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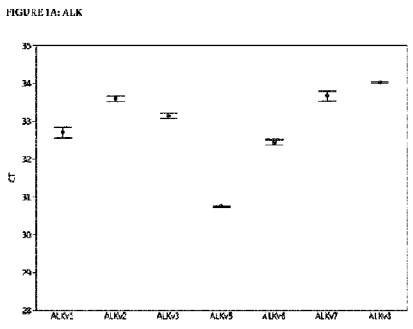
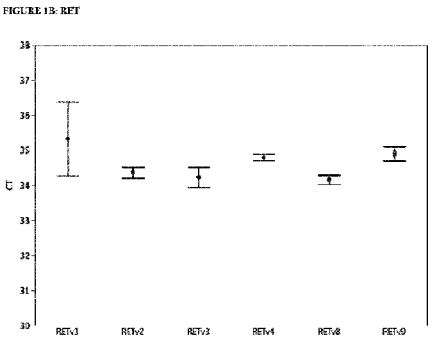
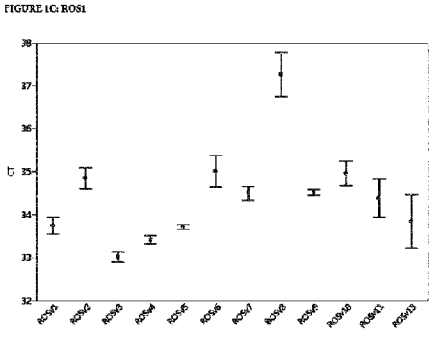
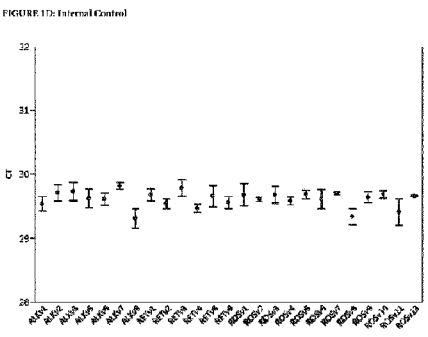
(54) **Title:** MULTIPLEX PCR DETECTION OF ALK, RET, AND ROS FUSIONS









(57) **Abstract:** Provided herein are methods and compositions for multiplex detection of a large number of actionable gene fusions with very high sensitivity and specificity. The present methods and compositions can detect ALK, RET, and ROS1 gene fusions, optionally in combination with other mutations and fusions.

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MULTIPLEX PCR DETECTION OF ALK, RET, AND ROS FUSIONS

BACKGROUND OF THE INVENTION

A number of cancers are associated with gene fusions (Yoshihara *et al.* (2015) *Oncogene* 34:4845). Perhaps the earliest reported example is the association of BCR-ABL with chronic myelogenous leukemia (CML) in the '60s (Nowell and Hungerford (1960) *J. Natl. Cancer Inst.* 25:85). Since then, hundreds more gene fusions have been reported for cancers in many different tissues (Presner and Chinnaiyan (2009) *Curr. Opin Genet. Dev.* 19:82).

Another example is the tyrosine receptor kinase ALK (anaplastic lymphoma kinase). EML4-ALK (echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase) fusions are associated with non-small cell lung cancer (NSCLC). In this case, the N terminal, extracellular portion of ALK is replaced by EML4 (KIF5B, HIP1, KLC1, TFG can also fuse with ALK in a similar manner). The expression of the resulting fusion gene is driven by the strong EML4 promoter, resulting in higher expression of the intracellular tyrosine kinase domain of ALK. In addition, EML4 forms a coiled-coil that results in ligand-independent dimerization, and constitutive activation of the ALK tyrosine kinase domain. Additional examples of activated kinase fusions involve RET (rearranged during transfection) and ROS1.

Detection of a gene fusion can be used to direct therapy. Most methods of detection require biopsy of tumor tissue, which is not feasible for many cancer patients, especially in later stages. Detection in biopsied tissue sections is typically carried out by fluorescence in situ hybridization (FISH) or immunohistochemistry (IHC). The tests have high false positive rates and background, in part because of shearing during the sectioning process. Skilled cytologists are thus required to observe multiple tissue sections, which necessitates a sizable biopsy from a weakened patient. Similarly, a difficulty with using RT-PCR is the amount and

quality of genetic material from tumor tissue, *e.g.*, in formalin fixed paraffin embedded (FFPE) form. *See, e.g.*, Liu *et al.* (2015) PLoSOne 10: e0117032.

Because detection is time and resource intensive, the testing rate is relatively low. Cancers associated with ALK fusions are very sensitive to ALK inhibitors such as crizotinib and ceretinib. Gene fusions with Rearranged during Transcription (RET), such as with KIF5B or CCDC6, are also sensitive to therapy, *e.g.*, with vandetanib (*see* Matsubara *et al.* (2007) *J. Thorac. Oncol.* 7:1872). The low rate of testing for gene fusions thus represents a great lost opportunity for treatment.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a multiplex assay composition comprising:

A. at least one primer set and probe that specifically amplify and detect at least one ALK fusion gene;

B. at least one primer set and probe that specifically amplify and detect at least one RET fusion gene; and

C. a primer set and probe that specifically amplify and detect an internal control.

In a second aspect, the present invention provides a method of identifying an individual with cancer comprising:

(a) contacting a biological sample from the individual with the composition of the first aspect;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining that at least one fusion gene is present if a fusion gene is detected in step (b);

(d) whereby the presence of at least one fusion gene in said individual's sample indicates sensitivity of said individual to a kinase inhibitor therapy if at least one fusion gene is present.

In a third aspect, the present invention provides a method of determining the likelihood of response of an individual with cancer to kinase inhibitor therapy comprising:

(a) contacting a biological sample from the individual with the composition of the first aspect;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining that at least one fusion gene is present if a fusion gene is detected in step (b); and

(d) determining that the individual will likely respond to the kinase inhibitor therapy.

In a fourth aspect, the present invention provides a method for determining the presence of at least one fusion gene in a biological sample from an individual with cancer comprising:

(a) contacting a biological sample from the individual with the composition of the first aspect;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining the presence of at least one fusion gene if a fusion gene is detected in step (b).

Provided herein are multiplex methods and compositions for detecting fusion genes, in particular those involving ALK, RET, and ROS1.

Provided herein are multiplex assay compositions comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; (C) at least one primer set and labeled probe that specifically amplify and detect at least one ROS1 fusion gene; and (D) a primer set and labeled

probe that specifically amplify and detect an internal control. Further provided are multiplex assay compositions comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (C) a primer set and labeled probe that specifically amplify and detect an internal control. Provided herein are multiplex assay compositions comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (B) a primer set and labeled probe that specifically amplify and detect an internal control.

In some embodiments, the at least one ALK fusion gene is selected from the group consisting of: EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon

20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20; the at least one RET fusion gene is selected from the group consisting of: KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12; and the at least one ROS1 fusion gene is selected from the group consisting of: CD74 exon 6-ROS1 exon 34, CD74 exon 6-ROS1 exon 32, EZR exon 10-ROS1 exon 34, TPM3 exon 8-ROS1 exon 35, SDC4 exon 4-ROS1 exon 32, SDC4 exon 2-ROS1 exon 32, SDC4 exon 2-ROS1 exon 34, SDC4 exon 4-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 35, and LRIG3 exon 16-ROS1 exon 35, in any combination.

In some embodiments, the composition comprises at least one primer set and probe that amplify and detect more than 2 ALK fusion genes, more than 2 RET fusion genes, and/ or more than 2 ROS1 fusion genes. In some embodiments, the composition comprises at least one primer set and probe that amplify and detect EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, CD74 exon 6-ROS1 exon 34, and EZR exon 10-ROS1 exon 34.

In some embodiments, the at least one ALK fusion gene include: EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20; the at least one RET fusion gene includes: KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12; and the at least one ROS1 fusion gene includes: CD74 exon 6-ROS1 exon 34, CD74 exon 6-ROS1 exon 32, EZR exon 10-ROS1 exon 34, TPM3 exon 8-ROS1 exon 35, SDC4 exon 4-ROS1 exon 32, SDC4 exon 2-ROS1 exon 34, SDC4 exon 2-ROS1 exon 32, SDC4 exon 4-ROS1 exon 32, SLC34A2 exon 13-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 32, SLC34A2 exon 4-ROS1

exon 35, and LRIG3 exon 16-ROS1 exon 35. That is, the assay composition includes primer sets and probes to amplify and detect all of the listed fusion genes.

In some embodiments, for the primer set to amplify at least one ALK fusion gene, the forward primer and reverse primer have sequences selected from the group consisting of SEQ ID NOs:1-50, and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, for the probe to detect at least one ALK fusion gene, the probe sequence is selected from the group consisting of SEQ ID NOs:182-186. The forward and reverse primer sequences and probe sequences can be used together in any appropriate combination to detect any 1, 2, 3, 4, 5, 6, or 7 ALK fusion variants in any combination. In some embodiments, for the primer set to amplify at least one RET fusion gene, the forward primer and reverse primer have sequences selected from the group consisting of SEQ ID NOs:83-145 and 187, and SEQ ID NOs:161-180, respectively. In some embodiments, for the probe to detect at least one RET fusion gene, the probe sequence is selected from the group consisting of:189-194. The forward and reverse primer sequences and probe sequences can be used together in any combination to detect any 1, 2, 3, 4, 5, or 6 RET fusion variants in any combination. In some embodiments, for the primer set to detect at least one ROS1 fusion gene, the forward primer and reverse primer have sequences selected from the group consisting of SEQ ID NOs:195-212, and SEQ ID NOs:213-226, respectively. In some embodiments, for the probe to detect at least one ROS1 fusion gene, the probe sequence is selected from the group consisting of:227-230 and 51. The forward and reverse primer sequences and probe sequences can be used together in any combination to detect any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 ROS1 fusion variants in any combination.

In some embodiments, the label on labeled probe that detects the internal control is different from the labels on the labeled probes that detect the fusion genes. In some embodiments, the labels on all of the labeled probes are different from each other. In some embodiments, a single labeled probe is used to detect all of the at least one ALK fusion genes. In some embodiments, a single labeled probe is used to detect all of the at least one RET fusion genes.

In some embodiments, a single labeled probe is used to detect all of the at least one ROS1 fusion genes. In some embodiments, the labeled probe is attached to a primer in the at least one primer set. In some embodiments, the labeled probe is separate from the primer set.

In some embodiments, where more than one ALK fusion gene is amplified and detected, all of the primer sets that amplify the ALK fusion genes include a single common primer. In some embodiments, where more than one ALK fusion gene is amplified and detected, the primer sets include unique primers. In some embodiments, where more than one RET fusion gene is amplified and detected, all of the primer sets that amplify the RET fusion genes include a single common primer. In some embodiments, where more than one RET fusion gene is amplified and detected, the primer sets include unique primers. In some embodiments, where more than one ROS1 fusion gene is amplified and detected, all of the primer sets that amplify the ROS1 fusion genes include a single common primer. In some embodiments, where more than one ROS1 fusion gene is amplified and detected, the primer sets include unique primers.

Further provided herein are multiplex assay compositions comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (C) a primer set and labeled probe that specifically amplify and detect an internal control. Also provided herein are multiplex assay compositions comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (B) a primer set and labeled probe that specifically amplify and detect an internal control. In some embodiments, at least one ROS1 fusion gene is amplified and detected in a separate multiplex assay. In some embodiments, the at least one ALK fusion gene is selected from the group consisting of: EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20; and the at least one RET fusion gene is selected from the group consisting of: KIF5B exon

15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12, in any combination. In some embodiments, the at least one ROS1 fusion gene is selected from the group consisting of: CD74 exon 6-ROS1 exon 34, CD74 exon 6-ROS1 exon 32, EZR exon 10-ROS1 exon 34, TPM3 exon 8-ROS1 exon 35, SDC4 exon 2-ROS1 exon 34, SDC4 exon 4-ROS1 exon 32, SDC4 exon 2-ROS1 exon 32, SDC4 exon 4-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 35, and LRIG3 exon 16-ROS1 exon 35.

In some embodiments, the composition comprises at least one primer set and probe that amplify and detect more than 2 ALK fusion genes and more than 2 RET fusion genes. In some embodiments, the composition comprises at least one primer set and probe that amplify and detect EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, and KIF5B exon 22-RET exon 12.

In some embodiments, the at least one ALK fusion gene include: EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20; and the at least one RET fusion gene includes: KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12.

In some embodiments, the label on labeled probe that detects the internal control is different from the labels on the labeled probes that detect the fusion genes. In some embodiments, the labels on all of the labeled probes are different from each other. In some embodiments, a single labeled probe is used to detect all of the at least one ALK fusion genes. In some embodiments, a single labeled probe is used to detect all of the at least one RET fusion genes. In some embodiments, the labeled probe is attached to a primer in the at least one primer set. In some embodiments, the labeled probe is separate from the primer set.

In some embodiments, where more than one ALK fusion gene is amplified and detected, all of the primer sets that amplify the ALK fusion genes include a single common primer. In some embodiments, where more than one ALK fusion gene is amplified and detected, the primer sets include unique primers. In some embodiments, where more than one RET fusion gene is amplified and detected, all of the primer sets that amplify the RET fusion genes include a single common primer. In some embodiments, where more than one RET fusion gene is amplified and detected, the primer sets include unique primers.

Examples of internal controls that can be used for the presently disclosed assays include, but are not limited to, SDHA (succinate dehydrogenase), LDHA (lactate dehydrogenase A), NONO, PGK (phosphoglycerate kinase 1), PPIH, HPRT1, beta-actin, GADPH, ACTB, and 16S rRNA.

In some embodiments, the composition further comprises a DNA polymerase, *e.g.*, a thermostable DNA polymerase such as Taq or a Taq derivative. In some embodiments, the composition further comprises reverse transcriptase. In some embodiments, the composition further comprises dNTPs. In some embodiments, the composition further comprises buffer amenable to polymerization by the DNA polymerase and reverse transcriptase.

In some embodiments, the composition further comprises a biological sample from an individual or group of individuals. In some embodiments, the individual has been diagnosed with cancer, *e.g.*, lung cancer (*e.g.*, non-small cell lung cancer (NSCLC), lung squamous cell carcinoma, lung adenocarcinoma), bladder carcinoma, glioblastoma, head and neck cancer, glioma, thyroid carcinoma, ovarian cancer, leukemia, lymphoma, prostate cancer, pancreatic cancer, renal cancer, or breast cancer.

