Video Article

# RNA Purification from Intracellularly Grown *Listeria monocytogenes* in Macrophage Cells

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## Abstract

Analysis of the transcriptome of bacterial pathogens during mammalian infection is a valuable tool for studying genes and factors that mediate infection. However, isolating bacterial RNA from infected cells or tissues is a challenging task, since mammalian RNA mostly dominates the lysates of infected cells. Here we describe an optimized method for RNA isolation of *Listeria monocytogenes* bacteria growing within bone marrow derived macrophage cells. Upon infection, cells are mildly lysed and rapidly filtered to discard most of the host proteins and RNA, while retaining intact bacteria. Next, bacterial RNA is isolated using hot phenol-SDS extraction followed by DNase treatment. The extracted RNA is suitable for gene transcription analysis by multiple techniques. This method is successfully employed in our studies of *Listeria monocytogenes* gene regulation during infection of macrophage cells [1-4]. The protocol can be easily modified to study other bacterial pathogens and cell types.

## Video Link

The video component of this article can be found at https://www.jove.com/video/54044/

## Introduction

Intracellular bacterial pathogens ─ bacteria causing infectious diseases and capable of growing and reproducing inside cells of human hosts ─ are a major health concern worldwide[5]. To invade and replicate within a mammalian cell, intracellular pathogens have acquired sophisticated virulence mechanisms and factors. While these mechanisms are fundamental to the ability to cause a disease, we know little about their regulation and dynamics. Since gene expression profiles of bacteria grown in liquid media do not reflect the actual environment within host cells, there is a growing need for transcriptome analyses of bacteria grown in their intracellular niches. Such analyses will enable the deciphering of specific bacterial adaptations triggered by the host and will help to identify new targets for therapeutic design. Transcriptome analysis of intracellularly grown bacteria is highly challenging since mammalian RNA outnumber bacterial RNA by at least ten fold. In this manuscript, we describe an experimental method to isolate bacterial RNA from *Listeria monocytogenes* bacteria growing inside murine macrophage cells. The extracted RNA can be used to study intracellular adaptations and virulence mechanisms of pathogenic bacteria by various techniques of transcription analysis, such as RT-PCR, RNA-Seq, microarray and other hybridization based technologies.

*L. monocytogenes* is the causative agent of listeriosis in humans, a disease with clinical manifestations targeting primarily immunocompromised individuals, elderly people and pregnant women[6]. It is a Gram-positive facultative intracellular pathogen that invades a wide array of mammalian cells that have been used for decades as a model in host-pathogen interactions studies[7]. Upon invasion, it resides initially in a vacuole or a phagosome (in the case of phagocytic cells), from which it must escape into the host cell cytosol in order to replicate. Several virulence factors have been shown to mediate the escape process, primarily the pore-forming hemolysin, Listeriolysin O (LLO) and two additional phospholipases[8]. In the cytosol the bacteria use the host actin polymerization machinery to propel themselves on actin filaments within the cell and to spread from cell to cell (**Figure 1**). All major virulence factors of *L. monocytogenes* involved in invasion, intracellular survival and replication, are activated by the master virulence transcription regulator, PrfA. [8-10].

In the last decade, several studies conducted by us and others have successfully applied methods for transcriptome analysis of intracellularly grown bacteria inside host cells[2,11-15]. Two main approaches are used to separate bacterial RNA from host-RNA which are based on: 1) Selective enrichment of bacterial RNA and 2) RNA isolation by differential cell lysis. The first approach relies on subtractive hybridization of total RNA extracts to mammalian RNA molecules (for example using commercially available kits) or selective capture of bacterial transcribed sequences (SCOTS)[11]. The second approach relies on differential lysis of bacteria and host cells, in which the host cells are lysed while bacterial cells remain intact. Bacterial cells are then separated from the host cell lysate, usually by centrifugation, and the RNA is extracted using standard techniques. The main problem using this approach is that together with the intact bacteria, host cells nuclei are also isolated, thus the RNA preparations still contain mammalian RNA. One way to overcome this problem is to separate intact bacteria from host cells nuclei using differential centrifugation, though this procedure usually takes time raising the concern of changes in gene expression profile during extraction. In this paper we present an improved and rapid bacteria RNA extraction protocol, which is based on cell differential lysis approach. First, *L.*

*monocytogenes* infected macrophage cells are lysed with cold water. Next, macrophage nuclei are removed by a brief centrifugation and intact bacteria are rapidly collected on filters, from which RNA is isolated using hot phenol-SDS extraction of bacterial nucleic acids.

