Optimizing Polymerase Chain Reaction (PCR) to Quantify Processed vs Unprocessed RNA Levels.

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Abstract

Proper mRNA processing, including the addition of a poly-A tail, is essential for transcription stability and export from the nucleus. Disruptions in this process can lead to mis-regulated gene expression and is associated with cancer. We use a Polymerase Chain Reaction (PCR) based method in yeast to assess mRNA processing efficiency while avoiding the use of radioactive materials. mRNA was isolated from yeast and converted to cDNA, then specific primers were used to amplify the cDNA using PCR, and the products analyzed using gel electrophoresis. Comparing band intensity suggests an approximate 1:2 ratio of processed RNA to total mRNA. These results support the use of PCR-based techniques for evaluating mRNA processing efficiency. This method is a safer, more accessible approach for studying RNA processing regulation and provides a foundation for future research into how mRNA processing is affected by various cellular conditions.

Introduction

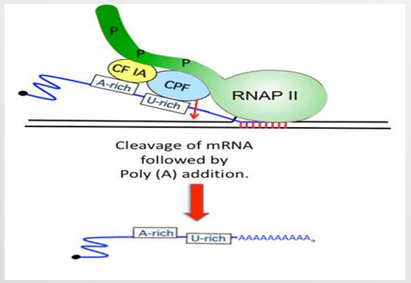


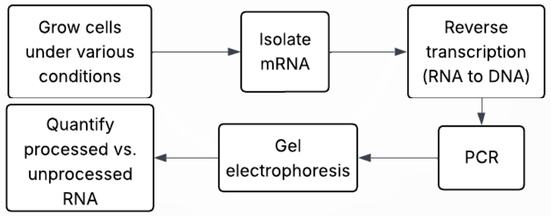
Figure 1: mRNA 3' end processing involves cleavage at a specific site of the newly transcribed mRNA followed by the addition of p(A) tail by a separate polymerase associated with the cleavage and polyadenylation (CPF) complex. This process occurs co-transcriptionally, through cleavage and polyadenylation factors that associate with the largest subunit of RNA polymerase II.

Proper mRNA processing is essential for gene expression, as it involves the addition of a poly-A tail, which stabilizes mRNA and facilitates its transport from the nucleus to the cytoplasm. Without processing, mRNA remains in the nucleus and cannot be translated into proteins. The importance of poly-A tail length, as highlighted in prior research[1], underscores its role in mRNA stability and regulation. Traditionally, radioactivity-based methods have been used to detect the efficiency of mRNA processing[2]. Improper mRNA processing has been correlated with cancer. Misprocessing can affect regulatory elements present on RNA and lead to the over-expression, particularly of oncogenes[3].

Our goal is to identify changes in mRNA processing efficiency under various cellular conditions. This study employs a PCR-based approach in Baker's Yeast, to avoid the challenges of working with radioactive materials. To calculate RNA processing efficiency, PCR based methods will be used to compare unprocessed and processed RNA levels. These findings will contribute to a broader understanding of RNA processing and its implications in disease development.

Methods and Materials

RNA is isolated from yeast and converted to cDNA for analysis as described below, prior to PCR and gel electrophoresis. Gel electrophoresis images illustrate differences in unprocessed versus total RNA levels.



PCR Reaction Setup:

PCR was done to amplify yeast mRNA using PDC1 primers (see figure 2). A master mix (MM) was prepared for each experiment and was used for each treatment group. The MM contained nucleotide-free water, 10x buffer with MgCl2, excess MgCl2, 10 µM dNTP, 10 µM forward PDC1 primer, 10 µM reverse span or total PDC1 primer, and enzyme blend. 49 µL of MM was added to each PCR tube followed by 1 µL of template.

