Gene Expression Signature in Peripheral Blood as a Marker of Parkinson's Disease

Application Note TRANS0114

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by the progressive depletion of dopaminergic neurons within the substantia nigra. The disorder clinically manifests as progressive symptoms of tremor, rigidity, bradykinesia and postural instability. PD usually occurs late in life – its incidence increases with age and has a prevalence of approximately 1.8% among people over the age of 65, translating to significant effects on health, morbidity and mortality in the overall population. Diagnosis of PD represents an ongoing challenge, as lengthy diagnostic procedures are required to establish certain diagnosis. For this reason, there is great interest in developing biomarker-based methods for the early and non-invasive detection of PD[1].

Whole-genome expression profiling studies surveying the peripheral blood of patients with PD have indicated altered patterns of expression in several genes[2]; however, due to the inherent limitations of the high-throughput methods used in such studies – including reduced statistical power and ability to detect low-abundance genes – it is often necessary to confirm the expression profiles of individual genes using a more targeted approach, namely, quantitative real-time PCR (qPCR)[3]. In this application, five genes were selected based on the results of past global gene expression studies, and a qPCR validation study was performed on samples taken from an independent subset of patients with PD and matched controls. The qPCR experiment was designed and prepared using the PIPETMAX® qPCR Assistant software and the PIPETMAX 268 automated pipetting instrument. The previously reported differences in expression of the proposed biomarker set were confirmed in this study, indicating the potential utility of such biomarkers in detecting PD in peripheral blood samples.

Materials & Methods

Samples

Peripheral blood samples were obtained from 16 healthy individuals and from 15 individuals who had been diagnosed with Parkinson's disease.

RNA Extraction and Reverse Transcription

Total RNA was extracted from peripheral blood samples using a Qiagen RNeasy® kit and quantified spectrophotometrically on a NanoDrop™. The extracted RNA was then treated with DNase I and reverse transcribed into cDNA using the Invitrogen SuperScript® VILO™ reagent kit.

qPCR

Samples were analyzed for five markers/genes: Marker gene 1, Marker gene 2, Marker gene 3, Marker gene 4, and Marker gene 5 (gene names are undisclosed, as data have not yet been published). In addition, the beta-actin gene (ACTB) was used as a reference gene (for normalization in data analysis). For each gene, samples were analyzed in 10 µl reactions (8 µl of TaqMan® Universal Master Mix II containing sequence-specific fluorescent probes and 2 µl of cDNA) on 384-well plates. Each sample was analyzed in two replicates and two dilutions (non-diluted and 10-fold). Two replicates of no-template-controls (NTCs) were used for each gene. An Applied Biosystems 7900HT Fast Real-Time PCR System was used to perform qPCR, and for all markers, commercially available TaqMan® Gene Expression Assays were used.

Sample Preparation and Tracking for qPCR

The qPCR experimental design was outlined using PIPETMAX® qPCR Assistant software, which generated two pipetting protocols for the PIPETMAX instrument: the first to prepare the sample dilutions and the second to prepare the 384-well qPCR plate. By importing an Excel .csv file containing a list of sample names into qPCR Assistant software, the qPCR plate layout was rapidly generated. In addition, the qPCR Assistant software prepared all calculations for the master mix set-up and sample dilutions (Figure 1A).

The PIPETMAX was used to prepare sample dilutions on 96-well plates and to transfer samples and master mixes (prepared manually) onto 384-well plates (Figure 1B). PDF guides prepared in the qPCR Assistant software were used to position labware on the PIPETMAX bed. When the reaction setup was complete, a thermocycler-specific exportable file containing plate specifications, sample names, and detector information was imported to the qPCR thermocycler's software.

