Identification of Factors Contributing to Variability in a Blood-Based Gene Expression Test

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Abstract

Background: Corus CAD is a clinically validated test based on age, sex, and expression levels of 23 genes in whole blood that provides a score (1–40 points) proportional to the likelihood of obstructive coronary disease. Clinical laboratory process variability was examined using whole blood controls across a 24 month period: Intra-batch variability was assessed using sample replicates; inter-batch variability examined as a function of laboratory personnel, equipment, and reagent lots.

Methods/Results: To assess intra-batch variability, five batches of 132 whole blood controls were processed; inter-batch variability was estimated using 895 whole blood control samples. ANOVA was used to examine inter-batch variability at 4 process steps: RNA extraction, cDNA synthesis, cDNA addition to assay plates, and qRT-PCR. Operator, machine, and reagent lots were assessed as variables for all stages if possible, for a total of 11 variables. Intra- and inter-batch variations were estimated to be 0.092 and 0.059 Cp units respectively (SD); total laboratory variation was estimated to be 0.11 Cp units (SD). In a regression model including all 11 laboratory variables, assay plate lot and cDNA kit lot contributed the most to variability ($p = 0.045; 0.009$ respectively). Overall, reagent lots for RNA extraction, cDNA synthesis, and qRT-PCR contributed the most to inter-batch variance (52.3%), followed by operators and machines (18.9% and 9.2% respectively), leaving 19.6% of the variance unexplained.

Conclusion: Intra-batch variability inherent to the PCR process contributed the most to the overall variability in the study while reagent lot showed the largest contribution to inter-batch variability.

Introduction

Peripheral blood gene expression profiling has been used to identify signatures which reflect a variety of pathological conditions, responses to pharmacological agents, and external environmental effects [1,2,3,4]. Examples include gene expression tests for auto-immune and inflammatory disorders such as lupus erythematosus, heart transplant rejection, and coronary artery disease (CAD) [5,6,7,8]. It has also been demonstrated that gene expression measurements can be affected by a number of ex vivo events that start at the time of blood collection and continue, if care is not taken, during downstream processing [9,10,11]. Although an increasing number of peripheral blood gene expression signatures have been described, very few have yet been rigorously validated and implemented into broad clinical practice.

We recently described the development and clinical validation of a peripheral blood gene expression test for the assessment of the likelihood of coronary artery disease in non-diabetic patients [8,12]. The test is a quantitative in vitro diagnostic performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified clinical laboratory that uses the gene expression profile of circulating peripheral blood cells to estimate the likelihood of significant coronary artery disease, defined as ≥50% stenosis in a major coronary artery by quantitative coronary angiography [8].

The test score is a function of the expression levels of 23 genes which are grouped into highly correlated terms reflecting biological processes or cell types [8]. The terms, some of which are sex-specific, are weighted and combined into an algorithm which also contains the patient’s age and sex [8]. The resulting output is a gene expression score (GES) which is related to the probability of obstructive CAD. For ease of clinical interpretation, the GES is linearly transformed into a reported score ranging from 1 to 40, with increasing values representing increased probability of obstructive CAD.

Herein we describe the variability present in the clinical laboratory across a 24 month period. We assess both intra-batch and inter-batch variability, and the contributions of laboratory personnel, machine, and reagent lot to the overall variability.
Results

The clinical laboratory process flow is depicted in Figure 1. The process contains a number of QC checkpoints and controls, including the whole blood control which is run with each batch of clinical samples (Fig. 1).

Total Variability

Total process variability was estimated using 895 whole blood control samples from the study period. The SD derived from this set of samples was 0.11 Cp units, or slightly less than 1 point on the reported GES scale (0.97 points on the 1–40 reported GES scale, 1.7% change in probability of obstructive disease, Table 1).

Intra-Batch Variability

In order to assess the variability inherent to the laboratory process, e.g. variability that was independent of operator, machine, and reagent lot, data was used from five batches, where the samples consisted solely of identical whole blood controls (combined N = 132). In processing these batches, single operators, machines, and reagent lots were used. The SD derived from this set of samples was 0.092 Cp units, representing ~70% of the overall variance (Table 1, Fig. 2A). Reproducibility of expression values of the 23 gene components of the GES were examined to determine gene-specific variances. Gene-specific SD was anti-correlated with levels of gene expression, increasing with decreased expression levels (Table 2, Fig. 3). When the individual gene-specific variances were weighted, using the component weight from the GES algorithm, AF289562 contributed the most to GES variance followed by CASP5 (23%, 10% respectively, Table 2). The sum of the weighted variances equaled that of the GES (0.092 Cp units), demonstrating that variability inherent to the PCR process was the major contributor to overall variability.

