# Optimal Thaw/Freeze Cycle Analysis for nCounter® Lung Gene Fusion Panel

## Overview

The nCounter Lung Gene Fusion Panel (catalog number XT-CSO-LGFU1-12) is configured for 12 reactions per kit. However, some customers desire to run smaller sample sizes in each batch of the experiment. Based on experimental data, we recommend that customers limit the thaw/freeze cycle to one time only to minimize the degradation of oligonucleotides and tags in the gene fusion panel. With regards to aliquoting, the best practice is to aliquot the TagSet and dilute probes in an appropriate amount then freeze the remainder at -80°C for future use, one time only.

## Materials and Methods

For RNA templates, we used a normal RNA reference (Clontech, Cat. No.: 636690), a ALK-RET-ROS1 Fusion RNA Reference Standard (Horizon Discovery, Cat. No.: HD784), and RNAs from two fresh frozen breast cancer tissues (017267T2(2) and 017723T2(2)). We extracted total RNA from the ALK-RET-ROS1 Fusion RNA Reference Standard (FFPE tissue) with the AllPrep DNA/RNA FFPE Kit (Qiagen, Cat. No.: 80234). Total RNA was extracted from two fresh frozen tissues of breast cancer obtained from Proteogenex (Culver City, CA) using the RNeasy Mini Kit (Qiagen, Cat. No.: 74104). Concentrations of RNA were adjusted to 20 ng/ul with RNase-free H2O for Clontech reference RNA, 017267T2(2), and 017723T2(2). The final RNA input was 130 ng per lung fusion assay for Clontech reference RNA, 017267T2(2), and 017723T2(2) (Table 1). The RNA concentration, measured by the Nanodrop (ThermoFisher), was 27.5 ng/ul in the ALK-RET-ROS1 Fusion RNA Reference Standard (HD784). The final total RNA input was 178.75 ng per lung fusion assay for the ALK-RET-ROS1 Fusion RNA Reference Standard (HD784) (Table 1). The maximal amount of RNA without any dilution was used for HD784 because of the inferior quality of RNA from FFPE tissues. All of the RNA templates were aliquoted into five 12-tube strip tubes for the time-point experiment shown in Table 1 below.

| No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RNA | Clontech Ref. RNA | | | HD784 | | | 017267T2(2) | | | 017723T2(2) | | |
| RNA input (ng) | 130 | | | 178.75 | | | 130 | | | 130 | | |

TABLE 1 RNA template for lung fusion reusability assays. Each RNA template was run in triplicate. 6.5 ul of RNA template was added to 8.5 ul of hybridization master mix.

To test the reusability of NanoString TagSets, we obtained six lung fusion kits (catalog number XT-CSO-LGFU1-12, including Probe A, Probe B, Probe P, and TagSet) from the NanoString Manufacturing Department. After thawing on ice, we combined five sets of TagSet, TE-diluted Probe A, Probe B, and Probe P into an individual tube with the labels of pooled TagSet, pooled Probe A, pooled Probe B, and pooled Probe P respectively.

We then split the pooled probes and TagSet evenly into four equal portions for the reusability experiments on Days 0, 1, 2, and 3. The first experiment on Day 0 used one aliquot of the pooled probes and TagSet immediately. The following experiments on Days 1, 2, and 3 used the aliquot of the pooled probes and TagSet, which went through the thaw/freeze cycle for 1, 2, and 3 times respectively. The exaggerated frequency of thaw/freeze cycle tested the tolerance of probes and TagSet to frequent thawing and freezing.