## Protocol

Note: During the entire experiment, macrophage cells are incubated at 37 °C in a 5% $CO_2$ forced-air incubator and taken out of the incubator only for experimental manipulations, which are performed in a Class II biological safety cabinet. Working with *L. monocytogenes* bacteria is according to biological safety level 2 regulations.

## 1. Cell Preparation and Bacterial Infection (Day 1 and 2)

1. Day 1
    1. Seed 2.0 x $10^7$ bone marrow derived macrophage cells (BMDM) on a 145 mm dish in 30 ml BMDM + Pen-Strep media (**Table 2**). Seed 3 plates for each bacterial strain to be analyzed. Incubate overnight at 37 °C in a 5% $CO_2$ forced-air incubator.
    2. Start an overnight bacterial culture of wild-type (WT) *L. monocytogenes* strain 10403S in 10 ml of brain heart infusion (BHI) medium, at 30 °C in a standard incubator. Place culture tubes slanted without shaking. Note: These growth conditions up-regulate the expression of the flagella genes, which promotes efficient infection.

2. Day 2
    1. Pre-warm BMDM medium (without Pen-Strep antibiotics) (**Table 2**) and phosphate buffered saline (PBS) at 37 °C.
    2. Wash macrophage monolayer twice with 25 ml of pre-warmed PBS to remove antibiotics. Add 30 ml fresh BMDM medium.
    3. Wash 1.5 ml of overnight bacterial culture with PBS by centrifuging bacteria at > 14,000 x g for 1 min, discarding the supernatant, and resuspending bacteria gently in 1.5 ml of PBS by pipetting. Repeat twice.
    4. Infect each macrophage plate with 0.5 ml of a washed overnight culture of wild-type *L. monocytogenes*. If using multiple plates, infect each plate 15 min apart. This time interval will enable harvesting each plate individually at the end of the infection.
    Note: The multiplicity of infection (MOI) is assayed by plating serial dilutions of bacterial culture used for infection on BHI agar plates and counting colony forming units (CFU) after 24 hr incubation of plates at 37 °C. Using 0.5 ml of WT *L. monocytogenes* strain 10403S results in a MOI of ~ 100 (100 bacteria CFU per macrophage cell). When analyzing bacterial mutants defective in intracellular growth, a higher MOI should be considered.
    5. Following 0.5 hr incubation at 37 °C, wash the infected cells twice with PBS, to remove unattached bacteria, and add 30 ml pre-warmed BMDM medium. Attached bacteria will internalize during next 0.5 hr.
    6. At 1 hr post infection, add 30 µl of gentamicin (1:1,000 to reach a final concentration of 50 µg/ml) to kill extra-cellular bacteria.
    7. Assemble the filter apparatus by placing a filter head on a collecting liquid flask with a vacuum outlet port. Then place a filter (0.45 µm) on a filter head, followed by a cylinder funnel and secure the different parts with a metal clamp. Prepare ice-cold RNase-free water.
    8. At 6 hr post-infection, harvest bacteria from infected cells as follows. Treat each plate individually.
    Note: Usually, *L. monocytogenes* completes 1-log of growth during 6 hr of infection in macrophage cells. Longer incubations could result in bacterial overgrowth and cell death of the macrophages, while shorter incubation periods could result in considerably lower bacterial loads. The MOI and the time of infection can be modified to reach optimal conditions.
        1. Wash infected cells with PBS once. Add 20 ml of ice-cold RNase-free water to lyse macrophage cells. Using a cell scraper, scrape the cells off the plate quickly but carefully.
        2. Collect lysed cells to a 50 ml conical tube. Vortex for 30 sec. Centrifuge at 800 x g for 3 min at 4 °C.
        3. Pass the supernatant through the filter apparatus using a vacuum system. Using tweezers, roll the filter and quickly transfer it to a 15 ml conical tube. Snap-freeze the tubes with the filters in liquid nitrogen.
        4. Assemble the filtration apparatus with a new filter for the next sample, as described in 1.2.7.
        5. Store the frozen filters at -80 °C for the next day. Alternatively, proceed directly to nucleic acid extraction.

