

## SUMMARY

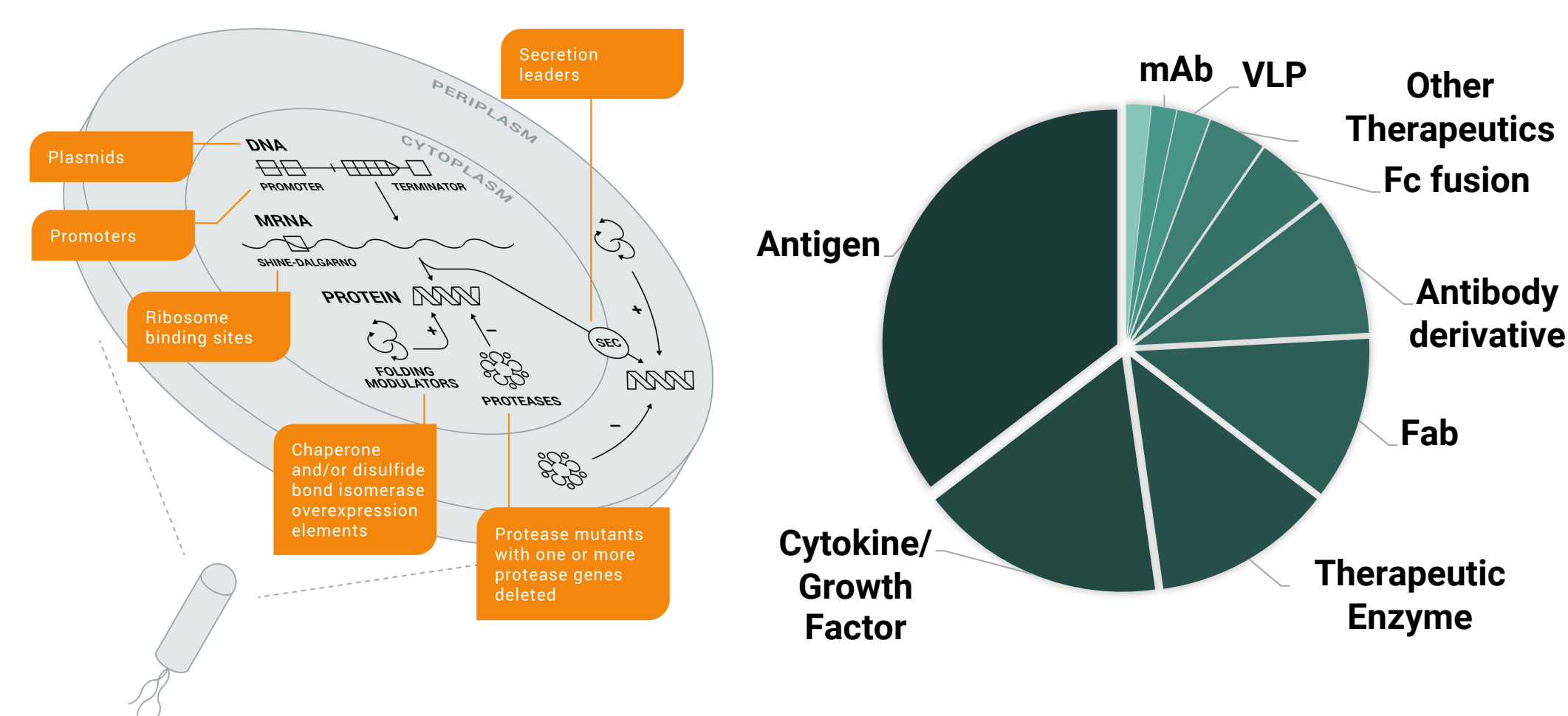
- A platform-based approach leveraging the Pfenex Expression Technology® was developed for implementing screening through lead optimization of novel ADCs.
- As an example of the workflow, an Anti-GPCR V<sub>H</sub>H therapeutic candidate modified to include a single ncAA (pAzF) substitution was developed.
- The site of ncAA incorporation was determined through rationale design using hydrogen-deuterium exchange (HDX) mass spectrometry
- pAzF feed strategies were evaluated at the 2L fermentation scale, identifying a continuous feed strategy that achieved very similar titers to the canonical VHH (~15 g/L)
- Higher pAzF concentrations were shown to be toxic, likely due to off-target amber suppression
- Peptide mapping of Y84pAzF V<sub>H</sub>H showed 100% pAzF incorporation. Intact mass spectrometry showed 100% click reaction conversion
- Binding activity was not affected by conjugation

## BACKGROUND

Non-canonical amino acids (ncAA) provide unique chemical functionalities that allow for controlled and selective modifications of therapeutic proteins. This technology underpins the development of increasingly complex bioconjugates, including site-specific ADCs and engineered proteins with enhanced half-life or dual-function capabilities.