The sixth kit was thawed and diluted with TE for Probes A, B, and P on Day 0 and then saved at -80°C for 30 days to test longer term storage (thaw/freeze cycle x 1). The summary of storage conditions and hybridizations is shown in Table 2 below.

| Four equal portion aliquots | Usage | Day of Experiment |
|---|---|---|
| Exp. 1 | Used immediately | Hybridized on Day 0 |
| Exp. 2 | Save at -80°C, thaw/freeze cycle x 1 | Hybridized on Day 1 |
| Exp. 3 | Save at -80°C, thaw/freeze cycle x 2 | Hybridized on Day 2 |
| Exp. 4 | Save at -80°C, thaw/freeze cycle x 3 | Hybridized on Day 3 |

| Original aliquot, thawed once | Usage | Day of Experiment |
|---|---|---|
| Exp. 5 | Thawed one set of Probes and TagSet and reused after 30 days of storage at -80°C (thaw/freeze cycle x 1) | Hybridized on Day 30 |

TABLE 2 Summary of storage conditions and hybridizations for five time points on Days 0,1, 2, 3, and 30.

All hybridizations for the lung fusion assays were performed according to the NanoString nCounter Gene Fusion Panel User Manual published in May 2016 (MAN-10028-01). Briefly, an aliquot of pooled TagSet and probes were thawed on ice and mixed with hybridization buffer as shown in Table 3 below. 8.5 ul of hybridization master mix was mixed with 6.5 ul of RNA templates in a 12-tube strip tube. The samples were hybridized at 67°C for 16 hours and immediately ramped down to 4°C. Complete hybridizations were transferred to the nCounter Analysis System for all five time-point experiments. The raw data files were analyzed using nSolver™ 4.0 Software with the Advanced Analysis module.

## Results

Initially we performed a correlation study of normalized counts for all time point experiments vs. Day 0. All raw counts were normalized to ERCC sum counts and converted to log2 scale for the correlation study. The data showed highly correlated ERCC normalized counts with great linearity across all four RNA templates (all R2 > 0.96 with slope ~ 1). The study did not show significant deviations of correlation in five time points of lung fusion assays (Figure 1).

| | x14 reactions |
|---|---|
| Hybridization buffer | 70 ul |
| Reporter Tagset | 28 ul |
| Diluted Probe A in TE | 7 ul |
| Diluted Probe B in TE | 7 ul |
| Diluted Probe P in TE | 7 ul |
| Total | 119 ul |

TABLE 3 Formula of hybridization master mix.

