Journal of Visualized Experiments

Quantitative Immunoblotting of Cell Lines as a Standard to Validate Immunofluorescence for Quantifying Biomarker Proteins in Routine Tissue Samples

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58735R2
Full Title:	Quantitative Immunoblotting of Cell Lines as a Standard to Validate Immunofluorescence for Quantifying Biomarker Proteins in Routine Tissue Samples
Keywords:	immunofluorescence; formalin-fixed paraffin-embedded; quantitative; immunoblot; Western blot; Pathology; protein; biomarker; Histology; immunohistology
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Kingston, Ontario, Canada

Cover Letter

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Tuesday, 3 July 2018

Journal of Visualized Experiments
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Dear JoVE editors,

I am pleased to submit our manuscript, titled "Immunofluorescence Microscopy for Objective Quantification of Biomarker Proteins in Formalin-Fixed, Paraffin-Embedded Tissue", for consideration for publication in JoVE. The methods outlined in the manuscript will provide a protocol for the objective quantification of biomarker proteins from formalin-fixed, paraffin-embedded tissue specimens suitable to a wide range of clinical and research applications. We believe the multi-component procedure described in this manuscript would lend itself well to representation in both written as well as video formats. For example, the explanation for the procedure for fixing and embedding cultivated cells in paraffin wax and for data normalization and comparison will be enhanced by the use of visual cues that can be incorporated into a video protocol.

The authors' contributions to the manuscript are as follows: Alison Moore wrote the manuscript, generated the figures, performed the immunoblotting experiments, and performed the data analysis. Lee Boudreau performed the immunofluorescence (IF) staining and consulted on IF findings. Shakeel Virk performed the image analysis to quantify the IF signals and consulted on IF findings. David LeBrun consulted on the IF findings and immunoblotting procedure and edited the manuscript. Throughout this process we have been in contact with three JoVE editors. Rachel Service reached out initially about writing the manuscript and sent the invitation to submit. Indrani Mukherjee was the second editor we were in contact with and helped to answer questions regarding timeline. Lyndsay Troyer is the current editor we are in contact with at the time of submission.

On behalf of my co-authors and myself, thank you for considering our work. We look forward to hearing back from you.

Sincerely,

David P. LeBrun, MD, FRCP(C)
Professor

TITLE:

Quantitative Immunoblotting of Cell Lines as a Standard to Validate Immunofluorescence for Quantifying Biomarker Proteins in Routine Tissue Samples

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KEYWORDS:

Immunofluorescence, formalin-fixed paraffin-embedded, quantitative, immunoblot, western blot, pathology, protein, biomarker, histology, immunohistology

SHORT ABSTRACT:

We describe the use of quantitative immunoblotting to validate immunofluorescence histology coupled with image analysis as a means of quantifying a protein of interest in formalin-fixed, paraffin-embedded (FFPE) tissue samples. Our results demonstrate the utility of immunofluorescence histology for ascertaining the relative quantity of biomarker proteins in routine biopsy samples.

LONG ABSTRACT:

Quantification of proteins of interest in formalin-fixed, paraffin-embedded (FFPE) tissue samples is important in clinical and research applications. An optimal method of quantification is accurate, has a broad linear dynamic range and maintains the structural integrity of the sample to allow for identification of individual cell types. Current methods such as immunohistochemistry (IHC), mass spectrometry, and immunoblotting each fail to meet these stipulations due to their categorical nature or need to homogenize the sample. As an alternative method, we propose the use of immunofluorescence (IF) and image analysis to determine the relative abundance of a protein of interest in FFPE tissues. Herein we demonstrate that this method is easily optimized, yields a wide dynamic range, and is linearly quantifiable as compared to the gold standard of quantitative immunoblotting. Furthermore, this method permits the maintenance of the

structural integrity of the sample and allows for the distinction of various cell types, which may be crucial in diagnostic applications. Overall, this is a robust method for the relative quantification of proteins in FFPE samples and can be easily adapted to suit clinical or research needs.

INTRODUCTION:

The need to quantify proteins in formalin-fixed, paraffin-embedded (FFPE) tissue biopsy samples exists in many clinical fields. For example, quantification of biomarker proteins in routine biopsy specimens is used to elucidate prognosis and inform treatment for cancer patients[1]. However, current methods are typically subjective and lack validation.

Immunohistochemistry (IHC) is used routinely in pathology laboratories and generally depends on a primary antibody directed at the target protein and a secondary antibody conjugated with an enzymatic label such as horseradish peroxidase[2]. Conventional IHC is sensitive, can make use of minute samples and preserves the morphological integrity of tissue samples thereby permitting assessment of protein expression within its relevant histological context. However, because the chromogenic signal generated by IHC is subtractive, it suffers from a relatively narrow dynamic range and offers limited potential for multiplexing[2–4]. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) preserves morphological integrity. However, this developing technology is associated with modest morphological resolution and requires significant calibration and normalization, impairing its feasibility for routine clinical use[5–7]. Alternative techniques to quantify protein in tissue samples include immunoblotting[8], mass spectrometry[9–11], and enzyme-linked immunosorbent assay (ELISA)[12], each of which begins with a homogenized lysate of sample tissue. Primary tissue samples are heterogeneous in that they contain a multitude of cell types. Therefore, techniques that entail homogenizing the samples do not permit quantification of a protein in a particular cell population of interest such as cancer cells.

Like IHC, IF is applicable to small FFPE samples and permits the retention of histological integrity[13]. However, thanks to the additive nature of fluorescence signals, IF is amenable to the application of multiple primary antibodies and fluorescent labels. Thus, a protein of interest may be relatively quantified within specific cells or cellular compartments (for example, nucleus *versus* cytoplasm) defined using other antibodies. Fluorescence signals also have the advantage of a greater dynamic range[13, 14]. The superiority, reproducibility, and multiplexing potential of IF applied to FFPE samples has been demonstrated[13–15].

Herein we describe the use of quantitative immunoblotting using established cell lines as a gold standard to ascertain the quantitative nature of IF coupled with computer-assisted image analysis in determining the relative abundance of a protein of interest in histological sections from FFPE tissue samples. We have applied this method successfully in a multiplex approach to quantify biomarker proteins in clinical biopsy samples[16–19].

PROTOCOL:

Approval to use primary human tissue samples was obtained from the Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (HSREB) at Queen's University.

1. Building a Cell-line Tissue Microarray (TMA)

1.1) Harvest and wash cells.

NOTE: This protocol has been tested on various established immortalized cell lines (*e.g.* HeLa, Jurkat, RCH-ACV).

1.1.1) For adherent cells, harvest approximately 1.3 x 10^7 cells once they reach approximately 80% confluency. Detach cells using a reagent appropriate for that cell line.

NOTE: Ethylenediaminetetraacetic acid (EDTA) is generally preferred over trypsin to reduce the risk of degrading surface proteins. If using trypsin, neutralize the trypsin using fetal bovine serum (FBS) immediately after harvesting the cells.

1.1.2) For suspension cells, harvest approximately 8 x 10^7 cells in log-phase of growth.

1.1.3) Collect the harvested/detached cells by centrifuging for 5 min at 225 x g in a 50 mL conical tube.

1.1.4) Decant the supernatant and re-suspend the pellet in 10 mL of 1x phosphate-buffered saline (PBS). Centrifuge for 5 min at 225 x g and decant the supernatant.

NOTE: 1X PBS is composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in distilled water; adjust pH to 7.4.