Variability within Individual Laboratory Processes

Variability in the clinical laboratory was assessed at the four major steps in the process: RNA extraction, cDNA synthesis, cDNA addition to assay plate, and qRT-PCR, with laboratory operator, machine, and reagent lot examined where possible (Table 3).

RNA extraction. During the study, six operators used four liquid handling robots interchangeably to extract RNA from whole blood; a total of twenty-five unique lots of RNA extraction kits were used during this period. Of the three variables, only RNA extraction kit lot contributed significantly to variability (p < 0.001, Table 3).

cDNA synthesis. Five operators used four liquid handling robots interchangeably to perform reverse transcription on the isolated RNA; a total of six unique cDNA kit lots were used during this period. Of the three factors, cDNA kit lot showed the strongest variability, followed by cDNA operator (p = 0.001; p = 0.012 respectively, Table 3). Multiple liquid handling robots introduced border-line significant variability into the process (p = 0.074, Table 3).

CDNA addition to assay plates. Sample addition was performed by eight operators using five liquid handling robots; neither operators nor machines introduced significant variability (Table 3).

qRT-PCR. Twenty four lots of assay plates were used during the study (see Methods S1 for a description of plate lot

| Type of Variability | Cp SD | GES SD | % Probability Change |
|---------------------|-------|--------|----------------------|
| Total Variability   | 0.11  | 0.97   | 1.7%                 |
| Intra-Batch Variability | 0.092 | 0.81   | 1.42%                |
| Clinical Variability | 1.19  | 10.5   | 18.4%                |

1SD given in Cp units and gene expression score points (GES).
2Percent change in probability of subject having obstructive CAD.

Figure 1. Depiction of sample flow and quality control points in the commercial laboratory. Process from whole blood sample to GES calculation consists of 4 laboratory steps and then quality control algorithm score calculation by a LIMS. Both sample controls (positive and negative) and process QC checks are used as indicated.

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manufacturing and Table S1 for plate manufacturing quality control metrics); qRT-PCR was performed by six operators using six different thermal cyclers. All three factors demonstrated significant variability, with plate lot contributing the most followed by operator and then machine (p < 0.001; p = 0.016; p = 0.032 respectively, Table 3).

Inter-Batch Variability: Contributions of Operator, Machine, and Reagent Lot

To identify factors contributing to inter-batch variability, we examined the eleven factors listed in Table 4; these were included in a model to determine the amount of variance due to operators, machines, or reagent lots across the entire process. The most significant variable in this model continued to be cDNA kit lot, which contributed to 16.9% of the inter-batch variance (p = 0.009, Table 4, Fig. 2B). Overall, reagent lots (RNA extraction kits, cDNA kits, and assay plates) contributed the most to inter-batch variance followed by operators and then machines (52.3%, 18.9% and 9.2% respectively, Fig. 2B), leaving 19.6% of the variance unexplained. When considered in the context of the overall variation, these laboratory-associated factors contributed to 24% of the overall variance, whereas 6% of the overall variance was unexplained (Fig. 2A).

Biological versus Technical Variability

The standard deviation (SD) across the 895 control samples was 0.11 Cp units (Table 1). In contrast, the SD across the 21,200 clinical samples run during the same period was 1.19 Cp units (10.5 points reported GES, 18.4% change in disease probability, Table 1), reflecting the biological variation in the tested population. As a quality control (QC) metric, guard-bands at ±3 points of control target reported GES were established, equating to a 6.8% change in the probability of having obstructive coronary artery disease. Of the 895 samples, 1.2% (11) fell outside this control range, resulting in the subsequent repeat of the batch of clinical samples associated with the failed control sample (Fig. 4).

Discussion

Multi-analyte laboratory developed tests (LDTs) are often complex in nature, containing multiple sample processing steps, operators, machines and types of reagents. As such, it is important to understand both sources and amounts of process variability, as high variability can lead to erroneous test results and potentially incorrect clinical actions. Corus CAD is a multi-gene test based on the expression levels of 23 genes in whole blood and provides an estimate of the likelihood of obstructive coronary disease in non-diabetic patients. The 23 genes were selected from a larger set of genes, identified through microarray and qRT-PCR-based studies using multiple, independent cohorts [12,13]. The genes are grouped into highly correlated meta-genes in order to minimize the impact of single gene variability [14,15]. The meta-genes represent biological processes (e.g. apoptosis, cell death, innate and active immune responses) that are known to be associated with CAD, as well as different cell types also associated with CAD (e.g. lymphocytes, neutrophils, monocytes) [12]. The test is run in a centralized, clinical laboratory and consists of multiple processing steps, by a number of operators, and with multiple reagents. Over a two year period, control samples were used to analyze intra- and inter-batch variability in the laboratory process.

