DATA NOTE

The identification of high-performing antibodies for TDP-43 for use in Western Blot, immunoprecipitation and immunofluorescence [version 2; peer review: 2 approved]

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Abstract

TAR DNA-binding protein 43 (TDP-43) is a DNA/RNA binding protein playing a critical role in the regulation of transcription, splicing and RNA stability. Mutations in TARDBP leading to aggregation, are suspected to be a characteristic feature of various neurogenerative diseases. The lack of well-characterized anti-TDP-43 antibodies acts as a barrier to establish reproducible TDP-43 research. In this study, we characterized eighteen TDP-43 commercial antibodies for Western blot, immunoprecipitation, and immunofluorescence using a standardized experimental protocol based on comparing read-outs in knockout cell lines and isogenic parental controls. We identified many well-performing antibodies and encourage readers to use this report as a guide to select the most appropriate antibody for their specific needs.

Keywords

Uniprot ID Q13148, TARDBP, TDP-43, antibody characterization, antibody validation, Western Blot, immunoprecipitation, immunofluorescence

This article is included in the Cell & Molecular Biology gateway.
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Author roles: Worrall D: Investigation, Visualization, Writing – Review & Editing; Ayoubi R: Investigation, Writing – Review & Editing; Fotouhi M: Investigation; Southern K: Writing – Original Draft Preparation, Writing – Review & Editing; McPherson PS: Conceptualization, Funding Acquisition, Resources, Supervision; Laflamme C: Conceptualization, Data Curation, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Review & Editing;

Competing interests: For this project, the laboratory of Peter McPherson developed partnerships with high-quality antibody manufacturers and knockout cell line providers. The partners provide antibodies and knockout cell lines to the McPherson laboratory at no cost. These partners include: - Abcam- ABclonal -Aviva Systems Biology -Bio Techne -Cell Signalling Technology -Developmental Studies Hybridoma Bank -GeneTex – Horizon Discovery – Proteintech – Synaptic Systems -Thermo Fisher Scientific.

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Introduction
TDP-43, encoded by the TARDBP gene, is a DNA/RNA-binding protein implicated in RNA metabolism and processing. Belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins that bind to RNA via highly conserved RNA recognition motifs, TDP-43 binds to UG-repeats with high specificity. Mutations in TARDBP that result in TDP-43 aggregation and neuropathology have been observed in distinct neurodegenerative diseases, known as TDP-43 proteinopathies. Various studies have identified a subset of amyotrophic lateral sclerosis (ALS) patients that possess TARDBP mutations, suggesting that TDP-43 gain of toxic function or loss of function is a causative factor in sporadic and/or familial ALS. Mechanistic studies would be greatly facilitated by the availability of high-quality antibodies.

Results and discussion
Our standard protocol involved comparing readouts from wild-type (WT) and TARDBP knockout (KO) cells. The first step was to identify a cell line(s) that expresses sufficient levels of TDP-43 to generate a measurable signal. To this end, we examined the DepMap transcriptomics database to identify all cell lines that express the target at levels greater than 2.5 log2 (transcripts per million (TPM) +1), which we have found to be a suitable cut-off (Cancer Dependency Map Portal, RRID: SCR_017655). Commercially available HAP1 cells expressed the TARDBP transcript at RNA levels above the average range of cancer cells analyzed. Parental and TARDBP knockout HAP1 cells were obtained from Horizon Discovery (Table 1).

For Western Blot experiments, we resolved proteins from WT and TARDBP KO cell extracts and probed them side-by-side with all antibodies in parallel (Figure 1).

For immunoprecipitation experiments, we used the antibodies to immunopurify TDP-43 from HAP1 cell extracts. The performance of each antibody was evaluated by detecting the TDP-43 protein in extracts, in the immunodepleted extracts and in the immunoprecipitates (Figure 2).

For immunofluorescence, as described previously, antibodies were screened using a mosaic strategy. In brief, we plated WT and KO cells together in the same well and imaged both cell types in the same field of view to reduce staining, imaging and image analysis bias (Figure 3).

In conclusion, we have screened TDP-43 commercial antibodies by Western Blot, immunoprecipitation and immunofluorescence and identified several high-performing antibodies under our standardized experimental conditions.

Methods
Antibodies
All TDP-43 antibodies are listed in Table 2, together with their corresponding Research Resource Identifiers (RRID), to ensure the antibodies are cited properly. Peroxidase-conjugated goat-antimouse and anti-rabbit antibodies are from...

