Preface

Hearing is a sensory modality critical to both language and cognitive development. In its absence, and without sensory input through another modality, such as the manual/visual modality of sign language, cognitive and language development can be severely impaired in the earliest formative years of a child. In its endeavor to discover the mechanisms underlying audition, the field of auditory science has provided rich comparative physiological studies, allowing insights into both the micromechanical and electrochemical world of this system. For many years, the auditory/vestibular sciences have been influenced by the discoveries of electrical engineers and sensory physiologists, who have provided insights into the functions of this dynamic system. The early discoveries in these fields, as well as advancements in microprocessing and materials technologies, provided a means whereby hearing could be regained partly through the use of a bionic device, known as a cochlear implant. Presently, this device and the auditory brainstem implant are the only ones to prosthetically replace brain function.

With the advent of molecular biology tools, such as RT-PCR, the auditory and vestibular fields have made great strides in understanding the genetic basis for various hearing and balance disorders over the past fifteen to twenty years. These technologies permitted the discovery of genes that control inner ear structure and function by overcoming the hurdle of working with small amounts of tissue, as found in the inner ear. The amplification of genes with RT-PCR provided a means to discover gene expression in the small, inner ear endorgans during development, as well as in damaged and normal sensory epithelia in the adult. The use of gene knockout animal models provided the means to verify the effects of genes critical to the development of this system, whereas *in situ* hybridization localized newly discovered gene transcripts. As these technologies continue to broaden the discovery of genes and their regulatory behavior, auditory and vestibular studies have begun to focus on proteins in terms of their interactions, structure, and how these factors relate to function.

In light of the dramatic changes in the auditory and vestibular sciences over these past fifteen plus years, this book describes RNA, protein, and imaging protocols that currently are in use and that have provided insights into genetic regulation, as well as insights into genes and pathogens involved in diseases of the ear. This overview provides a perspective of basic research with both mammalian and non-mammalian animal models, as well as protocols applicable to clinical studies. The chapters in Part 1 include basic protocols of RNA isolation and expression, followed by methods to study cell lineage, gene delivery, and the identification and use of stem cells. This section ends with techniques that are applicable to clinical studies of genes, pathogens, and cancers that lead to hearing loss in humans. Part 2 focuses on the study of inner ear proteins and more specifically on their interactions, including techniques such as the yeast-two hybrid assay, coimmunoprecipitation, plasmon resonance, and protein tagging for mass spectrometry. The final section, Part 3, describes imaging techniques

useful for the study of ions, protein-protein interactions, and imaging of proteins at the atomic level.

While the chapters are written by specialists in the auditory and vestibular fields, the techniques described herein will be useful to those exploring genes and proteins in other systems as well, especially where tissues are scarce and where a comparative approach lends itself to discovering the underlying causes of human disorders.

Chapter 2

Synthesis of Biotin-Labeled RNA for Gene Expression Measurements Using Oligonucleotide Arrays

Ana E. Vázquez, Liping Nie, and Ebenezer N. Yamoah

Abstract

Using gene arrays, it is currently possible to simultaneously measure mRNA levels of many genes in any tissue of interest. Undoubtedly, comprehensive measurements of gene expression as part of carefully designed experiments will continue to further our understanding of audition and have the potential to open up new avenues of research. This chapter describes a reliable protocol to prepare high-quality biotin-labeled RNA target, specifically for oligonucleotide array experiments. The procedure includes isolation of high-quality total RNA, synthesis of double-stranded cDNA engineered for in vitro transcription with T7 RNA polymerase, subsequent in vitro transcription in the presence of biotin-labeled ribonucleotides, and fractionation of the RNA to ~ 500 bp fragments, suitable for oligonucleotide array experiments. Because the membranous labyrinth is composed of functionally interdependent cellular structures, which themselves contain numerous, highly differentiated cell types, comprehensive analysis of gene expression in the cochlea is best complemented by immunohistotochemical studies or, if no suitable antibodies are available, by in situ hybridization studies. Either one of these techniques will identify the specific cell types that express the genes of interests.

Key words: Gene expression, oligonucleotide microarrays, gene chips, membranous labyrinth, cochlea.

