ensure that each SuperSelective primer would selectively initiate synthesis on its mutant target sequence without initiating synthesis on closely related sequences (such as the wild-type sequence and related mutant sequences), the following design criteria were adhered to: i) the 5′-anchor sequence needs to be sufficiently long (usually between 20 and 30 nucleotides in length), and its nucleotide sequence needs to be sufficiently strong (its hybrid with the template DNA fragments having a melting temperature at least 4°C higher than the 60°C annealing temperature of the PCR assay); ii) the foot sequence needs to be either 9 or 10 nucleotides long for use in the presence of 70 mmol/L tetramethylammonium chloride,[13] with its interrogating nucleotide(s) at the 3′ end; and iii) the bridge sequence must not be complementary to the corresponding intervening sequence in the template, must not form secondary structures at the annealing temperature, must not be complementary to any other sequence in the entire human genome, and must form a single-stranded bubble with the intervening sequence, so that the circumference of the bubble is sufficiently long to assure the desired selectivity, without being so long that the generation of amplicons is inordinately delayed.

Consequently, the key parameters to vary in designing these primers are the length and identity of the bridge sequence and the length of the corresponding intervening sequence in the template (which is determined by the locations in the template to which the anchor sequence and the foot sequence hybridize). Moreover, the resulting bubble need not be symmetric. The bridge sequence can be longer than the intervening sequence, or vice versa.[12] For example, in designing the primers for the detection of the two different mutations that can occur at different positions in exon 20 of the *EGFR* gene, a 22-nucleotide bridge sequence and a 13-nucleotide intervening sequence were chosen for the T790M primer, and a 13-nucleotide bridge sequence and a 20-nucleotide intervening sequence were chosen for the C797S primer.

Putative primer sequences were examined with the aid of the OligoAnalyzer 3.1 computer program (Integrated DNA Technologies) to ensure that under our PCR assay conditions they would be unlikely to form internal hairpin structures and would be unlikely to form self-dimers or to form heterodimers with the tailed conventional reverse primers. In addition, each putative primer sequence was compared with the sequence of human genomic DNA, utilizing the latest version of the Basic Local Alignment Search Tool (BLAST) from the US National Center for Biotechnology Information. The process usually begins with a SuperSelective primer design containing a 14-nucleotide bridge sequence that is located across from a 14-nucleotide intervening sequence. A monoplex PCR assay with the mutant target template and with the related wild-type template is then performed to assess the selectivity of the primer design. On the basis of these results, the SuperSelective primer sequence can then be modified, by altering either the length of the foot sequence by a single nucleotide (which has a relatively large effect on the C_T value) or the length of the bridge sequence or the intervening sequence, or both (where each nucleotide added or subtracted from the circumference of the bubble has a relatively small effect on the C_T value), thereby assuring that the primer will be highly selective.

In addition, when a set of SuperSelective primers is to be used together in a multiplex PCR assay, the bridge sequence of each primer (including the bridge sequence of the SuperSelective primer for the reference gene) can be fine-tuned (by increasing or decreasing the length of each primer's bridge sequence,[12] and/or by making nucleotide substitutions in the bridge sequence that modify its flexibility through the choice of nearest-neighbor nucleotides that stabilize each other[27]), so that all of the SuperSelective primers in the set will produce approximately the same C_T value when amplifying the same number of target sequences. In general, it was only necessary to test about four different SuperSelective primer designs to identify a highly selective, fine-tuned primer for the detection and quantitation of each rare mutant DNA target sequence.

Multiplex PCR Assays Utilizing DNA Isolated from Human Liquid Biopsy Samples

Cell-free DNA fragments were isolated from 1-mL plasma in human liquid biopsy samples by the Memorial Sloan Kettering Cancer Center utilizing a Nextprep-Mag cfDNA Automated Isolation Kit and a Chemagic 360 Nucleic Acid Extractor (PerkinElmer, Waltham, MA), and from other human liquid biopsy samples by ATGen utilizing a Quick-cfDNA Serum & Plasma Kit (Zymo Research, Irving, CA). Each cell-free DNA sample was further purified utilizing a Zymo DNA Clean and Concentrator Kit, in which the cell-free DNA was bound to a silica membrane, washed with a buffer to remove inhibitors of DNA polymerase, and then eluted from the membrane into 15 μL of nuclease-free water. To further eliminate the inhibitory effects of DNA polymerase inhibitors that remain in cell-free DNA isolated from liquid biopsy samples, the PCR assays were performed in the presence of bovine serum albumin.

These PCR assays were performed in 50-μL volumes that contained: 50 mmol/L KCl; 2.5 mmol/L $MgCl_2$; 20 mmol/L

Tris-HCl (pH 8.4); 250 μmol/L deoxy-ATP; 250 μmol/L deoxy-CTP; 250 μmol/L deoxy-GTP; 250 μmol/L deoxy-TTP; 0.5% Tween 20; 70 mmol/L tetramethylammonium chloride; 300 ng/μL bovine serum albumin (Thermo Fisher Scientific); 0.1 units/μL Platinum *Taq* DNA polymerase; 60 nmol/L of each of eight different SuperSelective forward primers; 500 nmol/L of each of five different tailed conventional reverse primers; 200 nmol/L of the CAL Fluor Gold 540-labeled molecular beacon for the detection of *EGFR* mutations G719C and G719S in exon 18; 200 nmol/L of the CAL Fluor Red 610-labeled molecular beacon for the detection of *EGFR* mutations ΔE746-A750 (1) and (2) in exon 19; 200 nmol/L of the fluorescein-labeled molecular beacon for the detection of *EGFR* mutation T790M in exon 20; 120 nmol/L of the Quasar 670-labeled molecular beacon for the detection of *EGFR* mutations L858R and L861Q in exon 21; and 30 nmol/L of the Quasar 705-labeled molecular beacon for the detection of the β-actin reference gene. For each assay, 40 μL containing all of the components listed above was added to each reaction tube cooled in an ice bath. Then, 10 μL containing the target DNAs was added to each tube and mixed, after which the PCR amplifications were performed.

After performing the multiplex SuperSelective PCR assays with blinded samples provided by the Memorial Sloan Kettering Cancer Center, and after the identities of the *EGFR* mutations that were thought to be present in those samples were revealed, droplet digital PCR (ddPCR) assays that are designed to detect a particular mutation were performed on portions of the Zymo-purified cell-free DNA samples to confirm the identity of the *EGFR* mutations present in those samples, and to quantitate the concentration of the *EGFR* mutant fragments and the *EGFR* wild-type fragments that were present in each sample. These ddPCR assays were performed on a Bio-Rad QX200 ddPCR instrument with Bio-Rad PrimePCR ddPCR Mutation Detection Assay Kits for *EGFR* L858R (catalog number dHsaMDV2010021), *EGFR* ΔE746-A750 (1) (catalog number dHsaMDV2010039), and *EGFR* T790M (catalog number dHsaMDV2010019). Each of these ddPCR assays utilizes conventional primers that amplify both the mutant and the wild-type sequences, and uses differently colored TaqMan probes to distinguish droplets containing mutant amplicons from droplets containing wild-type amplicons.

Results

Design of Multiplex SuperSelective PCR Assays

To demonstrate the selectivity and sensitivity of multiplex SuperSelective PCR assays, an assay was designed and tested that targets eight different somatic mutations in the *EGFR* gene (whose presence in non−small-cell lung cancer patients dictates different therapeutic decisions) and that simultaneously targets a wild-type reference gene so that the relative abundance of each somatic mutation in the sample can be estimated. Table 1 lists the sequences of the nine different SuperSelective primers, five different tailed conventional reverse primers, and five differently colored interprimer-specific molecular beacons that are present in these assays. In addition, Supplemental Table S1 shows the sequences of the mutant targets, the wild-type sequences from which these mutant sequences arose, and the sequences of the resulting amplicons, highlighting the relationship between the different elements of each SuperSelective primer, and where those elements end up in the resulting amplicon sequences. The assay was not designed for clinical use. Rather, the assay was purposely designed to demonstrate the ability of SuperSelective primers to discriminate point mutations in the same codon, point mutations separated from each other in the same exon, and similar deletions that occur in the same exon.

