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Transcriptome Analysis Methods: From the Serial Analysis of Gene Expression and Microarray to Sequencing new Generation Methods

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Abstract: Up-to-date research in biology, biotechnology, and medicine requires fast genome and transcriptome analysis technologies to investigate cellular state, physiology, and activity. Gene expression is the process of generating messenger RNA copies of a gene. The transcriptome, which contains the mRNA of the cell, reflects the cell's overall gene expression pattern. Understanding the nature and frequency of each RNA molecule in a given cell under certain circumstances is necessary to examine the transcriptome. Microarray and serial analysis of gene expression are two primary techniques researchers use in transcriptome studies. Here, microarray technology and next-generation sequencing of transcripts are states of the art. Since microarray technology is limited to RNA, quantifying transcript levels and sequence information, RNA-Seq provides nearly unlimited possibilities in modern bioanalysis. Sequencing of RNA, or RNA-Seq, is now a standard method to analyze gene expression and uncover novel RNA species. In addition, aspects of RNA biogenesis and metabolism can be interrogated with specialized techniques for cDNA library preparation. The present study will introduce and compare new high-performance methods used in examining the transcriptome. This also presents a detailed description of next-generation sequencing, describes the impact of this technology on transcriptome analysis, and explains its possibilities to explore the modern RNA world.

Keywords: Transcriptome; Gene expression; Microarray; RNA sequencing; cDNA

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1. Introduction

Up-to-date biology, biotechnology, and medicine research require fast genome and transcriptome analysis technologies to investigate cellular state, physiology, and activity. Gene expression generates messenger RNA copies of a gene [1]. The transcriptome, which contains the mRNA of the cell, reflects the cell's overall gene expression pattern. A simple observation of the cell's information flow represents the gene's information pathway to the mRNA and proteins [2]. The gene must be transcribed to the mRNA by RNA polymerase to produce the proteins needed, and then, the mRNA can be translated into proteins by ribosomes. Specific

proteins are expressed at different levels depending on the cell type and biological status [3-5]. In addition, due to the apparent relationship between gene expression and protein translation, recognizing the surface of mRNA may provide an indirect way to obtain some information about the current state of the cell [6,7], such as comparing the gene expression between patient and healthy cells can determine the molecular basis of the disease [8-10]. A transcriptome containing the cell mRNA reflects the gene expression's general pattern in the cell [11,12]. The transcriptome study can have a very complex composition, including hundreds of thousands of different mRNAs, each of which contributes to the general population [13,14]. Therefore, it is necessary to identify the existing mRNAs to examine the transcriptome.

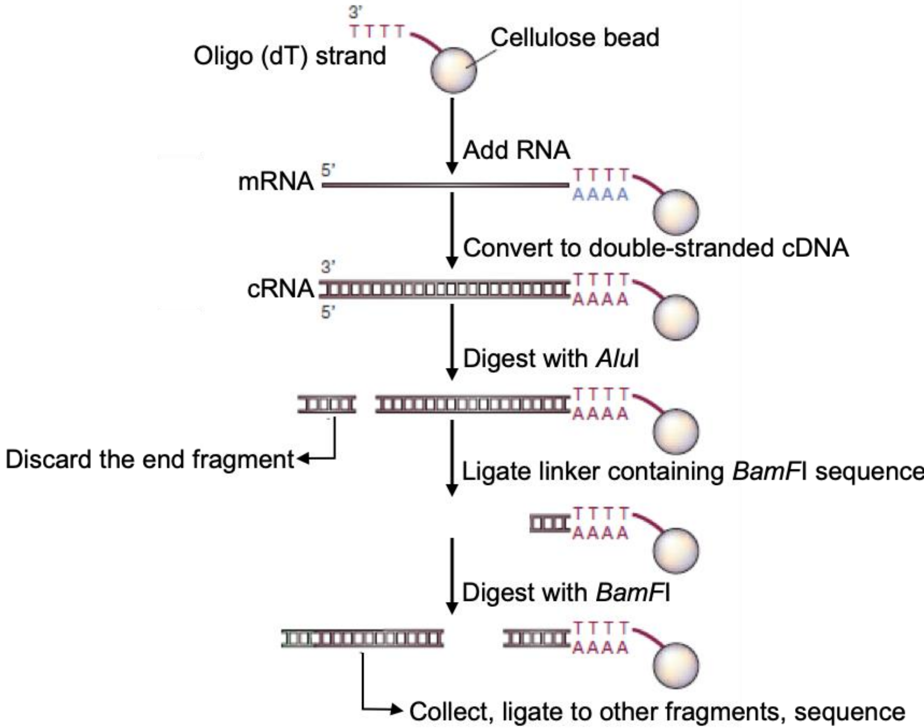

Figure 1. Serial analysis of gene expression (SAGE).