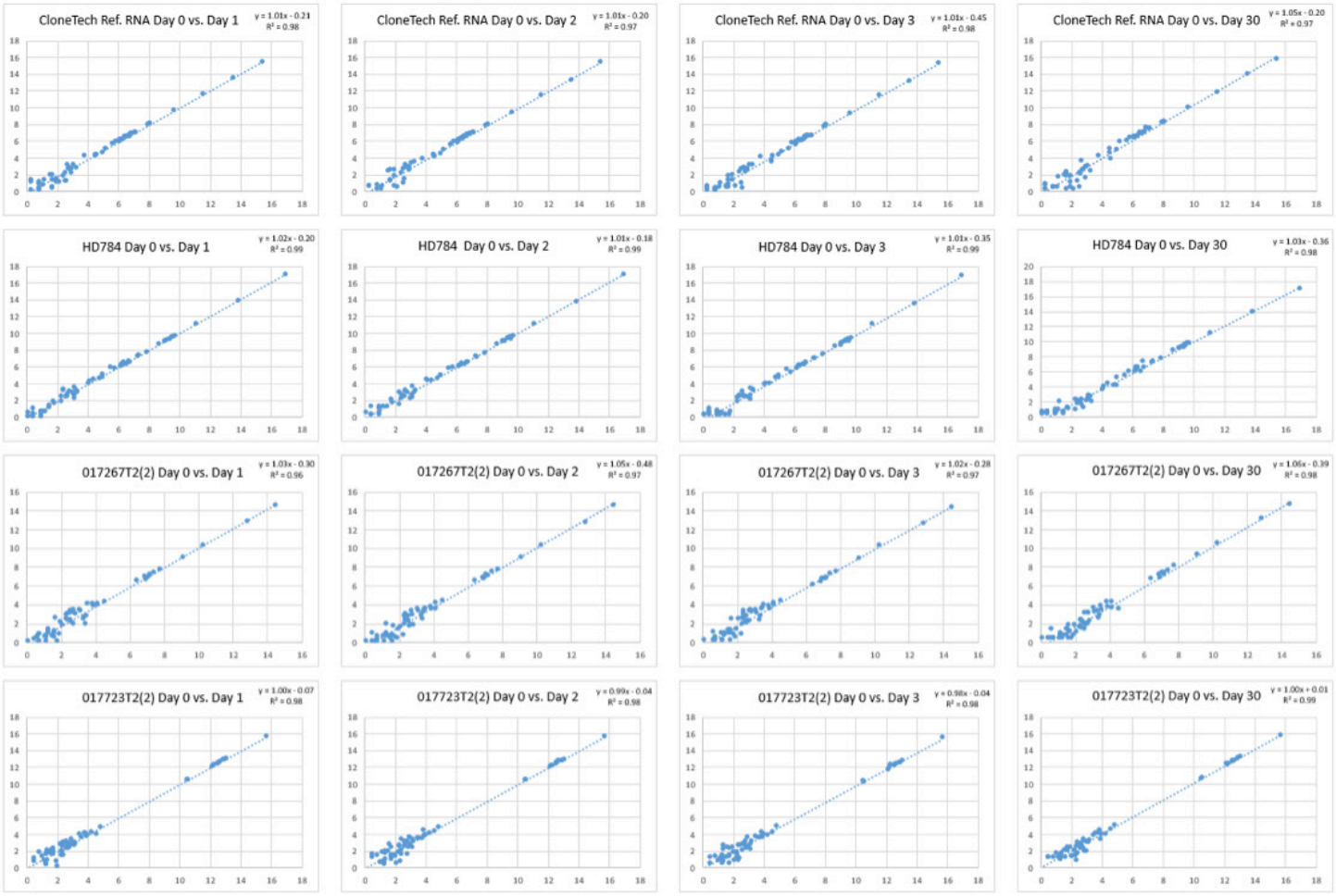
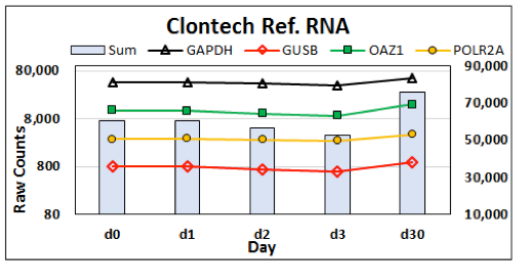



FIGURE 1 Correlation plots of two RNA standards (Clontech Reference RNA and ALK-RET-ROS1 Fusion RNA Reference Standard HD784) and RNA samples from two fresh frozen tissues (017267T2(2) and 017723T2(2)) on Day 1, Day 2, Day3, and Day 30 vs Day 0. All ERCC normalized counts have been transformed to the log 2 scale.

We then compared the ERCC normalized counts of four housekeeping genes (GAPDH, GUSB, OAZ1, and POLR2A) across the various time points to determine if the ERCC normalized counts would differ among the experiments. All the normalized counts of four housekeeping genes at different time points were compared to the normalized counts on Day 0. The normalized counts did not differ significantly between Day 0 and Day 1 after one thaw/freeze cycle and subsequent storage at -80°C for one day (using the student t-test with p-value 0.05 as the cut-off for statistical significance). This result suggests that the TagSet and diluted probes tolerate one thaw/freeze cycle. However, after the TagSet and probes underwent two to three cycles of thaw/freeze, the ERCC normalized counts decreased as shown by p-values less than 0.05 using the student t-test. The ERCC normalized counts on Day 30 with one cycle of thaw/freeze and 30 day storage at -80°C seemed to have significantly higher counts in Clontech Ref. RNA and the two RNA samples from fresh frozen tissues. This difference may be attributed to lot-to-lot variations since the Day 30 experiment used an independent TagSet and not the pooled TagSet used for the Day 0 through Day 3 time points. Alternatively, the difference may be due to changes in RNA quality after 30 days of storage in the -80°C freezer.

