Small molecule modulation of RNA splicing for drugging “undruggable” targets: taking advantage of Evotec’s high-throughput RT-qPCR platform

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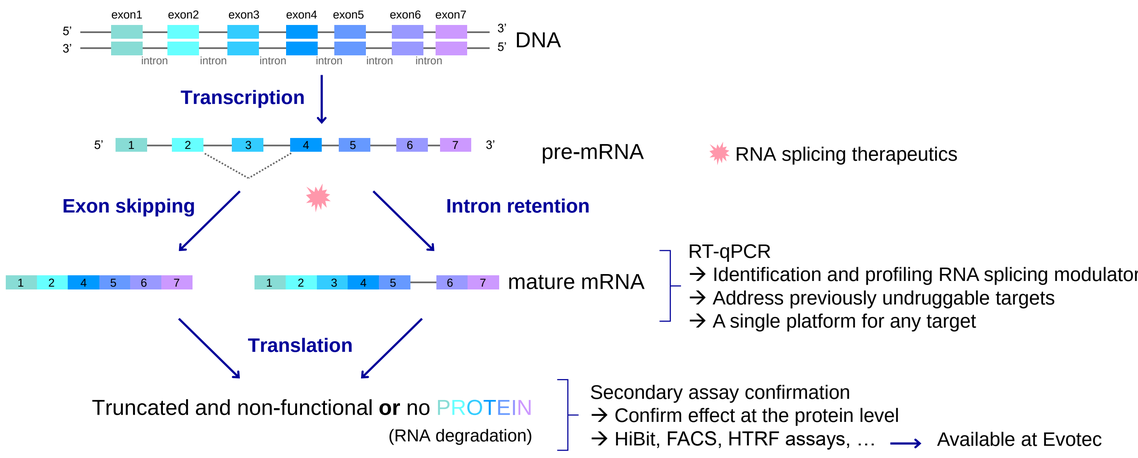
Only a limited number of disease-relevant protein targets are considered druggable by small molecules. Thus, it remains critical to find alternative strategies for “undruggable” targets. In most cases, the goal is to decrease the amount of the target to abolish its activity. Two main strategies are favored: induction of the target degradation or inhibition of the target expression. This latest approach can be reached by decreasing the messenger RNA (mRNA) coding for the target. In multicellular eukaryotes, introns are removed from pre-mRNA and exons are stitched together to produce mature mRNA transcript, used downstream as a template for protein translation. This essential step, called pre-mRNA splicing, is a key regulatory step in gene expression.

There are currently two main classes of validated RNA-splicing therapeutics: antisense oligonucleotides (ASOs) as well as small molecules. Lately, interfering with mRNA splicing has become a new avenue to drug ‘undruggable’ proteins. The goal is to modulate expression of pathogenic proteins by generating alternatively spliced RNA isoforms. This will lead to production of truncated and non-functional proteins or RNA degradation by nonsense-mediated mRNA decay (NMD) pathway.

Quantitative reverse transcription PCR (RT-qPCR) is the gold standard to identify and profile RNA splicing modulators. At Evotec, we can leverage our fully automated multiplex RT-qPCR platform to accelerate identification of small molecule or ASO modulators of RNA splicing. RT-qPCR is compatible with the most advanced cell models such as patient derived cells, primary cells, induced pluripotent stem cells or organoids. Our current optimized RT-qPCR process, miniaturized to 384-well plate format, allows to screen up to 15,000 compounds per day and to follow up to 4 genes in a single well by multiplexing. It thus represents a cost-effective alternative to reporter-gene assays. In addition to process automation, we present a highly scalable and automated data analysis workflow using Genedata Screener software. This software is used for rapid characterization of large sets of molecules to identify splicing modulators, highlight cytotoxic compounds and estimate compound activity from changes in concentration-response curves. Finally, gene expression hits are typically confirmed via different protein-based assays, such as HTRF or HiBit tagging system. Evotec has a strong track record at developing customized RT-qPCR assays and supported screening and profiling activities on dozens of RNA splicing targets representing several million compounds.

