Post-Computational Design Experimental Reference Guide

> ***Workflow Overview:***
>
> *1. Design mutation site combinations based on input antibody sequences and CDR design parameters;*
>
> *2. Predict complex structures and rank site combinations;*
>
> *3. Screen sequences meeting target criteria according to experimental conditions.*

Overview

This guide is intended to direct R&D personnel in the wet-lab validation of antibody CDR mutants generated through computational design.

- **1.1 Objective:** Screen candidate molecules with sequence identity below a specified threshold while maintaining or improving affinity/function.
- **1.2 Strategy:** Select the appropriate synthesis and validation strategy based on the number of mutants per CDR region.

Mutants per CDR	Recommended Strategy	Synthesis Method	Experimental Platform
< 20	Direct recombinant expression	Gene synthesis / Site-directed mutagenesis	Mammalian cells (CHO/HEK293)
20 – 100	Library construction & screening	Column-synthesized primers	Phage Display
> 100	Library construction & screening	Oligo Pool (chip-synthesized oligonucleotide pool)	Phage Display

1.3 Core Rationale

- **Pre-experiment:** Establish baseline data for the parental antibody.
- **Small-scale mutations (<20):** Directly construct full-length antibodies or Fab fragments for point-by-point validation; select the best CDR combination to form a "new parental" antibody.
- **Large-scale mutations (>20):** Using the "new parental" scaffold, employ phage display technology for high-throughput screening of high-diversity CDRs.

Phase 1: Parental Pre-experiment

A reliable test baseline must be established before processing any mutants.

- **2.1 Purpose:** Ensure the parental antibody exhibits detectable activity in the designated assay system and serves as the benchmark for subsequent screening.
- **2.2 Procedures:**
  - **Display efficiency verification (if phage screening is planned):** Construct parental scFv/Fab phage (scFv format recommended) and assess phage display level.
  - **Binding activity verification:** ELISA/SPR/BLI: Determine the parental EC_{50} or K_D against the target (BLI recommended); FACS: If the target is a membrane protein, verify the parental binding curve on positive cells.
- **2.3 Decision Point:** If the parental antibody originates from display screening with well-documented data, this step may be skipped. If the parental antibody fails to reproduce binding activity, experimental conditions must be optimized first (e.g., antigen quality, buffer formulation) before proceeding to mutant screening.

Phase 2: Small-Scale Mutant Validation (<20 Mutants/CDR)

For CDR regions with a limited number of mutations (e.g., H1=15, L1=10), a medium-to-low throughput recombinant expression strategy is employed.

- **3.1 Vector Construction & Expression**
  - **Format selection:**

    - hIgG1 (recommended): Long half-life, high stability, convenient for purification and downstream assays.
    - Fab/scFv: Suitable for rapid screening, but note that affinity may differ from full-length.
  - **Construction strategy:** Each recombinant plasmid contains a single mutation in one CDR.
  - **Expression system:** ExpiCHO or Expi293 transient transfection system recommended.
  - **Scale:** 4–10 mL culture volume typically yields sufficient protein for initial screening (0.5–2 mg).
- **3.2 Activity Assessment & Screening**
  - **Purification:** Protein A/G affinity purification; one-step SEC or SDS-PAGE confirmation of purity >90% recommended.
  - **Detection methods:**
    - Soluble antigen: Biacore (SPR) or Octet (BLI) for single- or multi-concentration kinetics.
    - Cell-surface antigen: Flow cytometry (FACS) for MFI measurement.
  - **Screening criteria:**
    - Yield & quality: Eliminate mutants with extremely low expression or severe aggregation.
    - Affinity: Select mutants with binding activity comparable to (≤3-fold) or better than the parental.
  - **Output:** Combine the best-performing mutant sequences from each CDR region to construct the "new parental" antibody as the scaffold for the next phase of large-scale screening.

Phase 3: Large-Scale Mutant Screening (>20 Mutants/CDR)

For CDR regions with high diversity, phage display technology is employed for high-throughput screening.