In some embodiments, the sample is enriched or isolated nucleic acid, *e.g.*, DNA or RNA. In some embodiments, the sample is RNA, *e.g.*, isolated from blood (*e.g.*, serum, plasma, other blood fraction), bronchoalveolar lavage, or tissue biopsy. In some embodiments, the

biological sample includes 100 nM or less of the polynucleotide comprising the fusion gene, *e.g.*, 0.01-100 nM, 0.01-25nM, 0.01-5 nM, 0.02-0.5 nM, or 0.02-0.1 nM.

Further provided are methods of treating an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; (C) at least one primer set and labeled probe that specifically amplify and detect at least one ROS1 fusion gene; and (D) a primer set and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected; and treating the individual if at least one fusion gene is present. Further provided are methods of treating an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (C) a primer set and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected; and treating the individual if at least one fusion gene is present. Further provided are methods of treating an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (B) a primer set

and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected; and treating the individual if at least one fusion gene is present.

In some embodiments, the treatment is with a kinase inhibitor, *e.g.*, a selective kinase inhibitor such as alectinib, crizotinib, ceritinib, lorlatinib, brigatinib, cabozantinib, apatinib, vandetanib, ponatinib, lenvatinib, DS6051b, or variants or combinations thereof. In some embodiments, the course of treatment includes radiation therapy or chemotherapy (*e.g.*, cisplatin, carboplatin, paclitaxel, docetaxel). In some embodiments, the treatment is with GSK1838705A, TAE-684, CEP-14083, AP26113, NMS-E628, sorafenib, vandetanib, motesanib, sunitinib, and XL-184 (*see, e.g.*, Mologni (2011) *Curr. Med. Chem.* 18:162).

In some embodiments, the individual is monitored throughout treatment, *e.g.*, to determine if the amount of fusion gene amplification product increases or decreases, or if a different fusion gene is detected. In some embodiments, the treatment is changed if the amount of fusion gene amplification product changes, or if a different fusion gene is detected. For example, if the amount of the originally detected fusion gene decreases but the cancer is progressing, treatment can be changed to be less targeted, *e.g.*, radio- or chemotherapy. If the individual's condition has improved, treatment can be reduced.

In some embodiments, the biological sample includes DNA or RNA, *e.g.*, separated or purified nucleic acids. In some embodiments, the biological sample is RNA from blood, *e.g.*, plasma, serum, or other blood fraction. In some embodiments, the amplification and detection are carried out using qRT-PCR.

In some embodiments, the individual is diagnosed with lung cancer (*e.g.*, non-small cell lung cancer (NSCLC), lung squamous cell carcinoma, lung adenocarcinoma), bladder carcinoma,

glioblastoma, head and neck cancer, glioma, thyroid carcinoma, ovarian cancer, leukemia, lymphoma, prostate cancer, pancreatic cancer, renal cancer, or breast cancer.

Further provided are methods for determining the presence of at least one fusion gene in a sample from an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; (C) at least one primer set and labeled probe that specifically amplify and detect at least one ROS1 fusion gene; and (D) a primer set and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected. Further provided are methods for determining the presence of at least one fusion gene in a sample from an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one ALK fusion gene; (B) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (C) a primer set and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected. Further provided are methods for determining the presence of at least one fusion gene in a sample from an individual, *e.g.*, an individual diagnosed with cancer, comprising contacting a biological sample from the individual with any of the multiplex assay compositions described herein (*e.g.*, comprising: (A) at least one primer set and labeled probe that specifically amplify and detect at least one RET fusion gene; and (B) a primer set

and labeled probe that specifically amplify and detect an internal control); carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample; determining that at least one fusion gene is present if a fusion gene is detected.

In some embodiments, the biological sample includes DNA or RNA, *e.g.*, separated or purified nucleic acids. In some embodiments, the biological sample is RNA from blood, *e.g.*, plasma, serum, or other blood fraction. In some embodiments, the amplification and detection are carried out using qRT-PCR.

In some embodiments, the individual is diagnosed with lung cancer (*e.g.*, non-small cell lung cancer (NSCLC), lung squamous cell carcinoma, lung adenocarcinoma), bladder carcinoma, glioblastoma, head and neck cancer, glioma, thyroid carcinoma, ovarian cancer, leukemia, lymphoma, prostate cancer, pancreatic cancer, renal cancer, or breast cancer.

In some embodiments, the method further comprises determining a course of treatment if at least one fusion gene is detected. In some embodiments, the treatment is with a kinase inhibitor, *e.g.*, a selective kinase inhibitor such as alectinib, crizotinib, ceritinib, lorlatinib, brigatinib, cabozantinib, apatinib, vandetanib, ponatinib, lenvatinib, DS6051b, or variants or combinations thereof. In some embodiments, the course of treatment includes radiation therapy or chemotherapy (*e.g.*, cisplatin, carboplatin, paclitaxel, docetaxel). In some embodiments, the treatment is with GSK1838705A, TAE-684, CEP-14083, AP26113, NMS-E628, sorafenib, vandetanib, motesanib, sunitinib, and XL-184.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows that the indicated ALK fusion variants (FAM) are detectable at 50 copies in 0.1ng WT RNA (n=3). Figure 1B shows that the indicated RET fusion variants (HEX) are detectable at 50 copies in 0.1ng WT RNA (n=3). Figure 1C shows that the indicated ROS1

fusion variants (JA270) are detectable at 50 copies in 0.1ng WT RNA (n=3). Figure 1D shows the internal control Ct values for each input RNA.

Figure 2 shows the limit of detection of ALK and RET fusions in a multiplex assay as described herein. The assay is able to detect 25 copies of fusion transcript diluted in UHR.

Figure 3 shows linearity data for representative ALK fusion variants

Figure 4 shows linearity data for representative RET fusion variants.

Figure 5 shows linearity data for representative ROS1 fusion variants

Figure 6 shows LOD data for a representative ALK fusion variant.

Figure 7 shows LOD data for a representative RET fusion variant.

Figure 8 shows LOD data for a representative ROS1 fusion variant.

DETAILED DESCRIPTION OF THE INVENITON

I. Introduction

The inventors have discovered a novel, quantitative, and multiplex method of detecting fusions between genetic regions. The presently disclosed methods require only a small amount of patient sample that can be gathered non-invasively, *e.g.*, circulating free RNA (cfRNA) from plasma.

Current tests require either biopsy or large amounts of plasma, due to the limited amount of circulating nucleic acids originating from a tumor. The presently described methods allow for an extremely sensitive (down to ~25 copies), one-tube assay to detect multiple gene fusions that are predictive of cancer and response to therapy. The present assays can be used for identification of a fusion variant, as well as monitoring and surveillance during treatment and/ or progression.

II. Definitions

A "genetic fusion" is hybrid chromosomal sequence formed by joining of two chromosomal locations that were previously separate. Fusion can occur between genes on the same chromosome (*e.g.*, interstitial deletion or chromosomal inversion) or on different chromosomes (*e.g.*, translocation).

A "fusion gene" is a hybrid gene formed by the joining of two genes that were previously separate, leading to a structural rearrangement and/or variant in the tumor genome. The fusion gene need not necessarily include coding sequence from both genes, but can include non-coding sequence from one of the genes, *e.g.*, promoter or 3' untranslated regions. The denomination of genes that comprise a fusion gene as "gene 1," "gene 2," "gene A," "gene B," etc., is used to distinguish between genes that make up the fusion and does not necessarily refer to the position of the genes in the fusion. The terms ALK fusion, RET fusion, and ROS1 fusion refer to fusion genes that include ALK, RET, and ROS1 as a member, respectively.

The terms "fusion site," "fusion point," "breakpoint" and like terms refer to the point in a genetic fusion where a nucleotide from one gene or genetic location is found adjacent to a nucleotide from another gene or genetic location.

The terms "target region," "target portion," "target fragment," and like terms refer to a region of a target nucleic acid sequence that is to be amplified and/or analyzed.

The terms "nucleic acid," "polynucleotide," and "oligonucleotide" refer to polymers of nucleotides (*e.g.*, ribonucleotides or deoxyribo-nucleotides) and includes naturally-occurring (adenosine, guanidine, cytosine, uracil and thymidine), non-naturally occurring, and modified nucleic acids. The term is not limited by length (*e.g.*, number of monomers) of the polymer. A nucleic acid may be single-stranded or double-stranded and will generally contain 5'-3' phosphodiester bonds, although in some cases, nucleotide analogs may have other linkages. Monomers are typically referred to as nucleotides. The term "non-natural nucleotide" or "modified nucleotide" refers to a nucleotide that contains a modified

nitrogenous base, sugar or phosphate group, or that incorporates a non-natural moiety in its structure. Examples of non-natural nucleotides include dideoxynucleotides, biotinylated, aminated, deaminated, alkylated, benzylated and fluorophor-labeled nucleotides.

The term "primer" refers to a short nucleic acid (an oligonucleotide) that acts as a point of initiation of polynucleotide strand synthesis by a nucleic acid polymerase under suitable conditions. Polynucleotide synthesis and amplification reactions typically include an appropriate buffer, dNTPs and/or rNTPs, and one or more optional cofactors, and are carried out at a suitable temperature. A primer typically includes at least one target-hybridized region that is at least substantially complementary to the target sequence. This region of is typically about 15 to about 40 nucleotides in length. A "primer pair" refers to a forward primer and reverse primer (sometimes called 5' and 3' primers) that are complementary to opposite strands of a target sequence and designed to amplify the target sequence. The forward and reverse primers are arranged within an amplifiable distance of each other on the target sequence, *e.g.*, about 10-5000 nucleotides, about 25-500, or about 60-120 nucleotides. A "primer set" refers to one or more primer pairs, or a combination of at least one forward primer and at least one reverse primer. For example, a primer set can include 3 forward primers and 1 reverse primer, so that 3 distinct amplification products can potentially be produced.

A primer set or primer pair that is specific for a sequence (or portion of a gene) that is 5' (or 3') of a fusion site (or breakpoint) refers to primers used to amplify a sequence that does not include the fusion site or breakpoint.

As used herein, "probe" means any molecule that is capable of selectively binding to a specifically intended target biomolecule, for example, a nucleic acid sequence of interest to be bound, captured or hybridized by the probes. Probes are typically labeled with a non-naturally occurring moiety, *e.g.*, a fluorophore, chromophore, affinity tag (*e.g.*, streptavidin or biotin), and/or a quencher.

The words "complementary" or "complementarity" refer to the ability of a nucleic acid in a polynucleotide to form a base pair with another nucleic acid in a second polynucleotide. For example, the sequence A-G-T (A-G-U for RNA) is complementary to the sequence T-C-A (U-C-A for RNA). Complementarity may be partial, in which only some of the nucleic acids match according to base pairing, or complete, where all the nucleic acids match according to base pairing. A probe or primer is considered "specific for" a target sequence if it is at least partially complementary to the target sequence. Depending on the conditions, the degree of complementarity to the target sequence is typically higher for a shorter nucleic acid such as a primer (*e.g.*, greater than 80%, 90%, 95%, or higher) than for a longer sequence.

The terms "identical" or "percent identity," in the context of two or more nucleic acids, or two or more polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides, or amino acids, that are the same (*e.g.*, about 60% identity, e.g., at least any of 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters, or by manual alignment and visual inspection. See e.g., the NCBI web site at ncbi.nlm.nih.gov/BLAST. Such sequences are then said to be "substantially identical." Percent identity is typically determined over optimally aligned sequences, so that the definition applies to sequences that have deletions and/or additions, as well as those that have substitutions. The algorithms commonly used in the art account for gaps and the like. Typically, identity exists over a region comprising a sequence that is at least about 8-25 amino acids or nucleotides in length, or over a region that is 50-100 amino acids or nucleotides in length, or over the entire length of the reference sequence.

The term "allele" refers to a sequence variant of a gene. One or more genetic differences can constitute an allele.

The term "kit" refers to any manufacture (*e.g.*, a package or a container) including at least one reagent, such as a nucleic acid probe or probe pool or the like, for specifically amplifying, capturing, tagging/converting or detecting RNA or DNA as described herein.

The term "amplification conditions" refers to conditions in a nucleic acid amplification reaction (*e.g.*, PCR amplification) that allow for hybridization and template-dependent extension of the primers. The terms "amplicon" and "amplification product" refer to a nucleic acid molecule that contains all or a fragment of the target nucleic acid sequence and that is formed as the product of in vitro amplification by any suitable amplification method. The borders of a given amplicon are typically defined by the position of the complementary portion of the forward and reverse primers used for amplification. Suitable PCR conditions are described in *PCR Strategies* (Innis *et al.*, 1995, Academic Press, San Diego, CA) at Chapter 14; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, Academic Press, NY, 1990)

The term "thermostable nucleic acid polymerase" or "thermostable polymerase" refers to a polymerase enzyme, which is relatively stable at elevated temperatures when compared, for example, to polymerases from *E. coli*. A thermostable polymerase is suitable for use under temperature cycling conditions typical of the polymerase chain reaction ("PCR"). Exemplary thermostable polymerases include those from *Thermus thermophilus, Thermus caldophilus, Thermus sp.* Z05 (*see, e.g.*, U.S. Patent No. 5,674,738) and mutants of the *Thermus sp.* Z05 polymerase, *Thermus aquaticus, Thermus flavus, Thermus filiformis, Thermus sp. sps17, Deinococcus radiodurans, Hot Spring family B/clone 7, Bacillus stearothermophilus, Bacillus caldotenax, Thermotoga maritima, Thermotoga neapolitana* and *Thermosipho africanus*, and modified versions thereof.

The term "sample" or "biological sample" refers to any composition containing or presumed to contain nucleic acid from an individual. The term includes purified or separated components of cells, tissues, or blood, *e.g.*, DNA, RNA, proteins, cell-free portions, or cell lysates. In some embodiments, analysis is conducted on plasma samples isolated from blood;

the terms "detected in patient's blood" and "detected in patient's plasma" are used interchangeably to mean that blood is obtained from the patient and plasma derived therefrom is used for the analysis. A sample can also refer to other types of biological samples, *e.g.*, skin, plasma, serum, whole blood and blood components (*e.g.*, platelets, buffy coat), saliva, urine, tears, seminal fluid, vaginal fluids, tissue biopsies, and other fluids and tissues, including paraffin embedded tissues. Samples also may include constituents and components of *in vitro* cultures of cells obtained from an individual, including cell lines.