Intra-batch test variability was estimated using data from five batches consisting of identical whole blood control samples (N = 132); yielding a standard deviation of 0.092 Cp units representing approximately 70% of the overall variance (Fig 2A, Table 1). Analysis of gene-specific variability allowed us to investigate individual contributions of each gene assay. Two
**Table 2.** Gene Expression Score Component Variability.

| Gene/Component | Mean Cp   | SD        | % Variability | Algorithm Weight |
|----------------|-----------|-----------|---------------|------------------|
| TSPAN16        | 33.23783329 | 0.114473  | <1%           | 0                |
| TMC8           | 27.0491705  | 0.623204  | 1.39%         | 0.069            |
| CD3D           | 28.49723576 | 0.066184  | 1.47%         | 0.069            |
| SPIB           | 31.67462259 | 0.106285  | 1.56%         | 0.04521          |
| RPL28          | 23.23034338 | 0.046649  | 2.33%         | 0.154            |
| HNRNPF         | 28.04762546 | 0.058873  | 2.35%         | 0.123            |
| ST00A8         | 22.36803951 | 0.052756  | 2.41%         | 0.14124          |
| CD79B          | 29.10833979 | 0.083717  | 2.49%         | 0.09179          |
| AQP9           | 25.77568108 | 0.06066   | 2.69%         | 0.137            |
| NCF4           | 26.35784643 | 0.063076  | 2.80%         | 0.137            |
| ST00A12        | 25.22470751 | 0.061538  | 2.82%         | 0.14124          |
| CLEC4E         | 27.10114833 | 0.061894  | 2.83%         | 0.123            |
| TLR4           | 27.6331357  | 0.051561  | 3.16%         | 0.189            |
| CXCR2          | 24.57673698 | 0.057652  | 3.53%         | 0.189            |
| TFCP2          | 30.10461938 | 0.01976   | 4.06%         | 0.123            |
| TNFRSF10C      | 24.88700233 | 0.07295   | 4.47%         | 0.189            |
| IL18RAP        | 27.81802168 | 0.057736  | 4.67%         | 0.24948          |
| SLAMF7         | 29.44608773 | 0.074932  | 4.93%         | 0.203            |
| KCNQ3          | 28.97177707 | 0.085435  | 5.23%         | 0.189            |
| KLRC4          | 29.5726601  | 0.082712  | 5.44%         | 0.203            |
| TNFAIP6        | 30.22744093 | 0.07769   | 6.28%         | 0.24948          |
| CASP5          | 31.80418539 | 0.123132  | 9.95%         | 0.24948          |
| AF289562       | 33.62904299 | 0.290804  | 23.17%        | 0.246            |

1 Despite high having a high SD, TSPAN16 contributes little to variability in the GES due to its low weight in the control sample used; in clinical samples TSPAN16 is weighted according to sex [8].

**Table 3.** Variability within Individual Laboratory Processes.

| Method and Variable | n   | p value | % Variance |
|---------------------|-----|---------|------------|
| RNA Extraction      | 4   | 0.843   | 0.10%      |
| RNA Extraction Kit Lot | 26 | <2 x 10^-16  | 18%        |
| RNA Operator        | 6   | 0.517   | 0.40%      |
| cDNA Synthesis      | 4   | 0.074   | 1.6%       |
| cDNA Kit Lot        | 6   | 8.5 x 10^-11 | 6.9%   |
| cDNA Operator       | 5   | 0.012   | 0.8%       |
| Sample Addition to Assay Plate | 5 | 0.149 | 0.8% |
| Sample Addition Operator | 8 | 0.358 | 0.9% |
| qRT-PCR              | 6   | 0.016   | 1.5%       |

1 p values in bold are <0.05.

**Table 4.** Components of Inter-Batch Variability Observed in the Study.

| Variable                  | p value |
|---------------------------|---------|
| RNA Extraction Machine    | 0.935   |
| RNA Extraction Kit Lot    | 0.327   |
| RNA Operator              | 0.480   |
| cDNA Synthesis Machine    | 0.435   |
| cDNA Kit Lot              | 0.009   |
| cDNA Operator             | 0.057   |
| Sample Addition Machine   | 0.214   |
| Sample Addition Operator  | 0.866   |
| qRT-PCR Machine           | 0.218   |
| qRT-PCR Assay Plate Lot   | 0.045   |
| qRT-PCR Operator          | 0.061   |

1 p values in bold are <0.05.
the lab over the same period (Table 1) [18]. Of the 895 whole blood control samples run during the study, only 11 fell outside quality control guard-bands requiring re-processing of the associated batch (Fig. 4). The overall variability of 0.11 Cp units equates to 0.97 points on the 1–40 GES scale, corresponding to a 1.7% change in the likelihood of obstructive coronary artery, an amount unlikely to alter clinical decision making (Table 1). These results demonstrate that with careful design and monitoring, complex gene-expression based LDTs such as this one can be successfully implemented in a clinical laboratory setting.

