A consensus platform for antibody characterization

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Method Article

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Abstract

Antibody-based research applications are critical for biological discovery. Yet, there are no industry standards to compare the performance of antibodies in various applications. We describe a knockout cell line-based antibody characterization platform, developed, and approved jointly by industry and academic researchers that enables the systematic comparison of antibody performance in western blot, immunoprecipitation, and immunofluorescence procedures. The scalable protocols consist of (i) the identification of appropriate cell lines for antibody characterization studies, (ii) development/contribution of isogenic knockout controls, validated at the protein level, and (iii) a series of antibody characterization procedures focused on the most common uses of antibodies in research. Guidelines for the assessment of antibody performance are presented along with the value of making the resulting data publicly available. This antibody characterization platform can be implemented with minimal technological limitations. The proposed antibody characterization platform may be performed readily on antibodies targeting a protein in roughly one month, with one person working less than full-time. Antibody characterization is needed to meet standards for resource validation and data reproducibility, increasingly required by journals and funding agencies.

Introduction

Antibodies are fundamental tools in biomedical research, yet the absence of standardized performance evaluation methods poses challenges for researchers selecting appropriate reagents. Current reliance on published descriptions and commercial quality control data lacks detailed characterization and data inclusion[1], hindering effective antibody selection. Additionally, the proliferation of commercially available antibodies targeting human proteins further complicates the process, often leading to time-consuming and inefficient searches for optimal reagents[2]. Moreover, instances of invalidated top-cited antibodies have tainted scientific literature[3-9], underscoring the need for standardized comparison methods to improve data quality and reproducibility.

To address these challenges, we introduce "Antibody Characterization through Open Science" (YCharOS), a collaborative effort among academia, leading antibody manufacturers, and knockout (KO) cell line providers. YCharOS has developed an antibody characterization platform based on KO cell lines as isogenic controls for common applications: western blot (WB), immunoprecipitation (IP), and immunofluorescence (IF). This collaboration enables the evaluation of approximately 80% of renewable antibodies accessible in commercial catalogs, with the remaining 20% outside our scope due to their availability from sources beyond our partnerships. This setup facilitates direct comparisons among antibodies targeting a specific protein.

A key strength of the YCharOS platform is the support of industrial partners, who contribute antibodies and KO cell lines, to enable comprehensive antibody characterization[10]. The iterative validation of KO cell lines and antibodies (ideally renewable) for their ability to recognize target proteins expressed at endogenous levels is another key component of the platform. The resulting data are shared openly[10],

benefiting the global biomedical community and promoting robust and reproducible research. By identifying specific antibodies and validating their performance, including the removal of non-specific antibodies from commercial catalogs[11], YCharOS contributes to improving the reliability of scientific data. Moreover, the consensus protocols employed by YCharOS facilitate the creation of a publicly accessible database containing trusted antibody characterization data[12], aiding researchers in antibody selection and reviewers in assessing antibody suitability. While independent researchers may not have access to as many antibodies for any given protein target, the feasibility of characterizing a wide array of available antibodies against a target protein has been demonstrated[4,5,7,13-15].

In the following sections, we detail the development of the YCharOS platform, its comparison with other antibody validation methods, experimental procedures, expertise and equipment requirements, data dissemination strategies, and limitations. Through these efforts, YCharOS aims to address the critical need for standardized antibody characterization methods, ultimately enhancing the reliability and reproducibility of scientific research.

Development of the Platform

The establishment of the YCharOS platform stems from a collaborative effort between academic and industry partners[16], aimed at addressing the critical need for standardized antibody characterization methods[1,17-19]. Co-authors of this article actively participate in the YCharOS public-private partnership, which forms the foundation of this endeavor. Central to the platform are protocols utilizing KO cell lines, with modifications to knockdown (KD) strategies when assessing antibodies targeting essential genes.

To ensure robust and comprehensive protocols, senior scientists from leading antibody manufacturers, including Abcam, Abclonal, Addgene, Aviva Systems Biology, Bio-Techne (comprising Novus Biologicals and R&D Systems), Cell Signaling Technology, Developmental Studies Hybridoma Bank, Genetex, Proteintech, and Thermo Fisher Scientific, collaborated in refining the methodologies. Furthermore, antibody manufacturers donate antibodies to the project, alongside contributions of KO cell lines from Abcam and Horizon Discovery (part of Revvity).

The platform's optimization (Fig. 1) enables the characterization of antibodies against a broad spectrum of human proteins, encompassing soluble, membrane-bound, and secreted proteins. Notably, as of March 2024, the platform has tested 859 antibodies targeting 96 human proteins.

A pivotal aspect of the YCharOS platform is its commitment to transparency and data sharing. To this end, all characterization data generated through collaborations are openly disseminated. Additionally, an analysis code for IF has been developed and made publicly available, facilitating the segmentation and direct comparison of fluorescence intensity between parental and KO cell lines.

Comparison with Other Methods

The International Working Group for Antibody Validation has recommended five antibody characterization methodologies[20]: 1) genetic strategies utilizing KO or KD cell lines as controls; 2) orthogonal strategies correlating antibody signals to known information about the protein of interest; 3) overlap of signals of two independent antibodies recognizing different epitopes in the same target; 4) antibody recognition of an overexpressed tagged version of the protein target; 5) employment of mass spectrometry to determine if the protein target captured by an antibody corresponds to the major signal in the immunoprecipitate[20]. Among these five recommended strategies, the genetic and orthogonal approaches are used approximately 90% of the time by antibody manufacturers, with 61% and 83% of antibodies validated using orthogonal strategies in WB and IF, respectively[11]. Genetic strategies account for 30% and 7% of validations in WB and IF, respectively[11]. Our analysis revealed discrepancies in the specificity of antibodies validated by suppliers, particularly with IF, where only 38% of antibodies validated by orthogonal strategies exhibited specificity[11]. This presents a compelling argument to prioritize genetic strategies for antibody validation.

Utilizing KO cell lines for antibody characterization offers several advantages, particularly in discerning between specific and non-specific binding. An antibody that selectively immunodetects its target protein in WB will produce a distinct band (or potentially multiple bands in presence of isoforms or posttranslational modifications) in the parental lysate that is absent in the KO lysate (Fig. 2a, case 1). A non-selective but specific antibody may recognize the target protein along with other unwanted proteins (Fig. 2a, case 2). Non-specific antibodies fail to recognize the target protein even in a cell line with confirmed target expression (Fig. 2a, case 3). Of the antibodies recommended for WB by their manufacturers, 35% detected their intended targets, as well as unwanted proteins, and 21% failed in detecting their intended target[11]. This finding underscores the importance of utilizing KO cell lines to accurately distinguish between undesired non-specific binding and genuine isoforms, post-translational modifications, or degradation of the target protein.

Despite the validation of edited gene modifications in KO lines through genomic PCR and DNA sequencing, our WB analysis revealed that approximately 14% of KO lines were not null for the target protein. Some KO lines resulted in truncated proteins rather than complete loss (Fig. 2b), emphasizing the necessity of WB screening using both wild-type (WT) and KO lysates to validate target protein expression and antibody specificity, as well as to validate the KO clone.

Experimental Design

The workflow employed by YCharOS involves three common laboratory procedures, WB, IP and IF (Fig. 1). Each antibody is tested for all applications regardless of the manufacturers' recommendations as the use of antibodies can be extended to applications not considered by the manufacturers.

Target protein expression in a particular cell line is assessed using RNA sequencing data available on the Cancer Dependency Map Portal (RRID:SCR_017655, "DepMap", depmap.org). The DepMap Portal

presents transcriptomic profiles of over a thousand cancer cell lines[21]. The target RNA expression value of 2.5 $\log_2$(transcripts per million (TPM)+1) has emerged as a minimal threshold that is likely to yield detectable protein levels suitable for antibody screening[11]. While cell line selection can be straightforward for proteins with high, ubiquitous expression, this step can be difficult for proteins expressed at lower levels or only in specific cell types. For this latter situation, we combine orthogonal and independent antibody validation strategies[20] to help in selecting an optimal cell line background for generating a KO cell line. Briefly, we select 4-8 available cancer cell lines with the highest RNA score, as well as one or two lines with an RNA score close to or equal to zero, and use at least two unique antibodies (i.e., different clone numbers if monoclonal antibodies) to assess protein expression in WB. This has proven useful when at least two antibodies show similar protein expression patterns in the cell lines with high RNA values and no signal in the lines with RNA close to zero, suggesting specificity to the target (Fig. 3a, case 1 and 2). A non-specific antibody would produce a different protein expression pattern than putative specific antibodies and would not correlate with RNA levels (Fig. 3a, case 3). The generation of a KO line remains essential to confirm the specificity of the signal and ascertain whether the selected parental cell line exhibits appropriate expression of the target protein (Fig. 3b). For instance, the SYT1 antibody utilized in case 1 selectively detects several SYT1 protein species, while the SY1 antibody in case 2 detects SYT1 along with undesired proteins, a scenario verifiable only using the KO cells and not through the orthogonal strategy (Fig. 3).

Sample preparation for WB and IP varies depending on whether the target of interest is an intracellular (Fig. 4, case 1) or secreted protein (Fig. 4, case 2). A secreted protein is defined as having a signal peptide and no transmembrane domains. It has been predicted that ~3000 human proteins are secreted (referred to as the secretome), representing ~15% of the human proteome[22]. These proteins are expected to be primarily identified in the conditioned medium of cell lines (Fig. 4, case 2). However, ~35% of the secretome may remain intracellular awaiting secretion in the absence of an appropriate releasing stimulus or are not released to the medium due to retention on the plasma membrane following secretion[22]. In the latter cases, the target protein can be detected both in the conditioned medium and in the cell lysate (Fig. 4, case 3). Subcellular annotation from UniProt (RRID:SCR_002380, uniprot.org) can be utilized to predict whether a protein is secreted, yet the identification of the target protein in the medium by mass spectrometry or using specific antibodies provides the definitive evidence that a protein can be secreted.

Cell preparation for Antibody Screening

The preparation of the parental and KO cell line samples for application testing can be carried out simultaneously or prior to each application. The procedures detailed below involve the use of adherent cell lines. One confluent 150 mm petri dish of the most common cancer cell lines corresponds to approximately 2 x 10^7 cells and 2 mg of protein lysate. Therefore, one confluent 150 mm dish is sufficient to test 14 antibodies by WB (20-50 µg per lane), two antibodies by IP (1.0 mg protein per IP) and 14 antibodies by IF (8,000 cells per well).

Procedure 1: Antibody Screening by WB

The radioimmunoprecipitation assay (RIPA) denaturing buffer extracts most intracellular proteins from culture cell lines, including cytoplasmic, nuclear, and membrane-bound proteins[23]. The commercial RIPA buffer used is composed of 25 mM Tris-HCl pH 7.8 (pH measured at 4°C), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. Secreted proteins are harvested directly from the cell culture medium. To this end, cells are grown in a serum-free medium for 18 hrs, media are collected, and debris in suspension are removed by centrifugation. The cleared media are then concentrated by filtration. The desired amount of WT/KO lysates or WT/KO cell media are run on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All antibodies are tested in parallel in WB.

Procedure 2: Antibody Screening by IP

Antibodies can be used to immunocapture target proteins from cell extracts or media. Antibody performance in IP can be assessed using mass spectrometry approaches[24], which for most laboratories is costly and time-consuming. To assess if an antibody can IP the target, IPs are performed using cell lysates generated in a non-denaturing buffer or using cell media, followed by WB (IP-WB) with KO-validated antibodies identified in the previous WB screening. A successful antibody should enrich its intended target in the IP, as compared to the starting material, and deplete it from the unbound fraction (Fig. 5). The unbound fraction is collected once incubation of the protein sample with the bead/antibody conjugate is complete.

Cell lysates (starting material) are prepared using a non-denaturing commercial lysis buffer (IP buffer) composed of 25 mM Tris-HCl pH 7.8 (pH measured at 4°C), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol. This buffer allows the efficient extraction of all targets tested thus far, including cytosolic, nuclear and membrane-bound proteins. Cell lysates are first incubated with antibody-bead conjugates. After incubation with the lysate, an aliquot of the unbound fraction is collected. Antibody-bead conjugates are then washed with lysis buffer to remove or minimize unbound and non-specifically bound proteins. Following the final wash, bound protein(s) are eluted from the beads. Similar volumes from the starting material and unbound fractions are run on SDS-PAGE side-by-side with the eluted fraction, followed by WB to detect the target protein. We were able to identify at least one antibody that can capture its intended protein for 73 out of 95 proteins (77% success rate).