Each SuperSelective primer and its related conventional reverse primer and molecular beacon probe were first tested in monoplex assays to confirm that each combination was sufficiently sensitive to detect as few as 10 target fragments. All of the primers and molecular beacons were then combined into a complete multiplex PCR assay, and experiments that explored the selectivity and sensitivity of each SuperSelective primer when present in this multiplex mixture were performed. Figure 3 shows the results of multiplex assays performed with samples that contained 10 copies of a linearized plasmid whose sequence included one of the eight different *EGFR* target mutations, as well as containing restriction enzyme-digested human genomic DNA from 1500 cells (ie, sufficient DNA to provide 3000 β-actin targets), which is the predominant amount of genomic DNA that is usually isolated from a 1-mL plasma sample.[28] Each assay was repeated three times. Despite there being two different mutations in each exon (either at the same position or relatively close to each other), the results show that each mutation in the pair is independently amplified. In every one of these assays, a signal arose in the color of the molecular beacon probe for the exon containing that particular mutant sequence; and an earlier signal arose in the color of the molecular beacon for the β-actin reference gene. Significantly, all of the other molecular beacons that were present in the assays only gave a background signal, confirming the specificity and selectivity of the assay. All of the positive signals were in response to the presence of a corresponding DNA target fragment in the sample; and in the absence of targets (Figure 3), no signal arose above background. These results confirm that when the nine different SuperSelective primers are used together, they do not cross-react, and they do not produce false-positive signals.

To illustrate the quantitative nature of these multiplex PCR assays, samples that contained 10, 100, or 1000 copies of each of the *EGFR* mutant target fragments, in addition to containing human genomic DNA fragments containing 10,000 copies of the β-actin reference gene were tested. Each assay was performed in duplicate. A

Table 1 Nucleotide Sequences of the Primers and Molecular Beacons Used in the Demonstration Multiplex *EGFR* PCR Assays

Target/description	Sequence
EGFR exon 18	
Forward primer **G719C** 28-20/13-8:1	5′-TCTCTTGAGGATCTTGAAGGAAACTGAA-CGTAGTCAACTTTGAACAAC-**AAGTGCTG**-**T**-3′
Forward primer **G719S** 28-20/13-8:1	5′-TCTCTTGAGGATCTTGAAGGAAACTGAA-CCTCTCCAACGAATCTCGAA-**AAGTGCTG**-**A**-3′
Tailed exon 18 reverse primer	5′-cggcta-TGTGCCAGGGACCTTACCTT-3′
Interprimer-specific molecular beacon	5′-(CAL Fluor Gold 540)-CGCTGG-*TGCGTTCGGCACGGTGTAT*-CCAGCG-(BHQ-1)-3′
EGFR exon 19	
Forward primer **ΔE746-A750 (1)** 29-14/14-8:2	5′-GGGACTCTGGATCCCAGAAGGTGAGAAAG-CCTCGGAACTACCA-**GCTATCAA**-**AA**-3′
Forward primer **ΔE746-A750 (2)** 29-14/14-9:1	5′-GGGACTCTGGATCCCAGAAGGTGAGAAAG-CGCGCGTTGGCAGA-**GCTATCAAG**-**A**-3′
Tailed exon 19 reverse primer	5′-cggcta-CCCCACACAGCAAAGCAGAAAC-3′
Interprimer-specific molecular beacon	5′-(CAL Fluor Red 610)-CGCCTC-*CGAAAGCCAACAAGGAAATCCTC*-GAGGCG-(BHQ-2)-3′
EGFR exon 20	
Forward primer **T790M** 24-22/13-8:1	5′-GCCGCCTGCTGGGCATCTGCCTCA-AAGAATCAACAAGCTACAACTC-**GCTCATCA**-**T**-3′
Forward primer **C797S** 21-13/20-9:1	5′-CTGCCTCACCTCCACCGTGCA-AGCACTCGCAGAA-**CCTTCGGCT**-**C**-3′
Tailed exon 20 reverse primer	5′-cggcta-CACCAGTTGAGCAGGTACTGG-3′
Interprimer-specific molecular beacon	5′-(Fluorescein)-CCGTGG-*CTGGACTATGTCCGGGAACACA*-CCACGG-(BHQ-1)-3′
EGFR exon 21	
Forward primer **L858R** 30-19/14-8:1	5′-AGGAACGTACTGGTGAAAACACCGCAGCAT-AAGAATCTACCGACACCAC-**TTTTGGGC**-**G**-3′
Forward primer **L861Q** 30-14/14-8:1	5′-CTGGTGAAAACACCGCAGCATGTCAAGATC-CAATTCCGCGACAA-**GGCCAAAC**-**A**-3′
Tailed exon 21 reverse primer	5′-cggcta-GGTCCCTGGTGTCAGGAAAATGCT-3′
Interprimer-specific molecular beacon	5′-(Quasar 670)-CGCCTG-*TGGGTGCGGAAGAGAAAGAATAC*-CAGGCG-(BHQ-2)-3′
Reference gene	
Forward primer **β-actin** 24-18/14-9:0	5′-CCAACCGCGAGAAGATGACCCAGG-CATAGCCAGCTAATGACC-**CCTCTTCTG**-3′
Tailed β-actin reverse primer	5′-cggcta-AGAGAACCAGTGAGAAAGGGC-3′
Interprimer-specific molecular beacon	5′-(Quasar 705)-CCGCTC-*CCTCCTTCCTGGCCTCCC*-GAGCGG-(BHQ-2)-3′

The structure of each of the nine SuperSelective forward primers is reflected in a descriptive code, whose meaning is exemplified as follows: for forward primer G719C 28-20/13-8:1, the target is *EGFR* mutation G719C; the anchor sequence is 28 nucleotides long; the bridge sequence is 20 nucleotides long; the intervening sequence in the (−) target template is 13 nucleotides long; and the foot sequence is 9 nucleotides long, 8 of which are completely complementary to both the mutant template and to the wild-type template, and the 3′-interrogating nucleotide is complementary to the mutant, but is not complementary to the wild type. The meaning of the different regions of the sequence listings is as follows: in the SuperSelective primers, the underlined nucleotides are in the bridge sequence, the bold nucleotides are in the foot sequence, and the bold underlined nucleotides are the 3′-interrogating nucleotide(s). In the tailed reverse primers, the lowercase nucleotides are in the tail sequence, which is not complementary to the (+) template sequence, but which is incorporated into the resulting (−) amplicon sequence. Each hairpin-shaped molecular beacon undergoes a structural reorganization when its probe sequence (italicized) binds to the interprimer sequence in its target (−) amplicon, resulting in a bright fluorescence signal in that molecular beacon's characteristic color.

series of dilutions of the genomic DNA fragments were also tested. Figure 4 shows the results. The observed C_T value of each reaction confirmed that the measured C_T values were inversely linearly proportional to the logarithm of the number of mutant target fragments present in the original sample.[25,26]

Selectivity and Sensitivity of Multiplex SuperSelective PCR Assays

Experiments were performed to determine whether these real-time multiplex SuperSelective PCR assays could detect and quantitate different amounts of a variety of *EGFR*

