Author's response to reviews

Title: A Novel Multiplex Polymerase Chain Reaction Assay for Profile Analyses of Gene Expression in Peripheral Blood

Authors:

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Version: 4 Date: 10 June 2012

Author's response to reviews: see over
Dear Professor Marinette Lacson,

We are indebted to you and the reviewers for the insightful and helpful comments that you provided for our manuscript entitled “A Novel Multiplex Polymerase Chain Reaction Assay for Profile Analyses of Gene Expression in Peripheral Blood” (manuscript 1876195980645702). Those comments enabled us to improve our article and helped to guide our research. We studied the comments carefully and revised the manuscript. The altered content is shown in blue font in the revised manuscript.

We calculated the positive and negative predictive value and added paragraphs in the discussion section on the perspectives for clinical application (see discussion section and additional file5. Table5). The manuscript has been edited by a very experienced medical editor working for International Science Editing whose first language is English.

A point-by-point response to the reviewers’ questions is provided below. We sincerely hope that the revised manuscript will now be suitable for publication in your excellent and highly respected journal. We look forward to hearing from you at your earliest convenience.

Yours sincerely

Yaping Tian
(E-mail: tianyp61@gmail.com)

Responses to reviewers’ comments

Reviewer #1: James Wingrove

**Question**

While the technical aspects of assay development and performance measurement in
the manuscript appear to be fairly solid and I appreciate the improvements the authors have made in the manuscript (e.g. addition of the replication set), I feel the clinical piece is less well developed and in some cases incorrect (e.g. the statement “Atherosclerosis is not considered important until a myocardial ischemia occurs”, first paragraph in Background, page 3, should be removed).

Response: The sentence “Atherosclerosis is not considered important until myocardial ischemia occurs” has been removed in the revised manuscript.

**Question**

The authors have now included an ROC analysis (Table 4) and conclude that the 4 gene model is a better classifier than any of the single genes alone. While the AUC is higher for the 4 gene model, in order to substantiate this claim the authors need to demonstrate this increase is significant (as well as provide 95% confidence intervals). What threshold was used to determine sensitivity and specificity? Was the threshold prospectively defined? What was the performance of the model in the replication set of subjects?

Response: A comparison of the diagnostic effects of single genes and the four markers is shown in the table below. The AUC value of single genes and the four markers was significantly increased (compared with area = 0.5). Although the AUC value of the four markers was higher than any other single gene, this increase was not significant. The reason may be the small study cohort. The threshold was defined as the maximum value of the Youden index (sensitivity + specificity – 1). The threshold values for different genes and the performance of the model are also shown in the table below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Threshold</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC (95% CI)</th>
<th>+PV</th>
<th>–PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>1.659</td>
<td>85.5</td>
<td>50.0</td>
<td>0.639 (0.539–0.730)</td>
<td>76.6</td>
<td>64.3</td>
</tr>
<tr>
<td>IL6</td>
<td>3.958</td>
<td>43.5</td>
<td>91.7</td>
<td>0.623 (0.523–0.716)</td>
<td>90.9</td>
<td>45.8</td>
</tr>
<tr>
<td>IL8</td>
<td>3.330</td>
<td>47.8</td>
<td>80.6</td>
<td>0.642 (0.542–0.733)</td>
<td>82.5</td>
<td>44.6</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.412</td>
<td>49.3</td>
<td>80.6</td>
<td>0.624 (0.524–0.717)</td>
<td>82.9</td>
<td>45.3</td>
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<tr>
<td>IL1B + IL6 + IL8 + MCP-1</td>
<td>0.485</td>
<td>50.7</td>
<td>77.8</td>
<td>0.669 (0.570–0.757)</td>
<td>81.4</td>
<td>45.2</td>
</tr>
<tr>
<td>Validation (IL1B + IL6 + IL8 + MCP-1)</td>
<td>0.403</td>
<td>66.67</td>
<td>73.33</td>
<td>0.831 (0.650–0.942)</td>
<td>73.5</td>
<td>68.7</td>
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AUC, area under the curve
+PV, positive predictive values
-PV, negative predictive values

**Question**

The AUC for the 4 gene model is modest at best, with poor sensitivity leading one to wonder about the clinical utility of the model; does it perform better than clinical factors in identifying patients with non-calcified plaque in this population. How does it compare to published diagnostic models using clinical factors (e.g. Diamond – Forrester)? How would such a model be used clinically? The higher specificity suggests as a rule-in test; what is the clinical action associated with the result?

