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(54) Title: NOVEL COMPLEX MUTATIONS IN THE EPIDERMAL GROWTH FACTOR RECEPTOR KINASE DOMAIN

(57) Abstract: Six new mutations were found in exon 19 of the EGFR gene, the exon that is often mutated in tumors. The invention comprises methods of detecting the mutations, methods of prognosis and methods of predicting response to treatment based on the presence of absence of the mutations.



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NOVEL COMPLEX MUTATIONS IN THE EPIDERMAL GROWTH FACTOR RECEPTOR KINASE DOMAIN

FIELD OF THE INVENTION

- 5 The invention relates to cancer diagnostics and companion diagnostics for cancer therapies. In particular, the invention relates to the detection of mutations that are useful for diagnosis and prognosis as well as predicting the effectiveness of treatment of cancer.

BACKGROUND OF THE INVENTION

- 10 Epidermal Growth Factor Receptor (EGFR), also known as HER1 or ErbB1, is a member of the type 1 tyrosine kinase family of growth factor receptors. These membrane-bound proteins possess an intracellular tyrosine kinase domain that interacts with various signaling pathways. Upon ligand binding, receptors in this family undergo dimerization and subsequent autophosphorylation of the tyrosine kinase domain. The
- 15 autophosphorylation triggers a cascade of events in intracellular signaling pathways, including the Ras/MAPK, PI3K and AKT pathways. Through these pathways, HER family proteins regulate cell proliferation, differentiation, and survival.

- A number of human malignancies are associated with aberrant expression or function of EGFR (Mendelsohn *et al.*, (2000), "*The EGF receptor family as targets for cancer therapy*,"
- 20 *Oncogene*, 19:6550-6565). In particular, it has been demonstrated that some cancers harbor mutations in the EGFR kinase domain (exons 18-21). In non-small cell lung cancer (NSCLC), these mutations were shown to promote anti-apoptotic pathways in malignant cells (Pao *et al.* (2004), "*EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib*",

P.N.A.S. 101 (36): 13306–13311; Sordella *et al.* (2004), "*Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways*", Science 305 (5687): 1163–1167).

Therapies targeting EGFR have been developed. For example, cetuximab (ERBITUX™) and panitumumab (VECTIBIX™) are anti-EGFR antibodies. Erlotinib (TARCEVA™) and
5 gefitinib (IRESSA™) are quinazolines useful as orally active selective inhibitors of EGFR tyrosine kinase. These drugs are most effective in patients whose cancers are driven by aberrant EGFR activity. A randomized, large-scale, double-blinded study of IRESSA™ (IRESSA Pan-Asia Study (IPASS)) compared gefitinib to the traditional chemotherapy as a first-line treatment in non-small cell lung cancer (NSCLC) (Mok *et al.* (2009), "*Gefitinib or
10 carboplatin-paclitaxel in pulmonary adenocarcinoma*", N Eng J Med 361:947-957). IPASS studied 1,217 patients with confirmed adenocarcinoma histology. The study revealed that progression-free survival (PFS) was significantly longer for IRESSA™ than chemotherapy in patients with EGFR mutation-positive tumors. The opposite was true for tumors where EGFR was not mutated: PFS was significantly longer for chemotherapy than IRESSA™. The
15 study demonstrated that to improve a lung cancer patient's chances of successful treatment, EGFR mutation status must be known.

Analysis of clinical outcome revealed that patients with tumors harboring mutations in the kinase domain of EGFR (exons 18-21) have better response to erlotinib than those with tumors expressing wild-type EGFR (U.S. Patent Nos. 7,294,468 and 7,960,118). These
20 mutations are predictive of response to tyrosine kinase inhibitors (TKIs) such as quinazolines erlotinib (TARCEVA™) and gefitinib (IRESSA™). Among the EGFR mutations, deletion of amino acids 746-750 is especially common in lung cancer patients (see U.S. Patent No. 7,294,468 and Kosaka *et al.* (2004), "*Mutations of the epidermal growth factor receptor gene in lung cancer, biological and clinical implications*", Cancer Res. 64:8919-23).
25 Kosaka *et al.* document a study involving 277 Japanese lung cancer patients. The Japanese

study revealed that EGFR mutations occurred in 40% of adenocarcinomas of the lung. About one-half of the mutations (20% of patients) are deletions around amino acids 746-750 (nucleotides 2238-2250).

Some mutations in the EGFR kinase domain are common, while others occur less frequently. However, it is essential that a clinical test for EGFR mutations target as many mutations as possible. This will assure that patients with rare mutations do not receive a “false negative” test result. If a rare mutation goes undetected, the patient with such a mutation will not receive potentially life-saving treatment. Therefore when a new mutation in the EGFR kinase domain is discovered, detecting this mutation has the potential of affecting the clinical outcome in some patients.

SUMMARY OF THE INVENTION

In one embodiment, the invention is an oligonucleotide that specifically hybridizes to a nucleic acid containing a mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1.

In another embodiment, the invention is a method of detecting a mutation in the epidermal growth factor receptor (EGFR) gene in a sample from a human, comprising: contacting the nucleic acid in the sample with an oligonucleotide capable of selectively hybridizing to a target nucleic acid containing a mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1; incubating the sample under conditions allowing selective hybridization of the oligonucleotide to the target nucleic acid; and detecting the hybridization.

In yet another embodiment, the invention is a method of treating a patient having a tumor possibly harboring cells with a mutation in the epidermal growth factor receptor (EGFR) gene, comprising requesting that the patient's sample be tested for the presence of the mutated EGFR gene characterized by a mutation selected from the list consisting of

5 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1; and if any of the mutations are reported as present, administering to the patient a compound that inhibits signaling of the mutant EGFR protein encoded by the mutated gene. Said embodiment includes a compound that inhibits signaling of the mutant EGFR protein encoded by the mutated

10 gene for use in the treatment of a disease related to or caused by a tumor possibly harboring cells with a mutation in the epidermal growth factor receptor (EGFR) gene, comprising requesting that the patient's sample be tested for the presence of the mutated EGFR gene characterized by a mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and

15 2264 C>A in SEQ ID NO: 1; and if any of the mutations are reported as present.

In yet another embodiment, the invention is a kit for detecting mutations in the human EGFR gene, including any mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, the kit comprising one or more oligonucleotides selected from

20 SEQ ID NOs: 11-40.

In yet another embodiment, the invention is a reaction mixture for detecting mutations in the human EGFR gene, including any mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, the reaction mixture comprising one

25 or more oligonucleotides selected from SEQ ID NOs: 11-40.