Procedure 3: Antibody screening by IF

In IF studies, fixation and cell permeabilization steps enable antibodies to reach their intracellular targets. Standardization of IF protocols is challenged by the diversity of fixation and permeabilization reagents and concentrations. A study comparing the suitability of six IF protocols with known specific antibodies targeting 18 proteins with distinct subcellular distributions revealed that fixation with paraformaldehyde (PFA) followed by permeabilization with Triton X-100 was adequate for detecting all proteins analyzed in their study, suggesting that a PFA/Triton X-100 based protocol is adequate for a significant number of human proteins[25]. The processes described here use 4% PFA with 0.1% Triton X-100 for permeabilization

and 0.01% for the later steps. While we recognize that this protocol will not be suitable for all human proteins, we were able to identify at least one specific antibody suitable in IF for 49 out of 82 intracellular proteins (60% success rate).

For IF, we use parental and KO cells labelled with fluorescent dyes of different wavelengths and then plated the cells together as a mosaic. Staining is performed with primary antibodies and a secondary antibody coupled to a fluorophore that emits at a different wavelength from that of the cell dyes. This mosaic strategy enables screening in a single well (or cover slip), thus avoiding imaging or user bias when comparing WT and KO cells in a single field of view.

The use of a high-content imaging system, designed to image numerous fields of view per well, enables rapid imaging of thousands of cells for all antibodies tested. The goal of the IF approach is not to determine the cellular location of the target protein but to determine whether there is a significant difference in overall signal coming from WT and KO cells. A larger collection of IF images make analysis more robust. We have developed a collection of scripts in Python and in ImageJ (RRID:SCR_003070) or FIJI (RRID:SCR_002285) made openly available on GitHub (github.com/ABIF-McGill/YCharOS_IF_characterization) to quantify and compare fluorescence from parental cells and KO cells. Generally, this quantitative analysis pipeline consists of object detection to generate masks of WT and KO cells, followed by background estimation and subtraction, followed by intensity measurement of antibody labeling intensity in each detected cell in each image. Antibody intensity in WT vs KO cells can be expressed as a ratio for each cell and plotted to compare antibody labeling intensity of several different antibodies for a given target. This more detailed analysis of numerous cells improves the comparison of performance between antibodies. Moreover, antibody titration is performed routinely on IF experiments, where two concentrations are tested, including the concentration recommended by the manufacturers, when available.

Expertise and Specialized Equipment Needed to Implement the Protocol

The protocols described here can be adapted to most standard molecular/cell biology laboratories. For cell culture, WB and IP, most trainees with prior biochemistry knowledge will be capable of performing these protocols or could learn them with appropriate training that would be of value for exploring or initiating a career in related research areas.

IF steps require training in microscopy and in fluorescence image analysis. While all analyses can be performed on standard desktop computers with minimal software requirements (including the open access FIJI software) using the provided analysis code, the cellpose (RRID:SCR_021716) segmentation code works best on a system with a Compute Unified Device Architecture (CUDA)-capable graphics card.

Data Dissemination and Uptake by the Research Community

YCharOS's data generation and dissemination are intended to benefit the global life sciences community, but its impact depends on real-world uptake of the data. To date, 859 different antibodies targeting 96 human proteins have been tested and characterization data are consolidated in the form of reports, with one report per protein. Reports are uploaded on ZENODO (RRID:SCR_004129), an open repository operated by the European Organization for Nuclear Research: https://zenodo.org/communities/ycharos/, and assigned a Digital Object Identifier (DOI). Datasets, which include raw data for all applications, can also be viewed and downloaded on ZENODO. To test the possibility of better outreach by indexation on PubMed, some ZENODO reports are converted into peer-review articles published in F1000 (www.f1000research/ycharos), accompanied by a guide to help interpret the antibody characterization data[10].

To ensure the proper identification of each antibody tested, each YCharOS report presents detailed antibody information, including antibody concentration, batch number and Research Resource Identifier (RRID). An RRID is a unique and persistent tag assigned to an antibody (and other research resources) that integrates the following detailed information in the case of antibodies: the target antigen, antibody clonality, catalogue number and supplier, clone ID, application(s) recommended by the manufacturers, host organism and availability of third-party validation data. Over 2.5 million antibodies are registered with an RRID and listed in the Antibody Registry (RRID:SCR_006397, antibodyregistry.org) and in the RRID portal (RRID:SCR_003115, https://scicrunch.org). RRIDs represent the gold standard for research reagent identification and are requested by over 1000 journals[26-28]. They facilitate access to third-party characterization data through the RRID portal, and the integration of characterization data with RRIDs via Biomed Resource Watch (https://scicrunch.org/ResourceWatch) could potentially establish the RRID portal as the primary centralized database for genetically validated antibodies[12].

Importantly, the participating antibody manufacturers, who have endorsed these protocols through extensive dialogue, and are represented as co-authors of this article, are also actively using the antibody characterization data in their marketing materials to help scientists select the most appropriate products for their research needs. In addition, these same companies are withdrawing or re-evaluating antibodies whose performance in these assays appears substandard[11], underlining the importance of informing antibody manufacturers in the latter case. Finally, targets for which better antibodies are needed are identified and perhaps designated for the development of new antibodies.

Limitations

Antibodies are among the most useful reagents in the biomedical sciences due to their ability to bind proteins or other antigens with high affinity and specificity, providing information on target abundance, cellular location, binding partners, modifications, and other biochemical or cellular features. Beyond the described protocols, antibodies find extensive application in techniques like flow cytometry, ELISA, and immunohistochemistry.

When faced with a multitude of antibody options for a specific protein, users can utilize the following guidelines for selection: i) prioritize recombinant or monoclonal antibodies with designated clone numbers to prevent duplicate purchases from different suppliers and ensure reagent renewability, ii) give preference to primary manufacturers with rigorous internal validation standards that offer refund policies if users demonstrate antibody specificity issues, iii) choose antibodies characterized using KO or KD cells, with data provided by manufacturers or referenced in published articles. Typically, commercial antibody vials contain 50 to 100 µg of purified antibody at concentrations ranging from 0.1 to 1.0 mg/ml, sufficient for conducting the described protocols.

Several factors can influence the performance of antibodies in different applications, with the abundance of the target protein in the cell line used being a critical factor. For example, MDA-MB231 cells display, according to DepMap, a *CD44* RNA level of 9.6 $\log_2$(TPM+1) and a ~10-fold increase in CD44 protein expression as compared to HAP1 as observed by WB (Fig. 6a, case 1). While some antibodies can detect CD44 in both cell types (Fig. 6a, case 1), others can only detect CD44 in the higher expressing, MDA-MB231 line (Fig.6a, case 2). This example illustrates the inconsistency of antibody evaluation based on the use of a single cell line – the CD44 antibody in case 2 would have been evaluated as non-specific using HAP1, but specific in MDA-MB231. Protein abundance also affects antibody performance in IF. For example, the THP-1 cell line presents with a *PLCG2* RNA level of 5.9 $\log_2$(TPM+1) according to DepMap. THP-1 is a monocyte-like cell line that grows in suspension and can be differentiated into adherent macrophage-like cells following a treatment with phorbol 12-myristate 13-acetate (PMA)[29]. As antibodies are routinely tested in IF on adherent cell lines, a PMA treatment therefore enables PLCG2 antibodies to be tested on THP-1 cells. A WB analysis with a KO-validated PLCG2 antibody reveals that while PMA treatment slightly reduces the PLGC2 protein level in THP-1, the THP-1 treated cells still exhibit a ~3-fold increase in PLCG2 protein level as compared to HAP1 (Fig. 6b, WB). The same PLCG2 antibody used in IF on HAP1 detects a signal similar between the WT and KO cells. However, the signal generated by the antibody in THP-1 treated with PMA is selective as evidenced by the high signal observed in WT cells and the absence of signal in KO cells (Fig. 6b, IF).

Despite the robustness of the WB, IP, and IF protocols described, differences in buffers, blocking reagents, antibody dilutions, and other protocol details can influence antibody performance[25,30]. Nevertheless, utilizing these protocols offers a productive strategy for assessing the application-specific performance of antibodies and optimizing their selection.

The absence of a universal, public collection of KO human cell lines hinders the ability of scientists to immediately use the described protocols. However, a significant portion of human genes have already been targeted and knocked out in cell lines generated by academic researchers. Cellosaurus (RRID:SCR_013869, https://www.cellosaurus.org/) is a knowledge resource that assigns an RRID identifier to cell lines used in biomedical research, including KO cell lines, whether generated by academic laboratories or industry[31]. Searching Cellosaurus (release 48, February 1, 2024) indicates that 13,644 KO cell lines covering 4,873 human genes have been generated with the majority being commercially

available. For example, Horizon Discovery has a collection of over 6,000 KO cell lines targeting more than 3,000 genes in the human HAP1 cell line. Horizon Discovery HAP1 KO lines are now part of Revvity's portfolio. Abcam has a catalogue of about 5,300 KO cell lines covering 2,915 human genes in various cell line backgrounds. ATCC recently made available 280 KO cell lines covering solute carrier protein superfamily members, that were generated by the RESOLUTE consortium[32]. While commercial KO cell lines are well-documented, those generated by individual researchers are often not registered, highlighting a gap in data accessibility. Adding a cell line to Cellosaurus can be done by writing to https://www.cellosaurus.org/contact.

While the platform presented is suitable for most proteins, it may require optimization for certain cases to achieve the desired signal-to-noise ratio. Notably, a KO-based methodology may not be applicable for evaluating antibodies targeting posttranslational modifications or essential genes. Hence, end users are strongly encouraged to conduct validation experiments in their own laboratories, as differences in protocols and cell lines can influence results.

Reagents

· 1.5 ml microtubes (Sarstedt, cat. no. 72.706)

· 15 ml conical tubes (Thermo Fisher Scientific, cat. no. 339658)

· 1x PBS sterile (Wisent, cat. no. 311-010-CL)

· 5 ml microtubes (Wards Sciences, cat. no. 470225-020)

· 50 ml conical tubes (Thermo Fisher Scientific, cat. no. AM12501)

· 96-well plates, clear flat-bottom (Revvity, cat. no. 6055300)

· Anti-mouse IgG for IP (HRP) (Abcam, cat. no. ab131368)

· BLUelf Prestained Protein Ladder (FroggaBio, cat. no. PM007-0500K)

· Boric acid (Fisher Scientific, cat. no. A73-3)

· Bovine serum albumin (Wisent, cat. no. 800-095)

· Bradford reagent (MilliporeSigma, cat. no. B6916)

· Cell culture dishes, 150 mm (Fisher Scientific, cat. no. 08-772-6)

· Cell scraper (Sarstedt, cat. no. 83.1830)

· CellTracker deep red dye (Thermo Fisher Scientific, cat. no. C34565)

· CellTracker green CMFDA dye (Thermo Fisher Scientific, cat. no. C2925)

· Centrifugal filter unit, Amicon Ultra-15 (Millipore, cat. no. UFC901096)

· Dako mounting medium (Dako, cat. no. S3023)

· DAPI (Thermo Fisher Scientific, cat. no. D3571)

· Dimethyl sulfoxide (DMSO; Sigma, cat. no. D8418)

· Fetal bovine serum (FBS; Wisent, cat. no. 080450)

· Goat anti-mouse Alexa-555 conjugated secondary antibody (Thermo Fisher Scientific, cat. no. A21424)

· Goat anti-mouse HRP conjugated secondary antibody (Thermo Fisher Scientific, cat. no. 62-6520)

· Goat anti-rabbit Alexa-555 conjugated secondary antibody (Thermo Fisher Scientific, cat. no. A21429)

· Goat anti-rabbit HRP conjugated secondary antibody (Thermo Fisher Scientific, cat. no. 65-6120)

· Invitrogen HiMark Pre-stained Protein Standard (Thermo Fisher Scientific, cat no. LC5699)

· IP lysis buffer (Thermo Fisher Scientific, cat. no. 87788)

· L-Glutamine (Wisent, cat. no. 609-065-EL)

· LDS Sample Buffer (4X) (Thermo Fisher Scientific, cat. no. NP0007)

· LSB, Laemmli SDS sample buffer, reducing (6X) (Thermo Fisher Scientific, cat. no. J61337.AD)

· MES-SDS running buffer (20X) (Thermo Fisher Scientific, cat. no. J62138.K2)

