**Journal of Visualized Experiments - Manuscript ID JoVE51248R1 “Adaptation of semi-automated circulating tumor cell (CTC) assays for clinical and preclinical research applications”. Lori E. Lowes, Benjamin D. Hedley, Mike Keeney, and Alison L. Allan.**

**Response to Reviewers’ Comments**

We thank the Editor and the Reviewers for their feedback and helpful comments. We have addressed specific Reviewers’ points as follows:

**Reviewer #1**

1. *The technique is only useful for scientists who have access to the CellSearch platform*

We agree with the reviewer, the protocols described in this manuscript are indeed targeted toward those that have access to the CellSearch platform or are considering collaborating with such a group.

1. *No alternative methods are mentioned/discussed*

The primary focus of this manuscript is the CellSearch system and therefore we believe that a detailed description of other techniques is outside the scope of the current manuscript. However, several excellent reviews on alternative CTC methods have been cited for the readers’ reference (see refs: 11-15), and a general overview of alternative approaches for human CTC analysis has been provided in the Introduction on page 4, paragraph 2. In addition several previously utilized CTC analysis techniques for *in vivo* animal modeling have also been mentioned and referenced on page 6, paragraph 1 of the revised manuscript.

**Reviewer #2**

1. *The manuscript is very well written and provides excellent detail regarding the proper use of the instrument as well as the incorporation of an additional novel marker. The submission is enhanced significantly by the inclusion of extensive quality control experiments, and a thoughtful discussion of the benefits and limitations of this instrument and the techniques. The figures provide good detail regarding expected results and the figure legends and representative data are very good. I have no major concerns with this manuscript.*

We thank the reviewer for this positive comment.

1. *The language of paragraph 2 of page 5 is a bit dense and was a little difficult to follow. Because the incorporation of additional biomarkers is an area of high interest for readers, it might help to make the first few sentences of this paragraph a little more "casual".*

This paragraph has been reworded for improved readability in the revised manuscript.

1. *typo pp12 "pixelated"*

This has been corrected in the revised manuscript.

**Reviewer #3**

We thank the reviewer for taking time to consider this manuscript and for the positive feedback.

**Reviewer #4:**

1. *The protocols detailed here actually are slight adaptations of the original protocols of the CellSearch systems and of the protocol of user-defined protein marker protocol detailed in ref 16 by the same authors. It appears that the crucial steps are the optimization of the immunostaining reactions with adequate controls (dilution of the antibodies, appropriate spiked or unspiked blood samples,?). Therefore, these optimization steps (which are briefly exposed in the second paragraph of the discussion) should be detailed in the "results" section as necessary procedures to be performed before using the standardized protocols proposed by the authors.*

A more detailed description of the appropriate steps necessary for optimization has been added to the results section (page 12, paragraph 2) of the revised manuscript.

1. *The protocol described to enumerate CTCs from preclinical models involves the use of the third channel for HLA staining. As a consequence, no empty channel is left for the detection of a user-defined antigen and so extra characterization of the CTCs is not possible. Though this is briefly mentioned by the authors, the fact that this protocol is set up for to enumerate human cells in mouse background and that it works only for enumeration should be better emphasized in the discussion and clearly exposed as a limitation of the method. Indeed, how important is it to enumerate CTCs in pre-clinical systems while metastasis quantification is easily feasible, unlike in human patients? It feels that characterizing CTC in pre-clinical systems is a major aspect rather than just enumeration?*

Please see the response to Reviewer #4, Comment #3, below.

1. *The authors state that "normal" epithelial mouse cells have been reported in pre-clinical systems. To our understanding, this suggests that the authors found EpCAM+/CK+/DAPI+/CD45- (but HLA-) cells in the non-spiked samples presented in figure 3. It would have been informative to have a quantification of these cells. Also, it would be interesting to mention what are the proposed hypotheses regarding their origin and regarding their presence in mice (but not in humans?).*

The reviewer is correct in his/her statement that EpCAM+/CK+/DAPI+/CD45-/HLA- cells were observed in non-spiked samples as depicted in Figure 3 of the manuscript, which we believe are murine skin epithelial cells. However, it should be noted that these cell numbers were typically very low (0.33±0.24 events/50µl; n=9), with higher cell numbers (up to 11 events/50µl) observed only when repeated attempts were required for adequate blood collection from the mice (i.e., multiple cardiac punctures). These cells are usually very easy to identify based on morphology alone (highly elongated, often with a long tail-like structure, and occasionally clustered), and therefore the HLA marker was simply utilized to confirm that these rare events were not CTCs.

