CHAPTER THREE

Genome-Wide Approaches to Monitor Pre-mRNA Splicing

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Contents

Abstract

Pre-mRNA processing is an essential control-point in the gene expression pathway of eukaryotic organisms. The budding yeast *Saccharomyces cerevisiae* offers a powerful opportunity to examine the regulation of this pathway. In this chapter, we will describe methods that have been developed in our lab and others to examine pre-mRNA splicing from a genome-wide perspective in yeast. Our goal is to provide all of the necessary information—from microarray design to experimental setup to data analysis—to facilitate the widespread use of this technology.

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1. Introduction

In the 30 years since Sharp and Roberts independently demonstrated the presence of split genes (Berget *et al.*, 1977; Chow *et al.*, 1977), it has become abundantly clear that pre-mRNA splicing and its regulation play an essential role in regulating gene expression in eukaryotic organisms (House and Lynch, 2008). By regulating the efficiency of splicing of specific transcripts during development, in specific tissues or in response to external stimuli, the expression levels of particular genes can be controlled. Similarly, splicing can control proteomic diversity by regulating splice site choice via the process known as alternative splicing. Remarkably, whereas the number of genes predicted to be encoded by the human genome has been steadily decreasing over the last 15 years, the fraction of genes known to be alternatively spliced has gradually increased over this same time and is now thought to be over 90% of genes in humans (Wang *et al.*, 2008). While great progress has been made in understanding the mechanistic details of splicing, many questions remain about the pathways used to regulate this process.

It was recognized early on that the components of the spliceosome and the basic mechanisms of splicing are highly conserved from yeast to humans. As such, the budding yeast *Saccharomyces cerevisiae* has played a pivotal role as a model organism for elucidating mechanisms of pre-mRNA splicing. While the underlying machinery is highly conserved between humans and yeast, the genome-wide distribution of introns is in fact quite different. Whereas over 90% of human genes are interrupted by at least one intron, only about 5% of *S. cerevisiae* genes contain a functional intron. In spite of this simplified architecture, one of the earliest recognized examples of regulated splicing was demonstrated in *S. cerevisiae*. In an elegant set of experiments, Roeder's group demonstrated that the Mer2 protein specifically modulates the splicing of the MER1 transcript during meiosis (Engebrecht *et al.*, 1991). The observation that a specific protein can play a pivotal role in the efficient splicing of a distinct set of transcripts highlights both the utility of splicing as a regulator of gene expression and also the need for genome-wide tools to assess global changes in splicing.

This chapter focuses on methods for studying genome-wide changes in pre-mRNA splicing in yeast. The last several years have seen the development of several distinct but related microarray platforms that allow for global analysis of splicing (Clark *et al.*, 2002; Juneau *et al.*, 2007; Pleiss *et al.*, 2007a,b; Sapra *et al.*, 2004; Sayani *et al.*, 2008). In this chapter, we will describe one such methodology using short oligonucleotide sequences that specifically detect each of the different splicing isoforms. The first oligonucleotide-based microarrays that were used to specifically probe changes in splicing status were developed by the Ares lab (Clark *et al.*, 2002).

We have used this approach to identify splicing responses to environmental stress and to evaluate effects of mutations in core spliceosomal components (Pleiss *et al.*, 2007a,b). The goal of this chapter is to guide you through the platform that we are currently using in our lab. While the details presented here are specific to our particular platform, we expect that the protocols can be readily adapted to meet the requirements of other platforms.

2. Microarray Design

The fundamental philosophy underlying splicing-sensitive microarrays is no different from standard gene expression microarrays in that short, complementary oligonucleotides are used to probe the abundance of a given RNA species. Whereas gene expression microarrays have oligonucleotides that target only coding regions of genes, the splicing-sensitive microarrays that we use include additional probes to both intron regions and the junction of the two ligated exons to distinguish changes in pre- and mature mRNA levels, respectively (Fig. 3.1). Using tools that are described below, we have designed sequences that target approximately 6000 genes, ~300 introns, ~300 junctions, tRNAs, snRNAs, snoRNAs, and other functional noncoding RNAs in the yeast genome. The sequences are readily available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, Accession number GPL8154) and can be downloaded and used by anyone to order microarrays from any of several different vendors. In this chapter, we will describe our work using custom Agilent microarrays which contain eight identical hybridization zones each printed with approximately 15,000 of these oligonucleotides. However, we see no reason why these sequences would not be transferrable to other commercial or homemade platforms.

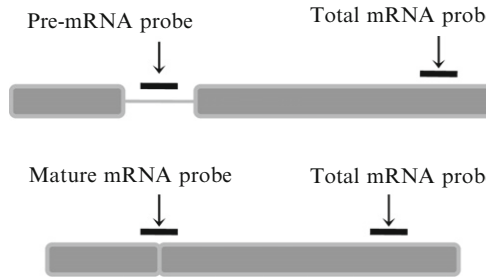

Figure 3.1 Microarray probe design. For each intron-containing gene, a minimum of three probes are designed. One measures changes in total RNA level by hybridizing to a region of the exon. A second measures changes in pre-mRNA level by hybridizing to a region of the intron. The third probe measures changes in mature mRNA levels by hybridizing to the junction of the two ligated exons.

If you are interested in learning how these microarrays are designed, continue reading the rest of this section. If not, order your microarrays and skip ahead to Section 3. As with all microarrays, a key component in the design of splicing-sensitive microarrays is the ability to probe the RNA of interest without cross-reacting with off-target or nonspecific RNAs. For probes that target the coding regions of genes (both intron-containing and intronless genes) this task is made relatively easy by numerous computational programs that have been specifically designed for this purpose. We have used the OligoWiz program (http://www.cbs.dtu.dk/services/OligoWiz/) to design 60 nt (nucleotide) probes to all ~6000 protein-coding genes in the yeast genome (Wernersson *et al.*, 2007). For intron-containing genes, where possible, probes were designed that target regions in both exons 1 and 2. However, because most yeast introns are located at the 5′ end of the gene, and exon 1 tends to be very short, probes have been designed to target a region only in exon 2 for most intron-containing genes (Fig. 3.1).

For intron-containing genes, OligoWiz can also be used to design probes targeting intronic regions. Because many yeast introns are short, we hoped to use shorter probe lengths to target the pre-mRNA species. However, it was unclear whether these probes would provide sufficient hybridization capacity. In our initial experiments with the Agilent platform, we tested both long (60 nt) and short (35 nt) probes targeted to the introns of all ~300 intron-containing genes and observed no significant loss of signal intensity when using the shorter probes. Therefore, all of the pre-mRNA specific probes on our microarray target a 35 nt region of the intron of interest. Likewise, because many functional RNAs like tRNAs and snoRNAs are also small, we designed probes to functional RNAs using 35 nt sequences.