· Nitrocellulose membrane (Bio-Rad, cat. no. 1620097)

· Normal goat serum (NGS) (Gibco, cat. no. 16210-064)

· Paraformaldehyde solution, 16% w/v in water (Beantown chemical, cat. no. 140770-10ml)

· Penicillin-Streptomycin, 100x (Wisent, cat. no. 450-201-EL)

· Peroxidase substrate, Femto (Thermo Fisher Scientific, cat. no. PI34096)

· Peroxidase substrate, regular (Thermo Fisher Scientific, cat. no. PI32106)

· PhenoPlate, 96-well, optically clear flat-bottom (Perkin Elmer, cat. no. 6055300)

· Pierce BCA protein assay kit (Thermo Fisher Scientific, cat. no. 23225)

· Poly-L-lysine (Sigma Aldrich, cat. no. P9155-5MG)

· Ponceau S powder (Thermo Fisher Scientific, cat. no. BP103-10)

· Precast Bis-Tris polyacrylamide gels, 12 wells, midi (Thermo Fisher Scientific, cat. no. WG1201BOX)

· Precast Tris-Acetate polyacrylamide gels, 12 wells, midi (Thermo Fisher Scientific, cat. no. WG1601BOX)

· Precast Tris-Glycine polyacrylamide gels, 12 wells, midi (Thermo Fisher Scientific, cat. no. WXP42012BOX)

· Prestained molecular weight marker (FroggaBio, cat. no. PM007-0500K)

· Protease inhibitor cocktail mix (Millipore Sigma, cat. no. P8340)

· Protein A Dynabeads (Thermo Fisher Scientific, cat. no. 10001D)

· Protein A–Peroxidase HRP (Millipore Sigma, cat. no. P8651)

· Protein G Dynabeads (Thermo Fisher Scientific, cat. no. 10004D)

· Protein L magnetic beads (Thermo Fisher Scientific, cat. no. 88850)

· RIPA lysis buffer (Thermo Fisher Scientific, cat. no. 89901)

· Skim milk powder, (Bioshop, cat. no. SKI400.1)

· Transfer buffer 10x, Tris-Glycine (Bio-Rad, cat. no. 1610771)

· Trichloroacetic acid (Fisher scientific, cat. no. SA433-500)

· Tris buffered saline with Tween 20 (TBST) 10x (Cell Signaling Technology, cat. no. 9997)

· Tris-Acetate SDS Running buffer (20x) (Thermo Fisher Scientific, cat. no. LA0041)

· Tris-Glycine SDS Running buffer (10x) (Bio-Rad, cat. no. 1610772)

· Triton X-100 (Thermo Fisher Scientific, cat. no. BP151-500)

· Trypsin (Wisent, cat. no. 325-542)

Reagent setup

· **Labelling culture medium** The appropriate type of complete medium but supplemented with only 5% serum.

· **Serum-free medium** The appropriate type of medium supplemented with all components except serum.

· **Borate buffer (0.15 M, pH 8.4)** Weigh 4.64 g of boric acid powder. Add the boric acid to a glass beaker containing 450 ml of distilled water and stir until the powder is completely dissolved. Adjust the pH to 8.4 using 10N NaOH. Complete to 500 ml with distilled water.

· **CellTracker deep red dye, 1000x solution** Dissolve 15 µg of CellTracker deep red dye with 40 µl of DMSO. Aliquot into 5 µl samples.

· **CellTracker green CMFDA dye stock, 1000x solution** Dissolve 50 µg of CellTracker green CMFDA dye with 20 µl of DMSO. Aliquot into 5 µl samples.

· **Complete IP lysis buffer** Add 10 µl of the protease inhibitor cocktail into 1.0 ml of ice-cold IP lysis buffer. Keep on ice and use immediately.

· **Complete RIPA lysis buffer** Add 10 µl of the protease inhibitor cocktail into 1.0 ml of ice-cold RIPA lysis buffer. Keep on ice and use immediately.

· **DAPI stock concentration (5 mg/ml)** Dissolve 10 mg of DAPI in 2.0 ml of deionized water. Aliquot and store at -20°C.

· **DAPI working concentration (5 µg/ml)** Add 5 µl of the DAPI stock concentration (5 mg/ml) to 5 ml of water. Prepare 1 ml aliquot at store at -20°C for years.

· **IF blocking buffer (1x PBS, 0.01% Triton X-100, 5% BSA, 5% NGS)** To 47.5 ml of IF incubation buffer, add 2.5 ml of NGS. Mix gently at 4°C and use immediately.

· **IF DAPI solution (1x PBS + 5 ng/ml DAPI)** Add 5 µl of DAPI working concentration (5 µg/ml) to 5 ml of 1x PBS.

· **IF fix buffer (0.5x PBS, 8%PFA, 20% sucrose)** Combine 5 ml of PBS 1x to 5 ml of PFA 16% in water. Dissolve 2 g of sucrose into a 10 ml solution made of 5 ml of PBS 1x and 5 ml of PFA 16% in water.

· **IF incubation buffer (1x PBS, 0.01% Triton X-100, 5% BSA)** To 80 ml of 1x PBS, add 10 µl of Triton X-100 and 5 g of BSA. Rock gently at 4°C until the BSA is completely dissolved. Complete at 100 ml with 1x PBS. Keep on ice and store at 4°C for 1 week.

· **IF permeabilization buffer (1x PBS, 0.1% Triton X-100)**. Add 50 µl of Triton X-100 to 50 ml of 1x PBS. Mix gently and store at 4 °C for 1 week.

· **Poly-L-lysine stock solution (1.0 mg/ml)** Dissolve 5 mg of poly-L-lysine in 4 ml of distilled water to make a stock at 1.0 mg/ml. Complete to 5 ml with distilled H2O.

· **Poly-L-lysine working solution (10 µg/ml)** Dilute the poly-L-lysine stock to 1:100 with 0.15M borate buffer (pH 8.4) to generate a final concentration at 10 µg/ml. Sterilize by filtration using a 0.2 µm filter unit.

· **Ponceau S working solution** Dissolve 1 g of Ponceau S powder in 485 ml of deionized water. Add 15 ml of trichloroacetic acid. Protect from light and store at room temperature.

· **Running buffer Tris-Acetate SDS 1x** Add 50 ml of Tris-Acetate SDS running buffer 20x to 950 ml of distilled water.

· **Running buffer Tris-Glycine SDS 1x** Add 100 ml of Tris-Glycine SDS running buffer 10x to 900 ml of distilled water.

· **TBST 1x** Add 100 ml of TBST 10x to 900 ml of distilled water.

· **Transfer buffer Tris-Glycine 1x (20% Methanol)** Add 150 ml of Tris-Glycine transfer buffer 10x to 1,050 ml of distilled water. Add 300 ml of Methanol before transfer.

· **WB blocking solution, used also for primary and secondary antibody preparation** Dissolve 5 g of non-fat milk powder in 100 ml of TBST1x. Prepare just before use.

Equipment

· Cell culture incubator (Forma Scientific, cat. no. 1998-081)

· Megafuge™ 16 Centrifuge (Thermo Fisher Scientific, cat. No. 75004270)

· Optima MAX-XP Ultracentrifuge (Beckman Coulter)

· TLA-100.3 Fixed-Angle Rotor (Beckman Coulter, cat. no. 349490)

· Open-Top Thickwall Polycarbonate Tube, 3.5 ml (Beckman Coulter, cat. no. 349622)

· DynaMag-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)

· Heat block (Fisher Scientific, cat. no. 11.718)

· High content microscopy system, ImageXpress (Molecular Device)

· iBright chemiluminescence instrument (Thermo Fisher Scientific, cat. no. CL1500)

· pH meter (Thermo Fisher Scientific, cat. no. STAR2116)

· Plate reader (Thermo Fisher Scientific, cat. no. A51119700C)

· Rotating mixer (Fisherbrand, cat. no. 88861041)

· Shaker, multiplatform (Fisherbrand, cat. no. 88861021)

· Sonicator (Thermo Fisher Scientific, cat. no. FB120A110

· Wet protein transfer system – Criterion blotter (Bio-Rad, cat. no. 1704070)

· XCell4 SureLock™ Midi-Cell electrophoresis system (Thermo Fisher Scientific, cat. no. WR0100)

Procedure

The described platform is intended to screen up to 14 antibodies at a time against a single protein target and can be adapted to test hundreds. All timings listed below do not include cell culture time. The cell culture requirement is sufficient to screen, in parallel, 14 antibodies directed against the same protein target, in 3 applications.

Procedure 1: Antibody screening by WB ● Timing 2 Days

1A) Protein extraction

Option 1: Cell lysate preparation for WB – *intracellular protein* ● Timing 1.5 hrs (Day 1)

i. Grow 2 x 150 mm dishes of parental cells and 2 x 150 mm dishes of KO cells to 80% confluence in complete medium (see the section “Cell preparation for Antibody Screening” for confluence instructions).

ii. Place the culture dish on ice, remove and discard medium.

iii. Wash the adherent cells three times with ~10 ml of ice-cold PBS 1x. Ensure total removal of PBS between washes using, for example, a vacuum.

iv. After the last wash, add 1.0 ml of RIPA lysis buffer supplemented with protease inhibitor cocktail mix to each 150 mm dish.

v. Use cell scrapers to detach adherent cells.

vi. Collect and pool the cell lysates from the same condition together into a 5 ml tube.

vii. Sonicate both cell lysates 3 x 5 sec at 40% amplitude.

viii. Rock for 30 min at 4 °C.

ix. Centrifuge at ~110,000 x *g* for 15 min at 4°C using a refrigerated ultracentrifuge.

CRITICAL STEP Ultra-speed centrifugation pellets insoluble contaminants that would otherwise adhere to the beads-antibody conjugate and subsequently interfere with the detection of the captured protein in WB. While maximum speeds achievable with a table-top centrifuge (15,000 - 20,000 x g) are commonly employed to clear lysates, we have observed that such centrifugation does not adequately remove insoluble particles.

x. Gently remove the tubes from the rotor and place them on ice.

xi. Transfer supernatants to fresh 1.5 ml microtubes kept on ice. Discard pellets.

PAUSE POINT Aliquots of parental and KO cell lysates can be stored at -20 °C for six months and at -80 °C for a year.

***Option 2*: Culture medium collection for WB – *secreted protein* ● Timing 1.5 hrs (Day 1)**

i. Grow 3 x 150 mm dishes of parental cells and 3 x 150 mm dishes of KO cells to 80% confluence in complete medium.

ii. Wash all dishes 3x with sterile warm PBS 1x under a laminar flow cell culture hood.

iii. Add 20 ml of warm serum-free medium to each 150 mm plate.

CRITICAL STEP A serum-free medium is used to avoid contaminating the pool of secreted cellular proteins with highly abundant exogenous proteins present in bovine serum.

iv. Incubate plates in an incubator at 37°C, 5% CO_2 for 18 hrs.

v. Collect media in 50 ml conical tubes on ice.

vi. Centrifuge the 50 ml tubes at 500 x *g* for 10 min at 4 °C to eliminate cells and large contaminants.

vii. Transfer the supernatants to new 50 ml conical tubes and centrifuge at 4,000 x *g* for 10 min at 4 °C to eliminate small contaminants.

viii. Transfer the supernatant to new 50 ml conical tubes on ice.

ix. Add 15 ml of cleared medium to each 15 ml centrifugal filter unit.

CRITICAL STEP Selection of the appropriate nominal molecular weight limit depends on the target protein. For example, the 10 kDa cutoff filter units can be used for proteins with a molecular weight higher than 10 kDa.

x. Centrifuge at 4,000 x *g* for 30 min at 4 °C. In each filter unit, a volume of ~500 µl of medium remains after centrifugation, resulting in a ~30-fold concentrated medium.

CRITICAL STEP If the protein concentration is too low and further centrifugation is required, 0.5 ml centrifugal filter units can be used.

xi. Collect the concentrated medium into 1.5 ml microtubes on ice.

xii. Add the corresponding volume of 100x protease inhibitor cocktail mix for a final concentration of 1x.

PAUSE POINT Aliquots of concentrated media can be stored at -20 °C for one year.

1B) Sample preparation for WB ● Timing 1.5 hrs (Day 1)

i. Measure protein concentration using a BCA protein assay kit for lysates (intracellular protein) and a Bradford reagent for media (secreted protein).