In yet another embodiment, the invention is the use of oligonucleotides selected from SEQ ID NOs: 11-40 in detecting mutations in the human EGFR gene selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1. In a variation of this embodiment, the invention is the use of detection of the mutations in the human EGFR gene selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, in diagnosis or prognosis of cancer. In a further variation of this embodiment, the invention is the use of detection of the mutations in the human EGFR gene selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1 in designing treatment of a cancer patient or predicting response of the cancer patient to the treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 (1A-1C) shows SEQ ID NO: 1, the cDNA sequence of wild-type EGFR.

FIG. 2 shows SEQ ID NO: 2, the amino acid sequence of wild-type EGFR.

FIG. 3 shows SEQ ID NO: 3, the wild-type sequence of nucleotides 2221-2260 of the EGFR gene; and SEQ ID NO: 4, the mutation 2236_2248>ACCC.

FIG. 4 shows SEQ ID NO: 3, the wild-type sequence of nucleotides 2221-2260 of the EGFR gene; and SEQ ID NO: 5, the mutation 2237_2244>CGCCC.

FIG. 5 shows SEQ ID NO: 6, the wild-type sequence of nucleotides 2221-2280 of the EGFR gene; and SEQ ID NO: 7, the mutation 2252_2277>AC.

FIG. 6 shows SEQ ID NO: 6, the wild-type sequence of nucleotides 2221-2280 of the EGFR gene; and SEQ ID NO: 8, the mutation 2240_2264>CGAAAGA.

FIG. 7 shows SEQ ID NO: 3, the wild-type sequence of nucleotides 2221-2260 of the EGFR gene; and SEQ ID NO: 9, the mutation 2239_2240 TT>CC.

5 FIG. 8 shows SEQ ID NO: 6, the wild-type sequence of nucleotides 2221-2280 of the EGFR gene; and SEQ ID NO: 10, the mutation 2264 C>A.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

10 To facilitate the understanding of this disclosure, the following definitions of the terms used herein are provided.

The term “n_m” or “n-m del” refers to a mutation that results in a nucleic acid lacking the nucleotides between positions “n” and “m.” The term “n_m>XYZ” refers to a complex mutation where the nucleic acid is lacking nucleotides between positions “n” and “m,” but
15 nucleotide sequence XYZ is inserted in their place. For example, the term “2236_2248>ACCC” refers to a mutation that results in a nucleic acid lacking nucleotides 2236-2248 but the nucleotide sequence ACCC is inserted in the place of the deleted nucleotides.

The term “nX>Y” refers to a mutation that results in a substitution of nucleotide X at
20 position “n” with the nucleotide Y. For example, the term “2264C>A” refers to a mutation that results in a substitution of a cytosine at position 2264 with an adenine. Similarly, the term “2239_2240TT>CC” refers to a mutation that results in a substitution of two thymines at positions 2239 and 2240 with two cytosines.

The term “allele-specific primer” or “AS primer” refers to a primer that hybridizes to more than one variant of the target sequence, but is capable of discriminating between the variants of the target sequence in that only with one of the variants, the primer is efficiently extended by the nucleic acid polymerase under suitable conditions. With other variants of the target sequence, the extension is less efficient, inefficient or undetectable.

The term “common primer” refers to the second primer in the pair of primers that includes an allele-specific primer. The common primer is not allele-specific, i.e. does not discriminate between the variants of the target sequence between which the allele-specific primer discriminates.

10 The terms “complementary” or “complementarity” are used in reference to antiparallel strands of polynucleotides related by the Watson-Crick base-pairing rules. The terms “perfectly complementary” or “100% complementary” refer to complementary sequences that have Watson-Crick pairing of all the bases between the antiparallel strands, i.e. there are no mismatches between any two bases in the polynucleotide duplex. However, duplexes
15 are formed between antiparallel strands even in the absence of perfect complementarity. The terms “partially complementary” or “incompletely complementary” refer to any alignment of bases between antiparallel polynucleotide strands that is less than 100% perfect (e.g., there exists at least one mismatch or unmatched base in the polynucleotide duplex). The duplexes between partially complementary strands are generally less stable
20 than the duplexes between perfectly complementary strands.

The term “sample” refers to any composition containing or presumed to contain nucleic acid. This includes a sample of tissue or fluid isolated from an individual for example, skin, plasma, serum, spinal fluid, lymph fluid, synovial fluid, urine, tears, blood cells, organs and tumors, and also to samples of *in vitro* cultures established from cells taken from an

individual, including the formalin-fixed paraffin embedded tissues (FFPET) and nucleic acids isolated therefrom.

The terms "polynucleotide" and "oligonucleotide" are used interchangeably.

“Oligonucleotide” is a term sometimes used to describe a shorter polynucleotide. An
5 oligonucleotide may be comprised of at least 6 nucleotides, for example at least about 10-12 nucleotides, or at least about 15-30 nucleotides corresponding to a region of the designated nucleotide sequence.

The term “primary sequence” refers to the sequence of nucleotides in a polynucleotide or oligonucleotide. Nucleotide modifications such as nitrogenous base modifications, sugar
10 modifications or other backbone modifications are not a part of the primary sequence.

Labels, such as chromophores conjugated to the oligonucleotides are also not a part of the primary sequence. Thus two oligonucleotides can share the same primary sequence but differ with respect to the modifications and labels.

The term "primer" refers to an oligonucleotide which hybridizes with a sequence in the
15 target nucleic acid and is capable of acting as a point of initiation of synthesis along a complementary strand of nucleic acid under conditions suitable for such synthesis. As used herein, the term "probe" refers to an oligonucleotide which hybridizes with a sequence in the target nucleic acid and is usually detectably labeled. The probe can have modifications, such as a 3'-terminus modification that makes the probe non-extendable by nucleic acid
20 polymerases, and one or more chromophores. An oligonucleotide with the same sequence may serve as a primer in one assay and a probe in a different assay.

As used herein, the term "target sequence", "target nucleic acid" or "target" refers to a portion of the nucleic acid sequence which is to be either amplified, detected or both.

The terms "hybridized" and "hybridization" refer to the base-pairing interaction of between two nucleic acids which results in formation of a duplex. It is not a requirement that two nucleic acids have 100% complementarity over their full length to achieve hybridization.

