

RNA Amplification: A snapshot of global gene expression

By: Bassam El-Fahmawi, PhD, Msc.
Technical Support Specialist, ^{MJS}BioLynx Inc.

High throughput exploitation of the human genome has become achievable with the availability of the complete human genome on a single microarray slide. Microarray technology has allowed for the expression profiling for multiple genes simultaneously (hundreds or thousands) which became an invaluable tool for the understanding of gene function and regulation. Recent advances in gene expression profiling utilizing cDNA and oligo-based microarrays have uncovered the involvement of several genes in disease prognosis, progression, response to treatment and validation of potential drug targets.

In general, global gene expression profiling utilizing cDNA microarrays requires a large amount of RNA. Traditionally, most microarray protocols require a minimum of 20µg of total RNA or 1-2 µg of mRNA, which is limiting especially if dealing with clinical samples. Just to name a few examples: samples obtained by sensitive selection techniques such as immunomagnetic bead selection (IMS) and laser capture micro dissection (LCM), needle or punch biopsies that are routinely used for pathological diagnosis or to monitor treatment progression. These samples are not only scarce and require technical expertise to obtain, but also result in nanograms of RNA as a starting material for analysis, rendering the study of gene expression for such samples with microarrays a challenge (Wang, 2005).

Two approaches have been developed to overcome this hurdle, one relies on intensifying the fluorescent signal through indirect labeling of cDNA or modifying the reverse transcriptase enzyme resulting in a higher incorporation efficiency of the Cy3/Cy5 dyes signal (Li, et. al., 2003; Wang, 2005). This approach has reduced the requirement of starting RNA material few folds but did not extend the utilization of microarray to sub-microgram levels.

The second approach is based on the pre-RNA amplification from nanograms to micrograms without losing the proportional gene expression representation in the starting material and thus minimizing bias. Due to amplification efficiency, linearity and reproducibility lowering the amount of total RNA needed for microarray analysis, RNA amplification is now the approach of choice for enhancing gene expression profiling in cDNA microarrays (Dafforn, et. al, 2004; Barker et. al., 2005; Wang, 2005).

RNA amplification strategies

The pre-amplification of RNA for cDNA microarrays was pioneered by Eberwine (Van Gelder, et. al., 1990; Eberwine, et. al., 1992). His main objective at that time was not focused on the study of pathogenomics from clinical samples but on amplifying enough material from a single cell for the analysis of individual or several genes.

Since then several methodologies for RNA amplification have been introduced based on two different approaches: linear isothermal amplification by in vitro transcription (IVT) of the cDNA, and PCR amplification of the entire population of cDNA following reverse transcription.

PCR-based amplification

Despite that PCR-based approach is a simple and an efficient method for RNA amplification, the fidelity of global transcript representation remains a major concern due to the exponential nature of PCR and the low fidelity of the DNA polymerase used (Wang, 2005). Several PCR-based approaches have been introduced which can be categorized as template switching (TS)-PCR, random PCR, and 3' tailing with 5' adaptor ligation PCR (Wang, 2005).

Limitations:

- DNA polymerase is biased: has low efficiency in the amplification of GC rich sequences as apposed to AT rich sequences.
- DNA polymerase has lower fidelity compared to RNA polymerase and thus creates errors and subsequently amplifies them.
- The exponential PCR reaction reaches saturation when excess input template quantities used, thus favoring the amplification of high abundant over low abundant transcripts. This will allow for the loss of the proportionality of the amplification process.

In recognition of these limitations, several recommendations have been put forward for improving PCR-based methodology performance for global gene expression profiling. These include the optimization of PCR cycle number and adjustment of template input amount to avoid reaching saturation. Furthermore the utilization of a high fidelity DNA polymerase will result in an amplified cDNA with minimal errors. Taking these recommendations in account will preserve the relative abundance of transcripts but the PCR approach requires an extensive optimization to ensure for optimal results. However, optimizing this method will result in efficient global amplification of less than 50 ng RNA starting material which is comparable to linear RNA amplification (Subkhankulova, T. and Livesey, 2006; Wang, 2005).

Linear isothermal RNA Amplification

The most commonly used mechanism for linear isothermal RNA amplification is based on T7 RNA polymerase-mediated IVT (Van Gelder, et. al., 1990; Eberwine, et. al., 1992; Dafforn, et. al, 2004; Barker et. al., 2005; Wang, 2005). Several protocols based on this technique have been developed and currently recommended for microarray analysis by several microarrays providers. Linear isothermal RNA amplification can increase the starting amounts of mRNA up to 1,000-fold in one round, but second and possibly third round might be required. Although the amplified RNA samples have been shown to generate reproducible microarray data when compared with non-amplified mRNA and closely approximate original samples it has its own limitations.

Limitations:

- While second or possibly third rounds of amplification may increase the assay sensitivity there is a potential for a 3' bias.
- The resulting microarray data can vary depending on the details of the amplification procedure and the amount of starting material.
- Time-dependent RNA degradation during IVT can introduce noise to the resulting microarray data.
- The procedure is lengthy, time consuming, and requires highly skilled operators.