1.2) Fix the cells in formalin and pellet them.

1.2.1) Suspend the cell pellet within the conical tube in 10 mL of 10% neutral buffered formalin (NBF).

NOTE: NBF can be prepared by diluting 1 mL of 37% formaldehyde stock solution in 9 mL of 1X PBS.

CAUTION: Use caution when handling formalin and formaldehyde. Use a fume hood for steps involving formalin and formaldehyde.

1.2.2) Incubate the cells on a rocker at 24 rpm at room temperature overnight (about 17 h).

1.2.3) Prepare a 1% solution of low melting point agarose in PBS (0.01 g of agarose per 1 mL PBS).

1.2.3.1) Prepare enough for 500 µL of agarose solution per cell line sample.

1.2.3.2) Dissolve the agarose in an 80 °C water bath or heat block, with occasional mixing for 2-5 min. Once dissolved, keep the solution in a 37 °C water bath or heat block to prevent hardening.

1.2.4) Pellet the fixed cells from step 1.2.2 by centrifuging for 5 min at 225 x g. Remove the supernatant and resuspend the cells in 500 µL of 1x PBS.

1.2.5) Transfer the cells into a 1.5 mL microcentrifuge tube and pellet by centrifuging for 5 min at 290 x g. Aspirate off all of the supernatant.

1.3) Cast cells in agarose.

1.3.1) Add ~500 µL of 1% agarose solution (from step 1.2.3) to each tube containing the cells. Gently, but quickly, pipette mixture up and down using a P-1000 micropipette to mix.

NOTE: Cut off the end of the pipette tip to enlarge the aperture and avoid forming bubbles. Keep the working agarose solution in the 37 °C water bath to prevent hardening.

1.3.2) Allow the agarose-cell solution to harden (5-10 min) at room temperature. Add 1 mL of 10% NBF to each microcentrifuge tube containing a sample and keep at room temperature until paraffin embedding (up to 24 h).

1.4) Prepare the agarose plug for paraffin embedding.

1.4.1) Aspirate off the NBF from the sample.

1.4.2) Remove the cell plug using a razor blade to cut the microcentrifuge tube, place the plug into a teabag and place the bag into a plastic tissue cassette. Store cassettes in 10% NBF at room temperature.

1.5) Process the samples overnight either manually or using an automated tissue processor, and embed in paraffin wax using standard histology methods.

NOTE: See Fischer *et al.*[20] for an example protocol.

1.6) Using a specialized “tissue arrayer” instrument, harvest duplicate 0.6-mm cores from the paraffin block from each cell line and insert them, in rows, into an empty recipient paraffin block in order to create a **cell line TMA**.

NOTE: Standard methods should be used such as those described in Fedor and De Marzo[21].

1.7) Incorporate cores from primary samples representing 2-3 additional tissue types (*e.g.,* tonsil, colon, testes, *etc.*) as positive or negative controls into the TMA. Choose tissues that are appropriate for the protein of interest.

1.8) Use a microtome to prepare two histological sections, approximately 4 to 6 µm thick, of the cell line TMA. Mount the section on a histology slide, dry it, and deparaffinize (as described in Fedor and De Marzo[21]).

2. Sample staining by Immunofluorescence

2.1) Optimize the dilution of primary antibody.

NOTE: Standard protocols exist for the optimization of IHC or IF for FFPE tissue sections such as in Kajimura *et al.*[22]. A brief overview of the approach is outlined here.

2.1.1) Prepare 4-5x dilutions of primary antibody to the protein of interest guided by the manufacturer's instructions.

2.1.2) Identify a control tissue type from an animal or human source that expresses the protein of interest in morphologically recognizable cell populations. Prepare sections of the tissue and mount them on a slide following standard immunohistochemistry procedure such as in Fedor and De Marzo[21].

NOTE: The number of sections required is the number of primary antibody dilutions plus one extra.

2.1.3) Using an automated or manual system for immunohistology, test the primary antibody dilutions from step 2.1.1 each on a slide from step 2.1.2. Omit the application of primary antibody from the extra slide and use it as a negative control. Additionally, stain all slides with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain.

2.1.4) Use a fluorescently tagged secondary antibody or, if greater sensitivity is required, a tyramide-based signal amplification system to label proteins of interest (see Stack *et al.*[13]).

NOTE: When multiplexing primary antibodies, a different fluorescent label is used for each protein.

2.1.5) Scan the immunostained slides using an appropriate instrument capable of generating a digital image file using excitation and detection wavelengths appropriate to the fluorophores that were used.

2.1.6) Use appropriate software to view the digital images and empirically choose the primary antibody dilution that optimizes signal intensity relative to background fluorescence.

2.2) Perform IF staining on the cell line TMA.

2.2.1) Use an automated or manual system for immunohistology to stain a slide of the cell line TMA with the optimized primary antibody dilution (as determined in step 2.1). Omit the

application of primary antibody from the second slide and use it as a negative control. Additionally, stain all slides with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain.

2.2.2) Scan the immunostained slides using an appropriate instrument capable of generating a digital image file using excitation and detection wavelengths appropriate to the fluorophores that were used.

2.2.3) Use an image analysis software package to identify the cellular compartment of interest (*i.e.,* cytoplasm *versus* nucleus) and quantify the mean fluorescence intensity (MFI) per cell.

NOTE: Various software packages can be used for this purpose and many are discussed in Stack *et al.*[13].

3. Quantitative Immunoblotting of Cell Lines

3.1) Prepare lysates of cells.

3.1.1) Harvest 2 million cells of each cell type, as described in steps 1.1.1 to 1.1.3.

3.1.2) Spin cells down for 5 min at 650 x g in a 50 mL conical tube. Decant the supernatant.

3.1.3) Wash cells with 10 mL of ice-cold PBS. Resuspend in 1 mL of ice-cold PBS and transfer to a 1.5 mL microcentrifuge tube. Centrifuge as per step 3.1.2, decant, and leave cells on ice.

3.1.4) Add about 200 µL of cold radioimmunoprecipitation (RIPA) lysis buffer with protease inhibitors (10 µL of 100x inhibitors per mL of RIPA lysis buffer) to the cells, vortex and incubate on ice for 15 min.

NOTE: The amount of lysis buffer required for effective lysis varies by cell line and can be determined empirically. RIPA buffer is composed of 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in distilled water.

3.1.5) To ensure relatively even loading on the immunoblots, quantify the total protein in a 20 µL sample from each lysate using an appropriate method such as the Bradford assay. Add about 40 µL of 6x Laemlli lysis buffer to the remainder (approximately 180 µL) of each cell lysate and boil at 100 °C in a heat block or water bath for 5 min.

NOTE: 6X Laemlli buffer is composed of 300 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 60% glycerol, 0.6% (w/v) bromophenol blue, 50 mM dithiothreitol (DTT) in distilled water.

3.1.6) Store the samples at -20 °C for up to two weeks.

3.2) Perform an immunoblot of all cells lines to determine which cell line has the most abundant protein of interest.

NOTE: We followed the procedure described in Mahmood, T. and Yang, PC.[23], with the following modifications.

3.2.1) Aliquot cell lysate (prepared in step 3.1) containing 10-50 µg of protein (depending on the abundance of the protein of interest) into microcentrifuge tubes. Add 2.5 µL of 6x Laemlli buffer and sufficient RIPA lysis buffer to make the volume up to 15 µL.