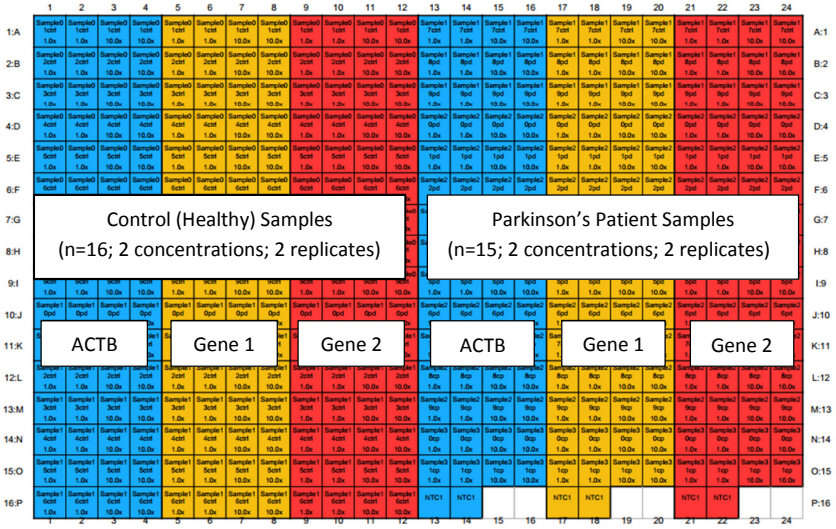

Figure 1A. qPCR plate layout prepared by PIPETMAX® qPCR Assistant software. (Layout of one of two plates containing Marker genes 3, 4, and 5 not shown)

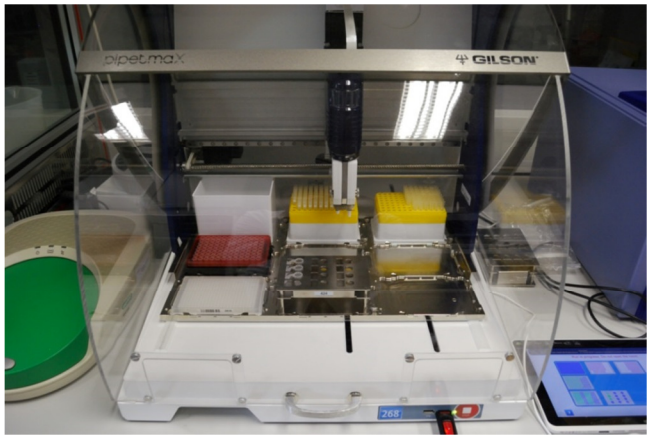

Figure 1B. PIPETMAX instrument performing the qPCR plate protocol run. No cross-contamination was observed during the run (all NTC controls were negative).

Data Analysis

Applied Biosystems SDS software was used to evaluate amplification curves. Cq data were then exported and analyzed using a relative quantification approach with efficiency correction[3] in Microsoft Excel to determine the relative quantity of transcripts for each marker gene. ACTB was used as the normalizer gene and one of the control samples was used as the calibrator sample. Relative quantities were log2 transformed and plotted. Gene expression differences between the patient and control samples were determined using a Welch's two sample t-test. Based on the expression profiling results of the five tested genes, a support vector machine (SVM) classier model was constructed, and its prediction accuracy was tested across 10 iterations of a 5-fold cross-validation scheme[4].

Results

Using real-time gene expression profiling on novel samples from patients with PD and matched healthy controls, significant gene expression alterations were detected for four of the five tested markers ($p<0.001$) (Figures 2 & 3). Results for all five markers were in agreement with the results from a genome-wide expression profiling study performed previously.

The discriminative power of this biomarker set was demonstrated by generating a disease prediction model and validating it using cross-validation tests. The 5-gene expression marker attained discriminatory AUC values above 0.85 (Figure 4), validating the potential utility of gene expression markers in predicting PD disease status.

