Fusion Gene Analysis

nCounter Elements™

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Fusion Gene Analysis Using nCounter Elements™

Introduction

nCounter Elements™ can be utilized to develop highly multiplexed assays for studying fusion genes in oncology and other fields. This white paper describes a comprehensive solution for analysis of gene fusions that is comprised of two broad design methodologies — 5'/3' imbalance designs and universal junction sequence designs (**FIGURE 1**). Evaluating 5'/3' imbalance enables detection of fusion events without prior knowledge of the partner gene, while junction designs enable detection of unique junction sequences. Combining these design methods enables the development of robust, highly multiplexed assays for fusion gene analysis.

Chromosomal rearrangements can form fusion genes and have an important role in the initial steps and progression of tumorigenesis. Such a gene often has a product with a novel function relative to the two genes that were fused into a single chimeric transcript. In recent years, fusion genes have been found in many hematological and solid tumors, demonstrating that chromosomal aberrations are a common cause of malignancy. An increasing number of fusion genes are being recognized as important diagnostic and prognostic indicators in human cancers, increasing the value of correctly identifying and quantifying the expression of unique fusion events.

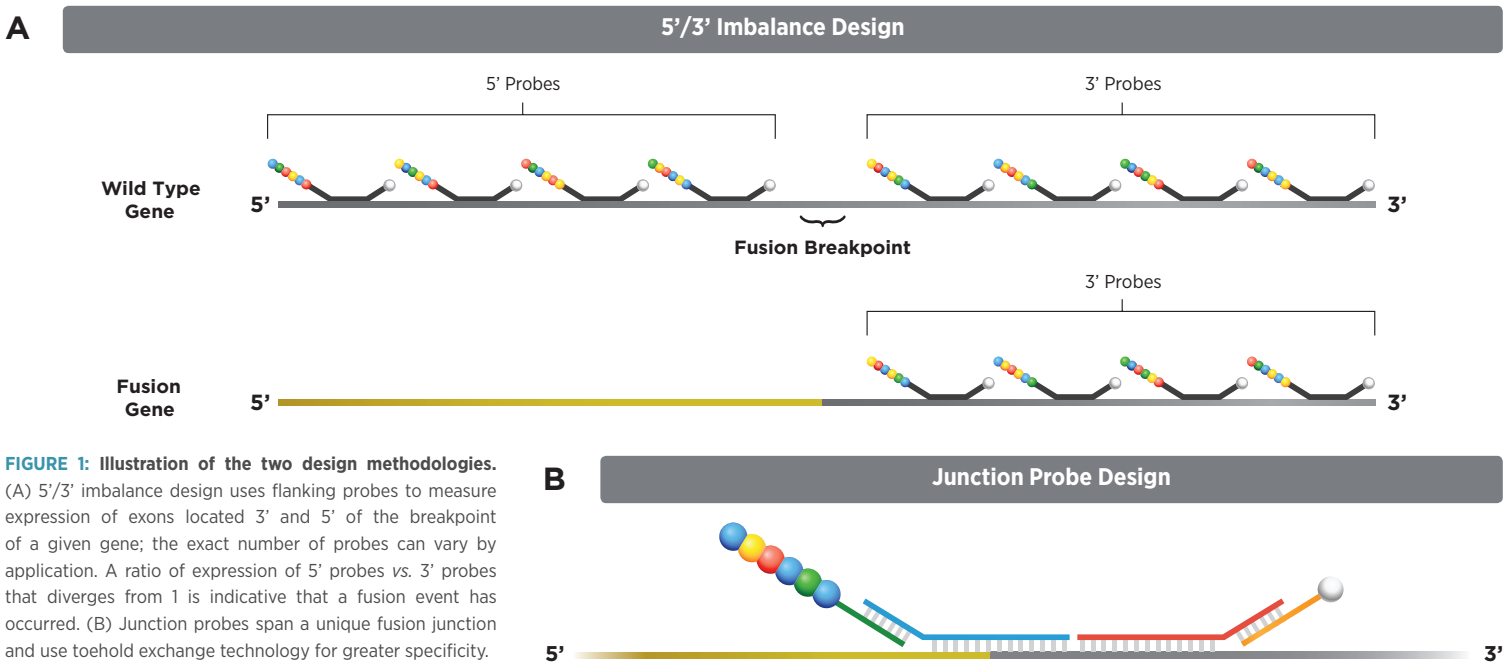

FIGURE 1: Illustration of the two design methodologies. (A) 5'/3' imbalance design uses flanking probes to measure expression of exons located 3' and 5' of the breakpoint of a given gene; the exact number of probes can vary by application. A ratio of expression of 5' probes *vs.* 3' probes that diverges from 1 is indicative that a fusion event has occurred. (B) Junction probes span a unique fusion junction and use toehold exchange technology for greater specificity.

Note: Results may vary depending on assay design, sample input, or other factors.

Overview of nCounter Elements

Elements chemistry uses a common Capture Tag and multiple Reporter Tags supplied by NanoString, which the user customizes through the addition of oligonucleotide probes (Probe A and Probe B for each target) that complement these tags and the targets of interest. Each Probe A recognizes a unique Reporter Tag and its assigned target, while each Probe B recognizes its target and the common Capture Tag. This is distinct from standard nCounter chemistry, in which the Reporter Probe and Capture Probe are manufactured with the target recognition sequences included.

5’/3’ Imbalance Designs