Materials and Methods

Ethics Statement

The whole blood used for the positive control in the study was collected from donors who had granted written consent. Ethics approval for this study was obtained from the Western Institutional Review Board (Protocol #20090362). Assessment of the gene expression scores from commercial samples was done in an anonymous fashion; the scores were analyzed without access to patient identifying information. No individual patient scores are reported, only the overall variance in the population.

Whole Blood Collection

Whole blood samples were collected in PAXgene® RNA Blood Tubes according to the manufacturer’s instructions (PreAnalytix). Commercial samples were shipped to the clinical laboratory at 4–8°C in temperature-controlled NanoCool containers (Nanopore).

RNA Preparation and Quantification

RNA was isolated by means of a magnetic bead based approach using the Agencourt RNAAdvance Blood kit (Beckman Coulter Genomics) and the Hamilton STAR automated liquid handler (Hamilton). Extraction was performed in 96-well plates containing 400 µl of whole blood per well. Genomic DNA removal was performed using the Ambion DNase I kit (Ambion) at 37°C for 25 min. Purified RNA was quantified by absorbance at 260 nm using the NanoDrop 8000 (Thermoscientific). After RNA extraction, the concentration and purity of the RNA were assessed by measuring A260/A280 ratio; to pass samples needed a RNA concentration between 15 and 50 ng/ul and a A260/A280 ratio between 1.5 and 2.5. Samples were re-extracted if they initially failed either metric. Upon a second failure for any single metric, samples were considered a no test and not reported.

Reverse Transcription

RNA was reverse-transcribed to cDNA using the High Capacity Reverse Transcription Kit (Life Technologies); normalization of RNA mass and reverse transcription set-up were performed on the Hamilton STAR liquid handling system. cDNA samples were diluted to a RNA equivalent of 1 ng/ul for downstream processing.

qRT-PCR Assays

The qRT-PCR assays used in the test incorporate DNA primers and Minor Grove Binding (MGB)-containing TaqMan probes (Life Technologies); assay design is described elsewhere [8,13].

Figure 4. Mean Deviation of GES from Target Score Across the Course of the Study. Solid black line = running mean deviation of GES across the 895 samples (x axis, chronological order samples were run; y axis, GES). Middle dashed line = target GES; upper and lower dashed lines = QC boundaries ±3 points target GES. 95% CI = grey area. doi:10.1371/journal.pone.0040068.g004
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qRT-PCR Assay 384 Well Plate Manufacturing

Each of the 23 genes in the test was assayed in triplicate for each sample. qRT-PCR reactions were performed in 10 μL volumes consisting of 8 μL of PCR reagents and 2 μL of cDNA sample (1 ng/μL). PCR reagents included Universal Master Mix (Life Technologies) TaqMan assay reagents, and DEPC-treated water; after sample addition the final reaction contained 900 nM each of unlabeled primers and 250 nM of probe. Plates were manufactured using liquid handling robots (Beckman Coulter). Plates were stored at −20°C until the time of sample addition. Plates were qualified if they met pre-defined qualification metrics (Methods S1, Table S1).

qRT-PCR

All qRT-PCR reactions were run using the Light Cycler 480 II (Roche). Cycling conditions for each plate included a 50°C incubation for 2 minutes followed by a 95°C incubation for 10 minutes. Each plate was processed through 45 cycles of 95°C for 15 seconds with a ramp time of 2°C/s and 60°C for 1 minute with a ramp time of 1°C/s. Fluorescence excitation was at 465 nm and fluorescence emission was monitored at 510 nm after each cycle for probe detection. Individual Cq values were calculated using software provided with LC480 II (Roche).