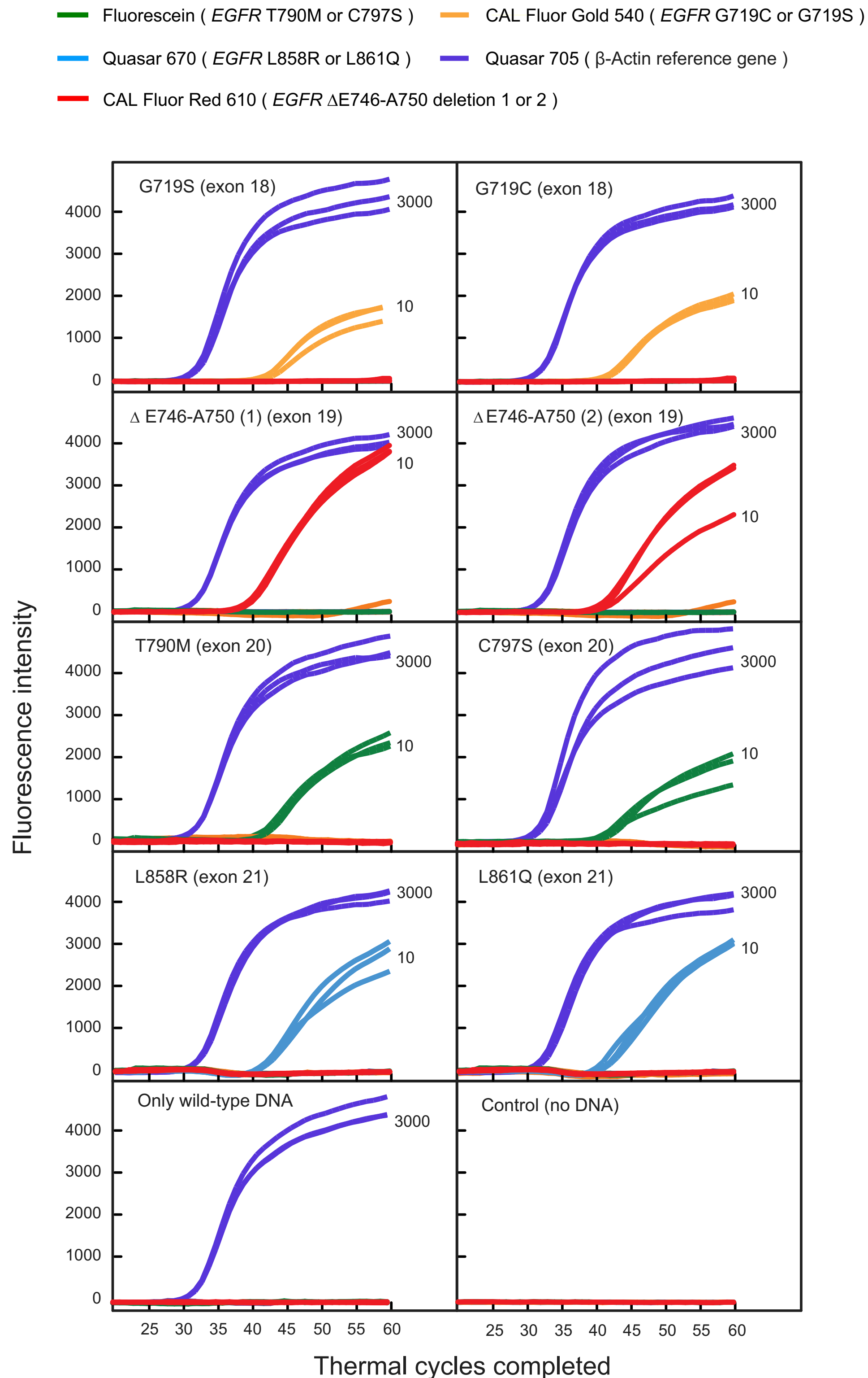

Figure 3 Confirmation of assay sensitivity. Ten different multiplex assays were performed in triplicate, each assay containing all nine different Super-Selective forward primers, five different tailed conventional reverse primers, and five differently colored molecular beacon probes. Nine of the samples contained human genomic DNA fragments that included 3000 copies of the wild-type β-actin reference gene; and eight of the samples also contained 10 copies of a linearized plasmid that included the sequence of a different *EGFR* target mutation. One sample served as a control, and did not contain any human genomic DNA, and did not contain any mutant target plasmids.

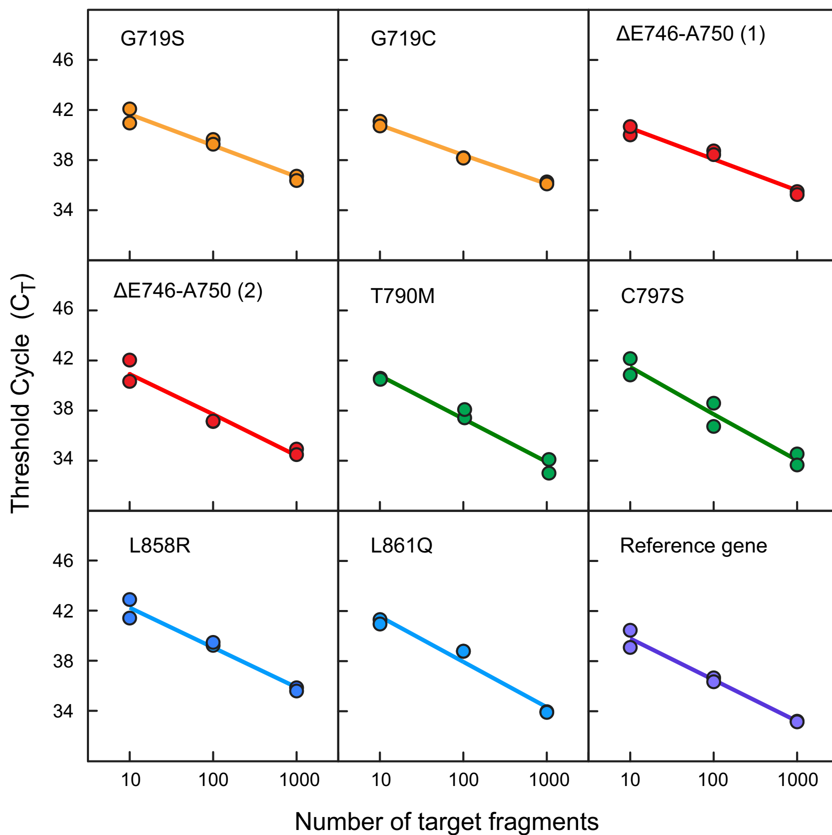

Figure 4 Quantitative nature of the response to different quantities of target sequence. Nine different sets of multiplex assays were performed in duplicate, each assay contained all nine different SuperSelective forward primers, five different tailed conventional reverse primers, and five differently colored molecular beacon probes. Eight of the sets contained human genomic DNA fragments from 5000 cells (ie, each of these samples contained 10,000 copies of the wild-type β-actin reference gene), and the samples in each of those sets contained 10, 100, or 1000 copies of a linearized plasmid that included the sequence of a different *EGFR* target mutation. A ninth set of reactions contained dilutions of the genomic DNA fragments, to confirm that SuperSelective primers for the β-actin reference gene provided comparable C_T values to the C_T values obtained from the mutant targets.

mutant DNA target fragments present in the same sample. Every assay contained all of the primers and all of the molecular beacons, and every assay was repeated five times to illustrate the reliability of the results. Five different combinations of *EGFR* mutant target DNA fragments were present in the samples. Figure 5 shows the results of these experiments. Significantly, despite the presence of 10,000 copies of human wild-type genomic DNA, signals arose only from those particular target mutations that were present in each sample. Moreover, in those samples where neither mutation in the same exon was present, yet 10,000 copies of the same wild-type exon were present, the fluorescence of the molecular beacon for those mutant DNA targets was suppressed. Yet, the presence of 10 copies of a mutant DNA target sequence always produced a reliable positive signal.