A "control" sample or value refers to a sample that serves as a reference, usually a known reference, for comparison to a test sample or test conditions. For example, a test sample can be taken from a test condition, *e.g.*, from an individual suspected of having cancer, and compared to samples from known conditions, *e.g.*, from a cancer-free individual (negative control), or from an individual known to have cancer and/ or a particular genetic abnormality (positive control). In the context of the present disclosure, an example of a negative control would be a biological sample from a known healthy (non-cancer, non-mutated) individual, and an example of a positive control would be a biological sample from a patient or cell line known to have a particular gene fusion. A control can also represent an average value or a range gathered from a number of tests or results. A control can also be prepared for reaction conditions. For example, a positive control for the presence of nucleic acid could include primers or probes that will detect a sequence known to be present in the sample, while a negative control would be free of nucleic acids. One of skill in the art will recognize that controls can be designed for assessment of any number of parameters. For example, a control can be devised to compare therapeutic benefit based on pharmacological data (*e.g.*, half-life) or therapeutic measures (*e.g.*, comparison of benefit and/or side effects). Controls can be designed for in vitro applications. One of skill in the art will understand which controls are valuable in a given situation and be able to analyze data based on comparisons to control values. Controls are also valuable for determining the significance of data. For example, if values for a given parameter are widely variant in controls, variation in test samples will not be considered as significant.

An "internal control" (IC) refers to a nucleic acid that is expected to be present in the sample, such as a housekeeping gene that is expressed or present at a fairly standard level across samples. The internal control can be used to standardize the amount and quality of nucleic acid in the sample with that of other samples and ensure that the amplification and detection reaction is functioning. Examples of internal controls include SDH (succinate dehydrogenase), LDHA (lactate dehydrogenase A), NONO, PGK (phosphoglycerate kinase 1), PPIH, HPRT1, beta-actin, GADPH, ACTB, and 16S rRNA.

The term "diagnosis" refers to a relative probability that a subject has a disorder such as cancer or certain type of cancer (*e.g.*, resulting from a gene fusion). Similarly, the term "prognosis" refers to a relative probability that a certain future outcome may occur in the subject. For example, in the context of the present disclosure, diagnosis can refer to classification of a cancer or the likelihood that an individual will be responsive to a particular therapy. The terms are not intended to be absolute, as will be appreciated by any one of skill in the field of medical diagnostics.

The terms "response to therapy," "response to treatment," "amelioration," and like terms refer to any reduction in the severity of symptoms. In the case of treating cancer, treatment can refer to, *e.g.*, reducing tumor size, number of cancer cells, growth rate, metastatic activity, reducing cell death of non-cancer cells, reduced nausea and other chemotherapy or radiotherapy side effects, etc. The terms "treat" and "prevent" are not intended to be absolute terms. Treatment and prevention can refer to any delay in onset, amelioration of symptoms, improvement in patient survival, increase in survival time or rate, etc. Treatment and prevention can be complete (undetectable levels of neoplastic cells) or partial, such that fewer neoplastic cells are found in a patient than would have occurred without the treatment. The effect of treatment can be compared to an individual or pool of individuals not receiving the treatment (*e.g.*, individuals having the same genetic fusion), or to the same patient prior to treatment or at a different time during treatment. In some aspects, the severity of disease is reduced by at least 10%, as compared, *e.g.*, to the individual before administration or to a

control individual not undergoing treatment. In some aspects the severity of disease is reduced by at least 25%, 50%, 75%, 80%, or 90%, or in some cases, no longer detectable using standard diagnostic techniques.

The terms "treat" and "administer," with reference to a patient, include recommending, providing, or prescribing a particular treatment to the patient, and are not limited to directly, physically treating the patient.

The term "threshold cycle" or "Ct" is a measure of relative concentration and is commonly used in real-time PCR (also referred to as qPCR). Ct refers to the intersection of an amplification curve and a threshold line. The threshold line is often set at a point when signal can be detected above background, or when an amplification reaction enters the exponential phase. Ct can be affected by concentration of target and amplification conditions, *e.g.*, the effect of conditions on detectable labels and amplification efficiency. A higher Ct corresponds to a longer time to reach the threshold, be it due to low target concentration or inefficient amplification.

The terms "individual," "subject," "patient," and like terms are used interchangeably and refer to humans, except where indicated. Other mammals can be considered subjects, such as non-human primates, as well as rabbits, rats, mice, dogs, cats, and other mammalian species. The term does not necessarily indicate that the subject has been diagnosed with a particular disease, but typically refers to an individual under medical supervision. A patient can be seeking treatment, monitoring, adjustment or modification of an existing therapeutic regimen, etc. A patient can include individuals that have not received treatment, are currently receiving treatment, have had surgery, and those that have discontinued treatment.

The terms "label," "tag," "detectable moiety," and like terms refer to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include fluorescent dyes, luminescent agents, radioisotopes (e.g., ^{32}P, ^{3}H), electron-dense reagents, or an affinity-based moiety, e.g., a "His tag" for purification, or a "strepavidin tag" that interacts with biotin.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. See, *e.g.*, Pfaffl, Methods: The ongoing evolution of qPCR, vol. 50 (2010); van Pelt-Verkuil *et al.* Principles and Technical Aspects of PCR Amplification, Springer (2010); Lackie, DICTIONARY OF CELL AND MOLECULAR BIOLOGY, Elsevier (4th ed. 2007); Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, Cold Springs Harbor Press (Cold Springs Harbor, N.Y. 1989). The term "a" or "an" is intended to mean "one or more." The terms "comprise," "comprises," and "comprising," when preceding the recitation of a step or an element, are intended to mean that the addition of further steps or elements is optional and not excluded.

III. Fusion genes

A number of cancer-associated fusion genes are known, and appear in all manner of cancers. Examples include lung cancer (*e.g.*, non-small cell lung cancer (NSCLC), lung squamous cell carcinoma, lung adenocarcinoma), bladder carcinoma, glioblastoma, head and neck cancer, glioma, thyroid carcinoma, ovarian cancer, leukemia, lymphoma, prostate cancer, pancreatic cancer, renal cancer, and breast cancer. Cancer-associated fusion genes commonly occur where one member of the fusion is a kinase involved in a pro-growth signaling pathway, and the other member contributes to elevated or constitutive expression or signaling. This is the case for fusions of ALK, RET, and ROS1. Common fusion partners for ALK are EML4 and KIF5B. Common fusion partners for RET are KIF5B, CCDC6, and NCOA4. Several genes are known to fuse with ROS1, including CD74, EZR, TPM3, SDC4, SLC34A2, and LRIG3 (*see, e.g.*, Yoshihara *et al.* (2015) *Oncogene* 34:4845).

The present compositions and methods focus on design of multiplex assays to detect ALK, RET, and ROS1 fusions. Invasive biopsy or excessive blood collection is often not feasible for cancer patients. The present compositions and methods allow for detection of several actionable gene fusions with a relatively small sample from the patient, which can be a non-invasive plasma sample.

The design of these highly multiplexed assays can vary. Where multiple ALK fusions are detected, for example, a common primer and probe that hybridize to sequences in the ALK gene near the fusion point, and primers specific for various fusion partners, can be used. Thus, for example, if 5 different ALK fusions are detected, the assay can include 15 oligonucleotides (10 primers and 5 probes) or 7 oligonucleotides (1 common primer, 1 common probe, and 5 specific primers).

In some embodiments, the multiplex assay detects 2, 3, 4, 5, 6, or 7 ALK fusions and 2, 3, 4, 5, or 6 RET fusions in a single amplification and detection reaction. In some embodiments, the multiplex assay further detects 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 ROS1 fusions in the same reaction. In some embodiments, the ROS1 fusions are detected in a separate amplification and detection reaction. In some embodiments, the amplification and detection reaction further includes an internal control (*e.g.*, a housekeeping gene).

The presence of ALK, RET and ROS1 fusions indicate that a cancer patient will be responsive to a selective kinase inhibitor. These include alectinib, crizotinib, ceritinib, lorlatinib, brigatinib, cabozantinib, apatinib, vandetanib, ponatinib, lenvatinib, DS-6051b, and variants or combinations thereof. The fusion status of a patient can be monitored throughout treatment to determine if the therapeutic approach can be changed, *e.g.*, to a different kinase inhibitor or more standard chemo- or radio-therapy.

IV. Preparation of sample

Samples for testing for genetic fusions can be obtained from any source, but are advantageously obtained in a non-invasive manner, *e.g.*, from blood or a blood fraction (*e.g.*, plasma, serum, platelets, etc.). Samples for the present methods can also be taken from urine, bronchoalveolar lavage, or tissue biopsy. Methods for isolating nucleic acids from biological samples are known, *e.g.*, as described in Sambrook, and several kits are commercially available (*e.g.*, High Pure RNA Isolation Kit, High Pure Viral Nucleic Acid Kit, and MagNA Pure LC Total Nucleic Acid Isolation Kit from Roche).

In some embodiments, DNA is prepared, and used as template for the presently disclosed amplification and detection methods. In some embodiments, RNA is prepared. When RNA is used as template for amplification by PCR, a reverse transcription step is required to prepare cDNA. A DNA polymerase such as Taq or another thermostable polymerase can then be used to carry out amplification.

In some embodiments, the sample is RNA is isolated from blood plasma. Depending on the condition of the patient, about 1-10 mL of plasma can be obtained for testing (usually about 2 mL). Kits for isolating circulating free RNA are commercially available, *e.g.*, from Norgen Biotek Corp or Qiagen.

As shown in the Examples, the presently disclosed methods for sample preparation and amplification/ detection with custom target-specific oligos are extraordinarily sensitive, and can be used to detect gene fusion mutations from as few as about 50 – and in some cases about 20 – copies in a sample diluted 1:4000 in wild type RNA background. This allows for detection of fusion variants in samples where the target sequence is very rare, *e.g.*, circulating cell-free RNA (cfRNA). Varying backgrounds of RNA and DNA in plasma do not detract from the specificity of detection even at low copy numbers.

V. Amplification and detection

Nucleic acid amplification can be carried out using any primer-dependent method. In some embodiments, the amplification is quantitative, so that the relative or actual abundance of a given amplification target can be determined by the amount of amplification product.

DNA-based methods can be used for the presently disclosed amplification and detection methods, *e.g.*, PCR. In some embodiments, real time or quantitative PCR is used (RTPCR or qPCR). qPCR allows for reliable detection and measurement of products generated during each cycle of PCR process. Such techniques are well known in the art, and kits and reagents are commercially available, *e.g.*, from Roche Molecular Systems, Life Technologies, Bio-Rad, etc. *See, e.g.*, Pfaffl (2010) Methods: The ongoing evolution of qPCR vol. 50. In some

embodiments, the amplification and detection are carried out in the presence of a dual labeled probe (*e.g.*, a TaqMan, CPT, LNA, or MGB probe) labeled with a quencher and a fluorophore (*see, e.g.*, Gasparic *et al.* (2010) *Anal. Bioanal. Chem.* 396:2023).

In some embodiments, a preliminary reverse transcription step is carried out (also referred to as RT-PCR, not to be confused with real time PCR). *See, e.g.*, Hierro *et al.* (2006) 72:7148. The term "qRT-PCR" as used herein refers to reverse transcription followed by quantitative PCR. Both reactions can be carried out in a single tube without interruption, *e.g.*, to add reagents.

RNA-based amplification methods can also be used, *e.g.*, transcription mediated amplification (TMA) or nucleic acid sequence based amplification (NASBA). *See, e.g.*, Fakruddin *et al.* (2013) *J Pharm Bioallied Sci.* 5:245; van Deursen *et al.* (1999) *Nucl. Acids Res.* 27:e15; Kamisango *et al.* (1999) *J Clin. Microbiol.* 37:310.

Some of the oligonucleotides used in the present assays (primers and probes) include alkyl base modifications to enhance selective amplification, in particular in a multiplex format.

A probe, or one or both primers in a primer pair can be labeled with any substance or component that directly or indirectly emits or generates a detectable signal. In some embodiments, the labels are fluorophores (dyes), many of which are reported in the literature and known to those skilled in the art, and many of which are commercially available. Fluorophores are described, *e.g.*, in Cardullo et al. (1988) Proc. Natl. Acad. Sci. USA 85: 8790; Hochstrasser et al. (1992) Biophysical Chemistry 45: 133; Selvin (1995) Methods in Enzymology 246: 300; Steinberg, Ann. Rev. Biochem., 40: 83- 114 (1971); and Wang et al., Anal. Chem. 67: 1197-1203 (1995).

The following are examples of fluorophores that can be used as labels: 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid; acridine; acridine isothiocyanate; 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl) phenyl]naphthalimide-3,5 disulfonate [0070] N-(4-anilino-1-naphthyl)maleimide;

anthranilamide; BODIPY; Brilliant Yellow; coumarin; 7-amino-4-methylcoumarin (AMC, Coumarin 120)/ 7-amino-4-trifluoromethylcoumarin (Coumaran 151); cyanine dyes; cyanosine 4',6-diaminidino-2-phenylindole (DAPI); 5',5''-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin; eosin isothiocyanate; erythrosin B; erythrosin isothiocyanate; ethidium; 5-carboxyfluorescein (FAM); 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF); 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE); fluorescein; fluorescein isothiocyanate; fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; phycoerythrin (including but not limited to B and R types); o-phthaldialdehyde; pyrene; pyrene butyrate; succinimidyl 1-pyrene butyrate; quantum dots; Reactive Red 4 (Cibacron Brilliant Red 3B-A); 6-carboxy-X-rhodamine (ROX); 6-carboxyrhodamine (R6G); lissamine rhodamine B sulfonyl chloride rhodamine; rhodamine B; rhodamine 123; rhodamine X isothiocyanate; sulforhodamine B; sulforhodamine 101; sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid; and lanthanide chelate derivatives.

Any of the listed fluorophores (dyes) can be used in the presently described assays to label a nucleic acid as described herein. Fluorophores can be attached by conventional covalent bonding, using appropriate functional groups on the fluorophore and/or nucleic acid.

