Detection of clinically relevant gene fusions with the HTG EdgeSeq ALK*Plus* Assay EU

Introduction

Numerous mutations have been shown to drive oncogenesis in non-small cell lung cancer (NSCLC). Measuring all of these events with standard methods is difficult given the small amount of material typically collected in the standard needle core biopsy or similar tissue collection method.

The HTG EdgeSeq ALK*Plus* Assay EU provides a next-generation sequencing (NGS)-based assay that measures mRNA gene rearrangement events in the ALK, ROS1, RET, and NTRK1 genes, activating mutations in HER2 and over-expression of MET. Using only a single section of formalin-fixed, paraffin-embedded (FFPE) lung tumor tissue, the HTG EdgeSeq ALK*Plus* Assay EU consolidates assessment of these mutations into a single assay, preserving valuable tissue for future research.

The RNA extraction-free assay is automated using the HTG EdgeSeq system. Data and information stored in the HTG EdgeSeq processor can be accessed by logging on in one of two modes: IVD mode or research use only (RUO) mode. When logged on in IVD mode laboratories can assess ALK status. When logged-on in RUO mode laboratories can access additional biomarker data for ALK[1], ROS1, RET, NTRK1, HER2 and cMET. This all-in-one test is available for use on the Illumina MiSeqDx and Illumina MiSeq next-generation sequencers.

Background

Gene fusions and rearrangements derive from a series of DNA-level events which cause two previously separate, distinct genes to be brought together forming a novel, new mRNA transcript. This novel protein may possess some, all, or none of the characteristics of the two parental genes. Gene fusions have been described throughout the scientific literature, however, the most commonly studied examples are those associated with human oncogenesis.

Perhaps the best described example of a fusion gene driving an oncogenic event in solid tumors is the anaplastic lymphoma kinase gene or ALK gene[3,4,5,6,7,8]. The expression of this gene, will stimulate cell proliferation through the JAK/STAT pathway, which in turn flows through the mTOR pathway of cell proliferation stimulation[9].

Expression of the the ALK gene, which encodes a kinase domain at its 3' end, is uncommon in most tissues, and is especially rare in non-hematopoietic lineages. Numerous DNA-level rearrangements, however, causes the ALK 3' kinase domain to be fused to a gene exhibiting much higher levels of expression, bypassing the tight gene expression regulation normally placed on this gene. The over-expression of the 3' kinase domain causes over-activation of the JAK/STAT[10] pathway, in turn causing uncontrolled cell proliferation, a hallmark of cancer.

Many of other clinically relevant gene fusions follow a similar pattern where a tightly regulated gene is dysregulated due to the DNA rearrangement event. Examples include MYC[11], ROS1[12], NTRK[13], and FGFR[14] gene fusions described in the scientific literature.

In the case of ALK, a drug which inhibits the functionof the 3' kinase domain has been developed, offering a highly-specific treatment option to patients with tumors driven by ALK expression. Known generically as crizotinib[15,16,17], the drug is administered to the 3-5% of patients whose tumors tes ALK positive. This targeted therapy has proven more effective on ALK positive tumors than standard therapies.

An interesting feature of crizotinib and other ALK inhibitors (e.g. cabozatinibalectinib and ceretinib) is that they also inhibit the function of kinases of similar structure to ALK, including the kinases activity of ROS1[12], RET[18,19], and NTRK[13]. While patients with tumors driven by these fusion events are less prevalent than ALK, there is a high likelihood that these patients will also benefit from therapeutic approaches incorporating these drugs. Recent NCCN guideline changes include assessing a patient's fusion status for many of these these events as well.

The most common laboratory testing methodology for fusion assessment utilizes two-color, break-apart FISH testing. This approach requires a significant amount of tissue (1 section per fusion assessed) and requires a highly variable, manual interpretation of the stained slide.

In addition to the previously described fusion genes, two other related markers are emerging as potential drivers of oncogenesis: cMET amplification and HER2 exon 20 insertions.

cMET is a well-studied marker of primary tumor to metastatic tumor conversion, however, it has recently been noted that overexpression of MET in primary tumors can itself be an oncogenic driver or resistance mechanism to other

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therapies. Many of the kinase inhibitors currently on market, including crizotinib and cabozatinib were originally developed to target cMET[20,21,22], and therefore have high affinity for this related kinase protein. Patients with primary tumors driven by cMET are likely to benefit from therapies utilizing the aforementioned drugs.

