Tools for Splice Modulation Drug Discovery: Spinach™ Splice Sensor Platform

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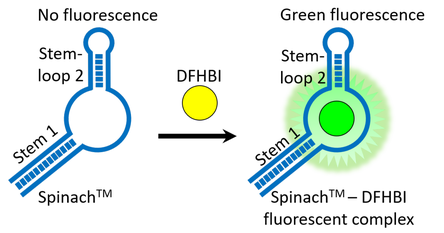
ABSTRACT

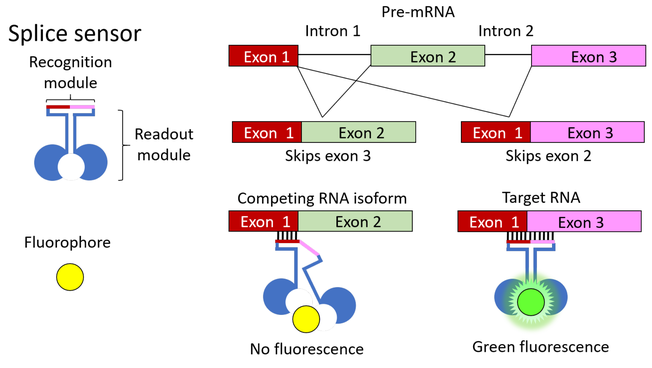
RNA splicing plays a central role in the generation of proteome diversity and in gene regulation. Splicing affects cellular processes, such as cell-fate and differentiation, acquisition of tissue-identity, and organ development. Consequently, defects in splicing are linked to many diseases, including spinal muscular atrophy, Duchenne muscular dystrophy, Parkinson's disease, and several types of cancer. Despite the significance of splicing in these diseases, drug discovery efforts targeting them are far and few. A critical bottleneck for such efforts is the lack of robust high-throughput assay tools to monitor endogenous spliced RNAs in the cell. Even though there are excellent tools such as RT-qPCR and RNA-seq to study RNA splicing, they are cost-prohibitive at this scale and are not readily adaptable for high-throughput screening (HTS) due to their complex and time-consuming methodology. While splice mini-gene offers the advantage of higher throughput, it lacks the ability to monitor the endogenous target RNA due its artificial design. Thus, there is an unmet need for simple and robust HTS-ready assay tools to monitor RNA splicing.

Addressing this need for robust assays for splice modulation drug discovery, Lucerna has developed an easy-to-use, HTS-ready splice sensor platform that can specifically detect any splicing event of interest. We have developed splice sensors against a variety of splicing targets, including pyruvate kinase isoforms M1 (PKM1) and M2 (PKM2), ciRS-7 circular RNA, and splicing targets involved in neurodegenerative diseases. The splice sensor platform displays rapid response times (<30 min), high selectivity (300-fold specific fluorescence), excellent sensitivity (can detect 10% change in splicing), and extended readout window (16h). In addition, the splice sensor platform can significantly reduce the false positives and error rates compared to other competitor assays due to its simpler homogenous workflow. In summary, the Spinach™ splice sensor platform offers robust, homogenous, and customizable assays for high-throughput splice modulating drug discovery.

ASSAY CONCEPT



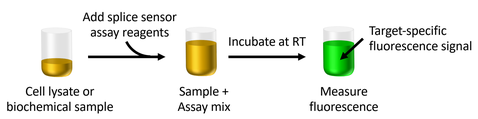
Spinach™ is a RNA mimic of the green fluorescent protein (GFP). The Spinach™ technology consists of RNA aptamers that bind and turn on the fluorescence of otherwise non-fluorescent dyes, such as DFHBI. By altering the aptamer sequences and dye structures, Spinach™ aptamers can be tuned to emit different colors. Spinach™ is genetically encodable with proven utility for imaging RNA in living cells and measuring cellular metabolites in HTS assay format.



Splice sensor is comprised of a readout module that is composed of a Spinach™ and a recognition module that targets the spliced RNA of interest. The recognition module is comprised of two RNA probes (dark red and pink) that are complementary to the exon sequences flanking the target splice site, i.e. exon 1 (dark red) and exon 3 (pink).

