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Advanced methods for the analysis of altered pre-mRNA splicing in yeast and disease

Huw B. Thomas and Raymond T. O'Keefe

Abstract

Splicing of pre-messenger RNA (pre-mRNA) transcripts is a fundamental process in all eukaryotes that provides a mechanism of increasing the proteomic diversity within a cell that can be tightly regulated in a dynamic manner. Whilst constitutive and alternative splicing are necessary for the correct development and regulation of cells/organisms, aberrant splicing is now associated with an increasingly varied number of human diseases, such as neurological and developmental diseases, and cancer. Studies of splicing mechanisms and regulation are often achieved in non-human model organisms such as yeast. Yeasts possess homologs to many of the core spliceosome components of higher organisms, including humans, and as such yeast are now a well-established model organism for understanding how differential splicing of transcripts can alter the phenotype of a cell or organism. Here we describe methods to investigate pre-mRNA splicing in yeast cells using modern RNA-Seq technology and bioinformatics software. Details of traditional validation methods are also described.

Key words pre-mRNA splicing, spliceosome, RNA-Seq, yeast

Running head: pre-mRNA splicing

1. Introduction

In eukaryotic cells, the identification and removal of intervening sequences from a newly transcribed pre-mRNA is a fundamental prerequisite of protein translation. Splicing of pre-mRNA transcripts is a highly regulated process catalysed by the spliceosome, a large ribonucleoprotein complex composed of five small nuclear ribonucleoproteins (snRNPs) and numerous other proteins [1]. Tightly regulated assembly and temporal rearrangement of the core components of the spliceosome lead to accurate splicing of pre-mRNA which can be dynamically regulated during development and in response to changing environmental conditions.

Much of the research into spliceosome structure, splicing-mechanisms and regulation has been achieved in yeast. Many of the core spliceosome components are homologous between yeast and higher eukaryotes, mirroring the level of structural complexity seen in human splicing. Fewer than 5% of yeast genes are subject to splicing but these spliced genes are highly expressed and the vast

majority of these genes are comprised of two exons separated by a single intron. This simplicity makes yeast an attractive system for detailed analysis of both constitutive and alternative splicing.

Modern advances in RNA sequencing (RNA-Seq) have led to a huge increase in sequencing speed, fidelity and ease of use. Coupled with an exponential fall in cost, this has resulted in a dramatic increase in RNA-Seq use and ubiquity among both basic and clinical research endeavours [2, 3]. A major advantage of RNA-Seq is the ability to interrogate the data in several ways reliant only on the downstream bioinformatics software used. For example, a single RNA-Seq dataset can be used to provide information on gene expression, transcriptional start sites, exon usage, and allele frequency, each with specific analytical software requirements, but all taking mapped RNA-Seq data as a starting input.

For studies of alternative splicing, many bespoke software packages are available which provide statistical quantification of splicing events between experimental conditions. However, a simpler process is to directly compare the expression levels of intron portions of transcripts with their surrounding exon portions. This comparison allows direct analysis of the levels of intron retention within a transcript under different conditions.

An increasing number of human diseases and disorders are being associated with mechanisms of splicing and components of the spliceosome [4–8]. Often despite mutations in core spliceosomal components that make up one specific snRNP particle, the resulting characteristics of the disorders are varied with very distinct or only partially overlapping phenotypes. For example, EFTUD2/SNU114, TXNL4A/DIB1, SNRNP200/BRR2 and PRPF8/PRP8 are all proteins associated with the U5 snRNP [9–11]. However, mutations in EFTUD2 and TXNL4A cause craniofacial disorders [4, 5] whereas mutations in SNRNP200 and PRPF8 cause degenerative retinal disease [12, 13]. Understanding how and why the genetic variations observed amongst ubiquitous spliceosome genes can lead to the scope of different diseases and disorders is of great value. However, in many cases access to patient samples is limited and alternative methods and model organisms must be used.

Well-established molecular techniques in yeast enable the engineering of yeast strains that closely resemble the genetic mutations seen in a disease/disorder of interest. These methods can be used as a prerequisite to analysis of global splicing patterns using RNA-Seq. For example, using site-directed mutagenesis and a plasmid shuffle technique [14] a variant form of a spliceosome gene can be expressed as the sole allele in a haploid yeast strain, thus investigating the effect of the mutation on global splicing fidelity.

Alternatively, replacing the promoter of a spliceosomal gene of interest with an inducible promoter allows the temporal knock-down in expression of a target gene [15]. This method allows characterisation of genes most sensitive to reduced levels of core spliceosome components and models several developmental disorders where reduced expression (haploinsufficiency) of core spliceosome components is predicted as the causative mechanism [16].