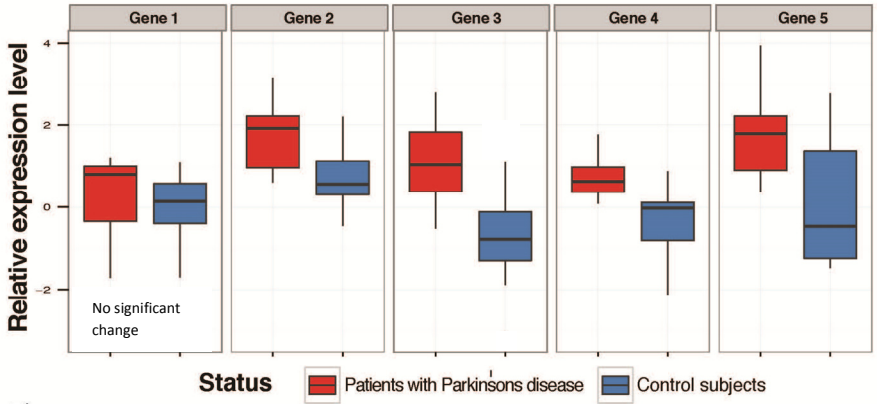

Figure 2. Box-plots summarizing the differential gene expression measured by qPCR. Significant differences in expression ($p<0.001$, according to Welch's t-tests) were observed for Marker genes 2-5 in patients with Parkinson's (red) compared with control subjects (blue).

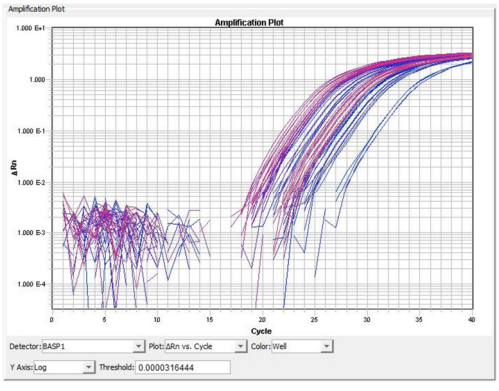

Figure 3. Representative amplification plot produced during the qPCR run. A wide range of amplification of Marker gene 4 (shown here) can be seen across the 15 patient (red) and 16 control (blue) samples.

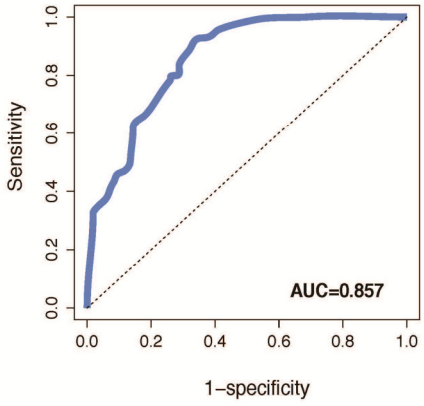

Figure 4. The ROC curve for predictive potential of the five gene expression biomarkers tested using qPCR.

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Summary

Four of the five biomarkers (Marker genes 2, 3, 4 and 5) analyzed in this study showed statistically distinct expression patterns in samples of peripheral blood from healthy individuals compared to those from individuals with Parkinson's disease. The qPCR approach employed here enabled a greater depth of analysis and validation of the gene expression differences previously observed in whole-genome expression profiling studies. By utilizing the PIPETMAX instrument paired with qPCR Assistant software, the process of designing and preparing the qPCR experiment was streamlined. Furthermore, the capacity of qPCR Assistant software to integrate with LIMS and other data management systems ensured sample traceability, as files created in qPCR Assistant were exported directly to the thermocycler used in this experiment. The disease prediction model generated based on the qPCR results indicated that this 5-gene marker has the capacity to predict disease status with a high level of accuracy. The reliable and reproducible pipetting afforded by the PIPETMAX contributed to the accuracy of this prediction model, whose success relies on the quality of the input data. Taken together, these results suggest the plausibility of gene expression profiling in peripheral blood for predicting Parkinson's disease status.

References

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