In the presence of target RNA, the two RNA probes of the recognition module bind the contiguous exon 1 and exon 3 splice junction. This enables the folding of Spinach™, its binding to fluorophore, and the emission of fluorescence.

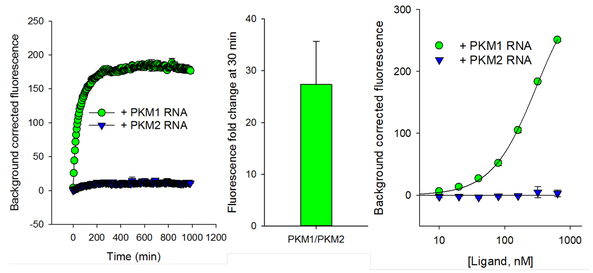
In contrast, only one of the RNA probes of the recognition module can bind the competing RNA (exon 1). This weak binding is unable to fold the Spinach™ into conformation that can bind the fluorophore and emit fluorescence..



The homogenous assay format of the Spinach™ splice sensor assay makes it easily adaptable for high-throughput screening.

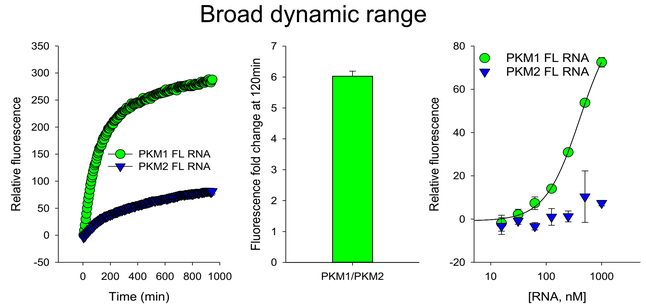
CASE STUDY: PYRUVATE KINASE

Fast and selective response



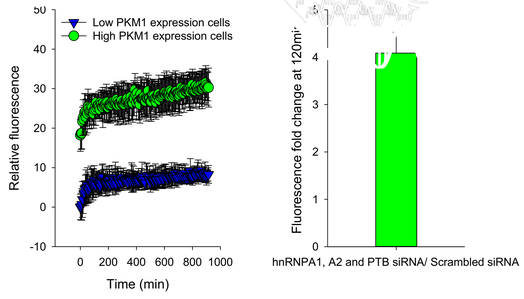
PKM1 splice sensor responded to the presence of PKM1 RNA with a rapid increase in fluorescence signal that was stable for over 16h. With nearly 27-fold PKM1-specific fluorescence and the ability to discriminate between PKM1 vs PKM2 as low as 10 nM, the PKM1 splice sensor demonstrated high selectivity and sensitivity.

Broad dynamic range



PKM1 splice sensor displayed a rapid and selective ~6-fold increase in fluorescence in the presence of PKM1 full-length (FL) RNA. The sensor can detect targets over a broad range of concentrations and was sensitive as low as ~2.5nM of PKM1 FL RNA.

Signal is increased when PKM1 RNA is suppressed

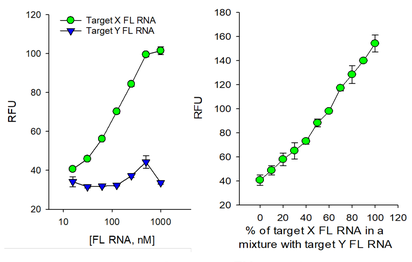


PKM1 splice sensor displayed a ~4-fold selective increase in fluorescence signal when mixed with cells expressing higher PKM1 levels, corroborating with independent qPCR results. Further, the fluorescence signal remained stable for over 14h in the presence of the proprietary stabilization reagent.

Similar to the PKM1 splice sensor, the PKM2 splice sensor also demonstrated a rapid increase in fluorescence in the presence of oligo RNA and PKM2 FL RNA (not shown) . In addition to demonstrating a stable fluorescence signal for over 16h, the PKM2 sensor showed a selective ~4-fold increase in fluorescence in the presence of its target, PKM2 FL RNA.