Cell culture
Both HAP1 WT and TARDBP KO cell lines used are listed in Table 1, together with their corresponding RRID, to ensure the cell lines are cited properly.11 Cells were cultured in DMEM high glucose (GE Healthcare cat. number SH30081.01) containing 10% fetal bovine serum (Wisent, cat. number 080450), 2 mM L-glutamate (Wisent, cat. number 609065), 100 IU penicillin and 100 μg/ml streptomycin (Wisent, cat. number 450201).

Antibody screening by Western blot
Western blots were performed as described in our standard operating procedure.12 HAP1 WT and TARDBP KO were collected in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) from Thermo Fisher Scientific (cat. number 0089901), supplemented with protease inhibitor from MilliporeSigma (cat. number P8340). Lysates were sonicated briefly and incubated for 30 min on ice. Lysates were spun at ~110,000 x g for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and Western blot. BLUeIelf prestained protein ladder from GeneDir4X (cat. number PM008-0500) was used.

Western blots were performed with precast midi 4-20% Tris-Glycine polyacrylamide gels from Thermo Fisher Scientific (cat. number WXP42012BOX) and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau staining which is scanned together with individual Western blots. Blots were blocked with 5% milk for
Lysates were rocked for 30 min at 4°C and spun at 110,000 × g in 1 ml of Pierce IP Lysis Buffer from Thermo Fisher Scientific (cat. number 87788), supplemented with protease inhibitor (Millipore Sigma, cat. number P8340). HAP1 WT lysates were prepared, and IP was performed using 2 μg of the indicated TDP-43 antibodies pre-coupled to Dynabeads protein G or protein A. Samples were washed and processed for Western blot with the indicated TDP-43 antibody. For Western blot, 80002-1-RR** was used at 1/1000. The Ponceau stained transfers of each blot are shown for similar reasons as in Figure 1.

**Monoclonal antibody, **Recombinant antibody.

1 hr, and antibodies were incubated overnight at 4°C with 5% bovine serum albumin (BSA) (Wisent, cat number 800-095) in TBS with 0.1% Tween 20 (TBST) (Cell Signaling Technology, cat. number 9997). Following three washes with TBST, the peroxidase conjugated secondary antibody was incubated at a dilution of ~0.2 μg/ml in TBST with 5% milk for 1 hr at room temperature followed by three washes with TBST. Membranes were incubated with ECL (Thermo Fisher Scientific, cat. number A44240).

**Antibody screening by immunoprecipitation**

Immunoprecipitation was performed as described in our standard operating procedure.[13] Antibody-bead conjugates were prepared by adding 2 μg to 500 μl of Pierce IP Lysis Buffer from Thermo Fisher Scientific (cat. number 87788) in a 1.5 ml microcentrifuge tube, together with 30 μl of Dynabeads protein A- (for rabbit antibodies) or protein G- (for mouse antibodies) from Thermo Fisher Scientific (cat. number 32106) prior to detection with the iBright™ CL1500 Imaging System (Thermo Fisher Scientific, cat. number A44240).

HAP1 WT were collected in Pierce IP buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) (Thermo Fisher Scientific, cat. number 87788), supplemented with protease inhibitor (Millipore Sigma, cat. number P8340). Lysates were rocked for 30 min at 4°C and spun at 110,000 × g for 15 min at 4°C. 0.5 ml aliquots at 2.0 mg/ml of lysate were incubated with an antibody-bead conjugate for ~2 hrs at 4°C. The unbound fractions were collected, and beads were subsequently washed three times with 1.0 ml of IP lysis buffer and processed for SDS-PAGE and Western blot on precast midi 4-20% Tris-Glycine polyacrylamide gels. Prot-A: HRP (Millipore Sigma, cat. number P8651) was used as a secondary detection system at a dilution of 0.4 μg/ml for an experiment where a rabbit antibody was used for both immunoprecipitation and its corresponding Western blot.