The terms "selective hybridization" and "specific hybridization" refer to the hybridization
5 of a nucleic acid predominantly (50% or more of the hybridizing molecule) or nearly
exclusively (90% or more of the hybridizing molecule) to a particular nucleic acid present in
a complex mixture where other nucleic acids are also present. For example, under typical
PCR conditions, primers specifically hybridize to the target nucleic acids to the exclusion of
non-target nucleic acids also present in the solution. The specifically hybridized primers
10 drive amplification of the target nucleic acid to produce an amplification product of the
target nucleic acid that is at least the most predominant amplification product and is
preferably the nearly exclusive (e.g., representing 90% or more of all amplification products
in the sample) amplification product. Preferably, the non-specific amplification product is
present in such small amounts that it is either non-detectable or is detected in such small
15 amounts as to be easily distinguishable from the specific amplification product. Similarly,
probes specifically hybridize to the target nucleic acids to the exclusion of non-target
nucleic acids also present in the reaction mixture. The specifically hybridized probes allow
specific detection of the target nucleic acid to generate a detectable signal that is at least the
most predominant signal and is preferably the nearly exclusive (e.g., representing 90% or
20 more of all amplification products in the sample) signal.

The present invention describes a novel mutation in the EGFR kinase domain that is useful
for cancer diagnosis and prognosis, designing a therapy regimen and predicting success of
the therapy.

The nucleotide numbering used herein is in reference to SEQ ID NO: 1, shown on Figure 1. Within SEQ ID NO: 1, the portion of the sequence between nucleotides 2221 and 2280, that encompasses the six mutations described herein is highlighted and underlined.

The amino acid numbering used herein is in reference to SEQ ID NO: 2, shown on Figure 2.

- 5 Within SEQ ID NO: 2, the signal sequence includes amino acids 1-24, the extracellular domain includes amino acids 24-645, the transmembrane domain includes amino acids 646-668, and the cytoplasmic domain includes amino acids 669-1210, of which the tyrosine kinase domain is amino acids 718-964, and the threonine phosphorylation site is amino acid 678.
- 10 The present study identified six novel mutations in the exon 19 (portion of the kinase domain) of the human EGFR gene. The mutations are illustrated in Figures 3-8. In the figures, the sequence deleted from the wild-type gene is underlined. The sequence inserted in the mutant gene in place of the deletion is shown in bold italics. The mutations are also summarized in Table 1.

15

Table 1: New mutations and wild-type sequences in exon 19 of the human EGFR gene

SEQ ID NO:		NUCLEOTIDE SEQUENCE
3	WT 2221-2260	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGA
4	Mut 2236_2248>ACCC	CCCGTCGCTATCAAG <i>ACCC</i> CAACATCTCCGA
5	Mut 2237_2244>CGCCC	CCCGTCGCTATCAAG <i>CGCCC</i> GAAGCAACATCTCCGA
6	WT 2221-2280	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTC
7	Mut 2252_2277>AC	CCCGTCGCTATCAAGGAATTAAGAGAAGCAA <i>ACTC</i>
8	Mut 2240-2264>CGAAAGA	CCCGTCGCTATCAAGGAAT <i>CGAAAGA</i> CAACAAGGAAATCCTC
9	Mut 2239_2240 TT>CC	CCCGTCGCTATCAAGGAA <i>CCA</i> AGAGAAGCAACATCTCCGA
10	Mut 2264 C>A	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAG <i>A</i> CAACAAGGAAATCCTC

The first mutation 2236_2248>ACCC is shown on Figure 3. Figure 3 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 3) and the corresponding fragment encoding the mutation 2236_2248>ACCC (SEQ ID NO: 4).

5 The second mutation 2237_2244>CGCCC is shown on Figure 4. Figure 4 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 3) and the corresponding fragment encoding the mutation 2237_2244>CGCCC (SEQ ID NO: 5).

The third mutation 2252_2277>AC is shown on Figure 5. Figure 5 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 6) and the corresponding fragment encoding the mutation 2252_2277>AC (SEQ ID NO: 7).

10 The fourth mutation 2240-2264>CGAAAGA is shown on Figure 6. Figure 6 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 6) and the corresponding fragment encoding the mutation 2240-2264>CGAAAGA (SEQ ID NO: 8).

The fifth mutation 2239_2240 TT>CC is shown on Figure 7. Figure 7 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 3) and the corresponding
15 fragment encoding the mutation 2239_2240 TT>CC (SEQ ID NO: 9).

The sixth mutation 2264 C>A is shown on Figure 8. Figure 8 shows the fragment of the nucleotide sequence of the wild-type EGFR (SEQ ID NO: 6) and the corresponding fragment encoding the mutation 2264 C>A (SEQ ID NO: 10).

In one embodiment, the present invention comprises oligonucleotides for detecting
20 mutations in exon 19 of EGFR selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1. In one embodiment, the invention comprises oligonucleotides (SEQ ID NOs: 11-40) for detecting the above mentioned mutations by allele-specific PCR (Tables 2), (Allele-specific PCR has been described in U.S. Patent No. 6,627,402). As

indicated in Table 2, oligonucleotides SEQ ID NOs: 12-14, 17-20, 23-25, 28-30, 33-35, 37 and 38 are selective, i.e. allele-specific primers. Some of these allele-specific primers contain internal mismatches with both the wild-type and mutant target sequence. Additional mismatches in allele-specific PCR primers have been shown to increase selectivity of the primers, see U.S. Patent Publication No. 2010/0099110. Other oligonucleotides in Table 2, SEQ ID NOs: 11, 15, 16, 21, 26, 27, 31, 32 and 36 are not selective, i.e. not allele-specific. These oligonucleotides direct amplification of both mutant and wild-type template. These selective and non-selective primers of the present invention are arbitrarily designated as “forward” primers. For exponential amplification, these primers may be paired with a second “reverse” primer that is not allele-specific, e.g. SEQ ID NO: 40. For probe based detection, a probe, e.g. SEQ ID NO: 39 may be used. It is understood by one of skill in the art that SEQ ID NOs: 39 and 40 are non-limiting examples. A different reverse primer and a different probe may also be used with a forward primer selected from SEQ ID NOs: 11-40.