3.2.2) Load a 5% stacking and an appropriate concentration resolving (*e.g.*, 10% for proteins between 15-100 kDa) SDS-PAGE gel with a protein ladder and the samples from step 3.2.1. Load 1x Laemlli buffer into empty wells. Run the power supply at appropriate settings, typically 125 V, for 80 min or until the bromophenol blue dye reaches the bottom of the plate.

3.2.3) Perform a semi-dry protein transfer as described in Wiedemann *et al.*[24].

NOTE: For the representative results, a nitrocellulose membrane (which does not need to be soaked in methanol), and cold Bjerrum Schafer-Nielsen (BSN) transfer buffer were used. BSN buffer is composed of 48 mM Tris, 39 mM glycine, 20% methanol in distilled water.

3.2.4) After transfer, use a razor blade to cut the membrane horizontally to separate the portion containing the protein of interest from an appropriate internal control protein, such as GAPDH.

3.2.5) Proceed to blocking and antibody incubations using the blocking buffer recommended by the manufacturer of the primary antibodies.

NOTE: Optimal primary antibody dilutions can vary dramatically depending on protein abundance and sensitivity. For the representative results below, the antibody to the protein of interest required a 1:1000 dilution while the GAPDH control required a 1:40,000 dilution. The dilution of secondary antibodies used was 1:3,000.

3.2.6) After antibody incubations and washing of the membrane, place the membrane strips in a clear plastic sheath such as a sandwich bag.

3.2.7) Prepare electrochemiluminescence (ECL) mixture following the manufacturer's instructions. Use a P-1000 pipette to cover the membrane with the ECL mixture, close the sheath, and incubate the membrane strips with the mixture in the dark at room temperature for 1-2 min.

3.2.8) Place the membrane sheath in a digital imaging platform. Use chemiluminescence and colorimetric marker detection to capture various exposures of the membrane.

NOTE: Exposure times will vary based on the amount of protein loaded, abundance of target protein, antibody affinity *etc.* Begin with an automatic exposure (typically a few seconds), and test exposure times above and below by increments of a few seconds.

3.2.9) Empirically, or using image analysis software, determine which cell line expresses the most target protein.

3.3) Find the linear dynamic range of each primary antibody using a serial dilution.

3.3.1) Perform steps 3.2.1-3.2.8 using a series of serial dilutions from the cell line which expresses the highest concentration of the target protein (identified in step 3.2.9).

3.3.2) Use image analysis software such as ImageJ to perform densitometry on the exposure images.

3.3.2.1) For example, using ImageJ, use the **Rectangular Selections** tool to select the first lane of the gel to quantify. Go to **Analyze| Gels| Select First Lane**. Use the mouse to move the resulting rectangle over to the next lane. Go to **Analyze| Gels| Select Next Lane**. Repeatedly move the rectangle to the next lane and select the lane for the remainder of the lanes.

3.3.2.2) Go to **Analyze| Gels| Plot Lanes**. Use the **Straight Line** tool to draw lines across the bases of each peak to remove background noise. Use the **Wand** tool to select each peak, and collect the density of each peak, henceforth referred to as band intensity, from the **Results** window.

3.3.3) Use the densitometry output to create a scatterplot of the band intensity *versus* the amount of total protein loaded for each primary antibody. Using a line of best fit and visual inspection, determine the location (intensity range) of the linear dynamic range of each antibody.

3.3.4) Choose a protein concentration that generates a value on the higher end of the linear range to be the concentration moving forward with all cell lines.

NOTE: Since this concentration is below the saturation level in the cell line with the greatest amount of this protein, there should be no danger of over exposing the bands for the other cell lines.

3.4) Perform an immunoblot using the protein concentration chosen in step 3.3.4 for all cell lines and repeat steps 3.2.1-3.2.8.

3.4.1) Perform densitometry on the digital scans as in steps 3.3.2 and 3.3.3. Choose the exposures that yield signals within the linear ranges for each antibody identified in step 3.3.4.

3.4.2) Using the band intensity signals from the ideal exposures from 3.4.1, calculate the ratio of target protein band intensity to loading control band intensity for each cell line. These ratio values indicate the relative abundance of the target protein of interest.

3.4.3) Perform a Pearson correlation test (can be done using a statistical software package) to correlate the values obtained from image analysis of the IF staining (step 2.2) to those obtained from immunoblotting (step 3.4.2).

REPRESENTATIVE RESULTS:

This protocol was used to confirm the ability of IF to determine the relative quantity of the anti-apoptotic protein Bcl-2 in cell lines made into FFPE tissue blocks. Quantifying Bcl-2 selectively in cancer cells can elucidate oncogenic mechanisms and can be useful in pathological diagnosis and in informing clinical management decisions[25]. More specifically, Bcl-2 plays a role in proper B-lymphocyte development and its expression is commonly investigated in the context of lymphoma[26–28]. **Figure 1** outlines the steps involved in the protocol. In an initial IF optimization step, various dilutions of the primary anti-Bcl-2 antibody were tested on human tonsil tissue using an automated immunohistology stainer as described in step 2.1. **Figure 2** contains images of the scanned and stained histology slides of human tonsil tissue that each received a different dilution of antibody. It can be seen that 1:50 is the optimal dilution that yielded strong signal and little background fluorescence. This dilution was then used on the cell line TMA as described in step 2.2. The TMA was also stained using DAPI to identify nuclei. A tyramide-based signal amplification kit was used to label Bcl-2 with a Cy5 fluorophore. Image analysis was used to quantify the cytoplasmic Cy5 fluorescence signal attributed to Bcl-2 in each cell line. Representative images of the staining can be seen in **Figure 3**. The immortalized cell lines chosen for this experiment included a variety of lymphoid-derived cells lines, namely 697, JeKo-1, Jurkat, RCH-ACV, Granta-519, REH, and Raji, in addition to HeLa, derived from a cervical carcinoma. The HeLa cells are known to express Bcl-2 at a very low level[29].

Based on an initial immunoblot of all eight cell lines, Granta-519 was determined to have the greatest abundance of Bcl-2 (not shown). Serial dilutions of the Granta-519 lysate were used in a subsequent immunoblot to find the linear dynamic range of the Bcl-2 and GAPDH (loading control) signals (**Figure 4A**). This immunoblot was exposed to the digital scanner for varying lengths of time. Densitometry using image analysis software was used to quantify the signal from each band, and these values were plotted against the amount of protein loaded (**Figure 4B**). From the data in **Figure 4B-top**, the dynamic range for Bcl-2 in this assay spans from a band intensity of nearly zero to 7500 (arbitrary units, blue line). The two higher exposure times fit a quadratic and non-linear equation, suggesting overexposure and saturation of the signal intensity. The range for GAPDH is from 3000 to 6500 (arbitrary units, **Figure 4B-bottom**). Values below 3000 (arbitrary units) dropped precipitously even when using a relatively low exposure time. A long exposure clearly results in saturation. From these graphs, it was determined that 12 µg would be a reasonable amount of protein to load when performing the immunoblot with all cell lines, since this amount of protein yielded Bcl-2 intensity values within the linear range for the Granta-519 cells, reducing the risk of overexposure for all other cell lines.

