Chapter 22

Expression Profiling to Identify Candidate Genes Associated with Allergic Phenotypes

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Abstract

Transcript profiling reveals valuable insights to molecular and cellular activity related to disease. Gene expression profiles provide clues as to how tissues or cells in a particular environment may respond to stimuli. Gene-targeted examination of transcript changes is accomplished by employing a quantitative PCR approach using cDNA prepared from isolated RNA.

Key words Transcript, Profiling, Quantitative PCR, Allergy

1 Introduction

Allergy is characterized by the presence and activity of many cell types, including eosinophils, mast cells, natural killer cells, macrophages, and neutrophils. In addition to leukocyte populations, additional specialized cell types also play a role during allergy-induced asthma, including smooth muscle cells, epithelia, and endothelia. Inflammatory factors, such as cytokines, chemokines, transcriptional regulators, cell surface markers, cell signaling activators, and mediators, influence allergic and immunogenic responses that may result in chronic inflammation, hyperactivity, and/or tissue remodeling. By examining cell- or tissue-specific molecular changes or profiles, mechanisms that initiate disease and/or propagate symptoms may be revealed. Additionally, gene expression profiling supports the identification of candidates for therapeutic targets that may either ameliorate or prevent disease.

Global transcript approaches in which >1,000 genes are assessed simultaneously include the use of genome-wide microarrays and direct sequencing from isolated nucleic acids or protein-bound DNA fragments. Common examples of these techniques include whole transcriptome shotgun sequencing or RNA sequencing (RNA-Seq) and chromatin immunoprecipitation (ChIP)-Seq.

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Additional levels of regulation may be discovered by analyzing alternatively spliced gene products and microRNA species. Overall, several reports utilizing technology-driven approaches and gene-targeted PCR applications have demonstrated the complexity of cellular interactions and responses to allergen and treatment in asthma and other allergic diseases [1–8]. As these high-throughput profiling methods require much more advanced technology, expensive equipment and reagents, expertise, and sophisticated data analysis capabilities, they will not be presented here.

Here, we present protocols for gene-targeted quantitative PCR (qPCR). These molecular approaches are utilized to obtain qualitative and quantitative data about gene expression in isolated cells or tissues. The first step in profiling gene expression is to obtain high-quality RNA. Next, reverse transcription (RT) is performed to produce complementary DNA (cDNA) that is used as a template for PCR amplification. Gene expression profiles or changes are determined by analyzing PCR products using sequence-specific probes plus forward and reverse primers (i.e., TaqMan®-mediated amplification) or the intercalation of a fluorescent molecule such as SYBR® Green with forward and reverse primers. These molecular approaches for gene analysis are relatively easy and straightforward to perform, which makes qPCR a critical technique for examining and validating potential therapeutic targets and/or diagnostic genes that are related to allergic disease.

2 Materials

1. Protective equipment: Fume hood or biological safety cabinet, gloves, goggles, lab coat.
2. Tissue or cell source.
3. Liquid nitrogen or dry ice plus a 95 % ethanol freezing bath.
4. Freezers: –80 and –20 °C.
5. RNaseZap® for surface decontamination.
6. Tissue disrupter: Homogenizer, tissue lyser, or bead mill with beads of appropriate size and density.
7. RNA isolation reagent: Phenol/guanidine thiocyanate, TRIzol®.
8. Alternative: RNA isolation kits or reagents; RNA*later*® for temporary tissue storage prior to RNA isolation.
9. Chloroform.
10. Isopropyl alcohol.
11. 70 % Ethanol.
12. 100 % Ethanol.
13. Sterile, deionized water (RNase-free).

