April 2, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7840-edited.doc).

Title: Methodical and pre-analytical characteristics of a Multiplex Cancer Biomarker Immunoassay
Author: Natalie Hermann, Katja Dreßen, Frank A. Schildberg, Christopher Jakobs, Stefan Holdenrieder
Name of Journal: *World Journal of Methodology*

ESPS Manuscript NO: 7840

The manuscript has been improved according to the suggestions of reviewers:
1 Language has been improved
2 Tables showing the extended imprecision have been included into the main paper
3 Revision has been made according to the suggestions of the reviewer as follows:

I. In this manuscript you tested the methodical and pre-analytical performance of a new multiplex cancer biomarker Magnetic Bead Panel Kit. This kit includes reagents for the detection of 24 biomarkers. You showed that the Human Cancer Biomarker Magnetic Bead Panel 1 assay is a stable and precise method for detection of most into the kit included biomarkers although single markers have to be interpreted with care. The experiments are carefully done and the interpretations are likely correct. Therefore, I believe that this manuscript is worth publication in World Journal of Methodology as it is.
   - *Thank you very much for these encouraging comments.*

II. The manuscript "Methodical and pre-analytical characteristics of a Multiplex Cancer Biomarker Immunoassay" approaches an issue of major importance, yet it needs to be improved for publication. The article is well written and we appreciate the good quality of their work, however further studies are needed to show the clinical applicability of the analysed kit, as the authors themselves have mentioned. Regarding the article content, we suggest the authors to give more details about the two serum pools (pool 1 and pool 2) used for the physiological external control.
   - *To create pool 1 37 residual and anonymized sera of the daily clinical routine diagnostics were mixed. The election criteria included present high levels of the inflammation parameter CRP and furthermore levels of the biomarkers AFP, β-HCG, CA 15-3, CA 125, CA 19-9, CEA, NSE and PSA well above average. Here patient history was not considered. Pool 2 is a mixture of two sera taken from young healthy women (mean age 23.5 years).*

Validation for clinical diagnostic use (IVD) requires a large cohort of patients and controls for specificity and sensitivity evaluation of each analyte (false positive, false negative results, etc).

- *For implementation into clinical diagnostics further studies evaluating its performance on a large cohort of patients with cancer disease and appropriate control groups which are relevant for differential diagnosis, i.e. healthy individuals and patients with organ related benign disease, are definitely required. Currently we are performing such clinical validation studies with cohorts of patients suffering from gastrointestinal, gynecological and urological cancers, respectively.*

The selected biomarkers panel is too large to be suitable in clinical routine for one single patient, in order to have a cost-effective analysis.

- *The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 purchased by Millipore offers a wide spectrum of applicable biomarkers, however, it is obviously not clinically relevant neither cost-effective to apply the complete panel in diagnostics. Nevertheless, most markers show a quite good performance thus the panel can be focused adopted on the biomarker pattern of clinical interest depending on the contemplated entity of disease.*
- *For the purpose of this study, we took the panel as it is offered from the manufacturer and tested it for its methodical quality. This step is only rarely done from an independent site but is crucial for the next step of clinical evaluations. Here, this procedure revealed the methodical shortcomings of some biomarkers and the necessity to include external pools on the basis of the same matrix as the clinical samples.*

We are also suggesting a few minor revisions in terms of spelling check and phrase topics. Considering these suggestions, we recommend the publication of the article after minor revisions are made.

- *The manuscript was completely revised by a native speaker.*

III. The manuscript is very well presented and highlights factors influencing the measurements of the key performance indicators of the multiplex cancer biomarker panel, which constitutes a highly interesting study. The findings from the comparison of the critical measurement parameters between physiological sera in parallel with synthetic internal controls will be of particular interest to a wide audience. The technical details are clearly defined and the interpretations are thorough with robust scientific conclusions. I recommend the publication of this manuscript. The only very minor editing detail I have spotted was a missing full stop at the end of the fourth paragraph of page 9 of the manuscript: "Observed concentration-based CVs ranged from 1.68% (MIF) to 36.09% (b-HCG) with 12 biomarkers measured with a CV below 5% and 4biomarkers exceeding the 10% mark (table 1)." Evidently, there has been thorough attention to detail during the preparation of this manuscript for review, which was a pleasure to read.