Table 2

Oligonucleotides for detecting new mutations in exon 19 of EGFR

Description	SEQ ID NO:	Sequence 5' -3'
Mutation 2240_2264>CGAAAGA		
Common Forward Primer	11	AATTCCCGTCGCTATCAAGGAA
Selective Forward Primer	12	TTCCCGTCGCTATCAAGGAATC
Selective Forward Primer	13	CCGTCGCTATCAAGGAATCGAA

Selective Forward Primer	14	GTCGCTATCAAGGAATCGAAAGACAA
Common Forward Primer	15	CAACAAGGAAATCCTCGATGTGAGT
Mutation 2252_2277>AC		
Common Forward Primer	16	GTCGCTATCAAGGAATTAAGAGAAGCA
Selective Forward Primer	17	GTCGCTATCAAGGAATTAAGAGAAGCAAA
Selective Forward Primer	18	CGCTATCAAGGAATTAAGAGAAGCAAAC
Selective Forward Primer	19	CTATCAAGGAATTAAGAGAAGCAAACCT
Selective Forward Primer	20	CTATCAAGGAATTAAGAGAAGCAAACCTC
Common Forward Primer	21	TCGATGTGAGTTTCTGCTTTGCT
Mutation 2236_2248>ACCC		
Common Forward Primer	22	AAAGTTAAAATTCCCGTCGCTATCAA
Selective Forward Primer	23	AGTTAAAATTCCCGTCGCTATCAAGA
Selective Forward Primer	24	GTTAAAATTCCCGTCGCTATCAAGAC
Selective Forward Primer	25	CCGTCGCTATCAAGACCCCA
Common Forward Primer	26	CAACATCTCCGAAAGCCAACAA
Mutation 2237_2244>CGCCC		
Common Forward Primer	27	AAAGTTAAAATTCCCGTCGCTATCAA
Selective Forward Primer	28	AAGTTAAAATTCCCGTCGCTATCAAGGC
Selective Forward Primer	29	CCGTCGCTATCAAGGCGC
Selective Forward Primer	30	CGCTATCAAGGCGCCCGA

Common Forward Primer	31	GAAGCAACATCTCCGAAAGCCAACAAGGA
Mutation 2264C>A		
Common Forward Primer	32	GGAATTAAGAGAAGCAACATCTCCGAA
Selective Forward Primer	33	ATTAAGAGAAGCAACATCTCCGAAAGA
Selective Forward Primer	34	ATTAAGAGAAGCAACATCTCCGAA <u>F</u> GA
Selective Forward Primer	35	ATTAAGAGAAGCAACATCTCCGAA <u>F</u> GAC
Mutation 2239_2240TT>CC		
Common Forward Primer	36	TTAAAATTCCCGTCGCTATCAAGGA
Selective Forward Primer	37	TAAAATTCCCGTCGCTATCAAGGAAC
Selective Forward Primer	38	AATTCCCGTCGCTATCAAGGAACC
Additional oligonucleotides		
Probe	39	<u>M</u> ATGGCTC <u>Q</u> TGAACCTCAGGCCACCTT <u>T</u> <u>P</u>
Reverse Primer	40	AGAGCAGAGCAGCTGCCAGA

Common primer = a primer that amplifies both mutant and wild-type nucleic acid

Selective primer = a primer that amplifies only the mutant and not the wild-type nucleic acid

5 E = N4-tert-butyl-benzyl-dC

F = N6-tert-butyl-benzyl-dA

M = FAM

Q = BHQ2

P = phosphate

10

For successful extension of a primer, the primer needs to have at least partial complementarity to the target sequence. Generally, complementarity at the 3'-end of the

primer is more critical than complementarity at the 5'-end of the primer (Innis *et al.* Eds. *PCR Protocols*, (1990) Academic Press, Chapter 1, pp. 9-11). This means that variations of the 5'-end, i.e. additions, substitutions or removal of nucleotides at the 5'-end, do not affect performance of a primer in a PCR assay. Therefore the present invention encompasses the
5 primers disclosed in Tables 2 as well as the variants of these primers with 5'-end variations.

Similarly, for successful probe hybridization, the probe needs to have at least partial complementarity to the target sequence. Generally, complementarity close to the central portion of the probe is more critical than complementarity at the ends of the probe (Innis *et al.* Chapter 32, pp. 262-267). This means that variations of the ends of the probe, i.e.
10 additions, substitutions or removal of a few nucleotides, do not affect performance of the probe in hybridization. Therefore the present invention encompasses the probe disclosed in Table 2 as well as the variants of these probes with terminal variations.

In other variations of this embodiment, the probe has a particular structure, including a protein-nucleic acid (PNA), a locked nucleic acid (LNA), a molecular beacon probe (Tyagi
15 *et al.* (1996), *Nat. Biotechnol.* 3:303-308) or SCORPIONS® self-probing primers (Whitcombe *et al.* (1999), *Nat. Biotechnol.* 8:804-807). A probe may be labeled with a radioactive, a fluorescent or a chromophore label. For example, the mutations may be detected by real-time allele-specific polymerase chain reaction, where hybridization of a probe to the amplification product results in enzymatic digestion of the probe and detection
20 of the digestion products (TaqMan™ probe, Holland *et al.* (1991) *P.N.A.S. USA* 88:7276-7280). Hybridization between the probe and the target may also be detected by detecting the change in fluorescence due to the nucleic acid duplex formation (U.S. Patent Publication No. 2010/0143901) or by detecting the characteristic melting temperature of the hybrid between the probe and the target (U.S. Patent No. 5,871,908).

Mutant EGFR gene or gene product can be detected in tumors or other body samples such as urine, sputum or serum. The same techniques discussed above for detection of mutant EGFR genes or gene products in tumor samples can be applied to other body samples. For example, cancer cells are sloughed off from tumors and appear in such body samples. State
5 of the art nucleic acid detection methods are capable of detecting mutant cells in a background of non-tumor cells in a wide variety of sample types.

In another embodiment, the invention is a method of treating a patient having a tumor possibly harboring cells with an EGFR gene having mutations in exon 19, selected from the group consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-
10 2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1; the method comprising requesting that the patient be tested for one or more of the above mentioned mutations in the patient's sample, and if the mutation is detected, administering to the patient a tyrosine kinase inhibitor (TKI) or an EGFR inhibitor. Said embodiment includes a tyrosine kinase inhibitor (TKI) or an EGFR inhibitor that inhibits signaling of the mutant
15 EGFR protein encoded by the mutated gene for use in the treatment of a disease related to or caused by a tumor possibly harboring cells with a mutation in the epidermal growth factor receptor (EGFR) gene, comprising requesting that the patient's sample be tested for the presence of the mutated EGFR gene characterized by a mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC,
20 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1; and if any of the mutations are reported as present. In variations of this embodiment, the tyrosine kinase inhibitors are EGFR kinase inhibitors such as for example, cetuximab, panitumumab, erlotinib or gefitinib.

