Chapter 11

Gene Expression Profiling of Celiac Biopsies and Peripheral Blood Monocytes Using Taqman Assays

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Abstract

Quantitative real-time PCR (qPCR) allows for highly sensitive, rapid, and reproducible quantification of mRNA: it has become an established technology for the quantification of gene expression with the 5′ nuclease assay using TaqMan® probes. It is used for a broad range of applications, including quantification of gene expression, measuring RNA interference, biomarker discovery, pathogen detection, and drug target validation. When studying gene expression with qPCR, scientists usually investigate changes—increases or decreases—in the quantity of particular gene products or a set of gene products. Investigations typically evaluate gene response to biological conditions such as disease states, exposure to pathogens or chemical compounds, organ or tissue location, and cell cycle or differentiation status. Here we describe this technique applied to molecular profiling of candidate genes in celiac biopsies and peripheral blood monocytes. Using data obtained by gene expression experiments, a discriminant equation has been developed that allows the correct classification of Celiac Disease (CD) patients compared to healthy controls, CD patients on a Gluten Free Diet (GFD), and other disease controls.

Key words Taqman assay, Gene expression, Celiac disease, Intestinal mucosa, Monocytes, Stepwise discriminant analysis

1 Introduction

Measurement of gene expression is becoming increasingly important in the study of diverse biological processes and understanding of disease pathogenesis [1]. Traditional methods, such as Northern blots and RNA protection assays, are limited by their requirement for large amounts of RNA and their time-consuming nature. By contrast, real-time reverse transcription-polymerase chain reaction (RT-PCR) requires minute amounts of RNA and is rapid and quantitative [2]. These assays require high reproducibility and precision, which may be impaired by inconsistencies in the procedures used to collect tissues and to isolate the RNA.

A common method to minimize these errors is to standardize the RNA or cDNA input amount and to simultaneously measure

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an RNA whose expression level is constant among samples [3, 4]. This RNA serves as an endogenous control and allows comparison of the data of the genes of interest among different samples. The critical steps which must be considered in a qPCR assay for a gene expression study are as follows:

- Sample Acquisition, Handling, and Preparation: these are the first potential source of experimental variability, especially for experiments targeting RNA, because mRNA profiles are easily perturbed by sample collection and processing methods. Nucleic acid extraction is a second critical step; the efficiency depends on adequate homogenization, the type of sample, target density, physiological status, genetic complexity, and the amount of biomass processed. These are all features which must be optimized to perform a reliable experiment.
- Quantification of RNA in the extracted samples is important, because it is mandatory that approximately the same amounts of RNA be used when comparing different samples.
- Reverse transcription: it is mandatory to standardize the amount of RNA reverse-transcribed, priming strategy, enzyme type, volume, temperature, and duration of the reverse transcription step.
- Assay performance: PCR efficiency, linear dynamic range, lower limit of detection (LOD), and precision have to be optimized.
- Data Analysis: includes an examination of the raw data, an evaluation of their quality and reliability, and the generation of reportable results.

We performed the experiments starting with RNA obtained from 48 fresh-frozen duodenal biopsies and 49 peripheral blood monocyte samples. Total RNA was reverse-transcribed into cDNA and, after retro-transcription, we carried out a linear pre-amplification step to enhance the low amount of RNA recovered from monocytes. Experiments were performed using the TaqMan® Gene Expression Assay, and the relative expression was calculated with the comparative Ct method.

Finally, we analyzed gene expression using discriminant analysis, which is performed to estimate the contribution of the expression of each gene to distinguish CD patients from healthy individuals and disease controls. The aim of this analysis is to weigh the discriminating capacity of each single gene to obtain a new composite variable, the discriminant score (D-score), which provides a group-specific score for each individual. Wilks' lambda is an estimate of the discriminant capacity ranging from 1 (complete overlap) to 0 (maximum distance). The variable that minimizes the overall Wilks' lambda is entered at each step. According to this analysis, only a few specific genes were selected for discriminating capacity,

giving a significant contribution to the Variance ratio F, with a first degree error always less than 0.001.

By multiplying the canonical unstandardized coefficients produced by the analysis to the actual values of the RQ of the candidate genes, a D-score was obtained for each individual. The discriminant score provides a probability of membership to the cases or to the controls groups for each individual. The highest membership probability for each case allows the classification into the diagnostic groups.

