The use of Internal Controls in RNA based Next Generation Sequencing panels to quantitate and standardize gene fusion detection

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Abstract

Introduction

Gene fusions and exon skipping variants are relevant biomarkers in treatment decision algorithms for non-small cell lung cancer (NSCLC) and can be detected using next generation sequencing (NGS) approaches. RNA based targeted NGS panels present significant advantages, especially in terms of analytical sensitivity, however, they are also challenging to standardize. We present here the use of Internal Standards (IS) added to patient samples that enable the calculation of transcript abundances and the determination of a limit of detection.

Methods

SNAQ™-SEQ ssRNA Internal Standards (IS) were designed for EML4-ALK, MET-Exon14 skipping (MET-EX14) and TMP3-NTRK1 native templates (NT); these standards included unique base changes flanking the fusion for bioinformatic separation. The IS mixture (110 copies each) was added to three patient RNA samples positive for either ELM4-ALK, MET-EX14, or IRF2BP2-NTRK1 fusions, and a NTC, sequenced using the Oncomine™ Comprehensive Assay v3 panel (Thermo Fisher Scientific), with fusion reads extracted using a modified analysis workflow of Ion Reporter™ software (version 5.16). IS reads were used to determine if SNAQ™-SEQ approach could provide a useful limit control and NT:IS ratio was used to demonstrate NT fusion abundance.

Results

All IS fusions were detected in each sample, the ELM4-ALK, MET-EX14 NT reads were 10-fold greater. No TMP3-NTRK1 NT reads indicate less than 110 starting copies. NT:IS ratio estimated EML4-ALK and MET-EX14 abundance as 7800 and 16,000 copies, respectively. With one exception, SNAQ™ estimated all other native fusions had sub 1 copy abundance (median 0.7, range 0.003-0.5) which suggests these are background noise. MET-EX14 fusion in the EML4-ALK sample had 14 copies, similar to LOD levels found in other quantitative nucleic acid detection technologies.

Conclusions

The sample containing low IS all had strong positive IS fusion read counts, indicating that ≥110 NT fusions should have been detected. The native ELM4-ALK and MET-EX14 fusions had >70-fold higher abundance than the IS spike-in. SNAQ™-SEQ IS ability to provide standardized abundance measurement could eliminate the less accurate read based thresholds, and instead allow NGS platforms to use established reporting range analytic validation like other quantitative RNA technologies.

Acknowledgements

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Internal Standards (IS) Design

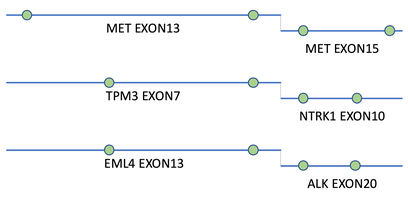

Figure 1. SNAQ™-SEQ internal standards for RNAseq abundance measurement of three fusions associated with non-small cell lung cancer (NSCLC). Internal standards (IS) of ssRNA synthetic to three oncogenic fusions, MET-MET exon 14 skipping, TMP3-NTRK1 and EML4-ALK containing indicated (text) flanking exons are depicted as RNAseq amplicons. Each ssRNA IS has four unique base changes (circles) to allow ThermoFisher's Ion Reporter bioinformatic pipeline to separate IS reads from the native template (IS) reads. The ratio between native fusion and internal standard fusion reads is used to calculate the native fusion abundance.

Native Template (NT) and IS fusion reads for Three Controls

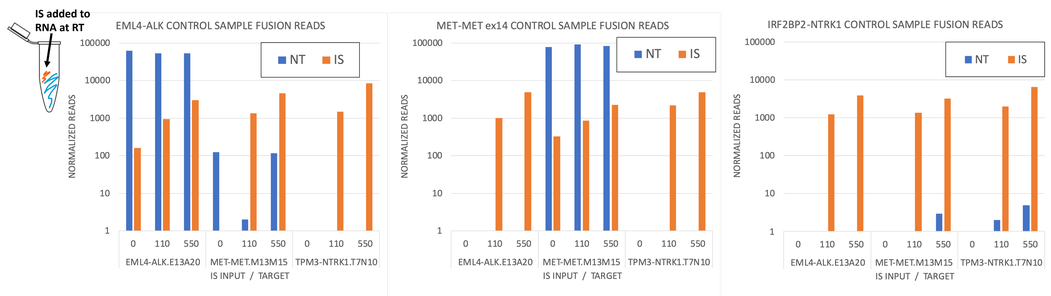

Figure 4. Normalized Fusion Reads of Three Control Samples. RNA from three control FFPE samples were spiked with SNAQ™-SEQ RNA mixture (x-axis, copies per sample), sequenced using Oncomine Comprehensive Assay v3, processed through Ion Reporter using a fusion alignment file that included IS sequences. Read counts for EML4-ALK.E13A20, MET-MET.M13M15, TPM3-NTRK1.T7N10 fusions were normalized to 1 million total reads (y-axis) for both native (NT) and internal standard (IS) and exported for analysis.