Most of the RNA information is derived from studies using biochemical methods, in which a small number of specific molecules are analyzed [5,15], such as the high-throughput methods [16,17]. Moreover, the expressed sequence tag (EST) method, which reveals the sequence and frequency of the relevant RNAs, has significantly identified new genes in the genomes [18]. However, the high cost of sequencing in this method has limited its use in the analysis of expression [19,20]. Also, the data obtained from this method appears to be semi-quantitative. On the other hand, the Serial Analysis of the Gene Expression (SAGE) method has significantly decreased the cost of the expression analysis based on each gene due to the sequencing of only one short identifier region in each cDNA (15 bp for the straightforward SAGE method and 21 bp for the long SAGE method) [21,22]. These findings indicate that the advent of new technology could be replaced the EST and SAGE methods for gene expression analysis mainly due to their cost-effectiveness for large-scale studies (Figure 1).

The gene expression analysis by DNA microarray is based on the hybridization of targets labeled by fluorescent, which are prepared of transcriptions by probes connected to a solid surface through printing or in-situ synthesis [23]. However, while this method can perform genomic transcriptions, the need for the availability of previous information on the sequence and the reference genome/transcriptome to design the microarray probes has limited

the development and use of this technology in the identification programs (Figure 2) [24-26]. Furthermore, mutual hybridization and background signals often lead to low specificity or low sensitivity for some genes [27,28]. Also, the RNA-seq technique can be used alone for transcriptome data extraction or other genomic functional methods to improve gene expression analysis [24,29]. Finally, the RNA-seq can be associated with different biochemical tests to analyze many other RNA biology aspects such as RNA-protein binding, RNA structure, or RNA-RNA interaction [30]. The present study will introduce and compare new high-performance methods used in examining the transcriptome.

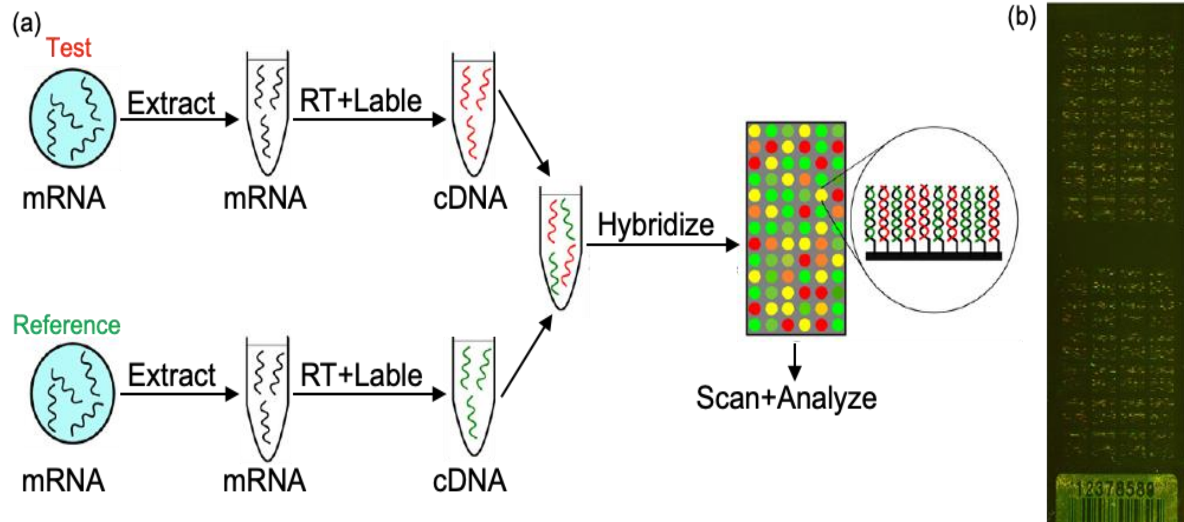

Figure 2. Spotted microarray experimental set-up. The extracting (target) mRNAs from cells under two different physiological conditions are converted to the cDNA by the process of reverse transcription and then labeled with different fluorescent colors such as cy3 or cy5. Equal amounts of targets are labeled and placed on glass slides containing cDNA amplicons or stabilized probes.