This approach has been challenged extensively by researchers and several modifications have been attempted to get it more user friendly and suitable for microarrays and QPCR studies for global gene expression profiling at a single-cell level. Several of these modified approaches is now commercially available for amplification and labeling of cDNA through several biotech leaders (Ambion, Genisphere, Agilent, Enzo Life Sciences, Arcturus, Invitrogen, Kreatech, Super Array, SBI, Strategen, Clonetech, NuGEN) (<http://www.biocompare.com/matrix/3213/RNA-Amplification-Kits.html>).

However, getting a snapshot of the gene expression profile from as little as a single-cell, the amplification method of choice needs to be capable of maintaining the proportional representation of RNA population including gene transcripts of low and high abundance. This reasonable representation will allow for a more advanced understanding of disease prognosis, progression, and treatment. This criteria is mostly met by Nugen's RNA amplification approach (www.nugeninc.com).

Nugen has introduced an innovative RNA amplification approach with a 4 hours turn around as opposed to the several days needed for the T7 based amplification with as little as 5 ng of starting material. This approach is an RNA-based, single primer, isothermal amplification, Ribo-SPIA™. This technology provides an elegant method for linear, isothermal amplification of the mRNA species in a total RNA population. In linear amplification, as compared to the exponential amplification of PCR, the relative representation of each transcript species in the original sample is maintained during and after amplification. Simply stated, replication is initiated and repeated up to 10,000 times off of each original transcript. The speed of replication is consistent across all transcripts, thus the distribution of transcript copies in the amplified product reflects the distribution of the transcripts in the original starting material.

Ribo-SPIA™ amplification is driven by the combination of a unique chimeric primer, an RNase H and a DNA polymerase (Fig 1). There are many advantages for this combination of components. First and foremost is the ability to efficiently and linearly amplify a very low amount of template, thus Ribo-SPIA™ based amplification is very robust; starting with as little as 5 ng of total RNA amplification levels of up to 10,000-fold can be achieved. Second, the DNA polymerase is a very processive enzyme, and allows for the initial step in the amplification process to be completed in just one hour, which is in stark contrast for the 14-16 hours required for T7 polymerase. Third, the amplified product is anti-sense single stranded cDNA. This is important for oligonucleotide microarray applications in that the cDNA is in the correct sense for hybridization, and to be used for validation of the microarray results by QPCR (http://www.nugeninc.com/html/02_technology1.html).

Parallel to the development of RNA amplification methodologies, sample preparation has been exposed to the same attention by researchers. A well accepted fact that the quality of the purified RNA starting material is essential for a successful and reproducible gene expression profiling.

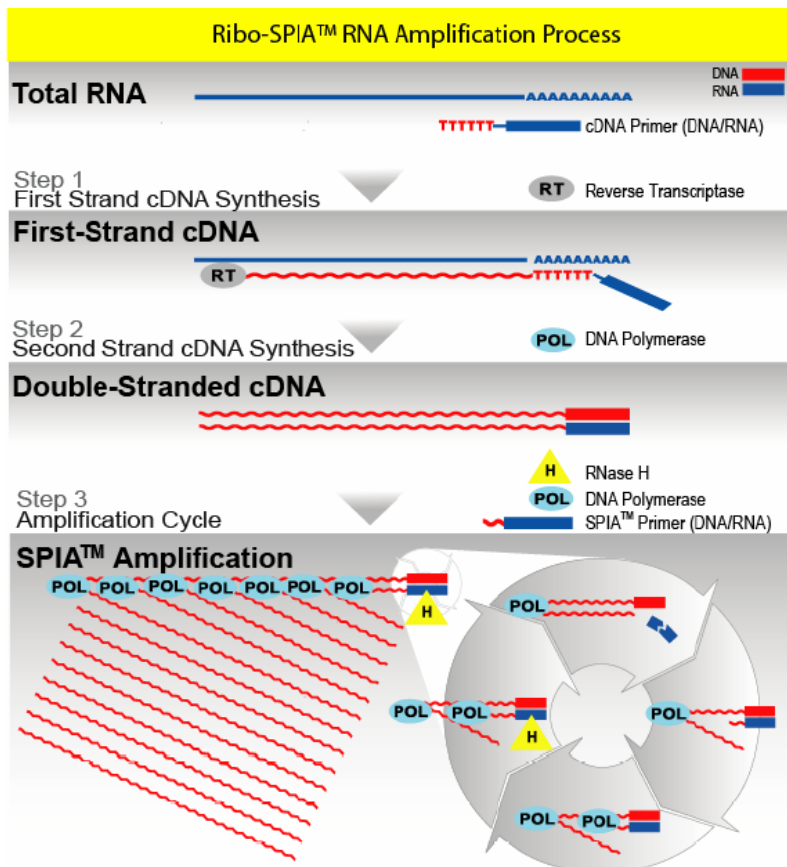


Fig. 1. Schematic drawing of the Ribo-SPIA process (courtesy of NuGEN Inc.).