Despite its potential, implementation has been limited by toxicity due to off-target incorporation of ncAAs and/or significantly reduced protein yields at fermentation scale.

This breakthrough delivers significant benefits including maintained production economics, enhanced conjugation specificity, and expanded protein functionality.



More than 200 leads, >500 unique proteins, and thousands of protein/peptide/library candidates have been expressed over 20 years using the Pfenex Expression Technology

**Figure 1:** *Pseudomonas fluorescens*, a Gram-negative bacteria, is the core of the Pfenex platform. Primrose has created thousands of unique expression strains that are constructed and screened in parallel using automated workflows. Pfenex's versatility is proven through six approved products and the expression of a diverse range of full-length aglycosylated antibodies and antibody derivatives (Fab, V<sub>H</sub>H, scFv).

## METHODOLOGY

### SDS-CGE Analysis:

- SDS-CGE analysis was performed using a LabChip GXII instrument (Revvity) with a HT Protein Express chip
- Reagents and samples were prepared following the manufacturer's protocol.



LabChip GXII instrument (Revvity)

### HDX Analysis:

- The D<sub>2</sub>O labeling reactions were carried out at 25°C with a minimum of triple replicates at 0.5, 5, and 1440 minute timepoints.
- MS Analysis was carried out using a Xevo G2-XS Q-TOF mass spectrometer with a nanoACQUITY HDX manager and Waters M Class ACQUITY μBinary Solvent Manager, and an auxiliary solvent manager.



Xevo G2-XS Q-TOF MS

### GPCR Binding Analysis:

- A cell-based binding assay utilizing a GPCR-overexpressing CCRF-CEM cells was employed to evaluate ncAA incorporated V<sub>H</sub>H binding to its target receptor.