**Response:** The AUC for the four gene model is modest at best, with poor sensitivity and high specificity. These results are meaningful for the assessment of CAD in laboratory-based studies. Most subjects with symptomatic CAD agree to undergo conventional angiography, but most of the subjects without symptomatic CAD accept conventional angiography reluctantly. Our model can be used in the assessment of subjects with asymptomatic CAD. If someone is initially diagnosed as having CAD, then imaging is necessary as the next step for the four-gene model.

The Diamond–Forrester method uses age, sex and symptoms to provide a quantitative assignment of the probability of coronary disease. CAD is distinguished by narrowing of the luminal diameters of vessels. However, many acute coronary syndromes are caused by plaque disruption and thrombosis rather than stenosis severity. Traditional clinical factors are CAD risk factors, and have not been used to distinguish the characteristics and compositions of plaques. Hence, we set up a non-invasive detection method using four cytokines. This technology might hold
promise in the identification of individuals with non-calcified plaques even though it has limitations.

**Question**

Unless the authors can substantially improve the manuscript in this area, I feel this portion should be removed, and the primary emphasis focused on the technical aspects of assay development and performance.

Response: We have simplified the technical aspects of the development and performance of the assay. Figures 1B–E of single RT-PCR capillary gel electrophoresis of the selected genes have been moved to the attached files. The figure showing the results of multiplex primer RT-PCR capillary gel electrophoresis before optimization (A) has also been moved to the attached files.

**Question**

Additionally, despite improvements, the manuscript still needs editing for grammar.

Response: We have revised the manuscript and paid special attention to the grammar.

**Question**

Quality of written English: Needs some language corrections before being published

Response: The manuscript has been edited by a very experienced medical editor working for International Science Editing whose first language is English.

**Reviewer #2: Tomasz Dziedzic**

**Question**

Unfortunately I still doubt if this protocol could be useful in clinical practice for
identifying subjects with asymptomatic coronary artery disease. In my opinion it is meaningless to measure 15 cytokines whereas only 4 of them differ between studied groups. The authors should justify why they think that test measuring 15 cytokines will be better than one measuring 4 cytokines.

Response: Studies have indicated that all 15 selected CAD-related genes might have an important role in the pathogenesis of atherosclerosis. However, there were few reports about the relationship between these genes. Therefore, we developed a new multiplex PCR assay to measure them together in one reaction system. We then compared which genes had the more important roles in relation to CAD. The results showed that only 4 from 15 analyzed genes were significantly different. In a subsequent study, we needed to measure only a four-cytokine panel to reveal clinically significant results instead of measuring 15 cytokines together.

**Question**

The authors should calculate positive and negative predictive values of the test using the cohort which already has been studied and confirm the results in independent sample. Without this kind of information, the only advantage of the manuscript is technical protocol to measure simultaneously the expression of 15 cytokines in blood. Such a protocol is publishable and suitable for journal focused on molecular methods, but without data about predictive values, the manuscript seems to be not feasible for journal dedicated to clinicians.

Response: The positive and negative predictive values of the test and the results in independent samples are shown in the table below. The AUC of our model for the four-gene model was modest at best, with poor sensitivity and high specificity. These results are meaningful for the assessment of CAD in laboratory-based studies. Subjects with symptomatic CAD agree to undergo conventional angiography, but most individuals without symptomatic CAD accept conventional angiography reluctantly. Our model can be used in the assessment of subjects with asymptomatic
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