14. Eppendorf tubes and cryovials.
15. Alternative: Spin columns.
16. Spectrophotometer (i.e., Nanodrop).
17. Pipettors (2, 10, 200, 1,000 μl).
18. 384-Well or 96-well PCR plate, depending on real-time PCR thermocycler used to evaluate expression; with optically transparent adhesive film for sealing of plate prior to loading into thermocycler.
19. 384-Well or 96-well thermocycler.
20. Enzymes: Reverse transcriptase, RNase inhibitor, Taq DNA polymerase.
21. Molecular reagents for reverse transcription: Primers [random hexamer and oligo d(T) nucleotide]; deoxynucleotide mix: 10 mM each of dGTP, dATP, dCTP, dTTP; 0.1 M dithiothreitol (DTT); 10× reaction buffer (supplied with enzyme from commercial sources or 500 mM KCl, 30 mM $MgCl_2$, 1 M Tris–HCl pH 9.3); reverse transcriptase.
22. Molecular reagents for qPCR: cDNA template; gene-specific primers (forward, reverse, probe); 10× PCR buffer supplied with DNA polymerase; SYBR® Green dye-containing PCR and enzyme reaction mix; dNTP mix: 10 mM each of dGTP, dATP, dCTP, dTTP.
23. Glycogen; amplicon-containing DNA plasmid to use as standardization control.

3 Methods

3.1 RNA Isolation

1. Dissect tissue from animal or isolate cells.
2. Flash freeze tissue or isolated cells and store at –80 °C (*see* **Note 1**).
3. Process tissue in phenol/guanidine thiocyanate reagent (*see* **Note 2**): Place 100–200 mg of frozen tissue in a 2 ml screw top cryovial with a ring in the cap; add beads (~25 % of space in cryovial) and 1 ml of TRIzol® reagent (Life Technologies). Then agitate for short pulses interspersed with periods of rest on ice, to prevent the heating of samples prior to complete tissue dispersal. (For example, with lung tissue: use 1.4 mm ceramic beads and shake in the bead mill for 40 s, rest for ≥20 s, and repeat with a 40 s pulse.) Transfer the homogenate to a fresh tube and continue with sample processing as directed by the manufacturer's protocol.
4. Follow the manufacturer's suggested protocol to isolate RNA from lysate by separating the aqueous from organic phases and

precipitating nucleic acid in isopropyl alcohol (*see* **Notes 3** and **4**).

5. Dissolve precipitated RNA in RNase-free water (or Tris–EDTA) and store RNA at –80 °C.
6. Assess spectral features (i.e., absorbance at 260 and 280 nm) to determine quantity and purity of RNA (*see* **Note 5**).

3.2 Production of cDNA by Reverse Transcription

1. Set up reactions by combining RNA, primer, RNase inhibitor, reverse transcriptase, deoxynucleotides, and reverse transcription buffer in an Eppendorf snap-cap tube (*see* **Note 6**).
2. Incubate according to the manufacturer's guidelines for the reverse transcriptase (*see* **Note 7**).
3. Store cDNA at –20 °C.

3.3 Quantitative PCR Amplification

1. Determine the gene or the gene targets of interest. Obtain commercially available primers or generate appropriate primers based on known gene sequences (*see* **Note 8**).
2. Set up reactions for qPCR by first aliquoting the template, which is usually diluted at least 2–5 fold in RNase-free water, into a PCR plate. Prepare the master mix for each target, containing primers plus Taq DNA polymerase in the reaction buffer. Aliquot the prepared master mix onto a PCR plate (*see* **Note 9**).
3. Perform PCR amplification using a 96-well or a 384-well thermocycler to determine either absolute or relative changes in expression. Include a melting curve step if using SYBR® Green dye incorporation. SYBR® Green dye is a useful way to assess the homogeneity of the amplified product, suggesting the production of a single species. Alternative multiplex profiling approaches using PCR profiling arrays can be performed (*see* **Note 10**).

3.4 Data Analysis

1. Determine the quantity of amplified product using the software package that is supplied with the thermocycler.
2. Utilize a plasmid standard that contains the amplified gene product to determine the absolute quantity of the target gene (*see* **Note 11**).
3. Simultaneously amplify an internal control target for each biological sample using primers to detect a housekeeping gene (*see* **Note 12**).
4. Determine the relative quantity using C_T analysis (*see* **Note 13**).