A second immunoblot of all cell lines was then performed as in step 3.4 and can be seen in **Figure 5A**. This blot was required since the initial blot contained bands with a signal intensity outside of the linear range. Image analysis software was used to determine the signal intensity of each band in the new blot. Only intensity values that were within the dynamic ranges determined above were used. The arrows on the right of **Figure 4B** demonstrate representative intensity values that were used and show where they fit within the linear range. The ratio of Bcl-2:GAPDH was then calculated for each cell line. This ratio, along with the fluorescence signal from IF can be seen in

Figure 5B. A Pearson correlation test demonstrated that the intensity ratios from immunoblotting were strongly and positively correlated with the intensity readings from quantitative IF ($r = 0.983$, $p < 0.001$; see **Figure 5C**).

Quantitatively assessing the amount of Bcl-2 proved to be particularly difficult as there was a wide range of expression of this protein across the eight tested cell lines. Using a long enough exposure to capture the signal of the low Bcl-2-expressing cell lines (>1 s) made it difficult to remain in the dynamic range for the high Bcl-2-expressing cell lines. In an attempt to quantify the faint bands produced by cell lines such as HeLa and Raji, several different exposure times, from 0.1 s to 5 s, were captured to determine the longest exposure time that could be used while remaining in the dynamic range for cells such as Granta-519 (see **Figure 6**). The nature of this immunoblotting technique limits the accuracy of signal detection as one approaches noise, suggesting it is optimally used to quantify proteins found at intermediate to high expression levels.

FIGURE LEGENDS:

Figure 1: Protocol workflow diagram. Immunofluorescence (IF) on a cell line tissue microarray (TMA) was run in parallel to quantitative immunoblotting (IB) of the same cell lines. Signals from each cell line are compared by Pearson correlation to validate the quantitative ability of the IF protocol.

Figure 2. Testing and optimizing immunofluorescence (IF) protocol. Sections of tonsil were incubated with different dilutions of anti-Bcl-2 antibody (1:50, 1:100, and no primary antibody). The slides were incubated with HRP-conjugated secondary antibody, the signal was amplified using a tyramide-Cy5 conjugate, and the slides were stained with DAPI. The slides were scanned at 20X magnification using filter sets appropriate to the maximal excitation/emission peaks of 350/470 nm and 649/666 nm for DAPI and Cy5, respectively. Respective exposure times were 160 ms and 200 ms. The field of view is centered over the same lymphoid follicle, which is expected to be Bcl-2 negative in the (central) germinal center and positive in the (peripheral) mantle zone. The 1:50 dilution gave a stronger specific fluorescence signal without increasing the nonspecific signal therefore it was used as the dilution going forward.

Figure 3. Immunofluorescence (IF) using cell line tissue microarray (TMA) sections. Sections of the cell line TMA were taken and incubated with a 1:50 dilution of anti-Bcl-2 or with no primary antibody. The slides were incubated with secondary antibody, the signal was amplified using a tyramide-Cy5 conjugate, and the slides were stained with DAPI. The slides were scanned at 20X magnification using filter sets appropriate to the maximal excitation/emission peaks of 350/470 nm and 649/666 nm for DAPI and Cy5, respectively. Respective exposure times were 250 ms and 625 ms. These representative images indicate that staining was successful, and that there is a range of Bcl-2 expression across the cell lines. The “no Bcl-2 antibody” negative control slide did not demonstrate any Cy5 signal, as expected. The representative image is from the 697 cell line. A core of hyperplastic tonsil tissue included on the TMA demonstrates an equivalent pattern to

that shown in **Figure 2**. The mean fluorescence intensity (MFI) for each cell line was determined by quantifying the Cy5 fluorescence signal using image analysis software.

Figure 4. Determining the dynamic linear range of the immunoblot (IB) signals. (**A**) IBs of serial dilutions of Granta-519 lysate. Each blot was exposed for a variety of times to find the linear dynamic range. (**B**) Band intensity for Bcl-2 (top) and GAPDH (bottom) *versus* amount of total protein loaded for IB in (A). Band intensities were quantified using the image analysis software ImageJ. For Bcl-2 (top), the signals remained linear throughout the range of dilutions when a low exposure (exp.) time (0.2 s) was used as supported by a Pearson's correlation coefficient (r value) of 0.994 ($p < 0.001$), but not for greater exposure times of 1 s and 2 min. For GAPDH (bottom), the data remained linear above an intensity of 3000 (arbitrary units) for low (0.2 s) and medium (2.2 s) exposure times as supported by Pearson's r values of 0.994 ($p < 0.001$) and 0.992 ($p < 0.001$), respectively, but not for a high exposure time (7 s). Arrows on the right indicate the band intensity for the specific cell line identified from the immunoblot in **Figure 5A**.

Figure 5. Quantitative immunoblotting (IB) of immortalized cell lines and comparison to immunofluorescence (IF). (**A**) IB of all cell lines. 12 μg of total protein was loaded into each well. (**B**) IB and IF signal intensities for each cell line. The IB signal is the ratio of Bcl-2:GAPDH band intensity from the blot in (A) as quantified by ImageJ. The IF signal is the fluorescence intensity of each cell line core on the TMA (**Figure 3**) as determined by image analysis. (**C**) Scatterplot of IF signal *versus* IB signal. Each data point represents a given cell line. The two methods produced linearly correlated results with a Pearson r value of 0.984 ($p < 0.001$).

Figure 6. Finding the optimal exposure time for quantitative immunoblotting (IB). IB of all cell lines is shown. Twelve micrograms of protein were loaded in each well. For each IB, various exposure times were used to capture an image where all bands remained within the linear dynamic range. The exposure times of the Bcl-2 and GAPDH strips respectively were: (**A**) 0.1 s and 0.1 s, (**B**) 1 s and 0.2 s, and (**C**) 5 s and 5 s. Panels **A** and **C** show examples of exposures where at least some of the bands on the blot were too faint or too strong, respectively.

DISCUSSION:

We have described a method that makes use of quantitative immunoblotting to demonstrate the utility of IF for ascertaining the relative abundance of a target protein in FFPE tissue samples. Current protein quantification methods are limited by their categorical nature, such as chromogenic IHC[2, 3], or by the need to homogenize samples, preventing investigation into the sample structure and cell populations, such as with immunoblotting and mass spectrometry[8–11]. Quantitative IF can transcend these limitations if the method is validated and applied carefully. By comparing the IF readouts to quantitative immunoblotting of immortalized cell lines, we were able to validate the semi-quantitative nature of the IF approach.

Primary tissue samples from patients or experimental animals are heterogeneous in that they contain multiple cell types. The application of several primary antibodies in multiplex IF permits the quantification of one or more proteins of interest in specific cell types or cell compartments[4, 13]. The optimization and application of multiplex IF is beyond the scope of this article but is

outlined in the following papers[17–19, 30]. The broad philosophy is to label cell populations and only quantify the protein of interest in the desired cell compartment, for example nuclei or cytoplasm, in the desired cell type. Once the quantitative nature of IF with a particular antibody has been verified by quantitative IB, the protocol can be upscaled to allow for rapid, high-throughput protein quantification in clinical samples. Clinical applications generally require the determination of relative, rather than absolute, protein abundance. However, the IF approach can be made formally quantitative if necessary. For example, a solution containing purified recombinant Bcl-2 protein could be prepared and quantified using a standard biochemical method. Serial dilutions could then be evaluated by quantitative immunoblotting and a standard curve generated to estimate the absolute protein content per cell in each cell line.

