
Methods and Platforms for the Quantification of Splice Variants' Expression

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Abstract. The relatively limited number of human protein encoding genes highlights the importance of the diversity generated at the level of the mRNA transcripts. As alternative RNA splicing plays a key role in mediating this diversity, it becomes critical to develop the tools and platforms that will deliver quantitative information on the specific expression levels associated with splice isoforms. This chapter describes the constraints generated by this global transcriptome analysis and the state-of-the-art techniques and products available to the scientific community.

1 Introduction

Gene profiling assays have become a standard approach to study physiological or pathophysiological processes as genes that are identified as being up- or down-regulated will point to distinct and relevant signaling pathways. Such analyses usually rely on technologies, such as RT-PCR Differential Display, Subtractive Hybridization Libraries, SAGE, and pan-genome microarrays that are often described as being able to scan the whole gene expression space. In fact, these technologies are not well-suited to detect and/or quantify isoforms generated by alternative RNA splicing, as they are all based on the 3' regions of mRNAs. It is now well admitted that alternative splicing is one of the main factors responsible for generating expression diversity at the mRNA level. Large-scale bioinformatics analyses and in-depth transcriptome analysis from selected chromosomes have reported or suggested high rates of alternative splicing, with over 60% of all human genes expressing multiple mRNAs (Lander 2001; Modrek and Lee 2002; Kampa et al. 2004). Alterations of the splicing patterns, by mutation or defects at the level of the spliceosome machinery, can lead to profound cellular deregulations and be the cause of human diseases (Faustino and Cooper 2003; Garcia-Blanco et al. 2004; see also Cooper et al., Stamm et al., Wirth et al., Graveley et al. in this review). *Cis-* or *trans-*acting mutations at the pre-mRNA level can affect RNA splicing and can also alter the ratio between the expression levels of two or more isoforms. Such ratios are thus critical parameters that need to be quantified as some isoforms can exert dominant negative or positive effects. For example, the

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ratio of Bcl-xS/Bcl-xL can determine whether cells undergo apoptosis (Rohrbach et al. 2005), and alteration of this ratio can change the ultimate fate of the cells (Taylor et al. 1999; Akgul et al. 2004). The importance of tau isoforms' ratios in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17; de Silva et al. 2003; Umeda et al. 2004) and of SMN isoforms' ratios in SMA (Feldkotter et al. 2002; Harada et al. 2002) constitute two other well documented examples.

Alternative splicing is also likely to provide the basis for two important medically related applications that will heavily rely on quantitative data. First, as alternative splicing can be deregulated in human diseases, it has the potential to generate novel biomarkers for disease detection and/or screening, prognosis, or treatment outcome. In addition, splice variants' expression ratios could also provide better biomarkers than the mere absolute levels of either variant as it has been demonstrated in the association between acetylcholine esterase splice variants and treatment outcome for Alzheimer's disease patients (Darreh-Shori et al. 2004). It can be anticipated that, as most diseases are complex and heterogeneous, a panel of individual markers will be required to optimize sensitivity and specificity, enhancing the critical issue to properly monitor their expression.

Second, splicing deregulations in human pathologies can point to altered or defective signaling pathways. Traditional pharmacology targeting selected gene products within these highlighted cascades could next be developed to generate, for instance, specific receptor agonists or enzyme inhibitors to somehow "mend" the defective pathway(s).

Novel strategies based on the correction of the deregulated splicing event itself at the RNA level are now being more and more documented. Whether such approaches rely on small molecular weight compounds (Slaugenhaupt et al. 2004; Solier et al. 2004; Soret et al. 2005; Tazi et al. 2005; see also Wirth et al. and Soret et al. in this book), antisense oligonucleotides targeting splice sites or regulatory regions (Sierakowska et al. 1999; Kalbfuss et al. 2001; Bruno et al. 2004; Scaffidi and Misteli 2005; see also Wilton et al in this review), more complex chimeric entities (Suter et al. 1999; Cartegni and Krainer 2003), or through gene therapy vectors (Goyenvalle et al. 2004), their performances will heavily depend upon the availability of appropriate platforms to quantify their efficacies and to adequately monitor their specificities vis-à-vis other splice events.