Sample prep and RNA purification

The majority of RNA isolation technologies require either fresh or frozen tissue or cells as starting material; however, the starting material for gene expression profiling is usually available in limited amounts. Also, RNA extraction and purification strategy adopted should be suitable for various types of cells and tissues with minimal RNA degradation and with no dramatic changes to the total RNA profile. Sample handling with precautions for RNases contamination improves the quality and quantity of RNA purified from starting material.

Innovations in RNA isolation techniques have enhanced the speed and purity of recovered RNA. Technologies for isolating RNA currently in use include the use of organic solvents, chaotropic agents, chromatography (silica, silica carbide, or glass based columns), and magnetic separation (Stull and Pisano, 2001).

The most popular RNA purification strategies are silica-based matrices which allow for rapid and efficient purification of RNA in the presence of chaotropic buffers that inactivate RNases. Recently, Macherey-Nagel has introduced a new silica-based kit, NucleoSpin® RNA/Protein Kit, suitable for gene expression profiling (microarray and QPCR), providing parallel isolation of total RNA and protein from the same starting material (Fig. 2). This will allow for gene expression profiling at both the transcription level and the translation level, resulting in better understanding of genes regulation and functions (<https://www.mn-net.com/web/MN-WEB-Biokatalog.nsf/Web/FramesE?Open>).

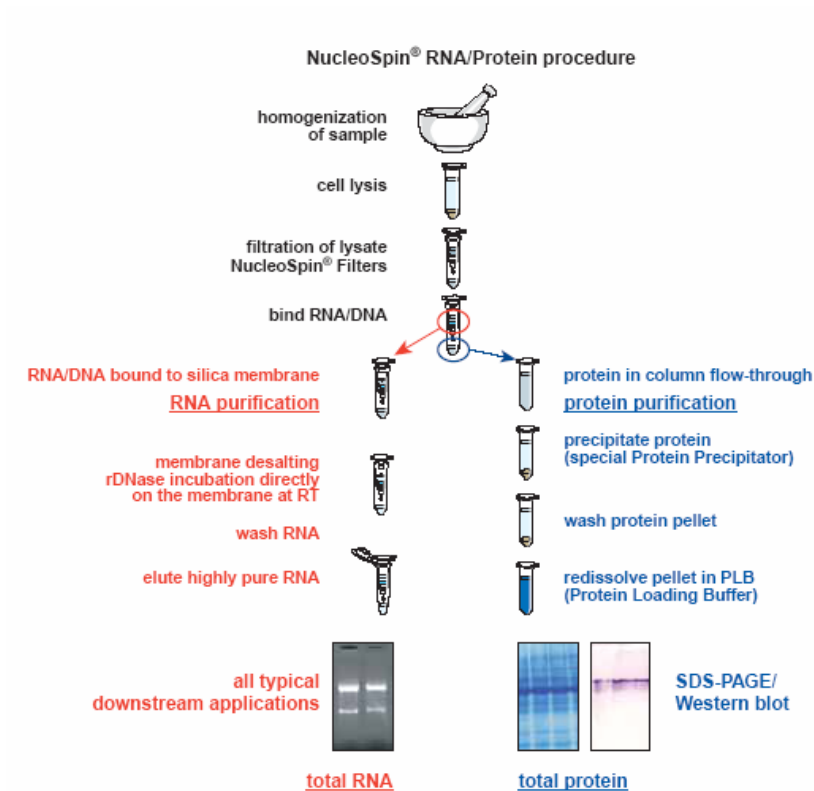


Fig. 2. NucleoSpin® RNA/Protein purification procedure illustration (courtesy of Macherey-Nagel).

In conclusion, advancing technologies for a more comprehensive gene expression profiling and having the means to validate the results is indispensable. Thus, the RNA amplification of choice has to be capable of amplifying RNA from as little as one cell maintaining the proportional representation of RNA population and generating micrograms quantities for microarray analysis and QPCR validation.

References:

Barker, CS., Griffin, C., Dolganov, GM., Hanspers, K., Yang, JY., and Erle, DJ. 2005. Increased DNA microarray hybridization specificity using sscDNA targets. *BMC Genomics*.6:57.

Biocompare website (www.biocompare.com)

Dafforn, A., Chen, P., Deng, G., Herrler, M., Iglehart, D., Koritala, S., Lato, S., Pillarisetty, S., Purohit, R., Wang, M., Wang, S., and Kurn, N. 2004. Linear mRNA amplification from as little as 5 ng total RNA for global gene expression analysis. *Biotechniques*. 37:854.

Li, J., Adams, L., Schwartz, SM., Bumgarner, RE. 2003. RNA amplification, fidelity and reproducibility of expression profiling. *C R Biol*. 326:1021.

Macherey-Nagel website (www.mn-net.com)

NuGEN Technologies, Inc. website (www.nugeninc.com)

Stull, D., and Pisano, J. 2001. Purely RNA. *The Scientist*, 22:29-31.

Subhankulova, T., and Livesey, FJ. 2006. Comparative evaluation of linear and exponential amplification techniques for expression profiling at the single-cell level. *Genome Biol.* 73:R18

Wang, E. 2005. RNA amplification for successful gene profiling analysis. *J. Trans. Med.* 3:28.