We applied our IF protocol to TMA sections representing biopsy samples from 66 cases of *de novo* diffuse large B-cell lymphoma[16]. Our results demonstrated acceptable run-to-run reproducibility (Pearson r = 0.837) and the expected, strong association between "Bcl-2-positive" status determined subjectively by visual scoring of conventional IHC and elevated expression determined objectively by IF ($p < 0.001$). Furthermore, Bcl-2 abundance by IF correlated with *Bcl-2* mRNA abundance in the same samples (Spearman $\rho = 0.69$, $p < 0.001$) and was significantly greater in cases with copy number gains of the *BCL2* gene ($p = 0.042$) or translocation of *BCL2* to the *IGH* locus ($p = 0.004$), as determined by fluorescence *in situ* hybridization. We obtained equivalent results in a separate cohort of cases. These findings provide additional evidence that supports IF as a valid method for determining the relative abundance of Bcl-2 in routine FFPE specimens. The use of IF to quantify several other biomarkers proteins in primary samples has been described previously[17, 18].

Optimization of this protocol is a two-fold process. First, IF with primary antibodies used must be optimized (step 2.1). Commercial antibodies commonly arrive with a suggested dilution for IHC protocols which should be used as a starting point along with a dilution or two above and below this (*i.e.,* if 1:100 recommended, add 1:50 and 1:150). After performing the IF and scanning the slide, determining which dilution is "optimal" entails visual inspection to identify the primary antibody dilution that generates the brightest signal without increasing background fluorescence. The second stage of optimization involves choosing the amount of protein to load for the immunoblot to ensure each band remains in the linear range (step 3.4). To ensure this, an immunoblot is performed using a serial dilution of the cell line that expresses the greatest abundance of target protein. From here, the band intensities *versus* protein amount can be plotted to determine the intensity ranges through which the signal remains linear. Since this range is established in the cell line with the most abundant target protein, for a given amount of protein all other cell lines will yield lower signals. When moving to an immunoblot of all cell lines (step 3.4), the dilution series is used to inform a protein amount to load that will yield strong signals without running the risk of overexposure beyond the linear range.

Despite the benefits of this protocol, the use of immunoblotting as a quantification verification method has its shortcomings. The primary concern revolves around the large range of expression for various proteins between specimens. It may be difficult to keep all samples within the limits of a linear range if some samples express the protein of interest to a much greater degree than

others. In the representative results shown above, varying exposures were captured to get an image where the extremes (high and low) are both within the linear dynamic range (**Figure 6**). The greater than expected IF signals seen for the HeLa and Jurkat cells in **Figure 5B** may indicate either the lowest possible IF signal for this system, or that the IB signals have reached the bottom of the linear detection limit and are less accurate. Either way, this indicates that IF values at that range are less likely to be accurate in this system, and that the technique is optimal of medium-to highly-expressed proteins. Additionally, the use of HRP-conjugated secondary antibodies is not optimal for quantitative purposes, as discussed earlier with respect to IHC[2, 3]. Using fluorescently tagged secondary antibodies for the immunoblot would provide a more robust confirmation that the IF is indeed linearly quantitative[31, 32], however, the Pearson r value of 0.984 obtained using the HRP-conjugated secondary antibodies in the above protocol was sufficient for the current purposes. Potential shortcomings associated with IF, such as autofluorescence and quenching vary based upon individual laboratory instrumentation, practices and tissue type, and can be minimized by various preventative measures[33, 34]. A very small amount of autofluorescence in the representative results can be seen in no Bcl-2 antibody sample in **Figure 3**, however this amount is trivial in comparison to the remaining IF signals.

The need to accurately quantify proteins of interest from FFPE tissue samples pertains to clinical and research purposes across many biological fields. The protocol presented here outlines the use of quantitative IF on FFPE tissue sections and validates its semi-quantitative nature by immunoblotting of immortalized cell lines. This method allows quantification across a high dynamic range, as well as the maintenance of the structural integrity of the tissue sample, which is not preserved in many other quantification methods[3, 8, 11]. Additionally, this protocol is easily adaptable and can be applied in research and clinical settings, especially for cancer-related research and prognosis.

ACKNOWLEDGMENTS:
This work was partially funded by the Fredrick Banting and Charles Best Canada Graduate Scholarship (A.M.).

