Author’s response to reviews

Title: Impact of sample acquisition and linear amplification on gene expression profiling of lung adenocarcinoma: laser capture micro-dissection cell-sampling versus bulk tissue-sampling

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Version: 3 Date: 31 December 2008

Author’s response to reviews: see over
Editorial Board  
c/o Joseph Dunckley  

MS: 7127581512017390

Please find below our response to the three reviewers’ comments.

We thank BMC and the reviewers’ for the time spent evaluating this manuscript and look forward to your timely response.

Cordially,

Eric W Klee

**Reviewer’s report**

**Title:** Impact of Sample Acquisition and Linear Amplification on Gene Expression Profiling of Lung Adenocarcinoma: Laser Capture Micro-dissection Cell-Sampling versus Bulk Tissue-Sampling  

**Version:** 1 **Date:** 24 June 2008  

**Reviewer:** Michael Emmert-Buck

**Reviewer’s report:**

Discretionary Revisions:

1) The authors address important issues in the manuscript. There are a variety of strategies available to investigators studying tissue specimens, each with its own strength and weakness. A more clear definition of the pros and cons of these approaches based on rigorous analysis is critical and will inform investigators as to which tools are most appropriate for a given study, as well as the associated pitfalls.

We agree with the reviewer that each strategy for studying tissue specimens has its own inherent strengths and weaknesses. We feel this manuscript highlights some of the observed experimental differences obtained when using different cell sampling methods for microarray analysis. However, to better introduce the subject to the readers, we have followed the reviewer’s recommendation and added the following two sentences within the Background to more clearly define the expected pro’s and con’s associated with LCM and bulk tissue sampling methods:

“LCM enables the precise extraction of target cells from a tissue sample consisting of a heterogeneous mixture of cell types. Bulk tissue sampling is faster and less expensive than LCM, however, sampled tissues often consist of a mixture of target and contaminating cell types.”

2) Amplification Bias:

When one considers gene expression analysis methodologies for both bulk and microdissected tissues, there are many areas of potential bias; tissue handling, tissue processing procedures, freezing and thawing, RNA purification, reverse transcription (RT), probe selection, and amplification. All labeled cDNA populations, amplified or not, are 'biased' to some degree and in fact may be
most biased by the RT step. However, the key point is if the bias is reproducible, and how it influences the ability to reliably detect differences in study populations. The manuscript would be improved if the authors commented on this point and discussed if they examined the reproducibility of their measurements with and without amplification.

We have included results in the Supplementary materials that address the question of reproducibility in the linear amplified and bulk microarrays. The following was added to the first paragraph of the Discussion: “An evaluation of replicate microarrays showed the inherent variability of the LCM amplified microarrays to be marginally higher than found for bulk tissue Affymetrix microarrays (Supplementary Materials). “

And added to the Supplementary Materials:
“Replicate analysis of the linear amplified microarrays was performed and compared to the correlation values computed from existing technical replicate analysis in bulk tissue specimens. Five samples were taken from a starting RNA extract, processed by linear amplification, and analyzed on microarray chips. We observed an average pair-wise correlation between the five replicate samples of 0.89 (SD 0.019). To compute pair-wise correlation in bulk tissue samples, raw cel-files were downloaded for five sample-A, technical replicates from the MAQC project [2]. These arrays were processed in the same manner as the LCM microarrays yielding an average pair-wise correlation of 0.92 (SD 0.003). It is important to note that the LCM samples included independent linear amplification steps, while the bulk samples were true technical replicates. This explains the slightly reduced pair-wise correlation for the LCM samples. “

3) The data and discussion of the unique tumor down regulations in the LCM samples was of interest and presented well in the manuscript.

We appreciate the reviewer’s positive comments

4) The authors should be more explicit in terms of sample size for each experiment, as well as how each sample was selected, i.e., randomly or based on similarities in morphology. (This was done in some cases, but not all.)

Sample size and selection criteria for the experiments were clarified in the Methods and Materials section, with the following changes made (in red):

Fresh frozen tissue samples were obtained from the Mayo lung tissue bank for ten patients with stage IIIA or IIIB adenocarcinoma of the lung and with surgical tumor resection. Normal lung tissue adjacent to tumor in six patients were also obtained from the Mayo lung tissue bank. All samples were fast frozen within 30 minutes after resection and stored in -80 °C. All cases were first reviewed by a pathologist (MCA) for their diagnosis accuracy and adequacy for a microarray study. Normal and tumor samples were selected if there was sufficient material to microarray profile for both laser capture microdissected samples and bulk tissue samples.