## 2. Nucleic Acids Extraction (Day 3)

Note: Perform all manipulations with phenol and chloroform solutions in a fume hood.

1. Prepare a 1:1 mix of acidified phenol: chloroform, 400 µl for each sample. Mix in separate tubes, and then withdraw the resulting aqueous layer by aspiration. Add 40 µl of 10% SDS.
2. Thaw the filter-containing tubes on ice to keep them cold. To each filter-containing tube add 650 µl of acetate-EDTA (AE) buffer (**Table 2**).
3. Perform the following steps as quickly as possible.
    1. Vigorously vortex the tube containing the filter so that the filter whisks to the periphery of the tube and the buffer fully washes the filter. Always keep the tube cold by placing it back on ice. Note: It may be necessary to additionally vortex the tube while inverted to fully wash the bacteria off the filter.
    2. Repeat for all tubes. It may be useful to spin-down the suspension shortly (1 min at 120 x g) at the end of the process.
4. Transfer the bacteria-containing buffer to a 1.5 ml microcentrifuge tube containing the SDS and phenol/chloroform mix (as prepared in 2.1). Repeat for all tubes. It may be necessary to spin the filter containing tubes (1 min at 120 x g) again to get residual buffer from the filter.
5. Place all the 1.5 ml tubes in a multi-tube vortex device, and vortex at full speed for 10 min.
6. Incubate the tubes in a 65 °C heat block for 10 min. Centrifuge at maximum speed (> 14,000 x g) for 5 min.
7. Transfer the aqueous layer (about 400 µl) from each tube to a new 1.5 ml tube containing 40 µl of 3 M sodium acetate (pH 5.2) and 1.0 ml of 100% ethanol. Vortex each tube thoroughly.
Note: Glycogen can be added at this step to improve visualization of the precipitated pellet.

8. Incubate the samples at -80 °C for 1 hr. Alternatively, incubate samples at -20 °C overnight. Then, centrifuge the samples at 4 °C for 20 min at maximum speed (> 14,000 x g).
9. Carefully aspirate off the ethanol (upper layer) from each tube. Add 500 µl cold 70% ethanol to each sample and vortex thoroughly. Centrifuge at 4 °C for 20 min at maximum speed (> 1 4,000 x g).
10. Carefully aspirate off the ethanol from each tube. Dry the samples for approximately 2 min using a vacuum evaporator with no heat. Do not over-dry the samples.
11. Add 25 µl RNase-free water to each sample. Incubate at room temperature for 20 min. Carefully vortex and spin-down.
12. Measure RNA concentration in the samples using a microvolume UV-Vis spectrophotometer. Expect to extract about 0.5 - 1 µg of nucleic acids per plate.
    Note: Technical replicates can be combined at this stage.

## 3. DNase Treatment

1. Set up the reaction according to **Table 1** in a microfuge tube. Incubate at 37 °C for 45 min. Add 450 µl RNase-free water and 500 µl of Phenol-Chloroform-IAA mix (**Table 2**), separate phases by centrifuging for 2 min at maximal speed (> 14,000 x g).
2. Transfer the aqueous layer to a new tube and add 500 µl chloroform-IAA mix (**Table 2**), vortex and separate phases by 2 min centrifugation at maximal speed (> 14,000 x g).
3. Transfer the aqueous layer to a new tube and add 1 ml of ethanol and 50 µl of 3 M sodium acetate (pH 5.2). Vortex.
   Note: Glycogen can be added at this step to improve visualization of the precipitated pellet.
4. Incubate for 1 hr at -80 °C. Alternatively, incubate samples at -20 °C overnight. Centrifuge at 4 °C for 20 min at maximal speed.
5. Carefully aspirate off the ethanol from each tube. Add 500 µl cold 70% ethanol to each sample and vortex thoroughly. Centrifuge at 4 °C for 20 min at maximum speed. Carefully aspirate off the ethanol from each tube.
6. Dry the samples for approximately 2 min using a vacuum evaporator with no heat. Do not over-dry the samples.
7. Add 12 µl of RNase-free water, incubate for 2 min at room temperature, vortex, and spin-down. The samples contain purified RNA, keep them on ice. Alternatively, dissolve RNA in RNase-free $H_2O$ with 0.1 mM EDTA or TE buffer (10 mM Tris, 1 mM EDTA).
8. Measure RNA concentrations using a microvolume UV-Vis spectrophotometer. Expect ~ 100 ng of RNA per sample (if technical replicates are combined).
   Note: RNA extracted according to this protocol is suitable for gene transcription analysis by multiple techniques. We usually employ RT-qPCR analysis to study *L. monocytogenes* gene transcription during infection of macrophage cells [1-4].

