Fc-Engineered Antibody Functional Characterization Protocol

ADCC | CDC | ADCP | Fcγ Receptor Binding | FcRn Binding

Standalone companion to the Fc-Engineered Antibody Expression Vector Construction Short Protocol

Version 2.0 • November 2026 • Research use only

1. Purpose and scope

This protocol describes adaptable in-vitro methods for characterizing Fc-mediated effector functions and receptor interactions of purified, Fc-engineered antibodies. It is intended for comparative research, not as a validated release test or a claim of clinical performance. Select assays according to the engineered Fc variant and target biology.

2. Assay selection guide

Fc-engineering objective	Priority measurements	Supporting measurements
Effector-null / reduced Fc activity	ADCC, CDC, FcγRIIIa and C1q binding	ADCP, antigen binding
ADCC-enhanced	ADCC; FcγRIIIa binding	ADCP, antigen binding
CDC/complement-modulated	CDC; C1q binding	C3b/C4b deposition, ADCC
FcRn/PK-modulated	FcRn binding at pH 6.0 and 7.4	pH-dependent release, stability
Structural/isotype-stabilized	Monomer content, aggregation, antigen binding	Selected Fc-function assays

3. General experimental design and controls

- Test a matched wild-type Fc antibody with identical variable regions, expression background, and purification history.
- Include an isotype-matched irrelevant antibody, no-antibody control, and assay-specific positive and negative controls.
- Confirm antigen binding and target-cell antigen density before attributing functional differences to Fc engineering.
- Measure concentration, purity, aggregation (e.g., SEC), and endotoxin where relevant; normalize by molar antibody concentration.
- Run a serial antibody concentration series spanning baseline through saturation; use at least technical duplicates or triplicates and independent biological replicates when feasible.
- Document cell passage, viability, effector donor or reporter lot, complement lot, receptor isoform/allotype, and instrument settings.

4. ADCC — Antibody-Dependent Cellular Cytotoxicity

4.1 Principle

Antibody-coated antigen-positive target cells engage FcγRIIIa (CD16a) on effector cells, resulting in target-cell killing or a reporter signal. Primary NK-cell assays measure cytotoxicity directly; reporter assays measure FcγRIIIa signaling and should not be described as direct target-cell lysis.

4.2 Materials

- Antigen-positive target cells and antigen-negative matched control cells; viable NK cells/PBMCs or validated FcγRIIIa reporter effector cells.
- Purified test and wild-type antibodies; cell culture medium and assay plates; appropriate viability/lysis readout or reporter substrate.
- Controls: target-only spontaneous signal, maximum-lysis control (for cytotoxicity assays), effector-only, irrelevant IgG, and positive reference antibody.

4.3 Example workflow

1. Confirm target-cell viability and surface antigen expression. Prepare a concentration series of engineered and wild-type antibodies in assay medium.

2. Dispense equal numbers of target cells into assay wells and add antibody dilutions; allow target coating under conditions optimized for the cell system.
3. Add effector cells at a prequalified effector-to-target ratio (commonly screened across approximately 5:1–20:1 for cell-killing assays); include target-only and effector-only controls.
4. Incubate under validated assay conditions. A short cytotoxicity assay often uses a several-hour incubation; reporter assays may require a longer vendor-specified interval.
5. Read target-cell lysis (flow cytometry, LDH, or luminescent viability) or reporter activity. Fit concentration–response curves and compare EC50 and maximal response.

4.4 Calculations and interpretation

Example percent specific lysis = 100 × (experimental release − spontaneous release) / (maximum release − spontaneous release). Use only when the chosen assay readout supports this normalization. Report donor variability and FcγRIIIa V158/F158 allotype when applicable. Reduced reporter signaling does not, by itself, establish absent cytotoxicity.

5. CDC — Complement-Dependent Cytotoxicity

5.1 Principle

Antigen-bound antibody recruits C1q and activates the classical complement pathway, which can result in target-cell lysis. CDC depends strongly on antigen density, epitope geometry, complement source, and target-cell complement regulators.

5.2 Materials

- Antigen-positive target cells and antigen-negative control; engineered and wild-type antibodies.
- Qualified complement source (e.g., human serum or a characterized complement reagent), plus matched heat-inactivated complement control.
- Viability or lysis assay; optional C1q/C3 deposition reagents.

5.3 Example workflow

1. Verify target-cell viability and antigen expression. Prepare a serial dilution of each antibody.
2. Combine target cells with antibody dilutions; include no-antibody and irrelevant-IgG wells.
3. Add a prequalified complement dilution to test wells, with parallel heat-inactivated or complement-free wells.
4. Incubate at 37°C for an assay-specific interval established in pilot testing; minimize variation in complement thawing and handling.
5. Measure viable cells or released lysis marker; optionally quantify C1q or C3 deposition by flow cytometry.

5.4 Calculations and interpretation

Report percent lysis or survival relative to matched complement-only and maximal-lysis controls, according to the readout. Fit EC50 and maximum lysis. Interpret C1q binding together with CDC because binding alone does not demonstrate complement-mediated killing.

6. ADCP — Antibody-Dependent Cellular Phagocytosis

6.1 Principle

FcγR-expressing phagocytes engulf antibody-opsonized targets. Uptake can be measured using fluorescently labeled cells or particles and quantified by flow cytometry or imaging.

6.2 Materials

- Antigen-positive fluorescent target cells or antigen-coated particles; monocyte-derived macrophages or validated phagocytic cells.
- Engineered and matched wild-type antibodies; fluorescence quenching or pH-sensitive uptake dye to distinguish internalized from surface-bound material.
- No-antibody, irrelevant-IgG, antigen-negative, and phagocytosis-inhibition controls as appropriate.