2 Materials

2.1 For All the Standard Working Procedures to Be Taken, See Note 1

1. Dynabeads® My Pure™ Monocyte kit (Life Technologies, Foster City, CA), superparamagnetic polystyrene beads coated with a monoclonal human anti-mouse IgG antibody.
2. Ambion® RiboPure™ kit (Life Technologies, Foster City, CA).
3. TRIZOL Reagent.
4. Bromochloropropane (BCP).
5. Glass fiber filter.
6. Low salt buffer.

2.2 RNA Quantification and Quality Control

1. Nanodrop® spectrophotometer.
2. Agarose.
3. 1× Tris/Borate/EDTA buffer (TBE).
4. High Capacity cDNA Reverse Transcription kit (Life Technologies, Foster City, CA).
5. Deoxynucleotide (dNTP) Solution Mix (100 mM).
6. 7900HT Fast Real-Time PCR system.
7. TaqMan® Gene Expression Assay (listed in Table 1).
8. TaqMan® Gene Expression Master Mix (Life Technologies, Foster City, CA).
9. cDNA.
10. SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA) software packages.

3 Methods

3.1 Monocyte Isolation

3.1.1 Dynabead Washing

1. Resuspend the Dynabeads in the vial to a homogenous suspension.
2. Transfer the desired volume of Dynabeads to a tube.
3. Add the same volume of Buffer 1 (PBS w/0.1 % BSA and 2 mM EDTA, pH 7.4), or at least 1 ml, and mix.

Table 1
List of taqman assays used for the gene expression analysis

Assay ID	Gene symbol	Gene name	Chromosome	NCBI reference sequence	Target exons	Amplicon length	Position
Hs99999908_m1	GUSB	Glucuronidase, beta	7	NM_000181.3	11–12	81 bp	1913
Hs00611823_m1	TAGAP	T-cell activation RhoGTPase activating protein	6	NM_054114.3	9–10	64 bp	1230
Hs00193878_m1	SH2B3	SH2B adaptor protein 3	12	NM_005475.2	2–3	81 bp	1089
Hs00175260_m1	RGS1	Regulator of G-protein signaling 1	1	NM_002922.3	3–4	115 bp	346
Hs00968436_m1	REL	v-relreticuloendotheliosis viral oncogene homolog (avian)	2	NM_002908.2	10–11	86 bp	1312
Hs00234713_m1	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	6	NM_006290.2	2–3	63 bp	363
Hs00998604_m1	TNFRSF14	Tumor necrosis factor receptor superfamily, member 14	1	NM_003820.2	6–7	102 bp	987
Hs00222327_m1	IL-21	Interleukin 21	4	NM_001207006.2	3–4	84 bp	406
Hs00944352_m1	LPP	LIM domain containing preferred translocation partner in lipoma	3	NM_001167671.1	8–9	85 bp	1593
Hs00361070_m1	KIAA1109	KIAA1109	4	NM_015312.3	43–44	66 bp	7360
Hs00542477_m1	TNFS14	Tumor necrosis factor (ligand) superfamily, member 14	19	NM_003807.3	4–5	69 bp	685
Hs99999150_m1	IL-2	Interleukin 2	4	NM_000586.3	2–3	89 bp	261

4. Place the tube in a magnet for 3 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume of Buffer 1 as the initial volume of Dynabeads.

3.1.2 Preparation of Mononuclear Cells (MNC) from Blood to Obtain Low Platelet Numbers

1. Dilute 10 ml of blood, collected in a preheparinized syringe with PBS pH 7.4 (to a total volume of 35 ml) at 18–25 °C Room Temperature (RT).
2. Add the diluted blood on top of 15 ml of Lymphoprep.
3. Centrifuge at $160 \times g$ for 20 min at RT. Allow to decelerate without brakes.
4. Remove 20 ml of supernatant to eliminate platelets.
5. Centrifuge at $350 \times g$ for 20 min at RT. Allow to decelerate without brakes.
6. Recover MNC from the plasma/Lymphoprep interface and transfer the cells to a 50 ml tube.
7. Wash the MNCs twice with Buffer 1 by centrifugation, first at $400 \times g$ for 8 min at 2–8 °C and second at $225 \times g$ for 8 min at 2–8 °C.
8. Count the collected cells with a Burker chamber (or other method) and resuspend the MNC at 1×10^8 MNC per ml in Buffer 1.
   For critical Steps for Cell Isolation, *see* **Note 2**.