Cell-Free DNA Fragments Isolated from Plasma

PCR analysis of cell-free DNA isolated from plasma is intrinsically difficult because of the nature of the sample. Cell-free DNA arises from nucleolytic digestion of chromosomal DNA in cells that have died, predominantly consisting of fragments averaging 166 bp in length,[29] because of the protective presence of nucleosomes that are bound to the chromosomal DNA. Consequently, it is important that the PCR primers for each target sequence hybridize to positions that are sufficiently close to each other to assure that most fragments will be amplified. Some of the primers designed for the demonstration assays that utilized plasmid DNA targets bound rather far away from each other, requiring that some of the target sequences be at least 131, 145, or even 154 nucleotides long (Supplemental Table S1). Therefore, two of the primers were redesigned so that the target sequences that could be detected were between 97 and 115 nucleotides in length. These changes include a new tailed conventional reverse primer for the amplification of *EGFR* L858R and L861Q in exon 20, a new tailed conventional reverse primer for the amplification of *EGFR* T790M in exon 21, and a new interprimer-specific molecular beacon for the detection of *EGFR* T790M amplicons (Table 2). Supplemental Table S2 illustrates the relative closeness of the primer binding sites in each target sequence, thereby enabling the amplification and detection of the relatively short cell-free DNA fragments that occur in liquid biopsy samples.

An additional problem associated with cell-free DNA fragments isolated from plasma is that the plasma often contains IgG, and other cellular components that arise from cells that break during the process of isolating the plasma, and these contaminants of isolated cell-free DNA can inhibit PCR by binding to the DNA polymerase.[30] This inhibition

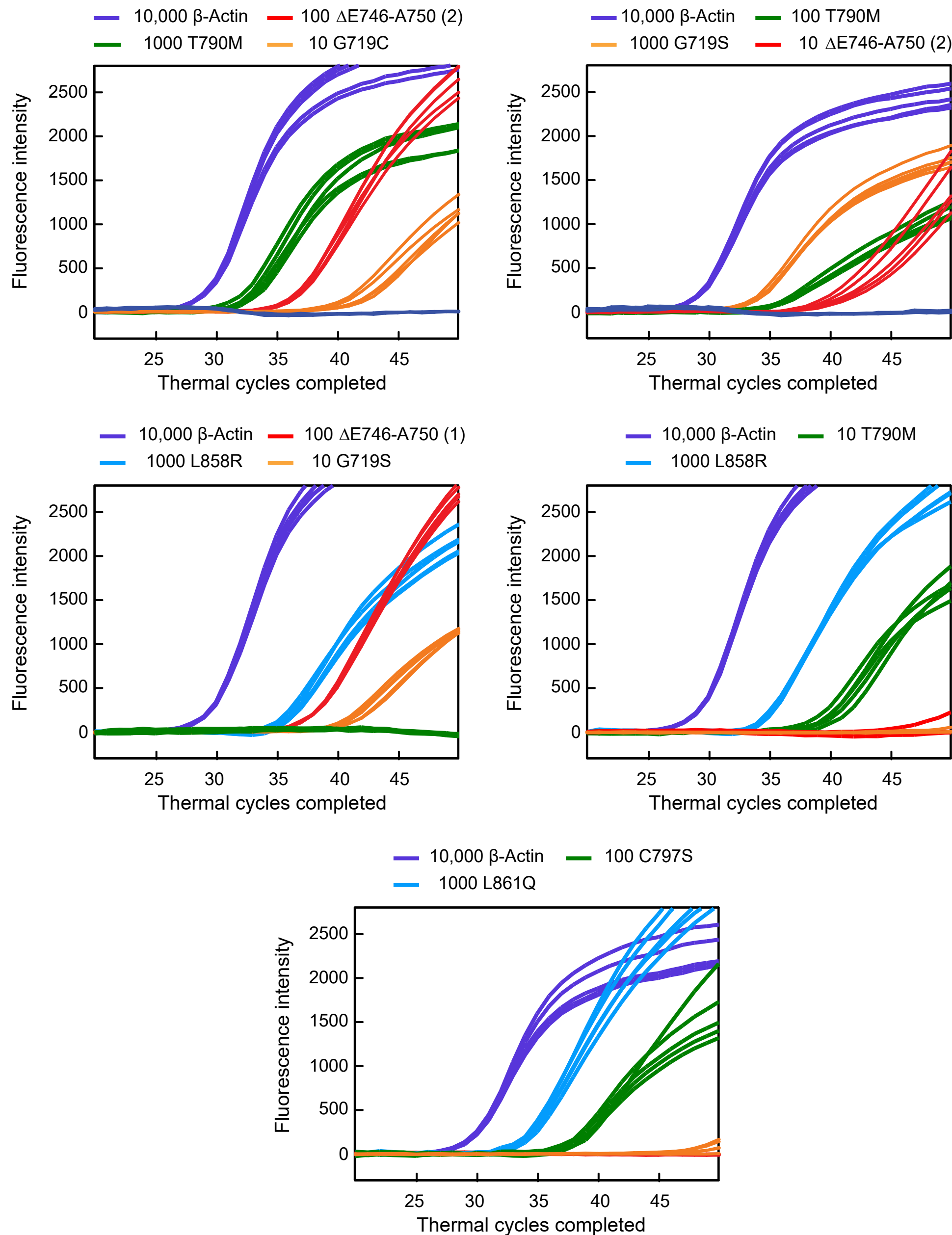

Figure 5 Examples of multiplex real-time SuperSelective PCR assays for the detection and quantitation of somatic mutations in the human *EGFR* gene. Every assay contained all nine SuperSelective forward primers, all five tailed conventional reverse primers, and all five differently colored molecular beacon probes, whose sequences are listed in Table 1. Every assay also contained fragments of human genomic DNA that included 10,000 copies of the β-actin reference gene; and every assay was repeated five times. Five different sets of linearized plasmids containing *EGFR* target sequences were used as templates for these PCR demonstration assays. The identity and quantity of the mutant DNA target fragments in each sample are indicated in the key above each graph.

can be overcome by further purifying the isolated cell-free DNA with a Zymo DNA Clean and Concentrator Kit, in combination with including 300 ng/μL bovine serum albumin in the PCR assays.[31]

Multiplex SuperSelective PCR Assays Utilizing Cell-Free DNA Isolated from Human Liquid Biopsies

To illustrate the specificity and sensitivity of SuperSelective PCR assays, six cell-free DNA samples isolated from the plasma of patients with non−small-cell lung cancer were obtained, one from a patient at the Hospital de Clínicas in Montevideo, and the other five from patients at the Memorial Sloan Kettering Cancer Center in New York. The mutations present in the cell-free DNA sample from the Montevideo patient were previously identified from a tissue sample utilizing a cobas *EGFR* Mutation Test v2 (Roche Molecular Systems, Pleasanton, CA). The mutations present in the cell-free DNA samples from Memorial Sloan Kettering Cancer Center had previously been

Table 2 Nucleotide Sequences of the Primers and Molecular Beacons Used to Analyze *EGFR* Mutations in Liquid Biopsy Samples