From the perspective of specificity, the most difficult oligonucleotides to design are those that target the mature mRNA species by hybridizing to the junction between ligated exons. Whereas the exon-targeting probes can be optimized by moving the targeted region anywhere within the coding sequence, by definition the oligonucleotides which probe changes in mature mRNA levels by targeting the junctions of ligated exons are restricted to the discrete sequences at the end and beginning of those neighboring exons. In designing these probes, we sought to identify the shortest length oligonucleotide which was sufficient to efficiently capture spliced mRNAs. Because of the varying sequence content in the exons of different intron-containing genes, we chose to vary the length such that the sequences upstream and downstream of the junction are energetically balanced (Fig. 3.2). Our initial experiments tested a variety of thermodynamic stabilities for every exon–exon junction in the genome. In these experiments, the best compromise between signal intensity and signal specificity was found for those junction probes that had ΔG° values

YML056c

$\Delta G°$	Exon 1	Exon 2	$\Delta G°$
10.2	CCAGTTACTG	AAGACGGTAA	10.8
13.8	TCCCAGTTACTG	AAGACGGTAAGT	13.8
16.5	CTTCCCAGTTACTG	AAGACGGTAAGTGT	17.0
18.8	GCTTCCCAGTTACTG	AAGACGGTAAGTGTC	18.5
22.6	TGGCTTCCCAGTTACTG	AAGACGGTAAGTGTCCA	22.3

YML085c

$\Delta G°$	Exon 1	Exon 2	$\Delta G°$
11.2	TATTAGTATTAATG	TCGGTCAAG	10.4
13.9	GTTATTAGTATTAATG	TCGGTCAAGCT	14.2
16.6	AAGTTATTAGTATTAATG	TCGGTCAAGCTG	15.9
19.6	AGAAGTTATTAGTATTAATG	TCGGTCAAGCTGGT	19.5
22.6	AGAGAAGTTATTAGTATTAATG	TCGGTCAAGCTGGTTG	22.4

YML017w

$\Delta G°$	Exon 1	Exon 2	$\Delta G°$
9.4	GAAGAAATGG	GAACAAATAATAC	11.2
13.6	GGGAAGAAATGG	GAACAAATAATACAT	13.8
16.4	CGGGAAGAAATGG	GAACAAATAATACATCT	16.8
19.1	AACGGGAAGAAATGG	GAACAAATAATACATCTAAT	19.8
22.7	GGAACGGGAAGAAATG	GAACAAATAATACATCTAATAAT	22.8

Figure 3.2 Design scheme for junction probes. For each intron-containing gene, a series of probes was created such that the hybridization energy derived from interactions with the upstream and downstream exons were thermodynamically balanced. For some genes, like YML056c, this yielded a nearly equal number of base pairs on either side of the junction. However, because of variable sequence content surrounding the junctions, other genes required longer base pairing regions either upstream (YML085c) or downstream (YML017w) of the exon–exon boundary. The boxed sequences correspond to the best performing probes in test hybridizations, and are included in the final microarray design.

closest to 17 kcal/mol on each side of the junction (Sugimoto *et al.*, 1996). While these parameters were used to design junction probes specific to the *S. cerevisiae* genes, the thermodynamic properties are such that these parameters are likely to be the optimal parameters for the design of junction probes for any organism.

Finally, the architecture of the Agilent microarray platform is such that 60 nt probes are printed with their 3′ ends covalently linked to the glass slide surface. Because the lengths of intron and functional RNA probes are fixed at 35 nt and our junction-specific probes vary from 24 to 36 nt, we included a stalk region at the 3′ end of these oligonucleotides to move the ''targeting region'' of the probes away from the glass in hopes of making them more readily accessible for hybridization. Several different stalk designs were tested. We settled on a sequence designed by Agilent to have low cross-reactivity with any genomic sequence. As expected, our initial experiments comparing probes containing stalks with those lacking them indicated that the stalks provided improved signal intensity with little or no loss in probe specificity.

3. Sample Preparation

The goal of this section is to describe all the steps needed to go from experimental design to hybridizing a sample on a microarray. Obviously, the details of experimental design will vary. For the purposes of this chapter we will describe a specific experiment comparing a yeast strain containing a temperature-sensitive mutation in a canonical splicing factor to a matched wild-type strain, which will be referred to as the experimental and reference strains, respectively, from here on. In our experience, the data from this type of experiment are most easily understood when a time course is followed after shifting the strains to the nonpermissive temperature. Defects in pre-mRNA processing can often be detected within minutes in an experiment like this. Below are the protocols for each of the major steps in the pathway: cell collection, RNA isolation, cDNA synthesis, fluorescent labeling, and microarray hybridization and washing.

3.1. Cell collection

The first step in an experiment is to collect appropriate cells from the experimental and reference strains. In our experience, microarrays are exceptionally sensitive assays that are able to detect subtle differences in growth and handling of samples. As such, we always collect actively growing cells in early to mid-log phase. Likewise, we work to standardize all experimental conditions to have equivalent volumes, flask sizes, growth media, shift conditions, etc. for both the experimental and reference strains. Our preferred method for harvesting cells is by vacuum filtration using mixed cellulose ester filters (Millipore Cat.#: HAWP02500, or equivalent) and a vacuum manifold apparatus (Millipore Cat.#: XX1002500, or equivalent). After collection, the filters can be placed in a 15 ml conical tube and immediately frozen in liquid nitrogen. This method provides a fast and simple mechanism for collecting rapid time points during a time course. We have also collected cells by centrifugation at $5000 \times g$ for 5 min, but disfavor this method because of the time involved in getting cells from growth condition to frozen cells.

The quantity of cells required for an experiment will depend upon your experimental conditions. In general, our protocol requires 40 μg of total RNA for both the experimental and reference samples. We routinely recover 20 μg of total cellular RNA from a single milliliter of cells grown in YPD with an optical density (OD) equal to 0.5 ($\sim 5 \times 10^6$ cells). Our yields of total RNA are typically twofold less for cells grown in synthetic media to the same OD.

3.1.1. Protocol for cell collection

(1) On day 1, start a 5 ml culture of both experimental and reference strains in YPD and allow them to grow overnight at the permissive temperature (25 °C).
(2) On day 2, for both strains, use the 5 ml overnight cultures to inoculate a fresh 50 ml culture to a starting OD of 0.1. Allow these to continue growing at 25 °C.
(3) When the two cultures have reached the appropriate cell density (between an OD of 0.5 and 0.75, ~3–4 h), collect 10 ml of cells by filtration. Immediately transfer the filter into a 15 ml conical tube, cap the tube tightly, and plunge it into liquid nitrogen.
(4) Transfer both the experimental and reference culture flasks to a shaking water bath at the nonpermissive temperature (37 °C). Collect additional 10 ml aliquots as described above after 5, 15, and 30 min. Cells can be stored at −80 °C until ready for RNA isolation.