HER2 (ERBB2) is also a well-studied marker, especially in the context of breast cancer. Gene amplifications have a well-established role in driving approximately 35 to 40% of primary breast tumors. In lung and other solid tumors, HER2 amplification is extremely rare, however a small subset (2% to 4%) of lung tumors express a mutated, non-amplified form of HER2 which, due to a small insertion of sequence, becomes constitutively active in stimulating cell proliferation[23,24,25]. Recent studies have shown that these patients will respond to a subset of the HER2 kinase inhibiting drugs currently on market. These patients generally fail standard lung tumor treatment therapies, including erlotinib treatment

The automated HTG EdgeSeq system

To provide researchers with a sensitive, accurate and reliable platform for tumor profiling, HTG has developed and optimized the HTG EdgeSeq system to allow multiplex probing of small biopsy samples, especially those from slides with formalin-fixed paraffin-embedded (FFPE) sections. The RNA extraction-free HTG EdgeSeq chemistry utilizes a modified the S1 nuclease protection assay (NPA) to produce a stoichiometric library of probes. The probes then have adaptors and molecular barcode sequences added through a simple PCR reaction. The resulting DNAs are then quantitated on next-generation sequencing (NGS) to identify the mRNA expression patterns indicative of gene fusions.

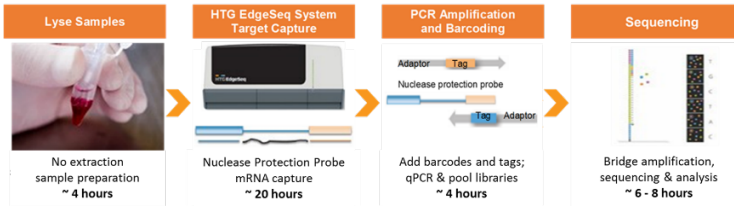

Figure 1: Workflow for the HTG EdgeSeq ALK*Plus* Assay EU.

Sample preparation reagents and materials are included with the HTG EdgeSeq kit. The heart of the HTG EdgeSeq system is the automated processor which uses proprietary reagents optimized for FFPE samples and can yield reproducible results with as little as 1.5 mm^2 of tissue from a 5-micron section. Using a microtiter-plate format, the HTG EdgeSeq can process 96 samples per day and thousands of mRNAs can be assessed in each sample. The total process from tissue dissection to report generation takes approximately two days, with roughly 3.5 hours of hands-on time required.

Detecting rearrangements in FFPE tissue

Figure 2 shows the RUO data generated from a FFPE biopsy of lung adenocarcinoma, grade G1, stage IIA. Consistent with previous testing performed via FISH analysis, an imbalance in the 3' to 5' expression was detected indicating a likely ALK rearrangement event was present in the sample; probes for the v3a and v3b variant subtype were also positive, identifying the specific fusion partner and site of the fusion event.

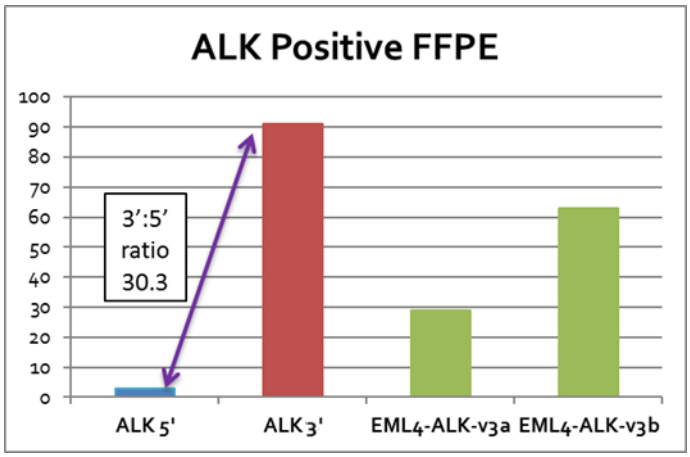

Figure 2: 3' to 5' exression imbalance indicating an ALK rearrangement event detected in FFPE tissue.

A similar comparison with FISH data was performed on a different lung cancer FFPE, this one featuring an apparent rearrangement in the RET gene.

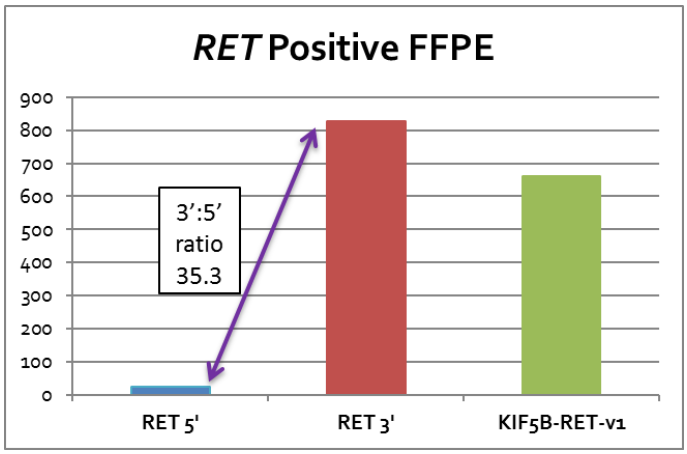

Figure 3: 3' to 5' exression imbalance indicating a RET rearrangement event detected in FFPE tissue.