1. Introduction

Significant improvements in molecular biology methods currently allow precise, simultaneous measurements of a large percentage of transcript levels in a tissue of choice. Optimization of RNA labeling methods, better quality arrays, as well as the development of high-density scanners have made microarray technology an efficient and reliable tool for quantifying levels of gene expression. As a result of these improvements, measurements of gene expression

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levels are now feasible at the level of the transcriptome, even if only very small amounts of tissue or a few cells are available *(1–3)*. Consequently, array experiments, monitoring changes of gene expression relevant to specific biological processes, are contributing to the progress of numerous research fields. Other molecular methods for measuring gene expression, such as Northern blot analysis or quantitative polymerase chain reaction, preclude the analysis of large numbers of genes. Additionally, Northern blot analysis requires the use of large amounts of RNA and, thus, is not a practical method for small sample sizes.

Presently, a number of platforms are used for global transcript analyses, including cDNA and, short (~ 25 nucleotides) and long (50–70 nucleotides) oligonucleotide arrays. Microarrays of spotted cDNAs are available and can be custom made or spotted on demand. These arrays have an advantage in that they can be designed and spotted, to ensure that complex transcriptomes of specific tissues are well represented or that the array is tailored to the specific scientific questions of interest. Because these arrays are usually prepared on slides by investigators, there is a lower cost associated with them. Collections of cDNAs for auditory research have been compiled into cDNA microarrays *(4, 5)*. For example, spotted cDNA arrays were utilized to study the effects of noise in the cochlea *(6, 7)*.

Oligonucleotide arrays are more costly than cDNA-spotted slides. The commercial oligonucleotide arrays, however, have the advantage that multiple independent measurements are made for each transcript, providing high measurement reliability. It is our experience that the technical variability in experiments, using oligonucleotide arrays, can be almost negligible. For example, high reproducibility was reported using an Affymetrix (Santa Clara, CA) zebrafish genome oligonucleotide array *(3)*. The correlation coefficient for comparisons between replicate samples of zebrafish hair cells and liver gene expression measurements were 0.95 and 0.99, respectively *(3)*, lending high confidence to this methodology. We have focused on the Affymetrix system, but various other biotechnology companies provide high-quality oligonucleotide arrays also. Commercially available oligonucleotide arrays now represent whole genomes of various organisms. For example, the GeneChip Mouse Genome 430A 2.0 Array (Affymetrix) contains most well-characterized genes and expressed sequence tags of GenBank®, dbEST, and RefSeq databases. Oligonucleotide microarrays have been used in studies of hair cell differentiation *(8)* and in studies of the effects of noise in the cochlea *(9)*.

Since changes in gene expression are at the core of many biological processes, such as development, differentiation, response to stress, and apoptosis, the ability to monitor gene expression changes is highly valuable to the understanding of these processes.

This chapter describes the preparation of target biotin-labeled RNA for short (~ 25 nucleotides) oligonucleotide arrays, including the following steps: RNA purification, synthesis of double-stranded cDNA containing a T7 promoter sequence, cDNA purification, in vitro transcription in the presence of biotin-labeled ribonucleotides, and fractionation of RNA. The RNA labeling protocol is based on the priming of cDNA synthesis with an oligo (dT) primer, containing the T7 promoter sequence. Thus, the cDNA obtained can be used for subsequent in vitro transcription with T7 RNA polymerase *(10)*. The T7 polymerase performs a linear amplification, and the in vitro RNA obtained reflects the abundance of each transcript in the initial RNA *(11–13)*.

Although outside of the scope of this chapter, our recommendation is to devote serious effort to the planning of the array experiments prior to tissue collection. The Gene Expression Omnibus (GEO) website, http://www.ncbi.nlm.nih.gov/geo/, is an invaluable resource for gene expression studies. Inner ear and other hearing-related gene expression datasets are currently easily accessed on GEO. Investigators are encouraged to familiarize themselves with GEO and to evaluate the datasets available prior to designing array experiments. Upon completion of the experiments, investigators are urged to submit their results so that they will be available for the benefit of the scientific community.