3.2. RNA isolation

In our experience, there are two factors that are crucial for efficiently isolating high-quality RNA for use in microarray experiments. The first critical factor is achieving efficient cell lysis. In our protocol, we affect cell lysis by using a combination of heat, exposure to phenol, exposure to SDS, and physical agitation. In our experience the most common reason for obtaining poor RNA yields results from insufficient vortexing during heating (step 2 below). The second crucial factor is maintaining the integrity of the RNA. In this regard, we focus on two aspects: temperature and time. During all of the steps listed below, the samples should be handled on ice. Likewise, where possible, all centrifugation steps should be performed in a refrigerated centrifuge. Additionally, we strive to minimize the amount of time that elapses between taking the cells out of the −80 °C freezer (step 1) and adding isopropanol to precipitate the RNA (step 8). Every effort should be made to move as expeditiously as possible until this point to ensure high-quality RNA. After the addition of isopropanol, the samples can be stored at −20 °C indefinitely.

An important improvement to both the reproducibility and integrity of our RNA preparations has come from the use of tubes containing Phase Lock Gel (5 Prime, http://www.5prime.com) to facilitate the separation of the organic and aqueous phases. Use of these tubes allows for nearly quantitative recovery of the aqueous phase and removes the inconsistency associated with manual aspirations at the interphase. Our protocols indicate $3000\times g$ spins to separate the aqueous and organic phases when using Phase Lock Gel, however, faster spins will give better separation. We routinely spin our samples in a Beckman X-15R centrifuge with a SX4750A rotor at top speed ($5250\times g$) at 4 °C for 5 min.

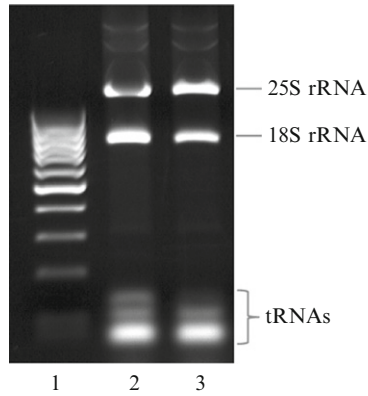

Figure 3.3 Typical banding pattern for purified total RNA. Two independent preparations of total cellular RNA have been separated by gel electrophoresis on a 1% agarose gel run in 1× TAE buffer. Loaded in lane 1 is 0.5 μg of GeneRuler 100 bp DNA Ladder (Fermentas). Lanes 2 and 3 are the total RNA samples. The locations of the 25S rRNA, 18S rRNA, and tRNA species are indicated.

The end of the RNA isolation procedure is the first opportunity to assess the quality of your experiment. We assess RNA quality in several ways. First, we determine the quantity of RNA isolated using a spectrophotometer and compare the results to the expectations described above. If the RNA isolation yield is significantly less than expected, we tend to discard this material, collect new cells and repeat the RNA isolation. Second, we examine the integrity of the isolated RNA by visualization on an agarose gel. While the quality of the mRNAs in the total RNA preparation cannot be directly assessed by the agarose gel, we use the bands corresponding to the ribosomal RNAs as a proxy for the integrity of the mRNAs. Figure 3.3 shows a typical banding pattern seen when 1 μg of RNA is separated on a 1% agarose/1× TAE gel. As an alternative, a higher resolution analysis of mRNA quality can be obtained using instruments such as the Agilent Bioanalyzer. It is perhaps worth a brief note that typical precautions should be used when handling RNA so as to avoid contamination with ribonucleases.

3.2.1. Materials for RNA isolation

15 ml Phase Lock Gel Heavy tubes (5 Prime Cat.#: 2302850)
AES buffer (50 m*M* sodium acetate (pH 5.3), 10 m*M* EDTA, 1% SDS)
Acid–phenol:chloroform (5:1) (pH < 5.5) (Ambion Cat.#: AM9720, or equivalent)
Phenol:chloroform:IAA (25:24:1) (Ambion Cat.#: AM9730, or equivalent)

Chloroform
3 *M* sodium acetate (pH 5.3)
Isopropanol
70% ethanol

3.2.2. Protocol for RNA isolation

(1) Remove conical tubes containing filters from the −80 °C freezer and place on ice. Immediately add 2 ml of acid–phenol:chloroform, then add 2 ml of AES buffer and vortex well. Cells will be easily removed from the filters by vortexing.
(2) Transfer the tubes to a 65 °C water bath and incubate for 7 min. Vortex thoroughly (5 to 10 seconds) once every minute.
(3) Transfer the tubes to ice and incubate for 5 min. During incubation, prepare one 15 ml Phase Lock Gel Heavy tube for each sample by spinning briefly at $\geq 3000 \times g$.
(4) Transfer the entire organic and aqueous contents to a prespun 15 ml Phase Lock Gel Heavy tube. Leave the filters behind if possible, but do not worry if they do transfer. Once the material is in the Phase Lock Gel Heavy tube, do not vortex. This fragments the Phase Lock Gel. Spin at $\geq 3000 \times g$ at 4 °C for 5 min.
(5) In the same 15 ml Phase Lock Gel Heavy tube, add 2 ml of phenol: chloroform:IAA to the supernatant. Mix by shaking, but do not vortex. Spin at $\geq 3000 \times g$ at 4 °C for 5 min.
(6) In the same 15 ml Phase Lock Gel Heavy tube, add 2 ml of chloroform to the supernatant. Mix by shaking, but do not vortex. Spin once again at $\geq 3000 \times g$ at 4 °C for 5 min.
(7) Prepare a new 15 ml conical tube with 2.2 ml of isopropanol and 200 μl of 3 *M* sodium acetate.
(8) Pour the supernatant from step 6 into the 15 ml conical tube with isopropanol. Mix by inverting several times.
(9) Transfer 2 ml of the isopropanol slurry into a 2 ml microcentrifuge tube and spin the RNA at top speed in a microcentrifuge ($\geq 14{,}000 \times g$) at 4 °C for 20 min. (The remainder of the RNA slurry can be stored at −20 °C for future use as this is the most stable storage method for RNA.)
(10) Carefully pour off the supernatant from the 2 ml tube so as not to disrupt the pellet. Add 2 ml of 70% ethanol to the pellet and mix by inverting several times. Spin again at top speed in a microcentrifuge ($\geq 14{,}000 \times g$) at 4 °C for 5 min.
(11) Repeat step 10 once.
(12) Carefully pour off the supernatant from the 2 ml tube so as not to disrupt the pellet, then briefly dry the RNA in a SpeedVac. Do not heat or overdry the samples.

(13) Dissolve the RNA in 50 μl of water. Determine the actual concentration using a spectrophotometer. Use a conversion factor of 1 $A_{260} = 40$ μg/ml RNA. This should give a concentration of approximately 2 mg/ml.

3.3. cDNA synthesis

After successful isolation of total RNA, the next step is to fluorescently label the experimental and reference samples for microarray hybridization. Several different methods exist, including direct RNA labeling (Wiegant *et al.*, 1999), generation of cRNA (Gelder *et al.*, 1990), or generation of cDNA (DeRisi *et al.*, 1997). It is worth noting that Agilent's protocols for gene expression microarrays take advantage of a linear amplification method using T7 RNA polymerase to generate direct-labeled cRNAs. While there are advantages and disadvantages to each of these methods, for reasons described below our protocols are instead designed and optimized for cDNA synthesis.