2. Study the transcriptome with Serial Analysis of Gene Expression (SAGE)

The SAGE technique allows the massive analysis of mRNA transcriptions without prior knowledge about the transcriptome [31]. The SAGE creates an extensive library of short oligonucleotide sequences (tag) with mRNA origin, prepared from a specific tissue or cell [32]. Each tag sequence directly indicates the frequency of the corresponding transcription [33]. This technique, which was first used at Johns Hopkins University, is a sequence-based method that shows genes' expression [34]. The SAGE produces gene expression profiles for a particular cell or tissue and identifies specific genes expressed in particular conditions (Figure 1)[35].

2.1. SAGE: Method of testing.

Instead of studying complete cDNAs, the SAGE produces short sequences of 12 bp, each representing an mRNA contained in the transcriptome [36]. This method allows these 12 bp sequences to identify the genes encoding the mRNA. The first step in producing 12 bp sequences is to stabilize the mRNA in the chromatography column, which binds the ends of the poly-A of the 3' terminus of these molecules to the oligo (dT) attached to cellulose grains in the column [37]. Then, the mRNA is transformed into a two-stranded cDNA and treated with restriction enzymes with a 4-nucleotide cutting site such as AluI [38]. As a result, the cDNA is regularly cut. The cDNA's last cut piece remains connected to the column cellulose aggregates, and the remaining parts are eliminated and removed [17]. In the next phase, a short connector connects to each cDNA's free end, an identification sequence for the BamFI enzyme

[39,40]. This unusual restriction enzyme cuts 10 to 14 downstream nucleotides instead of cutting inside the identification sequence [41]. Therefore, treatment with BamFI leads to extracting pieces with an average length of 12 bp from each cDNA's end. The parts are gathered and connected to create a catenane to be sequenced. Every single sequence can be specified within the catenane since they are separated by BamFI positions [42]. The steps are schematically illustrated in (Figure 1).

3. Study the Transcriptome with Microarray Technique

DNA microarrays are used in transcriptomic studies, with small slides with hundreds to tens of thousands of DNA molecules attached [29,43]. The DNA is capable of binding to complementary sequences generated from mRNA transcriptions, which facilitates the determination of different transcripts values of the mRNA in the cell (Figure 2)

The DNA microarrays allow molecular biologists to examine the mRNA's transcription level related to tens of thousands of genes. Thus, they create a window for performing the genome's internal functions at the transcription level [43]. Microarrays have helped study multiple diseases and regulate many biological mechanisms and organisms' cellular cycles [44]. In DNA microarrays, the probes are DNA oligomers capable of interacting with targeted complementary DNA sequences [45]. This leads to various DNA microarrays, although there are two general groups. The first group consists of microarrays that the single-stranded DNA oligomer probe (ssDNA) directly synthesizes on the substrate (in situ synthesis) [46,47]. The second group consists of microarrays. An ssDNA amplicon oligomer probe or a dsDNA (double-stranded DNA) is placed on the substrate, usually referred to as spotted arrays [48].

3.1. Types of microarray.

3.1.1. Affymetrix or GeneChip.

The Affymetrix array is known as GeneChips among traditional arrays, in which the DNA oligomers are synthesized in situ [46]. The GeneChips uses a photolithographic mask to determine the probe's position in the array to bind (connect) the next nucleotide to the lengthening oligomer [49]. The GeneChips have short probes (with 25 nucleotides) with multiple probe sequences for each target. All of them what is known as probe collections and contain the perfect matches (PM) for the examined sequence as well as probes containing a mismatch (MM) base at the midpoint position to determine non-specific targets [50]. Photolithographic techniques to produce arrays lead to highly reproductive and highly-ordered probe areas at the array level [51]. However, this is the same strategy that makes customized arrays more expensive. Affymetrix has concentrated on generating arrays for organisms that are widely used. However, in recent years, the selection of organisms for which an array has expanded considerably [50,52].