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

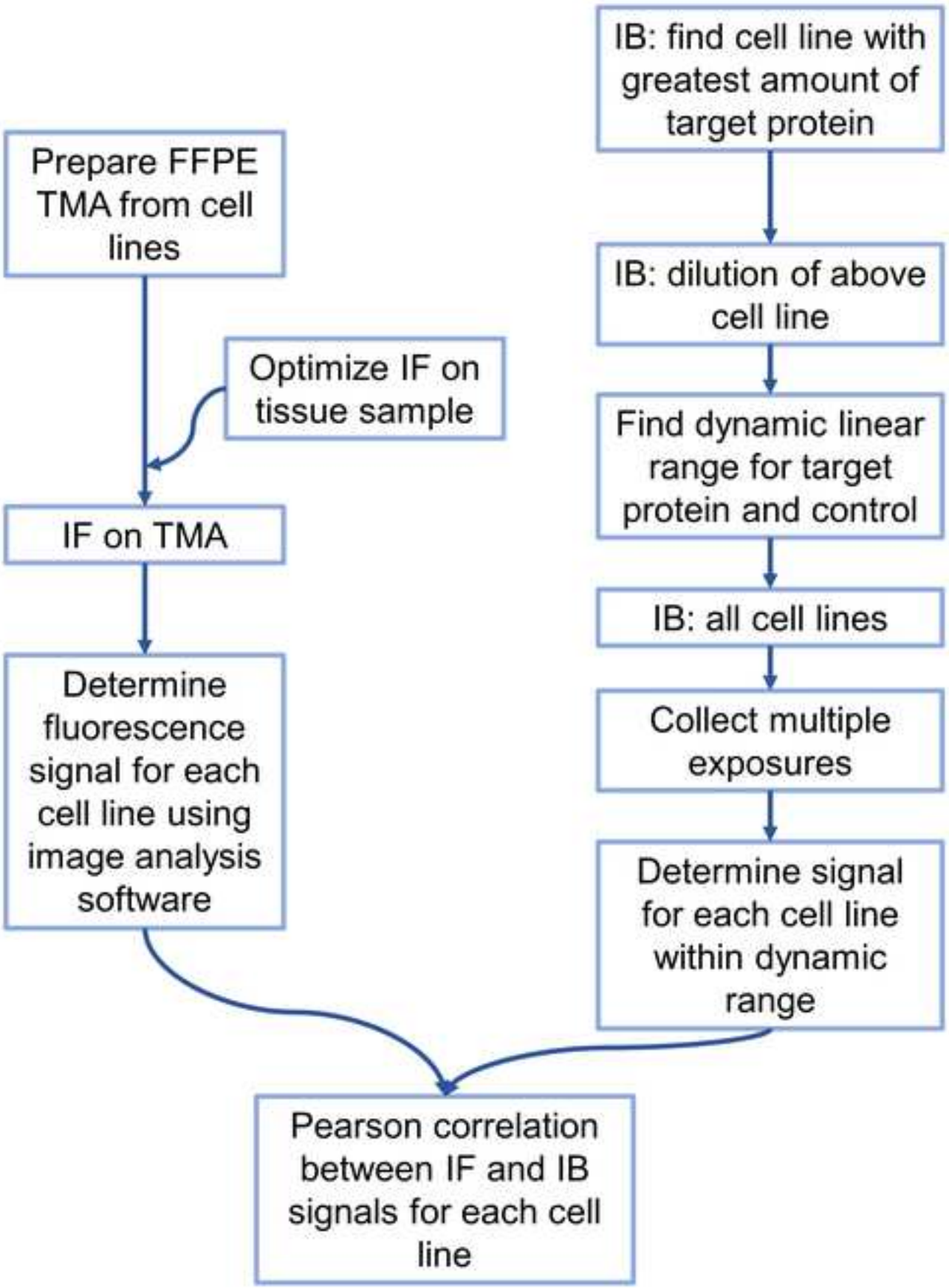


Figure 2

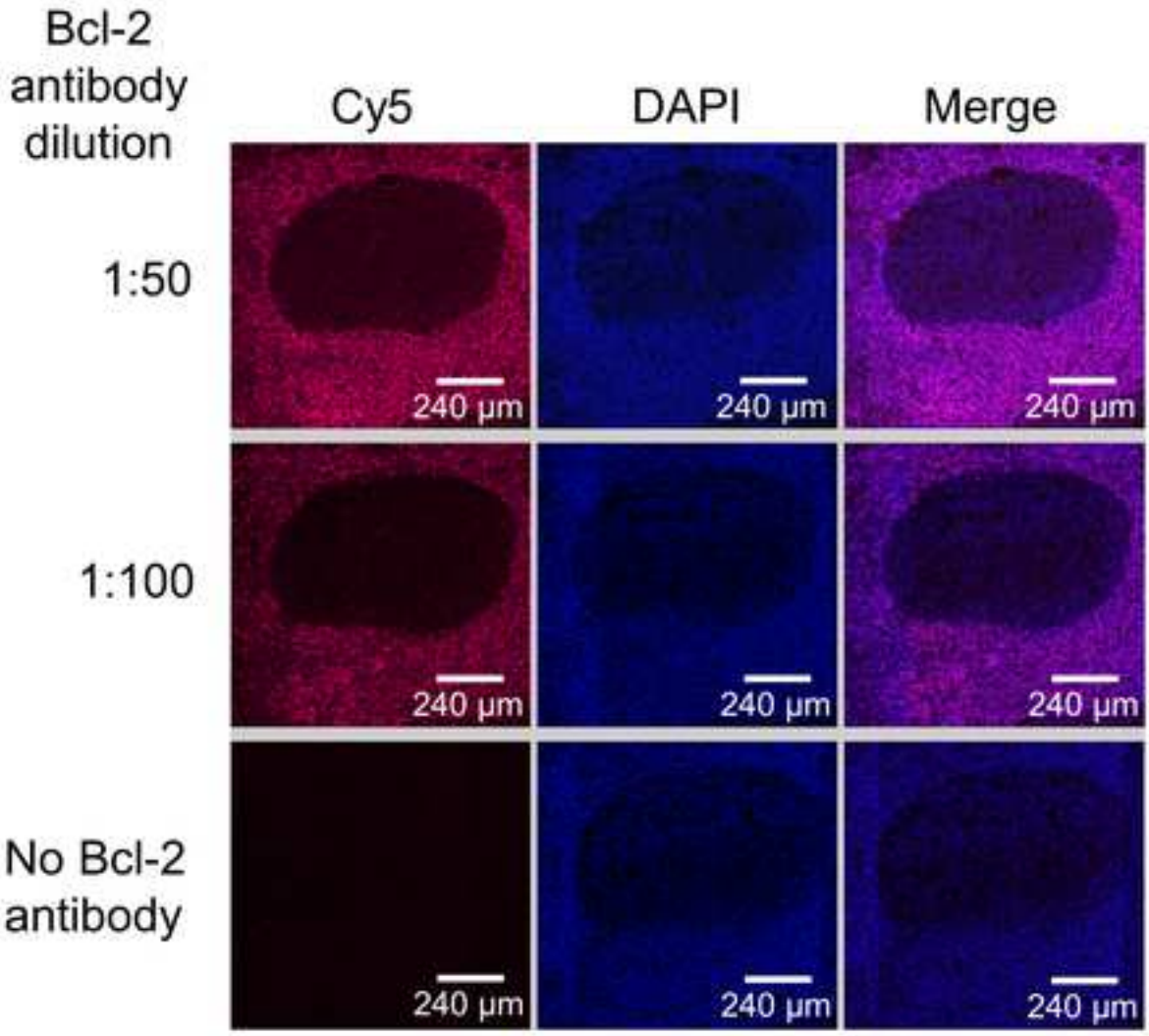
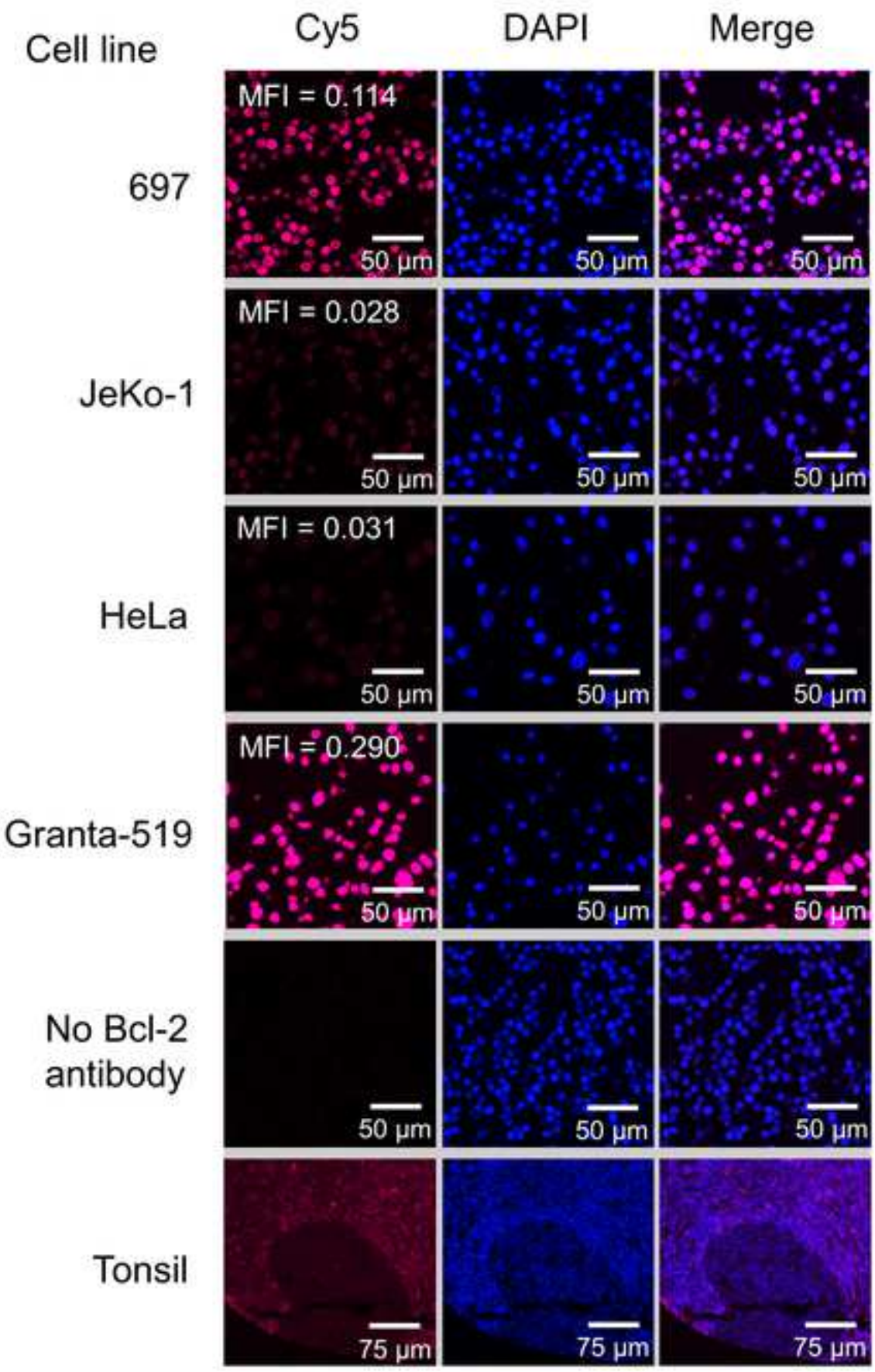