2. Materials

2.1. Membranous Labyrinth Tissue Collection

1. RNase-free dissection tools. Clean all tools with RNase *AWAY*.
2. RNA *later* RNA stabilizing reagent (QIAGEN Inc., Valencia, CA).

2.2. RNA Isolation

For RNA isolation, we routinely use solutions and columns from the RNeasy Protect Mini Kit (cat. no. 74124, QIAGEN Inc, Valencia, CA).

1. β-mercaptoethanol.
2. RLT buffer (RNeasy Protect Mini Kit).
3. DNase I stock solution: Prepare using RNase-free DNase Set (QIAGEN Inc, Valencia, CA). Before using, dissolve the solid DNaseI (1500 Kunitz units) in 550 μL of RNase-free water and mix gently by inverting the tube only; do not vortex. Divide into 10 μL aliquots and store at –20 °C for up to 9 months.
4. Buffer RDD (RNeasy Protect Mini Kit).
5. Ethanol.
6. Buffer RPE with ethanol added (RNeasy Protect Mini Kit).
7. Tris buffer: 10 m*M* Tris-HCl, pH 8.0.

2.3. Synthesis of Biotin-Labeled RNA Target for Array Analysis

1. MessageAmpII aRNA Kit (cat. no. 1751, Ambion, Inc., Austin, TX) (*see* **Note 1**).
2. 10 m*M* Biotin-11-CTP (Perkin Elmer Life Sciences, Waltham, MA).
3. 10 m*M* Biotin-16-UTP (Roche Molecular Biochemicals Indianapolis, IN).
4. 100% Ethanol.
5. RNase-free water: Prepare by adding 0.1% volume of diethyl pyrocarbonate to nanopure water, shake vigorously to mix well, incubate overnight at 37 °C, and autoclave the next day.
6. 70% ethanol prepared with RNase-free water.
7. 3 *M* NaOAc, pH 5.2, RNase free.
8. 1X Tris-Borate-EDTA (TBE) electrophoresis buffer.

2.4. Fragmentation of In Vitro Transcribed Biotin-Labeled RNA

1. 5X RNA fragmentation buffer; use RNase-free reagents: 200 m*M* Tris-acetate, pH 8.1, 500 m*M* KOAc, 150 m*M* MgOAc. Combine 4 mL of 1 *M* Tris-acetate, pH 8.1, with 0.64 g of MgOAc and 0.98 g of KOAc. Mix thoroughly and filter through a 0.2 μm filter. Aliquot and store at room temperature.

2.5. Gel Electrophoresis to Evaluate the Progression of the Fragmentation Reaction

1. 1X TBE electrophoresis buffer.
2. DNA electrophoresis loading dye.

2.6. Equipment

1. Homogenizer (e.g., PowerGen125, Thermo Fisher Scientific, Pittsburgh, PA).
2. Eppendorf 5804 R centrifuge (Eppendorf of North America, Westbury, NY).
3. Water bath or hot plate.
4. Spectrophotometer.
5. Thermalcycler.
6. Gel electrophoresis apparatus.

3. Methods

3.1. Membranous Labyrinth Tissue Collection

1. Dissect the membranous labyrinth from each cochlea quickly under RNase-free conditions, and place the tissues into 100 μL of RNA*later* (*see* **Notes 2** and **3**).

3.2. RNA Isolation

For RNA isolation, we routinely use the QIAGEN RNeasy Protect Mini Kit, including the in-column DNase digestion specifically as follows:

1. Calculate and prepare the total amount of β-mercaptoethanol/RLT buffer solution needed for all extractions (600 μL of β-mercaptoethanol/RLT buffer solution will be needed for each extraction). Prepare sufficient amount of this solution by adding 10 μL of β-mercaptoethanol per 1 mL of RLT buffer and mix thoroughly by vortexing.
2. Remove the RNA*later* buffer from the tissues.
3. Add 600 μL of β-mercaptoethanol/RLT buffer solution to the tissue (*see* **Note 4**).
4. Homogenize immediately for 40 s using three-fourth of the maximum speed of the PowerGen 125 homogenizer.
5. Centrifuge for 3 min at maximum speed in a microcentrifuge.
6. Remove the supernatant to a new tube.
7. Add 1 volume (600 μL) of 70% ethanol to the lysate and mix immediately.
8. Place the RNease column in a 2 mL collection tube, pipette up to 700 μL of the sample into the column, and centrifuge for 15 s at ≥ 8000 g. Discard the flow-through.
9. Apply the rest of the sample volume by loading aliquots of up to 700 μL, successively, onto the column. Centrifuge after each loading, as in **step 8, Section 3.2**, and repeat until the entire sample is loaded onto the column.
10. Pipet 350 μL of buffer RW1 into the RNeasy column and centrifuge for 15 s at ≥ 8000 g.
11. Add 70 μL of buffer RDD to 10 μL of DNase stock solution and mix by gently inverting the tube. Then, pipette this mix directly onto the RNeasy silica-gel membrane and incubate at room temperature for 15 min.
12. Pipet 350 μL of buffer RW1 into the RNeasy mini column and centrifuge for 15 s at ≥ 8000 g.
13. Place the RNeasy column into a new 2 mL collection tube and add 500 μL of buffer RPE (with ethanol added), and centrifuge for 15 s at ≥ 8000 g to wash the column. Discard the flow-through.
14. Wash with RPE buffer (with ethanol added) again. Repeat **step 13, Section 3.2**, discard the flow-through, and centrifuge again for 1 min at ≥ 8000 g to thoroughly dry the column.
15. Elute the RNA: Transfer the column to a new tube and add 30 μL of warm (50–55 °C) RNase-free water, wait for 1 min, and centrifuge for 1 min as in **step 14, Section 3.2**.
16. Prepare a 1:100 dilution of the obtained RNA in Tris buffer (*see* **Note 5**). Determine the amount of RNA, using a spectrophotometer.

3.3. Synthesis of Biotin-Labeled RNA Target for Array Analysis

We routinely use the MessageAmp™ II aRNA Amplification System (cat. no. 1751 Ambion, Inc., Austin, TX) to synthesize biotin-labeled RNA from the purified RNA obtained above. We recommend following Ambion's method exactly for:

1. Reverse transcription for first-strand cDNA synthesis (*see* **Note 6**).
2. Second-strand cDNA synthesis.
3. Purification of double-stranded cDNA.
4. In vitro transcription to synthesize biotin-labeled aRNA (*see* **Note 7**).

3.4. Quantitation of aRNA Yield

Prepare a 1:100 dilution of the RNA in Tris-HCl buffer and measure the RNA concentration (*see* **Note 8**).

3.5. Fragmentation of In Vitro Transcribed Biotin-Labeled RNA

1. Precipitate the labeled RNA and solubilize the RNA pellet to obtain a $0.64\,\mu g/\mu L$ concentration with RNase-free water.
2. Set up fragmentation reactions: $32\,\mu L$ of RNA ($\sim 21\,\mu g$) and $8\,\mu L$ of fragmentation buffer (*see* **Note 9**). Set a thermocycler in incubation mode to hold at $94\,°C$, use a timer, allow fragmentation at $94\,°C$ for 35 min exactly, and place the tube on ice following the incubation.
3. Load $1\,\mu g$ of fragmented RNA on a 1% agarose gel to evaluate the progress of the fragmentation reaction (*see* **Note 10**).

3.6. Perform Hybridization Experiments to Test Array for Evaluation of Target Quality

It is recommended to further evaluate the quality of the in vitro–transcribed RNA for gene-expression profiling by performing a hybridization experiment to Test3 Arrays. The Test3 Array is an economically priced array. The efficiency of the labeling reaction should be evaluated using these arrays, by inspecting the signal intensity of the housekeeping genes for the organism of interest (*see* **Note 9**). The 3′–5′ signal ratios for genes of the organism of interest should be between 1 and 3.