Herein we describe the methods required to undertake analysis of such yeast strains using modern RNA-Seq techniques to quantify precisely the exonic and intronic portions of the yeast transcriptome. Knowing how the expression of introns and exons alter over time can inform on not only the splicing efficiency for that transcript but also if increased retention of introns affects the stability of the overall transcript by leading to increased targeting of transcript by nonsense mediated decay pathways.

2. Materials

2.1 Yeast culture

2.1.1 YPD medium

Yeast extract (1% w/v); Peptone (2% w/v); Dextrose (2% w/v)

2.1.2 YPGal medium

Yeast extract (1% w/v); Peptone (2% w/v); Galactose (2% w/v)

2.2 RNA extraction

2.2.1 RiboPure™ RNA purification kit, yeast (Ambion® Thermo Fisher)

2.2.2 RNaseZAP™

2.3 Bioinformatic analysis (software and reference files)

2.3.1 FastQ Screen

2.3.2 FastQC [17]

2.3.3 Trimmomatic [18]

2.3.4 STAR: Ultrafast universal RNA-Seq aligner [19]

2.3.5 sacCer3 genome assembly for *S.cerevisiae* (April 2011)

2.3.6 HTSeq [20]

2.3.7 DeSeq2 [21]

2.3.8 Annotated yeast reference file (*S.cerevisiae.gff*)

2.3.9 GraphPad Prism

2.3.10 Multiple experiment viewer (MeV) [22]

2.4 cDNA synthesis

2.4.1 SuperScript™ IV First-Strand synthesis system (ThermoFisher): 5x SuperScript IV buffer; DTT (0.1 M); dNTPs (10 mM); Ribonuclease inhibitor (e.g. RNasin or RNaseOut); *E.coli* RNaseH (5000 U/mL)

2.5 PCR amplification

Phusion High-Fidelity DNA polymerase; 5x Phusion High-Fidelity buffer; dNTPs (10 mM); Forward validation primer (10 μM); Reverse validation primer (10 μM); Nuclease-free water (see **Note 1**)

2.6 Agarose gel electrophoresis

2.6.1 Agarose

2.6.2 1X Tris acetate EDTA Buffer (TAE) pH 8.5: Tris base 40 mM; EDTA 2 mM; Acetic acid 20 mM

2.6.3 SafeView DNA stain (NBS biologicals)

3. Methods

3.1 Yeast culture

Incubate yeast strain(s) in an appropriate volume of growth medium, for example YPD (or YPGal if using a galactose-inducible strain) and incubate at 30°C with constant shaking (see **Note 2**).

3.2 RNA isolation and preparation

1. Follow manufacturer's recommended protocol for purification of RNA from yeast cultures grown under your specific conditions using the RiboPure™ yeast RNA isolation kit. For best results: isolate cells during exponential growth phase and use close to maximum recommended number of cells (3×10^8) per filter cartridge (see **Notes 3 and 4**). Include optional DNase treatment step to ensure no contaminating DNA is carried forward into downstream applications.
2. Quantify purity of extracted RNA by measuring the 260/280 nm wavelength ratio using a spectrophotometer (i.e. NanoDrop™) (see **Note 5**).
3. Quantify the integrity of the RNA using an automated electrophoresis machine such as the Agilent Bioanalyser or TapeStation (see **Note 6**).
4. RNA is now ready for enrichment and library preparation prior to being sequenced on the Illumina platform (core facility or external contractor) (see **Note 7**).

3.3 Bioinformatic analyses

5. Run raw sequencing data (typically in .FASTQ format) through quality control analysis software such as FastQ Screen and FastQC to highlight any sample contamination and low quality sequencing (respectively).
6. Trim low sequencing quality bases from the 3' end of reads using Trimmomatic (see **Note 8**).
7. Map reads to appropriate reference genome assembly (for example UCSC's sacCer3 assembly for *S.cerevisiae*) using STAR and appropriate default settings (see **Note 9**).
8. Quantify the mapped reads using htseq-count. For alternative splicing analysis two separate read counts will need to be performed. The first quantifying all reads that fall within coding (exonic) regions of the genome and the second

quantifying all reads that fall within intronic regions. Use recommended/default settings and an appropriately annotated reference sequence to define coding and non-coding regions (for example *S.cerevisiae.gff* available from yeastgenome.org).