Figure 3



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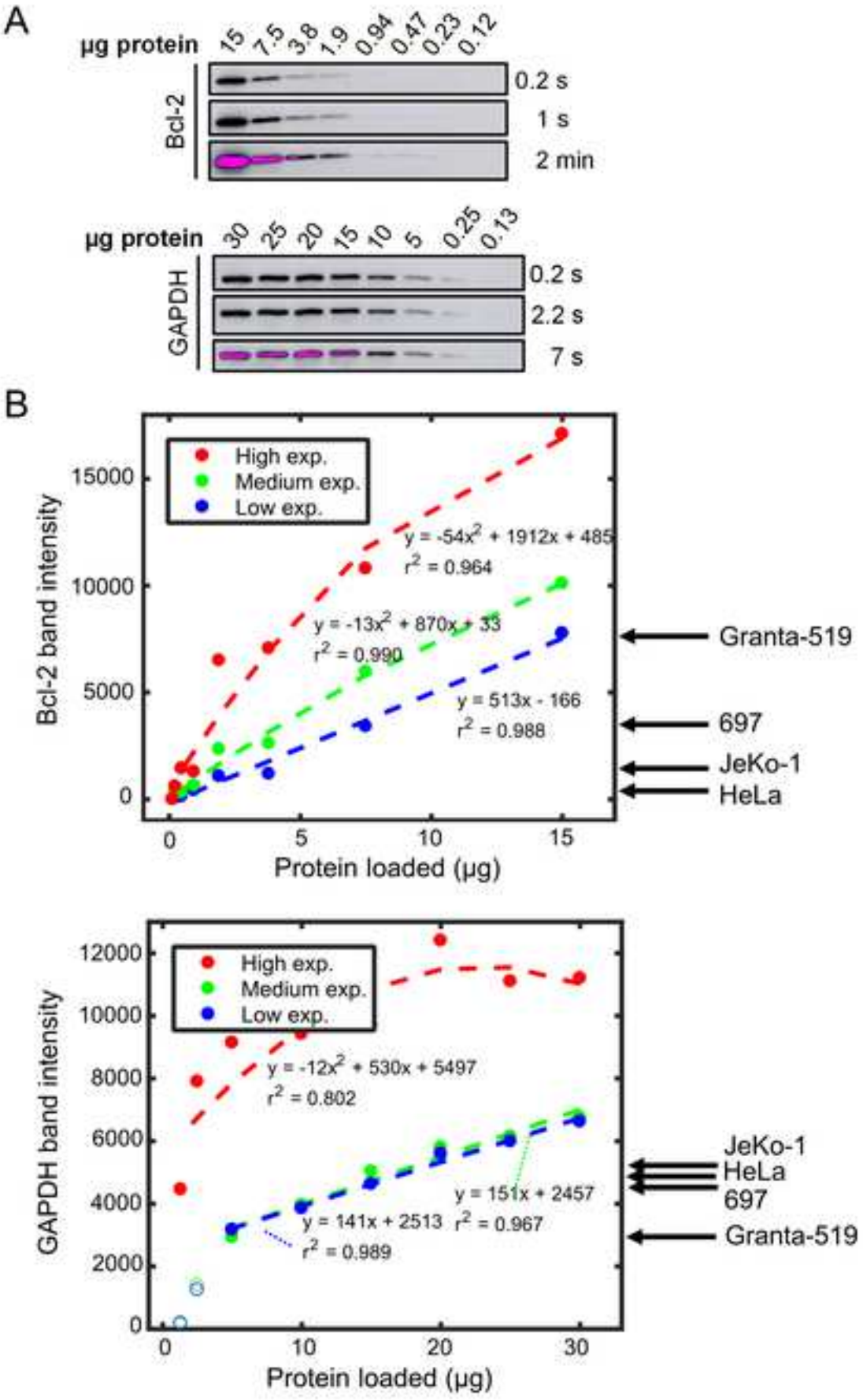