**Antibody screening by immunofluorescence**

Immunofluorescence was performed as described in our standard operating procedure.[8] HAP1 WT and TARDBP KO were labelled with CellTracker™ green (Thermo Fisher Scientific, cat. number C2925) or CellTracker™ deep red
**Figure 3.** TDP-43 antibody screening by immunofluorescence. HAP1 WT and TARDBP KO cells were labelled with a green or a far-red fluorescent dye, respectively. WT and KO cells were mixed and plated to a 1:1 ratio in a 96-well plate with a glass bottom. Cells were stained with the indicated TDP-43 antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody including DAPI. Acquisition of the blue (nucleus-DAPI), green (identification of WT cells), red (antibody staining) and far-red (identification of KO cells) channels was performed. Representative images of the merged blue and red (grayscale) channels are shown. WT and KO cells are outlined on both channels with green and magenta dashed lines, respectively. Antibody dilutions were chosen according to the recommendations of the antibody supplier. Exceptions were given for antibodies 12892-1-AP, MA5-32627**, A19123**, 89789**, 89718** and ab133547** which were titrated to 1/400, 1/1000, 1/900, 1/30, 1/10 and 1/700, respectively, as the signals were too weak when following the supplier’s recommendations. When the concentration was not indicated by the supplier, which was the case for antibodies 80001-1-RR**, GTX630196* and ab254166**, we tested antibodies at 1/700, 1/1000 and 1/500, respectively. At these concentrations, the signal from each antibody was in the range of detection of the microscope used. Antibody dilution used: 10782-2-AP at 1/400, 12892-1-AP at 1/250, 800001-1-RR** at 1/700, 80002-1-RR** at 1/250, MA5-27828* at 1/500, NBP1-92695* at 1/1000, 711051** at 1/500, MA5-27828* at 1/1000, MA5-32627** at 1/500, A19123** at 1/1000, 89789** at 1/900, 89718** at 1/30, GTX630196* at 1/1000, GTX630197* at 1/1000, ab109535** at 1/800, ab254166** at 1/500. Bars = 10 μm. *Monoclonal antibody, **Recombinant antibody.
# Table 2. Summary of the TDP-43 antibodies tested.

<table>
<thead>
<tr>
<th>Company</th>
<th>Catalog number</th>
<th>Lot number</th>
<th>RRID (Antibody Registry)</th>
<th>Clonality</th>
<th>Clone ID</th>
<th>Host</th>
<th>Concentration (μg/μl)</th>
<th>Vendors recommended applications</th>
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<td>Proteintech</td>
<td>10782-2-AP</td>
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<td>-</td>
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<td>2300002</td>
<td>AB_2882933</td>
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<td>11N20</td>
<td>rabbit</td>
<td>0.25</td>
<td>Wb</td>
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<td>16A22</td>
<td>rabbit</td>
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<td>IF</td>
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<tr>
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<td>MAB7778*</td>
<td>CHGW0121061</td>
<td>AB_2920573</td>
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<td>671834</td>
<td>mouse</td>
<td>0.50</td>
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<td>mouse</td>
<td>1.00</td>
<td>Wb, IF</td>
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<td>AB_10859634</td>
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<td>Wb, IF-Methanol</td>
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<td>DB9</td>
<td>mouse</td>
<td>0.47</td>
<td>Wb</td>
</tr>
</tbody>
</table>

Wb=Western blot; IF=immunofluorescence; IP=immunoprecipitation.

*Monoclonal antibody.

**Recombinant antibody.

†Refer to RRID recently added to the Antibody Registry (on January 2023), they will be available on the Registry website in coming weeks.
The nuclei were labelled with DAPI (Thermo Fisher Scientific, cat. number C34565) fluorescent dye, respectively. WT and KO cells were plated on glass coverslips as a mosaic and incubated for 24 hrs in a cell culture incubator at 37°C, 5% CO₂. Cells were fixed in 4% paraformaldehyde (PFA) (Beantown chemical, cat. number 140770-10 ml) in phosphate buffered saline (PBS) (Wisent, cat. number 311-010-CL) for 15 min at room temperature and then washed 3 times with PBS. Cells were permeabilized in PBS with 0.1% Triton X-100 (Thermo Fisher Scientific, cat. number BP151-500) for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum (Gibco, cat. no 16210-064) and 0.01% Triton X-100 for 30 min at room temperature. Cells were incubated with IF buffer (PBS, 5% BSA, 0.01% Triton X-100) containing the primary TDP-43 antibodies overnight at 4°C. Cells were then washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 555-conjugated secondary antibodies in IF buffer at a dilution of 1.0 μg/ml for 1 hr at room temperature with DAPI. Cells were washed 3 × 10 min with IF buffer and once with PBS.

Images were acquired on an ImageXpress micro widefield high-content microscopy system (Molecular Devices), using a 20×/0.45 NA air objective lens and scientific CMOS camera (16-bit, 1.97 mm field of view), equipped with 395, 475, 555 and 635 nm solid state LED lights (Lumencor Aura III light engine) and bandpass emission filters (432/36 nm, 520/35 nm, 600/37 nm and 692/40 nm) to excite and capture fluorescence emission for DAPI, CellTracker™ Green, Alexa fluor 555 and CellTracker™ Red, respectively. Images had pixel sizes of 0.68 × 0.68 microns. Exposure time was set with maximal (relevant) pixel intensity ~80% of dynamic range and verified on multiple wells before acquisition. Since the IF staining varied depending on the primary antibody used, the exposure time was set using the most intensely stained well as reference. Frequently, the focal plane varied slightly within a single field of view. To remedy this issue, a stack of three images per channel was acquired at a z-interval of 4 microns per field and best focus projections were generated during the acquisition (MetaExpress v6.7.1, Molecular Devices). Segmentation was carried out on the projections of CellTracker™ channels using CellPose v1.0 on green (WT) and far-red (KO) channels, using as parameters the ‘cyto’ model to detect whole cells, and using an estimated diameter tested for each cell type, between 15 and 20 microns. Masks were used to generate cell outlines for intensity quantification. Figures were assembled with Adobe Illustrator.

