Dear Review Editor at JoVE,

Thank you for your consideration of our article JoVE58735, entitled “Quantitative Immunoblotting of Cell Lines as a Standard to Validate Immunofluorescence for Quantifying Biomarker Proteins in Routine Tissue Samples”. We have reviewed the comments from the editor and peer reviewers and made the appropriate modifications to the manuscript. We have documented the changes in the revised manuscript using the “Track changes” function of Microsoft Word.

**Editorial comments**

* Spelling and grammar issues have been corrected.
* All centrifuge speeds are reported in x g.
* Commercial language has been removed from the manuscript text.
* Personal pronouns have been removed.
* The protocol has been revised to only include action items in the imperative, safety notices, and sparse “Notes”.
* A number of changes have been made so as to make it clearer to the reader that the primary purpose of this manuscript is to describe a protocol for using quantitative immunoblotting to investigate the quantitative nature of immunofluorescence histology. These changes include:
  + Revising the title;
  + Adding more detail to certain steps of the protocol;
  + Relying on citation of published literature to provide technical details for procedures that are peripheral to this work, including standard histology and immunohistology;
  + Acknowledging that some steps (i.e. button clicks, settings) will vary depending on the use of particular instrumentation or software.
* The protocol steps have been modified to only include 2-3 action steps and a maximum of 4 sentences.
* Highlighting has been revised according to the guidelines provided in the comments.
* Figure 4 now says “s” instead of “sec”.
* Figure 1 is referenced in the manuscript.
* Available lot numbers and RRIDs of antibodies have been included where the information was available.

**Comments from Reviewer 1**

* The MALDI-MSI technique has been referred to in the Introduction.
* The 3 papers cited on lines 97-98 were indeed from our research group. The names have been removed.
* Information on the 8 cell lines used is now provided in the “Representative results” section. This information is not included in the protocol because the attributes of the particular cell lines that we used are not fundamental to the protocol. We have left the protocol general to be used by readers with different cell lines of choice.
* The cell line used in Figure 3 for the no Bcl-2 antibody is now indicated in the figure legend.
* The catalog number for trypsin has been included.
* Typos have been corrected.

**Comments from Reviewer 2**

* We have changed the wording throughout the manuscript to better reflect the nature of the protocol. We have indicated that the method is for relative, rather than absolute, quantification of proteins and added paragraph that describes how one could make the technique quantitative if so desired.
* We also addressed concerns related to IF on FFPE tissues, such as that of autofluorescence, but as the focus of this paper is not to identify a new method of performing IF, we have not expounded at length.
* We have cited and present results from a separate manuscript, currently in preparation by our group that describes the relative quantification of Bcl-2 protein by IF in a cohort of primary biopsy samples. As we point out in the revised manuscript, these additional results document the run-to-run reproducibility of the IF-based assay, the correlation of these results with *Bcl-2* transcript abundance, and statistically significant associations with conventional immunohistochemistry for Bcl-2 and copy number gain or translocation of the *Bcl-2* gene ascertained in the same samples by fluorescence *in situ* hybridization.
* We have added a brief discussion on potential discrepancies between the IF and IB signals at low intensity values.

Please let me know if you believe any additional changes need to be made or if we have not sufficiently addressed the concerns outlined above.

Sincerely,

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