5) In the Results Section, under the subheading 'Probe Set Distance to 3' End of Transcript …', the authors should replace the apostrophe, when denoting the 3’ end of the transcript, with (’). This should be done in other sections when the 3’ end is discussed/used.

All improper uses of the apostrophe have been replaced by the correct symbol.

6) In the Discussion Section, paragraph 1, line 9, the sentence should read
and may reflect differences

The correction has been made.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I am one of the original inventors of laser capture microdissection and can receive royalty payments through the NIH technology transfer program.
Reviewer’s report  
Title: Impact of Sample Acquisition and Linear Amplification on Gene Expression Profiling of Lung Adenocarcinoma: Laser Capture Micro-dissection Cell-Sampling versus Bulk Tissue-Sampling  
Version: 1 Date: 14 July 2008  
Reviewer: Tucker Patterson  
Reviewer’s report:  
Major Compulsory Revisions  
The authors present a very comprehensive gene expression study comparing cancer cells versus benign cells collected from the same tissue sample using laser capture microdissection (LCM). Bulk tissue samples were also similarly compared (cancer vs. benign) as well as the effects of linear amplification on gene expression. However, one of the difficulties in interpreting the data presented results from the large amount of information being presented. The authors do present evidence that the differences in gene expression found between benign and cancer cells using LCM are much greater than the differences due to linear amplification bias. They also demonstrate that by using LCM, more cancer versus benign cell differences can be observed than when using bulk tissue samples.

Unfortunately there is a plethora of information that is formulated into Venn diagrams which make the data difficult to interpret at times. Using either an alternative or additional method (graphic or diagram) to present the data would help to clarify the results.

To provide some additional clarification of the data presented in Figures 2 and 4, we have included two additional sections in the Supplemental materials. These sections present the data from the Venn diagrams in tabular format with an alternative description of the observations made from the Venn diagrams. These supplemental material sections have been referred to within the appropriate portions of the Results.

The manuscript is confusing at times due to the many comparisons being made and the similar terminology used in these comparisons. Some minor nomenclature changes would also assist in helping to present this very interesting data in a better light and make it more understandable to the reader. The supplemental material included helps to a certain extent, but is not completely adequate in clarifying the information being presented.

It was difficult to modify the nomenclature used for presenting the four categories evaluated (bulk vs LCM; normal vs cancer; amplified vs non-amplified, upregulated vs downregulated), however, several sentences were reworked to improve the clarity of the results being discussed, as described below:

Results, paragraph 1:  
An average of 749 probesets in normal tissue and 684 probesets in cancer tissue were expressed at significantly different levels in the bulk and LCM datasets. -> When comparing the bulk data to the LCM data, significant changes in expression levels were found for an average of 749 probesets across all six normal tissues samples and 684 probesets across all ten cancer tissues samples.
There were 114 probesets in the normal tissue and 153 probesets in the cancer tissue with consistently altered expression in all samples. Many consistent alterations were identified, with 114 / 749 probesets altered in all six normal tissue samples, and 153 / 684 probesets altered in all ten of the cancer tissue samples.

Results paragraph 2:
Comparing the average expression values in all bulk and all LCM samples, stratified by cancer or normal status, identified 387 probesets in normal tissue and 500 probesets in cancer tissue with significantly varied expression levels. First, the average probeset expression value across all six bulk normal samples was compared to the average probeset expression value across all six LCM normal samples, and likewise for the cancer samples, identifying 387 probesets in normal tissue and 500 probesets in cancer tissue with significantly varied expression levels.

Using a Bonferroni corrected T-test ($\alpha=0.05$) to compare the microarray groups identified 577 probesets in normal tissue and 3616 probesets in cancer tissue, with significantly divergent expression. Second, a Bonferroni corrected T-test ($\alpha=0.05$) was used to compare the microarray groups identified 577 probesets in normal tissue and 3616 probesets in cancer tissue, with significantly divergent expression.

