# oncoReveal™ Fusion LBx: Single-tube multiplexed PCR-based NGS assay for detection of multiple gene fusions from cell free RNA

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## ABSTRACT

**Introduction.** Rapid advancement of liquid biopsy (LBx) has demonstrated significant promise in improving diagnostic and precision medicine via noninvasive methods. Much of this development has been focused on cell-free DNA (cfDNA), with less focus on cell-free RNA (cfRNA). Gene fusions are difficult to target by cfDNA due to large introns and repetitive sequences. In contrast, cfRNA is less influenced by these hurdles and can provide functional interpretation in addition to genetic variants from cfDNA. Here, we present oncoReveal™ Fusion LBx, an {RUO (Research User Only), single-tube, multiplexed, PCR-based NGS cfRNA fusion assay, targeting >150 fusion transcripts. We also developed a companion analysis module within Pillar's PiVAT® (Pillar Biosciences Variant Analysis Toolkit) and assessed its ability to capture vanishingly small counts of fusions in both plasma and FFPE (Formalin Fixed Paraffin Embedded) samples.

**Methods.** Gene fusion targets were designed using Pillar's VersaTile® software, targeting specific breakpoints of fusion transcripts from 18 cancer driver genes with >80 partners in total. Due to the lack of cfRNA fusion positive clinical samples, Horizon Discovery's Structural Multiplex cfDNA Reference Standard and Seraseq® Fusion RNA Mix v4 fusion positive reference samples were used to assess panel performance, covering 2 and 17 of the designed fusion targets, respectively. The Horizon cfDNA sample was run at 20ng input. For the Seraseq® sample, small RNA input from 2.5ng to 0.025ng were tested in the panel, with expected fusions ranging from 260 to 2 copies in the sample. Fusion input after dilution was estimated based on the manufacturer's stated starting RNA concentration (25ng/ul). Reverse transcribed cDNA from plasma samples of healthy donors, and from a normal cell line were tested as negative controls. All the samples were sequenced on Illumina's MiSeq™ platform and analyzed using Pillar's PiVAT® secondary analysis bioinformatics software.

**Results.** Each designed primer and its product were validated by in-silico amplification to ensure the product size is suitable for cfRNA interrogation. All expected fusion targets were identified by PiVAT® in the cfDNA sample. For the FFPE sample, we were able to detect fusions with as low as 0.25ng RNA input, equating to ~20 copies per target, demonstrating a 100% sensitivity at that input level. Notably, the assay demonstrated 100% specificity at low input ranges and negative controls in calling in-frame fusions.

**Conclusion.** Our data demonstrates high sensitivity and specificity of the oncoReveal™ Fusion LBx (RUO) panel in detecting gene fusions in cell-free and FFPE samples. Limited availability of commercial cfRNA standard samples limits a comprehensive assessment of the panel. However, the ability to detect fusion events from cfRNA samples represents a crucial step forward in the molecular profiling of cancer.

## EXPERIMENT DESIGN

- The oncoReveal Fusion LBx research-use-only (RUO) panel covers specific breakpoints of fusion transcripts from 18 driver genes with 83 partners (table 1).
- The panel was tested on positive standard reference samples as well as control, negative samples
- Positive standard reference samples were diluted from 2.5ng RNA input to 0.025ng input
- All analysis were performed on PiVAT
- **Positive standard reference samples:**
- Seraseq® Fusion RNA Mix v4 (number of fusions = 17),
  - Horizon® Structural Multiplex cfDNA Reference Standard (number of fusions = 2)
- **Normal samples:**
  - Human cfDNA normal sample, Coriell NA24385 normal cell line, Horizon ® Wild type

| Sample Type | Replicates | Sample Class |
|---|---|---|
| Coriell NA24385 normal cell line | 2 | Negative |
| In-house normal | 2 | Negative |
| Horizon® Wild type | 2 | Negative |
| Horizon® Structural Multiplex cfDNA (20ng) | 2 | Positive |
| Seracare Fusion v4 (0.025ng) | 1 | Positive |
| Seracare Fusion v4 (0.25ng) | 1 | Positive |
| Seracare Fusion v4 (2.5ng) | 2 | Positive |