Data availability
Underlying data
Zenodo: Dataset for the TDP-43 antibody screening study, https://doi.org/10.5281/zenodo.7665963.16
Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgments
We would like to thank the NeuroSGC/YCharOS/EDDU collaborative group for their important contribution to the creation of an open scientific ecosystem of antibody manufacturers and knockout cell line suppliers, for the development of community-agreed protocols, and for their shared ideas, resources and collaboration. We would also like to thank the Advanced BioImaging Facility (ABIF) consortium for their image analysis pipeline development and conduction (RRID: SCR_017697). Members of each group can be found below.


ABIF consortium: Claire M. Brown and Joel Ryan.

An earlier version of this article can be found on Zenodo (https://doi.org/10.5281/zenodo.7249802).

References


Open Peer Review

Current Peer Review Status: ✔️ ✔️

Version 1

Reviewer Report 14 June 2023

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Various antibodies against TDP-43 are now commercially available from several companies, but their validation is still insufficient. This study comprehensively validated the performance of various antibodies, which will provide important information for researchers when they select TDP-43 antibodies for their studies. The procedures are detailed and carefully described, and together with the references, are well reproducible.

Pathological TDP-43 is generally aggregated, fragmented, and hyperphosphorylated, which may affect antibody recognition. Thus, although it would be beyond the scope of this study, future validation using human patients’ samples, as recommended by the reviewer #1, or of the recognition sites of each antibody would make this data even more valuable.

The references 9, 12, and 13 were found to be available on Zendo, but it is difficult to reach them because of a lack of direct links. It is recommended that the DOI be appended.

Is the rationale for creating the dataset(s) clearly described?
Yes

Are the protocols appropriate and is the work technically sound?
Yes

Are sufficient details of methods and materials provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Due to the continuing significance of morphological assay to clarify TDP-43 proteinopathy, the characteristics of the antibody are crucial to drawing convincing and replicable results. Numerous antibodies against TDP-43 are commercially available to meet the needs. As is often the case, however, the results could be misleading unless the antibodies are used with a sufficient understanding of their properties. However, such information is hardly accessible until our use.

In this work, the authors comprehensively investigated the specificity and affinity of commercially available antibodies against TDP-43 by analyzing Western blotting, immunoprecipitation, and immunofluorescence. Another advantage of their work is adopting HAP1 cells through careful screening from transcription levels and TARDBP-KO cells. They successfully suggested several antibodies, which are conformation- and sequence-specific with high reactivity. Experimental protocols are clearly written, and the data presentation is compelling. This work deserves considerable attention because such information may help researchers minimize the time for optimization and acquire solid and replicable results. The weak points of their work is a lack of experiments using ALS-linked TDP-43 mutations or immunohistochemistry of ALS patients. However, those are beyond their scope, which awaits future validation.
Are the datasets clearly presented in a useable and accessible format?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Motor neuron disease, protein misfolding, antibody generation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response 21 Mar 2023**

**Kathleen Southern**

Thank you to Makoto Urushitani for your review of our publication. Although there is an abundance of commercial antibodies available for TDP-43, there lacks guidance to help researchers find the appropriate antibody for their experimental need. Accordingly, we too agree that the characterization of antibodies is a determinative factor when drawing convincing and replicable results. As such, the YCharOS initiative has set out to solve the antibody reproducibility crisis by characterizing commercially available antibodies for human proteins.

In terms of scoring the antibodies based on performance, we have found that for the most part, scientists interested in our reports have the expertise to interpret the antibody characterization data. Moreover, because we tested the antibodies under one set of conditions, and the scoring/recommendation would be valid only under this precise experimental setup and in the cell line used. That said, YCharOS reports serve as an invaluable guide pointing scientists to appropriate antibodies for their experimental needs.

In the future, we hope to study the ALS protein-network in ALS patients, using the YCharOS reports as a guide to select the appropriate antibodies. As for now, our aim for this publication is to assist researchers in selecting high-quality TDP-43 antibodies for studying TDP-43 proteinopathies.

**Competing Interests:** No competing interests were disclosed.
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