In a variation of this embodiment, the method further comprises querying for one more of
25 the following mutations: G719A, G719C, K745-A750 del K ins, E746V, E746K, L747S,

E749Q, A750P, A755V, S768I, L858P, L858R, E746-R748 del, E746-S752 del V ins, L747-E749 del, L747-A750 del P ins, L747-T751 del, L747-T751 del P ins, L747-P753 del S ins, L747-S752 del, R748-P753 del, T751-I759 del T ins, S752-I759 del, P753-K757 del, D770-N771 del NPG ins, D770-N771 del SVD ins, P772-H773 dup, P772-H773 del V ins, M766-
5 A767 del AI ins, S768-V769 del SVA ins, G779S, P848L, G857V, L858R, L861Q, L883S, D896Y, and E746-A750 del AP ins; and if one or more of the mutations are present, administering to the patient a compound that inhibits signaling of the mutant EGFR protein encoded by the mutated gene. The nucleotide changes causing the mutations listed above and methods of detecting them are disclosed in U.S. Patent Nos. 7,294,468 and
10 7,960,118 and U.S. App. Ser. No. 13/280,976, filed on October 25, 2011 (mutation E746-A750 del AP ins). Multiple mutations can be detected simultaneously or separately by using hybridization to multiple probes, for example in a dot-blot or nucleic acid array format, multiplex PCR, for example multiplex allele-specific PCR and multiplex PCR followed by a probe melting assay with each probe characterized by a mutation-specific
15 melting temperature. Multiple mutations may also be detected by high-throughput sequencing for example, using a method involving emulsion PCR amplification of single molecules adhered to a solid support, subsequent sequencing by synthesis and bioinformatic analysis of the sequence data, such as the method developed by 454 Life Sciences, Inc. (Branford, Conn).

20 In another embodiment, the invention is a method of determining an altered response of a patient having a malignant tumor to tyrosine kinase inhibitors (TKIs) or EGFR inhibitors. The method comprises querying the patient's sample for the presence of one or more mutations in exon 19 of EGFR selected from the group consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and
25 2264 C>A in SEQ ID NO: 1; and if the mutation is found, determining that the treatment is likely to be successful. In variations of this embodiment, the tyrosine kinase inhibitors are

EGFR kinase inhibitors or EGFR inhibitors are, for example, cetuximab, panitumumab, erlotinib or gefitinib.

In a variation of this embodiment, the method further comprises querying for one more of the following mutations: G719A, G719C, K745-A750 del K ins, E746V, E746K, L747S,
5 E749Q, A750P, A755V, S768I, L858P, L858R, E746-R748 del, E746-S752 del V ins, L747-E749 del, L747-A750 del P ins, L747-T751 del, L747-T751 del P ins, L747-P753 del S ins, L747-S752 del, R748-P753 del, T751-I759 del T ins, S752-I759 del, P753-K757 del, D770-N771 del NPG ins, D770-N771 del SVD ins, P772-H773 dup, P772-H773 del V ins, M766-A767 del AI ins, S768-V769 del SVA ins, G779S, P848L, G857V, L858R, L861Q, L883S,
10 D896Y and and E746-A750 del AP ins; and if one or more of the mutations are present, determining that the treatment with tyrosine kinase inhibitors is likely to be successful.

In yet another embodiment, the invention is a kit containing reagents necessary for detecting the one or more mutations in exon 19 of EGFR selected from the group consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA,
15 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1. The kit may comprise oligonucleotides such as probes or amplification primers specific for the mutated sequence but not the wild type sequence. Optionally, one or more allele-specific primers, common primers and probes in the kit may be selected from Tables 2. The kit further comprises reagents necessary for the performance of amplification and detection assay, such as the components
20 of PCR, a real-time PCR, or transcription mediated amplification (TMA). In some embodiments, the mutation-specific oligonucleotide is detectably labeled. In such embodiments, the kit comprises reagents for labeling and detecting the label. For example, if the oligonucleotide is labeled with biotin, the kit may comprise a streptavidin reagent with an enzyme and its chromogenic substrate. In variations of this embodiment, the kit
25 further includes reagents for detecting at least one more mutation in the EGFR gene,

selected from the following: G719A, G719C, K745-A750 del K ins, E746V, E746K, L747S, E749Q, A750P, A755V, S7681, L858P, L858R, E746-R748 del, E746-S752 del V ins, L747-E749 del, L747-A750 del P ins, L747-T751 del, L747-T751 del P ins, L747-P753 del S ins, L747-S752 del, R748-P753 del, T751-I759 del T ins, S752-I759 del, P753-K757 del, D770-N771 del NPG ins, D770-N771 del SVD ins, P772-H773 dup, P772-H773 del V ins, M766-A767 del AI ins, S768-V769 del SVA ins, G779S, P848L, G857V, L858R, L861Q, L883S, D896Y and and E746-A750 del AP ins.

In yet another embodiment, the invention is a reaction mixture for detecting mutations in the human EGFR gene, including one or more mutations selected from the list consisting of

10 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, the reaction mixture comprising one or more oligonucleotides selected from SEQ ID NOs: 11-40.

In yet another embodiment, the invention is the use of oligonucleotides selected from SEQ ID NOs: 11-40 in detecting one or more mutations selected from the list consisting of

15 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1. In a variation of this embodiment, the invention is the use of detection of one or more mutations selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, in diagnosis or prognosis of cancer. In

20 a further variation of this embodiment, the invention is the use of detection of one or more mutations selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240-2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1 in designing treatment of a cancer patient or predicting response of the cancer patient to the treatment.

*Example 1**Identifying mutations in lung cancer patient samples*

Tissue samples were obtained from lung cancer (NSCLC) patients. The samples were preserved as formalin-fixed, paraffin embedded tissue (FFPET). Nucleic acids were isolated
5 from the samples and subjected to direct sequencing on the Genome Sequencer FLX instrument (454 Life Sciences, Branford, Conn.).

The 2236_2248>ACCC mutation was detected in the average of 24.6% of the total of 3,550 reads from a sample. The 2237_2244>CGCCC mutation was detected in the average of 27.7% of the total of 4205 reads from a sample. The 2252_2277>AC mutation was detected
10 in the average of 24.6% of the total of 5368 reads from a sample. The 2240-2264>CGAAAGA mutation was detected in the average of 32.2% of the total of 3394 reads from a sample. The 2239_2240 TT>CC mutation was detected in the average of 74.3% of the total of 3120 reads from a sample. The 2264 C>A mutation was detected in the average of 32.8% of the total of 3394 reads from a sample. Only fraction of the reads was found to
15 contain a mutation reflecting the fact that the samples are mixtures of tumor and non-tumor cells.

*Example 2**Detecting the mutations using allele-specific oligonucleotides*

In this example, the mutant and wild-type targets were represented by plasmids containing
20 the mutant and wild-type inserts respectively. The targets were amplified using either mutation-specific primers or, in control reactions, non-selective "common" primers.