Target/description	Sequence
EGFR exon 18	
Forward primer **G719C** 28-20/13-8:1	5′-TCTCTTGAGGATCTTGAAGGAAACTGAA-CGTAGTCAACTTTGAACAAC-**AAGTGCTG**-**T**-3′
Forward primer **G719S** 28-20/13-8:1	5′-TCTCTTGAGGATCTTGAAGGAAACTGAA-CCTCTCCAACGAATCTCGAA-**AAGTGCTG**-**A**-3′
Tailed exon 18 reverse primer	5′-cggcta-TGTGCCAGGGACCTTACCTT-3′
Interprimer-specific molecular beacon	5′-(CAL Fluor Gold 540)-CGCTGG-*TGCGTTCGGCACGGTGTAT*-CCAGCG-(BHQ-1)-3′
EGFR exon 19	
Forward primer **ΔE746-A750 (1)** 29-14/14-8:2	5′-GGGACTCTGGATCCCAGAAGGTGAGAAAG-CCTCGGAACTACCA-**GCTATCAA**-**AA**-3′
Forward primer **ΔE746-A750 (2)** 29-14/14-9:1	5′-GGGACTCTGGATCCCAGAAGGTGAGAAAG-CGCGCGTTGGCAGA-**GCTATCAAG**-**A**-3′
Tailed exon 19 reverse primer	5′-cggcta-CCCCACACAGCAAAGCAGAAAC-3′
Interprimer-specific molecular beacon	5′-(CAL Fluor Red 610)-CGCCTC-*CGAAAGCCAACAAGGAAATCCTC*-GAGGCG-(BHQ-2)-3′
EGFR exon 20	
Forward primer **T790M** 24-22/13-8:1	5′-GCCGCCTGCTGGGCATCTGCCTCA-AAGAATCAACAAGCTACAACTC-**GCTCATCA**-**T**-3′
Tailed exon 20 reverse primer	5′-cggcta-CAATATTGTCTTTGTGTTCCCGG-3′
Interprimer-specific molecular beacon	5′-(Fluorescein)-CCGTGG-*CTGGACTATGTCCGGGAACACA*-CCACGG-(BHQ-1)-3′
EGFR exon 21	
Forward primer **L858R** 30-19/14-8:1	5′-AGGAACGTACTGGTGAAAACACCGCAGCAT-AAGAATCTACCGACACCAC-**TTTTGGGC**-**G**-3′
Forward primer **L861Q** 30-14/14-8:1	5′-CTGGTGAAAACACCGCAGCATGTCAAGATC-CAATTCCGCGACAA-**GGCCAAAC**-**A**-3′
Tailed exon 21 reverse primer	5′-cggcta-CCACCTCCTTACTTTGCCTCCT-3′
Interprimer-specific molecular beacon	5′-(Quasar 670)-CGCCTG-*TGGGTGCGGAAGAGAAAGAATAC*-CAGGCG-(BHQ-2)-3′
Reference gene	
Forward primer **β-actin** 24-18/14-9:0	5′-CCAACCGCGAGAAGATGACCCAGG-CATAGCCAGCTAATGACC-**CCTCTTCTG**-3′
Tailed β-actin reverse primer	5′-cggcta-AGAGAACCAGTGAGAAAGGGC-3′
Interprimer-specific molecular beacon	5′-(Quasar 705)-CCGCTC-*CCTCCTTCCTGGCCTCCC*-GAGCGG-(BHQ-2)-3′

The structure of each of the eight SuperSelective forward primers is reflected in a descriptive code, whose meaning is exemplified as follows: for forward primer G719C 28-20/13-8:1, the target is *EGFR* mutation G719C; the anchor sequence is 28 nucleotides long; the bridge sequence is 20 nucleotides long; the intervening sequence in the (−) target template is 13 nucleotides long; the foot sequence is 9 nucleotides long, 8 of which are completely complementary to both the mutant template and to the wild-type template; and the 3′-interrogating nucleotide is complementary to the mutant, but is not complementary to the wild type. The meaning of the different regions of the sequence listings is as follows: in the SuperSelective primers, the underlined nucleotides are in the bridge sequence, the bold nucleotides are in the foot sequence, and the bold underlined nucleotides are the 3′-interrogating nucleotide(s). In the tailed reverse primers, the lowercase nucleotides are in the tail sequence, which is not complementary to the (+) template sequence, but which is incorporated into the resulting (−) amplicon sequence. Each hairpin-shaped molecular beacon undergoes a structural reorganization when its probe sequence (italicized) binds to the interprimer sequence in its target (−) amplicon, resulting in a bright fluorescence signal in that molecular beacon's characteristic color.

identified by next-generation sequencing analysis performed by Resolution Bioscience (Kirkland, WA). However, the cell-free DNA samples isolated from these five patients were provided to us in a blinded manner. All six samples were further purified using a Zymo DNA Clean and Concentrator Kit, and multiplex SuperSelective PCR assays were performed with a portion of each of the six samples to identify and quantitate the *EGFR* mutations present in each sample.

The results of these assays are shown in Figure 6. All six samples gave a positive signal from the β-actin reference gene, and the threshold value of the β-actin signals reflected the amount of cell-free DNA in each sample. Samples 1, 2, 3, and 4 were positive for different *EGFR* target mutations, but samples 5 and 6 did not give signals for any of the *EGFR* target mutations. After these results were obtained, the previously determined identities of the five blinded samples (2, 3, 4, 5, and 6) were revealed. It turned out that

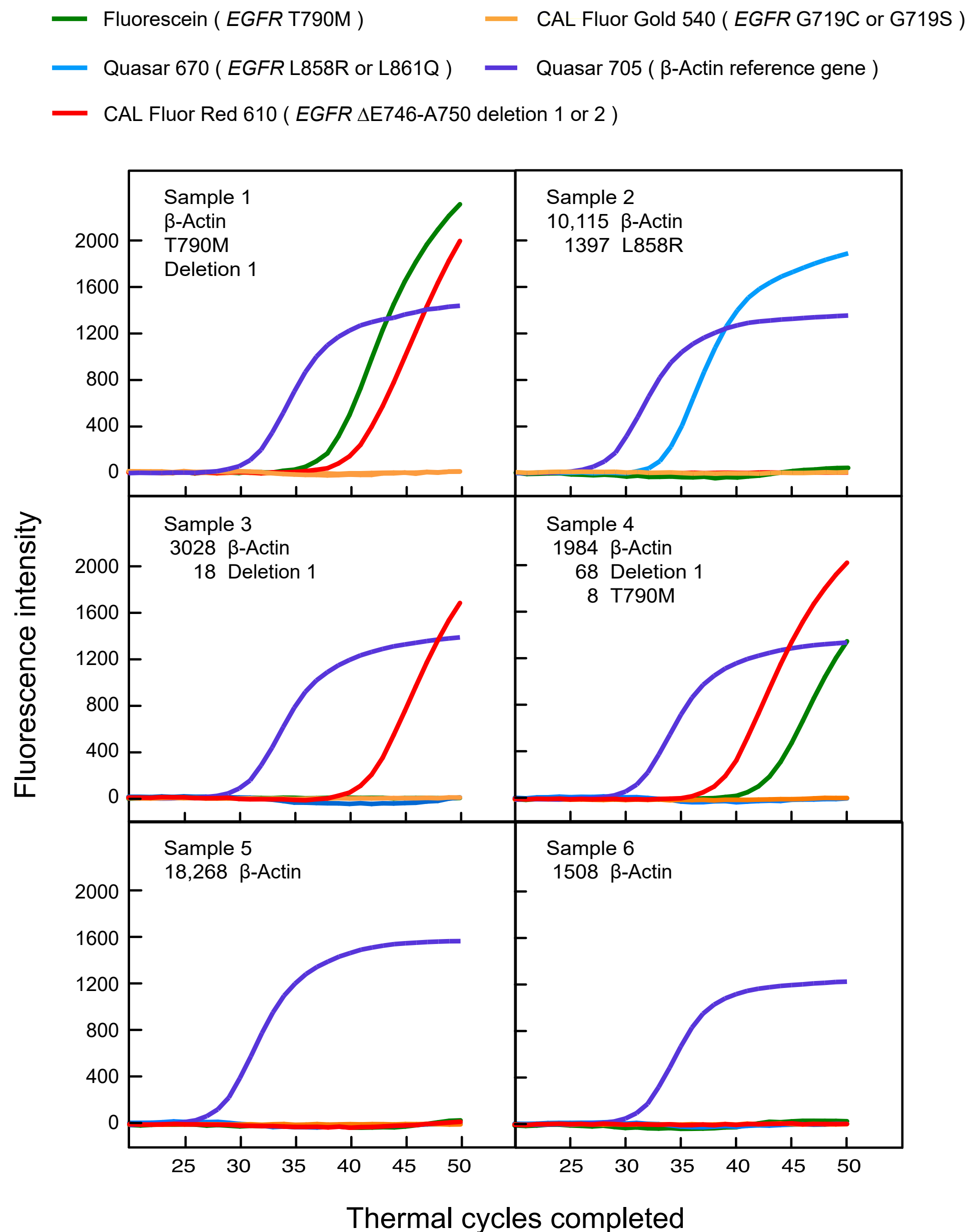

Figure 6 Multiplex real-time PCR assays utilizing cell-free DNA fragments isolated from the plasma in liquid biopsy samples obtained from patients with non—small-cell lung cancer. Every assay contained all eight SuperSelective forward primers, all five tailed conventional reverse primers, and all five differently colored molecular beacon probes, whose sequences are listed in Table 2. The isolated cell-free DNA in each sample was further purified with a Zymo Clean and Concentrator Kit, and a portion of that purified cell-free DNA was used as templates for a multiplex real-time PCR assay. Each of the five panels showing the results obtained from the blinded samples lists the quantity of each type of target sequence present, as subsequently determined by droplet digital PCR analysis of a remaining portion of the purified cell-free DNA.