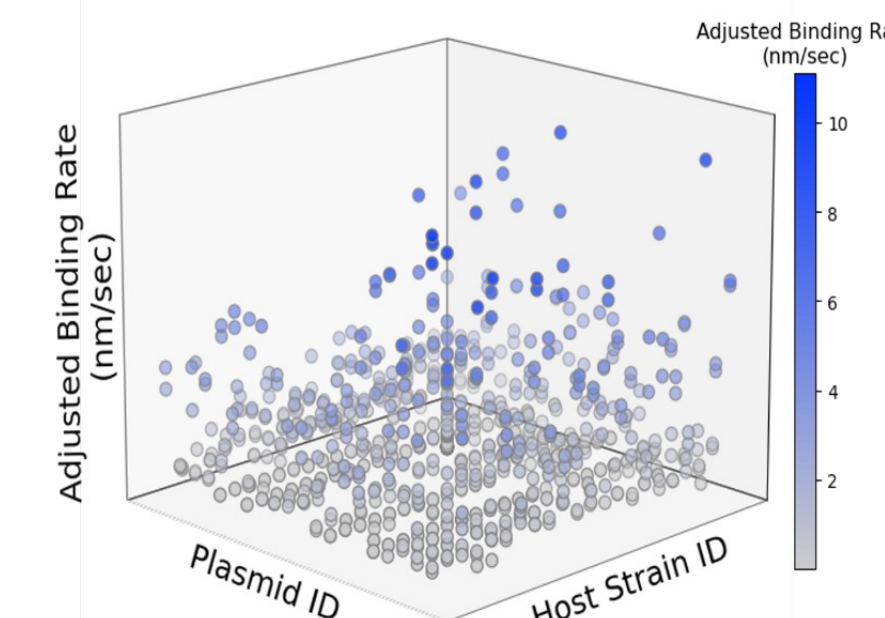
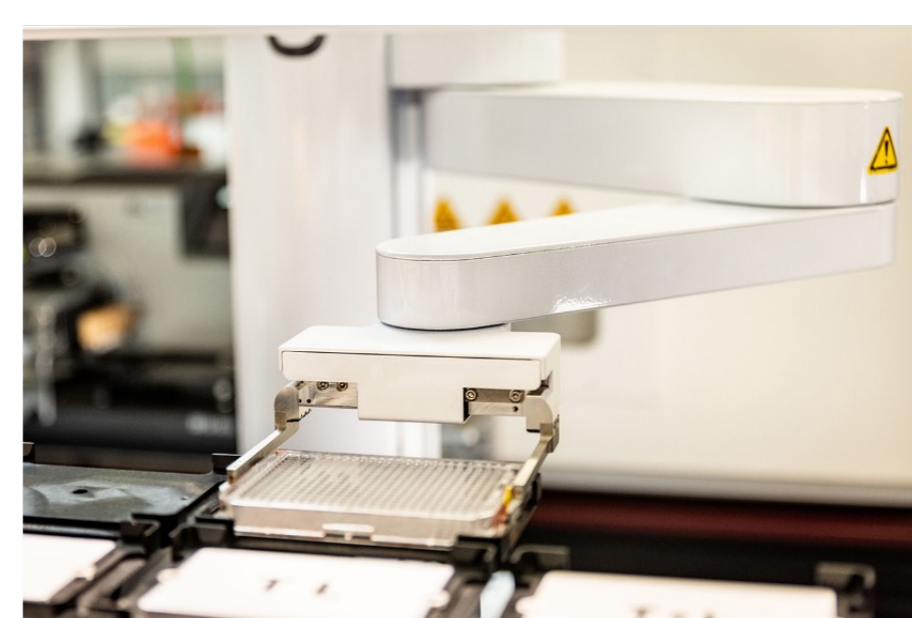
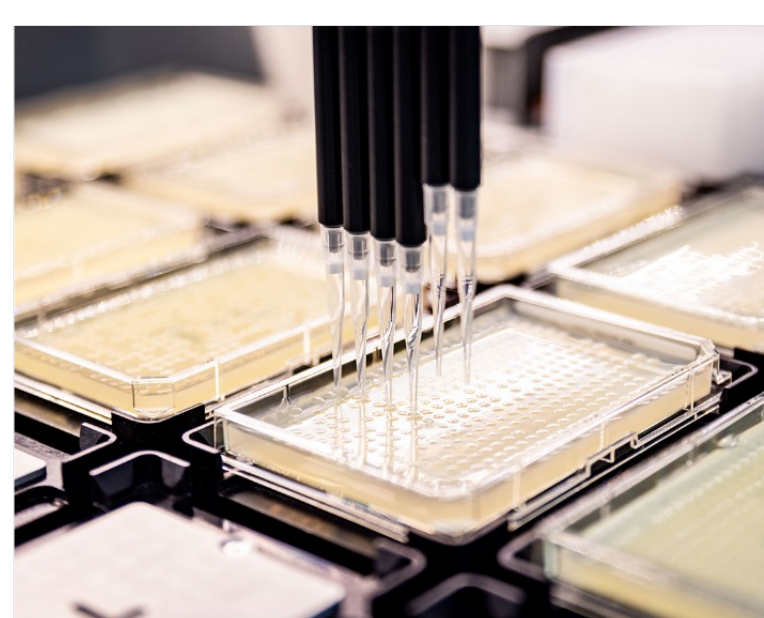
### 2L Fermentation:

- Cultures were operated under controlled conditions for pH, temperature, and dissolved oxygen using continuous feeds.
- Samples were withdrawn throughout the growth and production phases to determine the OD<sub>575nm</sub> and V<sub>H</sub>H titer of the culture.