Fusion events can be detected with a flanking nCounter Elements probe design that compares the ratio of gene expression upstream and downstream of the fusion junction (**FIGURE 1A**). Each fusion partner may have promoters with varied strength or activity, and after fusion some exons will be influenced by new promoters (those associated with their fusion partner). The reciprocal fusion gene may even be lost due to an unbalanced translocation event. Therefore, a ratio of 5’/3’ expression for a given gene that diverges from 1 suggests that a fusion event has occurred. This approach can be utilized for discovery of new fusion genes[1] or to develop robust assays for characterization of high-value samples[2,3].

Suehara *et al.* (2012) validated the use of nCounter technology for discovery of novel fusion genes on the basis of 5’/3’ imbalance. They first examined several cell lines as well as samples from patients with lung adenocarcinoma containing known fusions. nCounter results were highly concordant with the results of other established protocols, including fluorescent *in situ* hybridization (FISH), RT-PCR, and immunohistochemistry. In a separate set of samples from patients who tested negative for known oncogenic drivers, Suehara *et al.* used the 5’/3’ imbalance assay to identify novel tyrosine kinase fusions. Screening 90 tyrosine kinases and 3 serine/threonine kinases, two 100-base regions were selected for each gene transcript. A 5’ probe pair targeted a region upstream of the kinase domain exons, and a 3’ probe pair targeted a region within those exons or further 3’. Aberrant 5’/3’ ratios were identified in two cases, involving either *RET* or *ROS1*, and once detected these novel fusions could be further characterized.

Lira *et al.* (2013) later developed a similar assay to characterize *EML4-ALK* fusion variants, which have been identified in approximately 5% of non-small-cell lung carcinomas and are highly responsive to crizotinib (Xalkori®, Pfizer). Multiple upstream and downstream probe sets were used to develop a very robust assay that performs well compared to standard clinical assays (FISH and IHC). The resulting approach was later expanded to include simultaneous detection of fusions involving *RET* or *ROS1*[3], which may also respond to crizotinib and encompass an additional 2% of non-small cell lung carcinomas.

Data provided to NanoString by Kindstar Global* demonstrates the effectiveness of a 5’/3’ imbalance design for detecting fusion events involving the *ALK* gene without knowledge of the fusion partner. For this assay, 4 probes were placed upstream and 4 probes were placed downstream of the fusion junction. **FIGURE 2** shows the counts generated by these probes for a wild type *ALK* and an *ALK* fusion sample. In the sample containing an *ALK* fusion, there is a clear imbalance in the expression levels of the 5’ probes compared to the 3’ probes. *ALK* fusion samples exhibited significantly greater expression of the 3’ exons only, indicating that a fusion event placed these exons under control of a different, more active promoter.