3.1.3 Isolation of Human Monocytes from MNC

1. Transfer 100 µl (*see* **Note 3**) MNC in Buffer 1 to a tube.
2. Add 20 µl Blocking Reagent. Add 20 µl Antibody Mix.
3. Mix and incubate for 20 min at 2–8 °C.
4. Wash the cells by adding 2 ml Buffer 1.
5. Mix the tube and centrifuge at $300 \times g$ for 8 min at 2–8 °C.
6. Discard the supernatant.
7. Resuspend the cells in 900 µl Buffer 1, precooled to 2–8 °C.
8. Add 100 µl prewashed Depletion MyOne Dynabeads and mix.
9. Incubate for 15 min at 2–8 °C and mix (*see* **Note 4**).
10. Resuspend the bead-bound cells by vigorous pipetting 5 times (*see* **Note 5**).
11. Add 1 ml Buffer 1, precooled to 2–8 °C.
12. Place the tube in the magnet (provided from kit) for 3 min and transfer the supernatant to a new tube.
13. Repeat **step 12**. The supernatant contains human monocytes.

3.2 RNA Extraction

Sample acquisition is crucial to minimize experimental variability in mRNA expression experiments that are deeply influenced by sample collection and processing methods.

Nucleic acid extraction is a critical step; choosing an effective and rapid method for tissue or cell disruption is also crucial. The most effective method is determined by the nature of the tissue, the storage method, and the size of the sample. Extraction efficiency depends on adequate homogenization, the type of sample (e.g., in situ tissue vs. log phase cultured cells), target density, physiological status (e.g., healthy, cancerous, or necrotic), genetic complexity, and the amount of biomass processed. To see more precautions to use before working with RNA, *see* **Note 6**. The described procedure, performed by Ambion® RiboPure™ kit, is designed for 5–100 mg tissue samples, $0.1–20 \times 10^6$ cultured cells, or up to 10 cm^2 of monolayer culture. For samples smaller than 5 mg or 0.1×10^6 cells, s*ee* **Note 7**.

3.2.1 Cell Disruption and Initial RNA Purification

1. Weigh frozen tissue, and if necessary, break it into pieces smaller than ~50 mg (keeping tissue completely frozen) and homogenize directly in TRI Reagent (*see* **Note 8**).
2. Homogenize samples in 10–20 volumes TRI Reagent (e.g., 1 ml TRI Reagent per 50–100 mg tissue) using standard homogenization procedures.
3. For monocytes (as for any other cells grown in suspension) pellet cells, remove media, then lyse in 1 ml of TRI Reagent per 5×10^6 cells by repeated pipetting or vortexing.
4. Incubate homogenates from both samples and cell cultures with lysis buffer for 5 min at RT. This incubation allows nucleoprotein complexes to completely dissociate.

3.2.2 RNA Extraction

1. Transfer 1 ml of homogenate to a labeled 1.5 ml microcentrifuge tube.
2. Add 100 μl of 1-bromo-3-chloropropane (BCP) (*see* **Note 9**). Cap tubes tightly and vortex at maximum speed for 15 s.
3. Incubate the mixture at RT for 5 min.
4. Centrifuge at $12{,}000 \times g$ for 10 min at 4 °C to separate the mixture into a lower, red, organic phase (phenol-BCP phase); an interphase; and a colorless, upper, aqueous phase. RNA remains in the aqueous phase while DNA and proteins are in the interphase and organic phase (*see* **Note 10**).
5. Transfer 400 μl of the aqueous phase (top layer) to a new, labeled 1.5 ml microcentrifuge tube.

3.2.3 Final RNA Purification

1. Add 200 μl of 100 % ethanol to 400 μl of aqueous phase from previous step.
2. Vortex immediately at maximum speed for 5 s to avoid RNA precipitation.

3. For each sample, place a Filter Cartridge in one of the Collection Tubes supplied. Transfer the sample to a Filter Cartridge-Collection Tube assembly and close the lid.
4. Centrifuge the assembly at 12,000 × *g* for 30 s at RT or until all of the liquid is through the filter. Discard the flow-through and return the Filter Cartridge to the same Collection Tube. The RNA is now bound to the Filter Cartridge.
5. Apply 500 μl of Wash Solution to the Filter Cartridge-Collection Tube assembly, and close the lid. Centrifuge for 30 s at RT or until all the liquid is through the filter. Discard the flow-through and return the Filter Cartridge to the same Collection Tube.
6. Repeat the last three steps for a second wash.
7. Centrifuge for 30 s at RT to remove the residual Wash Solution.
8. Add 100 μl of Elution Buffer to the filter column.
9. Incubate at RT for 2 min. Centrifuge for 30 s to elute the RNA from the filter. The RNA will be in the elute, in the Collection Tube.