3.1.2. Nimblegen.

The Nimblegen arrays are also similar to GeneChips with the advantage that they do not need to build new photolithographic masks to design a new array like the GeneChips [53]. Another critical difference in the Nimblegen technology is long oligonucleotides (60 mers) compared with the 25-mers oligonucleotides used by Affymetrix [54]. Theoretically, it leads

to the further specificity of the hybridization of targets with the probes on the slide, reducing the probability of mutual hybridization of targets.

3.1.3. Spotted arrays.

Compared to the in situ arrays mentioned above, the spotted arrays are synthesized through the synthetic oligomer's mechanical deposition (generally 50-70-mers) or the cDNA amplicons [55,56]. The cDNA amplicons can be synthesized through reverse transcription of all the studied organism's mRNAs to generate the expressed sequence tags (ESTs). They will then be multiplied by polymerase chain reaction (PCR), purified, and indicated on the microarray slides [57]. In early microarrays, the cDNA libraries were produced for many different organisms, and further cDNA synthesis was done quickly, providing a source of access to probe materials.

4. Comparison of SAGE and Microarray

The microarray method requires information about the sequence of mRNAs, which is measured before that. In contrast, the SAGE can detect unknown gene transcriptions without prior knowledge of sequences during the analysis and can provide a statistical report of the population of the mRNAs present in the cell [58]. The serial analysis of gene expression (SAGE) is an efficient method for producing a snapshot of the mRNA population in the studied sample as short tags matching some of these transcriptions. In addition, the SAGE technique has used different organs to detect the transcriptome from other tissues and cells [59].

5. Study of the Transcriptome with the RNA-Seq technology

The first decade of this millennium has witnessed the emergence of a massive parallel sequencing known as the Next Generation of Sequencing (NGS) [56,60]. Deep sequencing, a revolution in biology and medicine, was rapidly used in RNA research extensively due to its ability to obtain a large amount of data quickly. RNA-Seq is the method of choice for studying gene expression and identifying new RNA species [61]. Compared to DNA microarray methods, RNA-Seq has less noise and a more dynamic range for detection [62]. Most importantly, the RNA-Seq directly illustrates the sequence's similarity, essential for analyzing unknown genes and new transcriptional isoforms [63]. Several different technologies have been investigated for RNA-Seq. While direct sequencing of RNA molecules is possible, most RNA-Seq experiments are performed using sequencing tools for DNA molecules due to the technical maturity of commercial tools designed to determine the DNA sequences [61].

5.1. cDNA library preparation.

Therefore, the cDNA library's preparation from RNA appears to be an essential step for the RNA-Seq approach. Each cDNA in an RNA-Seq library comprises a cDNA with a specific size associated with the adjacent adapter sequence as required for replication and sequencing on a particular platform. Preparing a cDNA library varies depending on the type of examined RNA and according to the size, sequence, structural characteristics, and frequency. The primary considerations include: i) How to obtain the studied RNA molecules; ii) convert RNA to two-stranded cDNAs with a specified size range: iii) How to place the adapter sequence at the end of the cDNA for replication and sequencing.

5.2. Selection of transcriptions containing poly (A).

The most common use of RNA-Seq has perhaps polyadenylated RNA sequencing [64]. In eukaryotic organisms, most proteins encoding RNAs (mRNAs) and many noncoding long RNAs (lncRNAs) (> 200 nucleotides) have a poly (A) tail [65]. The Poly (A) tail provides the technical potential for enriching the RNAs containing poly (A) from the total cellular RNA, which accounts for about 1 to 5 percent of the reservoir [66]. The RNAs containing poly (A) can be selected with magnetic or cellulose grains coated with the oligo-dT molecules [67]. Also, the polyadenylated RNAs can be selected using the Oligo dT primer for reverse transcription (RT) [68].

5.3. rRNA depletion.

