ONLY GOOD ANTIBODIES

A Framework for Planning Antibody Validation

Principles and guidance for researchers, supervisors, and reviewers

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Why plan antibody validation?

Antibodies are among the most widely used tools in biomedical research, but they do not always bind exclusively to their intended targets. Over half of commercial antibodies fail rigorous independent testing, and the vast majority of published papers using poorly performing antibodies present no relevant validation data.

The cost of using an antibody that does not work as assumed is not limited to the immediate experiment. It can misdirect an entire research programme, waste funding, and consume animal and human biological samples in experiments whose conclusions are unreliable.

This document sets out a practical framework for planning antibody validation and documenting your reasoning. It applies to researchers planning experiments, supervisors reviewing plans, reviewers assessing manuscripts, and funders evaluating applications.

The framework is built on three principles: what you need from an antibody depends on your scientific question; your controls should match your experimental context as closely as possible; and your validation should be done in the exact assay system you intend to use. It applies proportionality, so that the most rigorous scrutiny is directed at the antibodies where the risk is highest.

1. What does your scientific question require from the antibody?

Not all antibody-dependent experiments require the same level of validation. The critical factor is what your scientific question actually demands from the antibody in terms of specificity. This determines how much evidence you need and what kind.

We distinguish three situations:

The question is about the specific protein target

When your research question is about whether a particular protein plays a role in a process or disease — including its role in a signalling pathway, disease mechanism, or biological process — you need high confidence that your antibody data relates directly to that protein. If the antibody is detecting something else, your conclusion is wrong.

Examples include testing whether a candidate drug target is upregulated in disease tissue, investigating a protein’s role in a signalling pathway, or quantifying expression of a specific protein across experimental conditions.

For these experiments, aim for the strongest validation evidence available — ideally genetic controls (knockout or knockdown) in the application and sample type you are using.

Characterisation using community-adopted markers

In this category, the antibody is not directed at your experimental target. It is a community-adopted tool for identifying a cell population, phenotype, or well-studied outcome measure — defining the context in which your experiment takes place, not the subject of it. For example, an anti-CD4 antibody used to identify T helper cells in a study about T cell function.

If there are community-adopted tools and methods for this purpose — for example, HCDM workshop-verified clones for CD markers, or OMAP-validated panels for spatial biology — it may be sufficient to adopt these tools, provided you are using the same clone as characterised by the consortium and you adopt it critically. Confirm you are using the correct clone, not just the same target name. Different clones labelled against the same target may have different binding characteristics.

This approach carries a risk of groupthink. For well-established populations with decades of cross-validation, the risk is lower. For less well-studied populations, the evidence base behind the markers may be thinner than assumed. Characterisation markers for rare cell types should be regarded with appropriate caution. Where phenotypic analysis is critical to your hypothesis, consider applying the same evidence standard as for target-specific questions.

The question does not depend on what the antibody is detecting

In some cases, the antibody serves a technical function where specificity for the stated target is not what matters. The most common example is loading controls in Western blotting, where the purpose is to confirm equal loading rather than to make a claim about a specific protein.

For these uses, consider whether better alternatives exist. Total protein staining methods (such as Ponceau S or Stain-Free gels) are increasingly recognised as more reliable than antibody-based housekeeping controls, which can vary with experimental conditions. If you are using an antibody purely as a process control, document what it is actually for.

2. Selecting controls

The central principle of control selection is that off-target binding depends on the expression levels of cross-reactive proteins in your specific sample. Controls should therefore match your experimental sample and protocol as closely as possible.

Positive controls

Options for positive controls, ranging in strength and feasibility:

- The wild-type cell line from a knockout pair. Excellent — but verify the knockout independently, as approximately 30% of commercial knockout cell lines may not be true knockouts. The knockout may have been produced in a cell line without robust expression of the target.
- A tagged construct for transient transfection. Useful for screening antibodies quickly, but overexpression is supra-physiological and cannot confirm detection at endogenous levels.
- A commercial overexpression lysate (e.g. from OriGene, approximately £200 for 20µg). Shows where your protein should appear on a blot and is useful for ruling out bad antibodies cheaply before committing to more expensive validation.
- An engineered cell line with gene insertion (or re-insertion) at endogenous expression levels, with or without a tag. CRISPR knock-in approaches produce the most physiologically relevant positive control.

- A cell line engineered using lentiviral transduction for stable exogenous expression, with some control over expression level. Less physiological than knock-in but more accessible.

When choosing a positive control cell line, confirm that it actually expresses your target protein. Proteomic datasets such as ProCan-DepMapSanger (available at cellmodelpassports.sanger.ac.uk, covering 949 cell lines) provide mass spectrometry-based protein quantification. RNA expression data from DepMap or the Cancer Cell Line Encyclopedia can also guide cell line selection, though a commonly used threshold of TPM ≥2.5 for likely expression is not definitive — mass spectrometry confirmation is stronger where available.