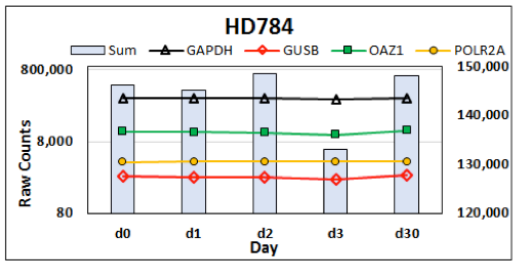
**Clontech Ref. RNA (636690 )**

| HK genes | Day 0 | Day 1 | Day 2 | Day 3 | Day 30 |
|---|---|---|---|---|---|
| GAPDH | 45031 | 45170 (0.66) | 42748 (0.001) | 39934 (0) | 55770 (0.002) |
| GUSB | 800 | 790 (0.67) | 707 (0.02) | 625 (0.0005) | 998 (0.005) |
| OAZ1 | 11808 | 11556 (0.13) | 10192 (0) | 9223 (0.0001) | 15605 (0.001) |
| POLR2A | 3000 | 3028 (0.55) | 2905 (0.23) | 2789 (0.01) | 3736 (0.005) |
| Sum | 60639 | 60547 | 56554 | 52572 | 76111 |



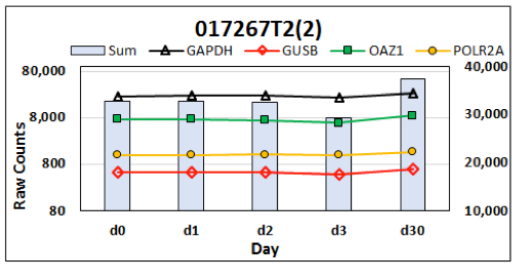

**HD784**

| HK genes | Day 0 | Day 1 | Day 2 | Day 3 | Day 30 |
|---|---|---|---|---|---|
| GAPDH | 128099 | 127537 (0.54) | 131337 (0.03) | 118147 (0.001) | 129008 (0.44) |
| GUSB | 855 | 794 (0.08) | 807 (0.09) | 683 (0.001) | 897 (0.1) |
| OAZ1 | 15133 | 14645 (0.06) | 14133 (0.004) | 12055 (0) | 16036 (0.01) |
| POLR2A | 2164 | 2164 (0.99) | 2171 (0.9) | 2176 (0.86) | 2172 (0.91) |
| Sum | 146251 | 145142 | 148450 | 133063 | 148115 |




**017267T2(2)**

| HK genes | Day 0 | Day 1 | Day 2 | Day 3 | Day 30 |
|---|---|---|---|---|---|
| GAPDH | 23462 | 23610 (0.67) | 23735 (0.42) | 21469 (0.004) | 26460 (0.002) |
| GUSB | 546 | 533 (0.45) | 534 (0.34) | 476 (0.02) | 633 (0.01) |
| OAZ1 | 7474 | 7414 (0.32) | 6946 (0.02) | 6188 (0.001) | 8962 (0) |
| POLR2A | 1259 | 1246 (0.8) | 1289 (0.42) | 1244 (0.63) | 1457 (0.04) |
| Sum | 32740 | 32804 | 32505 | 29380 | 37515 |




**017723T2(2)**

| HK genes | Day 0 | Day 1 | Day 2 | Day 3 | Day 30 |
|---|---|---|---|---|---|
| GAPDH | 53013 | 52246 (0.18) | 52134 (0.11) | 48393 (0) | 56032 (0.01) |
| GUSB | 1503 | 1423 (0.06) | 1405 (0.04) | 1217 (0.001) | 1658 (0.01) |
| OAZ1 | 8697 | 8379 (0.01) | 7781 (0.01) | 6921 (0) | 9590 (0) |
| POLR2A | 1446 | 1486 (0.18) | 1433 (0.77) | 1376 (0.01) | 1528 (0.02) |
| Sum | 64658 | 63535 | 62754 | 57909 | 68809 |