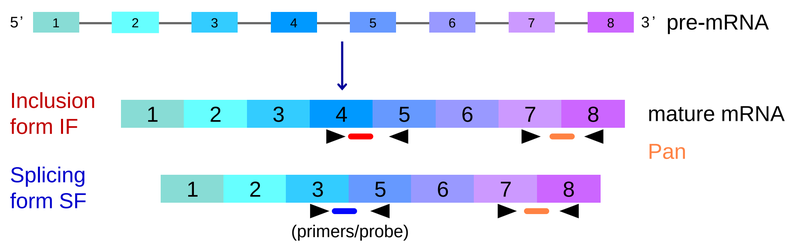
Pre-mRNA splicing modulation as a therapeutic approach

Principle of screening for mRNA splicing modifiers



RT-qPCR, a method of choice for characterization of alternative RNA splicing

Goal: Identify compounds inducing skipping of a specific exon



RT-qPCR advantages	RT-qPCR limitations
Drug « undruggable » targets	Specific equipment and data analysis workflow
Multiplexing (up to 3 variants + HKG)	Lower throughput (384w and >1h reading/plate)
Use unmodified cellular models	Variability / Costs

Multiplexing (up to 4 genes/well)

Tetraplex (2 splice isoforms [IF, SF] + Pan + ref. gene [HKG])

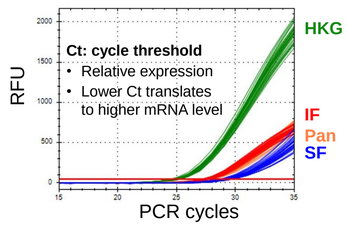


Figure description:
Amplification of up to 3 variants and a housekeeping gene/well

High throughput RT-qPCR screening

Accelerated workflow at Evotec Toulouse: One step RT-qPCR

Cell lysis → **cDNA production + qPCR amplification**

- Same sensitivity and specificity than two-step RT-qPCR
- Expeditive process / less error prone
- Low volumes (2µL) / Lower cost per sample
- Highly compatible with HTS automation

Figure description: We have optimized our RT-qPCR HTS protocol to provide maximal performance and throughput while reducing assay volumes, costs and risk of errors. RT-qPCR is performed directly from cell lysates as a one-step reaction

Fully automated RT-qPCR platform at Evotec Toulouse

Unique ET3 platform _ A miniaturized, accelerated and cost-optimized RT-qPCR platform

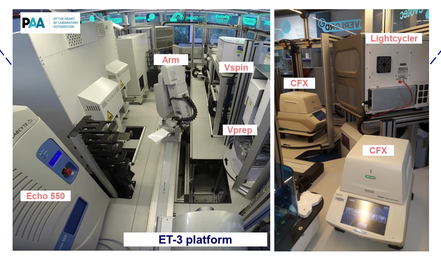


Figure description:
Established screening processes for 384-well plate assays. Automated screening system consists of incubators, washer/dispensers, Echo550 acoustic liquid handler, 4 real time thermocyclers 384w

RT-qPCR screening workflow

Overview of Evotec’s RT-qPCR screening cascade

Small molecule screening libraries
- Evotec collection (450K compounds)
  - 20K fragments
  - 30K natural products
- Aptuit collection (400K compounds)
- External customer collections

Cell line optimization and scale up ↔ **RT-qPCR assay development**
Target + housekeeping gene(s) for screening