3.7. Perform Hybridization Experiments to Oligonucleotide Microarray of Choice

The hybridization, washing, staining, and scanning of oligonucleotide arrays are usually performed at a microarray core facility. These protocols are outside of the scope of this chapter. Information about these procedures can be obtain at http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf

3.8. Data Analysis, Validation, Data Sharing, and Annotation

Data analysis, validation, data annotation, and sharing are all important components of all array experiments. However, these are outside the scope of this chapter. Briefly, array data analysis may be performed using one of the various software packages available without cost such as DNA-Chip Analyzer software (dChip v.4/14/06, http://biosun1.harvard.edu/complab/dchip/) and BRB array tools from the National Cancer

Institute (NIH) http://linus.nci.nih.gov/BRB-ArrayTools.html. Alternatively, data analysis software packages may be obtainable from the microarray core facilities (*see* **Notes 2, 10, 11**). After completion of array experiments, validation of the data, for a subset of genes of interest to the investigator, should be performed using quantitative PCR, immunohistochemistry, or in situ hybridization. After validation, the data should be submitted to GEO (http://www.ncbi.nlm.nih.gov/geo/) (see **Note 11**).

4. Notes

1. Make sure to store each of the components of the MessageAmpII aRNA at the temperatures indicated. Do not store enzymes in a frost-free freezer.
2. Gene expression experiments must be planned carefully. We recommend becoming familiar and evaluating the array data that is already available at the GEO website, http://www.ncbi.nlm.nih.gov/geo/, which may be relevant to the scientific questions being evaluated. We also recommend that for each experimental condition, tissues be collected and pooled into at least three independent but representative groups (a larger number of replicates should not be required). Isolate total RNA from each of these groups of tissues independently; the RNA samples and thus the resulting arrays within the same group will then be biological replicates.
3. For gene expression studies, the membranous labyrinth of each mouse should be dissected quickly under RNase-free conditions. We routinely place the tissues into 100 μL of RNA*later* (QIAGEN Inc.). Tissues should be stored in RNA*later* at 4 °C overnight and then placed in −20 °C the next morning until further use.
4. Perform **steps** 4–8 continuously and quickly, and always at room temperature. Make sure all centrifugation steps are carried out between 22 °C and 25 °C, not cold. This method yields approximately 2.5 μg of total RNA from 16 pooled mice membranous labyrinths. The RNA can be stored at −20 °C. However, it is recommended to continue the procedure as soon as possible.
5. For quantitation purposes, dilute the RNA in buffer and not in water, because in water the absorption will not be the same and the measurement will be inaccurate. The OD_{260}/OD_{280} ratio should be between 1.7 and 2. There are more sophisticated methods of obtaining RNA concentration measurements, but some of them require expensive instrumentation. However, it is our experience that spectrophotometer measurements of RNA in buffer are adequate for the purpose of this protocol.

6. The manufacturer (Ambion, Inc., Austin, TX) recommends using 1 μg of RNA. We find that this is not required. Occasionally, we have used 400 ng to obtain high-quality and sufficient target-labeled RNA for more than one array (amounts as low as 250 ng are likely to yield labeled RNA for more than one array). Each array requires 5 μg for the test array and 15 μg of biotin-labeled RNA for the experimental array.
7. Perform in vitro transcription for 14 h.
8. The RNA concentration in the fragmentation reaction should be between 0.5 and 2 μg/μL.
9. Agarose gel electrophoresis and ethidium bromide staining for visualization is sufficient to evaluate the progress of the fragmentation reaction. After fragmentation, a change in the size of the labeled RNA should be apparent. The size of the fragmented RNA should be ~ 200 bp or no larger than the 500 bp DNA marker. We recommend performing a hybridization to a "Test3 Array" and evaluating the detection signal as well as the 3′–5′ signal ratios for the genes of the organism of interest.
10. With any software of choice, it is our recommendation to compute the gene expression levels, the group means and standard errors, by pooling arrays (three arrays of each experimental group are adequate for many experiments). This approach helps to account for measurement inaccuracies and allows for the elimination of outliers (http://biosun1.harvard.edu/complab/dchip). The arrays within the same group are biological replicates.
11. Array data relevant to hearing research is available at GEO (http://www.ncbi.nlm.nih.gov/geo/). Investigators are urged to familiarize themselves with the data already available and relevant to their field of interest, and to submit their results so that they will be accessible to the scientific community.

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