CRITICAL STEP Precise monitoring of protein concentration is key to interpret antibody specificity by WB. Measurement of protein concentration in triplicate allows more precise and reproducible quantification for both the BCA and Bradford assay.

ii. Adjust the protein concentrations so that equal amounts of protein are loaded in each lane.

CRITICAL STEP Antibody signal (band intensity) follows a linear and proportional relationship with the protein concentration used to test every antibody/ target pair[33]. To reach this linear range of detection, we load a defined amount of protein depending on the putative abundance of the target in the chosen parental cell line. To do so, we first search the protein target in PAXdb[34] (RRID:SCR_018910, pax-db.org) to determine protein abundance in the selected cell line. PaxDB provides protein abundance in parts per million, or ppm. We use 50 µg of lysate or medium for low abundance proteins between 10 and 1000 ppm and 20 µg of lysate or medium for high abundance proteins with over 1000 ppm. Even with these considerations, primary antibody titration may be required later.

1C) Protein electrophoresis ● Timing 2 hrs (Day 1)

i. Select the appropriate type of polyacrylamide gels based on the target protein size. 12-well midi precast gels running under denaturing conditions are suggested here (Table 1).

CRITICAL STEP Various gel chemistries are commercially available with various advantages and disadvantages. While larger proteins are often considered harder to detect, we found that the commonly used Tris-Glycine (TG) gels are suitable for proteins up to 500 kDa. As an example, Plectin (PLEC), a large protein expected at 532 kDa, was detected using a KO-validated antibody on a TG (Fig. 8a, left WB), a Bis-Tris (BT) gel (Fig.8a, middle WB) combined with the MOPS buffer and on a Tris-Acetate (TA) gel (Fig.8a, right WB). BT and TA gels provided a better separation and resolution of PLEC than the TG. However, the TG gel was still revealed to be suitable for detecting PLEC and is chosen for standardization purposes in this large effort on antibody characterization as it covers the largest molecular weight spectrum. TG and

BT gels were transferred in 1x Tris/Glycine buffer as [CL1] detailed in Table 2. TA gel was transferred following the same parameters as the BT gel. All transfer conditions were inspired from manufacturers' recommendations and then further optimized.

On the other hand, standard transfer conditions from the TG gel were not suitable for transferring small proteins successfully (Fig. 8b, left WB). Therefore, BT gels combined with MES buffer are required for our procedures as this chemistry was highly efficient at separating and resolving the small 9 kDa protein, FCER1G (Fig. 8b, right WB).

ii. Prepare a master mix for both parental and KO cell lysates at the same protein concentration to facilitate sample loading into gels.

iii. Add loading sample buffer to a final concentration of 1x. Depending on the gel chemistry chosen above, samples must be prepared with the compatible type of loading sample buffer (Table 1). Similarly, prepare a master mix of molecular weight markers; volume can be increased with RIPA buffer to match the volume of cell lysate master mix. Chose the appropriate type of molecular weight marker for your protein. Two types of molecular weight markers are listed in Table 1; the BLUelf Prestained Protein Ladder covers proteins from 5 – 245 kDa while the HiMark Pre-Stained High Molecular Weight Protein Standard is suggested for proteins above 245 kDa.

iv. Heat the master mixes of protein samples and molecular weight markers for 10 min at 65°C in a heat block to help dissolve the SDS and/or glycerol in the loading sample buffer, which facilitates the loading into the gel.

CRITICAL STEP For some proteins, boiling samples can create artifacts. The G protein-coupled receptor S1PR1 runs as two major bands (~44, 48 kDa) with additional minor bands detected below and above both major bands (Fig. 7, left panel). Boiling the samples led to an intense artifactual smear above 245 kDa and a reduction of the signal at ~44 and 48 kDa (Fig. 7, right panel).

v. Pulse spin the samples and molecular weight master mixes using a microcentrifuge, and load samples into a 12-well polyacrylamide gels in the order suggested in Fig. S1a. A total of 4 antibodies can be tested on each 12-well gel. To test 14 antibodies, 4 x 12-well gels are required.

vii. Follow the conditions recommended by the manufacturer to run the gel using the appropriate running buffer (Table 1), until the dye front reaches ~3 mm from the bottom.

1D) Protein transfer to nitrocellulose membrane ● Timing 1.5 hrs (Day 1)

i. Transfer proteins from the gel to a membrane; nitrocellulose membranes are used here. The Bio-Rad criterion blotter is the wet transfer system employed. Transfer conditions are detailed in Table 2.

ii. Take the membrane out of the sandwich and wash twice with deionized water in a container.

iii. Stain all transferred proteins by covering the membranes with the Ponceau S solution.

iv. Incubate for 1 min; Ponceau S staining solution can be reused several times.

v. Wash off excess Ponceau S with deionized water until the area of membranes not covered by proteins becomes white.

vi. Dry the Ponceau S-stained membranes on Whatman filter paper.

vii. Label membranes properly with a suitable smudge proof pen.

viii. Scan membranes using a regular paper scanner.

ix. Trim membrane strips containing each a molecular weight marker and a WT/KO lysate pair.

PAUSE POINT Dried membranes can be stored at room temperature for months.

1E) Blocking and primary antibody incubation ● Timing 1 hr and O/N (Day 1 and 2)

i. Rehydrate membrane strips and remove Ponceau S staining by incubating the membrane in TBST 1x for 5 min.

ii. Block the membrane with the WB blocking solution for 1 hour at room temperature.

iii. During the blocking step, prepare the primary antibody solution in WB blocking solution.

CRITICAL STEP The initial concentration of primary antibody tested is in accordance with the manufacturer's recommendations. Antibodies are titrated only when the signal falls outside of the linear dynamic range.

iv. Incubate each membrane strip with the corresponding primary antibody dilution. A container or a resealable flat plastic bag can be used for the incubation.

v. Rock overnight at 4°C.

1F) Secondary antibody incubation and signal detection ● Timing 3 hrs (Day 2)

i. Discard antibody solution and wash membrane strips in a container with TBST 1x for 10 min under constant rocking. Repeat twice more for a total of 3 washes.

ii. During washes, dilute the corresponding HRP-conjugated secondary antibody in WB blocking solution to a concentration of 0.2 - 0.5 µg/ml.

iii. Rock membrane blot strips with the corresponding HRP-conjugated secondary antibody in a container or a sealed plastic bag for 1 hr at room temperature.

iv. Wash 3 x times in TBST 1x for 10 min with constant rocking.

v. After the last wash, place membrane strips on a clean surface and incubate with the peroxidase substrate for 1 min, then remove excess.

vi. Place membrane strips in a chemiluminescence imaging system and follow the manufacturer's guidelines for signal detection. The iBright imaging system is suggested here.

CRITICAL STEP Signal strength varies depending on the antibody and its concentration. Several exposures must be typically taken to observe the bands of interest at intensities comparable between the different antibodies.

vii. Name images properly and export the different exposures for later figure preparation.

viii. Analyze the band pattern to assess antibody specificity (Fig. 2a).

CRITICAL STEP Identification of a cell line with confirmed endogenous expression of the target, assessed using a KO-validated antibody, is essential to proceed with IP-WB and IF procedures.

PAUSE POINT The next procedures can be started at any time after screening antibodies by WB was successful.

Procedure 2: Antibody screening by IP ● Timing 3 days

2A) Protein extraction

Option 1: Cell lysate preparation for IP – *intracellular protein* ● Timing 1.5 hrs (Day 1)

i. Grow a 150 mm dish of parental cells to generate enough lysate for two IPs (7 dishes if 14 antibodies are tested).

ii. Place each culture dish on ice and discard medium.

iii. Wash the adherent cells three times with ~10 ml of ice-cold PBS 1x. Ensure total removal of PBS between washes with, for example, a vacuum.

iv. After the last wash, add 1.0 ml of ice-cold IP lysis buffer supplemented with 1x protease inhibitor cocktail mix to each 150 mm dish.

v. Use cell scrapers to gently detach adherent cells from the petri dish.

vi. Collect and pool parental cell lysates into a 5 or 15 ml tube, depending on the number of dishes used.

vii. Rock cell lysates for 30 min at 4 °C.

viii. Centrifuge at ~110,000 x *g* for 15 min at 4°C (as with WB).

ix. Gently remove the tubes from the rotor and place them on ice.

x. Pool the supernatants into the same tube kept on ice. Discard pellets.

CRITICAL STEP Freshly prepared lysates must be used for the IP experiment. Freezing the protein sample might affect the epitope to be recognized by the antibodies tested.

Option 2: **Culture media collection for IP – *secreted protein* ● Timing 1.5 hrs (Day 1)**

i. Grow a 150 mm dish of parental cells for each IP (15 dishes if 14 antibodies are tested).

ii. As described in the WB procedure **1A *option 2***, wash and grow the cells without serum for 18 h, then collect and concentrate media.

iii. Combine all concentrated media from parental cells into the appropriate tube.

iv. Add the corresponding volume of 100x protease inhibitor cocktail mix for a final concentration of 1x.

CRITICAL STEP Freshly collected and concentrated media must be used for the IP experiment.

2B) Sample preparation for IP ● Timing 1.5 hrs (Day 1)

i. Measure protein concentration using a BCA protein assay kit (lysate) or Bradford reagent (medium).

ii. For lysates, adjust protein concentration to 2.0 mg/ml with IP lysis buffer. 1 mg (500 µl at 2.0 mg/ml) is used later for each IP.

For concentrated media, the concentration is usually at ~1 mg/ml. 500 µg (500 µl at 1.0 mg/ml) is used later for each IP.

iii. Save enough lysate for starting material sample.

CRITICAL STEP 4% starting material is suggested here. 20 µl of lysate at 2.0 mg/ml (40 µg) or 20 µl of medium at 1.0 mg/ml (20 µg) are kept aside to run side-by-side with each IP. For 14 antibodies, save ~300 µl for starting material.

2C) Antibody-beads conjugation ● Timing 1.5 hrs (Day 1)

i. Select the appropriate type of magnetic beads for each antibody (Table 3)[35].

ii. Resuspend the beads slurry.

iii. Label enough microtubes (one microtube per antibody to be tested) and add 30 µl of magnetic beads slurry to 1.0 ml of IP lysis buffer.

iv. Add 2.0 µg of the corresponding primary antibody.

TROUBLESHOOTING When the antibody concentration is not provided by the manufacturer, follow their volume recommendation for IP, if applicable. If not, test a certain volume of the antibody and keep a record of the volume tested.

v. Maintain constant agitation on a rotating mixer for 1 hr at 4°C.

vi. Place tubes on the DynaMag-2 magnet and allow 15 sec for the beads to attach to the magnet.

vii. Vacuum out the buffer to remove unbound antibodies.

viii. Add 1.0 ml of IP lysis buffer and allow release of the beads by taking the tube off the magnet.

ix. Wash the beads by inverting the tube multiple times to resuspend.

x. Repeat to wash a second time and remove the excess unbound antibodies.

CRITICAL STEP Do not let the beads dry out at any step.

2D) Immunoprecipitation ● Timing 2 hrs (Day 1)

i. Remove buffer from the antibody-bead conjugate using the magnet.

ii. Add 500 µl of lysate at 2.0 mg/ml or 500 µl of medium at 1.0 mg/ml to each tube of antibody-conjugated beads.

iii. Incubate antibody-beads conjugates with the lysate for 1 hr at 4°C with constant agitation on a rotating mixer.

iv. Place each microtube on DynaMag-2 and allow at least 15 sec for the beads to converge toward the magnet.

v. From each tube, collect 20 µl of samples which represent the unbound fraction (proteins that did not bind to the antibody-bead conjugate).

vi. Pipet each unbound fraction in a labelled microtube, set aside on ice.

vii. Vacuum out any remaining samples from each tube on the DynaMag-2.

viii. Wash the magnetic beads 3x in 1 ml IP lysis buffer supplemented with protease inhibitor cocktail mix.

ix. After last wash, elute with 30 µl of the appropriate loading sample buffer diluted to 1x in IP lysis buffer.

x. Add loading sample buffer to the starting material and unbound fractions. Similarly, prepare molecular weight marker sample (volumes should be completed with IP buffer to match sample volumes).

CRITICAL STEP The final concentration of loading sample buffer must be identical in all samples (usually 1x).

xi. Heat all samples for 10 minutes at 65°C in a heat block.

PAUSE POINT Samples can be left at room temperature for one day or stored at -20°C for several weeks.