Most non-polyadenylated RNAs, including prokaryotic mRNAs, the mRNAs isolated from formalin-fixed samples, the formalin-fixed paraffin-embedded (FFPE) specimens, and the transcriptions lacking poly (A) in the eukaryotic cells are studied [69]. An essential issue in sequencing these RNAs is how to eliminate ribosomal RNAs (rRNAs), which have the highest RNA frequency in the cell but are less considered in most studies [17]. Several methods have been developed for their destruction in the RNA reservoir. An approach to eliminating the rRNAs is based on specific tail probes, which can be hybridized with rRNAs [70]. The unwanted rRNAs or their cDNAs are hybridized with biotinized DNAs or locked nucleic acid (LNA) probes and then separated by streptavidin grain [71].

Similarly, the rRNAs are targeted by noncoding oligo-DNAs and digested by RNase H [72]. This method is also known as probe-directed degradation (PDD) [73]. Although this approach has less difficulty than hybridization, it requires consistent coverage of rRNAs and unique probe sets designed for different species [74]. Recently, a sequence-based non-alternate method has been developed that addresses some of these issues. In this method, all cDNAs, including rRNA and other RNAs, are conjugated and bound to the rRNA probes [75]. Then, the hybridized sequences are digested by a duplex-specific nuclease (DSNs). As a result, they become useless for replication [76]. However, this approach requires large amounts of total RNA, which can be challenging when working with clinical specimens.

Another approach to rRNA depletion involves the use of specific and that bind to the examined RNA molecules along the RT, thus preventing the action of rRNAs [17]. In eukaryotic cells, RNA purification containing poly (A) based on the oligo-dT granules is the selective approach for most applications due to its ease of use and relatively low cost [77]. However, for low-input samples, the oligo-dT initiation generally provides better results. Both methods of poly (A) selection can effectively address the contamination of intron sequences. If an RNA sample is partially degraded or the user intends the noncoding RNAs, rRNA depletion is usually necessary through PDD or NSR initiation [78]. The PDD is better for samples with higher inputs, while the NSR initiation is mainly used for low-input RNAs.

5.4. Fragmentation.

After selecting RNA containing poly (A) or rRNA depletion, the RNA samples are usually divided into fragments with different sizes before the RT [17]. This is necessary due to the size limitation of most current sequencing systems, such as less than 600 bp in the Illumina sequence finder [79]. The RNAs can be fragmented with alkaline solutions, divalent cations such as Mg++ and Zn++, or enzymes such as RNase III [80]. The fragmentation with alkaline

solutions or divalent cations is usually done at high temperatures, like 70 °C, to reduce the RNA structure's effect on its fragmentation. Instead, the healthy RNAs can be reversely transcribed to fragment the whole cDNA length [81]. The conventional method for cDNA fragmentation requires an acoustic cut, which has less automation capability than RNA segmentation. In short, the full length of the two-stranded cDNAs can be degraded by DNases [82]. The latter method's development using a method based on the transposon, called Tagmentation, has simplified the cDNA fragmentation and simultaneously adds an adapter sequence [83]. In this method, a type of transposon of an active Tn5 performs the double-stranded DNA fragmentation. Connecting the adapter's oligonucleotide couples to both ends in a fast reaction (~ 5 minutes) [84]. However, it is noteworthy that the Tn5 and other methods based on cDNA decomposition enzymes require an accurate DNase enzyme ratio. The optimization of this method is more difficult than RNA fragmentation [85]. Consequently, RNA segmentation is still an approach often used to prepare the RNA-Seq library [86].

5.5. Adapters and directionality, amplification, and molecular labels.

In a standard protocol of the RNA-Seq library, the cDNAs synthesized with the desired size from the reverse transcription of fragmented RNAs with random hexamers or fragmented full-length cDNAs are bound to DNA adapters before replication and sequencing [87]. Due to more sequence detection constraints, the cDNA libraries must be replicated with PCR before sequencing. However, there is no optimal dataset processing instruction for various applications and analysis types to be used in the RNA-seq [86]. Therefore, the researchers design the test depending on the studied organism and their research objectives and adopt different analytical strategies [85]. A crucial prerequisite for analyzing the success of RNA-seq is the capability of the generated data to respond to the examined biological issues. This can be realized firstly by designing a good test plan, namely selecting a library type, the sequencing depth, and a number of proper replications for the biological system studied, and secondly, by designing the proper implementation of the sequencing test and ensuring that the obtained data would not be contaminated with unnecessary errors [86].