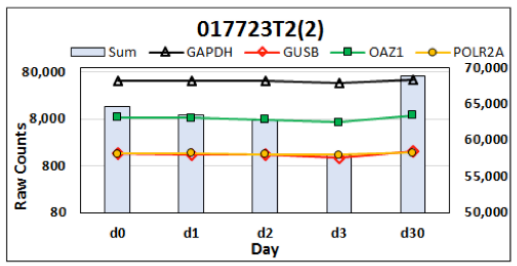



FIGURE 2 Changes of ERCC normalized counts for four housekeeping genes and their sum counts over 5 time points on Day 0, Day 1, Day 2, Day3, and Day 30 in two reference RNA standards (Clontech Reference RNA (636690) and ALK-RET-ROS1 Fusion RNA Reference Standard HD784) and RNA from two fresh frozen tissues of breast cancer (017267T2(2) and 017723T2(2)).

* Numbers in parenthesis: p-values of student t-test in comparison with the counts on Day 0. All p-values less than 0.05 are in pink.

We further analyzed the data with nCounter Advanced Analysis Software (nCounterAdvancedAnalysis_2.0.71). No fusion genes were detected in the two fresh frozen tissues, therefore, subsequent analyses focused on the negative control (Clontech reference RNA) and the positive control (ALK-RET-ROS1 Fusion RNA Reference Standard HD784). Figure 3 shows the heatmap of fusion junction probes. All of the positive controls across five time points show higher log2 counts on three fusion genes including EML4-ALK, CCDC6-RET, and SLC34A2-ROS1 (highlighted in red).