2E) WB assessment of antibody performance by IP

● **Timing 4.5 hrs (Day 2), O/N and 3 hrs (day 3)**

i. Select the same polyacrylamide gel as selected for the WB screening (Table 1).

ii. Load samples on 12-well polyacrylamide gels in the order suggested in Fig. S1b. A total of 3 antibodies can be evaluated from a single 12-well gel. To test 14 antibodies, 5 x 12-well gels are required.

iii. The following WB is done using KO-validated antibodies selected from the WB screening (**procedure 1**).

CRITICAL STEP Immunoglobulins from the antibodies used in the IP are eluted with the loading sample buffer containing reducing agents. For rabbit polyclonal antibodies, the heavy chain runs at 50 kDa, and the light chain at 25 kDa. For other types of antibodies, the molecular weight of these chains might vary. The same primary antibody is ideally used in WB to assess the performance of all antibodies tested by IP.

TROUBLESHOOTING When the proteins of interest have a molecular weight close to that of immunoglobulin chains, the antibody used in WB may cross-react with these chains, masking the protein signal. These cross-reactions can also create noise elsewhere in the IP pathway (Fig. 9a, case 1 and Fig. 9b, case 4). Two options are proposed to solve this problem: **1)** use a secondary detection system that should not react with unstructured immunoglobulins to avoid these cross-reactivities (Fig. 9a, case 2 and Fig. 9b, case 5) or **2)** where possible, choose a primary KO-validated antibody raised in a different host than the antibody used in IP, ideally allowing minimal detection of immunoglobulins from different species (Fig. 9a, case 3 and Fig. 9b, case 6).

iv. Perform the WB as detailed in **procedure 1E, F**.

v. Analyse the data by comparing the WB signal in the starting material, unbound fraction and immunoprecipitate (Fig. 5).

Procedure 3: Antibody screening by IF ● Timing 2 d

3A) Prepare parental/KO mosaic in 96-wells for IF ● Timing 2 hrs (Day1)

i. Coat each well of a 96-well, clear flat bottom plate with 100 µl of poly-L-lysine working solution. The suggested 96-well plate, Revvity cat. no 6055300, is compatible with cell imaging and most high-content imaging systems.

ii. Incubate one hour at room temperature.

iii. Wash each well twice with 100 µl of sterile water.

iv. Wash 150 mm dishes of 80% confluent parental and KO lines with 10 ml of warm PBS 1x.

v. Add 5.0 ml of warm trypsin to both the parental and the KO cell dishes.

vi. Incubate dishes at 37°C, 5% CO_2 for 2 min in a cell culture incubator.

vii. Confirm that cells have detached from the plate by using a bright field microscope, or visually inspect the plate. If cells are still attached, continue the incubation in the incubator until they have detached. Incubation time will vary between cell lines.

viii. Inactivate trypsin by adding 5.0 mL of complete culture media.

ix. Collect parental and KO cells in separate 15 ml canonical tubes.

x. Centrifuge at 1,500 x *g* for 3-5 min to pellet cells.

xi. Discard the supernatant.

xii. Resuspend the parental cell pellet with 2.0 ml of labelling culture media containing 5 µM of CellTracker Green CMFDA Dye.

xiii. Resuspend the KO cell pellet with 2.0 ml of labelling culture media containing 1 µM of CellTracker Deep Red Dye.

xiv. Incubate the cell suspensions in a cell culture incubator for 30 min with the lid slightly open. Gently tap the bottom of each tube every 5 min to put the cells back in suspension.

xv. Centrifuge both 15 ml tubes at 1,500 x *g* for 3-5 min.

xvi. Discard the supernatant.

xvii. Resuspend each labelled cell pellet with complete medium and count the cells.

xviii. Prepare a master mix with sufficient parental:KO cells at a ratio of 1:1 to plate cells in each well of the 96-well plate.

CRITICAL STEP For most cancer cell lines, 10,000 parental cells combined with 10,000 KO cells (20,000 cells in total) leads to 50-60% confluence in a well of a 96-well plate, which is ideal for subsequent imaging.

xix. Incubate cells in 100 µl of complete cell culture medium overnight at 37°C in the cell incubator.

Fixation in 96 well plates

xx. Add 100 µl of pre-warmed (37°C) IF fix buffer on top of the culture medium. Final concentration of PFA is 4%.

xxi. Incubate for 15 min at 37°C.

xxii. Aspirate and wash 3x with 100 µl PBS at room temperature.

xxiii. Plates can be stored at 4°C for a few days. Protect plates from light. Seal the plate using parafilm to avoid evaporation.

3B) Primary antibody staining ● Timing 24 hrs (Day 2)

i. Prepare content of each well. A total of 33 wells is required to test 14 antibodies, at two different concentrations, together with necessary controls.

CRITICAL STEP Wells #1-28 are dedicated for testing primary antibodies, whereas wells #29-33 are dedicated for controls. Wells #31 & #33 address bleed-through from the channel 1, 2 and 4 into channel 3. Well 32 controls DAPI emission bleed through into Channel 3.

ii. Incubate the necessary wells from the 96-well plate prepared in Step 1 with 100 µl of IF permeabilization buffer for 10 min at room temperature.

iii. Wash wells three times with 100 µl of 1x PBS.

iv. Incubate wells with 100 µl of IF blocking buffer for 30 min at room temperature.

v. During the incubation, prepare a 100 µl dilution of each primary antibody in IF buffer.

CRITICAL STEP After performing hundreds of immunofluorescence experiments, we found that 1.0 µg/ml is an appropriate first concentration to test as it generally provides an adequate signal falling within the detection range of a microscope. When an antibody is recommended for IF by the manufacturer, the recommended concentrated is tested, together with 1.0 µg/ml. If the recommended concentration is 1.0

µg/ml, 2.0 µg/ml is also tested. Where an antibody is not recommended for IF, 1.0 µg/ml and 2.0 µg/ml are tested. Antibody titration should be performed if signal obtained falls outside the linear range of detection.

vi. Incubate wells with the appropriate antibody dilution overnight at 4°C, or with IF buffer for control conditions.

3C) Secondary antibody labelling ● Timing 2 hrs (Day 3)

i. Wash wells three times for 10 min with 100 µl of IF buffer.

ii. Incubate wells with secondary antibody dilution containing either 0.1 µg/ml of goat secondary antibodies coupled to Alexa 555 in IF buffer, or with IF buffer alone for specific control wells.

iii. Wash wells three times for 10 min with 100 µl of IF buffer.

iv. Incubate wells with 1x PBS containing 5 ng/ml of DAPI, except specific control wells.

v. Wash twice with 100 µl of 1x PBS.

3D) Cell imaging ● Timing 2 hrs (Day 3)

i. Image wells (see raw images in Fig. 10a).

CRITICAL STEP We use an ImageXpress micro widefield high-content microscope equipped with 395, 475, 555 and 635 nm solid-state LED lights and bandpass filters to excite and capture separately DAPI, CellTracker Green CMFDA, Alexa568 (Alexa fluor 555) and CellTracker Deep Red respectively. The filter cube specifications are the following: 1) excitation (The excitation spectra are based on the emission band of the light sources (Lumencore AuraIII): blue - 395/25, green - 475/28, red - 555/28, far red - 635/22, 2) emission: blue - 432/36, green - 520/35, red - 600/37, far red - 692/40. The objective used is a water Apo LambdaS LWD with magnification of 20X, NA 0.95. The LED light or Illumination power is attenuated or optimized based on the signal expression for different targets for each channel. Camera is a 16-Bit CMOS 1.97mm FOV are used with a binning of 2, with a calibration (binned) of 0.6792 X 0.6792 um. The target maximum intensity is set at 33000. The Z-series is set at 2D projection image only. The average number of WT and KO cells imaged per condition is minimally 500.

CRITICAL STEP Sequential imaging setup is preferred to avoid any bleed through between channels. To control for bleed through when imaging 3 or 4 channels, four different controls are needed for each imaging experiment. Use the same setting for imaging the control samples. Image the single labelled control with all filters sets and carefully analyze potential bleed-through in the unlabelled channels. Adjust the emission spectra for each channel so that there is no/minimal bleed through into the unlabelled channels. To control for auto fluorescence, image an unlabelled cell sample that has gone through the staining protocol, in each of the four channels.

3E) Image analysis ● Timing 2 hrs (Day 4)

i. Image analysis can be done either visually or using the following analysis process.

CRITICAL STEP Antibody performance can be assessed visually. Antibodies that can immunolocalize their protein target will generate a specific signal in the parental cells, and a signal in KO cells comparable to the image background (area outside a cell). For a larger initiative, the automatic cell segmentation and quantification allow a more robust and reliable comparison of antibody performance between antibodies since antibody signals can be measured from thousands of cells.

ii. Using Fiji, inspect cell mask channels in images from several wells to make sure the cell mask staining is visible above background and noise, and determine an approximate cell diameter (in pixels) using the ellipse tool.

iii. To segment cells, run the cellpose segmentation[36] pipeline on cell mask and DAPI images. It is highly recommended to set up cellpose in a conda environment on a CUDA-capable GPU-equipped system, and to use a script to batch process all images from all wells from a plate. Use the cell diameter estimated above (in ii) as an input parameter and choose the cytosol-specific ('cyto') model. The output images after running cellpose are labeled masks of cells detected in each image, and can later be used as masks for antibody staining intensity quantification (Fig. 10b). For this step, we provide a script written in python (cellpose_batch_ycharos_IMX_images.py).

iv. In the raw images, estimate the base image background in the antibody channel. This can be done with a minimum intensity projection of images of an empty well, or by generating a minimum intensity projection of several sparsely seeded wells. This can be generated using a helper script (minimum_intensity_projection_images.ijm). Calculate the median intensity of this base background.

v. The following steps (v, vi, and vii) can be performed using a Fiji script (main_ycharos_IMX_images_script_Fiji.ijm) For each antibody image, generate a thresholded binary image by first calculating a pixel intensity value threshold using the Otsu method and converting the intensity image to a binary masks image.

vi. For each antibody image calculate the median intensity for all pixels outside of cellpose and Otsu thresholded objects and divide that intensity by the base background median intensity. Multiply the base background image by that ratio and subtract that resulting image from the antibody image. The resulting image is a background-subtracted antibody staining image, based on the background image obtained from an empty well, scaled to within image background intensities.

vii. In the background-subtracted antibody images, measure the intensity and dimensions statistics (mean, sd, median, area, xy coordinates, etc) for each mask.

viii. Generate cropped images with cell mask outlines overlaid on antibody images and on DAPI images, respectively (Fig. 10c, d), using a helper script in Fiji (crop_and_make_figure_panels.ijm)

ix. With the data table, calculate the ratio mean parental intensity divided by mean KO intensity for each image, plot these ratios for each image, for each antibody tested (Fig. 10e). This can be calculated in any data analysis software, here we have provided an R script for this purpose using data tables generated in previous scripts (calculate_ratios_and_plotting_template.R).

Troubleshooting

Time Taken

Anticipated Results

We describe standardized, industry-approved protocols for comparing and evaluating the performance of a set of antibodies targeting selected human proteins in WB, IP and IF. Initial characterization data assessment allows for the identification of non-specific or poorly performing antibodies, facilitating their exclusion from future antibody selection by users. We strongly advocate for users to communicate feedback to antibody suppliers regarding underperforming antibodies, as most suppliers will evaluate user data and take proactive measures to withdraw or amend antibody descriptions accordingly.

It is inevitable that different antibodies will exhibit varying degrees of selectivity towards their intended targets. Whenever feasible, users should prioritize the use of recombinant antibodies, as these are renewable products that contribute to reducing the need for animal-based antibody production. Drawing from our prior research, we anticipate that widespread adoption of these protocols can facilitate the identification of selective, renewable antibodies for approximately 50-75% of human proteins, depending on the application[11].

While the standardized protocols described herein may not yield optimal performance for all tested antibodies, we recommend users select one or two top-performing antibodies and optimize various parameters relevant to their chosen application and cell type. It is imperative to consider the endogenous protein expression level when determining the most suitable antibody concentration. Significant differences in protein expression between the cell line used for antibody characterization and the user's cell line may necessitate antibody titration.

The focus here remains on antibodies being tested against human targets. This step is essential before employing them on proteins from other species, which needs further validation using KO lines from the specific of interest.

The consensus protocols provided for antibody validation empower researchers to generate robust, reproducible, industry-standard data that can be readily disseminated for the benefit of the global biomedical community.