Testing revealed an RET translocation and KIF5B-RETv1 fusion junction (Figure 3). Confirmatory FISH analysis was attempted by several accredited laboratories, but results

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passing quality-control standards could not be obtained.

To confirm the presence of the RET rearrangement, qPCR testing was performed and in a very weak but detectable RET fusion of the same variant was detected. While informative, the CT values obtained were well beyond what would be considered reportable by commercial testing labs.

This highlights an important advantage of the HTG EdgeSeq ALK*Plus* Assay EU is its ability to work with FFPE material which fails in other testing approaches.

Detecting rearrangements in cell lines

Multiple cell lines with known gene rearrangements found in lung cancer have been assessed by HTG EdgeSeq ALK*Plus* Assay EU. Here the results for a NTRK1 rearrangement positive cell line are displayed to demonstrate that the same 3' to 5' paradigm observed in FFPE tissue applies to cell lines (Figure 4.) The probe for the TPM3-NTRK1 fusion described for this cell line in the literature also produced signal, confirming the correct identification of the rearrangement.

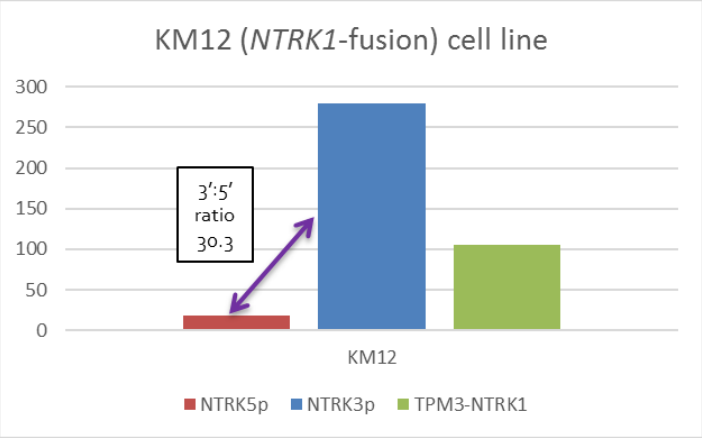

Figure 4: 3' to 5' exression imbalance indicating an NTRK1 rearrangement event detected in KM12 cell line.

Probes for a several small insertion events in the HER2 (ERBB2) gene are present in the HTG EdgeSeq ALK*Plus* Assay EU. About 4% of NSCLC patients have these mutations in exon 20 of HER2 which causes constitutive activation of the kinase activity; research indicates that tumors with exon 20 activating insertions may be susceptible to specific therapies targeting this kinase.

In the following example (Figure 5), a cell line reported to be homozygous for a 3-base insertion in exon 20 (H1781) was tested and compared to a different NSCLC cell line, H3122, which is reported to be non-mutated in the HER2/ERBB2 locus.

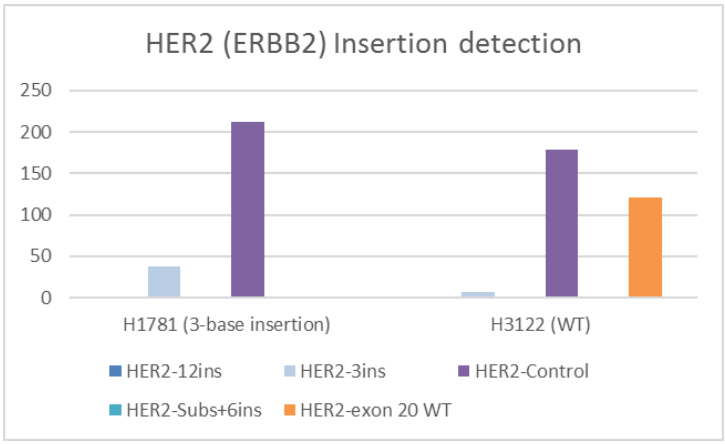

Figure 5: Detection of three-base insertion in a cell line harboring HER2 insertion event

Signal was obtained for both cell lines with the HER2-Control probe, indicating expression of the HER2/ERBB2 gene was occuring. When the insertion-specific probes for HER2 were examined, signal was obtained on a probe to a three-base insertion probe for the H1781 cell line, but not the H3122 cell line. As confirmation of the homzygous nature of the insertion in H1781, no signal was obtained for the probe targeting the non-mutated (HER2-exon 20 WT) probe, while substantial signal was obtained for the H3122 cell line.

Expression of MET

Over expression of the MET gene has been shown to convey resistance to a variety of treatments for NSCLC, and less frequently, a driver of oncogenesis.