## Representative Results

The model system is shown in **Figure 1** and includes macrophage cells infected with *L. monocytogenes* bacteria, which replicate in the macrophage cytosol. **Figure 2** represents the experimental scheme. **Figure 3** represents typical results of such RT-qPCR analysis of virulence genes during WT *L. monocytogenes* growth in macrophages in comparison to growth in rich laboratory medium BHI. The results show the transcription levels of two major virulence factors of *L. monocytogenes; hly* encoding LLO toxin and *actA* encoding actin assembly protein, which are robustly induced upon infection of macrophages.

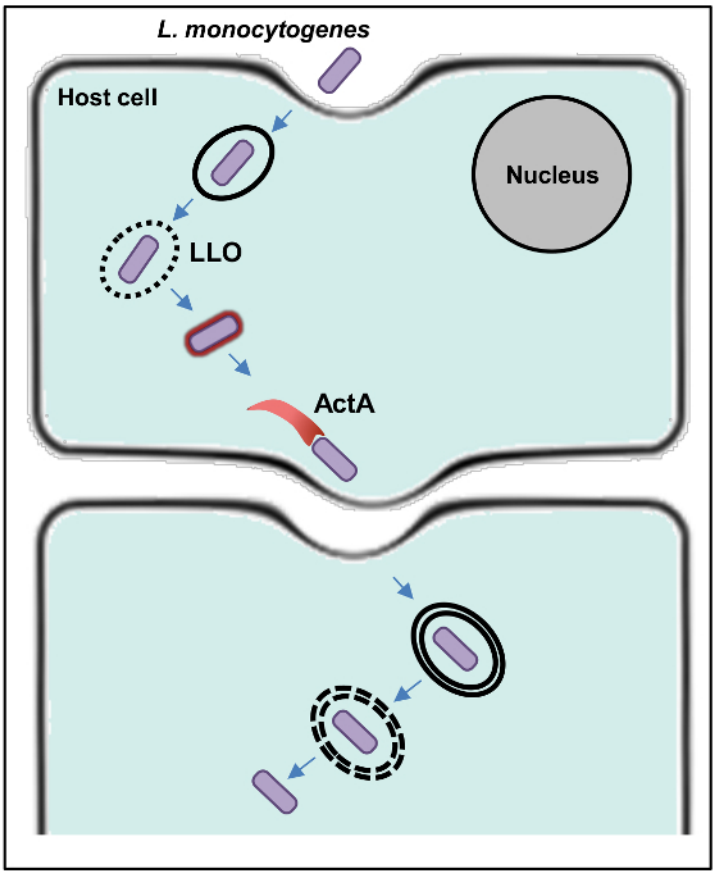


**Figure 1: General Overview of *L. monocytogenes* Lifestyle as an Intracellular Pathogen.** *L. monocytogenes* invades host cells by expressing specialized proteins termed internalins that induce active internalization into non-phagocytic cells, whereas phagocytic cells phagocytose the bacteria. Upon invasion, *L. monocytogenes* is initially found within an endosome/phagosome vacuole from which it rapidly escapes using primarily the listeriolysin O toxin (LLO). Within the host cell cytosol *L. monocytogenes* replicates rapidly, and spreads from cell to cell using actin based motility via its ActA protein. All mentioned virulence factors are regulated by the master virulence activator, PrfA. Please click here to view a larger version of this figure.