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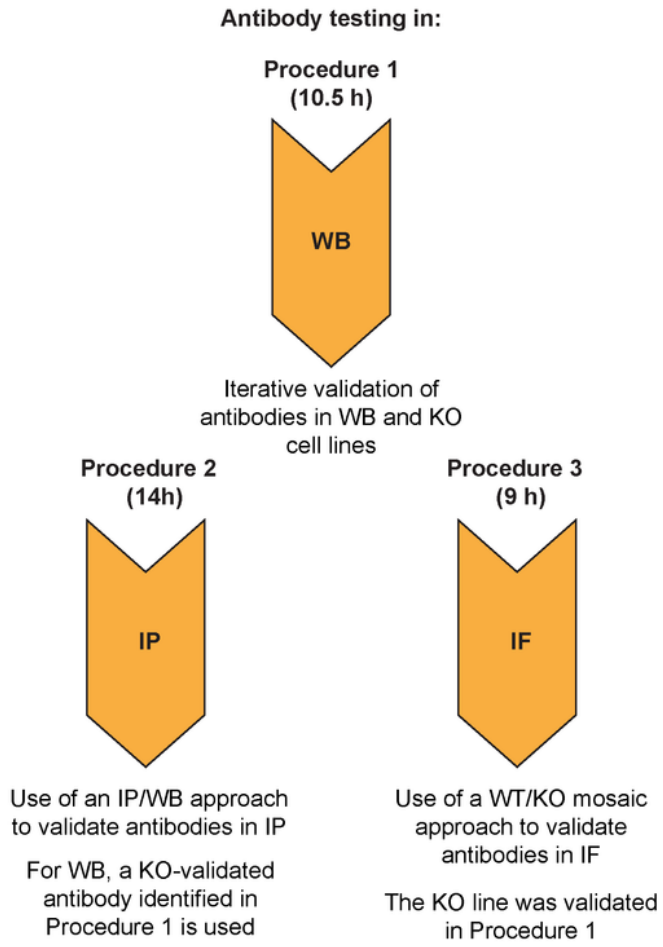
Figures



Ayoubi et al., Figure 1

Figure 1

Experimental design of the antibody characterization workflow.

All antibodies are tested in all three applications. Antibodies are first tested in WB to iteratively validate the KO lines and the antibodies (Procedure 1). Antibodies are next tested in IP followed by WB to evaluate their performance to capture their intended target (Procedure 2). The antibody selected for WB in Procedure 2 was previously validated in Procedure 1. Antibodies against intracellular proteins are next screened in IF (Procedure 3).

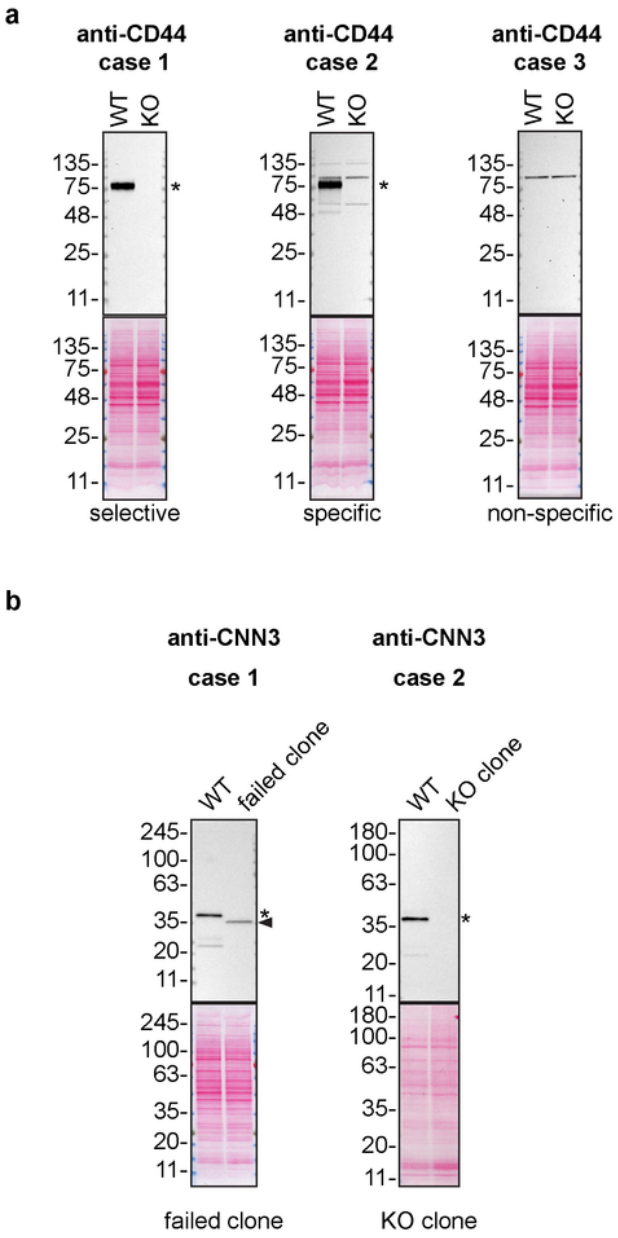
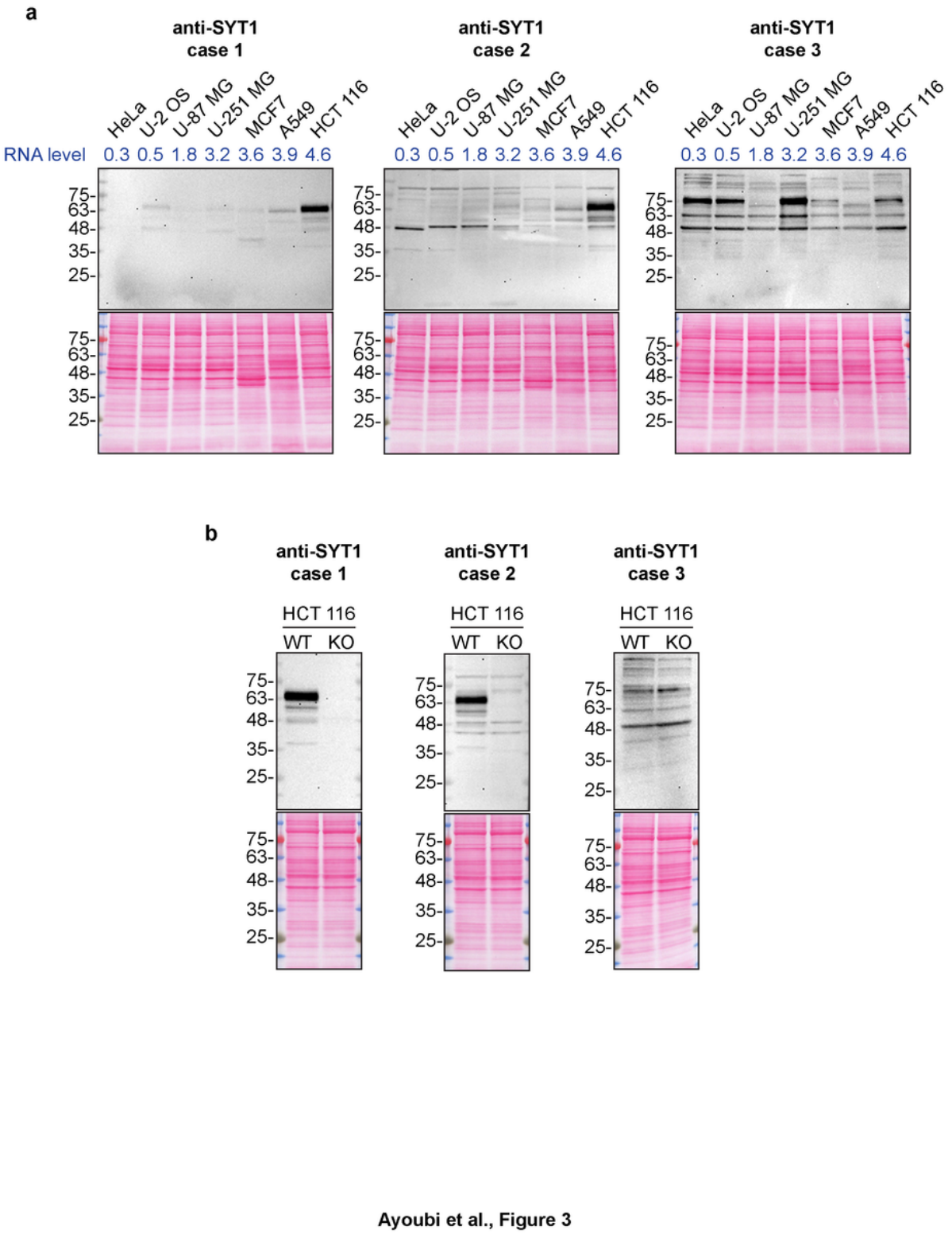


Figure 2

Interpretation of antibody performance in WB

For each presented WB, the antibody related chemiluminescent signal is shown at the top of its corresponding ponceau S-stained membrane. **a)**Three selected antibodies against the CD44 protein (Uniprot ID: P16070), expected at 82 kDa, are presented to illustrate various types of target specificity in

WB. In case 1, the antibody selectively detected CD44 as determined by the presence of a band in the WT lysate and the complete absence of any band in the KO lysate (star). In case 2, the antibody specifically detected CD44 as determined by the absence of the main band in the KO lysate (star), but also detected unwanted proteins (bands present in both WT and KO lysates). In case 3, the antibody failed to recognize CD44 as the band detected in the WT lysate is also detected in the KO lysate. **b)** A selective antibody against the CNN3 protein (Uniprot ID: Q15417) was used to characterize two independent commercial *CNN3* KO clones generated in the same cell line background. CNN3 was detected at ~40 kDa in the WT lysate (star). In case 1, a truncated ~35 kDa protein was detected in the lysate derived from the putative *CNN3* KO clone, defined here as a failed clone (arrowhead). In case 2, the antibody did not detect any form of residual CNN3 protein. 4-20% TG gels were used.



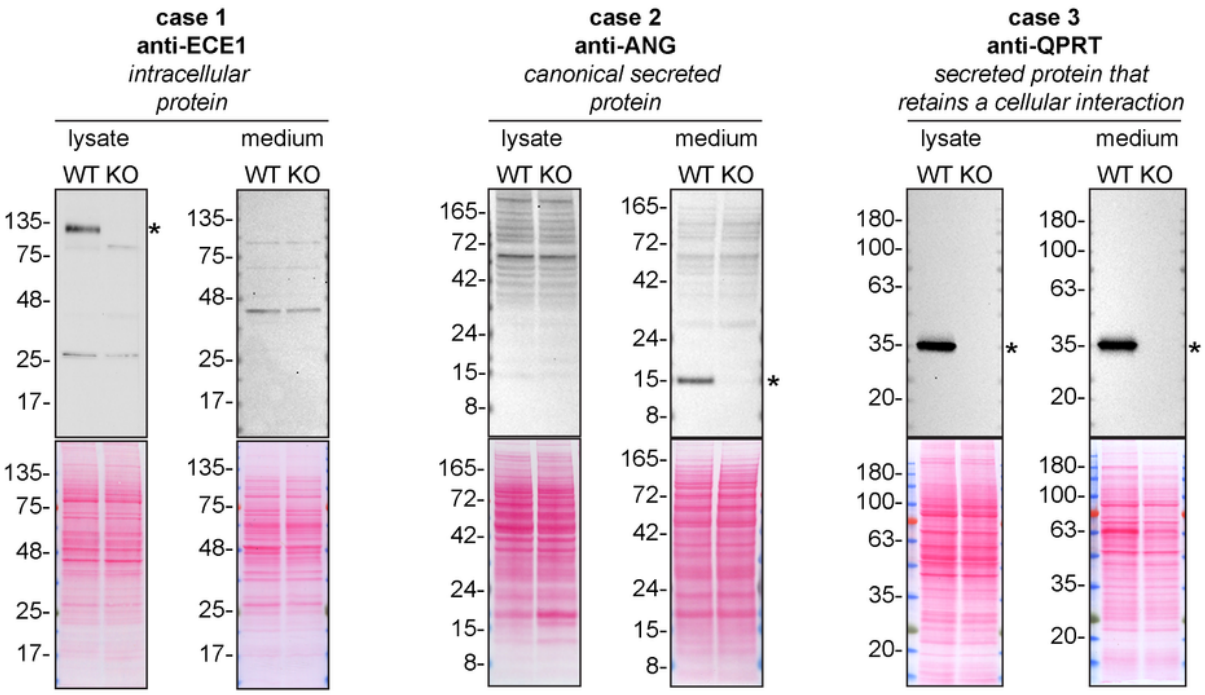
Ayoubi et al., Figure 3

Figure 3

Identification of an adequate cell line background for KO generation

WBs are presented as in Figure 2. **a)** The identification of an adequate cell line for the SYT1 protein (Uniprot ID: P21579, *SYT1* is the corresponding gene). Seven cancer cell lines were selected with RNA expression spanning from 0.3 to 4.6 $\log_2$(TPM+1). The RNA levels, in $\log_2$(TPM+1), were extracted from

DepMap.org and presented in blue below the corresponding cell line. Lysates were prepared, processed by SDS-PAGE and probed with three unique primary SYT1 antibodies (case 1, 2 and 3). In case 1 and case 2, both antibodies putatively identified Synatotagmin-1 at ~66 kDa in HCT116 as they provide a similar banding pattern with absence of signal in cells with low RNA value. In case 3, the antibody provided a signal that does not correlate with the signal of the other two antibodies. **b)** An HCT116 *Syt1* KO line was generated and used to validate that HCT116 expresses the endogenous SYT1 protein and the specificity of the SYT1 antibodies.