blinded samples 5 and 6, which did not contain *EGFR* target mutations, were purposely provided as controls from patients identified as possessing a somatic mutation in the *TP53* tumor suppressor gene, but no *EGFR* mutations. Blinded sample 2 was previously known to possess *EGFR* L858R, and blinded samples 3 and 4 were previously known to possess *EGFR* ΔE746-A750 (1). However, blinded sample 4 also gave a positive signal for *EGFR* T790M, and *EGFR* T790M had not previously been identified as being present in that patient's circulating tumor DNA. Significantly, the T790M signal in that SuperSelective PCR assay arose at a late C_T, indicating that *EGFR* T790M was a minor component in that cell-free DNA sample, and therefore was not likely to have been previously identified by next-generation sequencing analysis.

To confirm these results with an independent technique, remaining portions of the cell-free DNA in each of the five blinded samples provided by the Memorial Sloan Kettering

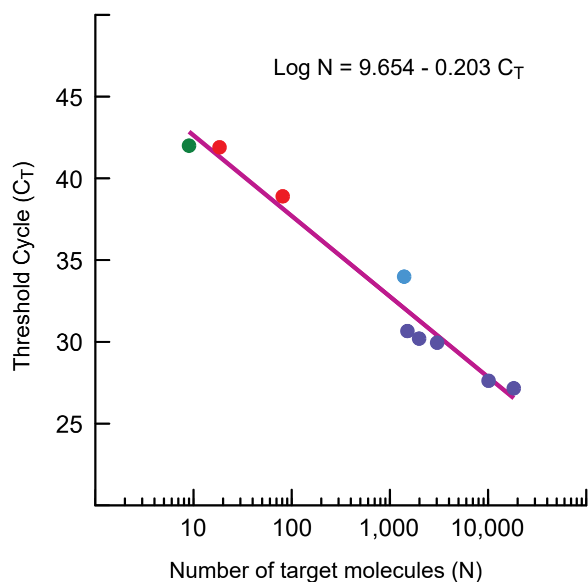

Figure 7 Plot of the C_T obtained for each detected target sequence versus the logarithm of the number of template fragments present in the sample added to initiate each multiplex PCR assay. The number of targets present in each sample was obtained by separate droplet digital PCR assays designed to detect particular mutations in the remaining portion of each of the five clinical samples provided by the Memorial Sloan Kettering Cancer Center. The resulting equation relating the logarithm of the number of target fragments to the observed C_T value for each target sequence can be utilized to determine the number of target fragments of each type present in assays performed with other clinical samples. The plotted values are as follows: *EGFR* T790M (green); *EGFR* ΔE746-A750 (1) (red); *EGFR* L858R (blue); and the β-actin reference gene (purple). On the basis of the formula in the figure, the observed C_T value of each mutation, and the observed C_T value of the reference gene, can be used to calculate the variable allele frequency of each mutation in the original liquid biopsy sample.

Cancer Center were analyzed by ddPCR assays to confirm the identity and quantity of each mutation, and to provide an estimate of the total quantity of cell-free DNA that was initially present in each SuperSelective PCR assay. The results confirmed the identity of the *EGFR* mutations in each sample, including the identity of the T790M resistance mutation. Furthermore, the logarithm of the quantity of each *EGFR* mutation in each sample, as well as the logarithm of the quantity of the β-actin reference gene in each sample (inferred from the total quantity of *EGFR* target fragments detected in the ddPCR assays), was plotted against the C_T determined for each positive signal in the SuperSelective PCR assays (Figure 7), and the results confirmed that the experimentally determined C_T values are inversely linearly proportional to the logarithm of the initial number of target molecules in the sample.[25,26] Significantly, even though the amount of cell-free DNA obtained from a liquid biopsy varies from sample to sample, the relative abundance of each somatic mutation can be directly estimated by comparison of the C_T value of that mutation to the C_T value of the reference gene, which reflects the amount of DNA in the sample.

On the basis of the ddPCR results individually performed for each mutation in the five blinded samples, the number of target DNA fragments initially present in each of these multiplex SuperSelective PCR assays performed with human clinical samples is listed in Figure 6. Eighteen molecules of *EGFR* ΔE746-A750 (1) were detected in sample 3, and eight molecules of *EGFR* T790M were detected in sample 4. Significantly, these were true-positive results, as signals for these mutations did not arise in samples known not to contain them. In particular, no false-positive signals arose in any of the samples, most notably in sample 5, whose β-actin reference gene signal indicated that 18,268 *EGFR* wild-type fragments were present in that sample. These results demonstrate the extraordinary specificity and sensitivity of multiplex SuperSelective PCR assays, even when the samples being analyzed contain cell-free DNA fragments isolated from liquid biopsies.

Discussion

The key conclusion of these experiments is that multiplex SuperSelective PCR assays can readily be designed for the detection and quantitation of a set of rare somatic mutations that are relevant to the selection of an effective personalized therapy for cancer patients. As new relevant somatic mutations are identified for different cancers, and as new targeted therapies are devised to treat these cancers, individualized multiplex PCR assays can be prepared that ignore the wild-type fragments, while identifying and quantitating the somatic mutations that are relevant to ongoing therapy.

The distinctive characteristic of these multiplex real-time PCR assays is that the suppression of signals from the abundant wild-type sequences is due to the extraordinary discriminatory ability of the SuperSelective primers, rather than from the less specific discrimination inherent in the use of hybridization probes, as occurs in conventional PCR assays. Essentially, the only difference between these assays and conventional multiplex real-time PCR assays is the substitution of SuperSelective primers for conventional primers. In all other respects, SuperSelective PCR assays are performed in the same manner: a DNA sample is prepared, added to an assay mixture, and then amplified. Therefore, SuperSelective PCR assays can be rapidly performed at points of care, and the results can immediately be utilized to inform a subsequent clinical course of action.