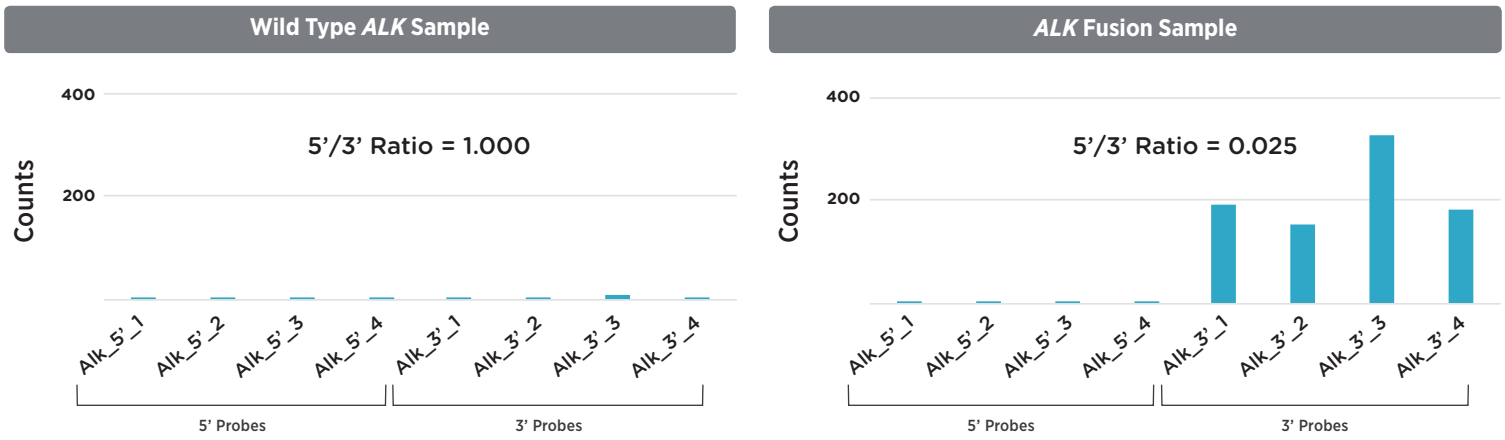

FIGURE 2: Differential expression of wild type and fusion *ALK* exons. Wild type *ALK* and *ALK* fusion samples were each assayed using four probes upstream and downstream of the fusion junction. Expression of the 3’ *ALK* exons is significantly altered in the *ALK* fusion.

Note: Results may vary depending on assay design, sample input, or other factors.

* Learn more about Wuhan Kindstar Diagnostics Co., Ltd. (“Kindstar Global”) by visiting **http://www.kindstarglobal.cn/aboutPro.html**.

Universal Junction Sequence Designs

nCounter Elements technology can also be used to characterize specific fusion junctions (**FIGURE 1B**) by utilizing a new technology, toehold exchange probes, described by Zhang *et al.* (2012). NanoString can design highly specific probes for virtually any fusion junction sequence as well as other splice variants of interest. Experiments utilizing this universal junction sequence design include an additional oligonucleotide in the hybridization; this adds a single pipetting step to the hybridization protocol for preparing 12 samples. Hundreds of junction probes can be multiplexed together in a single reaction. Toehold exchange designs that span the fusion junction can be incorporated into the existing user-supplied probes used with nCounter Elements reagents, enabling detection and discrimination of unique fusion events.

A multiplexed assay containing probes for 7 distinct *BCR-ABL* fusions was run using total RNA from two cell lines, SUP-B15 and K562, each known to contain a specific fusion gene (**FIGURE 3**). Human reference RNA was also included in the assay as a negative control.

Robust counts were obtained only for the correct fusion in each cell line. No cross-hybridization was detected for any of the incorrect probes, *i.e.*, those that shared one fusion partner but not the other. This indicated that the assay afforded highly specific detection of unique junction sequences. Furthermore, the assay was able to measure the relative expression of each fusion gene in the two cell lines.

The nCounter Analysis System does not require amplification, and data exhibit an extremely high signal-to-noise ratio. Samples with as little as 10% of fusion-containing RNA can be detected. To demonstrate this high sensitivity, 5 ng of total RNA extracted from several fusion-expressing cell lines was mixed with Human Reference RNA in a 1:10 ratio, and 50 ng of the combined RNA sample was run in a multiplexed nCounter Elements fusion assay (**FIGURE 4**).