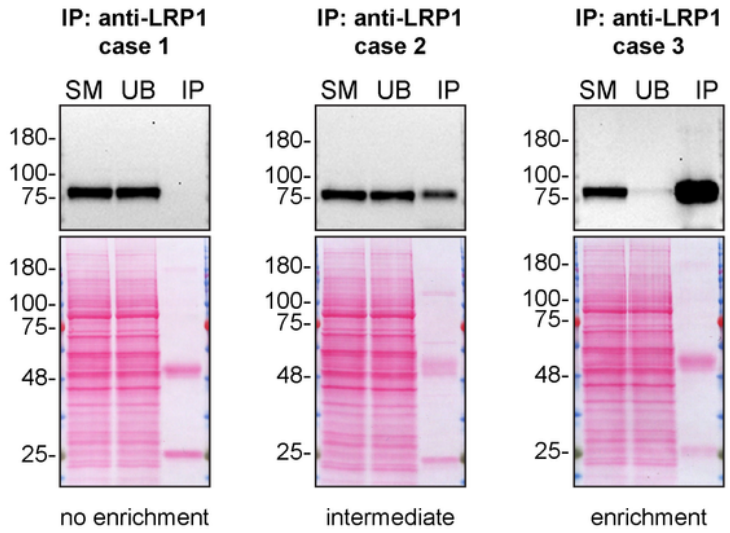


Ayoubi et al., Figure 4

Figure 4

Antibody performance correlates with sample preparation

WBs are presented as in Figure 2. From a cell line endogenously expressing the corresponding intended target, proteins were prepared from both cell lysates and conditioned medium. Protein targets were searched through Uniprot to determine whether they are predicted to be secreted or not. **a)** In case 1, the antibody targets ECE1 (Uniprot ID: P42892), a predicted intracellular protein. ECE1 was detected exclusively in the cell lysate sample (star). 4-20% TG gels were used. **b)** In case 2, the antibody targets Angiogenin (Uniprot ID P03950), a predicted canonical secreted protein. Angiogenin was only detected in the medium (star). 10% BT gels with MES running buffer were used. **c)** In case 3, the antibody targets the protein QPRT (Uniprot ID: Q15274), predicted to be secreted and to retain an intracellular distribution. QPRT was detected both in cell lysate (star) and medium (star). 4-20% TG gels were used.



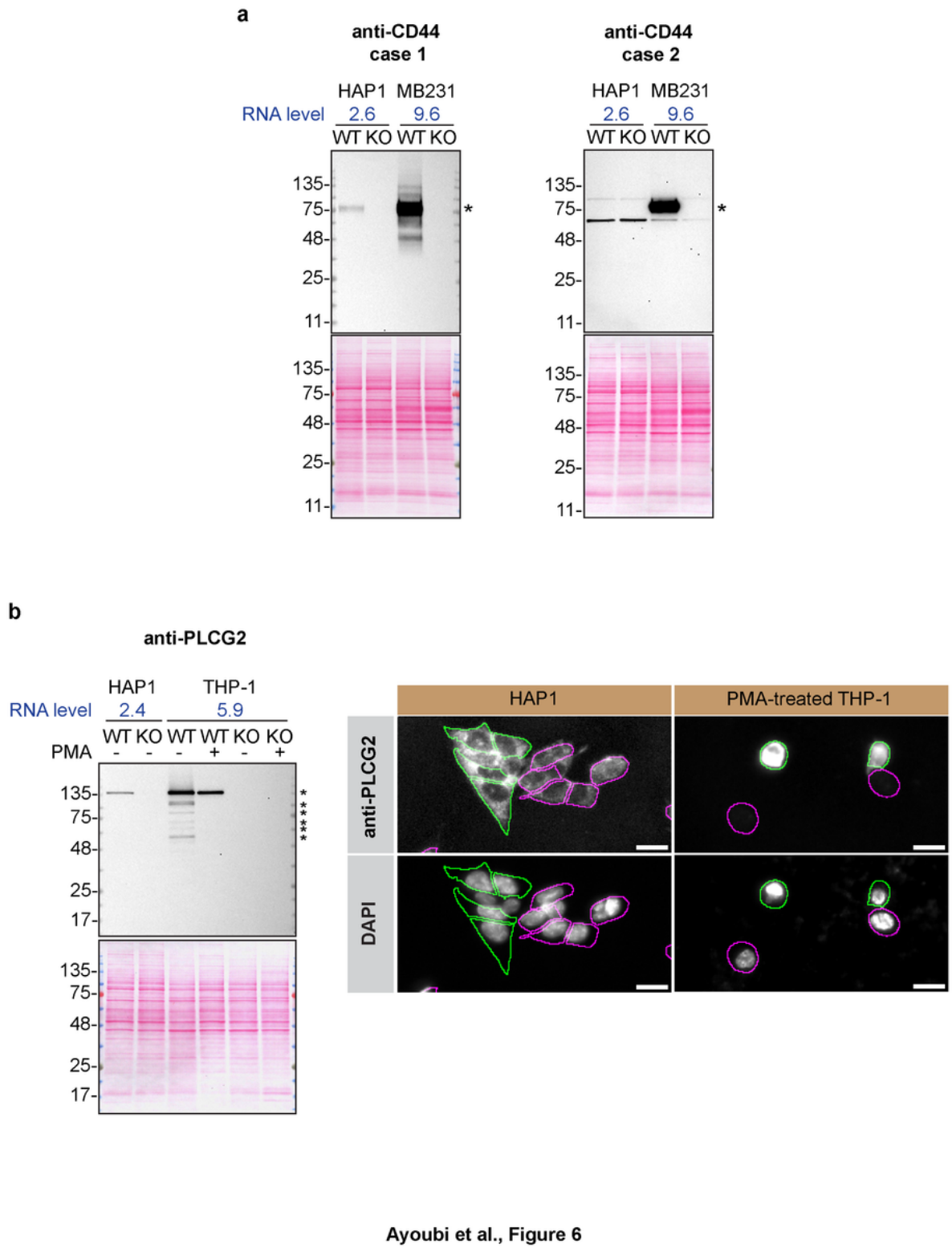
Ayoubi et al., Figure 5

Figure 5

Interpretation of antibody performance by IP

WBs are presented as in Figure 2. Three selected antibodies directed against the LRP1 protein (Uniprot ID: Q07954) illustrates different degrees of capture efficiency in IP. A selective LRP1 antibody in WB was used to detect the LRP1 protein level between three distinct fractions, namely the SM (4%), UB (4%) and

the IP. In case 1, the antibody did not capture the target protein as determined by the absence of signal in the IP fraction and unchanged level of the LRP1 protein in the UB. In case 2, the antibody captured the target protein to slightly below the level of the SM and failed to deplete LRP1 from the UB. In case 3, the antibody enriched its intended target in the IP several folds over the SM and mostly depleted LRP1 from the UB. This antibody successfully immunocaptured its intended target in the conditions used. 4-20% TG gels were used. SM=4%, UB=4%.

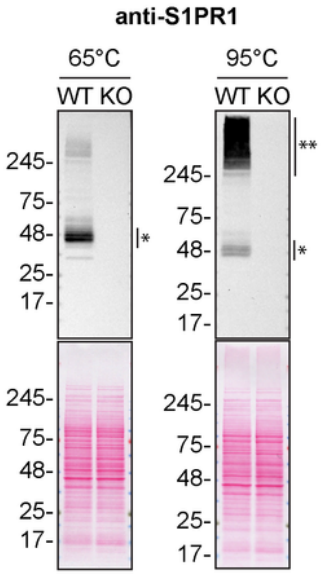


Ayoubi et al., Figure 6

Figure 6

Protein abundance influences antibody performance

WBs are presented as in Figure 2. **a)**Two antibodies against the CD44 protein (Uniprot ID: P16070) were selected to illustrate the effect of protein abundance on antibody performance. Both selected CD44 antibodies are different from those shown in Figure 2. RNA levels corresponding to both cell lines were extracted from DepMap.org and presented as in Figure 3. In case 1, the antibody was able to selectively detect CD44 in both cell lines (star). In case 2, the antibody detected CD44 in MB231, but not in HAP1 (star). 4-20% TG gels were used. MB231=MDA-MB231. **b)** The intracellular protein PLCG2 (Uniprot ID: P16885) was selected to illustrate the effect of protein abundance on antibody performance in IF. The same PLCG2 antibody was used in WB and in IF. PLCG2 was detected in WB (stars) using lysates from HAP1 WT and *PLCG2*KO as well as THP-1 WT and *PLCG2* KO, treated or not with PMA. The RNA levels are showed as in a). The PLCG2 antibody was tested on HAP1 (left IF) and PMA-treated THP1 (right IF). WT (green outline) and KO (purple outline) cell lines were plated as a mosaic and were segmented post-image acquisition. The gray-scale antibody channel is shown (top panels), together with the corresponding DAPI stain (nucleus, bottom panels). THP-1 are small cells that adopt a round shape. 4-20% TG gel was used.



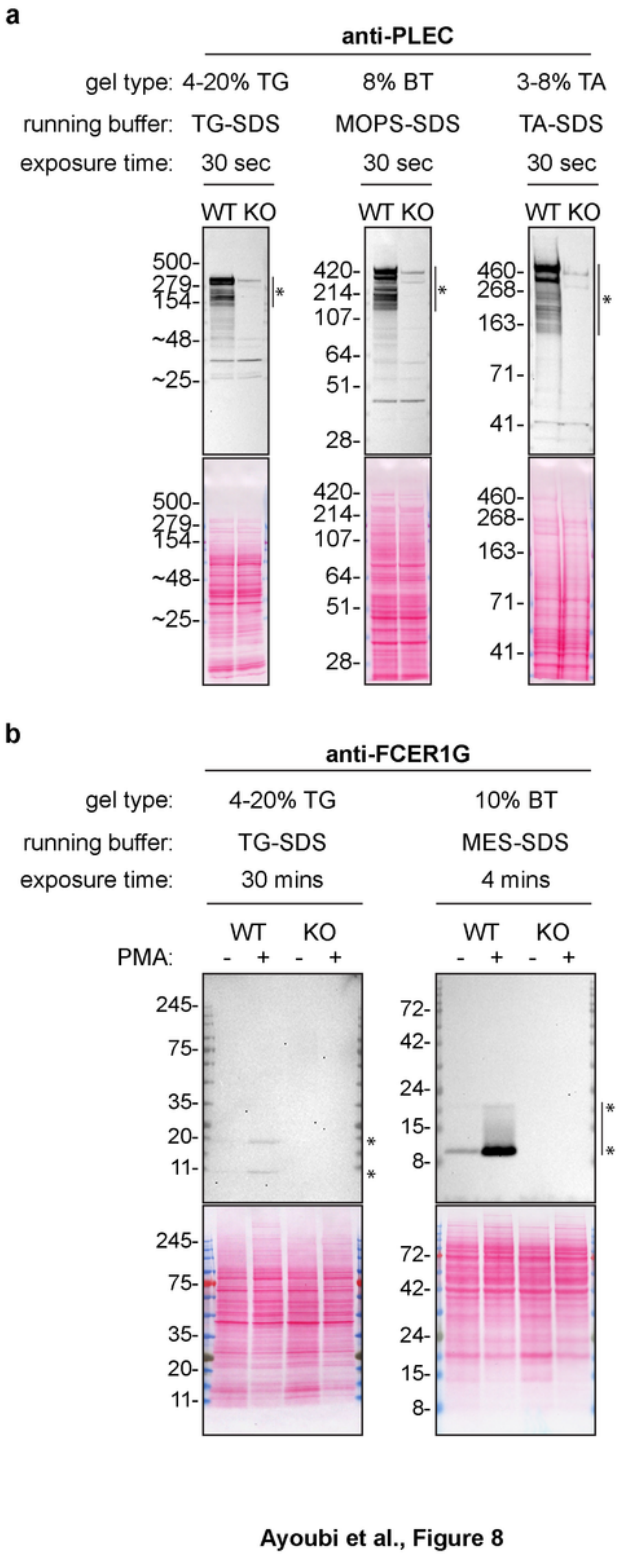
Ayoubi et al., Figure 7

Figure 7

Boiling protein samples creates an aggregation artifact

WBs are presented as in Figure 2. Lysates of a cell line expressing endogenous levels of S1PR1 (Uniprot ID: P21453), a transmembrane protein, were produced and were either heated at 65°C or 95°C for 10 min.