Figure 5

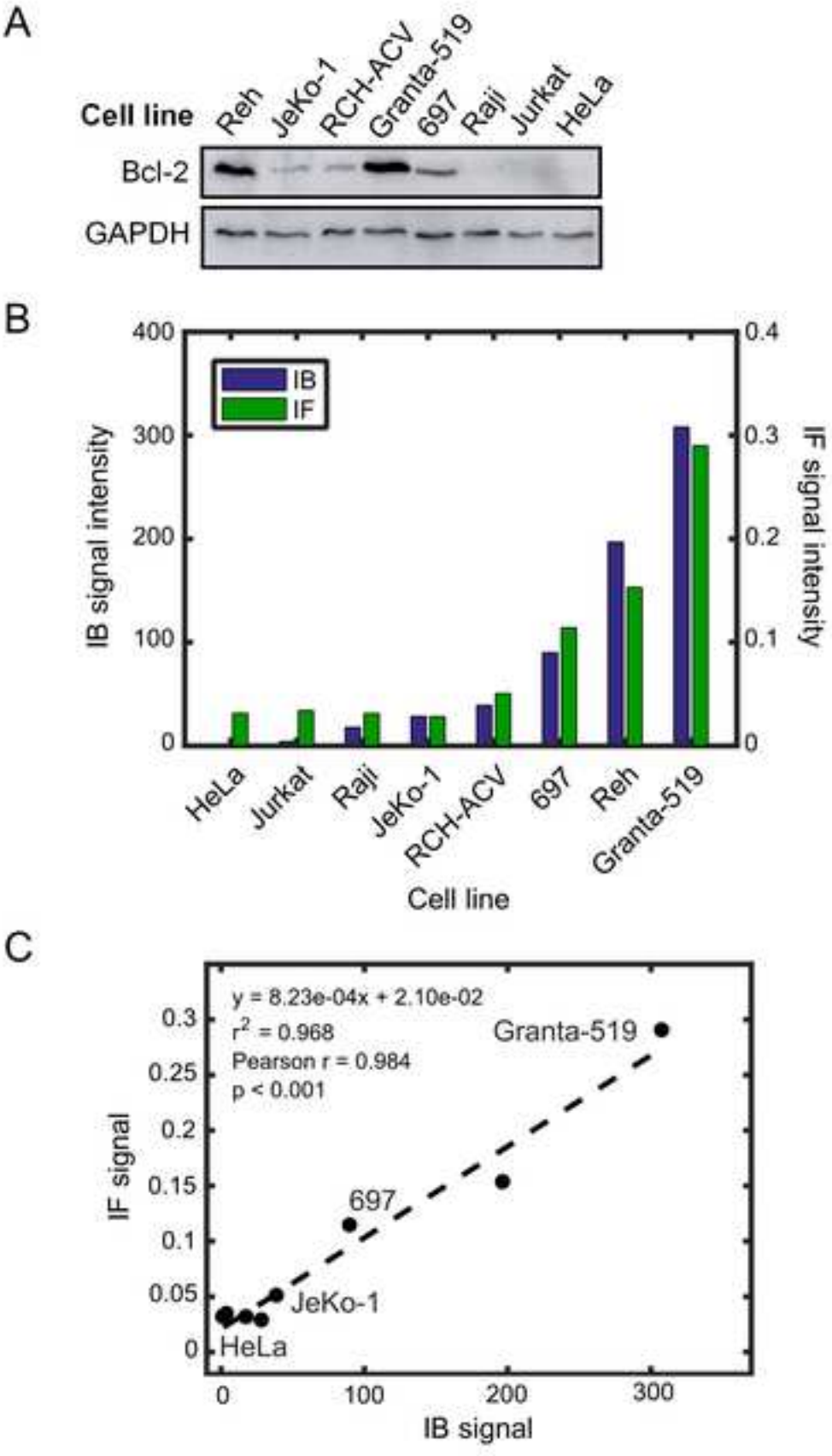
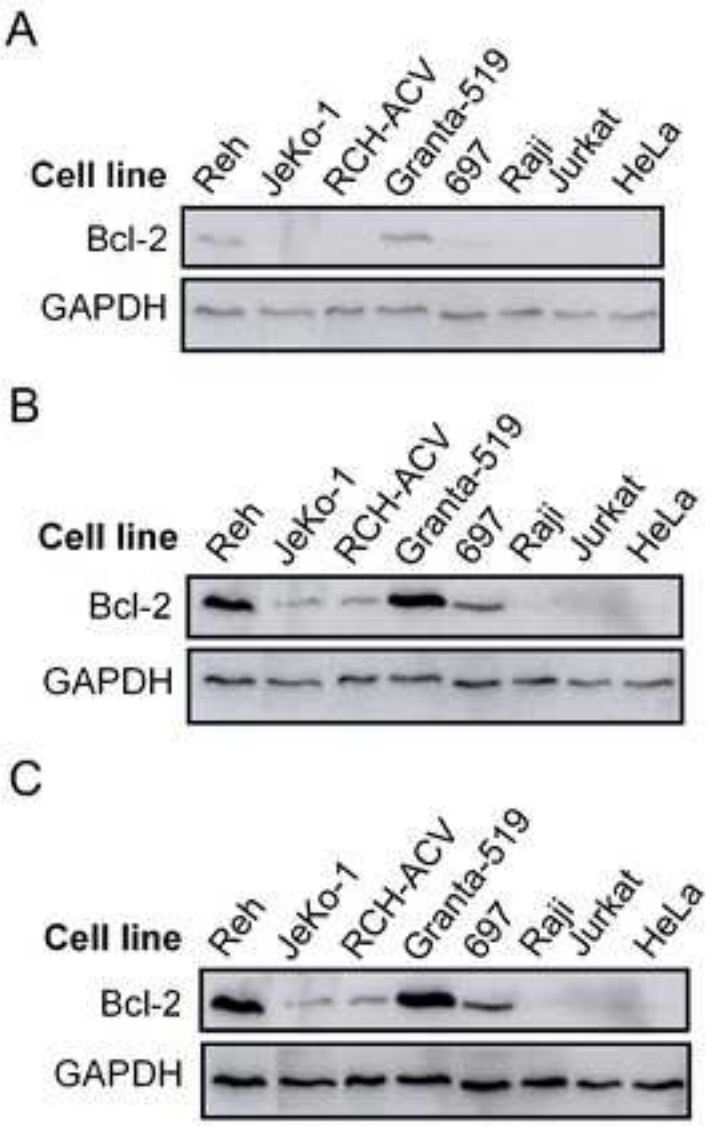


Figure 6



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
697	DSMZ	ACC 42	Cell line
JeKo-1	ATCC	CRL-3006	Cell line
Jurkat	ATCC	TIB-152	Cell line
RCH-ACV	DSMZ	ACC 548	Cell line
Granta-519	DSMZ	ACC 342	Cell line
REH	DSMZ	ACC 22	Cell line
Raji	ATCC	CCL-86	Cell line
HeLa	ATCC	CCL-2	Cell line
Trypsin/EDTA solution	Invitrogen	R001100	For detaching adhesive cells
Fetal bovine serum (FBS)	Wisent Inc.	81150	To neutralize trypsin
Neutral Buffered Formalin	Protocol	245-684	For fixing cell pellets
UltraPure low melting point agarose	Invitrogen	15517-022	For casting cell pellets
Mouse monoclonal anti-human Bcl-2 antibody, clone 124	Dako (Agilent)	cat#: M088729-2, RRID: 2064429	To detect Bcl-2 by immunoflourencence and immunoblot (lot#: 00095786
Ventana Discovery XT	Roche	-	For automation of immunofluorescence staining
EnVision+ System- HRP labelled polymer (anti-mouse)	Dako (Agilent)	K4000	For immunofluorescence signal amplification
EnVision+ System- HRP labelled polymer (anti-rabbit)	Dako (Agilent)	K4002	For immunofluorescence signal amplification
Cyanine 5 tyramide reagent	Perkin Elmer	NEL745001KT	For immunofluorescence signal amplification
Aperio ImageScope	Leica Biosystems	-	To view scanned slides
HALO image analysis software	Indica Labs	-	For quantification of immunofluorescence
Protease inhibitors (Halt protease inhibitor cocktail, 100X)	Thermo Scientific	1862209	To add to RIPA buffer

Ethylenediaminetetraacetic acid (EDTA)	BioShop	EDT001	For RIPA buffer
NP-40	BDH Limited	56009	For RIPA buffer
Sodium deoxycholate	Sigma-Aldrich	D6750	For RIPA buffer
Glycerol	FisherBiotech	BP229	For Laemlli buffer
Bromophenol blue	BioShop	BRO777	For Laemlli buffer
Dithiothreitol (DTT)	Bio-Rad	161-0611	For Laemlli buffer
Bovine serum albumin (BSA)	BioShop	ALB001	For immunoblot washes
Protein ladder (Precision Plus Protein Dual Color Standards)	Bio-Rad	161-0374	For running protein gel
Filter paper (Extra thick blot paper)	Bio-Rad	1703969	For blotting transfer
Nitrocellulose membrane	Bio-Rad	162-0115	For blotting transfer
Trans-blot SD semi-dry transfer cell	Bio-Rad	1703940	For semi-dry transfer
Skim milk powder (Nonfat dry milk)	Cell Signaling Technology	9999S	For blocking buffer
Tween 20	BioShop	TWN510	For wash buffer
GAPDH rabbit monoclonal antibody	Epitomics	2251-1	Primary antibody of control protein (lot#: YE101901C)
Goat anti-mouse IgG (HRP conjugated) antibody	abcam	cat#: ab6789, RRID: AB_955439	Secondary antibody for immunoblot
Goat anti-rabbit IgG (HRP conjugated) antibody	abcam	cat#: ab6721, RRID: AB_955447	Secondary antibody for immunoblot (lot#: GR3192725-5)
Clarity Western ECL substrates	Bio-Rad	1705060	For immunoblot signal detection
Amersham Imager 600	GE Healthcare Life Sciences	29083461	For immunoblot signal detection
ImageJ software	Freeware, NIH	-	For densitometry analysis

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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,

Alison Moore
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