3.3 RNA Quantification and Quality Control

There are several quantification procedures in common use that produce different results, including spectrophotometry (NanoDrop; Thermo Scientific), microfluidic analysis (Agilent Technologies' Bioanalyzer, Bio-Rad Laboratories' Experion), capillary gel electrophoresis (Qiagen's QIAxcel), or fluorescent dye detection (Ambion/Applied Biosystems' RiboGreen). We recommend comparison of the data obtained with the different methods [5].

We routinely control the quantity of RNA using the Nanodrop® spectrophotometer. The total RNA isolated with previously described procedures should have an A260/A280 ratio of 1.8–2.1. However, RNA with absorbance ratios outside of this range may still function well for quantitative-Reverse PCR (qRT-PCR) or other amplification-based downstream applications. RNA quality may also be analyzed by Agarose gel electrophoresis in Tris/Borate/EDTA buffer (TBE). After the spectrophotometric quantification, we routinely control 100 ng of each RNA sample on 1 % agarose gel electrophoresis, with a reference control size Marker.

3.4 Reverse Transcription

2 μg of total RNA extract (as previously described) from each biopsy and 100 ng RNA from monocytes were reverse-transcribed into cDNA with the High Capacity cDNA Reverse Transcription kit. This kit allows the quantitative conversion of 0.1 to 10 μg of total RNA to cDNA, with a concentration range between 0.002 and 0.2 μg/μl.

3.4.1 Prepare the 2× Reverse Transcription Master Mix: Allow the Kit Components to Thaw on Ice, per 20 µl Reaction add the Following Quantity

1. 10× RT Buffer: 2 µl.
2. 10× RT Random Primers: 0.8 µl.
3. 25× dNTP Mix (100 mM): 2 µl.
4. MultiScribe™ Reverse Transcriptase 50 U/µl: 1 µl
5. Nuclease-Free Water: 4.2 µl.

3.4.2 Preparing the cDNA Reverse Transcription Reaction

Pipette 10 µl of 2× RT master mix into each well of a 96-well reaction plate or individual tube. Pipette 10 µl of RNA sample into each well, pipetting up and down twice to mix. Seal the plates or tubes and load the thermal cycler (*see* **Note 11**).

3.4.3 Performing Reverse Transcription: Program the Thermal Cycler Conditions

1. Step 1: 25 °C × 10 min
2. Step 2: 37 °C × 120 min
3. Step 3: 85 °C × 5 min
4. Step 4: 25 °C × ∞
5. Set the reaction volume to 20 µl, load the reactions into the thermal cycler and start the reverse transcription run.

3.5 qPCR Using TaqMan Assay

Experiments are performed on the 7900HT Fast Real-Time PCR system using the TaqMan® Gene Expression Assay, and approximately 40 ng of cDNA as described in the protocol.

1. For each sample (to be run in triplicate), pipette the following reagents into a nuclease-free 1.5-ml microcentrifuge tube:
   - 20× TaqMan® Gene Expression Assay: 1 µl
   - 2× TaqMan® Gene Expression Master Mix: 10 µl
   - cDNA template (1–100 ng): 4 µl
   - Nuclease-Free Water: 5 µl
2. Cap the tube and invert it several times to mix the reaction components. Centrifuge the tube briefly. Transfer 20 µl of PCR reaction mix into each well of a 48-, 96-, or 384-well reaction plate. Seal the plate with the appropriate cover. Centrifuge the plate briefly. Load the plate into the instrument.
3. The SDS software (Life Technologies, version 1.4 or 2.4) is used to analyze the raw data and then additional statistical analysis is performed on GraphPad Prism 5.01®.
4. The relative expression is calculated using the comparative ΔCt method. The ΔΔCt method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in Ct values (ΔCt) between the target gene and the reference gene is calculated, and the ΔCts of the different samples are compared directly. The expression of each gene is normalized to an endogenous housekeeping gene. The ideal endogenous control should have a constant RNA transcription

level under different experimental conditions and be sufficiently abundant across different tissues and cell types. Although any gene that is stably expressed under the defined experimental conditions can serve as a normalization gene, the selection is most commonly made from constitutively expressed mRNA housekeeping genes, or ribosomal RNAs such as 18S rRNA. Genes such as HPRT [6], GUS, and B2M have shown relative stability across a number of tissues. Thus, there is no universal control gene and it is important to identify the most appropriate endogenous control for a particular cell type and experimental condition. GUSb was chosen as reference gene after it had been determined as the most stable reference gene out of 5 candidates (β-actin, B2M, GAPDH, GUSb, and HPRT1). All gene expression experiments were conducted according to MIQE guidelines [7].

