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Single-Tube Mutation Scanning of the Epidermal Growth Factor Receptor Gene using Multiplex LATE-PCR and Lights-On/Lights-Off Probes

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Abstract

Background: Numerous mutations in exons 18-21 of the epidermal growth factor receptor (*EGFR*) gene determine the response of many patients with Non-Small Cell Lung Carcinoma (NSCLC) to anti-EGFR Tyrosine Kinase Inhibitors (TKIs). This paper describes a single closed-tube assay for simultaneous mutational scanning of *EGFR* exons 18-21 using Linear-After-The-Exponential (LATE)-PCR and Lights-On/Lights-Off probes.

Methods: The assay first co-amplifies all four exons as separate single-stranded DNA products using LATE-PCR. The amplicons are then interrogated at endpoint along their length using sets of Lights-On/Lights-Off probes of a different color for each exon. The four resulting fluorescent signatures are unique for each underlying DNA sequence. Every mutation in a target potentially alters its unique fluorescent signature thereby revealing the presence of the mutation.

Results: The assay readily detects mutations which cause sensitivity or resistance to TKIs and can distinguish these clinically important genetic changes from silent mutations which have no impact on protein function. The assay identifies as little as 5% mutant sequences in mixtures of normal DNA and mutant DNA prepared from cancer cell lines. Proof-of-principle experiments demonstrate mutation identification in formalin-fixed, paraffin-embedded NSCLC biopsies.

Conclusion: The LATE-PCR *EGFR* assay described here represents a new type of highly informative, single-tube diagnostic test for mutational scanning of multiple gene coding regions and/or multiple gene targets for personalized cancer therapies.

Keywords: LATE-PCR; Lights-On/Light-Off probes; *EGFR* gene mutation scanning; Personalized cancer medicine; Molecular diagnostics; Single-tube assay

Introduction

Insights into the biology of cancer have led to highly effective therapies that target gene products that are specifically altered in a particular tumor [1,2]. As a result, physicians can design optimal treatment regimes in accord with the status and dynamics of each patient's disease. This personalized treatment approach is technically challenging for cancer genes with numerous actionable mutations spread over multiple exons. Molecular profiling of such genes requires diagnostic tests capable of identifying each and every one of the possible targeted mutations in a format that is appropriate for routine clinical use [3,4]. This paper describes a single closed-tube PCR assay that simultaneously interrogates over 700 nucleotides in exons 18-21 of the epidermal growth factor receptor (*EGFR*) gene for more than 30 mutations of all types that define the responses of Non-Small Cell Lung Carcinomas (NSCLC) to anti-EGFR targeted therapies.

EGFR, a member of the Erb-B family of receptor tyrosine kinases, regulates the Ras/Raf/mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase-(PI3K)-PTEN-AKTmTOR signaling pathways that control cell proliferation and survival. In normal cells, ligand binding to EGFR causes homodimerization or heterodimerizaton of the receptor with other members of the Erb-B family leading to activation of the EGFR tyrosine kinase domain. This event triggers a phosphorylation signaling cascade that regulates genes controlling cell division and cell death. In NSCLC cells, EGFR over-expression or mutations that constitutively activate these signaling pathways result in uncontrolled tumor growth and survival. This finding led to the development of the anti-EGFR Tyrosine Kinase Inhibitors (TKIs) Gefitinib (Iressa®) and Erlotinib (Tarceva®). These drugs compete with ATP for binding to the EGFR tyrosine kinase domain and block the aberrant EGFR tyrosine kinase activity in cancer cells [5,6].

Treatment of NSCLC with TKIs reduces tumor size and correlates with increased progression-free survival over standard chemotherapies [7,8]. The success of these treatments, however, is strictly dependent on the presence of TKI-sensitizing mutations in the EGFR tyrosine kinase domain encoded by exons 18-21 in the absence of dominant TKI-resistant mutations [9-11]. TKI-sensitizing *EGFR* mutations occur in 10%-15% of primary NSCLC tumors in the U.S. and Europe and in up to 50% of NSCLC tumors in Asia. TKI-sensitizing mutations include a family of over 27 different deletions in *EGFR* exon 19, the L858R mutation in *EGFR* exon 21, and three allelic mutations in codon 719 in *EGFR* exon 18. Together these mutations encompass 90%-95% of all TKI-sensitizing mutations. A single mutation in *EGFR* exon 20, T790M, accounts for ~50% of TKI-resistant cases. Other less frequent TKI-resistant mutations (<1%-5%) include a family of three nucleotide insertions between codons 770 and 771 and a point mutation in codon 768 in *EGFR* exon 20. Additional mutations scattered along *EGFR*

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exons 18-21 collectively accounts for the remaining 5%-10% TKI-sensitizing mutations and additional TKI-resistant cases [6,12-15]. Thus, detection of all TKI-sensitizing and resistant *EGFR* mutations in individual NSCLC patients including detection of novel variants requires scanning the entire ~700 base pairs coding regions of *EGFR* exons 18-21 for mutations [3,4].

The most common methods for mutation scanning of *EGFR* exons 18-21, such as dideoxysequencing [3,4,16], high resolution melting analysis [17], and COLD PCR [18,19], are limited because these methods only interrogate one exon/amplicon at a time and/or involve formation of heteroduplexes of mutant and wild-type DNA. Complete mutation scanning of *EGFR* exons 18-21 by any of these methods requires a minimum of four separate PCR amplifications and may not be suitable for analysis of clinical samples that are in very limited supply and must undergo multiple molecular tests. Alternative methods for *EGFR* exon 18-21 mutation scanning such as the use of Surveyor DNA endonuclease in combination with high-performance liquid chromatography [20], denaturing High-Performance Liquid Chromatography (dHPLC) [21], PCR single strand conformation polymorphism analysis [22], or more recently massive parallel sequencing [23] lack the simplicity and convenience of a single closed tube assay [4]. These methods involve sample transfer after PCR amplification which risks laboratory or sample contamination with PCR products. Other PCR strategies designed to specifically detect the most frequent *EGFR* TKI-sensitive and TKI-resistant mutations (*e.g.*, Amplification-Refractory Mutation System (ARMS) PCR [24], use of allele-specific hybridization FRET probes of different colors [4,25], pyrosequencing [26], primer extension [27], and loop-mediated isothermal amplification (LAMP) [28], among other strategies) require the use of multiple tubes to achieve analysis of just a subset of all possible cancer-related mutations.