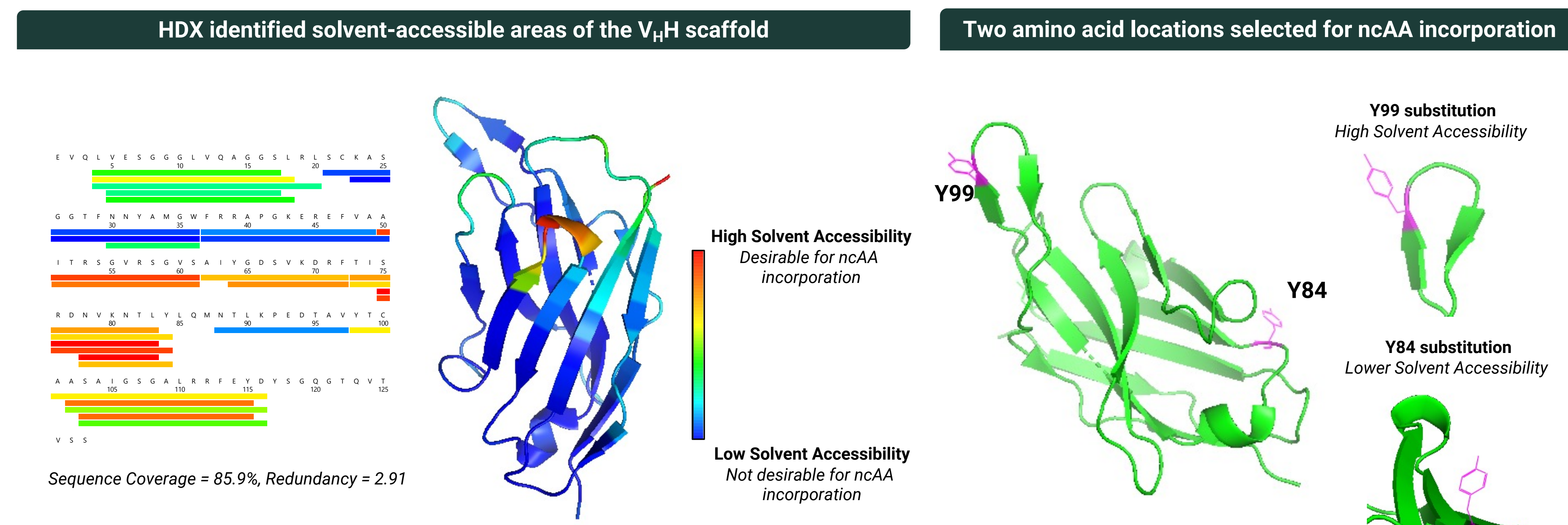
2L Fermentation Optimization

## High-throughput expression and analytics accelerates candidate down-selection



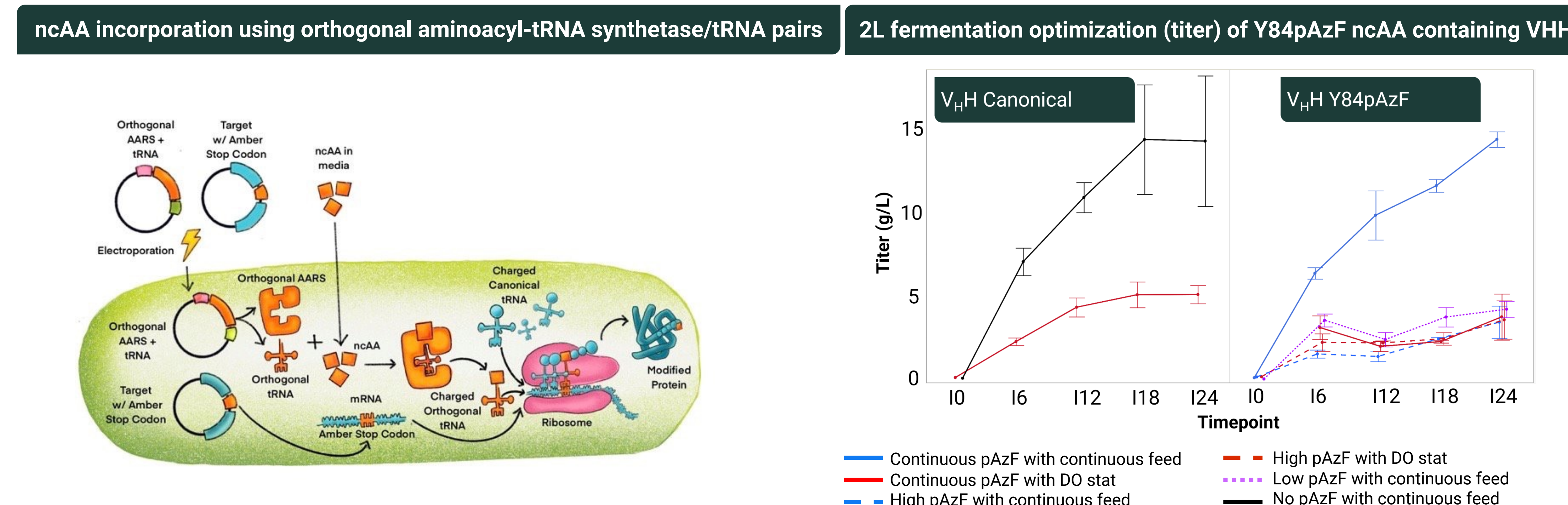
Strain Building	Sample Processing	Binding Assessment and Characterization	Analysis
<ul style="list-style-type: none"> <li>• Combinatorial strain array construction</li> <li>• 96-well growth &amp; induction</li> <li>• Reach ODs of 30-50 → multiple analytical methods per well</li> </ul>	<ul style="list-style-type: none"> <li>• Culture sampling</li> <li>• Lysate fractionation</li> <li>• BLI or ELISA Sample Preparation</li> </ul>	<ul style="list-style-type: none"> <li>• Binding: Automated BLI and/or ELISA</li> <li>• Productivity: On-deck protein titer determination</li> <li>• Developability: On-deck protein aggregation assessment</li> </ul>	<ul style="list-style-type: none"> <li>• Automated data processing workflows to expedite analysis</li> <li>• LIMS platform to ensure data integrity and traceability</li> </ul>