Assay transfer/miniaturization and robotic adaptation
384w format

RT-qPCR pilot screening
~5K compounds — 2 compound concentrations n=3

RT-qPCR primary screening
up to 400K compounds — Multiplex reactions n=1

Hit confirmation — n=3 → **QC**

Dose-response evaluation → **MedChem review**

Molecular profiling
more genes, TLDA, Omics …

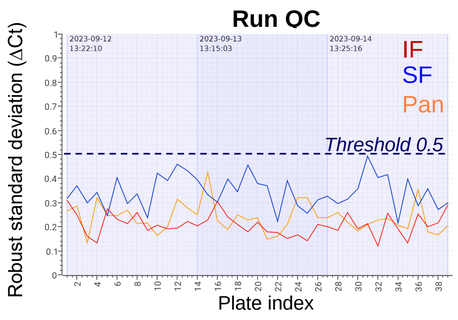
Counter-screen Orthogonal screen

Secondary assays
protein level, HTRF

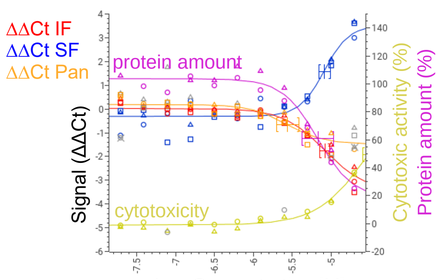
Integration into Evotec drug discovery platform

Data analysis using Genedata Screener

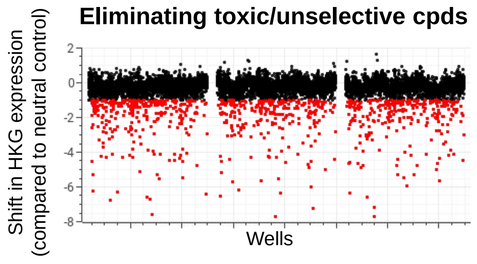
Run QC



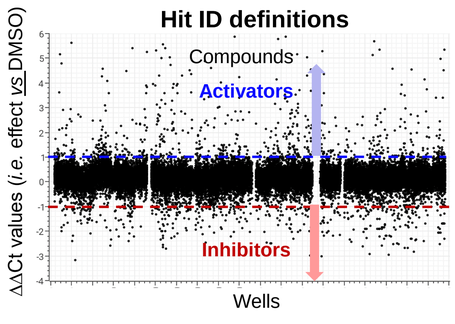
Dose response curve fitting



Eliminating toxic/unselective cpds



Hit ID definitions



Comparative analysis between two targets

Selectivity towards Target 1 and Target 2

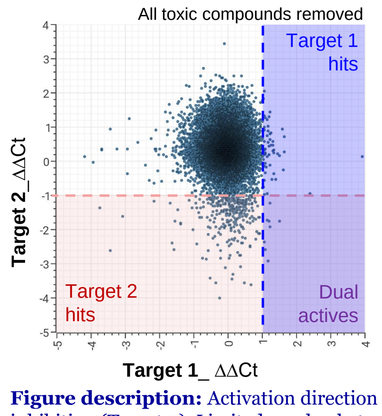


Figure description: Activation direction (Target 1) *vs* inhibition (Target 2). Limited overlap between the two targets: in 31K compound library, only one compound is active and confirmed on both targets

RT-qPCR workflow and track records

HTS campaigns performed at Evotec Toulouse on RT-qPCR platform

Our current optimized RT-qPCR process, miniaturized to 384-well plate format allows to screen:

- Up to 15,000 compounds per day
- Up to 4 genes (3 target genes + 1 reference gene) in a single well

Since 2016, a large number of assays have been developed:

- 17 RT-qPCR screening campaigns
- 2.2 millions of compounds tested in total (130K compounds/screen in average)

Conclusions

- RNA target is an appreciated alternative to many proteins that are still considered challenging therapeutic candidates
- Evotec’s RT-qPCR platform allows to quantify cellular mRNA, or non-coding RNA, following treatment with drug candidates, e.g. small molecules, antisense oligonucleotides, interfering RNAs or proteins
- RT-qPCR is compatible with all cell types, adherent or in suspension, and even with the most advanced models such as induced pluripotent stem cells or organoids, allowing to develop assays in physiologically relevant models
- A high throughput screening approach to gene expression profiling with established processes for 384-well plates allowing to screen up to 15,000 compounds per day on 4 different genes
- The Evotec platform constitutes a powerful tool for the analysis of splicing modulation, making it possible to measure in parallel the modulation on the WT and cryptic form of a gene of interest and to monitor the effects of toxic compounds via the reference gene
- Screening analysis with Genedata, specifically developed and optimized for the treatment of HTS data flow and adapted for quantitative PCR, has been standardized and is routinely used at Evotec during screening campaigns

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