## ASSAY DESIGN

### Advantages of Targeting cfRNA

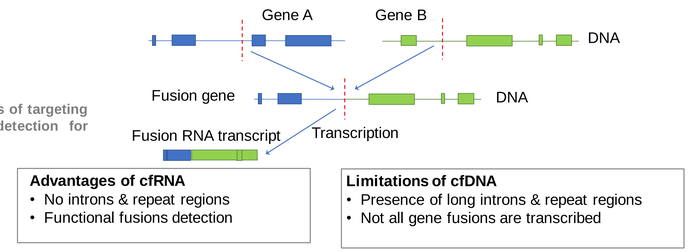


Figure 1. Advantages of targeting cfRNA for fusion detection for liquid biopsy panel.

### Breakpoint Targeting to Improve Fusion Detection

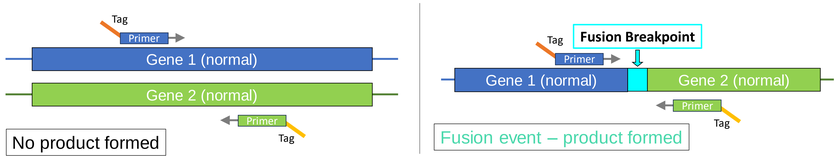


Figure 2. Overview of the oncoReveal™ Fusion LBx cfRNA library preparation to detect fusions. Breakpoints are targeted with multiplex PCR primers that only form product in the presence of fusion.

## SCHEMATIC OF PiVAT's ALGORITHM

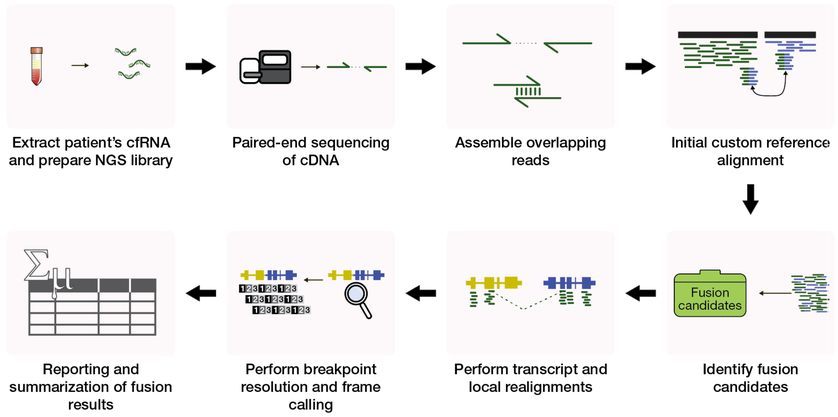


Figure 3. Schematic representation of PiVAT's fusion calling algorithm. The panel targets cfRNA obtained from patient's blood, it is first reverse transcribed to cDNA. The cDNA is then processed through the oncoReveal™ Fusion LBx (RUO) panel to generate Illumina sequencing library, which is then sequenced in a paired-end manner. The sequenced data undergoes several quality processing steps to reduce overall noise in the data. Only the paired-end reads passing PiVAT's internal filters, are assembled pairwise to generate a merged read. The merged reads are aligned to a custom reference sequence to identify fusion candidates by clustering reads based on their alignment. The fusion candidates undergo further transcript and local realignment to identify the correct transcript for each read and read rescue, respectively. Frame calling and breakpoint identification is performed in the final step with read count reported per fusion call. All results are summarized in a report generated for the user.