4 Notes

1. To preserve tissue, flash freeze in a cryovial in liquid nitrogen (or using ethanol plus a dry ice chilling bath). Store samples at –80 °C. Alternatively, RNA*later*® or a similar reagent can be

used to store fresh tissue prior to disruption. RNA*later*® rapidly permeates fresh tissue, inactivates RNases, and stabilizes RNA within tissues or cells, thereby eliminating the requirement for immediate freezing of biological samples during the harvest or the isolation. Tissue can be stored in RNA*later*® at 4 °C for 1 month or –20 °C indefinitely prior to tissue disruption.

2. Many alternatives exist for tissue/cell disruption. The first factor is the solubilizing extraction agent [9], which may include but is not limited to TRIzol® or TRIsure® (Bioline). A 1:5 volume ratio of tissue to extraction reagent is typically utilized. Alternatively, RNA isolation kits based on spin columns using affinity of nucleic acids to silica-based matrices and specific for nucleic acids of interest are commercially available. The second factor entails the physical disruption method. Tissue homogenizers with blades grind tissues, with adverse consequences of foaming, aerosol formation, and challenges with cleaning in between sample application. As an alternative, bead mills are used to disrupt samples and release nucleic acids by "cracking" specimens against glass, ceramic, or steel beads during vigorous agitation. Bead mills permit high-throughput processing with multiple samples in individual cryovials while minimizing foaming effects. The choice of beads depends on the density and fibrous nature of the tissue from which the RNA will be extracted. A third low-technology approach entails pulverizing frozen tissue in liquid nitrogen using a mortar and pestle. However, this approach requires the maintenance of the frozen state of samples and increased time for processing of individual samples.
3. Follow laboratory safety procedures by using hazardous chemicals in a fume hood. Always wear personal protective equipment (i.e., gloves, goggles, lab coat) when handling hazardous reagents such as phenol and chloroform.
4. Prior to RNA work, the lab bench area should be carefully cleaned and treated with 70 % ethanol or RNaseZap®. Similar reagents can be used to reduce RNase contamination from surfaces. If possible, maintain a region of the lab or the bench dedicated to RNA work. Always use aerosol-barrier tips and ultrapure water. Diethylpyrocarbonate or DEPC-treated water to remove nucleases is not necessary for these protocols.
5. Nanodrop spectrophotometers are very easy to use and require only 1 μl of undiluted RNA for analysis. The absorbance ratio (260 nm/280 nm) for RNA should be 1.8–2.0. The RNA concentration is determined (automatically) by the following: μg/ml RNA = absorbance at 260 nm × dilution × 40 μg/ml.
6. Generating complementary DNA can be accomplished in several ways. Total RNA can be reverse transcribed in the presence of random hexamer and nonamer or using oligonucleotide d(T)

primers that preferentially bind to polyadenylated RNA species. An example of an RT reaction of 25 μl consists of the following: RNA template (0.1 to 2 μg), 1.25 μl of RNase inhibitor, 10 mM of primer, dNTP mix (10 mM each of dGTP, dATP, dCTP, dTTP), reverse transcriptase, 2.5 μl of 0.1 M DTT and 2.5 μl of 10× PCR buffer, and water.

7. Incubation of the cDNA reaction mix can be accomplished using a thermocycler, heated chamber, hybridization oven, or water bath. Use the established temperature and time (including activation and inactivation steps) according to the specifications for the enzyme. For example, 37 °C for 50 min is optimal for M-MLV reverse transcriptase, while SuperscriptIII reverse transcriptase conditions specify 50 °C for 50 min.
8. Since the sequences of most genomes and genes have been identified, there are many commercial sources for primers, with Applied Biosystems® at Life Technologies being a primary source. The online database of reagents can be searched using several criteria, including species, gene ID, name, or even sequence. Bioinformatics tools to obtain target sequences include Basic Local Alignment Search Tool (BLAST) from NCBI, Ensembl Genome Browser, and UCSC Genome Browser. However, primers can be designed de novo and examples of primer design algorithms include http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Primer design, and Primer Express. In general, primer and probe sets should have the following features: the oligonucleotide primers should bind across exon junctions to insure that RNA-derived cDNA is amplified over contaminating genomic DNA; the melting temperature of the probe should be the highest, with the optimal differences being 10 °C greater than the forward and reverse primers that have T_m of 55–65 °C; the amplified product should be 60–150 bp long; and the length of the primers should be similar (~20 bp) with no guanosines located at the 5′ terminus of the probe, which would prevent the addition of the fluorescent label.
9. qPCR reaction setup is directed according to the reagents used. In general, it is critical to assay multiple biological replicates and technical replicates (i.e., three reactions for each condition and each template) in addition to non-template and internal reference controls (*see* **Note 12**). A TaqMan®-based detection method uses two primers plus probe labeled with a 5′ fluorescent reporter tag and a 3′ quencher in a prepared reaction mix to assess amplification of a specific PCR product. For example, a 15 μl total volume reaction would contain 7.5 μl of 2× PCR master mix, 0.6 μl of each of primer (10 μM), 0.15 μl of probe (10 μM), template, and water. A SYBR® Green-based detection method uses two primers with a dye