- **4.1 Mutant Library Primer Design & Synthesis**
  - **Scaffold selection:** Use the "new parental" determined in Phase 2 as the template.
  - **Synthesis strategy comparison:**
    - Column-synthesized primers: for 20–100 mutant CDRs.
    - Oligo Pool (chip-synthesized oligonucleotide pool): for >100 mutant CDRs.
- **4.2 Library Construction**

- **Fragment assembly:** Use overlap extension PCR (SOE-PCR) to splice mutant CDR fragments with the scaffold. Format: scFv recommended (Linker: $(G_4S)_3$). Key point: Both ends of the scFv fragment must include homology arms (>15 bp) matching the phagemid vector for homologous recombination.
- **Vector ligation:** Recommended: Homologous recombination (e.g., Gibson Assembly / In-Fusion), far more efficient than traditional restriction enzyme ligation. Alternative: Double restriction enzyme digestion ligation (SfiI/NotI, etc.).
- **Transformation & library establishment:** Electroporation: TG1 electrocompetent cells preferred; ensure transformation efficiency $>1\times10^8$ to cover library diversity. Chemical transformation: Use only when diversity is extremely low ($<1\times10^5$).
- **Library QC:**
  - Library size: Calculate total transformants via serial dilution plating.
  - Accuracy: Randomly pick 24–48 single clones for Sanger sequencing.
  - Acceptance criteria: Correct reading frame rate >50% (ideal >70%), with designed mutation sites present.
  - Insert rate: Colony PCR to detect empty vector rate.

- **4.3 Phage Display Screening**
  - **Screening rounds:** Typically 3–4 rounds of panning.
  - **Antigen format & strategy:**
    - **Soluble antigen:** Solid-phase panning: Coat immunotubes/microplates (easy epitope exposure, but may cause antigen denaturation). Solution-phase panning: Biotinylated antigen + magnetic bead pull-down (preserves native antigen conformation; recommended).
    - **Transmembrane/complex antigen:** Cell-based panning: Overexpressing cell line (+) vs. null cell line (−). Biomimetic membrane system: Use Nanodiscs or VLPs to present antigen, combined with magnetic bead selection.
    - **Cross-panning strategy:** Screening is not limited to a single strategy. In addition to pure solid-phase, solution-phase, or cell-based panning, cross-panning approaches (solid/solution, solid-phase/cell) can be employed to enrich antibodies with high specificity and native conformation recognition.
  - **Selection pressure:** Progressively decrease antigen concentration across rounds (e.g., 100 nM → 10 nM → 1 nM) or increase wash stringency to enrich high-affinity clones.
- **4.4 Hit Identification**

- **Enrichment detection (Polyclonal ELISA/FACS):** Assess binding signal of each round's phage pool to confirm enrichment trend.
- **Monoclonal screening:** Pick 96–384 single clones from the round showing clear enrichment (typically R3 or R4). Prepare monoclonal phage supernatants for ELISA or FACS-based primary screening.
- **Sequencing & analysis:** Sanger-sequence positive clones, analyze CDR sequences, remove duplicates, and determine unique hits.

Phase 4: Final Validation (Candidate Molecule Confirmation)

Perform detailed validation of the unique hits, analogous to Phase 2.

- **5.1 Full-length/Fab recombinant expression:** Convert scFv sequences back into mammalian expression vectors.
- **5.2 Purification & QC:** SEC-HPLC analysis of purity and aggregate proportion.
- **5.3 Precise affinity determination:**
  - Measure $k_{on}/k_{off}/K_D$ using Biacore/Octet, or determine EC_{50} by FACS.
  - Perform head-to-head comparison with the parental antibody.
- **5.4 Functional assays:** Depending on project requirements, conduct in vitro efficacy studies (e.g., blocking assays, cell killing assays) or epitope binning experiments.

Frequently Asked Questions & Considerations

6.1 Low QC sequencing accuracy:

- Check primer synthesis quality (especially for long primers).
- Optimize PCR conditions to reduce non-specific amplification.
- For Oligo Pools, verify that amplification cycle number is not too high, which may introduce bias.

6.2 No enrichment after panning:

- Antigen failure: Re-validate antigen activity.

- Over-stringent washing: Aggressive washing in R1 may cause loss of rare clones.
- Low display efficiency: Check helper phage titer or helper phage ratio.

6.3 Monoclonal sequence homogeneity:

- This may indicate "biological advantage" amplification (a clone with a growth advantage that does not necessarily bind well). Consider increasing antigen selection pressure in subsequent rounds or switching to a different panning strategy.