To estimate whether the second round of RNA amplification induced a bias in the expression data, RNA from two normal-bulk and two cancer-bulk samples were linearly amplified. To estimate whether the second round of RNA amplification, required for LCM sample processing, induced a bias in the expression data, RNA from two normal-bulk and two cancer-bulk tissue samples were linearly amplified.

Comparisons were then repeated using the two-sample average expression levels in these samples, for the bulk, LCM, and linear amplified bulk microarrays, stratified by cancer and normal status. As evident in Figure 2, very few probesets were identified with varied expression levels between the LCM and linear amplified bulk samples. Substantially more divergent probesets were found when comparing the bulk data to the LCM data, and when comparing the bulk data to the linear amplified bulk data. Using the two-sample average probeset expression values from the linear-amplified bulk, bulk, and LCM microarrays, the previous comparisons were repeated. As evident in Figure 2, very few probesets were identified with substantially different expression levels in the LCM samples compared to the linear amplified bulk samples. However, when comparing expression levels in the LCM samples to that in the bulk samples, or when comparing expression levels in the linear-amplified bulk samples with that in the bulk samples, there were substantially more divergent probesets identified.

Results paragraph 6:
There were substantially more probesets commonly identified between the bulk and linear amplified bulk samples, than between either of these sample-sets and the LCM sample-set. There were substantially more probesets commonly identified between the bulk and linear amplified bulk samples, than between the bulk and LCM sample-sets, or between the linear-amplified bulk and LCM sample sets.

Results paragraph 7:
To further evaluate the amplification bias impact on the differential expression analysis, probesets with significantly altered expression levels between the bulk and linear
amplified bulk datasets were compared to probesets uniquely identified, in the LCM or in the bulk datasets (but not both), as differentially expressed. -> To further evaluate the effect amplification bias has on the selection of differentially expressed genes, probesets expressed at significantly different levels in the bulk and linear amplified bulk samples were compared to probesets possessing cancer-to-normal differential expression levels in either the LCM or the bulk samples, but not both.

Methods Section:
The authors indicate 5000 pulses were captured but do not include how many cells were collected. Is the reader to assume one cell per pulse? It is certainly possible to collect several cells with one pulse depending on the LCM settings. This should be specified as well as how many slides were needed to collect the required number of cells.

We have added the following to the Methods section:
Each pulse targeted 3-5 cells. Due to tumor cell pleomorphism, it is difficult to standardize the number of cells captured per pulse. The number of slides used per case varied from 2 to 10, as every case and every tumor block had different sized tumors.

Although 10 ng of RNA was linearly amplified, it cannot be assumed that all samples were similarly amplified. What was the amount of amplified RNA generated and how much RNA was hybridized for each sample?

The amount of amplified material ranged from 84 µg to 134 µg for the LCM samples and 130 µg to 145 µg for the linear amplified bulk samples. A total of 15 µg of cRNA was hybridized to the chips for the LCM samples and 15 µg for the bulk tissue samples. This information has been added to the methods section.

What type of scanner was used?
A GeneChip Scanner 3000 7G was used. This has been added to the Methods and Materials section.

Minor Essential Revisions
The manuscript is well-written except for some minor grammatical issues that are outlined below and will greatly improve the readability of the paper if incorporated.
1. when the references are listed within the text there are several instances of a failure to space preceding the reference
   This has been corrected.
2. many sentences in the text begin with numerals; the numbers should either be spelled out or the sentences rearranged so they do not begin with numerals
   All instances of sentences starting with numerals have been corrected.
3. LCM sampled should be hyphenated (LCM-sampled) as well as LCM-based
   These have been corrected.
4. first line of page 6 should read "….distance from the gene 3’ end….." – insert “the” Done.
5. last sentence of page 7; tight associated should read “tight association”
   Corrected.
6. first paragraph of Discussion, next to last sentence should read “differences in cell population”
   Corrected
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests.
Reviewer’s report
Title: Impact of Sample Acquisition and Linear Amplification on Gene Expression Profiling of Lung Adenocarcinoma: Laser Capture Micro-dissection Cell-Sampling versus Bulk Tissue-Sampling
Version: 1 Date: 18 November 2008
Reviewer: Yuanyuan Xiao
Reviewer’s report:
I have the following queries for the authors:
Major Compulsory Revisions:

1) I would like to see a plot of log-intensities vs distances from probeset to 3' end for LCM, bulk and linear amplified bulk data, and this will help prove that long distance to 3' end results in lower hybridization intensities.