How relevant is a splice isoform? Has it been generated simply by leakage of the spliceosome machinery? Is it likely to bear a functional impact? The availability of robust and high-throughput tools to scan and quantify the whole transcriptome, including splice variant will clearly empower the scientists to resolve some of these questions. These tools and technologies will be described in this chapter. This review will mainly focus on the technologies available to detect and quantify the expression of known or predicted splice variants at the RNA level. Known or predicted variants

indicate that nucleotide sequence information is available to constitute the basis of the measurement of the expression level. Obviously, if an isoform mRNA gets properly translated, its product can be detected with traditional immunological assays and this will be briefly discussed at the end of this review.

2

General Principles: Specificity is the Key Issue

Alternative splicing produces an extra layer of complexity when dealing with specificity. Not only the selected assay needs to be able to discriminate from other genes that may be homologous, but it needs to discriminate the variant from other isoforms. Indeed, the co-expression of several splice variants in any given biological sample will be the rule more than the exception. This represents a technical challenge as isoforms will share a high degree of homology. The different types of splicing events will dictate the strategy that needs to be developed (Fig. 1). Insertional events such as intron retention (IR), novel alternative exons (NE), or certain uses of 5' or 3' cryptic sites (3' or 5' ASD/ASA) will generate additional sequence

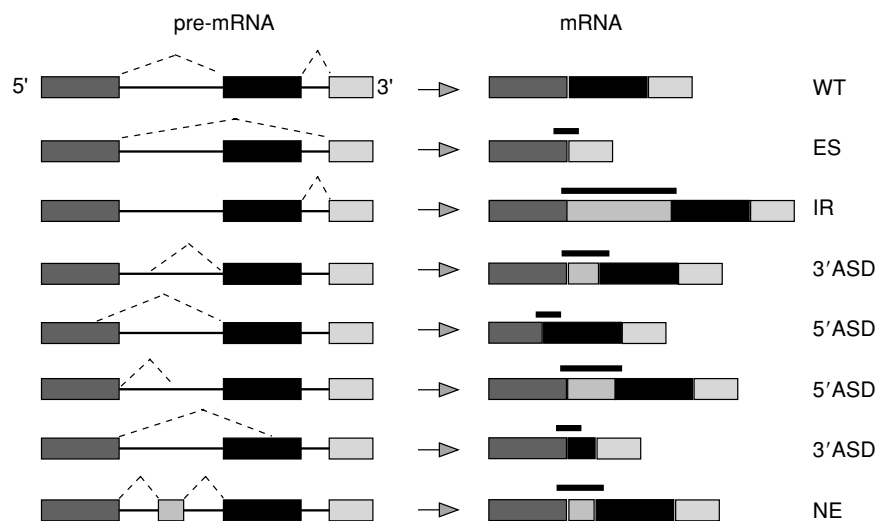


Fig. 1. Types of alternative splicing and effects at the mRNA level. *WT*: wild-type, *ES*: exon skipping, *IR*: intron retention, *ASD*: alternative splice donor (3' and 5'), *ASA*: alternative splice acceptor (3' and 5'), and *NE*: novel exon. The *black bar* above each *mRNA* isoform delineates the specific sequence created by the splicing event. Exonic and junction PCR primers or microarray probes can be designed in those sequences to monitor the alternative isoforms

information as compared to a wild-type form. Such sequences could serve as the basis for the required specificity. Deletion events caused by exon skipping events (ES) or by the remaining uses of cryptic splice sites (3' or 5' ASD/ASA) will not provide such novel sequences. In this case, one can only rely on novel junction sequences to discriminate from the wild-type counterpart.

These general principles apply to the techniques that will now be described. They have been classified into two groups, low/medium throughput and high throughput.