## PANEL TARGET LIST

| Driver Gene | Partner Gene |
|---|---|
| ALK | EML4, CLTC, HIP1, KIF5B, KLC1, STRN, TFG, TPM3, TPR, MSN |
| BRAF | KIAA1549, MKRN1, FAM131B, AKAP9 |
| EGFR | SEPT14, PSPH, RAD51 |
| ERG | TMPRSS2 |
| FGFR2 | BICC1, CASP7 |
| FGFR3 | TACC3, BAIAP2L1 |
| MET | KIF5B |
| NRG1 | CD74, SLC3A2, VAMP2 |
| NTRK1 | TPM3, TFG, LMNA, SQSTM1, CHTOP, ARHGEF2, NFASC, IRF2BP2, PPL, BCAN, SCYL3, TP53, CD74, MPRIP, TPR |
| NTRK2 | STRN, AFAP1, NACC2, BCR, TRIM24, QKI, PAN3, SQSTM1 |
| NTRK3 | ETV6, BTBD1, EML4, SQSTM1, TFG, RBPMS |
| PBX1 | TCF3 |
| PPARG | PAX8, CREB3L2 |
| PRKACA | DNAJB1 |
| RAF1 | ESRP1, SRGAP3 |
| RET | CCDC6, CUX1, KIF5B, NCOA4, TRIM33, PRKAR1A |
| ROS1 | CCDC6, CD74, CLTC, EZR, GOPC, LRIG3, MSN, SDC4, SLC34A2, TFG, TPM3 |
| TFE3 | SFPQ, ASPSCR1, CLTC, PRCC, NONO |
| MET exon14 skipping, EGFR variant III (EGFRvIII) | |

**Table 1. Panel target list.** The table lists the fusion events targeted in our oncoReveal™ Fusion LBx panel. For each fusion pair, driver and partner genes are listed. In addition to intergenic fusion pairs, MET exon 14 skipping and EGFR variant III are also targeted.

## PERFORMANCE SUMMARY

| Sample Type | Fusion counts* | # of Reps. | Sample Class | PPA | NPA | LOB |
|---|---|---|---|---|---|---|
| Coriell NA24385 normal cell line | | 2 | Negative | N/A | 100.0% | 0.00 |
| In-house normal | | 2 | Negative | | 100.0% | 0.00 |
| Horizon® Wild type | | 2 | Negative | | 100.0% | 0.00 |
| Horizon® Structural Multiplex cfDNA | | 2 | Positive | 100.0% | 100.0% | N/A |
| Seracare Fusion v4 (2.5ng) | 145-477 | 2 | Positive | 100.0% | 100.0% | |
| Seracare Fusion v4 (0.25ng) | 14-48 | 1 | Positive | 100.0% | 100.0% | |
| Seracare Fusion v4 (0.025ng) | 1-5 | 1 | Positive | 94.1%* | 100.0% | |

LoB = FP / Sample; NPA = (1 – FP x 100 / (FP + TN)); PPA = TP x 100 / (TP+FN)
* Fusions not detected by PiVAT but detected by comparator methods.

**Table 2. Summary of performance metrics from secondary analysis pipeline.** The table summarizes the limit of blank (LoB), negative percent agreement (NPA), and positive percent agreement (PPA) for the normal and positive standard reference samples tested in this study. The panel has very high sensitivity up to 0.25ng RNA dilution. Fusion calls in lower dilutions are detected but fail to pass read count threshold in PiVAT. However, when all the samples are processed through an independent comparator method (STAR-fusion), most of the fusion are detected with identical NPA. STAR-fusion consistently miss MET exon 14 skipping and EGFRvIII event detection.

## CONCLUSIONS

- We present here oncoReveal™ Fusion LBx, an (RUO) cfRNA, liquid biopsy-based panel that targets >150 fusion transcripts.
- We demonstrate high sensitivity and specificity of our panel in cell-free and FFPE samples, down to 0.025ng RNA input
- Limited availability of commercial cfRNA standard samples limits a comprehensive assessment of the panel.
- However, the ability to detect fusion events from cfRNA samples represents a crucial step forward in cancer research and the profiling of liquid biopsy samples.