This paper describes a single closed-tube multiplex assay for comprehensive mutational scanning of *EGFR* exons 18-21. The assay is based on two existing technologies: Linear-After-The Exponential (LATE)-PCR, a form of non-symmetric PCR amplification that efficiently generates single-stranded DNA [29,30], and Lights-On/Lights-Off probes, a class of hybridization probes for mutation scanning of multiple DNA targets in the same reaction [31]. As proof-of-concept, the assay detected both common and rare *EGFR* mutations using cancer cell lines and formalin-fixed, paraffin-embedded NSCLC biopsies whose mutational status was either known in advance or independently confirmed using a Dilute-'N'-Go sequencing protocol specifically designed for LATE-PCR products [32,33].

Materials and Methods

LATE-PCR primers

Separate sets of LATE-PCR primers for *EGFR* exons 18-21 exhibiting similar priming efficiencies under the same amplification conditions were designed using Visual OMP software [34] (version 6.6.0; DNA Software, Inc, Ann Arbor, MI) in accordance with LATE-PCR design criteria [29,30]. Excess and limiting LATE-PCR primers for each amplicon had similar melting temperatures, about 72ºC and 75°C respectively, as calculated by Visual OMP software, Table 1. The lengths of the resulting amplicons were kept below 220 nucleotides to facilitate amplification of low quality genomic DNA typically recovered from formalin-fixed, paraffin-embedded clinical samples [35]. Primers were purchased from Biosearch Technologies (Novato, CA, USA). The primers for exons 18, 19, and 21 are located in intron sequences flanking each exon and amplify normal and mutant sequences for each exon with similar efficiency (Supplementary information S1). Exon 20 has a higher GC content than exons 18, 19, and 21 (60%). In order to generate an amplicon for exon 20 with a melting temperature that matched that of the other amplicons, one of the exon 20 primers is placed in a region inside of exon 20 that has no known mutations according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database [12]. Primers sets were first optimized for specificity and efficiency in monoplex LATE-PCR reactions before being integrated into the multiplex assay.

Lights-On/Lights-Off probes

Four sets of differently colored Lights-On/Lights-Off probes, one for each exon, were designed to hybridize to LATE-PCR single-stranded products over the same wide temperature range at the end of amplification (Table 2). Each set spanned the non-primer sequence of an amplicon (Supplementary data S1). Some probes included nucleotide mismatches to their target sequences to adjust the probe melting temperature. Lights-On probes were labeled with a fluorophore and a quencher at opposite ends. Some Lights-Off probes contained a quencher at the 5' end and were blocked at their 3' end with a C3-linker. Other Lights-Off probes contained only a quencher at the 3' end. Some particularly short Lights-Off probes had two quencher moieties at opposite ends that interacted with the quencher or fluorophore moieties from adjacently hybridized probes to increase the stability of bound probes. Certain Lights-On probes were designed to have dual On/Off functions. These probes hybridize to the target such that the quencher of one probe is adjacent to the fluorophore of another Lights-On probe. By this method binding of the second Lights-On probe acts as a Lights-Off probe to the already bound adjacent Lights-On probe (Supplementary information S1).

Lights-On/Lights-Off probes were designed taking into account the unique constraints imposed by the target sequences and secondary structures of the four *EGFR* single-stranded amplicons. The nucleotides for codons 800-823 in exon 20 were not included in the amplicon because this sequence forms a secondary structure of particularly high melting temperature that prevents the binding of probes at lower temperatures. Lights-On/Lights-Off probes were constructed to bind at temperatures below the primer annealing temperature of the reaction (72ºC) in accordance to LATE-PCR principles [29]. Lights-Off probes

Primer Name	Tm (°C)	Primer Sequence
Limiting primer exon 18	75	5' CCCAGAGGCCTGTGCCAGGGACCTTAC 3'
Excess primer exon 18	72	5' CTTGTCTCTGTGTTCTTGTCCCCCC 3'
Limiting primer exon 19	75	5' CCATGGACCCCCACACAGCAAAGCAGAAACTCAC 3'
Excess primer exon 19	72	5' GCCAGTTAACGTCTTCCTTCTCTCTCTGTCATA 3'
Limiting primer exon 20	74	5' TGGGAGCCAATATTGTCTTTGTGTTCCCGGACATAGT 3'
Excess primer exon 20	72	5' GTGCCTCTCCCTCCCTCCAG 3'
Limiting primer exon 21	75	5' AGGAAAATGCTGGCTGACCTAAAGCCACCTCCTTAC 3'
Excess primer exon 21	72	5' CTCACAGCAGGGTCTTCTCTGTTTCAG 3'

Table 1: Assay primer sequences.

Probe	Tm (°C)	Probe Sequence	5' Mod.	3' Mod.
Exon18 ON	71	5'GGTTGATCTTTTTGAATTCAGTTTCCTTCAAGATCCTCCC 3'	FAM	BHQ-1
Exon18 OFF	65	5' CGGAGCCCAGCACT 3'	BHQ-1	BHQ-1
Exon18 ON	69	5' CAAAGAGAGCTTGGTTGGGAGCTTTG 3'	BHQ-1	FAM
Exon18 ON	59	5' GGCTCCACTGGGTGTAAGCC 3'	BHQ-1	FAM
Exon18 OFF	61	5' AGGCTCCACAAGCTG 3'	BHQ-1	C3
Exon19 ON	69	5' GGACCTTCTGGGATCCAGAGTCCCCC 3'	Cal-Red	BHQ-2
Exon19 OFF	62	5' ACGGGAATTTTAACTTTCTC 3'	-	BHQ-2
Exon19 ON	59	5' CCGCTTTCGGAGATGTTGCTTGG 3'	BHQ-2	Cal-Red
Exon19 OFF	57	5' CTCTTAATTTCTTGATAGCG 3'	BHQ-2	BHQ-2
Exon19 ON	62	5' TTATCGAGGATTTCCTTGTTGGAA 3'	BHQ-2	Cal-Red
Exon20 ON	59	5' GCAGATACCCAGTAGGCGG 3'	Quasar	BHQ-2
Exon20 OFF	55	5' CTGCATGGTGAAGGTGAG 3'	BHQ-2	BHQ-2
Exon20 OFF	64	5' GCATGAGCCGCGTGATGAG 3'	BHQ-2	BHQ-2
Exon20 ON	69	5' CCAGGGGGCAGCCGAAGG 3'	BHQ-2	Quasar
Exon21 OFF	50	5' CCAGCATTATGGCTCGCCC 3'	BHQ 1	C3
Exon21 ON	70	5'TTAAAATCTGTGATCTTGGCATGCTGCGGTGAA 3'	Cal-Or.	BHQ-1
Exon21 ON	56	5' TTTTTGTCTCCCCCTGCATGGTATTCTTAA 3'	BHQ-1	Cal-Or.
Exon21 OFF	44	5' TCTCTTCTGTACCC 3'	BHQ-1	C3
Exon21 ON	59	5' CCACGGTCCCCCAAGTAGTTTATGCCGG 3'	Cal-Or.	BHQ-1
Exon21 OFF	42	5' CTAGGTCTTGGTGGATTGAGCG 3'	BHQ-1	BHQ-1
Exon21 ON	55	5' CCCACCAGTATGTTCCTGGTTGGG 3'	BHQ-1	Cal-Or.