CASE STUDY: CNS TARGET

Portable sensor design

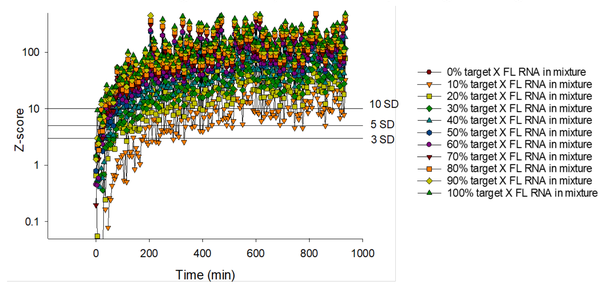


Due to the modular design of the Spinach™ platform, a splice sensor directed against a confidential CNS target X (external collaboration) was developed by switching to a recognition module specific for target X. Its alternative splice form, target Y, was used as a control.

Target X splice sensor shows a highly selective and dose-dependent increase in fluorescence in the presence of target X and is able to discriminate between 16nM of target X and target Y FL RNA, indicating high sensitivity. A statistically significant difference in fluorescence signal ($p<0.05$) observed even with a ~10% change in target X FL RNA in a mixture with target Y FL RNA.

This work was supported by SBIR funding from NCI.

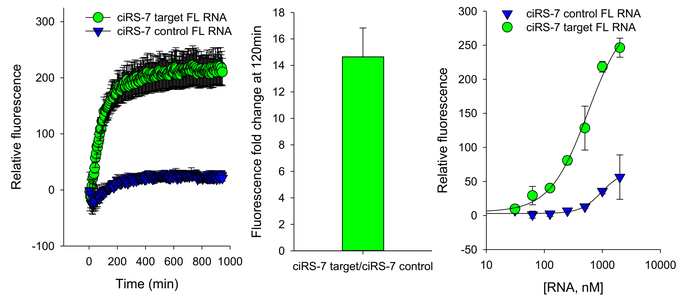
Detects significantly smaller splicing changes



Even a 10% increase in the target X FL RNA in a mixture with target Y elicits a significant fluorescence response from target X splice sensor.

CASE STUDY: CIRCULAR RNA

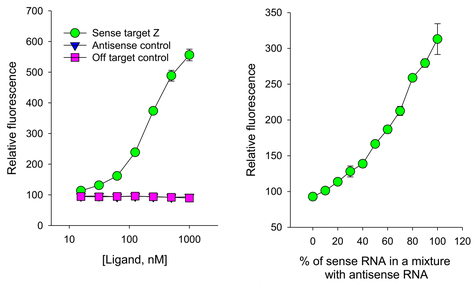
Detects back-spliced transcripts



To demonstrate the versatility of the splice sensor platform, we created a splice sensor targeting the back-splice junction of ciRS-7, a circular RNA (circRNA) well-known to be involved in cancer. ciRS-7 sensor displayed a rapid increase in fluorescence in the presence of ciRS-7 target FL RNA. In addition to displaying a selective ~14-fold increase in fluorescence in the presence of ciRS-7 target FL RNA, the ciRS-7 sensor can detect targets over a broad range of concentrations and was sensitive to as low as ~62.5nM of ciRS-7 target FL RNA.

CASE STUDY: INTRONIC RNA

Selectively differentiates sense and antisense introns



In an adaptation of the splice sensor platform, we developed intronic sensors targeting the sense and antisense strands of a confidential intron target Z (external collaboration). The sense strand intronic sensor (SS sensor) showed a highly selective and dose-dependent increase in fluorescence in the presence the target and was sensitive as low as 16 nM of the target. When the SS sensor was added to a mixture of varying ratios of target Z intron sense : antisense strands, a statistically significant difference in fluorescence signal ($p<0.05$) was observed even with a ~10% change in target Z sense RNA in the mixture. Similar results were obtained for the intronic sensor targeting antisense strand of target Z (not shown).

SUMMARY

Fast, selective, stable	Read in 30 min, robust target-specific fluorescence, >16h readout window
Customizable, target any RNA isoform of interest	Detect endogenous mRNAs, protein free, no artificial systems
Super detection resolution	Can detect as low as 10% splicing changes
Versatile, HTS adaptable, mix-and-read	Biochemical and cell-based applications, multiplex capability

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