As noted above, a dual labeled probe can be used for detection. The dual labeled probe can comprise a fluorophore, such any of the fluorophores listed above, and a quencher. Suitable quenchers include but are not limited to DDQ-I, Dabcyl, Eclipse, Iowa Black FQ, BHQ-1,

QSY-7, BHQ-2, DDQ-II, Iowa Black RQ, QSY-21, and BHQ-3. For fluorophores having an emission maximum between 500 and 550 nm (*e.g.*, FAM, TET, and HEX), a quencher with an absorption maxima between 450 and 500 nm can be selected (*e.g.*, dabcyl or BHQ-1). For fluorophores having an emission maximum above 550 nm (*e.g.*, rhodamine and Cy dyes), a quencher with an absorption maxima above 550 nm can be selected (*e.g.*, BHQ-2). *See, e.g.*, Johansson (2003) *Meth. Mol. Biol.* 335:17 for considerations in selecting dye-quencher pairs.

Detection devices are known in the art and can be selected as appropriate for the selected labels. Detection devices appropriate for quantitative PCR include the cobas® and Light Cycler® systems (Roche), PRISM 7000 and 7300 real-time PCR systems (Applied Biosystems), etc.

VI. Kits

In some embodiments, reagents and materials for carrying out the presently disclosed methods are included in a kit. In some embodiments, the kit includes components for obtaining, storing, and/ or preparing sample. Such components include, *e.g.*, sterile needles and syringes, EDTA-lined tubes, buffers (*e.g.*, for binding nucleic acid to, and elution from a matrix), RNase inhibitors, and/ or DNase, etc.

In some embodiments, the kit includes forward primer(s) and reverse primer(s) for amplifying ALK fusion variant(s) having sequences selected from the group consisting of SEQ ID NOs:1-50, and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes probe(s) for detecting ALK fusion variant(s) having sequences selected from the group consisting of SEQ ID NOs:182-186. The forward and reverse primer sequences and probe sequences can be used together in any appropriate combination to detect any 1, 2, 3, 4, 5, 6, or 7 ALK fusion variants in any combination. In some embodiments, the kit includes forward primer(s) and reverse primer(s) for amplifying RET fusion variant(s) having sequences selected from the group consisting of SEQ ID NOs:83-145 and 187, and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes probe(s) for detecting

RET fusion variant(s) having sequences selected from the group consisting of:189-194. The forward and reverse primer sequences and probe sequences can be used together in any combination to detect any 1, 2, 3, 4, 5, or 6 RET fusion variants in any combination. In some embodiments, the kit includes forward primer(s) and reverse primer(s) for amplifying ROS1 fusion variant(s) having sequences selected from the group consisting of SEQ ID NOs:195-212, and SEQ ID NOs:213-226, respectively. In some embodiments, the kit includes probe(s) for detecting ROS1 fusion variants having sequences selected from the group consisting of:227-230 and 51. The forward and reverse primer sequences and probe sequences can be used together in any combination to detect any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 ROS1 fusion variants in any combination.

In some embodiments, the kit includes a forward primer and reverse primer for amplifying an EML exon 13- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:1-10 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an EML exon 20- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:11-20 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an EML exon 6- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:21-30 and SEQ ID NOs:52-61 and 181, respectively. the kit includes a forward primer and reverse primer for amplifying an EML exon 2- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:31-35 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an EML exon 18- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:36-40 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a KIF exon 24- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:41-45 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a

KIF exon 17- ALK exon 20 fusion variant having sequences selected from the group consisting of SEQ ID NOs:46-50 and SEQ ID NOs:52-61 and 181, respectively. In some embodiments, the kit includes a probe for detecting an ALK fusion having a sequence selected from group consisting of SEQ ID NOs:182-186.

In some embodiments, the kit includes a forward primer and reverse primer for amplifying a KIF exon 15- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:83-97 and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a KIF exon 16- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:98-107 and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a KIF exon 22- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:108-117 and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a KIF exon 23- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:118-127 and 187, and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a CCDC exon 1- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:128-135 and 118, and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an NCO exon 6- RET exon 12 fusion variant having sequences selected from the group consisting of SEQ ID NOs:136-145 and SEQ ID NOs:161-180, respectively. In some embodiments, the kit includes a probe for detecting a RET fusion having a sequence selected from group consisting of SEQ ID NOs:189-194.

In some embodiments, the kit includes a forward primer and reverse primer for amplifying a CD74 exon 6- ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NOs:195-197 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a CD74

exon 6- ROS1 exon 32 fusion variant having sequences selected from the group consisting of SEQ ID NOs:195-197 and SEQ ID NOs:213-215, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an EZR exon 10- ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NO:208 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying a TPM3 exon 8- ROS1 exon 35 fusion variant having sequences selected from the group consisting of SEQ ID NOs:211-212 and SEQ ID NOs:216-221, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SDC4 exon 4- ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NOs:200-202 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SDC4 exon 2- ROS1 exon 32 fusion variant having sequences selected from the group consisting of SEQ ID NOs:198-199 and SEQ ID NOs:213-215, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SDC4 exon 2-ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NOs:198-199 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SDC4 exon 4- ROS1 exon 32 fusion variant having sequences selected from the group consisting of SEQ ID NOs:200-202 and SEQ ID NOs:213-215, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SLC34A2 exon 13- ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NOs:203-205 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SLC34A2 exon 13- ROS1 exon 32 fusion variant having sequences selected from the group consisting of SEQ ID NOs:203-205 and SEQ ID NOs:213-215, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an SLC34A2 exon 4- ROS1 exon 32 fusion variant having sequences selected from the group consisting of SEQ ID NOs:206-207 and SEQ ID NOs:213-215, respectively. In some

embodiments, the kit includes a forward primer and reverse primer for amplifying an SLC34A2 exon 4- ROS1 exon 34 fusion variant having sequences selected from the group consisting of SEQ ID NOs:206-207 and SEQ ID NOs:222-226, respectively. In some embodiments, the kit includes a forward primer and reverse primer for amplifying an LRIG3 exon 16- ROS1 exon 35 fusion variant having sequences selected from the group consisting of SEQ ID NOs:209-210 and SEQ ID NOs:216-221, respectively.

In some embodiments, each of the primer sets is packaged in separate tubes, *e.g.*, to be added in ratios to be determined by the user. In some embodiments, one or more or all of the primer sets are packaged in a single tube with predetermined ratios.

The kit can also include enzymes, such as reverse transcriptase and or DNA polymerase. In some embodiments, the DNA polymerase is a thermostable DNA polymerase capable of amplifying in thermocycling conditions, *e.g.*, Taq or a Taq derivative. In some embodiments, the kit includes dNTPs. In some embodiments, the kit includes buffers conducive to polymerization/ amplification by the selected polymerases.

In some embodiments, the kit includes controls, *e.g.*, a polynucleotide that is wild type at the genetic fusion to be detected (*i.e.*, no genetic fusion), or a polynucleotide that includes the genetic fusion to be detected.

The kit can also include consumables such as sample tubes or vials; reaction containers (*e.g.*, tubes, multiwell plates, microfluidic chips or chambers, etc), as well as directions for use or reference to a website.

VII. Examples

A. Example 1: Multiplex assays for detection of ALK, RET, and ROS1 fusion panel

In this example, we tested a multiplex, quantitative RT-PCR method to detect ALK, RET, and ROS1 fusions (ALK/ RET/ ROS1 panel). Four different sets of primers and probes are used in a single-tube (or vessel, well, chamber, compartment) assay to reduce the amount of sample needed to achieve measurable, reliable results. These four sets correspond to (i) ALK

(detected with one or more probes labeled with a first label), (ii) RET (detected with one or more probes labeled with a second label), (iii) ROS1 (detected with one or more probes labeled with a third label), and (iv) an internal control (detected with a probe labeled with a forth label). The labels can be selected from those disclosed herein and in some embodiments are distinguishable from one other. In the present example, ALK fusions are detected with a FAM-labeled probe, RET fusions are detected with a HEX-labeled probe, ROS1 fusions are detected with a JA270-labeled probe, and the internal control is detected with a Cy5.5-labeled probe.

The coverage of the highly multiplexed assay is shown in Table 1 with the fusion variant number indicated in parenthesis.

Table 1

Label	**Gene**	**Fusion**	**Coverage**	**Oligonucleotides**
FAM	ALK	EML4 exon 13-ALK exon 20 (V1) EML4 exon 20-ALK exon 20 (V2) EML4 exon 6a/b-ALK exon 20 (V3) EML4 exon 2-ALK exon 20 (V5) EML4 exon 18-ALK exon 20 (V8) KIF5B exon 17-ALK exon 20 (V6) KIF5B exon 24-ALK exon 20 (V7)	7 fusions 94% ALK fusions	15 primers 2 probes
HEX	RET	KIF5B exon 15-RET exon 12 (V1) KIF5B exon 16-RET exon 12 (V2) KIF5B exon 22-RET exon 12 (V3) KIF5B exon 23-RET exon 12 (V4) CCDC6 exon 1-RET exon 12 (V8) NCOA4 exon 6-RET exon 12 (V9)	6 fusions 97% RET fusions	
JA270	ROS1	CD74 exon 6-ROS1 exon 34 (V2) CD74 exon 6-ROS1 exon 32 (V1) EZR exon 10-ROS1 exon 34 (V10) TPM3 exon 8-ROS1 exon 35 (V13) SDC4 exon 4-ROS1 exon 34 (V5)	12 fusions 95% ROS1 fusions	11 primers 3 probes

		SDC4 exon 2-ROS1 exon 32 (V3) SDC4 exon 2-ROS1 exon 34 (V14) SDC4 exon 4-ROS1 exon 32 (V4) SLC34A2 exon 13-ROS1 exon 34 (V7) SLC34A2 exon 13-ROS1 exon 32 (V6) SLC34A2 exon 4-ROS1 exon 32 (V8) SLC34A2 exon 4-ROS1 exon 34 (V9) LRIG3 exon 16-ROS1 exon 35 (V11)		
CY5.5	IC	IC	N/A	2 primers 1 probe

The multiplex may include various gene fusion detection combinations, and in some embodiments, fewer fusions are assayed and detected. An example of an assay format for detection ALK and RET fusions is shown in Table 2. Fusions in ROS1 can be detected separately, or in a parallel assay, for example, as shown in Table 3.

Table 2

Label	**Gene**	**Fusion**	**Coverage**	**Oligonucleotides**
FAM	ALK	EML4 exon 13-ALK exon 20 (V1) EML4 exon 20-ALK exon 20 (V2) EML4 exon 6a/b-ALK exon 20 (V3) EML4 exon 2-ALK exon 20 (V5) EML4 exon 18-ALK exon 20 (V8) KIF5B exon 17-ALK exon 20 (V6) KIF5B exon 24-ALK exon 20 (V7)	7 fusions 94% ALK fusions	15 primers 2 probes
HEX	RET	KIF5B exon 15-RET exon 12 (V1) KIF5B exon 16-RET exon 12 (V2) KIF5B exon 22-RET exon 12 (V3) KIF5B exon 23-RET exon 12 (V4) CCDC6 exon 1-RET exon 12 (V8) NCOA4 exon 6-RET exon 12 (V9)	6 fusions 97% RET fusions	
CY5.5	IC	IC	N/A	2 primers 1 probe

Table 3

Label	Gene	Fusion	Coverage	Oligonucleotides
FAM	ROS1	CD74 exon 6-ROS1 exon 32 (V1) SDC4 exon 2-ROS1 exon 32 (V3) SDC4 exon 4-ROS1 exon 32 (V4) SLC34A2 exon 13-ROS1 exon 32 (V6) SLC34A2 exon 4-ROS1 exon 32 (V8)	12 fusions 95% ROS1 fusions	11 primers 3 probes
HEX	ROS1	CD74 exon 6-ROS1 exon 34 (V2) EZR exon 10-ROS1 exon 34 (V10) SDC4 exon 4-ROS1 exon 34 (V5) SLC34A2 exon 13-ROS1 exon 34 (V7) SLC34A2 exon 4-ROS1 exon 34 (V9) SDC4 exon 2-ROS1 exon 34 (V14)		
JA270	ROS1	TPM3 exon 8-ROS1 exon 35 (V13) LRIG3 exon 16-ROS1 exon 35 (V11)		
CY5.5	IC	IC	N/A	2 primers 1 probe

The oligonucleotides shown in Tables 4-6 can be selected for use in the assays. The first set of forward and reverse primers amplifies across EML4-ALK and KIF5B-ALK fusions. The primers are designated with the gene name (*e.g.* EML for EML4), exon (*e.g.*, 13 for exon 13), and designation (*e.g.*, F1 for Forward 1). The symbols <t_bb_dA>, <t_bb_dC>, <t_bb_dT>, <t_bb_dG> refer to p-tert butylbenzyl modified A, C, T, and G, respectively. Forward and reverse primers can be used in single pairs or in any combination to amplify different fusion products, as will be appreciated by one of skill in the art. In the present example, the number of oligonucleotides in the reaction was minimized, as indicated in Tables 1-3. The reverse primers in all reactions served as primers for the reverse transcriptase reactions.