The requested graphs have been provided below for all probesets with a clearly identifiable 3' end, based on the criteria described in the manuscript. Independent plots were generated for bulk, linear amplified bulk, and LCM normal samples (cancer sample graphs can be provided, but there was no significant difference in the plot distributions).

We had previously evaluated these plots for inclusion in the manuscript, but chose not to include them. However, given the reviewer’s interest in the figure, we have added to the supplemental materials to provide an additional perspective on this data.

The reviewer’s request has aptly led to the conclusion that 3' distance is not necessarily the only factor that might be causing the linear amplification bias in the expression. Therefore, we have modified the text in the discussion section to clearly state that the 3' distance may at least “partially” explain the variation in expression.
2) It will be helpful to produce box-plots of distances from probeset to 3' end for differentially expressed genes (between diseased and normal tissues) in LCM, linear amplified bulk and bulk data. Separate up- and down-regulated genes. This will further verify whether this differential expression is reflective of amplification bias or cell population bias.

Below is a copy of the requested box-plots. The graph highlights a trend in the spread of the data points (regarding 3' distance), that diminishes with both the amplification process and cell selection process. It also shows that the down-regulated probesets exhibit a tighter spread of data (regarding 3' distance), and that for up-regulated probesets, there is a slightly larger variation versus 3' distance than observed for down-regulated probesets. We have chosen to also include this box-plot in the supplemental materials section that addresses the effect of 3' distance on expression signals and differential probeset selection.
3. Can the authors please explain the diametrical effect of amplification on genes that are up-regulated in cancer and genes that are down-regulated in cancer? The authors conclude that the former is subject to amplification bias but the latter do not.

This is an astute observation by the reviewer. We note in the discussion that “the largest impact of the amplification bias was observed in the upregulated probesets identified only in the bulk dataset”. We believe this bias is primarily observed in the bulk data and not in the LCM data as we expect the amplified (LCM) signals to be attenuated by the linear amplification process, thereby reducing the likelihood we can detect differential expression. As for the difference in the number of upregulated and down-regulated probesets identified in the bulk samples and reported in Table 1, we feel this may reflect two events. First, if the overlap between the set of probesets with observed amplification bias and the set of up-regulated (or down-regulated) probesets is purely by chance, then there is some sampling error as to the number of probesets that would randomly fall in this intersection of groups. Second, the number of uniquely identified down-regulated genes in the bulk tissue is considerably smaller than up-regulated, given the large number of down-regulated probesets identified in the LCM samples.

The fact that there are more down-regulated genes in LCM samples has led the author to conclude that LCM is more sensitive in identifying differentially expressed genes; however, it is conceptually inconsistent that this sensitivity is
not applicable in up-regulated genes in cancer.

This is an interesting observation that is drawn out of the histology of the normal and tumor samples. We can reasonably expect to sample benign tissue without including significant amounts of infiltrating tumor cells using both LCM and bulk tissue sampling techniques. However, tumors consist of a complex arrangement of tumor cells infiltrating around and in between regions of normal cells. Therefore, when bulk sampling tumors, it is reasonable to expect to obtain both tumor cells and a percentage of benign cells. However, when using LCM, you are specifically selecting for a pure population of tumor cells, and do not have significant benign cells sampled. Now, given this is true, we can reasonably expect the following:

(i) Identifying genes up-regulated in tumors compared to benign tissue, should be relatively resistant to the effect of contaminating normal cells in the tumor tissue sample, as by default, the normal cells should have low-expression levels for the gene of interest.

(ii) Identifying genes down-regulated in tumors compared to benign tissue, however, should be more sensitive to the effects of contaminating benign cells in the tumor tissue sample, as these cells will be expressing the target gene at high levels and thus causing a masking of the “loss” of signal from the tumor cells.

Minor Essential Revisions:
Typo on page 14 under Data Preprocessing and Analysis, 4-fold change should be log2(4) instead of log2(2); similar mistake in the first paragraph on page 15.

In fact, the log2(2) was the correct notation. The manuscript has been updated to correctly state a 4x change, and not a 4-fold change.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests:
I declare that I have no competing interests