9. Calculate and quantify differential expression of genes, transcripts, exons or introns using DeSeq2 (see **Note 10**)

10. Once a quantified value for each intron and/or exon of interest has been achieved, the data can be visually represented in a variety of way which best suits the aim of the specific study. A common method of representing global patterns of expression is to use hierarchical clustering of quantified expression values and visualisation in a heatmap image. Use Multiple experiment Viewer (MeV) to perform hierarchal clustering using default settings, an example of which is shown in Fig. 1. Direct comparisons of intron vs exon expressions for individual transcripts of interest can be achieved using dual y-axis graphs using graphical/statistical analysis software such as GraphPad/Prism. If looking at alternative splicing at multiple time points then comparison of intron vs exon expression over time can reveal several different patterns of expression relating to splicing efficiency and transcript stability. Fig. 2 gives examples of the patterns of expression seen between exon and intron portions of transcripts when spliceosome function is disrupted by temporal knock-down of a core spliceosome component.

3.4 Data validation by RT-PCR

11. Using purified RNA collected from step 4, convert up to 5 µg of yeast RNA for each experimental condition to cDNA using the SuperScript IV First-Strand synthesis system following the manufacturer's recommended protocol (see **Note 11**).

12. Anneal 1 µL of primers (50 µM Oligo d(T)₂₀, 50 ng/µL random hexamers or 2 µM gene specific primers may be used) to RNA by mixing RNA with 1 µL of primers and 1 µL of 10 mM dNTPs and incubate at 65°C for 5 min before cooling on ice.

13. Add 1 μ L SuperScript IV reverse transcriptase and 4 μ L of 5X buffer to 1 μ L of DTT (100 mM) and 1 μ L RNase inhibitor (e.g. RNasin®). Incubate sample at 50-55°C for 10 min before stopping the reaction by incubating sample at 80°C for a further 10 min. Following the reaction, any left-over RNA may be removed by addition of 1 μ L of *E. coli* RNaseH (5000U/ μ L) and incubating at 37°C for 20 min.

14. Newly synthesised cDNA from step 11 can be stored at -20°C or used immediately for PCR amplification.

15. Using primers specifically designed to validate results from bioinformatics analysis, perform a standard PCR reaction using the Phusion High-fidelity DNA polymerase (see **Note 12**). Mix 4 μ L of 5X Phusion-HF buffer with 0.4 μ L dNTPs (10 μ M), 0.2 μ L Phusion DNA polymerase, 1 μ L of each validation primer (10 and add to 1 μ L of cDNA from step 10 (20-100 ng/ μ L). Add nuclease-free water to the reaction up to a volume of 20 μ L (see **Note 1**) and incubate in a thermal cycler using the following protocol:

1. 98°C for 30 sec
2. 98°C for 10 sec
3. Calculated annealing temperature for 20 sec (see **Note 13**)
4. 72°C for 15-30sec/kb
5. Repeat steps 2-4 for 34 cycles
6. 72°C for 10 min

16. Analyse amplified PCR product(s) by running on a suitable percentage agarose gel. For example: for a PCR product 200-1000 base pairs, use 1% w/v agarose in 1x TAE buffer plus 0.01% SafeView DNA-stain. Using a BioRad Mini-Sub Cell GT gel electrophoresis chamber run the gel at a constant voltage of 65-75 V for 40-60 min (other electrophoresis equipment may require different voltage and/or running time).

15. Image gel using a UV-image station

16. Quantify density of bands and calculate ratio of spliced transcript vs unspliced transcript and compare with ratio quantified by RNA-Seq.

4. Notes

1. Use a cellulose nitrate filter unit with 0.2 μm pore size to remove potential nuclease contamination from de-ionised water. This avoids the need to use DEPC treatment.
2. If comparing splicing between strains with different levels of temporal knock-down of a core spliceosome component e.g. 4h knock-down vs 8h knock-down then starting OD of yeast cultures should be altered such that final ODs of strains are as similar as possible at the point of RNA extraction despite different incubation times. In addition, at least 3 replicates should be processed to ensure consistency and accuracy of downstream RNA-Seq data.
3. RNA is much less stable than DNA and subject to degradation by RNases present in the environment. Therefore, care should be taken to eliminate risk of degradation by firstly treating the working area and instruments (e.g. pipettes) with an anti-RNase decontaminant such as RNaseZap™. Secondly, extracted RNA should be kept on ice between steps, wherever possible, and finally, for best results, isolated RNA should be snap frozen (with liquid nitrogen) and stored at -80°C (unless being used for downstream procedures immediately).
4. If higher final concentrations of RNA are required the final elution step of the RiboPure™ protocol can be repeated with the original eluted flow-through to increase the yield of RNA from the filter cartridge without having to increase the final volume. For example 2 x 50 μL elution steps using preheated elution solution, followed by a third elution with the collected 100 μL elute.
5. Success of subsequent RNA-Seq and quality of downstream data is heavily dependent on the purity and integrity of the original RNA sample. Purity of the

RNA sample can be determined by NanoDrop™ quantification of the 260/280 nm wavelength ratio, with a ratio lower or higher than 2.0 indicating potential contamination with solvents (e.g. Phenol from extraction kit) and/or protein (from original cell culture).