Table 4: Oligonucleotides for use in amplification and detection of ALK fusions

Probe dye (for example)	Forward primer	SEQ ID NO	Sequence
FAM	EML13F1	1	ACACCTGGGAAAGGACCTAAA
	EML13F2	2	CACACCTGGGAAAGGACCTAAA
	EML13F3	3	CCACACCTGGGAAAGGACCTA
	EML13F4	4	CCACACCTGGGAAAGGACCT
	EML13F5	5	CCACACCTGGGAAAGGACC
	EML13F6	6	CCACACCTGGGAAAGGAC
	EML13F7	7	CCCACACCTGGGAAAGGAC
	EML13F8	8	GCCCACACCTGGGAAAGGA
	EML13F9	9	AGCCCACACCTGGGAAAG
	EML13F10	10	GAGCCCACACCTGGGAAA
	EML20F1	11	CTCGGGAGACTATGAAATATTGTACT
	EML20F2	12	TCGGGAGACTATGAAATATTGTACT
	EML20F3	13	CGGGAGACTATGAAATATTGTACT
	EML20F4	14	CTCGGGAGACTATGAAATATTGTAC
	EML20F5	15	ACTCGGGAGACTATGAAATATTGTA
	EML20F6	16	AACTCGGGAGACTATGAAATATTGTA
	EML20F7	17	TAACTCGGGAGACTATGAAATATTGTA
	EML20F8	18	TAACTCGGGAGACTATGAAATATTGT
	EML20F9	19	TAACTCGGGAGACTATGAAATATTGTA
	EML20F10	20	ACTCGGGAGACTATGAAATATTGTAC
	EML6F1	21	AAGCATAAAGATGTCATCATCAACCAA
	EML6F2	22	AGCATAAAGATGTCATCATCAACCAA
	EML6F3	23	GCATAAAGATGTCATCATCAACCAA
	EML6F4	24	CATAAAGATGTCATCATCAACCAAG
	EML6F5	25	GCATAAAGATGTCATCATCAACCAAG
	EML6F6	26	GCATAAAGATGTCATCATCAACCA
	EML6F7	27	GCATAAAGATGTCATCATCAACC
	EML6F8	28	AGCATAAAGATGTCATCATCAACC

	EML6F9	29	AAGCATAAAGATGTCATCATCAACC
	EML6F10	30	AAGCATAAAGATGTCATCATCAAC
	EML2F1	31	CTCAGTGAAAAAATCAGTCTCAAG
	EML2F2	32	CTCAGTGAAAAAATCAGTCTCAAGT
	EML2F3	33	TCAGTGAAAAAATCAGTCTCAAGTA
	EML2F4	34	TCAGTGAAAAAATCAGTCTCAAGTAA
	EML2F5	35	CAGTGAAAAAATCAGTCTCAAGTAAAG
	EML18F1	36	CAGCTCTCTGTGATGCGCTA
	EML18F2	37	CTCTCTGTGATGCGCTACT
	EML18F3	38	TCTCTGTGATGCGCTACTCAA
	EML18F4	39	GCTCTCTGTGATGCGCTAC
	EML18F5	40	CTGTGATGCGCTACTCAATAG
	KIF24F1	41	AGAAGAGGGCATTCTGCACA
	KIF24F2	42	GAGGGCATTCTGCACAGA
	KIF24F3	43	GAGGGCATTCTGCACAGAT
	KIF24F4	44	GAAGAGGGCATTCTGCACAG
	KIF24F5	45	GGGCATTCTGCACAGATTG
	KIF17F1	46	GAACTAGTCCAGCTTCGAGCA
	KIF17F2	47	TGAAGAACTAGTCCAGCTTCGA
	KIF17F3	48	CTAGTCCAGCTTCGAGCACAA
	KIF17F4	49	AAGAACTAGTCCAGCTTCGAG
	KIF17F5	50	GTCCAGCTTCGAGCACAAG
	Reverse primer		
	ALK20R1	52	GCTCTGCAGCTCCATCTG
	ALK20R2	53	GGCTCTGCAGCTCCATCT
	ALK20R3	54	GGGCTCTGCAGCTCCATC
	ALK20R4	55	GGGCTCTGCAGCTCCAT
	ALK20R5	56	GGGCTCTGCAGCTCCA
	ALK20R6	57	TGCAGCTCCATCTGCATGG
	ALK20R7	58	GCAGCTCCATCTGCATGG
	ALK20R8	59	CAGCTCCATCTGCATGGC
	ALK20R9	60	AGCTCCATCTGCATGGC
	ALK20R10	61	GCTCCATCTGCATGGCT

	ALK20R11	181	TGCAGCTCCATCTGCATGGCT TGCAGCTCCATCTGCATGG<t_bb_dC>T
	Probe		
	ALK20RP9_Q6	182	<DYE-Thr>CCGCCG<BHQ_2>GAAGCACCAGGAGC
	ALK20P4	183	<DYE-Thr>TACCGCC <BHQ_2>GGAAGCACCAGGAGCTGCA
	ALK20P5	184	<DYE-Thr>TACCGCC <BHQ_2>GGAAGCACCAGGAGCTGC
	ALK20P6	185	<DYE-Thr>TACCGCC <BHQ_2>GGAAGCACCAGGAGCTG
	ALK20P7	186	<DYE-Thr>TACCGCC <BHQ_2>GGAAGCACCAGGAGCT

Table 5: Oligonucleotides for use in amplification and detection of RET fusions

Probe dye (for example)	**Forward primer**	**SEQ ID NO**	**Sequence**
HEX	KIF15F1	83	GAATTGCTGTGGGAAATAATGATG
	KIF15F2	84	GAATTGCTGTGGGAAATAATGAT
	KIF15F3	85	ATTGCTGTGGGAAATAATGATGTAAAG
	KIF15F4	86	TTGCTGTGGGAAATAATGATGTAAAG
	KIF15F5	87	TGCTGTGGGAAATAATGATGTAAAG
	KIF15F6	88	GCTGTGGGAAATAATGATGTAAAG
	KIF15F7	89	GAATTGCTGTGGGAAATAATGATGTAAA
	KIF15F8	90	GAATTGCTGTGGGAAATAATGATGTAA
	KIF15F9	91	AATTGCTGTGGGAAATAATGATGTAAA
	KIF15F10	92	ATTGCTGTGGGAAATAATGATGTAAA
	KIF15F11	93	ATTGCTGTGGGAAATAATGATGTAA
	KIF15F12	94	AATTGCTGTGGGAAATAATGATGTA
	KIF15F13	95	ATTGCTGTGGGAAATAATGATGTA
	KIF15F14	96	GAATTGCTGTGGGAAATAATGATGTA
	KIF15F15	97	GAATTGCTGTGGGAAATAATGATGT
	KIF16F1	98	CATGTCAGCTTCGTATCTCTCAA

	KIF16F2	99	ATGTCAGCTTCGTATCTCTCAA
	KIF16F3	100	CATGTCAGCTTCGTATCTCTCA
	KIF16F4	101	GCATGTCAGCTTCGTATCTCTC
	KIF16F5	102	CATGTCAGCTTCGTATCTCTC
	KIF16F6	103	GCATGTCAGCTTCGTATCTCT
	KIF16F7	104	GCATGTCAGCTTCGTATCTC
	KIF16F8	105	CAGCATGTCAGCTTCGTATC
	KIF16F9	106	TAGCAGCATGTCAGCTTCGTA
	KIF16F10	107	AGCAGCATGTCAGCTTCG
	KIF22F1	108	AGGACCTGGCTACAAGAGTTAA
	KIF22F2	109	GGACCTGGCTACAAGAGTTAA
	KIF22F3	110	GGACCTGGCTACAAGAGTTAAA
	KIF22F4	111	AGGACCTGGCTACAAGAGTTAAA
	KIF22F5	112	AGGACCTGGCTACAAGAGTTA
	KIF22F6	113	GGACCTGGCTACAAGAGTTA
	KIF22F7	114	GACCTGGCTACAAGAGTTAAAAAG
	KIF22F8	115	ACCTGGCTACAAGAGTTAAAAAG
	KIF22F9	116	AGGACCTGGCTACAAGAGTT
	KIF22F10	117	GGACCTGGCTACAAGAGTT
	KIF23F1	118	TTGAACAGCTCACTAAAGTGCACAAA
	KIF23F2	119	TGAACAGCTCACTAAAGTGCACAAA
	KIF23F3	120	GAACAGCTCACTAAAGTGCACAAA
	KIF23F4	121	AACAGCTCACTAAAGTGCACAAA
	KIF23F5	122	ACAGCTCACTAAAGTGCACAAA
	KIF23F6	123	GAACAGCTCACTAAAGTGCACAA
	KIF23F7	124	AACAGCTCACTAAAGTGCACAA
	KIF23F8	125	ACAGCTCACTAAAGTGCACAA
	KIF23F9	126	GAACAGCTCACTAAAGTGCACA
	KIF23F10	127	AACAGCTCACTAAAGTGCACA
	KIF23F13	187	TTGAACAGCTCACTAAAGTGCA
	CCDC1F1	128	TGCGCAAAGCCAGCGT
	CCDC1F2	129	CGACCTGCGCAAAGCCA
	CCDC1F3	130	GACCTGCGCAAAGCCAG

	CCDC1F4	131	CCTGCGCAAAGCCAGC
	CCDC1F5	132	ACCTGCGCAAAGCCAGC
	CCDC1F6	133	CTGCGCAAAGCCAGCGT
	CCDC1F7	134	GACCTGCGCAAAGCCAGC
	CCDC1F8	135	CGACCTGCGCAAAGCC
	CCDC1F14	188	CAAAGCCAGCGTGACCA
	NCO6F1	136	TGTATCTCCATGCCAGAGCAG
	NCO6F2	137	GTATCTCCATGCCAGAGCAG
	NCO6F3	138	CTGTATCTCCATGCCAGAGCA
	NCO6F4	139	GCTGTATCTCCATGCCAGAG
	NCO6F5	140	GGCTGTATCTCCATGCCAGA GGCTGTATCTCCATGCCAG<t_bb_dA>
	NCO6F6	141	GGCTGTATCTCCATGCCAG
	NCO6F7	142	AGGCTGTATCTCCATGCCA
	NCO6F8	143	GAGGCTGTATCTCCATGCCA
	NCO6F9	144	AGAGGCTGTATCTCCATGC
	NCO6F10	145	GAGAGGCTGTATCTCCATGC
	Reverse primer		
	RET12R1	161	AGAGTTTTTCCAAGAACCAAGTTCT
	RET12R2	162	CTAGAGTTTTTCCAAGAACCAAGTTCT
	RET12R3	163	CTAGAGTTTTTCCAAGAACCAAGTTC
	RET12R4	164	CTAGAGTTTTTCCAAGAACCAAGTT
	RET12R5	165	CTAGAGTTTTTCCAAGAACCAAGT
	RET12R6	166	CTAGAGTTTTTCCAAGAACCAAG
	RET12R7	167	TAGAGTTTTTCCAAGAACCAAGTTCTT
	RET12R8	168	GAGTTTTTCCAAGAACCAAGTTCTT
	RET12R9	169	AGTTTTTCCAAGAACCAAGTTCTT
	RET12R10	170	GTTTTTCCAAGAACCAAGTTCTT
	RET12R11	171	TAGAGTTTTTCCAAGAACCAAGTTCT
	RET12R12	172	TAGAGTTTTTCCAAGAACCAAGTTC
	RET12R13	173	AGAGTTTTTCCAAGAACCAAGTTC
	RET12R14	174	AGAGTTTTTCCAAGAACCAAGTT
	RET12R15	175	AGAGTTTTTCCAAGAACCAAGT

	RET12R16	176	CTCCTAGAGTTTTTCCAAGAACCAA
	RET12R17	177	CTCCTAGAGTTTTTCCAAGAACCA
	RET12R18	178	TCCTAGAGTTTTTCCAAGAACCAA
	RET12R19	179	CCTAGAGTTTTTCCAAGAACCAA
	RET12R20	180	GAGTTTTTCCAAGAACCAAGTTCT
	Probe		
	RET12P3_HEX	189	<DYE_Thr>ATCCAAA<BHQ_2>GTGGGAATT CCCTCGGAAGAAC
	RET12P4_HEX	190	< DYE_Thr>CCAAAGT<BHQ_2>GGGAATT CCCTCGGAAGAAC
	RET12P8_HEX	191	< DYE_Thr>TCCAAAG<BHQ_2>TGGGAATT CCCTCGGAAGAA
	RET12P14_HEX	192	< DYE_Thr>CCAAAGT<BHQ_2>GGGAATT CCCTCGGAAGAACTT
	RET12P18_HEX	193	< DYE_Thr>TCCAAAG<BHQ_2>TGGGAATT CCCTCGGAAGAACTT
	RET12P13_HEX	194	< DYE_Thr>ATCCAAA<BHQ_2>GTGGGAATT CCCTCGGAAGAACTT

Table 6: Oligonucleotides for use in amplification and detection of ROS1 fusions

Probe dye (for example)	**Forward primer**	**SEQ ID NO**	**Sequence**
JA270	CD74ex6F2	195	CACTGACGCTCCACCGAA
	CD74ex6F1	196	AAGCCCACTGACGCTCCA
	CD74ex6F3	197	ACTGACGCTCCACCGAAA
	SDC4ex2F1	198	GAGCTGTCTGGCTCTGG<t_BB_dA>
	SDC4ex2F2	199	TGTCTGGCTCTGGAGATCT TGTCTGGCTCTGGAGAT<t_bb_dC>T
	SDC4ex4F1	200	TTGAGAGAACGGAGGTCCT
	SDC4exF2	201	TGAGAGAACGGAGGTCCT
	SDC4ex4F3	202	TTGAGAGAACGGAGGTCCTG
	SLC34A2ex13F1	203	ATAACCATTAGCAGAGAGGCT

	SLC34A2ex13F2	204	AACCATTAGCAGAGAGGCTCA
	SLC34A2ex13F3	205	ATAACCATTAGCAGAGAGGCT
	SLC34A2ex4F1	206	AGTAGCGCCTTCCAGCT
	SLC34A2ex4F2	207	GCCTTCCAGCTGGTTGGA
	EZRex10F2	208	GAAGACAAAGAAGGCAGAGAGA
	LRIG3ex16F1	209	TTCTTACCACAACATGACAGTAGT
	LRIG3ex16F2	210	TCTTACCACAACATGACAGTAGTG
	TPM3ex8F1	211	GAAAAGACAATTGATGACCTGGA GAAAAGACAATTGATGACCTGG<t_BB_dA>
	TPM3ex8F5	212	AAGCTGGAAAAGACAATTGATGAC
	Reverse primer		
	ROS1ex32R1	213	GTATTGAATTTTTACTCCCTTCTAGTAATTTG
	ROS1ex32R2	214	GTATTGAATTTTTACTCCCTTCTAGTAATTT
	ROS1ex32R3	215	GTATTGAATTTTTACTCCCTTCTAGTAATT
	ROS1ex35R1	216	TATAAGCACTGTCACCCCTT
	ROS1ex35R2	217	ATAAGCACTGTCACCCCTT
	ROS1ex35R3	218	TATAAGCACTGTCACCCCT
	ROS1ex35R4	219	CTTTGTCTTCGTTTATAAGCACTGTCA
	ROS1ex35R5	220	AACTCTTTGTCTTCGTTTATAAGCACTGT
	ROS1ex35R6	221	AGCCAACTCTTTGTCTTCGTTTATAAGCA
	ROS1ex34LArev1	222	CAGTGGGATTGTAACAACCAGAAAT
	ROS1ex34LArev2	223	GTCAGTGGGATTGTAACAACCAGA
	ROS1ex34LArev3	224	GTCAGTGGGATTGTAACAACCA
	ROS1ex34LArev4	225	CAGTGGGATTGTAACAACCAGAAA
	ROS1ex34LArev5	226	CAGTGGGATTGTAACAACCAGAA
	Probe		
	ROS1EX32P2	227	< DYE_Thr>TGGAGTCCCAAA<BHQ_2> TAAACCAGGCATTCCCA
	ROS1EX34P1	228	< DYE_Thr>TGATTTTTGGAT<BHQ_2> ACCAGAAACAAGTTTCATAC
	ROS1EX32P3	229	<DYE_Thr>TGGAGTC<BHQ_2> CCAAATAAACCAGGC<t_BB_dA>TTCCCA

	ROS1EX34P3	230	<DYE_Thr>TGATTTT<BHQ_2> TGGATACCAGAAACAAGTTTCATAC
	ROS1EX35P1	51	< DYE_Thr>TCTGGCATAGAA<BHQ_2> GATTAAAGAATCAAAAAAGTGCCAAG

We have tested this method using RNA from EML4-ALK positive cell lines NCI-H2228 and EML4-ALK Fusion Variant 1 cell line from Horizon Discovery, CCDC6-RET cell line LC2AD, as well as from NSCLC formalin fixed paraffin embedded tissue (FFPET) and plasma specimens.