- *Thank you very much.*

IV. The manuscript treats a problem of major importance and has general merits; however it has to be improved for publication.

The language needs serious improvements.

- *The manuscript was completely revised by a native speaker.*

The description of work and selection of data has some flows.

The selected panel: it is true that Merck-Millipore offers this panel with 24 markers (with the possibility to select between total PSA and free PSA). However, it is very unlikely to have all these markers at the same time. It's understood that the purpose was a general evaluation of the components, which is a bit different than a real validation of a clearly focused biomarker panel.

- *Yes, this is correct. The purpose of this study was the evaluation of the methodical quality of the biomarker panel as it is offered from the manufacturer. Here, this procedure revealed the methodical shortcomings of some biomarkers and the necessity to include external pools on the basis of the same matrix as the clinical samples. Of course, the next step will be a clinical evaluation to identify the most sensitive and specific marker patterns for cancer detection.*

The samples were run on a pool of sera; it is not clear how many sera were pooled? Also, it seems that the pool involved sera from different pathologies, so as to create a mixture of markers.

- *This is now described in detail as also commented under point II*

The selection of data to calculate the CV and recovery based on ST7 is atypical, since usually this concentration is most often outside the linearity range. I suggest using another concentration (S6, S5) for this purpose.

- *Here, we determined the CVs and recoveries for ST7, as this is the least manipulated sample of all standards. In most of the calibration curves, this point was still in the linearity range; furthermore it represents an important point of the FI/concentration curve, which is the basis for the final calculation of marker concentration. As we had calculated CVs and recoveries for the whole dilution series ST7-ST1, we included now the results for ST5 instead of ST7 to meet your point.*

Some pre-analytical conditions were thoroughly investigated, establishing the optimal conditions for best results for most analytes.

Some variants would be useful in the assay, for instance, setting higher the number of events; 50 is low and can be a source of the problems.

- *Within the Bio-Plex® 200 system it is possible to vary the minimal number of events needed for measurement. With 50 events as minimum per bead (which was the recommended number of the manufacturer) we chose a compromise between a time-effective measurements and sufficiently precise results, though. It is obvious, that the accuracy of measurements increases with higher number of events. Nevertheless, our findings could achieve acceptable CV values in most cases despite the low minimum number of events set. Therefore we did not compare absolute number of detected events and their calculated CVs.*

Examination of the pre-analytical conditions was made thoroughly; thus, contribution in the analytical process reproducibility and a maximization of the reliability for most samples was achieved.

However, it looks like the only parameter to estimate intra- and inter-assay imprecision was Mean FI, while other elements (for instance, number of events) were not considered.

- *Yes, we kept the minimum number of events constant and tested the imprecisions using FI and concentrations.*

I don't understand how the recovery% was calculated for VEGF and sFASL; the data presented report concentrations below the limits of detection for both, in all experiments.

- *Indeed the concentration was measured below the limit; nevertheless, FI levels were measured for all markers except VEGF and could be evaluated. The extrapolation limits for the calculated concentration are predefined by the formula of the curve, which does not exclude the possibility to assess the measurement accuracy even below the limits. Such FI values are not translated into concentration by the system.*
- *The 50% dilutions of pool 1 samples were run in each of the 5 plates. The dilution of 20 markers fell into the accepted recovery of 70% – 130%. Range of all markers was between 53.19% (β-HCG) and 136.24% (FGF2) (see figure 1). Here, concentration levels calculated by extrapolation were included. VEGF was the only biomarker without any calculable levels of concentration.*

The test for plasma run comparatively with serum – one case? The effective concentrations in serum and plasma would be of more use.

- *Finally, biomarkers were tested in serum and EDTA-plasma samples that were taken in parallel from these (mentioned before) two donors.*
- *We consider recoveries to be clearer in representation of an overall result than absolute concentrations, which are less comfortable for the reader.*

4 References and typesetting were corrected

5 Comments have been included

6 Core tip has been included

7 Abbreviations were complemented by full words when mentioned the first time

We would appreciate very much if the revised version of our manuscript is now accepted for publication in the *World Journal of Methodology*.

Yours sincerely,

Stefan Holdenrieder (MD)