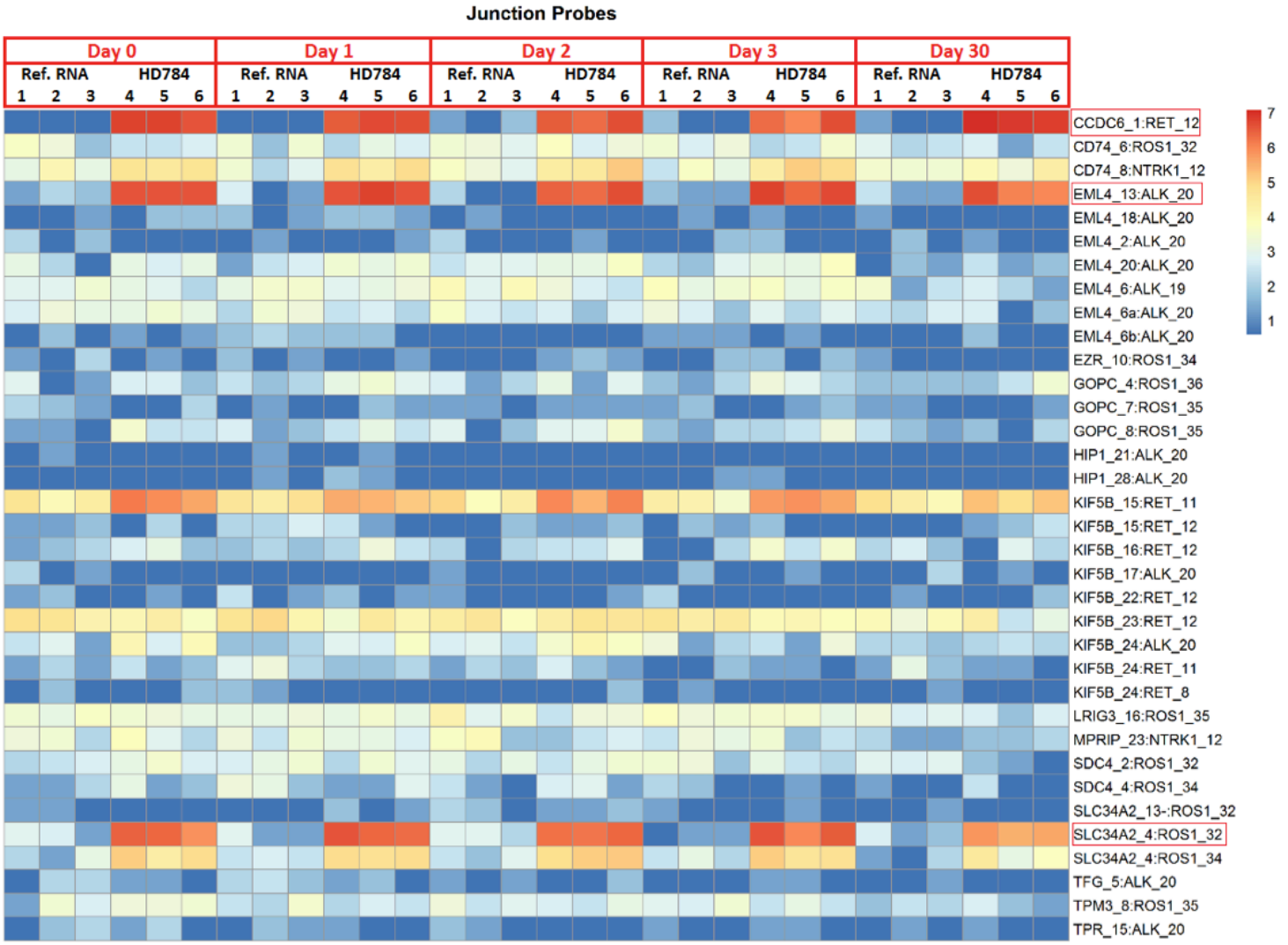



FIGURE 3 Heatmap of fusion junction probes. Three positive probes are highlighted in red. Numbers 1-3 are triplicate experiments on the Clontech reference RNA across five time points (Day 0, 1, 2, 3 and 30). Numbers 4-6 are triplicate experiments on the ALK-RET-ROS1 Fusion RNA Reference Standard (HD784) across five time points (Day 0, 1, 2, 3 and 30).

*Heatmap is colored by the log2 count according to the legend.

Figure 4 shows the heatmap of imbalanced end probes, which display significant differences in log2 counts (all p-values < 0.05) between 3P and 5P probes on ALK, RET, and ROS1 in the positive control (HD784). However, there were no significant differences (all p-values > 0.05) between 3P and 5P probes of ALK, RET, and ROS1 in the negative control (Clontech reference RNA). The summarized fusion calls are shown Figure 5.

The fusion calls were all negative (green) across the five timepoints in the negative control (Clontech reference RNA), and ALK, ROS1, and RET fusions were detected in the positive control (pink, HD784). There is no deviation of fusion calls in the negative and positive control samples in all triplicate experiments and all five time points.

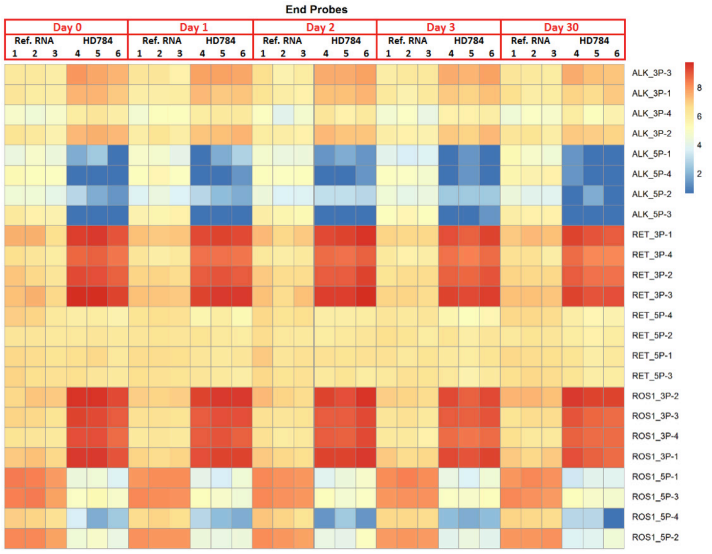



FIGURE 4 Heatmap of imbalanced end probes. Numbers 1-3 are triplicate experiments on the Clontech reference RNA across five time points (Day 0, 1, 2, 3 and 30). Numbers 4-6 are triplicate experiments on the ALK-RET-ROS1 Fusion RNA Reference Standard (HD784) across five time points (Day 0, 1, 2, 3 and 30).