In the case of plasma, we extracted cfRNA using the Roche High Pure FFPET RNA extraction kit with MagNA Pure Lysis Buffer and Esperase enzyme. Because the yield of cfRNA is too low to be measured accurately, we input a fixed volume (1/24 of total) of the extracted plasma cfRNA into the qRT-PCR.

The reaction conditions were as follows. For each reaction, 25 uL of input RNA was added to a RT-PCR reaction mix comprising forward and reverse primers, labeled probe, buffer, dUTP, dTTP, dATP, dGTP, UNG, and Z05 enzyme to a final volume of 50uL. The reactions were run in multiplex, each with primers and probes specific for every fusion variant indicated in Table 1.

Results were confirmed using a Next Generation Sequencing assay that detects the fusion variants covered in the qRT-PCR assay.

Maximum Ct (threshold cycle) was set at 38, meaning that a signal must be detectable over background within a Ct of 38. Data is shown in Figures 1A, 1B, 1C, and 1D. The input RNA for each reaction was known to have the indicated fusion variant. Each reaction was repeated three times.

Figure 1A shows that each ALK fusion variant is detectable at 50 copies. Figure 1B shows that each RET fusion variant is detectable at 50 copies. Figure 1C shows that each ROS1

fusion variant is detectable at 50 copies. Figure 1D shows that the reaction efficiency and input was equivalent, as indicated by the Internal Control Ct's.

B. Example 2: Sensitivity of ALK and RET Fusions in Titered Transcripts

We tested the multiplex qRT-PCR for the limit of detection of the ALK and RET fusion variants shown in Example 1, Table 1. We tested the multiplex assay by titering ALK or RET fusion positive transcripts into 0.1 ng Universal Human RNA (UHR) at 250, 100, 50, or 25 copies. The amplification and detection reactions were repeated 3 times.

As shown in Figure 2, all of the ALK and RET fusion variants tested was detectable down to 25 copies.

C. Example 3: Linearity Studies and Further Limit of Detection (LOD) Studies

Further studies were carried out to determine the linearity of detection for ALK, RET, and ROS1 fusions, as shown and described in Figures 3-5.

Sensitivity, or Limit of Detection (LOD) studies are shown and described in Figures 6-8. The LOD for each assay is shown in Tables 7 and 8. All 7 ALK, 6 RET, and 13 ROS1 fusion variants are detectable down to less than 10 copies. The predominant fusion variants are marked with an *.

Table 7

Fusion	**Hit Rate**	**LOD for 95% Probability**
E13:A20*	12/12 all levels tested	<6.25 copies
E20:A20*	12/12 all levels tested	<6.25 copies
E6:A20*	12/12 all levels tested	<6.25 copies
E2:A20	11/12 at 6.25 copies	6.45 copies
K17:A20	12/12 at 6.25 copies (11/12 at 12.5 copies)	4.78 copies
K24:A20	12/12 all levels tested	<6.25 copies
E18:A20	12/12 all levels tested	<6.25 copies

K15:R12*	11/12 at 6.25 copies	6.45 copies
K16:R12*	11/12 at 6.25 copies	6.45 copies
K22:R12	12/12 all levels tested	<6.25 copies
K23:R12	11/12 at 6.25 copies	6.45 copies
C1:R12*	12/12 all levels tested	<6.25 copies
N6:R12	12/12 all levels tested	<6.25 copies

Table 8

Fusion variant	**Hit Rate**	**LOD for 95% Probability**
C6:R32	12/12 all levels tested	<6.25 copies
C6:R34*	12/12 all levels tested	<6.25 copies
SD2:R32	11/12 at 6.25 and 12.5 copies	11.07 copies
SD4:R34	12/12 all levels tested	<6.25 copies
SL13:R32	12/12 all levels tested	<6.25 copies
SL13:R34	12/12 all levels tested	<6.25 copies
SL4:R32	12/12 all levels tested	<6.25 copies
SL4:R34	11/12 at 6.25 copies	6.45 copies
E10:R34*	11/12 at 6.25 copies	6.45 copies
L16:R35	11/12 at 12.5 copies; 12/12 at 6.25 copies	4.78 copies
T8:R35	12/12 all levels tested	<6.25 copies
SD2:R34	12/12 all levels tested	<6.25 copies

CLAIMS

1. A multiplex assay composition comprising:

   A. at least one primer set and probe that specifically amplify and detect at least one ALK fusion gene;

   B. at least one primer set and probe that specifically amplify and detect at least one RET fusion gene; and

   C. a primer set and probe that specifically amplify and detect an internal control.

2. The composition of claim 1 further comprising

   D. at least one primer set and probe that specifically amplify and detect at least one ROS1 fusion gene;

3. The composition of any one of claims 1 and 2, wherein the at least one ALK fusion gene is selected from the group consisting of: EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20.
4. The composition of any one of claims 1 to 3, wherein A includes at least one primer set and probe that amplify and detect more than 2 ALK fusion genes.
5. The composition of any one of claims 1 to 4, wherein A includes at least one primer set and probe that amplify and detect EML4 exon 13-ALK exon 20, EML4 exon 20-ALK exon 20, EML4 exon 6a/b-ALK exon 20, EML4 exon 2-ALK exon 20, EML4 exon 18-ALK exon 20, KIF5B exon 17-ALK exon 20, and KIF5B exon 24-ALK exon 20.
6. The composition of any one of claims 1 to 5, wherein the at least one RET fusion gene is selected from the group consisting of: KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12.
7. The composition of any one of claims 1 to 6, wherein B includes at least one primer set and probe that amplify and detect more than 2 RET fusion genes.

8. The composition of any one of claims 1 to 7, wherein B includes at least one primer set and probe that amplify and detect KIF5B exon 15-RET exon 12, KIF5B exon 16-RET exon 12, KIF5B exon 22-RET exon 12, KIF5B exon 23-RET exon 12, CCDC6 exon 1-RET exon 12, and NCOA4 exon 6-RET exon 12.
9. The composition of any one of claims 2 to 8, wherein the at least one ROS1 fusion gene is selected from the group consisting of: CD74 exon 6-ROS1 exon 34, CD74 exon 6-ROS1 exon 32, EZR exon 10-ROS1 exon 34, TPM3 exon 8-ROS1 exon 35, SDC4 exon 4-ROS1 exon 34, SDC4 exon 2-ROS1 exon 34, SDC4 exon 2-ROS1 exon 32, SDC4 exon 4-ROS1 exon 32, SLC34A2 exon 13-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 32v2, SLC34A2 exon 4-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 35, and LRIG3 exon 16-ROS1 exon 35.
10. The composition of any one of claims 2 to 9, wherein D includes at least one primer set and probe that amplify and detect more than 2 ROS1 fusion genes.
11. The composition of any one of claims 2 to 10, wherein D includes at least one primer set and probe that amplify and detect CD74 exon 6-ROS1 exon 34, CD74 exon 6-ROS1 exon 32, EZR exon 10-ROS1 exon 34, TPM3 exon 8-ROS1 exon 35, SDC4 exon 4-ROS1 exon 34, SDC4 exon 2-ROS1 exon 32v2, SDC4 exon 2-ROS1 exon 32, SLC34A2 exon 13-ROS1 exon 34, SLC34A2 exon 13-ROS1 exon 32v2, SLC34A2 exon 4-ROS1 exon 32, SLC34A2 exon 4-ROS1 exon 35, and LRIG3 exon 16-ROS1 exon 35.
12. The composition of any one of claims 1 to 11, further comprising a thermostable DNA polymerase.
13. The composition of any one of claims 1 to 12, further comprising reverse transcriptase.
14. The composition of any one of claims 1 to 13, further comprising a biological sample from an individual.
15. The composition of claim 14, wherein the biological sample includes RNA from plasma.

16. A method of identifying an individual with cancer comprising:

(a) contacting a biological sample from the individual with the composition of any one of claims 1-13;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining that at least one fusion gene is present if a fusion gene is detected in step (b);

(d) whereby the presence of at least one fusion gene in said individual's sample indicates sensitivity of said individual to a kinase inhibitor therapy if at least one fusion gene is present.

17. A method of determining the likelihood of response of an individual with cancer to kinase inhibitor therapy comprising:

(a) contacting a biological sample from the individual with the composition of any one of claims 1-13;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining that at least one fusion gene is present if a fusion gene is detected in step (b); and

(d) determining that the individual will likely respond to the kinase inhibitor therapy.

18. The method of claim 17, wherein the kinase inhibitor therapy is selected from the group consisting of alectinib, crizotinib, ceritinib, lorlatinib, brigatinib, cabozantinib, apatinib, vandetanib, ponatinib, lenvatinib, DS6051b, or a variant thereof.

19. A method for determining the presence of at least one fusion gene in a biological sample from an individual with cancer comprising:

(a) contacting a biological sample from the individual with the composition of any one of claims 1-13;

(b) carrying out amplification and detection under conditions that allow formation and detection of an amplification product in the presence of at least one fusion gene in the biological sample;

(c) determining the presence of at least one fusion gene if a fusion gene is detected in step (b).

20. The method of any one of claims 16-19, wherein the biological sample includes DNA or RNA.

21. The method of any one of claims 16-19, wherein the biological sample is RNA from plasma of the individual.

22. The method of any one of claims 16-19, wherein the amplification and detection are carried out using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