The correct fusion was clearly detected in each cell line. Fusion probe counts ranged from approximately 50 to 250, well above the calculated background threshold level of 9 counts. Cross hybridization rates of all other fusion probes from the same fusion families were below background (data not shown).

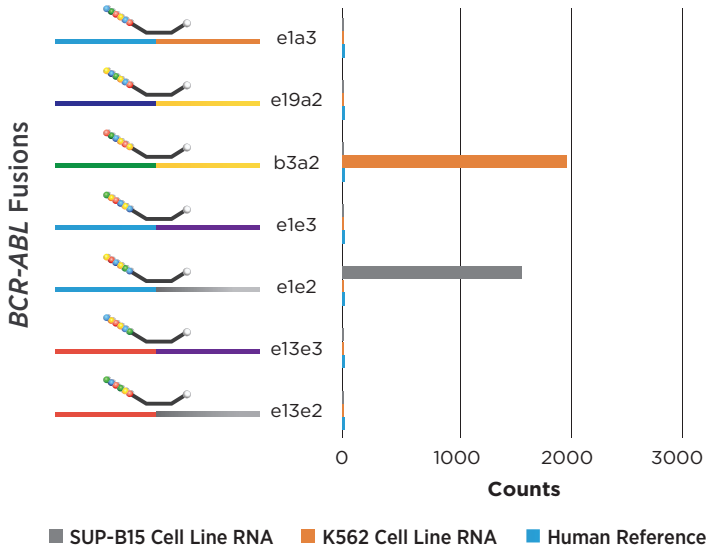

FIGURE 3: Multiplexed analysis of *BCR/ABL* fusions. Robust counts were obtained only for the correct fusion in each cell line, using human reference RNA as a negative control.

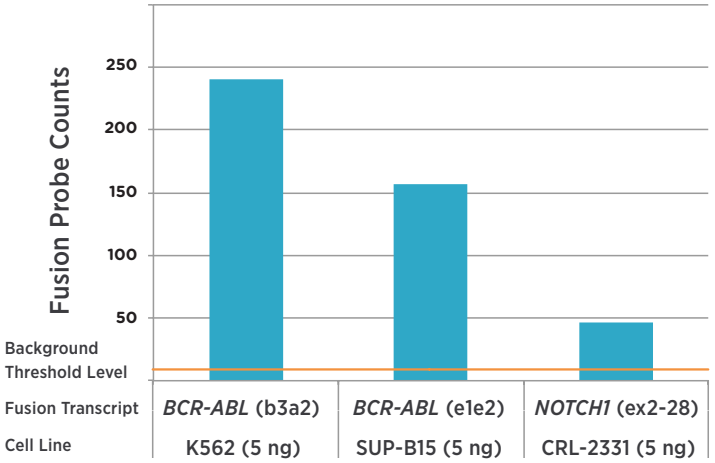

FIGURE 4: Fusions detected in the presence of 90% normal RNA. Counts for each fusion target were well above the background threshold even when RNA from fusion-containing cell lines was mixed with human reference RNA.

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Toehold Exchange Technology

Traditional probe designs for fusion junctions risk cross-hybridization with sequences that are only partially complementary. In the absence of a perfect complementary target, single-stranded probes can exhibit low-level einteractions with other, highly similar targets. This phenomenon often impacts gene fusion families because a probe for one family member may match either the 5' or 3' exon at the fusion junction of a different family member. To detect unique fusion junctions with nCounter Elements, the design of Probe A — one of two user-supplied oligonucleotide probes — is modified using toehold exchange technology, and a protector oligonucleotide is added that provides a perfect complement to Probe A and competes with off-target interactions. These modifications to the 5' end of each Probe A enable specific detection of exon-exon junction sequences with very low cross-hybridization (typically less than 0.5%) to transcripts containing either exon alone.