Internal PAX Pool Blood Control

Approximately 2.5 mL of blood was collected in PAXgene® RNA Blood Vacutainer Tubes from consented donors (Western IRB Protocol #20090458). The blood/PAXgene reagent mixture from donor tubes were pooled together. Large volumes (>5 liters) were pooled per lot, requiring proper mixing to ensure solution homogeneity. After pooling and mixing, each pool was distributed into approximately 2000 1.5 ml aliquots, incubated at room temperature for a minimum of 120 min, and stored frozen at −80°C. To ascertain pool homogeneity, fifteen aliquots per lot (spanning the sequence of aliquots prepared) were assayed and the whole blood control score target was computed; batch control samples needed to be with +/+ 3 points of this score in order for the batch of associated clinical samples to pass quality control. Pools were qualified if they met the pre-defined qualification metrics (Methods S1).

Statistical Methods

All analysis was performed using R, version 2.13. Standard methods were used to estimate means, SD's, and correlations. Gene expression pre-processing and score calculations were performed as previously described (8). Analysis of variance (ANOVA) models were used to estimate the proportion of variation attributable to each factor.

Supporting Information

Table S1 RT-qPCR Plate Stability Metrics.

Methods S1

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Author Contributions

Conceived and designed the experiments: MRE, JAW SR PB AMJ SED. Performed the experiments: RN MHD MD. Analyzed the data: MRE PB AMJ JAW. Wrote the paper: JAW MRE SR.

References

1. Goodison S, Sun Y, Urquidi V (2010) Derivation of cancer diagnostic and prognostic signatures from gene expression data. Bioan. 10: 2193-2197.
2. Lampe JW, Stepaniants SB, Mao M, Radich JP, Dai H, et al. (2008) Signatures of environmental exposures using peripheral leukocyte gene expression: tobacco smoke. Cancer Epidemiol Biomarkers Prev 17: 443-453.
3. Llaverias G, Pou J, Ros E, Zamzon D, Cofán M, et al. (2008) Monocyte gene-expression profile in men with familial combined hyperlipidemia and its modification by atorvastatin treatment. Pharmacogenomics 9: 1035-1045.
4. Nakamura S, Kobayashi M, Sugino T, Kajimoto O, Matoba R, et al. (2010) Effect of exercise on gene expression profile in unfractionated peripheral blood leukocytes. Biochem Biophys Res Commun 391: 846-851.
5. Barchler EC, Batiwalla FM, Karypis G, Gaffney PM, Ortmann WA, et al. (2003) Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 100: 2610-2615.
6. Bennett L, Palacky AK, Arce E, Cantrell V, Borvak J, et al. (2005) Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 197: 711-725.
7. Deng MC, Eisen HJ, Melra MR, Billingham M, Marboe CC, et al. (2006) Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. Am J Transplant 6: 150-160.
8. Rosenberg S, Elashoff MR, Beincke P, Daniels SE, Wingrove JA, et al. (2010) Multicenter validation of the diagnostic accuracy of a blood-based gene expression test for assessing obstructive coronary artery disease in non-diabetic patients. Ann Intern Med 153: 425-434.
9. Dumeaux V, Olsen KS, Niel G, Paulsen RH, Borresen-Dale AL, et al. (2010) Deciphering normal blood gene expression variation: The NOWAC post-genome study. PLoS Genet 6: e1000873.
10. McPhail S, Goralski TJ (2005) Overcoming challenges of using blood samples with gene expression microarrays to advance patient stratification in clinical trials. Drug Discov Today 10: 1485-1487.
11. Barchler EC, Batiwalla FM, Karypis G, Gaffney PM, Moser K, et al. (2004) Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation. Genes Immun 5: 347-353.
12. Elashoff MR, Wingrove JA, Beincke P, Daniels SE, Tingley WG, et al. (2011) Development of a blood-based gene expression algorithm for assessment of obstructive coronary artery disease in non-diabetic patients. BMC Med Genomics 4: 26.
13. Wingrove JA, Daniels SE, Sehnert AJ, Tingley W, Elashoff MR, et al. (2008) Correlation of peripheral-blood gene expression with the extent of coronary artery stenosis. Circ Cardiovasc Genet 1: 31-36.
14. Brunet JP, Tamayo P, Golub TR, Mesirov JP (2004) Metagenes and molecular pattern discovery using matrix factorization. Proc Natl Acad Sci U S A 101: 4146-4149.
15. Park MY, Hastie T, Tibshirani R (2007) Averaged gene expressions for regression. Biostatistics 8: 212-227.
16. Karrer EE, Lincoln JE, Hegenhout S, Bennett AB, Bostock RM, et al. (1995) In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. Proc Natl Acad Sci U S A 92: 3814-3818.
17. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29: 23-39.
18. Cronin M, Sanghi C, Liu ML, Pho M, Dutta D, et al. (2007) Analytical validation of the Oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response prediction in node-negative, estrogen receptor-positive breast cancer. Clin Chem 53: 1084-1091.