## HDX-MS guides rational ncAA site selection in lead V<sub>H</sub>H therapeutics



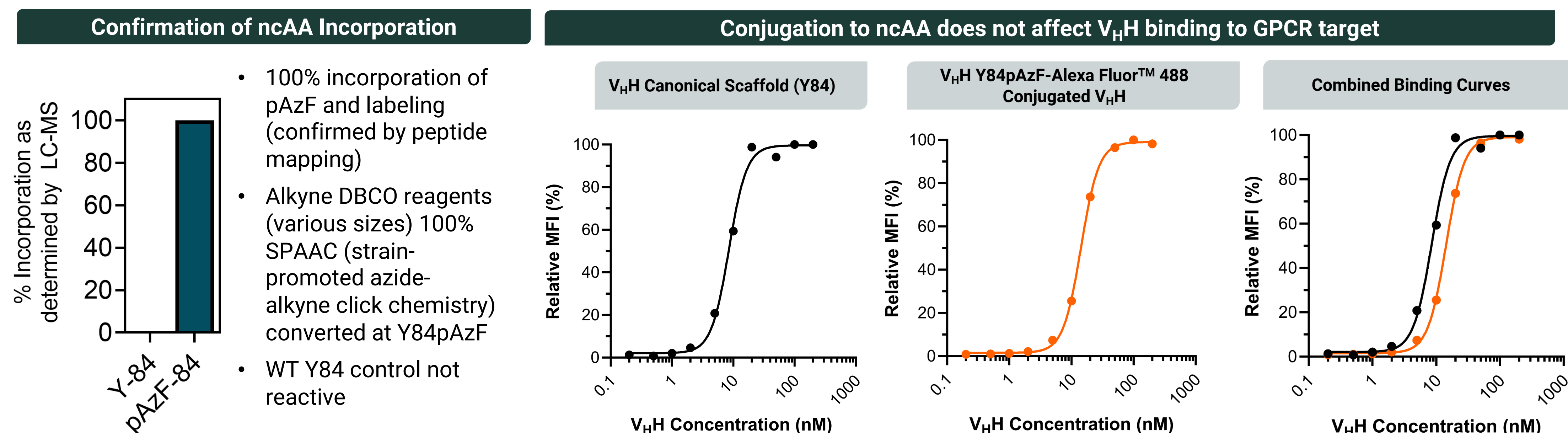
**Figure 2.** HDX-MS analysis identifies solvent-exposed regions of proteins suitable for ncAA incorporation. **Left:** Schematic of the HDX workflow: POI is analyzed by HDX, data is mapped to the primary sequence, and results are used to pinpoint highly accessible regions for ncAA incorporation. **Middle:** HDX solvent accessibility map of a V<sub>H</sub>H protein, with highly exposed regions shown in red and buried regions in blue. **Right:** Two tyrosines from the HDX analysis, Y84 (moderate accessibility) and Y99 (high accessibility), were selected for ncAA incorporation into a V<sub>H</sub>H scaffold.

## 15g/L V<sub>H</sub>H titers maintained with or without ncAA incorporation



**Figure 3.** **Left:** Schematic of ncAA incorporation using orthogonal aminoacyl-tRNA synthetase/tRNA pairs. **Right:** 2L fermentation optimization of Y84pAzF ncAA containing V<sub>H</sub>H. Various pAzF feed strategies were evaluated; similar titers were achieved between the canonical (black) to the non-canonical V<sub>H</sub>H (blue) with an optimized pAzF feed strategy.

## Conjugation of the ncAA does not affect binding to the GPCR



**Figure 4.** **Left:** Summary of LC-MS analysis of ncAA incorporated VHH; **Right:** Binding Affinity by Flow Cytometry. Canonical V<sub>H</sub>H Y84 achieves 8.5 nM affinity (left, black) and Y84pAzF-Alexa Fluor 488 V<sub>H</sub>H achieves 14.2 nM (middle, orange). Right panel overlays both curves.

## CONCLUSION

We have successfully bridged a critical gap in genetic code expansion technology by demonstrating that ncAA's can be incorporated into therapeutic antibodies at pre-production scale without loss of yield and cell viability. This breakthrough directly addresses the commercialization bottleneck that has limited ncAA therapeutics to niche applications. By removing ncAA toxicity from our system, we have broadened the opportunities for site-specific ADCs and provides the know-how to develop additional strains with additional ncAA chemistries.