F. Hoffmann-La Roche AG

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

FIGURE 1A: ALK

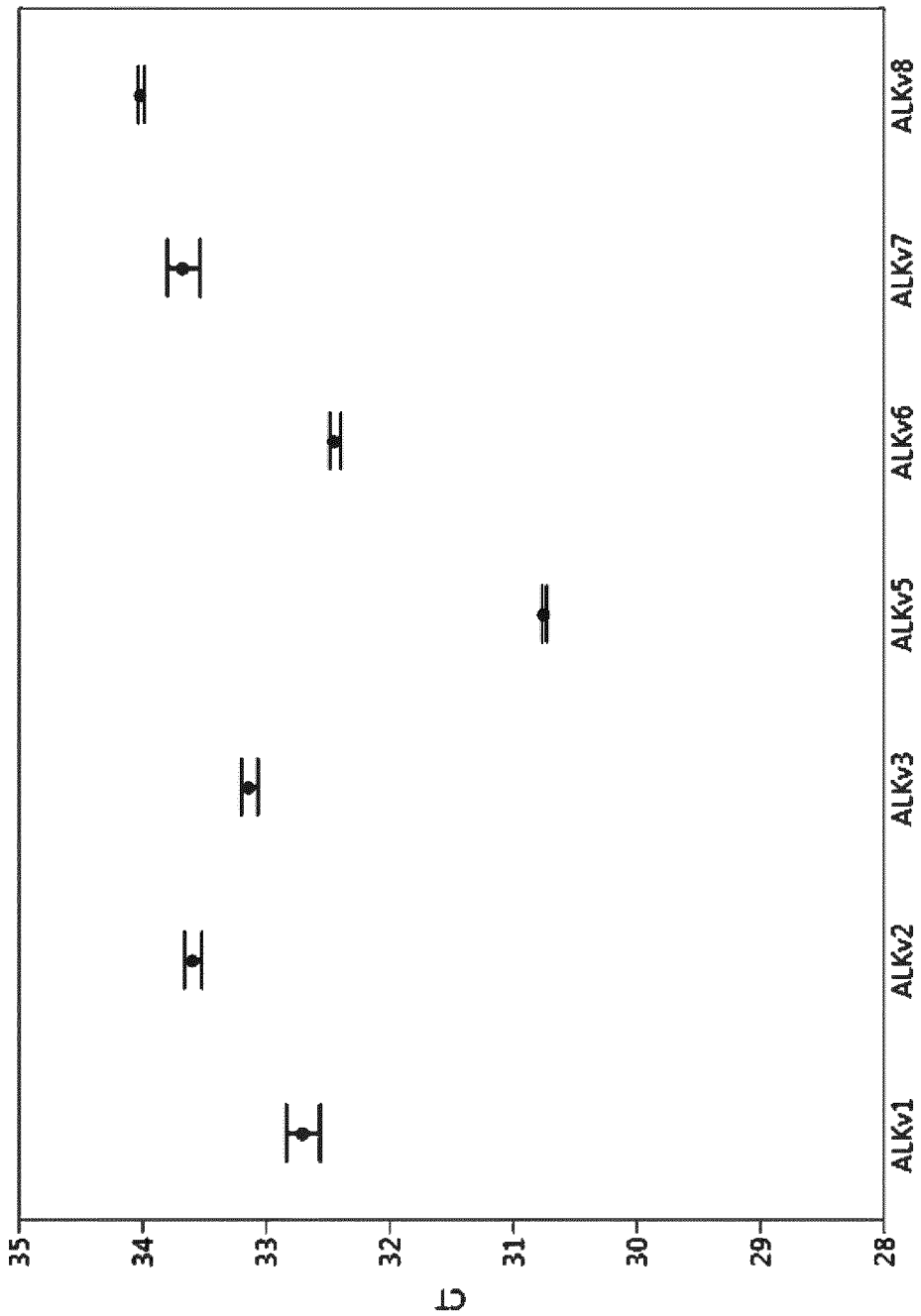


FIGURE 1B: RET

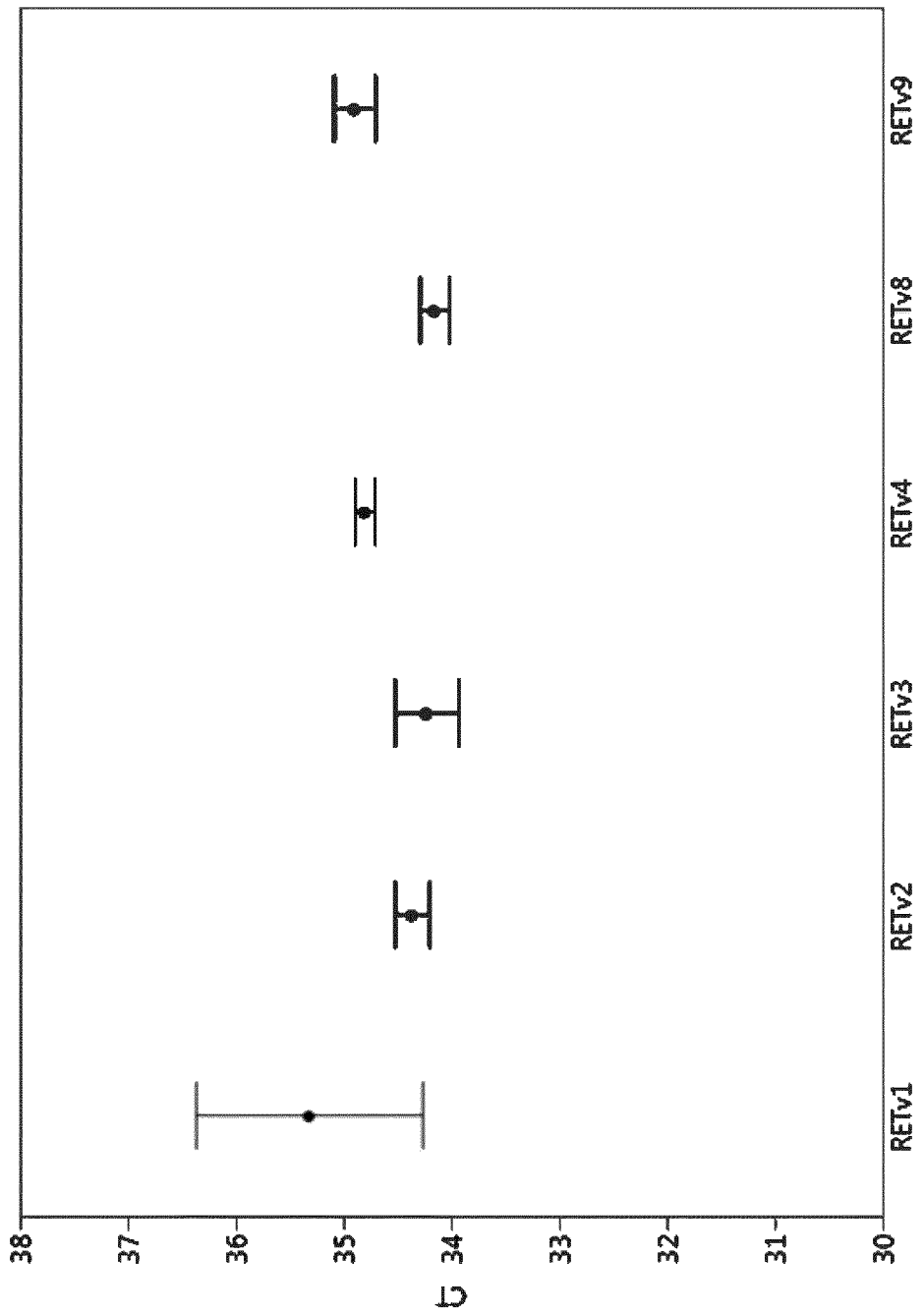
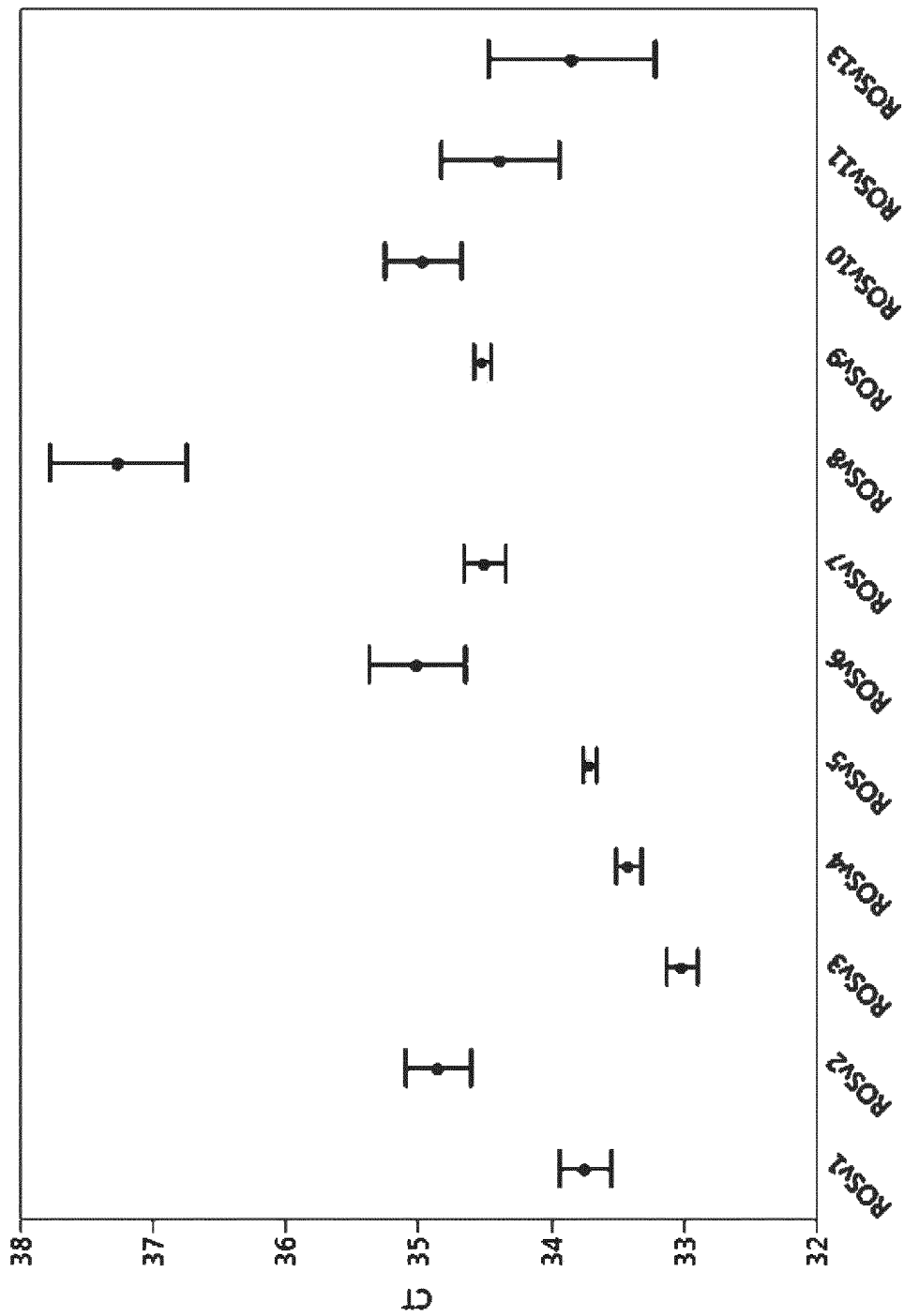


FIGURE 1C: ROS1



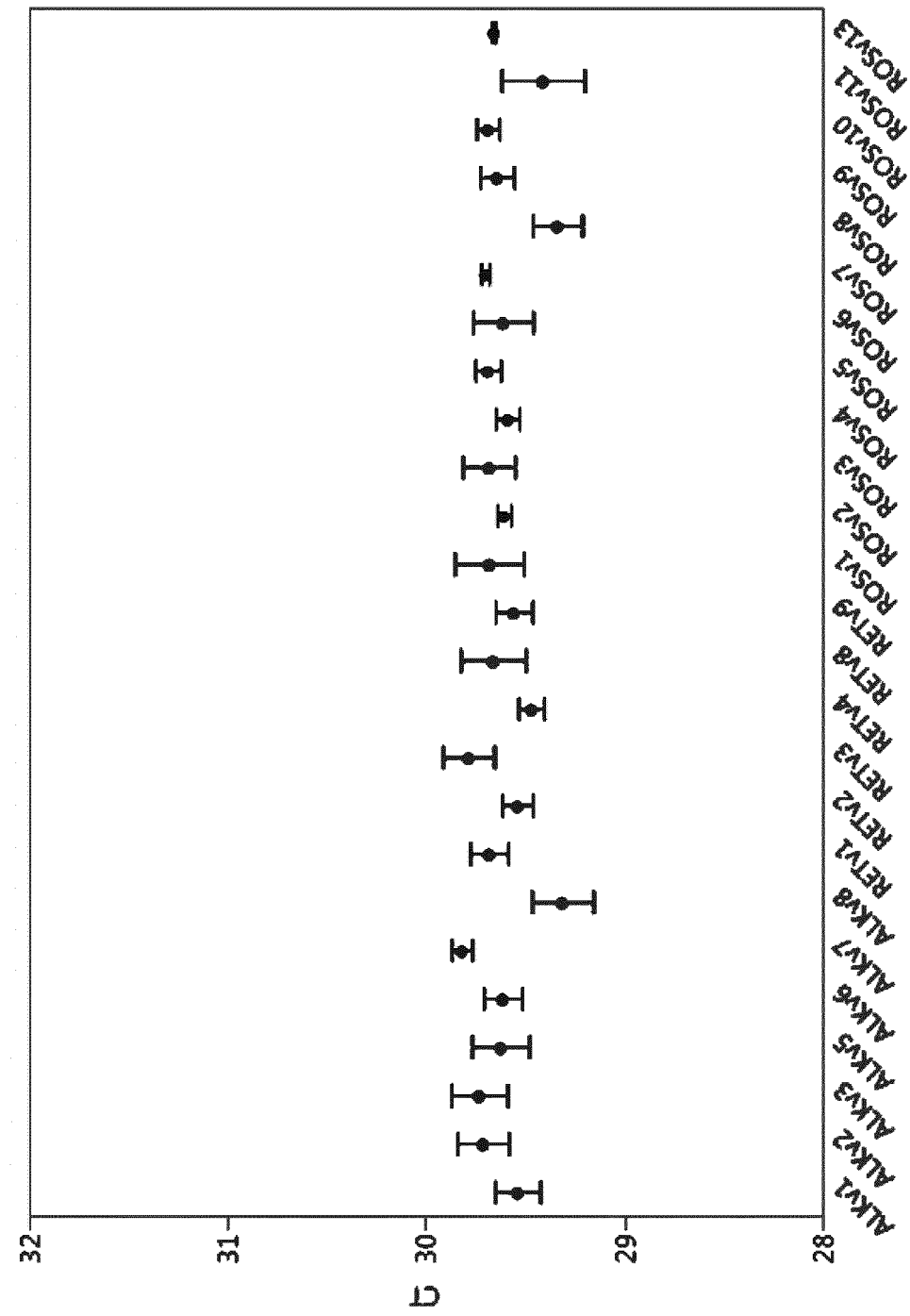
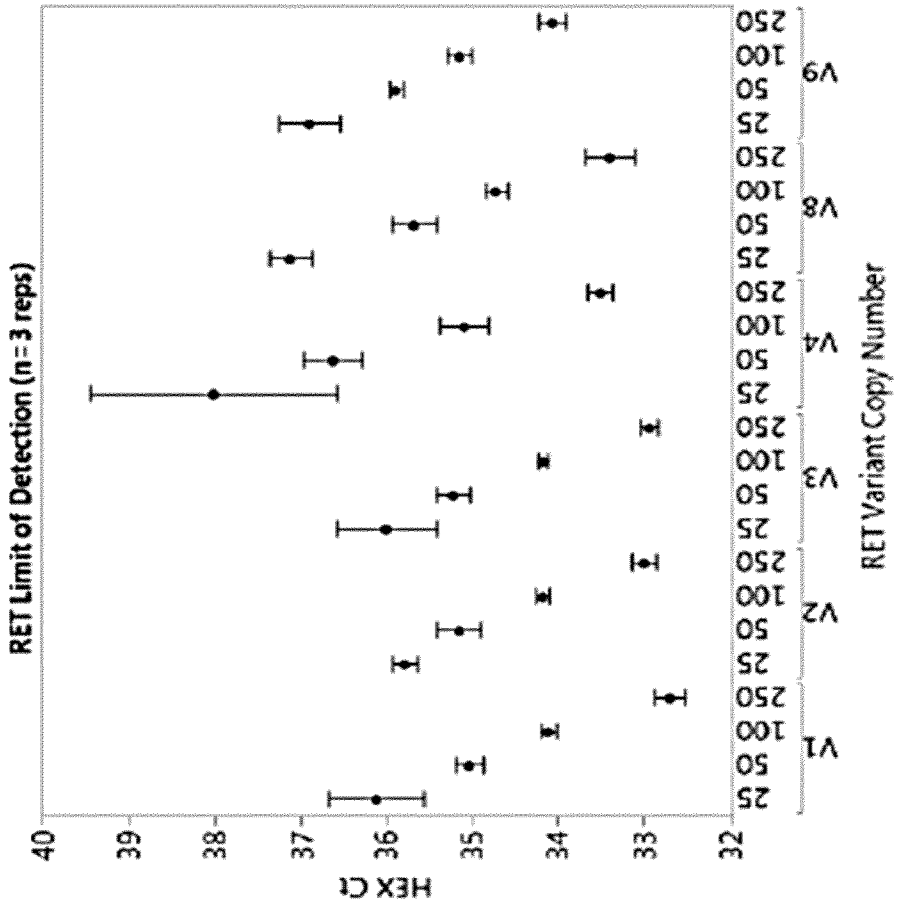


FIGURE 1D: Internal Control

FIGURE 2


RET Limit of Detection (n = 3 reps)
HEX Ct
40
39
38
37
36
35
34
33
32
V1
V2
V3
V4
V8
V9
25
50
100
250
RET Variant Copy Number