While traditional expression microarrays (and Agilent's gene expression protocols) use oligo-dT sequences to prime their cDNA (and cRNA) reactions, we instead use random 9-mer oligonucleotides. There are two main reasons for choosing this method. First, we are interested in looking at RNAs independent of their poly-(A) tail status. We presume that many pre-mRNA species may lack fully developed poly-(A) tails, and such species may be undetectable when priming with oligo-dT. The second reason is related to the fact that most *S. cerevisiae* introns are located at the 5′ end of their transcripts. Therefore, to distinguish between pre- and mature mRNA species, cDNAs must be produced that correspond to the 5′ end of the transcript, the efficiency of which is greatly reduced if all priming events take place at the poly-(A) tail. While random priming helps to alleviate this issue, the potential downside of random priming is the production of cDNAs corresponding to the highly abundant ribosomal RNA species. For a given intron-containing gene there are on the order of one million copies of rRNA for every single copy of pre-mRNA, thereby highlighting the need for highly specific probe design. Nevertheless, we find that the combination of our probe design and priming strategy does produce highly specific data. For example, as a measure of the potential cross-reactivity of the ribosomal cDNA species, we have examined microarrays comparing wild-type strains to strains containing complete deletions of intron-containing genes. Whereas robust signal is detected on the intron-specific probes for the wild-type strain, we find the signal intensity for the deletion strains are significantly reduced to levels near background. This suggests that there is minimal cross-hybridization of the ribosomal cDNAs to our specific probes.

In general, there are two different methods by which fluorescent dyes can be incorporated into cDNA: either by inclusion of a fluorescently

labeled nucleotide analog which can be directly incorporated by reverse transcriptase, or by inclusion of a derivatized nucleotide analog containing a reactive chemical group which can be used to covalently attach fluorescent dyes in subsequent reactions. Our protocols utilize the latter method, including aminoallyl-dUTP in the reverse transcription reaction, which is highly reactive to *N*-hydroxysuccinimidyl (NHS) ester derivatized fluorophores. The advantage to this method is that the aminoallyl-dUTP is efficiently incorporated by reverse transcriptase, whereas fluorescently labeled nucleotide analogs are often poor substrates for polymerases. Because cellular RNAs contain modified nucleotides with potentially reactive primary amines, our protocol includes an RNA hydrolysis step after cDNA synthesis to facilitate their removal. As will be described later, the cDNA purified from this protocol can be efficiently reacted with fluorescent dyes.

The total amount of fluorophore incorporated into a sample is an important factor for optimal microarray signal and can be controlled by adjusting the ratio of aminoallyl-dUTP to dTTP in the cDNA synthesis reaction. Our initial experiments using Agilent microarrays indicated that the specific fluorescent activity of the hybridized sample needed to be significantly lower than what has been typically used for spotted microarrays (DeRisi *et al.*, 1997; Pleiss *et al.*, 2007b). At high concentrations of aminoallyl-dUTP, we observed optical interaction between the two fluorophores, presumably because of the overlap between their emission and absorption spectra. This problem was alleviated by reducing the ratio of aminoallyl-dUTP to dTTP. The different requirements for Agilent and spotted microarrays presumably reflect the differences in the density and orientation of the oligonucleotide probes in these two formats.

For both the experimental and reference samples, a single splicing microarray requires 20 μg of total RNA as starting material. We always perform our microarray experiments as technical repeats where the orientation of the dyes is reversed, resulting in so-called ''dye-flipped'' replicates. As indicated earlier, this means that 40 μg of total RNA are needed for both experimental and reference samples for a replicate set of hybridizations. We set up a single cDNA synthesis reaction for the entire 40 μg of total RNA, which will be divided later for fluorescent labeling and hybridization. Important considerations in setting up the cDNA reactions are the concentration of total RNA and random primers. Our best results have been achieved using a final concentration of total RNA at or below 0.5 mg/ml and random primers at 0.25 mg/ml. At RNA concentrations higher than this, the efficiency of cDNA synthesis drops off significantly.

For the protocol listed below we purify recombinant MMLV reverse transcriptase and make our own buffers. However, commercial enzymes can also be used. In considering different commercial enzymes it is important to use an enzyme like Superscript Reverse Transcriptase (Invitrogen)

that has the RNaseH activity disrupted. However, most commercial enzymes are packaged with a reaction mix that contains buffer, salt and $MgCl_2$ together. When we hybridize our primers to our RNA, we find that it is important to leave out the $MgCl_2$ to avoid stabilizing RNA structures and therefore recommend using homemade buffers for this step. Under the conditions described below we typically achieve cDNA synthesis yields that are between 30% and 50% conversion by mass (12–20 μg of cDNA from 40 μg of starting RNA). As this is a second opportunity for quality control, yields significantly below this expectation warrant a repeat of the cDNA synthesis step or there will be insufficient signal for the microarray experiment.

After a successful cDNA synthesis reaction it is important to purify the products away from any unincorporated aminoallyl-dUTP prior to fluorescent labeling. In the protocol below we use a commercial kit from Zymo Research that is designed to purify oligonucleotides from unincorporated dNTPs. An alternative that we have found to be both cost-effective and high quality is to use 96-well Glass Fiber DNA binding plates. Such plates are made by several manufacturers and are widely available. We have commonly used plates from Nunc (Cat.#: 278010) along with homemade cDNA binding buffer (5 *M* guanidine–HCl, 30% isopropanol, 90 m*M* KOH, 150 m*M* acetic acid) and wash buffer (10 m*M* Tris–HCl (pH 8.0), 80% ethanol).

3.3.1. Materials for cDNA synthesis

Total RNA for experimental and reference samples
10× RT buffer = 0.5 *M* Tris–HCl (pH 8.5), 0.75 *M* KCl
10× dN_9 = 5 mg/ml dN_9 oligonucleotides
10× $MgCl_2$ = 30 m*M* $MgCl_2$
10× DTT = 0.1 *M* DTT
10× dNTP's (+aa-dUTP) = 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 9.8 m*M* TTP, 0.2 m*M* aminoallyl-dUTP
Reverse transcriptase
RNA hydrolysis buffer = 0.3 *M* NaOH, 0.03 *M* EDTA
Neutralization buffer = 0.3 *M* HCl
DNA Clean and Concentrator—25 kit (Zymo Research, Cat.#: D4006)

3.3.2. Protocol for cDNA synthesis

Using the volumes in the protocol listed in Table 3.1 as a guide, but adjusted for actual RNA concentrations, do the following:

(1) Anneal the primers to the total RNA by heating in a 60 °C water bath for 5 min. (Note that at this stage the RNA and primer concentrations (1 and 0.5 mg/ml, respectively) are twice the values that they will be

Table 3.1 Experimental setup for cDNA synthesis reaction

Reagent	Stock concentration	Volume used (μl)	Volume used per 8 rxns (μl)
RNA/primer mix			
RT buffer	10×	5	
dN_9	5 mg/ml	5	
Total RNA	2 mg/ml	20	
Water		20	
Total		50	
Enzyme/dNTP mix			
RT buffer	10×	5	44
$MgCl_2$	10×	10	88
DTT	10×	10	88
dNTP's (+aa-dUTP)	20×	5	44
Reverse transcriptase	20×	5	44
Water		15	132
Total		50	440

in the final cDNA synthesis reaction. Note also that $MgCl_2$ is omitted from this step, but buffer and salt are included, which we find increases the yield by about 20% relative to annealing in water alone.)