Each 15 µL reaction contained 10,000 copies of the target DNA and standard PCR reagents including nucleoside triphosphates, DNA polymerase, uracil-N-glycosylase, and 0.1µM

each of forward and reverse primer, and 0.05 μ M probe (all selected from Table 2),
 Amplification and analysis were done using the LightCycler™ 480 instrument (Roche
 Applied Science, Indianapolis, Ind.) Reactions were cycled using the following profile:
 50°C for 5 minutes, followed by 2 cycles of 95°C for 10 seconds and 62°C for 30 seconds,
 5 followed by 55 cycles of 93°C for 10-seconds and 62°C for 30 seconds.

Results for each forward primer are shown in Table 3. Amplification is represented by
 “cycles to threshold” or C_t value. The higher C_t represents a less efficient amplification, e.g.
 by a mismatched primer. ΔC_t represents the difference between the wild-type and the
 mutant target amplification. The presence of ΔC_t indicates that the mutation is detected
 10 and the value of ΔC_t represents selectivity of the assay.

Table 3

Detecting mutations by allele-specific PCR

FWD PRIMER SEQ ID NO:	mut C_t^*	wt C_t	ΔC_t
Mutation 2240_2264>CGAAAGA			
11	22.2	22.6	0.4
12	22.3	30.1	7.8
13	22.5	36.7	14.2
14	21.9	33.1	11.3
15	22.5	22.5	0 (control)
Mutation 2252_2277>AC			
16	22.1	22.5	0.4 (control)
17	22.2	22.6	0.4 (control)

18	24.5	39.4	14.9
19	22.3	27.7	5.4
20	22.6	29.5	6.9
21	21.2	22.3	1.1 (control)
Mutation 2236_2248>ACCC			
22	22.8	23.0	0.2 (control)
23	22.6	29.8	7.2
24	22.5	47.4	24.9
25	22.7	NA	>32.3
26	22.4	22.4	0 (control)
Mutation 2237_2244>CGCCC			
27	23.1	22.9	-0.2 (control)
28	22.7	28.4	5.6
29	22.6	NA	>32.4
30	22.4	NA	>32.6
31	22.8	23.0	0.2 (control)
Mutation 2264C>A			
32	23.1	23.0	-0.1 (control)
33	23.5	39.0	15.5
34	24.7	NA	>30.3
35	24.8	36.5	11.7

Mutation 2239_2240TT>CC			
36	22.2	22.8	0.6 (control)
37	22.5	25.3	2.8
38	22.4	38.0	15.5

* each C_t is an average of two experiments

NA: not amplified

Control - common, not allele-specific forward primer

5

While the invention has been described in detail with reference to specific examples, it will be apparent to one skilled in the art that various modifications can be made within the scope of this invention. Thus the scope of the invention should not be limited by the examples described herein, but by the claims presented below.

10

CLAIMS

What is claimed is:

1. An oligonucleotide that specifically hybridizes to a nucleic acid containing a mutation
5 selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC,
2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ
ID NO: 1.
2. The oligonucleotide of claim 1, selected from SEQ ID NOs: 12-14, 17-20, 23-25, 28-30,
10 33-35, 37 and 38.
3. A method of detecting a mutation in the epidermal growth factor receptor (EGFR)
gene in a sample from a human, comprising:
 - (a) contacting the nucleic acid in the sample with an oligonucleotide capable of
15 selectively hybridizing to a target nucleic acid containing a mutation selected
from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC,
2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in
SEQ ID NO: 1;
 - (b) incubating the sample under conditions allowing selective hybridization of the
20 oligonucleotide to the target nucleic acid, and
 - (c) detecting the hybridization.

4. The method of claim 3, further comprising after step (b), amplification of the target nucleic acid, wherein the hybridization in step (b) results in extension of the selectively hybridizing oligonucleotide.
5. The method of claims 3 or 4, wherein detection in step (c) takes place during each round of amplification.
6. The method of claims 3 or 4, wherein detection in step (c) takes places after the last cycle of amplification.
7. The method of claim 3, wherein the oligonucleotide is selected from SEQ ID NOs: 12-14, 17-20, 23-25, 28-30, 33-35, 37 and 38.
8. A compound that inhibits signaling of the mutant EGFR protein encoded by the mutated gene for use in the treatment of a disease related to a tumor possibly harboring cells with a mutation in the epidermal growth factor receptor (EGFR) gene, comprising requesting that a patient's sample be tested for the presence of the mutated EGFR gene characterized by a mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1; and if any of the mutations are reported as present.
9. Compound of claim 8, wherein said compound is cetuximab, panitumumab, erlotinib or gefitinib.

10. The compound of claim 8, further comprising requesting that the patient's sample be also tested for the presence of the mutated EGFR gene characterized by one or more of the mutations G719A, G719C, K745-A750 del K ins, E746V, E746K, L747S, E749Q, A750P, A755V, S768I, L858P, L858R, E746-R748 del, E746-S752 del V ins, L747-
5 E749 del, L747-A750 del P ins, L747-T751 del, L747-T751 del P ins, L747-P753 del S ins, L747-S752 del, R748-P753 del, T751-I759 del T ins, S752-I759 del, P753-K757 del, D770-N771 del NPG ins, D770-N771 del SVD ins, P772-H773 dup, P772-H773 del V ins, M766-A767 del AI ins, S768-V769 del SVA ins, G779S, P848L, G857V, L858R, L861Q, L883S, D896Y and E746-A750 del AP ins.
- 10
11. A kit for detecting mutations in the human EGFR gene, including any mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ ID NO: 1, the kit comprising one or more oligonucleotides selected from SEQ ID
15 NOs: 11-40.
12. The kit of claim 11, comprising one or more oligonucleotides selected from SEQ ID NOs: 12-14, 17-20, 23-25, 28-30, 33-35, 37 and 38 and/or further comprising nucleic acid precursors, nucleic acid polymerase and reagents and solutions necessary to
20 support the activity of the nucleic acid polymerase.
13. A reaction mixture for detecting mutations in the human EGFR gene, including any mutation selected from the list consisting of 2236_2248>ACCC, 2237_2244>CGCCC, 2252_2277>AC, 2240_2264>CGAAAGA, 2239_2240 TT>CC and 2264 C>A in SEQ
25 ID NO: 1, the reaction mixture comprising one or more oligonucleotides selected from SEQ ID NOs: 11-40.