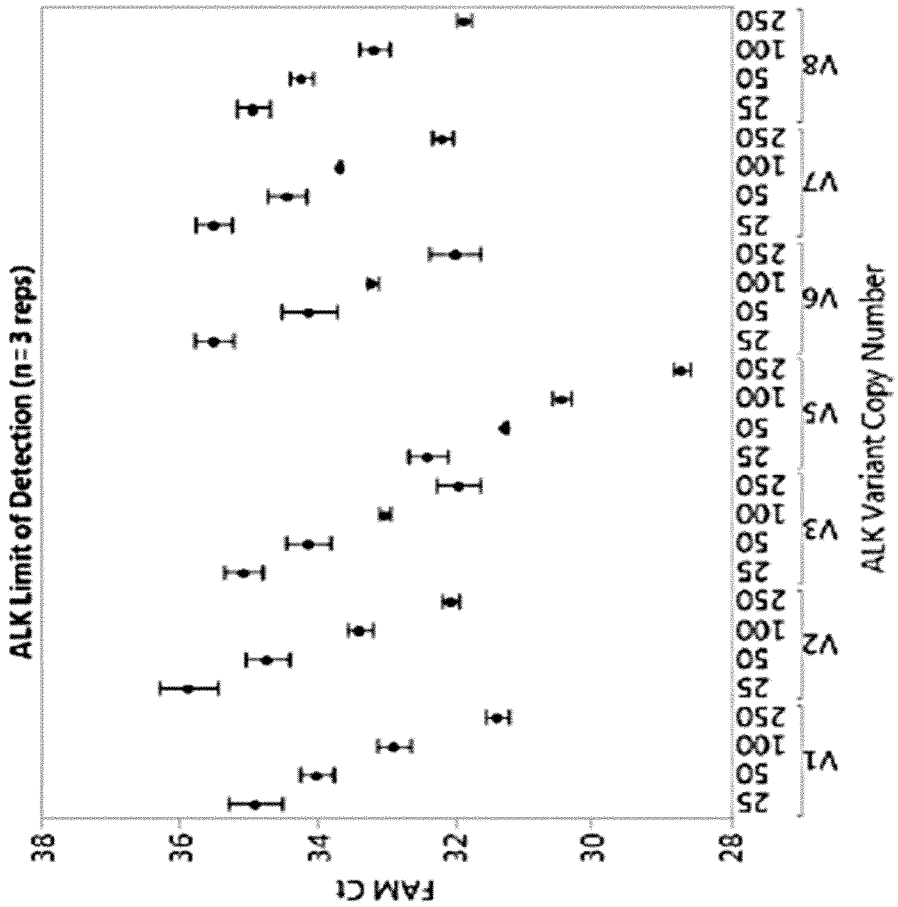
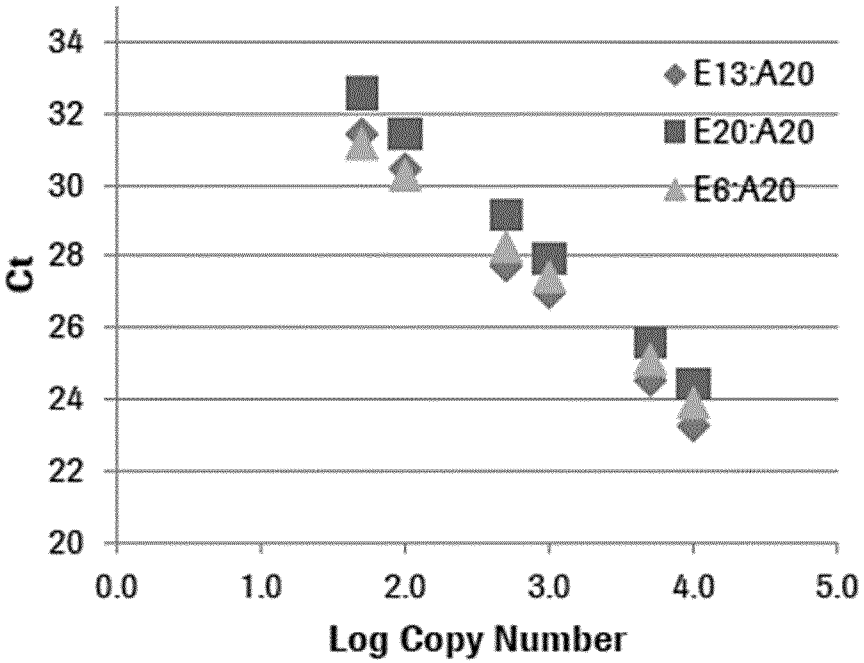

ALK Limit of Detection (n = 3 reps)
FAM Ct
38
36
34
32
30
28
V1
V2
V3
V5
V6
V7
V8
25
50
100
250
ALK Variant Copy Number

Figure 3

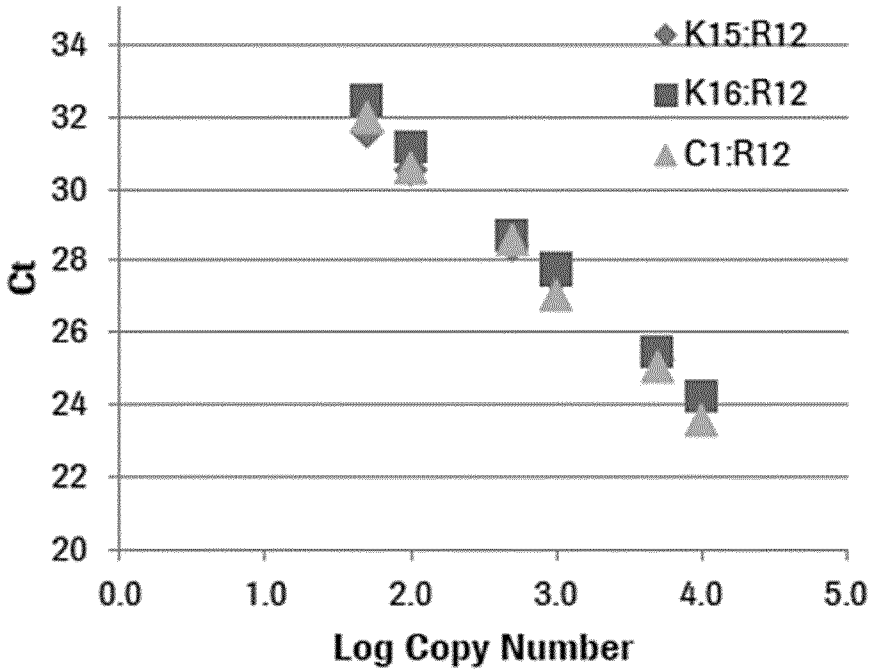
- Each fusion RNA transcript tested in 0.5ng UHR background
  - 5 levels spiked into UHR: 10000, 5000, 1000, 500, 100, and 50
  - 3 replicates per level; input copy number estimated by ddPCR
- Representative ALK variants shown



Fusion	Linearity Slope
E13:A20	-3.52
E20:A20	-3.51
E6:A20	-3.11
E18:A20	-3.43
E2:A20	-3.51
K17:A20	-3.57
K24:A20	-3.59

Figure 4

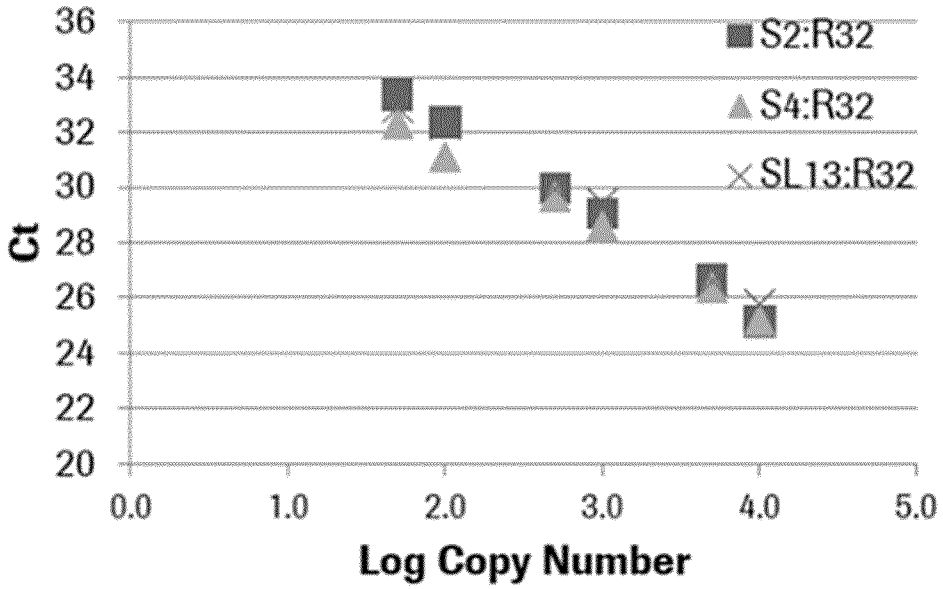
- Each fusion RNA transcript tested in 0.5ng UHR background
  - 5 levels spiked into UHR: 10000, 5000, 1000, 500, 100, and 50
  - 3 replicates per level; input copy number estimated by ddPCR
- Representative RET variants shown



Fusion	Linearity Slope
K15:R12	-3.18
K16:R12	-3.49
K22:R12	-3.26
K23:R12	-3.28
C1:R12	-3.54
N6:R12	-3.28

Figure 5

- Each fusion RNA transcript tested in 0.5ng UHR background
  - 5 levels spiked into UHR: 10000, 5000, 1000, 500, 100, and 50
  - 3 replicates per level; input copy number estimated by ddPCR
- Representative ROS1 variants shown



Fusion	Linearity Slope
C6:R32	-3.25
S2:R32	-3.50
S4:R32	-3.00
SL13:R32	-3.18
SL4:R32	-3.02
C6:R34	-3.02
S4:R34	-3.08
SL13:R34	-3.14
SL4:R34	-2.93
E10:R34	-3.25
S2:R34	-3.33
T8:R35	-3.27
L16:R35	-3.37

Figure 6

- Each fusion RNA transcript tested in 0.5ng UHR background
  - UHR background increased to test assay robustness; previously tested at 0.1ng
  - 5 levels spiked into UHR: 100, 50, 25, 12.5 and 6.25 copies per PCR rxn
  - 12 replicates per level; input copy number estimated by ddPCR
- Representative ALK variant shown

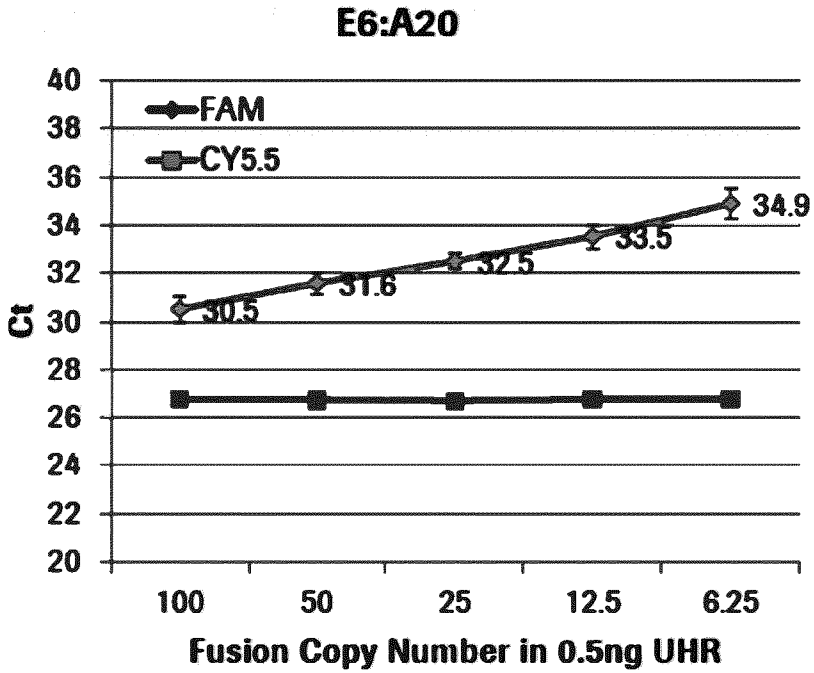


Figure 7

- Each fusion RNA transcript tested in 0.5ng UHR background
  - UHR background increased to test assay robustness; previously tested at 0.1ng
  - 5 levels spiked into UHR: 100, 50, 25, 12.5 and 6.25 copies per PCR rxn
  - 12 replicates per level; input copy number estimated by ddPCR
- Representative RET variant shown

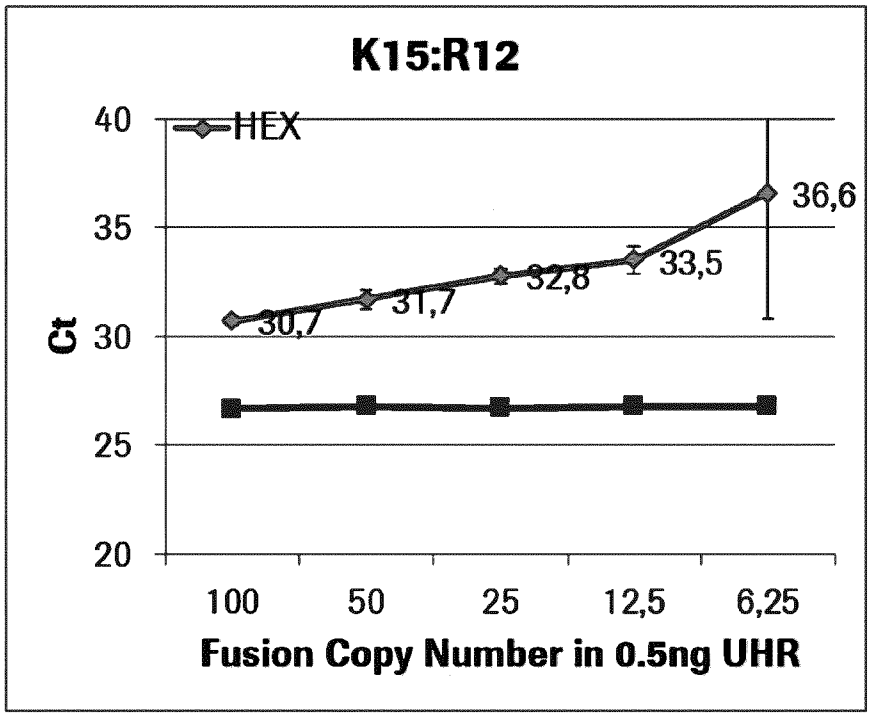


Figure 8

- Each fusion RNA transcript tested in 0.5ng UHR background
  - UHR background increased to test assay robustness; previously tested at 0.1ng
  - 5 levels spiked into UHR: 100, 50, 25, 12.5 and 6.25 copies per PCR rxn
  - 12 replicates per level; input copy number estimated by ddPCR
- Representative ROS1 variant shown