(2) Immediately after heating the samples, transfer them onto ice for an additional 5 min to allow the primers to anneal.

(3) While the RNA/primer mix is cooling, make the enzyme/dNTP mix as described in Table 3.1.

(4) Add 50 μl of enzyme/dNTP mix to the annealed RNA/primer mix (the RNA should now be at its final concentration of 0.5 mg/ml). Briefly vortex and spin the tubes, then allow them to incubate at 42 °C. In our experience the reaction is >90% complete after 2 h; however, we routinely incubate the samples overnight.

(5) To hydrolyze RNA prior to purification of the cDNA, add 50 μl of RNA hydrolysis buffer, vortex, and spin down. Place this mix in a 60 °C water bath for 15 min, then transfer to ice.

(6) Neutralize the solution by adding 50 μl of neutralization buffer. Vortex and spin down.

(7) Each column in a DNA Clean and Concentrator—25 kit can bind a maximum of 25 μg of cDNA. Therefore, a single cDNA reaction, which starts with 40 μg of total RNA and yields about 20 μg of cDNA, can be purified with a single column. Follow the manufacturer's instructions for purification of single stranded cDNA until the elution step. Proceed with elution as described in step 8.

(8) Transfer the column to a clean 1.7-ml tube. Add 35 μl of water directly onto the filter. Wait 30 s, then spin the samples in the

microfuge at top speed ($>14{,}000\times g$) for 1 min (spinning at $10{,}000\times g$ is effective for elution and can help to prevent the lids of the collection tubes from snapping off).

(9) Add a second 35 μl aliquot of water directly onto the filter, wait another 30 s, and spin the samples in the microfuge at top speed ($>14{,}000\times g$) for 1 min.

(10) After discarding the column, vortex the eluate well and spin down. Quantitate cDNA yield using a spectrophotometer. Although this is a cDNA sample, we continue to use a conversion factor of 1 $A_{260} = 40$ μg/ml cDNA. Using this conversion factor, expect to recover between 12 and 20 μg of cDNA from 40 μg of starting RNA.

(11) Split the eluate into two equal aliquots ($\sim$33 μl) in 1.7 ml tubes and dry in the SpeedVac. These samples will subsequently be labeled with the two different dyes to be used as matched replicates for the microarray.

3.4. Fluorescent labeling of cDNA

Many different vendors sell fluorescent dyes which have both the appropriate spectral properties and the appropriate derivatizations to react with the primary amine of the aminoallyl modified nucleotide. We have used both Cy dyes from GE Healthcare (Cy3 and Cy5) and Alexa dyes from Invitrogen (Alexa 555 and 647), and have found largely overlapping results. We presume that similar dyes from other vendors could also be used. A major concern for both choosing and handling these fluorescent dyes is that there is ample evidence in the literature that significant oxidation of both Cy5 and Alexa 647 can result from the levels of ozone that are commonly present in the air. Methods for mitigating ozone levels will be discussed in Section 3.6. A possible alternative is to use a new dye from GE Healthcare called Hyper5 which is a modified version of Cy5 that is reportedly stable to ozone (Dar *et al.*, 2008).

A single tube of NHS ester derivatized Cy3 or Cy5 contains a sufficient amount of fluorophore to label 16 cDNA samples. Because the NHS ester is highly unstable we do not store opened dye packages. Therefore, the actual volume of DMSO that we use to dissolve a single dye aliquot is determined by the number of samples. For example, in this protocol where eight different hybridizations are being performed (a four-point time course with dye-flipped replication), a single dye pack should be dissolved in 42 μl of DMSO (enough for eight 5 μl aliquots).

3.4.1. Materials for fluorescent labeling of cDNA

0.1 *M* sodium bicarbonate (pH 9.0)
DMSO (Fluka, Cat.#: 41647)

Cy3 NHS ester (GE, Cat.#: PA23003)
Cy5 NHS ester (GE, Cat.#: PA23005)
DNA Clean and Concentrator—25 kit (Zymo Research, Cat.#: D4006)

3.4.2. Protocol for fluorescent labeling of cDNA

(1) Dissolve dried down cDNA in 5 μl of 0.1 *M* sodium bicarbonate. To ensure that all of the cDNA is resuspended in this small volume, vortex well and spin down several times.
(2) Dissolve the Cy3 and Cy5 dyes in 42 μl of DMSO. Because it is difficult to see whether all of the dye is dissolved, vortex well and spin down several times.
(3) To one of the two tubes of experimental cDNA, add 5 μl of Cy3 dissolved in DMSO. To the other tube of experimental cDNA add 5 μl of Cy5 dissolved in DMSO. Do the same for your reference cDNA samples.
(4) Incubate the reactions in the dark in a 60 °C water bath for 1 h. While many dye labeling protocols incubate at room temperature, we observe a significant increase in labeling efficiency at elevated temperatures.
(5) To the 10 μl labeling reaction add 100 μl of DNA binding buffer from the DNA Clean and Concentrator kit, and then proceed according to manufacturer's instructions until the elution step. Proceed with elution as described in step 6.
(6) Transfer the column to a clean 1.7 ml tube. Add 35 μl of water directly onto the filter. Wait 30 s, then spin the samples in the microfuge at top speed ($>14{,}000\times g$) for 1 min (spinning at $10{,}000\times g$ is effective for elution and can help to prevent the lids of the collection tubes from snapping off).
(7) After discarding the column, quantitate cDNA yield using a spectrophotometer. Continue to use a conversion factor of 1 $A_{260} = 40$ μg/ml cDNA. Using this conversion factor, expect to recover $\geq 50\%$ of the cDNA that was included in the labeling reaction. Note that it is in theory possible to monitor Cy3 and Cy5 incorporation at this step; however, with this protocol the expected absorption levels for these dyes are close to background.
(8) Pool the appropriate Cy3- and Cy5-labeled samples for each hybridization, and dry in the SpeedVac. *Important note*—make sure that the appropriate samples are combined at this step. This is the easiest moment to ruin a great experiment. For example, the Cy3-labeled experimental sample should be combined with the Cy5-labeled reference sample, and *vice versa*.
(9) After the samples have dried in the SpeedVac, it is important to resuspend the samples in water quickly, because Cy5 is highly sensitive to ozone. Resuspend each pellet in 25 μl of water. Vortex well to

ensure proper mixing. We typically proceed immediately to hybridization. But if necessary, samples can be flash frozen in liquid nitrogen and stored in the dark at −80 °C indefinitely.