3

Low/Medium Throughput Techniques

Until the introduction of PCR, the quantitative analysis of alternative transcripts was limited to technologies such as northern blotting or ribonuclease protection assays, both having severe limitations. Northern blotting is very time-consuming, requires a large amount of RNA, is only suitable for highly expressed transcripts, and may not be able to differentiate close isoforms (Streuli et al. 1987). RNase protection assay is also a heavy technique, more efficient to monitor small sequence variations but very limiting for low abundant transcript quantification (Saccomanno et al. 1992). The introduction of amplification steps in quantification methods permitted one to overcome the limitations associated with these conventional methods.

3.1

RT-PCR-Based Platforms

Whether they are splice variants or not, the successful analysis of transcripts by qualitative, semi-quantitative, or quantitative RT-PCR is highly dependent on several parameters, including RNA quality, reverse transcription, reagent dispensing, and the selection of normalization methods/genes. Appropriate guidelines and processes to avoid potential pitfalls have been described in the following reviews (Wilhelm and Pingoud 2003, Bustin and Nolan 2004, Godfrey and Kelly 2005).

For the sake of simplicity, we will refer to a situation in which two isoforms are being expressed (often one of these isoforms will be considered the wild-type form). Irrespective of the type of splicing event, there will always be a “long” and a “short” isoform. For instance, for an exon-skipping event, the long form will be the wild-type and the short form the exon-skipped variant. For an intron retention event, the wild-type will this time be the “short” form and the “long” form will be the intron-retaining variant.

3.1.1

Semi-quantitative RT-PCR

Semi-quantitative PCR is an appropriate method to rapidly obtain a first set of data on the relative levels of expression between the two variants in one or more samples, or on the differential expression between two or more samples. PCR primers are either selected on both sides of the splice event (Fig. 2, primer set a) or selected in sequences specific to the long form (Fig. 2, primer set b) or common to both forms (Fig. 2, primer set c). PCR primers' efficacy and specificity are usually first verified on a gradient of annealing temperatures. End-point PCR at the selected temperature is next performed with a number of PCR cycles ensuring that the assay will stay in the exponential phase (usually between 20 and 30). PCR amplicons are next visualized by gel electrophoresis and can be further quantified by densitometry or via an Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto). Correction by internal normalization controls with comparable expression level is required. More sophisticated quantifications of the PCR amplicons have recently been described. First, pyrosequencing has been applied to the analysis of G protein $G\alpha_s$ subunit splice variants (Frey et al. 2005). This technology, initially developed for single nucleotide polymorphism (SNP) analysis relies on the differential incorporation of a deoxynucleotide by DNA Polymerase in primer extension assays, the PCR amplicons acting as

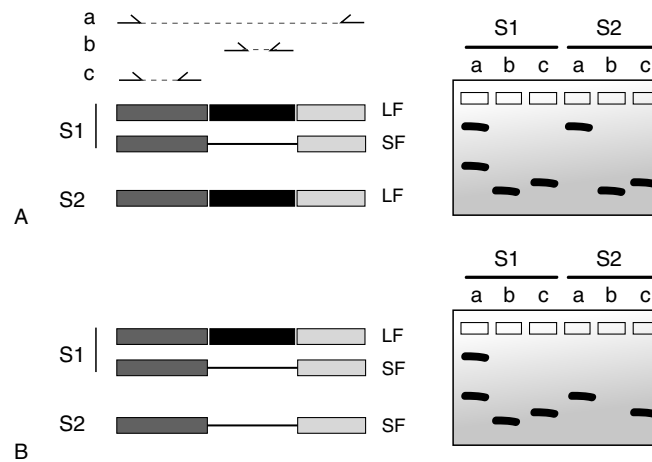


Fig. 2A, B. Semi-quantitative RT-PCR. **A:** Sample 1 *S1* contains both long and short forms while sample 2 *S2* contains only the long form. **B:** Sample 1 *S1* contains both long and short forms while sample 2 *S2* contains only the short form. Primers set *a* will amplify both variants and produce different size amplicons that can be resolved by gel electrophoresis. Primers set *b* will only amplify the long form. Primers set *c* will amplify both forms but produce one unique amplicon

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