14. The reaction mixture of claim 13, comprising one or more oligonucleotides selected from SEQ ID NOs: 12-14, 17-20, 23-25, 28-30, 33-35, 37 and 38 and/or further comprising nucleic acid precursors, nucleic acid polymerase and reagents and solutions necessary to support the activity of the nucleic acid polymerase.
- 5

FIGURE 1A

1 ATGCGACCCCTCCGGGACGGCCGGGGCAGCGCTCCTGGCGCTGCTGGCTGCGCTCTGCCCCG
61 GCGAGTCGGGCTCTGGAGGAAAAGAAAGTTTGCCAAGGCACGAGTAACAAGCTCACGCAG
121 TTGGGCACTTTTGAAGATCATTTTCTCAGCCTCCAGAGGATGTTCAATAACTGTGAGGTG
181 GTCCTTGGAATTTGGAAATTACCTATGTGCAGAGGAATTATGATCTTTCCTTCTTAAAG
241 ACCATCCAGGAGGTGGCTGGTTATGTCTCATTGCCCTCAACACAGTGGAGCGAATTCCT
301 TTGGAAAACCTGCAGATCATCAGAGGAAATATGTACTACGAAAATTCCTATGCCTTAGCA
361 GTCTTATCTAACTATGATGCAAATAAAACCGGACTGAAGGAGCTGCCCATGAGAAATTTA
421 CAGGAAATCCTGCATGGCGCCGTGCGGTTTCAGCAACAACCCGTGCCCTGTGCAACGTGGAG
481 AGCATCCAGTGGCGGGACATAGTCAGCAGTGACTTTCTCAGCAACATGTCGATGGACTTC
541 CAGAACCACCTGGGCAGCTGCCAAAAGTGTGATCCAAGCTGTCCCAATGGGAGCTGCTGG
601 GGTGCAGGAGAGGAGAACTGCCAGAACTGACCAAAATCATCTGTGCCCAGCAGTGTCTCC
661 GGGCGCTGCCGTGGCAAGTCCCCCAGTGACTGCTGCCACAACCAGTGTGCTGCAGGCTGC
721 ACAGGCCCCCGGAGAGCGACTGCCTGGTCTGCCGCAAATTCGAGACGAAGCCACGTGC
781 AAGGACACCTGCCCCCACTCATGCTCTACAACCCACCACGTACCAGATGGATGTGAAC
841 CCCGAGGGCAAATACAGCTTTGGTGCCACCTGCGTGAAGAAGTGTCCCCGTAATTATGTG
901 GTGACAGATCACGGCTCGTGCGTCCGAGCCTGTGGGGCCGACAGCTATGAGATGGAGGAA
961 GACGGCGTCCGCAAGTGTAAGAAGTGCGAAGGGCCTTGCCGCAAAGTGTGTAACGGAATA
1021 GGTATTGGTGAATTTAAAGACTCACTCTCCATAAATGCTACGAATATTAAACACTTCAAA
1081 AACTGCACCTCCATCAGTGGCGATCTCCACATCCTGCCGGTGGCATTAGGGGTGACTCC
1141 TTCACACATACTCCTCCTCTGGATCCACAGGAAGTGGATATTCTGAAAACCGTAAAGGAA
1201 ATCACAGGGTTTTTGCTGATTACAGGCTTGGCCTGAAAACAGGACGGACCTCCATGCCTTT
1261 GAGAACCTAGAAATCATACGCGGCAGGACCAAGCAACATGGTCAGTTTTCTCTTGCACTC
1321 GTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCTCAAGGAGATAAGTGATGGAGAT
1381 GTGATAATTTACAGGAAAACAAAATTTGTGCTATGCAAATACAATAAACTGGAAAAAACTG
1441 TTTGGGACCTCCGGTCAGAAAACAAAATTATAAGCAACAGAGGTGAAAACAGCTGCAAG
1501 GCCACAGGCCAGGTCTGCCATGCCTTGTGCTCCCCGAGGGCTGCTGGGGCCCGGAGCCC
1561 AGGGACTGCGTCTCTTGCCGGAATGTCAGCCGAGGCAGGGAATGCGTGGACAAGTGCAAC

FIGURE 1B

1621 CTTCTGGAGGGTGAGCCAAGGGAGTTTGTGGAGAACTCTGAGTGCATACAGTGCCACCCA
1681 GAGTGCCTGCCTCAGGCCATGAACATCACCTGCACAGGACGGGGACCAGACAACTGTATC
1741 CAGTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATG
1801 GGAGAAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGC
1861 CATCCAACTGCACCTACGGATGCACTGGGCCAGGTCTTGAAGGCTGTCCAACGAATGGG
1921 CCTAAGATCCCGTCCATCGCCACTGGGATGGTGGGGGCCCTCCTCTTGCTGCTGGTGGTG
1981 GCCCTGGGGATCGGCCTCTTCATGCGAAGGCGCCACATCGTTCGGAAGCGCACGCTGCGG
2041 AGGCTGCTGCAGGAGAGGGAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAAC
2101 CAAGCTCTCTTGAGGATCTTGAAGGAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCC
2161 GGTGCGTTCGGCACGGTGTATAAGGGACTCTGGATCCCAGAAGGTGAGAAAGTTAAATTT
2221 CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTC
2281 GATGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGTGCCGCCTGCTGGGCATC
2341 TGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTCCTGGAC
2401 TATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAG
2461 ATCGCAAAGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCC
2521 AGGAACGTACTGGTGAAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAA
2581 CTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTGCCATCAAGTGG
2641 ATGGCATTGGAATCAATTTTACACAGAATCTATACCCACCAGAGTGATGTCTGGAGCTAC
2701 GGGGTGACTGTTTGGGAGTTGATGACCTTTGGATCCAAGCCATATGACGGAATCCCTGCC
2761 AGCGAGATCTCCTCCATCCTGGAGAAAGGAGAACGCCTCCCTCAGCCACCCATATGTACC
2821 ATCGATGTCTACATGATCATGGTCAAGTGCTGGATGATAGACGCAGATAGTCGCCCAAAG
2881 TTCCGTGAGTTGATCATCGAATTCTCCAAAATGGCCCGAGACCCCAGCGCTACCTTGTC
2941 ATTCAGGGGGATGAAAGAATGCATTTGCCAAGTCCTACAGACTCCAACCTCTACCGTGCC
3001 CTGATGGATGAAGAAGACATGGACGACGTGGTGGATGCCGACGAGTACCTCATCCCACAG
3061 CAGGGCTTCTTCAGCAGCCCCCTCCACGTCACGGACTCCCCTCCTGAGCTCTCTGAGTGCA
3121 ACCAGCAACAATTCCACCGTGGCTTGCAATTGATAGAAATGGGCTGCAAAGCTGTCCCATC
3181 AAGGAAGACAGCTTCTTGACGCGATACAGCTCAGACCCACAGGCGCCTTGACTGAGGAC

