

# **Research Proposal: Experimental Investigation of Multifunctional Artificial Enzymes with Unnatural Amino Acids**

## **Research Question**

Can we create bifunctional artificial enzymes by strategically combining successful mutations from specialized variants and exploring dual incorporation of different UAAs?

## **Objectives (4-week timeframe)**

1. Generate hybrid variants combining key mutations from hydrazone-specialized (RMH) and Friedel-Crafts-specialized (RGN) enzymes
2. Create and test variants incorporating both pAF and aY at distinct positions within the LmrR scaffold
3. Evaluate synergistic effects between distal mutations identified in prior studies for enhanced dual catalysis

**Methodology**

**Week 1: Design and Construction of Hybrid Variants**

- Design hybrid variants combining key mutations from RMH (A92R, N19M, F93H) and RGN (L18R, F93G, S95G)
- Construct variants with single UAA incorporation (either pAF or aY at position 15)
- Prepare site-directed mutagenesis for dual UAA incorporation (pAF at position 15 and aY at a second position)
- Transform expression systems and initiate protein production

**Week 2: Protein Expression and Purification**

- Express hybrid variants in the presence of appropriate UAAs using the established incorporation system
- Purify proteins using established protocols (Ni-NTA chromatography)
- Verify UAA incorporation using mass spectrometry
- Assess protein stability through thermal denaturation experiments

**Week 3: Catalytic Activity Screening**

- Test all variants for hydrazone formation activity using the NBD-H/4-HBA assay
- Test all variants for Friedel-Crafts alkylation using standard indole/cinnamaldehyde substrates
- Identify variants that maintain dual catalytic activities
- Perform preliminary kinetic analysis of promising candidates

**Week 4: Substrate Scope and Data Analysis**

- Evaluate substrate scope of the best-performing bifunctional variants
- Examine the influence of distal mutations (F54L, N88Q) on catalytic activities
- Analyze structure-function relationships based on existing crystal structures
- Prepare research summary and proposals for future work

**Expected Outcomes**

- 1.Identification of at least one hybrid variant that maintains both catalytic activities
- 2.Understanding of how mutations from specialized variants interact in hybrid constructs
- 3.Preliminary data on the feasibility of dual UAA incorporation in artificial enzymes
- 4.Insights into how distal mutations influence different catalytic activities

**Significance**

This work would bridge the gap between specialized enzyme variants, potentially creating novel bifunctional artificial enzymes. Understanding how mutations from different evolutionary trajectories interact could provide valuable insights for future enzyme engineering efforts, while dual UAA incorporation would expand the catalytic toolkit available for artificial enzyme design.

# Research Proposal: Creating Multifunctional Artificial Enzymes Using Unnatural Amino Acids

## Research Question

Can we make one enzyme that can do both reactions by combining the best features from the two specialized enzymes and by using both UAAs in the same protein?

## Goals for 4 Weeks

1. Make hybrid enzymes by mixing the best mutations from the two specialized enzymes.
2. Create versions with both UAAs (pAF and aY) in different spots on the protein.
3. Test if combining these features makes the enzyme work better for both reactions.

## Plan

### Week 1: Making the Enzymes

- Design and build new enzyme versions by combining useful mutations.
- Make versions with one or both UAAs in the protein.
- Start growing bacteria to produce the proteins.

### Week 2: Protein Production

- Grow and purify the new enzymes.
- Check if the UAAs are correctly added using mass spectrometry.
- Test if the proteins are stable.

### Week 3: Activity Testing

- Test each enzyme for both hydrazone and Friedel-Crafts reactions.
- See which enzymes can do both reactions well.
- Do basic measurements to see how fast and efficient they are.

### Week 4: Further Testing and Analysis

- Try different starting materials (substrates) with the best enzymes.
- See how extra mutations affect activity.
- Analyze results and prepare a summary for future work.

### What We Expect to Find

- At least one enzyme that can do both reactions.
- Learn how the different mutations work together.
- Find out if having both UAAs in one enzyme is possible and helpful.
- Get ideas for making even better artificial enzymes in the future.

### Why This Matters

If successful, this project will show how to make enzymes that can do more than one job. This could help scientists design new proteins for chemistry and medicine.

### What We Need

- Lab space for protein work
- Materials for making mutations and adding UAAs
- Equipment for testing enzyme activity and checking proteins