However, in many situations, such as the analysis of liquid biopsy samples from cancer patients, PCR assays will only be useful if a relatively large number of different mutant targets can be detected and quantitated in a single run. The amount of information that can be obtained in a single assay is usually limited by the number of colors that can be distinguished by the spectrofluorometric thermal cycler. In the assays that were performed, only five different fluorescent colors could be distinguished by the PCR instrument, and one of those

colors was dedicated to the detection of the reference gene. Newer PCR instruments have been developed that distinguish more colors, such as Cepheid's GeneXpert thermal cycler (that utilizes wavelength-shifting molecular beacons[32] to enable 10 different fluorescent signals to be distinguished).[33] Moreover, digital PCR instruments are being developed that can distinguish multiple colors (such as Stilla Technologies' six-color digital PCR instrument)[34]; and because each droplet or well in a digital PCR instrument performs a miniature PCR screening assay that identifies a single DNA target fragment, color-coded molecular beacons will enable digital PCR assays to simultaneously identify and quantitate a large number of different targets.[35]

In clinical situations, it is often desirable to identify which targeted therapy will be effective, rather than identifying the particular somatic mutation that suggests the use of that therapy. There are often several different relevant somatic mutations that can occur, and any one of the mutations in that group suggests the same targeted therapy. This realization can be incorporated into the design of multiplex SuperSelective PCR assays. There can be a separate SuperSelective primer for the generation of amplicons from each somatic mutation in a group of mutations that suggests the same therapy. And there can be different molecular beacon probes for the different amplicons that can be generated from those mutations. However, all of the molecular beacons for lighting up those different amplicons can be labeled with the same colored fluorophore. Consequently, a positive signal in that color will identify the targeted therapy that can be used for that patient, even though the particular somatic mutation responsible is not identified. In this manner, SuperSelective PCR assays, even if performed in a spectrofluorometric thermal cycler that can only distinguish five fluorescent colors, can identify five different groups of mutations, each group indicating which targeted therapy will be effective, and perhaps, which targeted therapy will be ineffective because of the presence of a member of a group of mutations that indicates resistance to a particular therapy. The specificity of multiplex SuperSelective PCR assays offers an advantage when assessing circulating tumor DNA at low concentrations, when nontumor sources, such as clonal hematopoiesis, may pervasively contaminate the results of next-generation sequencing.[36]

Because of their simplicity, specificity, and sensitivity, SuperSelective PCR assays may be useful for a variety of medical applications that are designed to detect rare mutant DNA fragments in the presence of abundant wild-type DNA fragments. For example, with improved precision, this technology has the potential to be used to monitor a patient after treatment, to detect the presence of minimal residual disease,[37] enabling early intervention. This requires further clinical studies with large sample sizes, including a comparison with tumor-informed next-generation sequencing technologies.[38] Another example occurs when a clinical sample indicates that a patient is infected with a particular bacterial species that can be treated with an effective antibiotic, but the presence of rare bacteria possessing a mutant gene resistant to that antibiotic would lead to overgrowth and failure of the therapy.[39] A rapid, low-cost SuperSelective PCR assay of the bacterial DNA could identify rare antibiotic-resistant species, so that an effective antibiotic could be prescribed. And, finally, detrimental inherited mutant genes that are present in a fetus are often identified by invasive amniocentesis. However, fetal cells escape into the mother's circulation, and DNA fragments from those fetal cells are transiently present in the mother's plasma.[40] Consequently, multiplex SuperSelective PCR assays performed on the DNA isolated from a noninvasive liquid biopsy of the mother's blood should enable detection of the presence of harmful mutations, obviating the need for amniocentesis. In summary, the availability of sensitive multiplex SuperSelective PCR assays is likely to have wide clinical utility.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.11.006*.

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Supplemental Table S1: Amplicons Generated from DNA Target Sequences Present in Linearized Plasmids

Target		(−) Strand Sequence (3´ to 5´)
***EGFR* Exon 18**	Wild-type template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**C**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
G719C	Mutant template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**A**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
97 nt-long target	Amplicon	AGAGAACTCCTAGAACTTCCTTTGACTT---GCATCAGTTGAAACTTGTTG--TTCACGAC-**A**-CGAGGCC-*ACGCAAGCCGTGCCACATA*-TTCCATTCCAGGGACCGTGT-ATCGGC
***EGFR* Exon 18**	Wild-type template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**C**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
G719S	Mutant template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**T**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
97 nt-long target	Amplicon	AGAGAACTCCTAGAACTTCCTTTGACTT---GGAGAGGTTGCTTAGAGCTT--TTCACGAC-**T**-CGAGGCC-*ACGCAAGCCGTGCCACATA*-TTCCATTCCAGGGACCGTGT-ATCGGC
***EGFR* Exon 19**	Wild-type template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTT-**CC**-**TTAATTCTCTTCG**--TT-GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
ΔE746-A750 (1)	Mutant template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTT-**TT**-------------------GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
111 nt-long target	Amplicon	CCCTGAGACCTAGGGTCTTCCACTCTTTC--GGAGCCTTGATGGT--CGATAGTT-**TT**-------------------GTAGAGG-*CTTTCGGTTGTTCCTTTAGGAG*-CTACACT-CAAAGACGAAACGACACACCCC-ATCGGC
***EGFR* Exon 19**	Wild-type template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTTC-**C**-**TTAATTCTCTTCGT**-T--GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
ΔE746-A750 (2)	Mutant template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTTC-**T**-------------------GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
110 nt-long target	Amplicon	CCCTGAGACCTAGGGTCTTCCACTCTTTC--GCGCGCAACCGTCT--CGATAGTTC-**T**-------------------GTAGAGG-*CTTTCGGTTGTTCCTTTAGGAG*-CTACACT-CAAAGACGAAACGACACACCCC-ATCGGC
***EGFR* Exon 20**	Wild-type template	CGGCGGACGACCCGTAGACGGAGT-------GGAGGTGGCACGT------CGAGTAGT-**G**-CGTCGAGTACG---GGAAGCCGA-C-GGAG-GACCTGATACAGGCCCTTGTGT-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC
T790M	Mutant template	CGGCGGACGACCCGTAGACGGAGT-------GGAGGTGGCACGT------CGAGTAGT-**A**-CGTCGAGTACG---GGAAGCCGA-C-GGAG-GACCTGATACAGGCCCTTGTGT-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC
131 nt-long target	Amplicon	CGGCGGACGACCCGTAGACGGAGT--TTCTTAGTTGTTCGATGTTGAG--CGAGTAGT-**A**-CGTCGAGTACG---GGAAGCCGA-C-GGAG-*GACCTGATACAGGCCCTTGTGT*-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC-ATCGGC
***EGFR* Exon 20**	Wild-type template	GACGGAGTGGAGGTGGCACGT----CGAGTAGT-G-CGTCGAGTACG---GGAAGCCGA-**C**-GGAG-GACCTGATACAGGCCCTTGTGT-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC
C797S	Mutant template	GACGGAGTGGAGGTGGCACGT----CGAGTAGT-G-CGTCGAGTACG---GGAAGCCGA-**G**-GGAG-GACCTGATACAGGCCCTTGTGT-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC
114 nt-long target	Amplicon	GACGGAGTGGAGGTGGCACGT--------AGCACTCGCAGAA--------GGAAGCCGA-**G**-GGAG-*GACCTGATACAGGCCCTTGTGT*-TTCTGTTATAACCGAG-GGTCATGGACGAGTTGACCAC-ATCGGC
***EGFR* Exon 21**	Wild-type template	TCCTTGCATGACCACTTTTGTGGCGTCGTA------CAGTTCTAGTGTCT-----AAAACCCG-**A**-CCGGTTTG-A-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG
L858R	Mutant template	TCCTTGCATGACCACTTTTGTGGCGTCGTA------CAGTTCTAGTGTCT-----AAAACCCG-**C**-CCGGTTTG-A-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG
154 nt-long target	Amplicon	TCCTTGCATGACCACTTTTGTGGCGTCGTA---TTCTTAGATGGCTGTGGTG---AAAACCCG-**C**-CCGGTTTG-A-CG-*ACCCACGCCTTCTCTTTCTTATG*-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG-ATCGGC
***EGFR* Exon 21**	Wild-type template	GACCACTTTTGTGGCGTCGTACAGTTCTAG------TGTCT-----AAAACCCG-A-CCGGTTTG-**A**-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG
L861Q	Mutant template	GACCACTTTTGTGGCGTCGTACAGTTCTAG------TGTCT-----AAAACCCG-A-CCGGTTTG-**T**-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG
145 nt-long target	Amplicon	GACCACTTTTGTGGCGTCGTACAGTTCTAG--------GTTAAGGCGCTGTT-----CCGGTTTG-**T**-CG-*ACCCACGCCTTCTCTTTCTTATG*-GTACGTCTTCCTCCGTTTCATTCCTCCACCGAAATCCAGTCGG-TCGTAAAAGGACTGTGGTCCCTGG-ATCGGC
β-Actin	Reference gene	GGTTGGCGCTCTTCTACTGGGTCC----ACTCACCGGGCGAT----GGAGAAGAC-CACCGGC-GGAGGAAGGACCGGAGGG-CCTCGACG-CGGGAAAGAGTGACCAAGAGA
111 nt-long target	Amplicon	GGTTGGCGCTCTTCTACTGGGTCC--GTATCGGTCGATTACTGG--GGAGAAGAC-CACCGGC-*GGAGGAAGGACCGGAGGG*-CCTCGACG-CGGGAAAGAGTGACCAAGAGA-ATCGGC