Abbreviations: BHQ-1, Black Hole Quencher 1; BHQ-2, Black Hole Quencher 2; C3, C3 spacer linker; Cal-Red, Cal-Red 510; Quasar, Quasar 610; Cal-Or., Cal Orange 560

Table 2: Light-On/Lights-Off probe sequences.

were also designed to bind to the most common mutations [6], to be 10-15 nucleotides long to enhance their allele-discrimination, and to generate mutant fluorescent signatures that deviate considerably from the normal reference signature [31]. Lights-On probes tended to be 20-25 nucleotides long and were preferentially used for binding to infrequent mutations. Lights-On probes of higher melting temperature were also designed to bind sequences that could form secondary structures that might interfere with probe binding at lower temperatures. Finally, whenever possible, probes were placed with the intention of generating the simplest reference fluorescent signature on normal targets. Mutant signatures thus stood out more distinctly by comparison.

The color for each Lights-On/Lights-Off probe set was also chosen according to the frequency and impact of the interrogated mutations. Thus, the Lights-On probes for exon 18 were labeled with FAM, the weakest fluorophore, because the frequency of TKI-sensitizing mutations in exon 18 is relatively low, <5% [6]. In contrast, the Lights-On probes for exon 20 were labeled with the brightest fluorophore, Quasar 670, since the most critical TKI-resistance mutations reside in this exon [6]. Other details on the design of Lights-On/Lights-Off probes have been described elsewhere [31,36-38].

Genomic DNA preparation from cancer cell lines and NSCLC biopsies

Various cancer cell lines containing *EGFR* mutations were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA). Genomic DNAs from these cell lines were prepared using Quantilyse, a proteinase K lysis solution optimized for PCR consisting of 10 mM Tris-Cl pH 8.3 , 5 µM SDS, and 0.1 mg/ml Proteinase K as described [39]. The resulting DNA samples were stored at 4°C to avoid freezing and thawing. The sequence of normal and mutant *EGFR* exons 18-21 in each cell line was confirmed by sequencing.

Normal genomic DNA with various allele configurations for the natural-occurring SNP rs1050171 [40] in *EGFR* exon 20 (NG_007726.3:g.167339G>A) [40] were either purchased from the Coriell Cell Repositories (Camden, New Jersey) or prepared from the ATCC cancer cell lines as described above and confirmed by sequencing. The following samples were used: DNA samples NA10851 (AA genotype), NA07348 (A/G genotype), and NA10855 (GG genotype) and cell lines HCC827 (AA genotype) and HCC4006 (GG genotype).

Genomic DNA from formalin-fixed, paraffin-embedded NSCLC biopsies with known normal sequences and mutations in *EGFR* exons 18-21 as determined by COLD-PCR/sequencing analysis [18] were kindly provided by Dr. Khalid Tobal (Molecular Oncology Unit, Guys' Hospital, London UK). These samples were prepared and characterized using the methods described by Pennycuick et al. [19].

LATE-PCR amplification conditions

Multiplex LATE-PCR amplifications were carried out in 25 µl reactions consisting of 1X Platinum Taq buffer (Invitrogen, Carlsbad, CA), 3 mM $MgCl_2$, 400 µM of each deoxynucleotide triphosphate, 50 nM of each limiting primer, 1 µM of each excess primer, 100 nM of each Lights-On probe, 300 nM of each Lights-Off probe, 2.5 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 1-1000 copies of genomic DNA. Amplification was carried out in a Stratagene MX3500P (Agilent Technologies, Santa Clara, CA) for 70 cycles. Each cycle consisted of denaturation at 99°C for 10 seconds, primer annealing at 72°C for 15 seconds, and primer extension at 75°C for 30 seconds - the denaturation temperature was set at 99°C instead of the customary 95°C because of the higher Tm of the double-stranded component of the exon 20 amplicon, 96°C. At the end of amplification there was a final extension step at 75°C for 3 minutes. For endpoint analysis, the reaction was slowly cooled down from 85°C to 35°C at 1°C degree decrements every 30 seconds to allow for hybridization of the Lights-On/Lights-Off probes. After 10 minute incubation at 35°C for equilibration of the bound probes, the reaction was then heated from 35°C to 85°C in 1°C increments every 30 second with fluorescent

acquisition at every degree. When necessary, samples were prepared for dideoxysequencing by the Dilute'N'Go method [32,33] and sequenced by GeneWiz (South Plainfield, NJ). Sequence analysis was conducted using the CLUSTAL online program [41]. The normal *EGFR* exon 18-21 sequences from NCBI Reference Sequence NG_007726.3 were used for comparison against all cell lines and clinical samples. Deletions were identified in sequence chromatographs as regions where deleted target sequence became out of frame relative to the reference sequence.