*Heatmap is colored by the log2 count according to the legend.

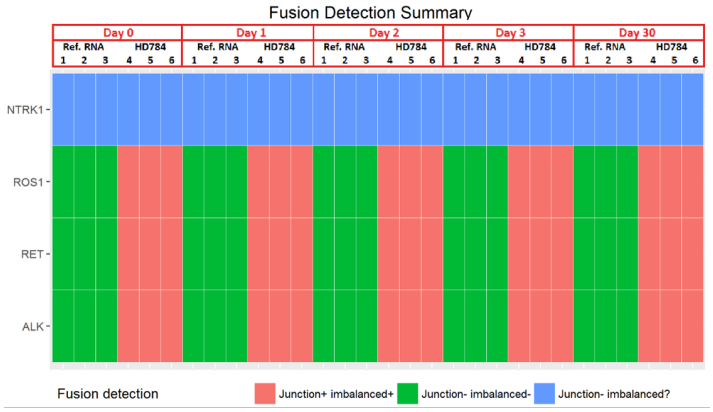



FIGURE 5 Summary of fusion calls. Numbers 1-3 are triplicate experiments on the Clontech reference RNA across five time points (Day 0, 1, 2, 3 and 30). Numbers 4-6 are triplicate experiments on the ALK-RET-ROS1 Fusion RNA Reference Standard (HD784) across five time points (Day 0, 1, 2, 3 and 30).

* Red: Both junction and imbalanced probes were positive.
Green: Both junction and imbalanced probes were negative.
Blue: Junction probe is negative; no imbalanced probe in the assay.

## Discussion

The experiments discussed herein tested conditions of the thaw/freeze cycle (up to three cycles) to determine the effect on the probes and TagSet. The ERCC normalized counts did not differ significantly between Day 0 (no thaw/freeze cycle) and Day 1 (thaw/freeze x 1) in all four housekeeping genes except OAZ1, which had higher counts on Day 1 in one of the fresh frozen tissues ((017723T2(2)). However, the ERCC counts of GAPDH and OAZ1 decreased significantly in all four sample types (p-values < 0.05 by the student t-test compared to Day 0) after more than two cycles of thaw/freeze on Day 2 and Day 3. The decreased counts suggest possible degradation of oligonucleotide probes and/or tags after frequent thaw and freeze. The counts in the Day 30 experiment were higher than the experiment on Day 0. The difference might be attributed to lot-to-lot variations because the TagSet used on Day 30 was a separate lot from the pooled TagSet for Days 0, 1, 2, and 3. However, there is no evidence of oligo degradation as the counts increased slightly in the Day 30 experiment.

The fusion calls did not exhibit a deviation in results across the five time points for the positive and negative control samples suggesting robustness of the lung fusion panel even with partially degraded oligonucleotides and/or tags after two to three thaw/freeze cycles. The results of fusion calls did not deviate from the baseline (Day 0) over the five time points in the negative and positive RNA reference standards.

## Recommendations

The fusion calls were not drastically influenced by thaw/freeze cycles up to three times with long-term storage at -80°C in this experiment. However, we recommend that customers limit the thaw/freeze cycle to one time only to minimize degradation of oligonucleotides and tags in the gene fusion panel based on the evidence of decreased ERCC counts in housekeeping genes with frequent thaw/freeze (Figure 2). It is best practice to aliquot the TagSet and diluted probes in an appropriate amount needed then freeze the remainder at -80°C for future use, one time only. The risk of oligonucleotide and tag degradation will be minimized by limiting the thaw/freeze cycle to one time only.

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