When the mutant target sequence is amplified, the complement of the SuperSelective primer's sequence is incorporated into the 3' end of the (-) amplicon, and the complement of that primer's unique bridge sequence (underlined) replaces the intervening sequence in the mutant (-) strand, thereby assuring that each different (-) amplicon is exponentially amplified without interference from the other SuperSelective primers. The tailed reverse primer sequence is incorporated into the 5' end of the (-) amplicon. Interprimer-specific sequences within the (-) amplicon (italicized) determine which differently colored molecular beacon lights up the amplicon. The nucleotides that distinguish the mutant sequence from their related wild-type sequence are bold and underlined.

Supplemental Table S2: Amplicons Generated from Cell-free DNA Target Fragments Isolated from Liquid Biopsies

Target		(−) Strand Sequence (3´ to 5´)
***EGFR* Exon 18**	Wild-type template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**C**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
G719C	Mutant template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**A**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
97 nt-long target	Amplicon	AGAGAACTCCTAGAACTTCCTTTGACTT---GCATCAGTTGAAACTTGTTG--TTCACGAC-**A**-CGAGGCC-*ACGCAAGCCGTGCCACATA*-TTCCATTCCAGGGACCGTGT-ATCGGC
***EGFR* Exon 18**	Wild-type template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**C**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
G719S	Mutant template	AGAGAACTCCTAGAACTTCCTTTGACTT-----AAGTTTTTCTAGT-------TTCACGAC-**T**-CGAGGCC-ACGCAAGCCGTGCCACATA-TTCCATTCCAGGGACCGTGT
97 nt-long target	Amplicon	AGAGAACTCCTAGAACTTCCTTTGACTT---GGAGAGGTTGCTTAGAGCTT--TTCACGAC-**T**-CGAGGCC-*ACGCAAGCCGTGCCACATA*-TTCCATTCCAGGGACCGTGT-ATCGGC
***EGFR* Exon 19**	Wild-type template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTT-**CC**-**TTAATTCTCTTCG**--TT-GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
ΔE746-A750 (1)	Mutant template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTT-**TT**-------------------GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
111 nt-long target	Amplicon	CCCTGAGACCTAGGGTCTTCCACTCTTTC--GGAGCCTTGATGGT--CGATAGTT-**TT**-------------------GTAGAGG-*CTTTCGGTTGTTCCTTTAGGAG*-CTACACT-CAAAGACGAAACGACACACCCC-ATCGGC
***EGFR* Exon 19**	Wild-type template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTTC-**C**-**TTAATTCTCTTCGT**-T--GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
ΔE746-A750 (2)	Mutant template	CCCTGAGACCTAGGGTCTTCCACTCTTTC--AATTTTAAGGGCAG--CGATAGTTC-**T**-------------------GTAGAGG-CTTTCGGTTGTTCCTTTAGGAG-CTACACT-CAAAGACGAAACGACACACCCC
110 nt-long target	Amplicon	CCCTGAGACCTAGGGTCTTCCACTCTTTC--GCGCGCAACCGTCT--CGATAGTTC-**T**-------------------GTAGAGG-*CTTTCGGTTGTTCCTTTAGGAG*-CTACACT-CAAAGACGAAACGACACACCCC-ATCGGC
***EGFR* Exon 20**	Wild-type template	CGGCGGACGACCCGTAGACGGAGT-------GGAGGTGGCACGT------CGAGTAGT-**G**-CGTCGA-GTACGGGAAGCCGACGGAGGA-CCTGATACA-GGCCCTTGTGTTTCTGTTATAAC
T790M	Mutant template	CGGCGGACGACCCGTAGACGGAGT-------GGAGGTGGCACGT------CGAGTAGT-**A**-CGTCGA-GTACGGGAAGCCGACGGAGGA-CCTGATACA-GGCCCTTGTGTTTCTGTTATAAC
105 nt-long target	Amplicon	CGGCGGACGACCCGTAGACGGAGT--TTCTTAGTTGTTCGATGTTGAG--CGAGTAGT-**A**-CGTCGA-*GTACGGGAAGCCGACGGAGGA*-CCTGATACA-GGCCCTTGTGTTTCTGTTATAAC-ATCGGC
***EGFR* Exon 21**	Wild-type template	TCCTTGCATGACCACTTTTGTGGCGTCGTA-----CAGTTCTAGTGTCT----AAAACCCG-**A**-CCGGTTTG-A-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCT-TCCTCCGTTTCATTCCTCCACC
L858R	Mutant template	TCCTTGCATGACCACTTTTGTGGCGTCGTA-----CAGTTCTAGTGTCT----AAAACCCG-**C**-CCGGTTTG-A-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCT-TCCTCCGTTTCATTCCTCCACC
115 nt-long target	Amplicon	TCCTTGCATGACCACTTTTGTGGCGTCGTA--TTCTTAGATGGCTGTGGTG--AAAACCCG-**C**-CCGGTTTG-A-CG-*ACCCACGCCTTCTCTTTCTTATG*-GTACGTCT-TCCTCCGTTTCATTCCTCCACC-ATCGGC
***EGFR* Exon 21**	Wild-type template	GACCACTTTTGTGGCGTCGTACAGTTCTAG-----TGTCT----AAAACCCG-A-CCGGTTTG-**A**-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCT-TCCTCCGTTTCATTCCTCCACC
L861Q	Mutant template	GACCACTTTTGTGGCGTCGTACAGTTCTAG-----TGTCT----AAAACCCG-A-CCGGTTTG-**T**-CG-ACCCACGCCTTCTCTTTCTTATG-GTACGTCT-TCCTCCGTTTCATTCCTCCACC
106 nt-long target	Amplicon	GACCACTTTTGTGGCGTCGTACAGTTCTAG-------GTTAAGGCGCTGTT----CCGGTTTG-**T**-CG-*ACCCACGCCTTCTCTTTCTTATG*-GTACGTCT-TCCTCCGTTTCATTCCTCCACC-ATCGGC
β-Actin	Reference gene	GGTTGGCGCTCTTCTACTGGGTCC----ACTCACCGGGCGAT----GGAGAAGAC-CACCGGC-GGAGGAAGGACCGGAGGG-CCTCGACG-CGGGAAAGAGTGACCAAGAGA
111 nt-long target	Amplicon	GGTTGGCGCTCTTCTACTGGGTCC--GTATCGGTCGATTACTGG--GGAGAAGAC-CACCGGC-*GGAGGAAGGACCGGAGGG*-CCTCGACG-CGGGAAAGAGTGACCAAGAGA-ATCGGC

When the mutant target sequence is amplified, the complement of the SuperSelective primer's sequence is incorporated into the 3' end of the (-) amplicon, and the complement of that primer's unique bridge sequence (underlined) replaces the intervening sequence in the mutant (-) strand, thereby assuring that each different (-) amplicon is exponentially amplified without interference from the other SuperSelective primers. The tailed reverse primer sequence is incorporated into the 5' end of the (-) amplicon. Interprimer-specific sequences within the (-) amplicon (italicized) determine which differently colored molecular beacon lights up the amplicon. The nucleotides that distinguish the mutant sequence from their related wild-type sequence are bold and underlined.