6. Integrity of RNA is traditionally measured on a scale of 1-10 with an RNA Integrity Number (RIN) value of 10 indicating the highest integrity possible. A value of 8 or above is generally considered appropriate for RNA-Seq experiments, with decreasing values indicating the RNA sample has degraded during the isolation process or is of low starting quality (e.g. from dead/senescent cells). Whilst good RNA-Seq data can be achieved from low RIN RNA, analysis of alternative/aberrant splicing events demands the highest read depth and read coverage possible.
7. Several options are often available in terms of methods for enrichment (such as poly-A selection or rRNA depletion) and library preparation (such as single or paired-end sequencing). Typically, poly-A selection provides excellent enrichment of the mRNA population, whilst paired-end sequencing provides the greatest amount of data with a higher rate of uniquely mapped reads, an important consideration for splice variant analysis.
8. Removing bases with low sequencing quality from the 3' ends of reads can improve the success rate of subsequently mapping reads to the reference genome. This step can also be used to remove any adapter or barcode sequences added during library preparation that would interfere with the mapping process.
9. For best results, use only reads that map uniquely to the reference sequence. For paired-end libraries, reads should be mapped as pairs.
10. Software such as DeSeq2 take the quantified mapped reads and calculates the log fold change and statistical significance of any differential expression of a defined

sequence (for example an exon, intron or coding sequence) between two experimental conditions. This analysis provides an accurate method of quantifying the level of intron retention or splicing efficiency of a transcript under a given experimental condition.

11. Depending on final elution volume in the final step of RNA isolation, often the RNA concentration will not need further dilution before beginning cDNA synthesis ($< 500 \text{ ng}/\mu\text{L}$). If multiple samples are to be processed in parallel, normalise all samples at this point by using an equal amount of starting RNA for each cDNA synthesis reaction. Thus, for the least concentrated sample no water need be added (i.e. using the maximum $11 \mu\text{L}$ of RNA), whilst all other samples will be made up to an $11 \mu\text{L}$ volume using a dilution of RNA and water to achieve an equal starting amount of RNA per $20 \mu\text{L}$ cDNA synthesis reaction.
12. For validating alternative splicing events, primers should be designed to amplify between two exons separated by an intron, such that two different PCR products could be produced, one representing unspliced transcripts (containing the intron sequence), and a second population of a shorter product representing spliced transcripts. Typically an unspliced product size of 300-500bp with a shorter (spliced) product by least 100bp is desirable when designing primers.
13. Use a T_m calculator specifically for the Phusion High-fidelity DNA polymerase to calculate the optimal annealing temperate for the validation primers.

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Fig. 1 Example heat-map of transcript intron expression levels under different conditions. Red represents high expression, black represents moderate expression, and green represents low/no expression. Each column represents the single intron region of a transcript. Each of the 9 rows represents a separate experiment with different experimental conditions. Heat-map was produced by Multiple experiment Viewer (MeV) using normalised expression values for each intron region and performing hierarchical clustering of values [22].

Fig. 2 Example graphs showing intron (blue bars) and exon expression (red bars) for a given transcript in yeast following temporal reduction in a core spliceosome component. Galactose condition represents normal expression of core spliceosome component, glucose conditions represent conditions of reduced expression of core spliceosome component for 4 and 8 h. Data quantified by RNA-Seq. **A.** Expression of introns increases with time whilst exon expression remains constant – increased intron retention. **B.** Both intron and exon expression levels alter over time proportionally – No change in splicing efficiency. **C.** Decrease in intron expression whilst exon expression levels stay constant – Increase in transcript splicing. **D.** Increase in intron expression and corresponding decrease in exon expression – Increase in intron retention and transcript degradation.

Fig. 3 Example of RT-PCR validating results for an RNA-Seq experiment. Left panel shows expression values for an intron portion of a transcript following knockdown of a spliceosomal component for 4h and 8h. Right-hand panel shows RT-PCR using validating primers to identify a corresponding increase in larger fragment relating to transcript where intron portion is retained in the pre-mRNA.