3.5. Microarray hybridization

The Agilent Custom 8×15K Microarrays are a single piece of 1×3 in. microscope glass with eight different hybridization zones. The individual hybridizations are kept separated by use of a matched gasket slide which, when sandwiched with the microarray slide, creates eight distinct hybridization compartments. All eight compartments need to be simultaneously hybridized. A detailed description of the microarray architecture and instructions for their use accompanies each microarray order and should be used as a supplement to the protocols described here.

Because dust is highly fluorescent, it is important to keep dust to a minimum during the hybridization procedure. We minimize the time that the microarray surfaces are exposed to air and always work on clean surfaces. Likewise, gloves should always be worn to avoid contamination of the glass. When possible the glass slides should be handled with tweezers. When this is not possible, the glass slides should only be handled by their edges.

3.5.1. Materials for hybridization

Agilent Custom 8×15K Microarray
2× hybridization buffer (Agilent, Cat.#: 5190-0403)
Eight chamber gasket slides (Agilent, Cat.#: G2534-60014)
Hybridization chamber (Agilent, Cat.#: G2534A)
Hybridization oven (Agilent, Cat.#: G2545A)

3.5.2. Protocol for hybridization

(1) Heat samples to 95 °C for 2 min. Place in a drawer or dark box for 5 min to cool, then spin down briefly.
(2) To each of the samples add 25 μl of 2× Agilent hybridization buffer. Mix by gently pipetting up and down. DO NOT VORTEX as this introduces bubbles that are problematic for the hybridization.
(3) Place a gasket slide in a hybridization chamber with the gaskets facing up.
(4) Load 40 μl of each sample into the appropriate gasket section. Avoid pipetting bubbles. To reduce the evaporation of the samples that are loaded first, the time to load eight samples should be minimized.
(5) After all eight sections have been loaded, carefully place the microarray slide onto the gasket slide. Note that it is important to ensure that the printed side of the microarray slide is exposed to the sample (see Agilent protocol).

(6) Close the hybridization chamber. Once assembled, rotate the entire chamber two or three times to wet the gasket linings. Ensure that any bubbles within the hybridization compartments can rotate freely. Gently tapping the chamber may help to release any stuck bubbles.
(7) Hybridize in a rotating oven at 60 °C for 16 h. In our experience hybridization times ranging from 14 to 18 h produce similar results.

3.6. Microarray washing

Prior to scanning the microarray, unhybridized cDNAs must be washed off of the glass surface. The design of the probes on the microarray is optimized for specificity at 60 °C. Because nonspecific species will begin to cross-hybridize at lower temperatures, an important consideration during the washing steps is minimizing the time between removing the microarray from the hybridization oven and washing away any unbound cDNAs.

A second important consideration during microarray washing is the capacity of atmospheric ozone to oxidize Cy5 dyes. This oxidation potential is most acute on a dried microscope slide after hybridization and washing. Several different mechanisms have been developed to mitigate the effects of ozone. The solution we chose was to create a chamber where ozone can be specifically removed from the air. Prebuilt chambers are commercially available which are of sufficient size to house a microarray scanner (Scigene, NoZone Workspace). Alternatively, ozone scavenging filters are available (Ozone Solutions, NT-40) which can be used to remove ozone from any chamber. We have built a simple chamber in our lab using Plexiglass which has sufficient working space for wash dishes and our microarray scanner (15 cubic feet). If changing the infrastructure in your lab is not a possibility, other options exist. For example, Agilent has developed a stabilizing wash solution (Agilent, Cat.#: 5185-5979). Likewise, Genisphere has developed a coating solution that stabilizes the optical properties of the Cy5 dye (Genisphere, Cat.#: Q500500).

3.6.1. Materials for microarray washing

Glass washing dishes with slide racks (Thermo, Shandon Complete Staining Assembly 121)
Wash I = 6× SSPE and 0.005% sarcosyl (or Agilent, Cat.#: 5188-5325)
Wash II = 0.06× SSPE and 0.005% sarcosyl (or Agilent, Cat.#: 5188-5326)

3.6.2. Protocol for microarray washing

(1) Prepare three glass wash dishes, two with Wash I (one without a slide rack, one with a slide rack), and one with Wash II (with a slide rack).

(2) Remove the hybridization chamber from the hybridization oven. Open the chamber such that the sandwich of the microarray and gasket slides remains intact. Quickly transfer sandwiched slides to the Wash I dish with no rack. With the sandwich completely submerged and supported with one hand, use tweezers to gently pry the two slides apart, allowing the gasket slide to drop to the bottom of the glass dish. Hold the microarray slide with your fingers being careful to touch only the stickers or sides of the glass.
(3) Keep the microarray slide submerged in Wash I and swish the microarray slide back and forth two or three times to remove most of the unhybridized sample.
(4) Quickly transfer the microarray slide into the rack in the second Wash I dish, taking care to minimize exposure of the microarray to air.
(5) Vigorously agitate the rack up and down for 1 min.
(6) Transfer the Wash I dish with microarray into the ozone-free chamber. Place Wash II glass dish with rack in ozone-free chamber. Quickly transfer the microarray slide from Wash I to Wash II.
(7) Gently agitate slide rack up and down, ensuring that all bubbles are washed off the microarray slide.
(8) With a pair of tweezers, grab the microarray slide by a corner with the stickers. Slowly remove the slide from Wash II. If this is done slowly enough (over 10 s) the microarray will come out dry because of the sheeting qualities of the wash solution.
(9) The microarray can either be scanned immediately or can be put into a dark box and protected from ozone until ready to be scanned.

4. Microarray Data Collection

The Agilent microarrays are printed on standard microscope glass (1×3 in.) and can be analyzed using any scanner than can accommodate this format. The features on an Agilent Custom 8×15K Microarray are approximately 60 μm in diameter. We get the highest quality data when we scan at a pixel size of 5 μm; this yields about 100 pixels of data for each feature. There are several companies that manufacture microarray scanners, including Agilent, Molecular Devices, and Tecan, which are capable of scanning at this resolution. We use an Axon 4000B for all of our data collection (Molecular Devices). The instruments listed above all use lasers to excite the Cy3 and Cy5 dyes and photomultiplier tubes (PMTs) to quantitate fluorescence intensity at each spot. Below are some general guidelines to facilitate microarray scanning, but because each of these instruments has different parameters, user guides should be consulted for specific details.

An important assumption underlying microarray experiments is that the total global signal is unchanged between the experimental and reference samples with only a small subset of features showing change in expression behavior. As such, an important consideration when scanning the microarrays is to equalize the total fluorescent signal intensity from the Cy3 and Cy5 samples. In the early days, this required a painstaking process of manually adjusting the PMT gain settings. Fortunately, newer versions of scanner control software allow the user to largely automate this step.