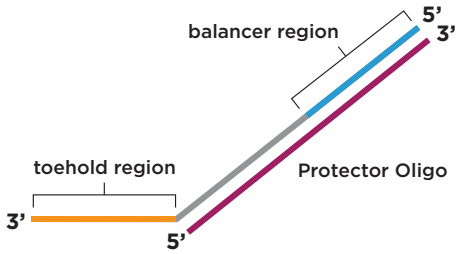

FIGURE 5: Diagram of toehold exchange probe design. Probe A forms a partially double stranded complex with a protector oligonucleotide. This complex contains a single-stranded toehold region, a double stranded balancer region, and a double-stranded region in the middle complementary to both the protector oligonucleotide and the target. (The Reporter Tag is not shown in this illustration.)

Combining the protector oligonucleotide with Probe A forms a partially double-stranded complex, which contains one double-stranded target-non-specific region (the "balancer"), one double-stranded target-specific region, and one single-stranded target-specific region (the "toehold"; **FIGURE 5**). Toehold and balancer regions are designed to be similar in length, base composition, and thermodynamic binding strength. As a result, the difference in the hybridization free energy between the protector:probe complex and the target:probe complex approximates zero, and an equlibrium is ultimately reached between hybridization with the protector oligonucleotide or the true target (**FIGURE 6A**) with an average 50% of probes bound to the target. In the presence of other, non-perfect matches, the difference in the hybridization free energy is much less favorable even if the target is partially complementary (*e.g.*, wildtype transcripts or another fusion variants). The vast majority of probes in such cases will remain bound to the protector oligonucleotide (**FIGURE 6B**).

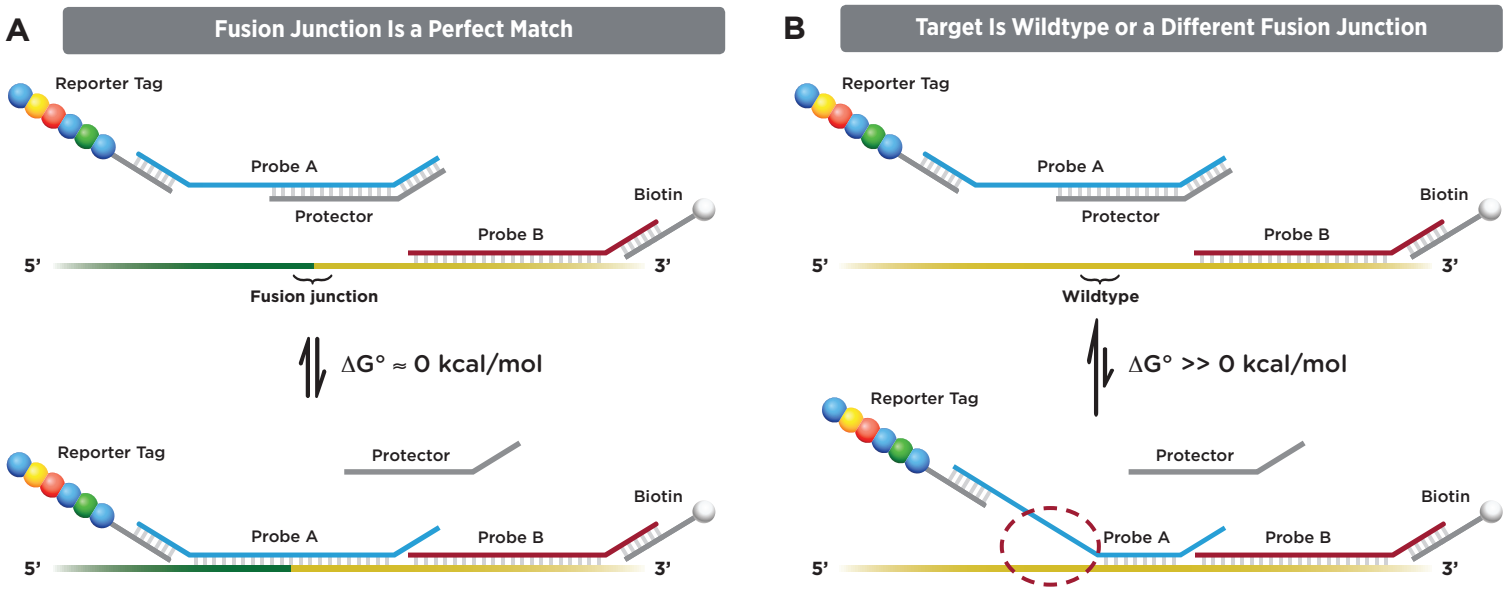

FIGURE 6: Equilibrium established between the Elements Fusion probes and the hybridization complex. (A) The difference in the hybridization free energy between protector:probe and target:probe is roughly zero, resulting in a large proportion of target:probe complexes. **(B)** Hybridization between the protector-bound probe and a non-specific sequence (indicated by a red circle) is highly unfavorable even if that sequence is partially complementary.

