Abstract

We have previously described a targeted genomic Laboratory Developed Test (LDT) that measures mutations in EGFR, KRAS and BRAF from blood-derived DNA using Droplet Digital™ PCR (ddPCR). This test supports the rapid delivery of molecular diagnostic results, with >95% of test results delivered within 72 hours of sample receipt. Our Laboratory has used this test as a key clinical need for the delivery of rapid test results that leads to faster treatment decisions. Additionally, the test is of utility for those patients for whom tissue is unavailable or insufficient for molecular testing. These needs are especially real for patients diagnosed with non-small cell lung cancer (NSCLC). In this report we will update on new test development using plasma-derived DNA and a set of multiplexed assays created in collaboration with the Bio-Rad Digital Biology Center – Pleasanton (Bio-Rad). These assays were designed to detect fusion transcripts resulting from rearrangements in ROS1 (8 variants) and RET (8 variants). We will also update on our EML4-ALK RNA test (3 variants). Design considerations, sensitivity and specificity, as well as reproducibility and robustness studies for these complex assays will be reviewed. Similar studies were conducted for the development of the commercially available EML4-ALK fusion test. EML4-ALK concordance studies compared the fusion found in blood with known positives and negatives found using FISH and PCR-based methods (n=24 evaluable matched pair samples). Clinical sensitivity, specificity and concordance were 85%, 100% and 92% respectively. We also report on RNA fusion test performance for all tests testing within our Clinical Laboratory (>1 year of testing). RNA fusion variant tests maintain the 72 hour concordance time established previously for cfDNA based testing, with 96% resulting in <72 hours from receipt of the sample. The robust detection of these rare but actionable RNA variants from plasma represents a molecular testing option of substantial value to patients and their physicians.

Results

Figure 1. Product Development Overview. The schematic highlights our framework for the development of Laboratory Developed Tests (LDTs). The process is maintained in our Quality Management System (ISO-13485:2015 certified) and can be scaled to accommodate products from CLIA through FDA classification.

Figure 2. Overview of Blood Sample Processing Steps for Fusion Variant Detection in the BioRad Laboratory. Sample testing is initiated when whole blood is drawn into the specimen collection kit and shipped to the BioRad Laboratory. Circulating RNA is recovered from multiple sources within the plasma, reverse transcribed with gene specific primer and concentrated. For ddPCR analysis, samples are processed using the BioRad QX200 ddPCR system and droplet counts are evaluated using QuantaSoft Software. The test results are documented and reported back to the physician. The process is designed to work within a 72 hour timeframe.

Figure 3. EML4-ALK, ROS1 and RET Fusion Variant Multiplexed Assay Design. Representative drug resistance distribution from positive analytic samples for A. EML4-ALK, B. ROS1 and C. RET fusion variant assays (top) and coverage of the final multiplexed assays in NSCLC (bottom).

Figure 4. Analytic Sensitivity and Specificity. Analytic standards to A. EML4-ALK (E13:A20), B. SDC4-ROS1 (S2:R34) and C. CCDS-RET (C1:R12) were diluted in a background of total human RNA. With each fusion variant the limit of detection was established at 0.2% variant frequency or lower using pre-defined 2 copy criteria for variants. All samples above the threshold also contain at least 150 copies of control gene. Specificity of each assay was all demonstrated by testing a cell-line which expresses an alternative fusion variant.

Figure 5. Precision Testing. A. Table indicating the structure of the accuracy testing apparatus. B. EML4-ALK C. ROS1 and D. RET performance, over three runs on the same day (intra-day), three runs on consecutive days (inter-day) and with two operators. Analytic standards were measured at three concentrations. Mean Copies +/- Standard Deviation are shown. No Reverse Transcriptase and No Template Controls were run along side each batch of samples and were all negative (data not shown).

Figure 6. Robustness Studies. The GeneStrat test positive control was run on 21 consecutive days (excluding weekends and holidays). The total number of copies detected by ddPCR for A. EML4-ALK, B. ROS1 and C. RET along with the internal control gene were shown. Mean Copies +/- Standard Deviation: EML4-ALK 190±64, RET: 28±12, ROS1: 423±143. No Reverse Transcriptase and No Template Controls were run along side each batch of samples and were all negative (data not shown).

Figure 7. Final Acceptance Test. The final evaluation of the ALK, ROS1 and RET tests before launch was performed to validate the end-to-end procedural steps in the workflow. A mock Test Request Form was generated and shipped using the specimen collection kit with NSCLC donor samples. Upon arrival at the BioRad Laboratory the sample was processed according to the SOPs by the operators performing through the entire process. Finally, a mock Test Result Report was generated from the test system and verified to accuracy of report fields.

Figure 8. GeneStrat RNA Test Turn Around Time (TAT). TAT was computed for all testing requiring an RNA variant (>3,500) over a 14 month period. Data excludes weekends, holidays, and samples held >72 hours due to incomplete clinical information on the Test Request Forms (TRF).

Summary

• Blood-based multiplexed tests for mRNA fusion variants EML4-ALK, ROS1 and RET were developed.

• The mRNA tests simultaneously measure 78% of EML4-ALK, 88% of ROS1 and 99% of RET fusion transcript variants in NSCLC.

• Analytic Limit of Detection of 0.2% fusion transcript in a background of normal cell-line RNA was achieved.

• Precision was demonstrated with analytic standards at three concentrations over three runs, three days and two operators.

• Robustness was demonstrated with positive controls over 21 consecutive days of testing.

• EML4-ALK was clinically validated with sensitivity of 85%, specificity of 100% and concordance of 92%.

• On-market test turn-around-time is 72 hours or less in 96% of cases.

• A blood-based PD-L1 RNA expression test is under development.

References


• COSMIC database: v73, released 14-NOV-2016. http://cancer.sanger.ac.uk/cosmic

Table 1. Clinical Validation of the EML4-ALK Test. Concordance studies compared known positives and negatives established with FISH and PCR based methods to the blood-based GeneStrat EML4-ALK fusion test (n=24 evaluable matched pair samples). Calculated sensitivity and concordance is based on the 78% of the total ALK mutations in NSCLC (E13:A20, E20:A20 and E70:A70) represented by the GeneStrat assay. Clinical specificity was established at 100% for both ROS1 and RET tests. Sensitivity has not been determined due to unavailability of samples with these rare mutations.