The concentration of each Lights-On probe was set at 100 nM, slightly lower than the anticipated maximal yield of single-stranded DNA amplicons generated in the LATE-PCR at terminal concentration at 70 cycles to guarantee complete binding of all the Lights-On probes and minimize differences among replicate fluorescent signatures [31,36-38], except for the experiment testing the limit of detection of the assay where the concentration of each Lights-On probe was set at 300 nM. The concentration of each Lights-Off probe was set three-fold higher than the concentration of the Lights-On probe to guarantee that every bound Lights-On probe will have a Lights-Off probe hybridized next to it [31,36-38] (*i.e.,* 300 nM for all experiments shown in this paper except for the experiment testing the limit of detection of the assay where it was 600 nM).

For experiments involving artificial mixtures of mutant and normal genomes, DNA from cell line HCC4006 carrying a TKI-sensitizing deletion in *EGFR* exon 19 was matched in concentration with genomic DNA from normal cells (as confirmed by Ct values generated by real-time LATE-PCR amplification with SYBR-Gold) and mixed in various ratios to generate samples with 50%, 10%, 5%, and 1% mutant DNA. Every sample contained a total of 10,000 genomes to guarantee that the lowest percentage of mutant genomes (1%) would be in large enough large numbers (100 copies) such that every replicate sample would be guaranteed to contain mutant genomic DNA. A similar set of mutant and normal genome mixtures was prepared for DNA from cell line NCI-H1975 carrying the TKI-resistant mutation T790M.

Data analysis

Fluorescent signatures were generated by plotting the negative first derivative of the raw fluorescent signals from the melting analysis relative to temperature (-dF/dT) as a function of temperature without any background signal subtraction or data normalization [31]. The only exception was the data in Figure 5B in which the fluorescent derivative values were normalized to the highest peak in the fluorescent signature that was common to all samples. For each exon, mutations were identified by comparing the shape of fluorescent signature from the test sample to the shape of the fluorescent signature from the reference normal sample. Differences in the shape beyond the noise among replicate samples due to temperature shifts in the position of the peaks and valleys of the fluorescent signatures identified the presence of a mutation [31]. Differences in fluorescent signature intensities without a temperature shift reflected differences in amplicon yield and do not indicate a mutation [38]. The Student's t-test was used to establish the statistical significance of the difference in the fluorescent signatures at specific temperatures that distinguished artificial mixtures of 5% mutant genomes, 95% normal genomes from samples with 100% normal DNA.

Results

Principle of the LATE-PCR *EGFR* assay

The LATE-PCR *EGFR* assay co-amplifies *EGFR* exons 18-21 as separate amplicons in a single tetraplex reaction. The four resulting single-stranded products are then simultaneously scanned for

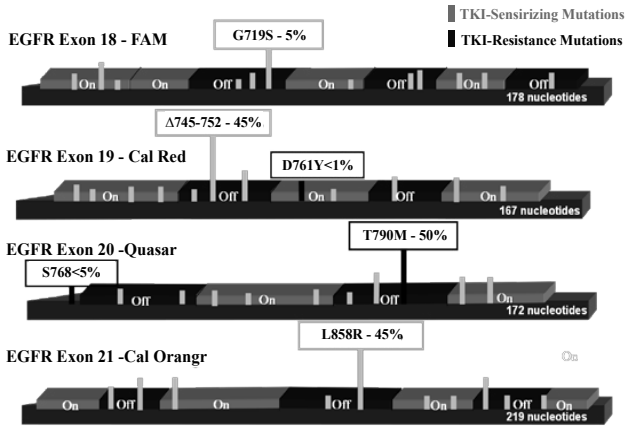

Figure 1: LATE-PCR *EGFR* assay design. This diagram illustrates the distribution of Lights-On/Lights-Off probes along their corresponding amplicon targets and the length of the amplicons (gray and black bars labeled "On" and "Off" correspond to Lights-On/Lights-Off probes; thick black lines correspond to amplicon targets; light grey vertical lines illustrate TKI-sensitizing mutations; black vertical lines illustrate TKI-resistant mutations; boxes with labels mark the approximate position of mutation hotspots and specify the name and frequency of the mutation; see also Supplementary information S1). Whenever possible, Lights-Off probes were designed to bind the sites of the hotspots mutations to generate the most distinctive mutant fluorescent signatures (mutations under Lights-Off probes have the greatest impact on fluorescent signatures) [31]

mutations at endpoint using sets of Light-On/Lights-Off probes of a different color for each exon (Figure 1 and Supplementary information S1). The probes hybridize at temperatures below the primer annealing temperature and do not interfere with amplification. Each Lights-On probe is labeled with a quencher and a fluorophore and generates a fluorescent signal when hybridized to its target sequence in the absence of an adjacent Lights-Off probe. Each Lights-Off probe is labeled with only a quencher and is designed to extinguish the signal from its paired Lights-On probe when hybridized to the adjacent target sequence at a lower temperature. Melting of the complete set of Lights-On/Lights-Off probes hybridized along the length of their target amplicon generates a fluorescent signature that is unique for the sequence of each amplicon. Any change from a reference normal fluorescent signature indicates the presence of a mutation. Mutations can be single nucleotide changes, deletions, or insertions anywhere in the target sequence. The fluorescent color and temperature of the change relative to the normal reference signature indicates the presence and approximate location of the mutation [31,36-38]. Each cancer-related fluorescent signature is unique and highly reproducible making it possible to identify mutations either by reference to previously characterized fluorescent signatures or by sequencing those signature that do not match any previously established fluorescent signatures.

Reference fluorescent signals from normal *EGFR* exon 18-21 sequences.