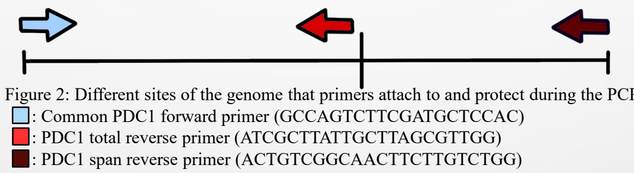


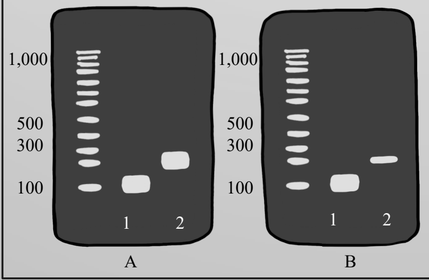
Figure 2: Different sites of the genome that primers attach to and protect during the PCR.
: Common PDC1 forward primer (GCCAGTCTTCGATGCTCCAC)
: PDC1 total reverse primer (ATCGCTTATTGCTTAGCGTTGG)
: PDC1 span reverse primer (ACTGTCGGCAACTTCTTGTCTGG)

PCR Conditions:

PCR amplification occurred through a standard BioRad Thermocycler. Initial denaturing occurred for 2 minutes at 95ºC, followed by denaturing at 95ºC for 30 s, annealing at 63ºC for 30 s, and extending for 1 minute at 72ºC. The process repeated for 20 cycles before storing indefinitely at 4ºC.

Gel Electrophoresis, Staining, and Imaging:

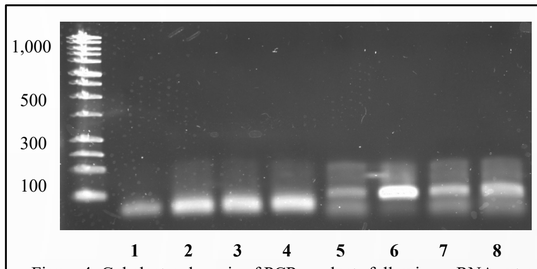
Agarose was dissolved into 1x TAE or TBE buffer in heat and cooled to make a gel. Loading dye was added to each sample and to the ladder. A 100 bp ladder was used for size reference before loading the samples. The gel ran at 105 V in the TAE or TBE buffer until the dye traveled down approximately 2/3 the way down. After gel electrophoresis, gel was stained with diluted Ethidium bromide before destained with distilled water. The DNA was visualized using UV light.



1: PCR product with primers for total RNA
2: PCR product with primers for unprocessed RNA

Figure 3 Expected results of experiment. (A) a ratio of 1 signifying all RNA remains unprocessed, suggesting a complete disruption in the RNA processing mechanism. (B) an unprocessed/total ratio of .5, indicating that half of the RNA was unprocessed.

Results



1: Negative control (water)
2: Positive control (genomic DNA)
3: RT sample
4: No RT sample
5: Negative control (water)
6: Positive control (genomic DNA)
7: RT sample
8: No RT sample

Figure 4: Gel electrophoresis of PCR products following mRNA extraction and cDNA synthesis. Lanes 1-4 show PCR products corresponding to total RNA and lanes 5-8 show PCR products corresponding to unprocessed RNA.

DNA was detected in all samples, suggesting some contamination with genomic DNA. However, the specificity of the primers was confirmed: primers designed to amplify total RNA yielded shorter fragments compared to those targeting unprocessed RNA. The bands in lanes seven and eight were approximately half as intense as those in lanes three and four, indicating an approximate 1:2 ratio between unprocessed and total mRNA.

This conclusion is supported by the observation that the intensity of the bands were similar between in lanes 2 and 6, which is expected since genomic DNA (nor reverse transcribed RNA) that were used in these samples remains unprocessed and should therefore produce the same amount of product with both sets of primers. If the genomic DNA bands had been less intense like the rest of the samples, it would have suggested a difference in primer efficiency rather than a true difference between processed and unprocessed RNA.

Discussion and Future Directions

This method is successful in identifying levels of unprocessed and processed RNA, but:

1.) PCR conditions still need to be modified to reduce smearing

2.) RNA purification and the RT process needs to be improved, because there is DNA contamination in the sample without RT reaction, which should not be the case.

3.) need to be very careful about possible ways that samples get contaminated

For future research, the PCR product bands should be quantified using specific software to determine the ratios of unprocessed vs. total mRNA and evaluate RNA processing efficiency under different conditions.

References

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3.Mayr, C., & Bartel, D. P. (2009). Widespread Shortening of 3′UTRs by Alternative Cleavage and Polyadenylation Activates Oncogenes in Cancer Cells. *Cell*, *138*(4), 673–684.

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