The reviewer is also interested in the proposed hypothesis regarding the origin of these cells. As mentioned above, we believe that these cells are mouse squamous epithelial cells and that the cell number increase observed with repeated cardiac puncture is due to increased disruption of epithelial cell layers in the surrounding area and therefore these cells would not typically be present in the normal mouse circulation. The reviewer also questions why these cells are not present in human samples. In actuality, contamination with human squamous epithelial cells has also been reported by the manufacturer and in the literature, typically resulting from needle contamination during blood collection. These cells are again very rare and tend to be easily identified based on morphology (very large with a high cytoplasmic to nuclear ratio and cornflake appearance in the cytoplasm). As mouse squamous epithelial cells are typically much smaller, we cannot necessarily use the same criteria to classify them as we would with human samples, and therefore HLA was employed for validation purposes. We have clarified this in the revised manuscript by substituting “mouse *squamous* epithelial cells” for “mouse epithelial cells” where appropriate.

Finally, the reviewer states that the inability for CTC characterization is a limiting factor in the usefulness of this assay; maintaining that CTC enumeration in a mouse background is already easily feasible and that CTC characterization is a major aspect missing from this protocol. Firstly, we disagree with this reviewer that CTC enumeration in a mouse background is “easily feasible”, since these cells are still very rare even in the smaller blood volume found in a mouse versus human. Although other techniques do exist for CTC enumeration, they are typically complicated, multi-step protocols, and none utilize the gold standard CellSearch protocol as employed for this manuscript. As small changes in CTC enumeration technique can alter CTC recovery, an assay that adequately models the current capabilities of the CellSearch assay is warranted for *in vivo* modeling. In addition, although we have added HLA to our protocol to ensure accurate CTC identification, contamination with mouse squamous epithelial cells is fairly minimal and that higher cell numbers are only observed when repeated attempts must be made in collecting mouse blood. Certainly if a user was confident enough in routinely identifying these cells based on morphology alone, then HLA could be omitted from this protocol to allow for on-system CTC characterization. In addition, as previously demonstrated using the CellSearch system, downstream CTC characterization is easily accomplished1,2 and therefore we anticipate that our assay would be amenable to this as well.

Information regarding these issues has been added to the Discussion section (page 16, paragraph 1) in the revised manuscript.

**Additional Changes to Revised Manuscript**

Following consultation with Janssen Diagnostics LLC (formerly Veridex LLC) since the initial submission of the manuscript, we have made 2 additional minor changes to the revised manuscript with regards to specific criteria for classification of events with dim CK/DAPI expression and pixelated images (Figure 1). These revisions have been made with track changes and can be found on page 12, paragraph 1 of the Results section. Figure 1 has also been modified slightly and uploaded as a revised PDF.

In addition, since the initial time of submission of this manuscript Janssen Diagnostics LLC has introduced a new commercially available tumor phenotyping reagent, IGF-1R. This has been added to the list of commercially available markers on page 5, paragraph 2 of the introduction.

Finally, we mistakenly omitted a funding source (Prostate Cancer Canada) from the Acknowledgements section, and this has been corrected in the revised manuscript.

**References**

1. Sieuwerts, A. M., Kraan, J., *et al.* Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast cancer research and treatment* **118** (3), 455–68, doi:10.1007/s10549-008-0290-0 (2009).

2. Flores, L. M., Kindelberger, D. W., *et al.* Improving the yield of circulating tumour cells facilitates molecular characterisation and recognition of discordant HER2 amplification in breast cancer. *British journal of cancer* **102** (10), 1495–502 (2010).