FIGURE 1C

3241 AGCATAGACGACACCTTCCTCCCAGTGCCTGAATACATAAACCCAGTCCGTTCCCCAAAAGG
3301 CCCGCTGGCTCTGTGCAGAATCCTGTCTATCACAATCAGCCTCTGAACCCCGCGCCCAGC
3361 AGAGACCCACACTACCAGGACCCCCACAGCACTGCAGTGGGCAACCCCGAGTATCTCAAC
3421 ACTGTCCAGCCCACCTGTGTCAACAGCACATTCGACAGCCCTGCCCACTGGGCCCAGAAA
3481 GGCAGCCACCAAATTAGCCTGGACAACCCTGACTACCAGCAGGACTTCTTTCCCAAGGAA
3541 GCCAAGCCAAATGGCATCTTTAAGGGCTCCACAGCTGAAAATGCAGAATACCTAAGGGTC
3601 GCGCCACAAAGCAGTGAATTTATTGGAGCATGA

(SEQ ID NO: 1)

FIGURE 2

1 MRPSGTAGAA LLALLAALCP ASRALEEKV CQGTSNKLTQ LGTFEDHFLS
51 LQRMFNNCEV VLGNEITYV QRNYDLSFLK TIQEVAGYVL IALNTVERIP
101 LENLQIIRGN MYEENSIALA VLSNYDANKT GLKELPMRNL QEILHGAVRF
151 SNNPALCNVE SIQWRDIVSS DFLSNMSMDF QNHLGSCQKC DPSCPNGSCW
201 GAGEENCQKL TKIICAQQCS GRCRGKSPSD CCHNQCAAGC TGPRESDECLV
251 CRKFRDEATC KDTCPPLMLY NPTTYQMDVN PEGKYSFGAT CVKKCPRNYV
301 VTDHGSCVRA CGADSYEMEE DGVRKCKKCE GPCRKVCNGI GIGEFKDSLS
351 INATNIKHFK NCTSISGDLH ILPVAFRGDS FTHTPPLDPQ ELDILKTVKE
401 ITGFLLIQAW PENRTDLHAF ENLEIIRGRT KQHGOFS LAV VSLNITSLGL
451 RSLKEISDGD VIISGNKNLC YANTINWKKL FGTSGQKTKI ISNRGENSCK
501 ATGQVCHALC SPEGCWGPEP RDCVSCRNVS RGRECVDKCN LLEGEPRFV
551 ENSECIQCHP ECLPQAMNIT CTGRGPDNCI QCAHYIDGPH CVKTCPAGVM
601 GENNTLVWKY ADAGHVCHLC HPNCTYGCTG PGLEGCPNG PKIPSIATGM
651 VGALLLLLVV ALGIGLFMR RHIVRKRTL RLLQERELVE PLTPSGEAPN
701 QALLRILKET EFKKIKVLGS GAFGTVYKGL WIPEGEKVKI PVAIKELREA
751 TSPKANKEIL DEAYVMASVD NPHVCRLGI CLTSTVQLIT QLMPFGCLLD
801 YVREHKDNIG SQYLLNWCVO IAKGMNYLED RRLVHRDLAA RNVLVKTPQH
851 VKITDFGLAK LLGAEEKEYH AEGGKVPIKW MALESILHRI YTHQSDVWSY
901 GVTWELMTF GSKPYDGIPA SEISSILEKG ERLPQPPICT IDVYMIMVKC
951 WMIDADSRPK FRELIIEFSK MARDPQRYLV IQGDERMHL SPTDSNFYRA
1001 LMDEEDMDDV VDADEYLIPQ QGFFSSPSTS RTPLLSLSA TSNNSTVACI
1051 DRNGLQSCPI KEDSFLQRYSDPTGALTED SIDDTFLPVP EYINQSVPKR
1101 PAGSVQNPVY HNQPLNPAPS RDPHYQDPHS TAVGNPEYLN TVQPTCVNST
1151 FDSPAHWAK GSHQISLDNP DYQODFFPKE AKPNGIFKGS TAENAEYLRV
1201 APQSSEFIGA

(SEQ ID NO: 2)

FIGURE 3

2221	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGA	2260	(SEQ ID NO: 3)
->	CCCGTCGCTATCAAG----- ACCCCA ACATCTCCGA		(SEQ ID NO: 4)

FIGURE 4

2221	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGA	2260	(SEQ ID NO: 3)
->	CCCGTCGCTATCAAG--- CGCCC GGAAGCAACATCTCCGA		(SEQ ID NO: 5)

FIGURE 5

2221	CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAAAGGAAATCCTC	2280	(SEQ ID NO: 6)
->	CCCGTCGCTATCAAGGAATTAAGAGAAGCAA----- ACCTC		(SEQ ID NO: 7)

FIGURE 6

2221 CCCGTCGCTATCAAGGAATTAAAGAGAAGCAACATCTCCGAAAGCCAAACAAGGAAATCCTC (SEQ ID NO: 6)
-> CCCGTCGCTATCAAGGAAT-----CGAAAGACAACAAGGAAATCCTC (SEQ ID NO: 8)

FIGURE 7

2221 CCCGTCGCTATCAAGGAATTAAAGAGAAGCAACATCTCCGA 2260 (SEQ ID NO: 3)
-> CCCGTCGCTATCAAGGAACCAAGAGAAGCAACATCTCCGA (SEQ ID NO: 9)

FIGURE 8

2221 CCCGTCGCTATCAAGGAATTAAAGAGAAGCAACATCTCCGAAAGCCAAACAAGGAAATCCTC (SEQ ID NO: 6)
-> CCCGTCGCTATCAAGGAATTAAAGAGAAGCAACATCTCCGAAAGACAACAAGGAAATCCTC (SEQ ID NO: 10)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/004627A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/147959 A1 (BELL DAPHNE W [US] ET AL BELL DAPHNE WINIFRED [US] ET AL) 6 July 2006 (2006-07-06) abstract SEQ ID NO: 690, 694; page 17, column 1, paragraph 2 - page 18, column 1, paragraph 6; figures 2-5 page 18, column 2, paragraph 3 - page 19, column 1, paragraph 5; tables 6,7 ----- -/--	1,2,11



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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Name and mailing address of the ISA/

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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/004627

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	US 2005/272083 A1 (SESHAGIRI SOMASEKAR [US]) 8 December 2005 (2005-12-08) abstract; figure 8 -----	1-14
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Information on patent family members

International application No

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