Expression of the MET transcript can be accomplished using the additional markers found within the assay.

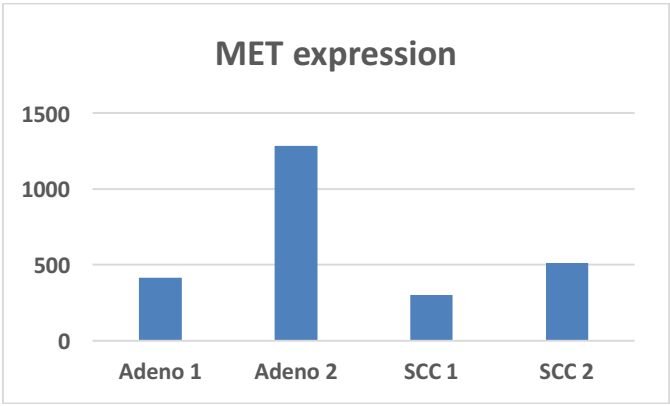

Figure 6: Expression of the MET gene in NSCLC.

Tissue subtyping: RNA biomarkers

Genes potentially useful for confirming tumor histopathology in small biopsy samples such as FFPE sections are present

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in the HTG EdgeSeq ALK*Plus* Assay EU. Probes for adenocarcinoma- and squamous cell-specific markers were used along with appropriate controls to displayed the expected patterns of expression when compared to cases previously classified by conventional pathology methods (see Figure 6.)

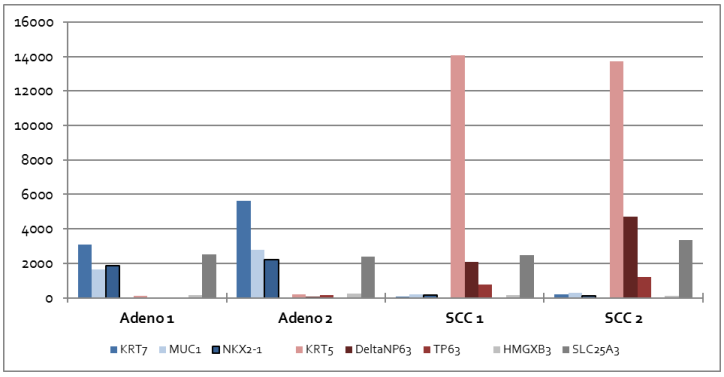

Figure 7: Probes differentiating adenocarcinoma and squamous cell carcinoma.

Rearrangements in heterogenous samples

Tumor biopsies often contain a substantial proportion of non-malignant cells which do not harbor the oncogenic mutation; effective testing methodologies must be able to identify a specific event in the context of normal, non-mutated tissue. Detection of a rearrangement positive sample in heterogeneous tissue matrix was simulated by mixing two cell lines in different ratios; one cell line possessed the rearranged copy of the ROS1 gene and the other a pair of non-altered (wild-type) ROS1 alleles. Reliable detection of the ROS gene fusion, as measured by 3' to 5' ratio was achieved in cell mixtures containing as little as 5% of fusion positive cells in a total population of 5,000 cells (Figure 8.)

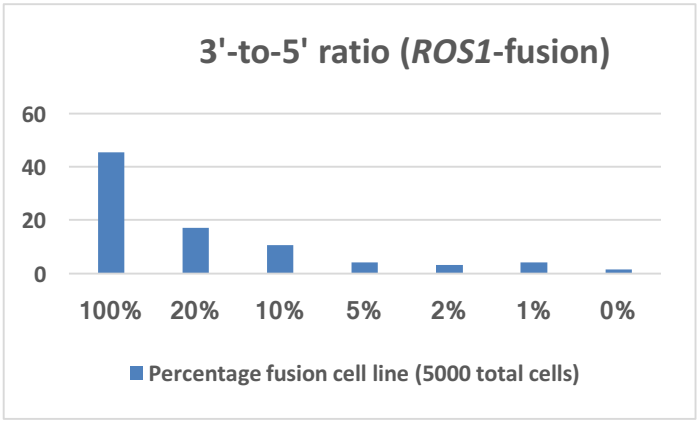

Figure 8: Detection of rearrangement events in heterogenous samples.

Conclusion

The HTG EdgeSeq ALK*Plus* Assay allows many rare, but clinically important, mutations driving NSCLC to be detected using a single section of FFPE tissue. The RNA extraction-free workflow is automated on the HTG EdgeSeq system, providing and simple and robust solution for mutation detection.

Data in this white paper were generated on the HTG EdgeSeq ALKPlus Assay EU or similar HTG assays using identical probe sequences.

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[1] ALK data available with CE-IVD version only. Not available in U.S. IVD version.
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