IS added to standard NGS workflow

NT fusion reads converted into fusion abundance

Patient sample fusion read count not affected by IS addition

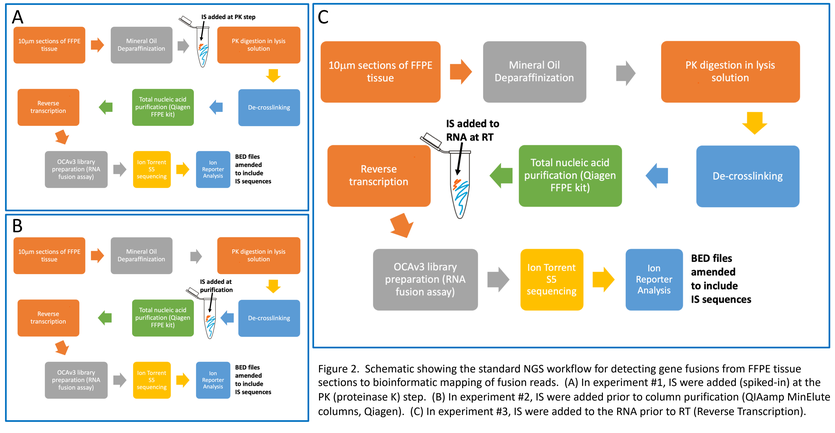

Figure 2. Schematic showing the standard NGS workflow for detecting gene fusions from FFPE tissue sections to bioinformatic mapping of fusion reads. (A) In experiment #1, IS were added (spiked-in) at the PK (proteinase K) step. (B) In experiment #2, IS were added prior to column purification (QIAamp MinElute columns, Qiagen). (C) In experiment #3, IS were added to the RNA prior to RT (Reverse Transcription).

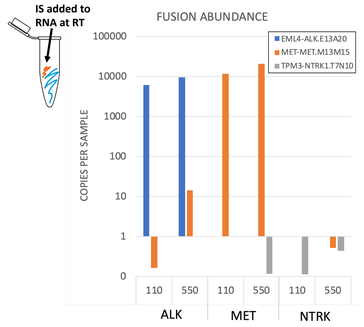

Figure 5. NT abundance in control samples. Samples labeled ALK, MET, NTRK were expected to contain fusions EML4-ALK.E13A20, MET-MET.M13M15, IRF2BP2-NTRK1, respectively. The indicated RNA fusions abundance (legend) was calculated using the NT:IS ratio times the IS input level (y-axis, 110 or 550 copies) and represent the fusion templates present at time of mixing IS controls with sample RNA.

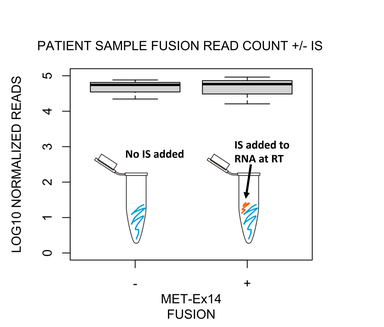

Figure 6. Patient sample fusion read count with and without IS added. Three independent patient samples containing MET ex14 skipping (MET-MET.M13M15) were processed with (+) and without IS (-) added to the RNA at RT step (110 copies). ANOVA analysis did not indicate a significant read normalized MET Ex14 fusion count difference.

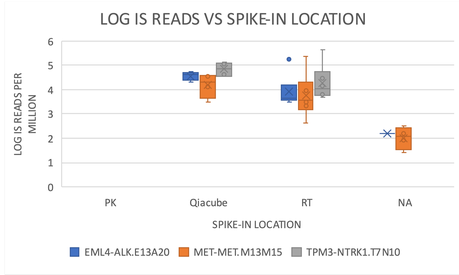

Figure 3. Internal Standard Yields Dependence on Step Added to Testing Protocol. SNAQ™-SEQ ssRNA Internal standards to indicated fusions (legend) were added at PK (proteinase K) step, prior to column purification, at RT (into purified RNA prior to reverse transcription), NA (Not Added to sample) added at 1100, 1100, 550, 0 copies each fusion, respectively. RNA samples were tested using the Oncomine Comprehensive Assay v3 using a modified fusion alignment file that included Internal Standard sequences, read counts normalized to 1 million total reads was exported and plotted (y-axis). Note that no IS reads were recovered when the IS was added at the PK step.

Conclusions and Next Steps

- SNAQ™-SEQ ssRNA Internal Standards (IS) were successfully added to the workflow for an RNA based NGS assay for fusion detection (Oncomine Comprehensive Assay v3).
- Unsupported modified Ion Reporter analysis workflow was able to map and analyze reads corresponding to SNAQ™-SEQ ssRNA Internal Standards (IS).
- IS reads were recovered when IS was added prior to column purification and at RT step, but not when IS was added at PK step.
- IS added at RT step were able to provide standardized abundance measurements for gene fusions and provide a limit of detection.
- IS added at RT step did not alter the patient sample fusion read count.

Next Steps:

- Routine incorporation of IS added at RT step into the workflow for fusion detection.
- Continue exploring the use of IS to control for the efficiency of nucleic acid extraction.
- Consider the use of IS in the cross-validation of different RNA based NGS fusion detection assays.