Single stars point at the major bands representing S1PR1, whereas the double star points at the aggregated form. 4-20% TG gel was used.

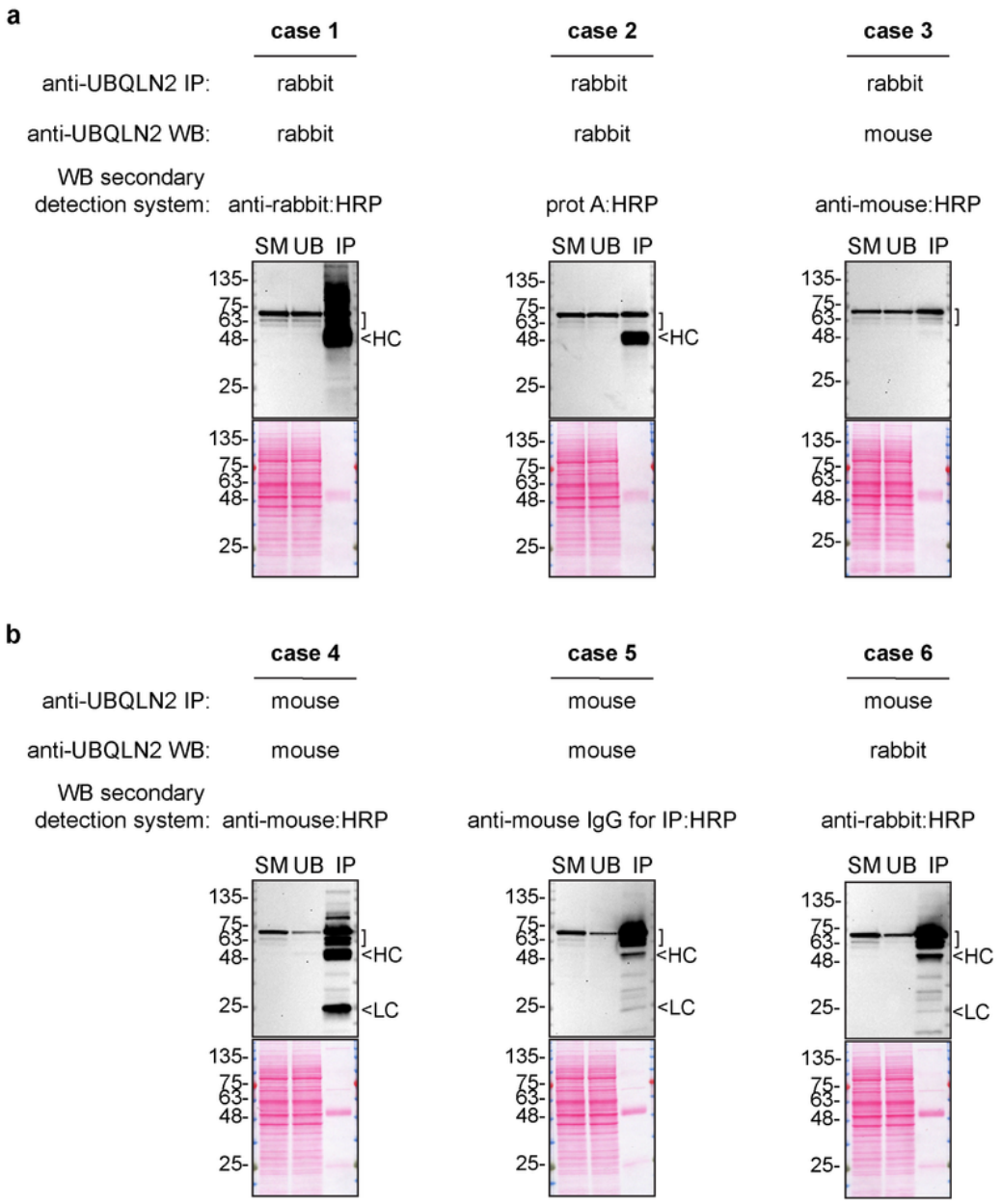


Ayoubi et al., Figure 8

Figure 8

Choice of SDS-PAGE chemistry in WB

WBs are presented as in Figure 2. The chemistry of the SDS-PAGE modifies the reading of the antibody signal. **a)** A KO-validated antibody against the large PLEC protein (Uniprot ID: Q15149, *PLEC* is the corresponding gene) was used in WB from WT and *PLEC* KO lysates ran on three gels with distinct chemistries, namely 4-20% TG, 8% BT (MOPS buffer) and 3-8% TA using TG-SDS, MOPS-SDS and TA SDS buffers, respectively. PLEC has 9 putative isoforms produced by alternative splicing with the canonical PLEC isoform expected at 532 kDa (Uniprot.org). The vertical line followed by a star indicates the region of the gels where the isoforms are identified. The *PLEC* KO cell line expresses residual PLEC protein isoforms. **b)** A KO-validated antibody against the small FCER1G protein (Uniprot ID: P30273) was used in WB of WT and *FCER1G* KO THP-1 lysates, each PMA-treated or not, ran on either 4-20% TG or 10% BT using TG-SDS or MES-SDS, respectively. PMA treatment was used to differentiate THP-1 into adherent macrophage-like cells. FCER1G is expected at ~10 kDa. The BT gels improved both the antibody-based signal and the resolution.



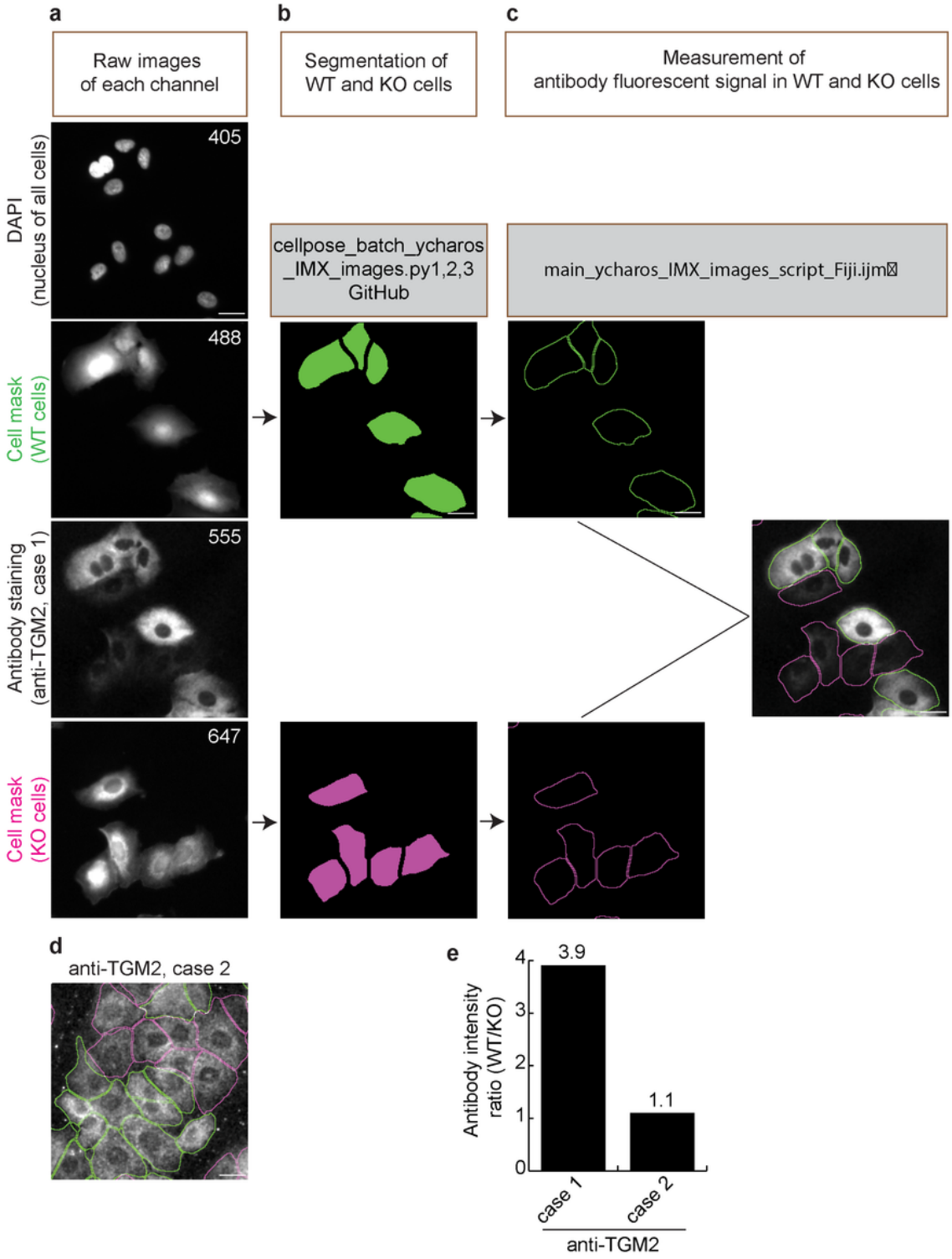
Ayoubi et al., Figure 9

Figure 9

Selection of secondary detection systems for IP-WB experiments

WBs are presented as in Figure 2. A rabbit **a)**or mouse **b)** antibody targeting human UBQLN2 (Uniprot ID: Q9UHD9) was used in IP in combination with different secondary WB detection systems. In case 1 and 2, a rabbit primary antibody was used in WB and detected using either a secondary anti-rabbit:HRP or prot

A:HRP, respectively. In case 3, a mouse primary antibody was used in WB coupled with a secondary anti-mouse:HRP. In case 4 and 5, a mouse primary antibody was used in WB and detected using either a secondary anti-mouse:HRP or anti-mouse IgG for IP:HRP, respectively. In case 6, a rabbit primary antibody was used in WB coupled with a secondary anti-rabbit:HRP. SM=4%, UB=4%, HC=heavy chain, LC=light chain, bracket indicates different UBQLN2 protein species identified in the IP.



Ayoubi et al., Figure 10

Figure 10

Semi-automated analysis of antibody performance in IF

Semi-automated image analysis of a mosaic culture of WT and KO cells was conducted using in-house developed codes that take advantages of the publicly available cellpose algorithm and FIJI (ImageJ) software. Two antibodies against the TGM2 protein (Uniprot ID: P21980) are presented. A *TGM2* KO line, validated by WB, was used. **a)** Images for all four channels corresponding to DAPI (nucleus), CellTracker Green CMFDA (WT cell mask), the antibody staining corresponding to anti-TGM2, case 1 (coupled to an Alexa 555 conjugated secondary antibody) and CellTracker Deep Red (KO cell mask) were acquired with an ImageXpress high-content microscope and prepared for analysis. **b)** A python script that executes cell segmentation using Cellpose1.0 was ran on both cell mask channels. **c)**An ImageJ macro was used to generate cell mask outlines, perform background signal subtraction on the antibody channel using minimum intensity projection. Processed images were overlayed with cell masks outlines in the antibody channel and intensity was quantified in the segmented cells. All scripts are openly available on the YCharOS GitHub page. Bars = 20 µm **d)** Same processes as in **a**, **b**, and **c** were applied to the anti-TGM2 antibody, case 2. **e)** Plot showing the antibody intensity ratio of WT to KO cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Ayoubietal.FiguresS1.png
- DataAvailability.docx
- Table1.docx
- Table2.docx
- Table3.docx
- Table4.docx