Normal *EGFR* exons 18-21 have a fixed DNA sequence except for exon 20 which contains a silent single nucleotide polymorphism consisting of an A and a G allele at the third position of *EGFR* codon 787 (SNP rs1050171, NG_007726.3:g.167339G>A) [42]. The LATE-PCR assay generated unique, highly reproducible fluorescent signatures for each normal sequence (Figure 2, panels A-D-normal DNA sequences were independently confirmed by sequencing). Each of the possible homozygous and heterozygous allele configurations of SNP rs1050171 had its own fluorescent signature in the corresponding region of exon

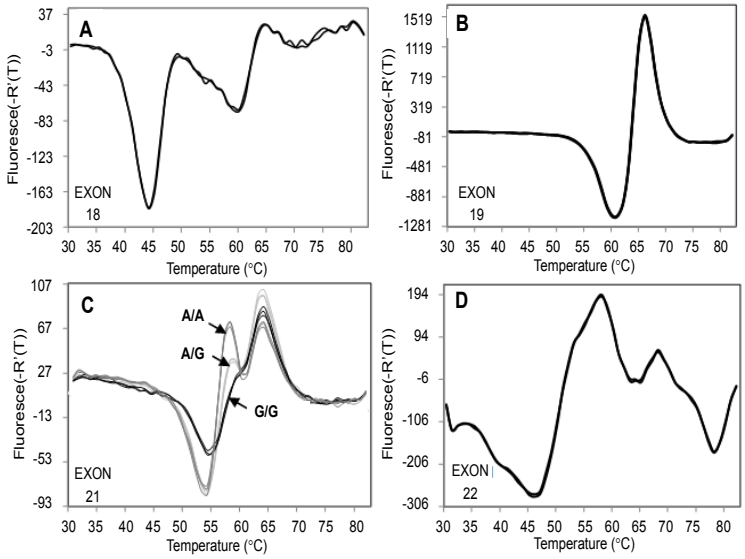

Figure 2: Reference fluorescent signatures from normal *EGFR* exon 18-21 sequences. This figure shows the fluorescent signatures for *EGFR* exons 18-21 from normal DNAs with different genotypes for the naturally-occurring rs1050171 single-nucleotide polymorphism in *EGFR* exon 20 (Panel A: exon 18; Panel B: exon 19; Panel C: exon 20; Panel D: exon 21 - normal *EGFR* sequences in these DNA samples were independently confirmed by sequencing). Each exon generated its own characteristic, highly reproducible fluorescent signature which was the same for all normal DNA samples tested (exons 18, 19, and 21; Panels A, B, and D) except for exon 20 where samples with different SNP rs1050171 allele configurations had unique fluorescent signatures (Panel C). These normal fluorescent signatures served as a reference for comparison against the fluorescent signatures from test samples.

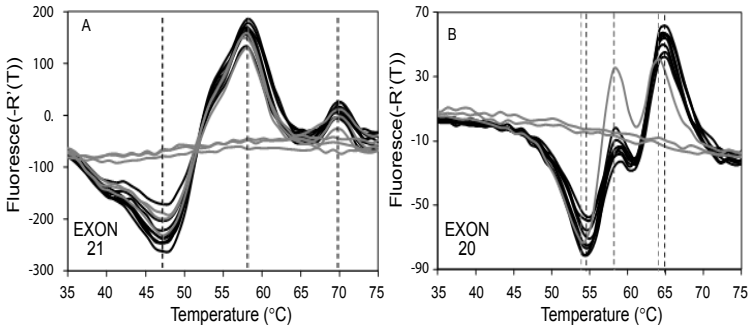

Figure 3: Assay limit of detection. This figure shows the fluorescent signatures for sets of three replicate samples from serial 10-fold dilutions of normal genomic DNA heterozygous for SNP in exon 20 (black lines 1000-10 targets, gray lines, <10 targets). Panel A: Fluorescent signatures for exon 21: The shape of the fluorescent signatures, as defined by the temperature of the peaks and valleys (see vertical black dashed lines) was not impacted by the number of starting targets in the amplification reaction. Some replicate reactions with less than 10 starting genomes did not received genomic DNA due to Poisson distribution statistics and did not generate fluorescent signatures. Panel B: Fluorescent signatures for exon 20. Reactions containing less than 10 genomes generated different fluorescent signatures due to uneven distribution of the individual SNP rs1050171 alleles in these samples due to Poisson statistics. These results show that the assay can resolve mixtures of DNA sequence variants by dilution and has a detection sensitivity of less than 10 starting DNA molecules. Note: Fluorescent signatures in this figure were noisier compared to fluorescent signatures of the rest of the figures in this paper due to use of excess concentrations of Lights-On probes, see Materials and Methods. Probes bound to the totality of single stranded DNA generate replicate fluorescent signatures whose amplitude varies according to the amplicon yield in each replicate. Such amplitude differences were not considered for mutation detection in this experiment.

20, thereby demonstrating the resolving power of the assay. The set of normal fluorescent signatures in Figure 2 served as a reference for comparison to the fluorescent signatures from experimental samples for mutation identification.

Limit of detection of the assay

Replicate assays containing 1000, 100, 10, or <10 copies normal genomic DNA at the start of the reaction were amplified and analyzed to determine whether the number of starting targets impacted any of the fluorescent signatures. Amplification was carried out for 70 cycles to generate similar concentrations of single-stranded DNA amplicons at endpoint despite a three-order of magnitude difference in the number of starting targets in the reactions [33]. As illustrated in Figure 3A for *EGFR* exon 21, replicate reactions with 10 to 1000 starting copies generated fluorescent signatures that were fully consistent with the normal target sequence. A subset of the reactions initiated with fewer than 10 gene copies failed to generate the expected fluorescent signature, probably because these reactions did not actually contain target DNA due to Poisson's distribution statistics [43]. Similar results were obtained for *EGFR* exons 18 and 19 (not shown). In the case of exon 20, more

than one fluorescent signature was observed in amplification reactions started with fewer than 10 genomes (Figure 3B). This result is fully consistent with the fact that the starting genomes were heterozygous for SNP rs1050171 (A/G alleles) as established by sequencing and as evidenced by this sample's fluorescent signature which matches the reference normal A/G genotype signature (Figure 2C). At the single-genome level, 50% of reactions would have an A allele and 50% of reactions would have a G allele. The particular aberrant signature obtained in Figure 3B corresponded to amplicons with only the A allele by comparison to the reference normal fluorescent signatures (compare Figure 3B to Figure 2C) as confirmed by sequencing. These results demonstrate that the LATE-PCR assay has a detection sensitivity of less than 10 starting DNA molecules. These results also show that the assay can resolve mixtures of DNA sequence variants by dilution.