In the presence of the intended target, Probe A can undergo strand exchange and hybridize to the biological target instead of the protector oligonucleotide. Hybridization of Probe A to the desired target is initiated at the 5' single-stranded toehold region, proceeds through a branch migration process, and is completed *via* spontaneous dissociation of the 3' balancer region to release the single stranded protector (**FIGURE 7**). While the toehold region is part of the target-specific portion of Probe A, the balancer region is carefully designed from a database of random unique sequences with no homology to known sequenced species indexed by the External RNA Controls Consortium (ERCC). This design results in highly specific detection of correct targets and makes off-target hybridization events very energetically unfavorable.

Conclusion

Highly multiplexed assays for detecting gene fusion events can be developed with nCounter Elements. Assays based on this approach are capable of accurately detecting and discriminating fusion events (including unique fusion junctions) in FFPE and fresh frozen patient tissue as well as cell lines. Evaluating 5'/3' imbalance enables detection of fusions involving a given gene without knowledge of its partner gene(s). Junction probe designs enable the specificity required for detecting unique fusion junctions and utilize toehold exchange technology to overcome the risk of cross-hybridization with similar sequences. Combining both methods in a single assay can provide orthogonal approaches to detecting the known fusion and the ability to detect novel fusion events.

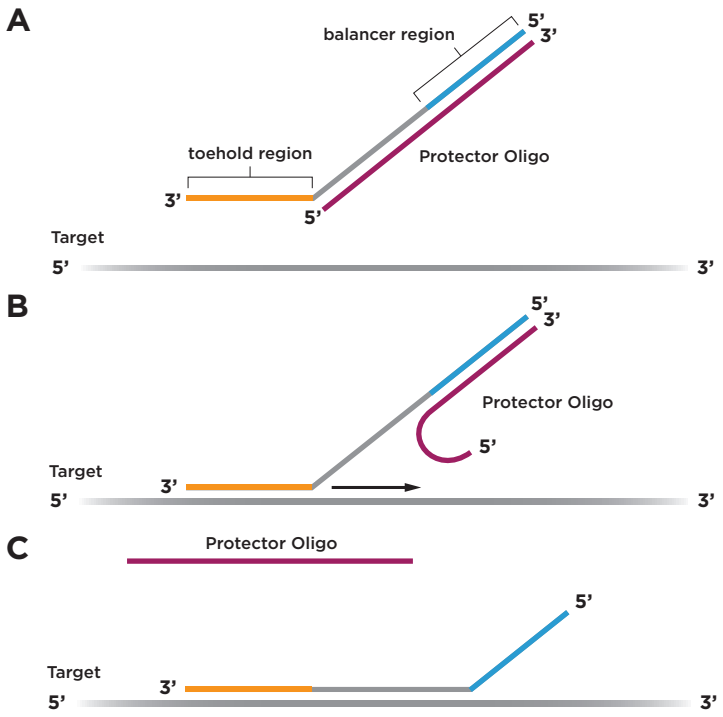

FIGURE 7: Toehold probe hybridization. (A) The target nucleic acid initially hybridizes with the target-specific, single-stranded toehold region. **(B)** Probe-target hybridization continues through branch migration (indicated by the black arrow) as the protector oligo dissociates. **(C)** The balancer region is becomes single-stranded after the probe hybridizes with the target. (The Reporter Tag is not shown in this illustration.)

References

1. Suehara *et al.* (2012) Identification of KIF5B-RET and GOPC-ROS1 fusions in lung adenocarcinomas through a comprehensive mRNA-based screen for tyrosine kinase fusions. *Clin Cancer Res* 18(24):6599-6608.
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4. Zhang *et al.* (2012) Optimizing the specificity of nucleic acid hybridization. *Nat Chem* 4(3):208-214.

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