Negative controls

Options for negative controls:

- A genetic knockout cell line in the cell type of interest. This is the gold standard but is not feasible for human tissue or for essential genes. Verify the knockout independently.
- A knockout cell line in a different cell type, used for initial antibody characterisation and selection. This is the YCharOS approach — open characterisation data is available through the OGA Antibody Database (onlygoodantibodies.co.uk). The characterisation identifies which antibodies are most likely to be selective, even though performance in your specific experimental context may differ.
- siRNA or shRNA knockdown. Useful when knockout is not feasible, but partial reduction in signal is harder to interpret. Confirm knockdown efficiency by RT-qPCR. Be aware that siRNA itself has off-target effects — if the signal does not reduce, the problem could be the knockdown, the antibody, or both.
- A cell line or tissue without expression of the target. Check proteomic or transcriptomic datasets to identify candidate negative controls. This is less conclusive than genetic controls because the absence of signal may reflect expression level rather than antibody specificity.
- For immunohistochemistry on human tissue: consider staining formalin-fixed paraffin-embedded (FFPE) cell pellets from knockout cell lines alongside your tissue sections, using the same protocol.

Additional controls for quantitative work

If you are developing quantitative or semi-quantitative assays, you may need controls that span a range of expression levels. These could include tissues or cell lines with graded expression (guided by RNA or proteomic data), or for ELISA-type assays, spiking recombinant protein into plasma at known concentrations. The principle remains the same: match your controls to your experimental context.

3. Carrying out the validation

Use the controls you have selected in the exact assay system you are using. If you are validating for immunofluorescence, run your positive and negative controls through your immunofluorescence protocol. An antibody that works in one application does not necessarily work in another, because the protein is presented differently (denatured vs. native, fixed vs. unfixed, intracellular vs. surface).

Protocol details matter. For flow cytometry, fixation and permeabilisation method can fundamentally change antibody performance. An antibody that works for surface staining

may fail after fixation. For intracellular targets, consider testing multiple fixation and permeabilisation protocols (e.g. PFA-saponin, PFA-Triton, methanol), as the optimal protocol depends on the specific antibody and target combination.

If the controls do not work — if you cannot demonstrate a clear difference between your positive and negative controls in the assay you intend to use — do not use that antibody in that assay.

Where possible, build in antibody-independent readouts to confirm the same story. If your antibody shows increased protein expression, does RT-qPCR show increased mRNA? If flow cytometry shows a shift, does single-cell RNA sequencing support the same conclusion? These complementary approaches strengthen any antibody-based finding.

4. Searching for existing evidence

Search for existing independent characterisation data before planning your own experiments.

Recommended sources include:

- The OGA Antibody Database (onlygoodantibodies.co.uk) — curated, searchable characterisation data with knockout controls across Western blot, immunoprecipitation, immunofluorescence, and flow cytometry.
- BenchSci — AI-indexed published images; filter by "genetic" verification to find knockout-validated data.
- CiteAb — citation-ranked antibody data; filter by knockdown or knockout verification.
- Labome — manually curated knockout validation data.
- HCDM (hcdm.org) — workshop-verified clones for CD markers in flow cytometry.
- Google Images — search for "[target] knockout validated antibody [application]" to find vendor and published data.

When evaluating existing data, look at the actual images rather than relying on tick-boxes or claims. Check whether the data is from the same application and sample type as your planned experiment. Manufacturer data with genetic controls is replicable more than 80% of the time, but always verify the specifics.

Prioritise recombinant antibodies where available. Large-scale independent testing shows recombinant antibodies have the highest success rates across applications (approximately 67% for Western blot, 48% for immunofluorescence), compared with monoclonals (41% WB, 31% IF) and polyclonals (27% WB, 22% IF).

5. Documenting your plan

A documented plan allows a supervisor to check whether the approach is proportionate, a reviewer to assess whether the evidence supports the claims, and a funder to evaluate whether the methodology is sound.

For each antibody-dependent experiment, record:

1. What you are trying to show and which application and sample type you are using.
2. What your scientific question requires from the antibody — high specificity for the target, comparability with community tools, or a technical function.
3. What existing evidence you found (or did not find) and where you searched.

4. Your positive and negative control strategy, including the specific materials you will use and how closely they match your experimental sample.
5. The antibody identity: vendor, catalogue number, lot number, clone name, RRID, host species, and the dilution or concentration used.
6. Any antibody-independent readouts you will use to corroborate the findings.
7. The outcome of validation experiments and your decision to proceed, reject, or test further.

An interactive version of this framework, which guides you through these prompts and generates a structured document, is available at onlygoodantibodies.co.uk/tools/validation-planner.

6. How this framework fits with other initiatives

This framework implements principles endorsed by a multi-stakeholder Delphi consensus study (Blades, Biddle, Froud et al., 2026) in which 32 international experts rated proposed interventions for improving antibody validation practices. The Delphi panel reached consensus that researchers should be trained in antibody validation, that institutions should embed validation expectations into research integrity frameworks, and that funders should require validation plans in grant applications.

The categorisation approach (target-specific, population-level, technical function) is a practical implementation of these principles, presented as a design framework for community testing and refinement.

Further resources

- IWGAV five-pillar framework (Uhlén et al., 2016, Nature Methods 13:823–827) — the standard vocabulary for describing validation approaches: genetic, orthogonal, independent antibody, recombinant expression, and capture mass spectrometry.
- YCharOS consensus protocol (free at protocols.io/view/a-consensus-platform-for-antibody-characterization-14egn9d16l5d; canonical version: Ayoubi et al., 2024, Nature Protocols) — detailed protocols for knockout-based antibody characterisation across Western blot, immunoprecipitation, immunofluorescence, and flow cytometry.
- EuroMAbNet practical guide (euromabnet.com/guidelines) — broader validation guidelines across applications, with video tutorials.

Contact and feedback

We welcome feedback on this framework. If you have suggestions, concerns, or would like to be involved in developing implementation guidance, please contact:

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