Whereas new software has improved the ability to equalize Cy3 and Cy5 signal, another important consideration is maximizing total signal intensity. For most microarray scanners the dynamic range is limited to about three orders of magnitude. By comparison, the difference in abundance of a rare pre-mRNA species and an abundant mature mRNA species can easily be greater than three orders of magnitude, meaning that no single scanning condition can generate reliable data for every RNA species. Agilent's microarray scanners automate the process of scanning each microarray twice; once using the lasers at full power and once at reduced power settings. The Agilent software then integrates the data from these two scans to increase the dynamic range of the experiment. In our experience, we have empirically identified conditions that maximize data collected from just a single scan. On our Axon 4000B, using the built in software, we set a saturation tolerance level equal to 0.1%. Using these settings and with the PMT gain values between 500 and 600 for both channels, only the most highly abundant RNA species in the cell are oversaturated, yet robust signal can be detected for the rare pre-mRNA species.

5. Microarray Data Analysis

In this section, we will divide the tasks for data analysis into two general parts: one is the technical details for processing the scans from the previous section, and the second is deriving biological meaning from an experiment. The first step is to extract quantitative measurements for both experimental and reference samples for each of the $\sim$15,000 features for all eight hybridizations. For this step in the data analysis pathway, processing a splicing-sensitive microarray is no different than processing a standard gene expression microarray. A description of the tools necessary for extracting data from a microarray experiment can be found in Chapter 2 of this volume, and should be consulted for this and subsequent sections. Included in that chapter are both the descriptions of the software that can be used and instructions for their implementation.

5.1. Data normalization

Having successfully extracted quantitative information for all of the features on the microarray, the next step is to mathematically normalize the data derived from the experimental and reference samples. As described in Chapter 2 of this volume, the Bioconductor package of data analysis software (Gentleman *et al.*, 2004) provides several different options for normalizing microarray data. We have compared the output when analyzing a single microarray using several different normalization algorithms and have found that the LOWESS normalization algorithm does the best job at addressing nonlinear behavior often seen in microarrays (Yang *et al.*, 2002). Therefore, we use the maNorm package within Bioconductor to implement LOWESS normalization across all of our data. The output from this analysis is a single value corresponding to the $\log_2$ transformed ratio of the Cy5 intensity to the Cy3 intensity for all 15,000 features.

5.2. Replication

Of the $\sim$15,000 features present on our splicing-sensitive Agilent microarrays there are $\sim$7000 unique features, each of which is replicated at either two or three distinct locations on each microarray. The next step in analyzing the data is to collapse these replicate data into a single averaged value. A spreadsheet program such as Microsoft Excel can be used to calculate averages as well as coefficients of variation for these measurements. Having compressed the data to a single value for each unique feature, the next task is to compare and average the values determined between the dye-flipped replicate experiments. It is important here to repeat that the standard output from the maNorm package is always presented as a ratio of Cy5 to Cy3 intensity. According to the design of our experiment, one microarray compares Cy5-labeled experimental sample with Cy3-labeled reference sample, whereas its corresponding replicate compares Cy5-labeled reference sample with Cy3-labeled experimental sample, which for the purposes of this section we will refer to as ''forward'' and ''flip'' experiments, respectively. Because of their orientations, the data output from the maNorm package for the ''forward'' and ''flip'' experiments are expected to be negatively correlated. Therefore, to determine the average behavior described by the dye-flipped experiments the ''flip'' value must be multiplied by -1 prior to averaging. At this point, the value associated with each of the $\sim$7000 unique features represents a composite value incorporating replication within a single microarray and between dye-flipped microarrays and constitutes as many as six independent measurements for each time point.

5.3. Splicing specific data

While no further steps are necessary to analyze the behavior of intronless genes, analyzing intron-containing genes requires additional steps. Whereas just one value is needed to describe the behavior of an intronless gene, even the simplest intron-containing gene has a minimum of three values associated with it, corresponding to the total, pre- and mature mRNA probes. None of these metrics by themselves is sufficient to understand the splicing behavior of a given transcript, but rather they must be considered in relation to one another. In their original experiments, the Ares group addressed this issue by creating splice junction and intron accumulation indexes; they divide the values for both the mature probe and the pre-mRNA probe by the value of the total mRNA probe, respectively (Clark *et al.*, 2002). By comparison, we have chosen to analyze the data by concurrently examining the behaviors of each of the three individual metrics (Pleiss *et al.*, 2007a,b). Database software such as Microsoft Access can be used to associate the total, pre- and mature mRNA specific probes with one another for a given intron-containing gene.

5.4. Extracting biological meaning

Congratulations! Having successfully collected experimental samples, isolated RNAs, converted RNA to cDNA, labeled and hybridized the cDNA to microarrays, extracted and normalized the data, you have now made it to the hard part—understanding the biology underlying your experiment. For traditional gene expression experiments, this is rather straightforward in the sense that one can look for transcripts that show either increased or decreased abundance. Those transcripts showing increased abundance either reflect genes whose transcription has increased or whose degradation has decreased. From the perspective of splicing, it is more difficult to describe what a defect should look like. A simple expectation might be that a defect in splicing would lead to accumulation of pre-mRNA levels with a concomitant decrease in mature mRNA levels. This expectation, however, presumes nearly equal steady state levels of pre- and mature mRNA. For genes that are efficiently spliced, where the mature mRNA level is much greater than the pre-mRNA level, a defect in splicing could be expected to show an accumulation of pre-mRNA with little or no change in mature mRNA. Our experience in analyzing these types of data demonstrates that both of these profiles can be seen among the different intron-containing genes in *S. cerevisiae*. As such, several different descriptors exist which may be used to identify a transcript whose splicing is altered in response to an experimental condition.

With these ideas in mind, the next challenge for any experiment is finding the important biological changes among the sea of data collected

in a microarray experiment. Without a doubt, this is the most challenging part of the microarray experiment because no single approach to interrogating the data will identify all of the genes whose behavior is modified. Rather, we find the best way to identify these genes is to look at the data from many different perspectives. For example, in our experience comparing data across an experimental time course is a powerful way to identify genes that are responding to an experimental condition. Two important software packages we use to organize and visualize our data, Cluster (Eisen *et al.*, 1998) and Java Treeview (Saldanha, 2004), are described in the Chapter 2 of this volume. We find that Cluster works particularly well for organizing splicing-specific information. For example, Fig. 3.4 shows the results of a time course examining a mutation in the canonical splicing factor Prp16 versus a wild-type reference. By concurrently examining the behavior of the total, pre-, and mature mRNA, transcripts can be identified whose splicing is affected by the experimental condition. This figure demonstrates the variety of behaviors possible for different transcripts, as described above, demonstrating the global patterns that result from a defect in splicing.