6.3 Example workflow

1. Label target cells/particles and verify signal and viability; prepare serial antibody dilutions.
2. Opsonize targets with antibody, then wash if required by the assay design.
3. Combine targets and phagocytes at a pilot-optimized ratio and incubate under conditions that preserve phagocyte viability.
4. Stop uptake, quench extracellular fluorescence or use an internalization-sensitive dye, and collect flow-cytometry or imaging data.
5. Quantify the fraction of phagocytes with internalized targets and uptake intensity, comparing engineered and wild-type antibodies.

6.4 Calculations and interpretation

Example phagocytic score = (% uptake-positive phagocytes) × (mean fluorescence intensity of uptake-positive cells), with background subtraction and a consistently defined gating strategy. Distinguish binding/adherence from true internalization.

7. Fcγ Receptor Binding — ELISA, BLI or SPR

7.1 Principle and receptor panel

Measure interaction with human FcγRI (CD64), FcγRIIa (CD32a; relevant allotypes), FcγRIIb (CD32b), and FcγRIIIa (CD16a; V158 and F158 as relevant). FcγRIIIb may be included for particular research questions. FcγR binding is context-sensitive to receptor glycosylation, avidity, and immobilization geometry.

7.2 ELISA screening workflow

1. Immobilize qualified recombinant FcγR or capture it using an appropriate tag-specific reagent; block nonspecific binding.
2. Add serial dilutions of purified antibodies and incubate under consistent conditions.
3. Wash and detect bound antibody using a noncompeting detection reagent; include blank and irrelevant-IgG controls.
4. Record background-corrected signal versus antibody concentration and compare apparent EC50 or relative binding.

7.3 BLI/SPR kinetic workflow

1. Select a capture format minimizing avidity artifacts (e.g., captured FcγR or antibody), and establish a stable baseline in running buffer.
2. Inject or dip into a dilution series of the soluble binding partner with reference and blank controls.
3. Monitor association and dissociation; regenerate or replace surfaces according to validated instrument methods.
4. Fit an appropriate binding model only when data quality supports it; report ka, kd, KD, fit residuals, and immobilization density.

For low-affinity FcγR interactions, equilibrium affinity or steady-state binding may be more defensible than a simple 1:1 kinetic fit. Do not compare apparent affinities across differing receptor formats without qualification.

8. FcRn Binding — pH-Dependent ELISA, BLI or SPR

8.1 Principle

FcRn binds IgG Fc more strongly at acidic endosomal pH (approximately 6.0) and typically releases it near physiologic pH (approximately 7.4). Binding changes are a mechanistic indicator, not a direct measurement of in-vivo serum half-life.

8.2 Materials and buffer conditions

- Qualified recombinant human FcRn/β2-microglobulin complex, matched wild-type and engineered antibodies.
- Assay-compatible acidic buffer (target pH ~6.0) and neutral buffer (target pH ~7.4), matched for ionic strength where feasible.
- ELISA, BLI or SPR platform with reference channels, blanks, and appropriate capture chemistry.

8.3 Example workflow

1. Equilibrate receptor and analyte in acidic assay buffer. Establish baseline and verify low nonspecific binding.
2. Measure binding of serial antibody concentrations at pH ~6.0; use reference subtraction and fit equilibrium or kinetic parameters as supported by data.
3. Repeat or perform a controlled buffer shift to pH ~7.4 to evaluate dissociation/release, avoiding artifacts from abrupt bulk refractive-index changes.

4. Compare engineered and wild-type Fc for acidic binding, neutral-pH residual binding, and release behavior; repeat with independent preparations.

8.4 Interpretation

Report KD at pH 6.0 when quantifiable, and percent residual binding or dissociation at pH 7.4. Stronger pH 6.0 binding is not necessarily advantageous if neutral-pH release is impaired. PK claims require additional appropriate studies.

9. Data reporting and acceptance considerations

Assay	Primary outputs	Minimum contextual information
ADCC	EC50, maximal lysis or reporter response	Cell identity, E:T ratio, donor/allotype, assay readout
CDC	EC50, maximum lysis; optional complement deposition	Complement source/lot, target antigen density
ADCP	% uptake-positive, phagocytic score	Phagocyte source, uptake discrimination method
FcγR binding	Apparent EC50 or KD/kinetic rates	Receptor isoform/allotype, assay format, model
FcRn binding	pH 6 KD; pH 7.4 release	Buffer pH, receptor construct, capture format

Before establishing formal acceptance criteria, qualify assay precision, dynamic range, inter-run variability, reference antibody performance, and suitability for the intended decision. Report uncertainty and do not infer effector activity from receptor binding alone.

10. Troubleshooting

Observation	Possible cause	Suggested check
Weak ADCC/CDC signal	Low antigen density, inactive effector/complement	Confirm antigen expression and positive control
High background	Nonspecific binding or target death	Use irrelevant IgG, blanks, viability checks
ADCP signal without internalization	Surface adherence	Quench external fluorescence or use pH-sensitive dye
Poor BLI/SPR fits	Avidity, mass transport, low affinity	Reduce surface density; consider steady-state analysis
Unexpected FcRn binding at pH 7.4	Buffer mismatch or slow release	Verify pH and reference subtraction

11. Documentation and limitations

Record protocol revision, reagent vendors/lots, instrument software, raw data, analysis settings, control performance, deviations, and conclusions. These procedures are general research workflows and require optimization and verification for each antibody, cell model, receptor reagent, and assay platform. The Fc-engineered expression vector kit does not include assay cells, complement, recombinant receptors, or detection reagents unless explicitly stated in the product documentation.

Prepared by Fusion BioLabs research team. This document does not claim that any listed assay or Fc variant has been validated by Fusion BioLabs.