3.6 Statistical Analysis

Using SPSS software, a discriminant multivariate analysis is performed on biopsy gene expression RQ values. Using this approach 5 genes (TNFAIP3, IL-21, c-REL, RGS1, and LPP) were selected for discriminating capacity. The multivariate equation is capable of discriminating celiacs from controls; in fact 92.9 % of individuals were correctly classified efficiently (95 % of controls and 90.9 % of celiacs).

By the same multivariate approach, the expression of 4 candidate genes from monocytes was selected, with a pattern quite similar to that observed in the duodenal tissue. *LPP, c-REL, KIAA1109, and TNFAIP3* genes help to discriminate cases from controls; indeed 91 % of controls and all CD patients were correctly classified.

Finally, we obtained four clustered D-scores, one for each group (Controls, CD, Crohn, and CD on GFD) with no overlap with the active celiacs. The D-Score of active celiac patients was negative in all cases, while it was positive for all the other groups on differentiated clusters. This score produces a group membership probability for each individual, allowing us to correctly classify all controls and CD patients; none of the controls, neither CD on GFD nor Crohn patients were misclassified as CD patients.

Our discriminant function is proposed in an attempt to improve the diagnosis of CD and as a support to limit invasive techniques. Esophago-gastro-duodenoscopy is still the gold standard for the diagnosis of CD, but it can decrease the patient's compliance and is indeed a major bottleneck in developing countries: a simple blood sample, which can be easily dispatched, may help to disseminate the diagnostic coverage to the majority of patients that cannot reach a specialized reference center [7]. In the near future, because of the new ESPGHAN protocol [8], we may have no information about the status of the traditional target tissue in many patients: gene expression on a blood sample may well add safety and sensitivity to a biopsy-free diagnostic protocol, thereby providing a good proxy of the mucosal status.

4 Notes

1. Prepare all solutions with ultrapure water and analytical grade reagents. Prepare and store all reagents at RT. Diligently follow all waste disposal regulations when disposing waste materials.
2. Use a mixer that provides tilting and rotation of the tubes to ensure that Dynabeads do not settle at the bottom of the tube. When incubating Dynabeads and cells, the incubation temperature must be 2–8 °C to reduce phagocytic activity and other metabolic processes. If the temperature is above 2–8 °C, the monocytes will engulf the beads and be depleted from the sample, giving a low recovery of monocytes. Never use less than 100 μl Dynabeads per 1×10^7 MNC sample. It is critical to follow the magnet recommendations to ensure a successful isolation.
3. The protocol described in Subheading 3.1 is based on 1×10^7 MNC; it can be scaled up from $1 \times 10^7 - 5 \times 10^8$ cells.
4. Mix well tube with gentle tilting and rotation several times.
5. Use a pipette with a narrow tip opening (e.g., a 1000 μl pipette tip or a 5 ml serological pipette).
6. Before working with RNA, it is always a good idea to clean the lab bench and pipettors with an RNase decontamination solution (e.g., Ambion RNase*Zap*® Solution). Wear laboratory gloves at all times during this procedure and change them frequently. Gloves protect you from the reagents, and they protect the RNA from nucleases that are present on skin. Use RNase-free pipette tips to handle Wash Solution and Elution Buffer, and avoid putting used tips into the kit reagents.
7. The procedure is compatible with tissues that have been stored in Ambion RNAlater® Solution. Total RNA yield is typically 100–500 μg per 100 mg of tissue, depending on the type of tissue.
8. Larger pieces of tissue, very hard or fibrous tissues, and tissues with a high RNase content must typically be ground to a powder in liquid nitrogen for maximum RNA yield.
9. Alternatively, use 200 μl of chloroform (without isoamyl alcohol) in place of BCP.
10. The volume of the aqueous phase is typically about 60 % of the volume of TRI Reagent used for homogenization.
11. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles. Place the plate or tubes on ice until you are ready to load the thermal cycler.

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