Assay verification with cancer cell lines

Following characterization of the reference fluorescent signatures, the assay was verified for detection of high frequency cancer-related *EGFR* mutations in several cell lines [6,12,13,44]. TKI-sensitizing mutations in these cell lines included members of a family of deletions in exon 19 encompassing codons 746-751 (a 9-nucleotide deletion ΔL747-E749 A750P c.2239_2247del 9, 2248 G>C in cell line HCC4006; a 15-nucleotide deletion ΔE746-T750 c.2235–2249 del 15 in cell line HCC827, and 18-nucleotide deletion, ΔE746-A751 S752I c.2236–2253 del 18, 2254 T>A, 2255 C>T in cell line HCC2935 - as a whole this family of deletions accounts for 45% of all TKI-sensitive mutations), a point mutation in exon 18 (mutation G719S c.2155G>A in cell line SW48, which together with other alleles at this position accounts for ~5% of TKI-sensitive mutations), and a point mutation in exon 21 (mutation L858R c.2573T>G in cell line NCI-H1975, which accounts for 40%-45% of all TKI-sensitive mutations) [6,12,13,44]. TKI-resistant mutations included the most common resistance point mutation in exon 20 (mutation T790M c.2369C>T in cell line NCI-H1975 corresponding to ~50% of TKI-resistant mutations) [6,12,13,44]. Normal and mutant *EGFR* sequences in each of these cancer cell lines were confirmed by sequencing.

The LATE-PCR *EGFR* assay generated unique fluorescent signatures for each of the deletions in exon 19 (Figure 4A). The assay also yielded distinctive fluorescent signatures from genomes carrying the G719S, the L858R, or the T790M point mutations (Figure 4B-D). In

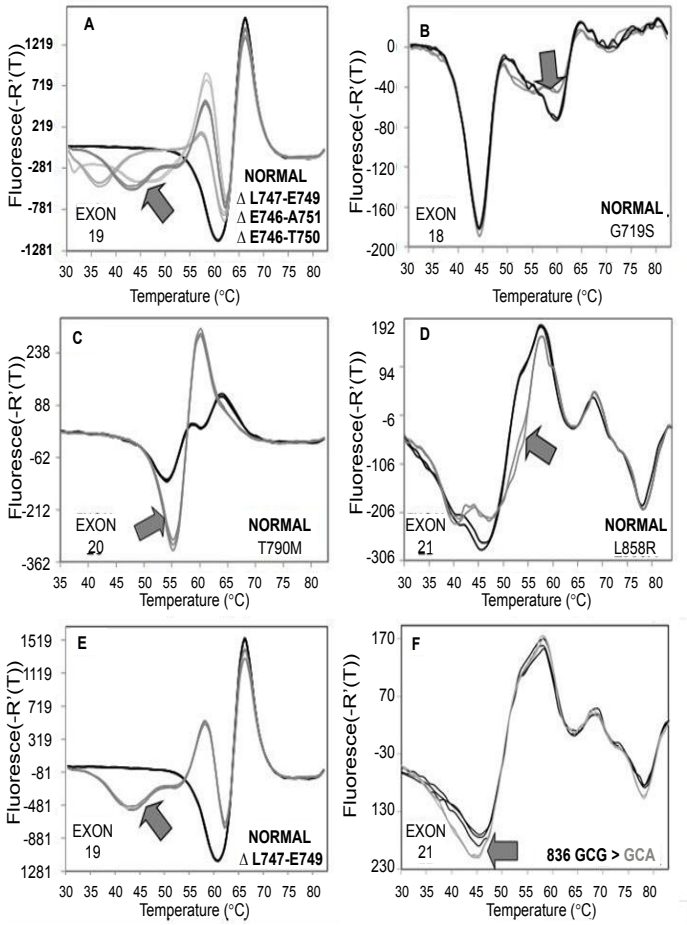

Figure 4: Fluorescent signatures from cancer cell lines with high frequency TKI- sensitizing and TKI- resistant mutations in *EGFR* exons 18-21. Black lines correspond to reference normal fluorescent signatures; gray lines correspond to fluorescent signatures from a cell line, arrows point to relevant differences indicative of mutations. All samples were processed in triplicate and plotted individually. In every instance *EGFR* mutations were confirmed by sequencing. Panel A: Each exon 19 deletion generates a unique fluorescent signature. Panel B-E: TKI-related mutations generate distinctive fluorescent signatures (Panel B, exon 18 G719S mutation in cell line SW48; Panel C, exon 20 T790M mutation in cell line NCI-H1975; Panel D, exon 21 L858R mutation in cell line NCI-1975; Panel E, exon 19 ΔL747-E749 deletion in cell line HCC4006). Panel F: Altered fluorescent signature from exon 21 in cell line HCC4006 due to a silent mutation in *EGFR* codon 836. This data demonstrates that silent mutations that do not affect the protein sequence generate their own fluorescent signature that distinguishes these mutations from true TKI-related mutations.

addition, analysis of the cancer cell line HCC4006 revealed that the assay identified a silent mutation that does not alter the protein sequence and discriminated it from true driver mutations. According to the supplier (ATCC), the HCC4006 cell line has a deletion mutation in *EGFR* exon 19 and is normal for *EGFR* exons 18, 20, and 21. LATE-PCR analysis of this cell line generated normal fluorescent signatures from exons 18 and 20 (not shown), the expected mutant fluorescent signatures from exon 19 (Figure 4E), and an altered fluorescent signature for exon 21 (Figure 4F) which was different from the L858R mutation commonly found in this exon (Figure 4D). Consistent with the LATE-PCR results, DNA sequence analysis of cell line HCC4006 established normal DNA sequences for *EGFR* exons 18 and 20, mutant DNA sequences for exon 19 (the 9 bp deletion ΔL747-E749 in exon 19, not shown) and a silent G-to-A mutation in the third position of codon 836 in exon 21, Figure 4F). Thus, a silent mutation in the probe target sequence that does not alter the protein sequence has its own unique fluorescent signature that distinguishes it from true driver mutations.

Detection of mutant sequences among excess normal DNA

Tumors often contain mixtures of cancerous and normal cells. To determine the detection sensitivity of the assay for mixtures of mutant and normal genomes, genomic DNA from cell line NCI-H1975 containing the T790M TKI-resistant mutation in exon 20 and from the cell line HCC4006 carrying the ΔL747-E749 TKI-sensitizing deletion in exon 19 were first matched in concentration to a sample of normal DNA and then separately mixed to generate artificial mixtures containing 50%, 10%, 5%, and 1% mutant DNA. The resulting fluorescent signatures of these mixtures, Figure 5, demonstrates that the assay distinguished as little as 5% of each mutant genome in 95% normal DNA ($p<0.002$ for both examples in Figure 5).