6. Comparison of RNA-Seq and Microarray

Comparing the RNA-Seq and Affymetrix platforms' datasets using a set of similar samples indicates a high correlation between the gene expression profiles produced by the two platforms [88]. However, RNA-Seq is superior in detecting the transcriptions with low frequency, distinguishing between the significant biological isoforms, and identifying genetic variants. The RNA-Seq also shows a more comprehensive dynamic range than the microarray approach, which allows the detection of differentially expressed genes (DEGs) with further changes [89,90]. Also, the two datasets' analysis reveals the advantage of avoiding the microarray probe's technical issues, such as mutual hybridization, non-specific hybridization, and the limited detection range of the probes [91,92]. Since the RNA-Seq dependent on the pre-designed probe is not complementary for sequence detection, it needs the frequency issues and the probe interpretation, making the data interpretation easier [91,93]. Despite the great benefits of the RNA-Seq method, the microarray is still the standard choice for researchers conducting transcriptional characterization tests. The RNA-Seq sequencing technology is new and more costly than the microarray approach for most researchers; its data storage is

challenging and has a more complex analysis [94,95]. As these barriers are resolved, the RNA-Seq platform is expected to become the dominant tool for transcriptome analysis.

7. Conclusions

Since discovering the RNA role as a critical intermediary between the genome and proteome, identification of transcription and gene expression measurement have been two different main activities in molecular biology. The SAGE technique quickly and accurately analyzes the relative frequency of all transcripts in a given sample (cell or tissue). The SAGE has been widely used in biology, medicine, and pharmacology. This method has been successfully used to compare expected and patient cells' expression profiles or two distinct treated samples. In summary, two samples are used in this technique that is connected and tagged with separate primers and then duplicated. After that, the primers are removed, forming the sticky ends of the cloned connectors, and sequenced in a vector. Then, an extensive computational analysis is performed. In the case of yeast transcriptome and cancer, the SAGE-based databases are accessible through the Internet.

The microarray context is an excellent example of the integration and convergence of several technologies, including automatic DNA sequencing, DNA replication using PCR, highly efficient oligonucleotide synthesis, the chemistry for labeling nucleic acids, and bioinformatics. A microarray can be considered a hybridization test or miniature gene detection. Instead of measuring signals in the experiment at the macro level, such as microtiter plates, membrane spraying, and test tubes, the microarray tests or single elements are measured at micron dimensions. The mRNAs from control and experimental samples are used to synthesize fluorescent-labeled cDNA or RNA probes, which are subsequently hybridized with the microarray components. The fluorescent signal of hybridized probes is measured with a laser scanner capable of detecting the release of various fluorescent colors. The control and test samples' signal intensity correlates directly with the initial concentration of mRNA in the cell or tissue. Therefore, it can be used to infer whether a specific gene's expression is positively or negatively set or remains unchanged.

Since its emergence about eight years ago, RNA-Seq has turned into a widely used method to study gene expression and examine the biogenesis aspects and RNA metabolism. Many methods have been developed to advance sequencing technologies, and new RNA-Seq processes are also expected to emerge in the future. We believe that many areas are particularly related to RNA analysis. First, the current sequencing systems have a size limit. Therefore, the instruments capable of long reading and a high reading output will be very useful, especially for the fragmentation of transcriptional isoforms, including those created by alternative initiation, AS, and alternative polyadenylation and gene fusions. Secondly, the current sequencing chemistry cannot work well in the case of homopolymers. This is especially suited for sequencing the poly (A) tail, which plays a major role in transcriptional metabolism. Third, the sequence sensitivity needs to be further improved to reduce or eliminate the replication. It is also important for single-cell analysis, where only a small fraction of the genes can be commonly analyzed. Finally, reviewing spatial information on RNA expression in the cells is of great importance in recent studies and appears to be a new frontier for RNA-Seq.

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Conflicts of Interest

The authors declare no conflict of interest.

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