that binds to double-stranded DNA to detect accumulating PCR products. In this case, a 10 μl reaction mix would contain the following: 5 μl of 2× SYBR® Green reaction mix, 0.4 μl of each of primer (10 μM), template, and water. Always prepare master mixes, just before loading onto a PCR plate following the addition of the diluted template. Always include an excess amount of master mix to compensate for any pipetting-induced errors.

10. PCR profiling arrays offer an alternative multiplex profiling approach that supports disease-specific or pathway-focused gene expression studies. PCR profiling arrays simultaneously determine the expression levels of about 80 genes. These pre-plated commercial PCR arrays are typically accompanied with ready-to-use master mixes. Likewise, these arrays provide expression profiling gene primers, housekeeping primers, and RNA quality control targets. Essentially, cDNA that is synthesized from 25 ng to 5 μg of total RNA is mixed with the prepared PCR master mix, aliquoted to wells in a 96-well plate, and then subjected to PCR amplification and analysis, following the manufacturers' protocols.
11. To estimate an absolute quantity of transcript, linear regression based on a known quantity of input molecules must be generated. One approach is to use a plasmid DNA-based standard that contains the PCR amplicon of interest cloned into a vector. The TOPO® TA cloning vector (Invitrogen/Life Technologies) has been specifically designed for subcloning PCR products that contain a single AT base overhang. The plasmid DNA is then harvested from bacteria and molecular amounts are quantified by spectral assessment at 260 nm. The number of molecules is determined based on the following: μg/μl (based on $OD_{260\ nm}) \times (1 \times 10^{-6}$ g/μg) × $(1 \times 10^{18}$ attomol)/(650 g/bp) × (bp of plasmid) = attomol/μl, where 1 attomol is 602,500 molecules. Prepare dilutions of 500 attomol/μl and then 15 attomol/μl using 0.1 mg/ml of glycogen, an inert carrier, dissolved in water. Subsequently four to five serial 5 to 15-fold dilution aliquots of the plasmid standard are prepared and analyzed to generate standard linear regression statistics. The most efficient PCR amplification will give a line with a slope of –3.3 and correlation coefficient R^2 of 0.99.
12. An internal control used as a normalization reference should be assessed for each sample. These typically consist of gene(s) that should be ubiquitously expressed in many cell types and for which expression does not vary with changes in conditions. These housekeeping genes (i.e., β-actin, tubulin, β2 microglobulin, hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein genes) should be analyzed for each cell type as their utility as internal

references is controversial [10–12]. Additionally, the 18S rRNA gene is also used as an internal reference even though this gene is transcribed in greater abundance by RNA polymerase III in contrast to RNA polymerase II-transcribed genes.

13. C_T (or threshold cycle) is a relative measure of PCR product generation and is determined where the amplification curve crosses the threshold of product detection. The comparative C_T method may be employed to determine relative changes in expression compared to an internal reference from the control with a change in condition [13, 14]. The relative amount of target is calculated to be $2^{-(\Delta\Delta C_T)}$ or the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control. In other words, first normalize gene expression of the target to a reference using the difference in C_T ($\Delta C_T = C_T$ target $- C_T$ reference). Next, determine the change with treatment or tissue type using the difference in ΔC_T ($\Delta\Delta C_T = \Delta C_T$ condition/tissue of interest $- \Delta C_T$ untreated control/base tissue).

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