Assay verification with clinical samples

As proof-of-principle for the use of the assay with clinical samples, genomic DNAs from ten Formalin-Fixed Paraffin-Embedded (FFPE) NSCLC biopsies of known *EGFR* mutational status for exons 18-21, as previously established by COLD-PCR/dideoxysequencing [18,19] were scanned for mutations using the LATE-PCR assay. The experimenters were blinded to the *EGFR* mutation status of the clinical DNA samples while performing and interpreting the results. Table 3 summarizes the results of the analyses. All ten clinical samples, representing a total of 40 *EGFR* amplicons (4 amplicons per sample) generated fluorescent signatures from all exons. These fluorescent signatures were initially categorized as either normal or altered based solely on comparison to the reference normal fluorescent signature for each corresponding exon (Table 2 and Supplementary information S2 and S3). Some of the altered fluorescent signatures were assigned to a specific class of mutations

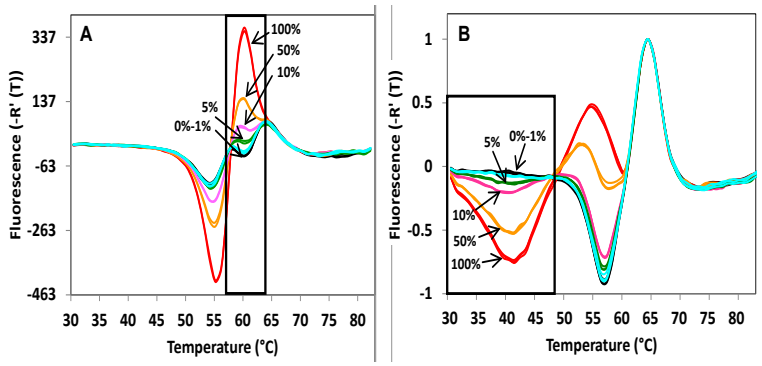

Figure 5: Detection of mutant *EGFR* sequences in mixtures of normal and mutant DNA. Artificial mixtures containing different ratios of normal and mutant genomes generate fluorescent signatures that distinguished as little as 5% mutant genomes in 95% normal DNA. All samples were processed in replicates and plotted individually. Panel A: Mixtures of normal DNA and DNA from cell line NCI-H1975 carrying the T790M mutation. Panel B: Mixtures of normal DNA and DNA from cell line HCC4006 genomic DNA carrying the 9-base pair exon 19 ΔL747-E749 deletion. Black box indicates the range of temperatures where the fluorescent signatures were most discriminating for the various DNA mixtures. Percentages in the panels correspond to the percentage of mutant genomes in each mixture. Replicate set of fluorescent signature for each mixture is labeled with a different color. The overlapping 1% and 0% fluorescent signatures in each panel were labeled blue and black, respectively. The maximal difference in the fluorescent signatures between the 5% and 10% mutant mixtures was statistically significant ($p<0.002$).

Test Samples	Exon 18	Exon 19	Exon 20	Exon 21
1068	Normal	Normal	**Altered**	Normal
629	Normal	**Deletion**	Normal	Normal
1012	Normal	**Deletion**	Normal	Normal
1020	**Altered**	Normal	Normal	Normal
417	Normal	Normal	**Altered**	**Altered**
1009	**Altered**	Normal	Normal	**Altered**
906	Normal	Normal	Normal	Normal
1035	Normal	**Normal**	Normal	**Normal**
1002	Normal	**Altered**	Normal	**Altered**
1124	Normal	**Altered**	Normal	**Altered**

Note: Normal and altered fluorescent signatures refer to fluorescent signatures from the clinical samples that either match or differ from the reference normal fluorescent signatures (see text for details). Deletions listed were identified based on the similarities between the altered and reference normal fluorescent signatures (Supplementary data S2). The only LATE-PCR result that was discordant with the known *EGFR* mutation status of these samples defined by COLD-PCR/dideoxysequencing is underlined; see text and Supplementary information S2 and S3 for details.

Table 3: Results from blinded analysis of clinical samples using the LATE-PCR *EGFR* assay.

based on their similarities to known references mutant fluorescent signatures from the cancer cell lines (Table 3 and Supplementary information S2). The remaining altered fluorescent signatures did not correspond to any of the mutant fluorescent signatures generated from the cancer cell lines (Supplementary information S2).

Once the samples were decoded based on their known sequences from COLD-PCR/sequencing or on confirmatory sequencing data (Supplementary information S2), the LATE-PCR assay correctly scored 38/40 amplicons (95%) as mutant or normal. The only two discordant results were from the same sample (sample 1035). These results were classified as false negatives since LATE-PCR identified all amplicons from this sample as normal while the COLD-PCR/dideoxysequencing reference method scored two of the four amplicons from this sample as mutants (according to COLD-PCR/dideoxysequencing exon 19 had a silent mutation and exon 21 had the L858R mutation, Table 3 and Supplementary data S2). The sample scored as normal by LATE-PCR did not have any sequence variants above the detection threshold of dideoxysequencing performed on the LATE-PCR products (data not shown). Thus, this false negative result could be attributed to a greater detection sensitivity of COLD-PCR/sequencing which enriches for mutations [18,19].

Discussion

The single-tube LATE-PCR assay described here simultaneously scans *EGFR* exons 18-21 for all possible mutations. Each mutation or set of mutations within an exon exhibits a unique fluorescent signature which distinguishes it from the corresponding reference normal fluorescent signature. Individual fluorescent signatures are reliable because they reflect the intrinsic thermodynamic interactions between a carefully designed set of probes and their target sequences under optimized assay conditions.