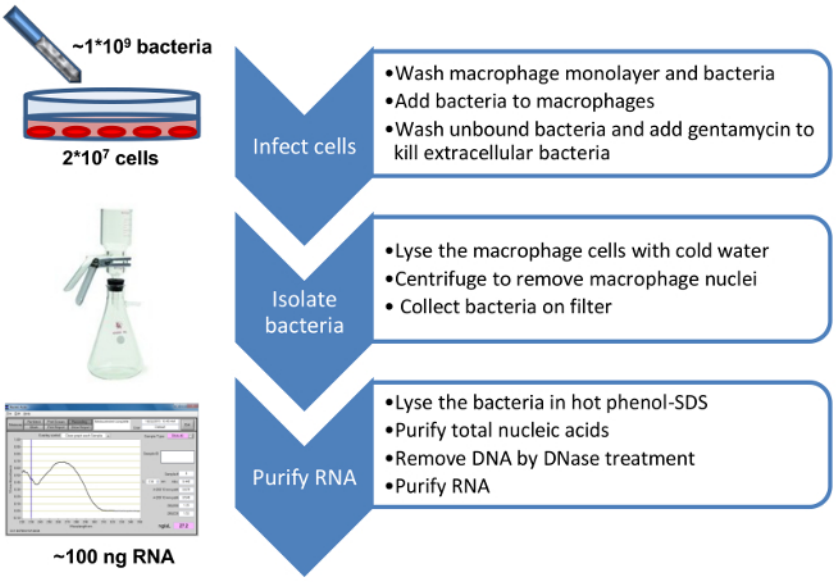


**Figure 2: A Flow Diagram Representing the Experimental Procedure.** The main steps include lysis of infected cells, separation of host cells nuclei and bacteria cells and RNA isolation. Critical steps are illustrated. Please click here to view a larger version of this figure.

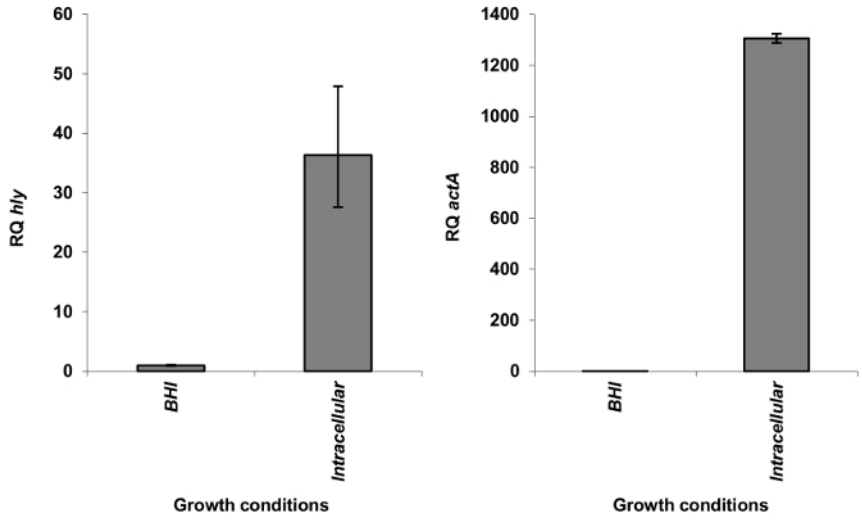


**Figure 3: Transcription Analysis of Virulence Genes during *L. monocytogenes* Intracellular Growth.** Transcription analysis of *hly* gene (encoding LLO) and of *actA* gene during *L. monocytogenes* 10403S intracellular growth in macrophage cells at 6 hours post infection in comparison to their levels during exponential growth in BHI medium using RT-qPCR analysis. Transcription levels are represented as relative quantity (RQ), relative to the transcription levels during growth in BHI. Transcription levels were normalized to the levels of 16S rRNA as a reference gene. The data is representative of 3 independent biological repeats (N=3). Error bars represent 95% confidence interval. Please click here to view a larger version of this figure.

| | |
|---|---|
| RNA | up to 2 µg (in max volume of 44 µl) |
| 10x DNase buffer | 5 µl |
| RNase-free water | complete to 50 µl total volume |
| DNAse | 1 µl (1 unit) |