6. Future Methodologies

Splicing-sensitive microarrays are a powerful tool for examining genome-wide changes in pre-mRNA splicing. However, as with all microarray technologies, the advent of high-throughput, short-read sequencing technologies promises to change the way splicing is studied from a genome-wide perspective (Wold and Myers, 2008). In theory, these short-read sequencing methodologies have an advantage over microarray technologies in that they take an unbiased approach to the experiment. Because microarrays require probes be designed to target-specific RNAs, they are by nature poor at discovering previously uncharacterized species. By directly sequencing total cellular RNA, short-read sequencing methodologies should be able to identify both previously uncharacterized RNAs and novel splicing events. Nevertheless, many of the same challenges that the splicing-sensitive microarray community faced must now be resolved in the context of short-read sequencing methodologies. For example, the most widely used current methods for sequencing cellular RNAs utilize poly-(A) selection schemes to remove ribosomal RNAs from the pool of sequenced samples. For the same reasons described at the beginning of this chapter we think it is likely that many of the interesting RNA processing events happen independent of the poly-(A) status of the RNA. Until such time as these methodologies have been developed for the sequencing technologies,

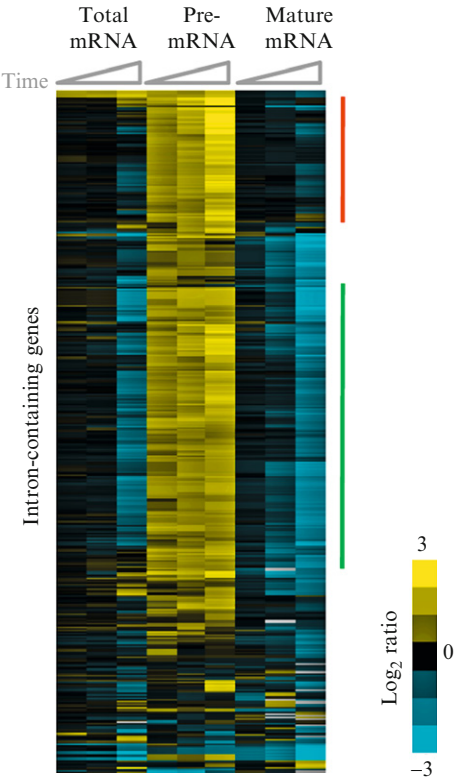

Figure 3.4 Genome-wide changes in pre-mRNA splicing. Results are presented from an experiment comparing a strain containing a cold-sensitive *prp16-302* mutation with a matched wild-type strain as both were shifted to the nonpermissive temperature. Data are shown from unshifted samples (grown at 30 °C), as well as after 10 and 60 min of incubation at 16 °C. Each horizontal line represents the behavior of a single intron-containing gene during this time course. Notice that some genes (indicated with a red bar) show a dramatic increase in pre-mRNA level with very little change in mature mRNA level, whereas other genes (indicated with a green bar) show a strong increase in pre-mRNA level concomitant with a strong decrease in mature mRNA level.

splicing-sensitive microarrays will continue to be a fast, cost-efficient, and effective way to examine genome-wide changes in pre-mRNA splicing.

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REFERENCES

Berget, S. M., Moore, C., and Sharp, P. A. (1977). Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* **74,** 3171–3175.

Chow, L. T., Gelinas, R. E., Broker, T. R., and Roberts, R. J. (1977). An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* **12,** 1–8.

Clark, T. A., Sugnet, C. W., and Ares, M. (2002). Genomewide analysis of mRNA processing in yeast using splicing-specific microarrays. *Science* **296,** 907–910.

Dar, M., Giesler, T., Richardson, R., Cai, C., Cooper, M., Lavasani, S., Kille, P., Voet, T., and Vermeesch, J. (2008). Development of a novel ozone- and photo-stable HyPer5 red fluorescent dye for array CGH and microarray gene expression analysis with consistent performance irrespective of environmental conditions. *BMC Biotechnol.* **8,** 86.

DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278,** 680–686.

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868.

Engebrecht, J. A., Voelkel-Meiman, K., and Roeder, G. S. (1991). Meiosis-specific RNA splicing in yeast. *Cell* **66,** 1257–1268.

Gelder, R. N. V., Zastrow, M. E. V., Yool, A., Dement, W. C., Barchas, J. D., and Eberwine, J. H. (1990). Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* **87,** 1663–1667.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., *et al.* (2004). Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol.* **5,** R80.

House, A. E., and Lynch, K. W. (2008). Regulation of alternative splicing: More than just the ABCs. *J. Biol. Chem.* **283,** 1217–1221.

Juneau, K., Palm, C., Miranda, M., and Davis, R. W. (2007). High-density yeast-tiling array reveals previously undiscovered introns and extensive regulation of meiotic splicing. *Proc. Natl. Acad. Sci. USA* **104,** 1522–1527.

Pleiss, J. A., Whitworth, G. B., Bergkessel, M., and Guthrie, C. (2007a). Rapid, transcript-specific changes in splicing in response to environmental stress. *Mol. Cell* **27,** 928–937.

Pleiss, J. A., Whitworth, G. B., Bergkessel, M., and Guthrie, C. (2007b). Transcript specificity in yeast pre-mRNA splicing revealed by mutations in core spliceosomal components. *PLoS Biol.* **5,** e90.

Saldanha, A. J. (2004). Java Treeview—extensible visualization of microarray data. *Bioinformatics* **20,** 3246–3248.

Sapra, A. K., Arava, Y., Khandelia, P., and Vijayraghavan, U. (2004). Genome-wide analysis of pre-mRNA splicing: Intron features govern the requirement for the second-step factor, Prp17 in *Saccharomyces cerevisiae* and Schizosaccharomyces pombe. *J. Biol. Chem.* **279,** 52437–52446.

Sayani, S., Janis, M., Lee, C. Y., Toesca, I., and Chanfreau, G. F. (2008). Widespread impact of nonsense-mediated mRNA decay on the yeast intronome. *Mol. Cell* **31,** 360–370.

Sugimoto, N., Nakano, S., Yoneyama, M., and Honda, K. (1996). Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. *Nucleic Acids Res.* **24,** 4501–4505.

Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S. F., Schroth, G. P., and Burge, C. B. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* **456,** 470–476.

Wernersson, R., Juncker, A. S., and Nielsen, H. B. (2007). Probe selection for DNA microarrays using OligoWiz. *Nat. Protoc.* **2,** 2677–2691.

Wiegant, J. C., van Gijlswijk, R. P., Heetebrij, R. J., Bezrookove, V., Raap, A. K., and Tanke, H. J. (1999). ULS: A versatile method of labeling nucleic acids for FISH based on a monofunctional reaction of cisplatin derivatives with guanine moieties. *Cytogenet. Cell Genet.* **87,** 47–52.

Wold, B., and Myers, R. M. (2008). Sequence census methods for functional genomics. *Nat. Methods* **5,** 19–21.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: A robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res.* **30,** e15.