We anticipate that the LATE-PCR assay will significantly reduce the time and the cost inherent to analyzing individual tumor samples for *EGFR* mutations by conventional dideoxysequencing. Most (85-90%) of lung cancer cancer patients in the United States and Europe do not have mutations in the four *EGFR* exons given the low frequency of cancer-related *EGFR* mutations in these populations [9-11]. This is even true for high prevalence subgroups, including women with adenocarcinomas and non-smokers with lung cancer, in which 70% of samples have normal *EGFR* exons [9-11]. The LATE-PCR assay has the potential to rapidly detect mutant *EGFR* exons for subsequent characterization by DNA sequencing, thereby avoiding spending resources on sequencing normal exons. Furthermore, as fluorescent signatures corresponding to specific mutations are gathered, they can be stored in an ever expanding database which may eventually allow immediate identification of any previously observed genotypes. This database may also be useful for mathematically predicting the fluorescent signatures of mixed mutant/normal genomes of varying proportions. As shown in Figure 3B, the presence of such mixtures could rapidly be tested by diluting the DNA in the sample down to the single molecule level.

The single-tube multiplexed assay is significantly more convenient and informative compared to other methods for *EGFR* mutation scanning. For once, it is the only mutation scanning method capable of interrogating multiple PCR products for all possible mutations at the end of amplification in a single closed-tube. In contrast, mutation scanning by dideoxysequencing and high resolution melting requires the use of four tubes to scan four exons. High resolution melting has the additional limitation that it only reveals the presence of a mutation but not its identity or location [45]. In the LATE-PCR assay, the fluorescent signature of each mutation immediately reveals its approximate location and may firmly stablished its identity by comparison to previously archived fluorescent signatures [31,36-38]. Next generation sequencing can simultaneously interrogate *EGFR* exons 18-21 for mutations but lack the simplicity and convenience inherent to a closed tube assay [23].

Assays based on mutation-specific ARMS PCR primers, Taqman, or FRET probes of different colors (molecular beacon, scorpion probes) simultaneously interrogate *EGFR* exons 18-21 in single multiplex reaction but only identify the most prevalent set of TKI-related mutations due to the limited number of detection colors available in existing PCR machines [4]. Moreover, since these three types of probes are sequence-specific, any silent mutations under a mutation-specific probe will cause these probes to fail to hybridize and generate a false negative result. The same silent mutations would generate a unique fluorescent signature in the LATE-PCR *EGFR* assay that would distinguish them from true driver mutations. Mutant fluorescent signatures can be further characterized by sequencing unlike the case of ARMS PCR, where all amplicons, including false-positive amplicons due to mispriming, contain the sequence of the mutation-specific primers used for amplification.

In proof-of-principle experiments, the LATE-PCR assay correctly identified all silent and disease-causing *EGFR* mutations in the various cancer cell lines tested as confirmed by sequencing. Particularly noteworthy was the ability of the assay to identify and distinguish among the various TKI-sensitizing deletions in *EGFR* exon 19 which differ in prognostic significance (Figure 4A) [46]. This is not possible with *EGFR* assays based on mutation-specific probes or mutation-specific primers where all exon 19 deletions are detected by the absence of amplification products. Such assays require additional controls to distinguish exon 19 deletions from PCR amplification failures. The assay was not verified for detection of EGFR insertion mutations because these mutations were not present in our tested samples. According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database [12], insertion mutations only correspond to 1.52% of all EGFR mutations. This type of mutations has been detected in LATE-PCR Lights-On/Lights Off assays of similar design as the assay described here used for scanning for mutations conferring antibiotic-resistance in *Mycobacterium tuberculosis* (Rice et al., manuscript in preparation)

The LATE-PCR assay also correctly identified the presence of a mutation in all but one of the FFPE NSCLC biopsies tested. The assay even detected EGFR mutations that it was not been optimized to detect because they were not present in the cancer cell lines used for assay development. The only exception was a sample where all the amplicons were scored as normal by LATE-PCR and by subsequent dideoxysequencing but as mutant by the reference COLD-PCR/sequencing method. This false negative result could be attributed to a greater detection sensitivity of COLD-PCR/sequencing, which enriches for *EGFR* mutations [18,19]. The LATE-PCR assay has a detection sensitivity of 5% mutant targets in 95% normal DNA, a sensitivity that exceeds that of conventional dideoxysequencing (25%-30%), is within the range of detection sensitivity of HRMA (2.5%-10%), but is below the sensitivity afforded by COLD-PCR enrichment (0.1%-1%) [18,19,47]. Thus, the LATE-PCR assay succeeded in all instances where the mutation was present within the detection sensitivity of the assay. Since the LATE-PCR assay is aimed at analysis of solid tumors for patient stratification, detection sensitivity could be enhanced by ensuring a sufficient percentage of tumor cells in the biopsied sample (>70%) using core needle biopsies or laser-catapulting micro-dissection. Use of such tumor-enriched samples would also permit characterization of samples

with altered fluorescent signatures identified by the LATE-PCR assay using the less sensitive but more commonly available conventional dideoxysequencing method. Alternatively, the LATE-PCR EGFR assay could be combined with PCR amplification protocols that enrich for mutant targets (e.g., COLD-PCR).

Results from analysis of the NSCLC FFPE biopsies also demonstrate the robustness of the LATE-PCR assay. Thus, the fact the normal fluorescent signatures from the clinical samples were identical to the reference normal fluorescent signals generated from intact genomic DNA (Supplementary information S3) shows that the assay was compatible with the different techniques of DNA extraction used for analysis of the cancer cell lines [39] and the clinical samples [19] as well as with the FFPE method of biopsy preservation, despite the damage that FFPE archiving causes to genomic DNA. The results reported here suggest that the assay will be capable of identifying the vast majority EGFR mutations but analysis of larger numbers of clinical samples will be needed to confirm this conclusion. Thus, the data presented here should be considered only proof-of-concept evidence.

In the future, the clinical utility of this assay as a mutation scanning method would be further complemented by incorporation of additional cancer genes into the multiplex assay. *EGFR* is not the only gene that determines the response to anti-EGFR TKIs; mutations in other members of EGFR's Erb-B family and in downstream effectors of EGFR can initiate malignant growth and dictate response to inhibitors [48,49]. Since the LATE-PCR *EGFR* assay is modular, additional genetic targets can easily be added to the system and probed outside of the temperature space currently used for the *EGFR* Lights-On/Lights-Off probes. Thus, additional cancer gene targets such as *KRAS, BRAF, and PIK3CA* can be included, amplified, and interrogated for mutations in the same tube as *EGFR*.

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