**Table 1: DNase Reaction Protocol.**

| **1. 500 ml BMDM+Pen-Strep media (Filter sterilized):** | |
|---|---|
| DMEM | 235 ml |
| FBS (inactivated 30 min at 54 °C) | 100 ml |
| M-CSF (L-929 conditioned medium) [19] | 150 ml |
| Glutamine | 5 ml |
| Sodium pyruvate | 5 ml |
| β-Mercaptoethanol | 0.5 ml |
| Penicillin/Streptomycin | 5 ml |
| Total | 500 ml |
| **2. 500 ml BMDM (Filter sterilized):** | |
| DMEM | 235 ml |
| FBS (inactivated 30 min at 54 °C) | 100 ml |
| M-CSF (L-929 conditioned medium) [19] | 150 ml |
| Glutamine | 5 ml |
| Sodium pyruvate | 5 ml |
| β-Mercaptoethanol | 0.5 ml |
| Total | 500 ml |
| **3. AE buffer** | |
| NaOAc pH 5.2 | 50 mM |
| EDTA | 10 mM |
| RNase-free water | |
| **4. phenol-chloroform-IAA** | |
| Phenol | 25 ml |
| Chloroform | 24 ml |
| Iso-amyl alcohol | 1 ml |
| **5. chloroform-IAA** | |
| Chloroform | 24 ml |
| Iso-amyl alcohol | 1 ml |

**Table 2: Recipes of Media and Buffers.**

## Discussion

The protocol described here represents an optimized method for isolation of bacterial RNA from *L. monocytogenes* bacteria growing intracellularly in macrophage cells. This protocol is based on cell differential lysis and includes two major steps for enrichment of bacterial RNA: macrophage nuclei sedimentation using centrifugation and a rapid collection of bacteria by filtration. These steps are followed by a standard RNA extraction procedure. While this protocol describes purification of listerial RNA, it can be easily modified to other bacterial pathogens. Although the method focuses on purification of RNA for transcriptional analysis, the principle used to separate the intracellularly growing bacteria from its host can also be applied for purification of other bacterial components, such as DNA, proteins, cell-wall, *etc.* Such genomic and biochemical analyses would provide a better understanding and characterization of the intracellular pathogen life-cycle during the course of infection.

The procedure results in ~100 ng of total bacterial RNA. Although RNA yields are relatively low, they are suitable for downstream analyses of gene transcription using multiple techniques, such as RT-qPCR, RNA-Seq and modern hybridization based technologies. To improve RNA yields, it is recommended to use fresh bacterial inoculums, as well as fresh phenol solution nuclease-free water and buffers to avoid possible RNase contaminations. Harvested bacteria and isolated RNA should be always kept on ice during the extraction process. The infection time can be adjusted depending on the biological question and the organism studied. To increase the amounts of RNA, the experiment can be scaled up to include more infection plates per each sample.

The main concern when performing such an experiment is the risk of changing the transcription profile of the bacteria during harvesting steps. Most bacteria respond to cold temperatures by activating cold shock stress responses, and thus bacterial harvesting should be performed as quickly as possible. Reagents and equipment should be prepared ahead of time, and each sample should be treated separately. The most critical step is to immediately freeze the filter-containing bacteria in liquid nitrogen. Failing to do so may dramatically reduce the quality of the isolated RNA. Another critical step in this protocol is the ethanol precipitation of the relatively small amounts of nucleic acids. Extra care should be taken

not to disrupt the invisible nucleic acids pellet. Glycogen can be added to the precipitation solution to improve visualization of the pellet during these steps.

For some subsequent applications, *e.g.,* RT-qPCR, removing DNA is critical. Although we do not routinely monitor the efficiency of the DNase treatment, a reduction of 3 - 5 fold in the total amount of nucleic acids following DNase treatment is indicative of efficient DNase treatment. Of note, any commercially available technique or kit for DNA removal from RNA samples is suitable. In addition, using quality control samples, such as a sample with no reverse transcription performed, is beneficial in such applications.

A major limitation of the protocol described here is the relatively low amount of RNA extracted, which is about 100 ng of total RNA. Thus, this technique is not suitable for classical hybridization techniques, such as Northern blot analysis, which require micrograms of RNA.

The recent technological progress in RNA sequencing (deep RNA-seq) enables the analysis of both the bacterial pathogen and the mammalian host transcription profiles together, without the need to separate their RNAs, a method named 'dual RNA-seq'[16-18]. Although dual RNA-seq analysis is ideal in studying host-pathogen interaction, it is very expensive and cannot be used frequently. Because bacterial RNA content in infected cells is particularly low, it is extremely difficult and expensive to get enough bacterial reads for an effective gene expression analysis, even with the high read depth provided by newest sequencing platforms. An additional drawback of this approach is the frequent use of an RNA or cDNA amplification step, which may lead to biased results of gene expression. The method presented here offers a compromise between information obtained and cost-effectiveness.

## Disclosures

The authors have nothing to disclose.

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