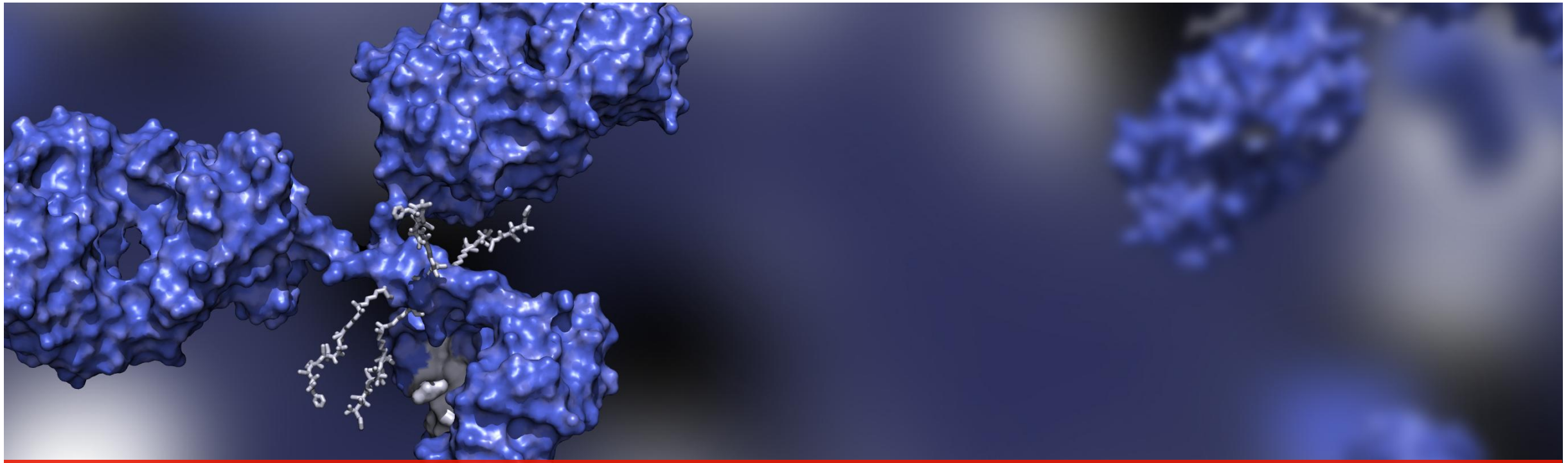


Diving Into the Complexities of Non-Natural Amino Acid Antibodies for ADCs with Innovative Mass Spec Approaches

Hirsh Nanda
Director – Analytical Sciences
Cell Engineering & Analytical Sciences, Therapeutic Development & Supply
Johnson & Johnson Innovative Medicine
Springhouse, PA

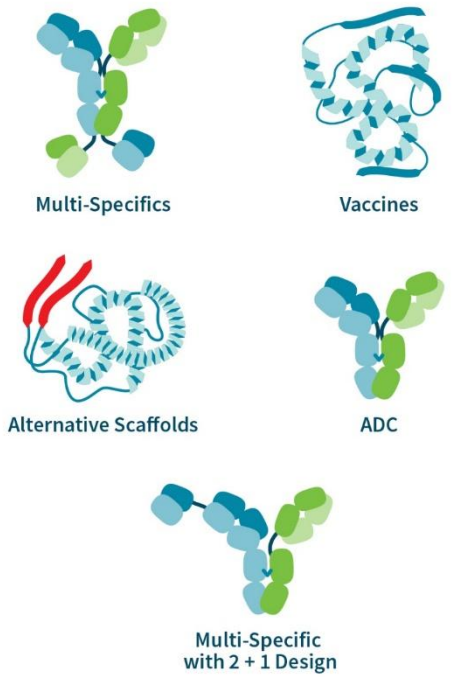
Antibody drug conjugate with four drug compounds linked to IgG immunoglobulin

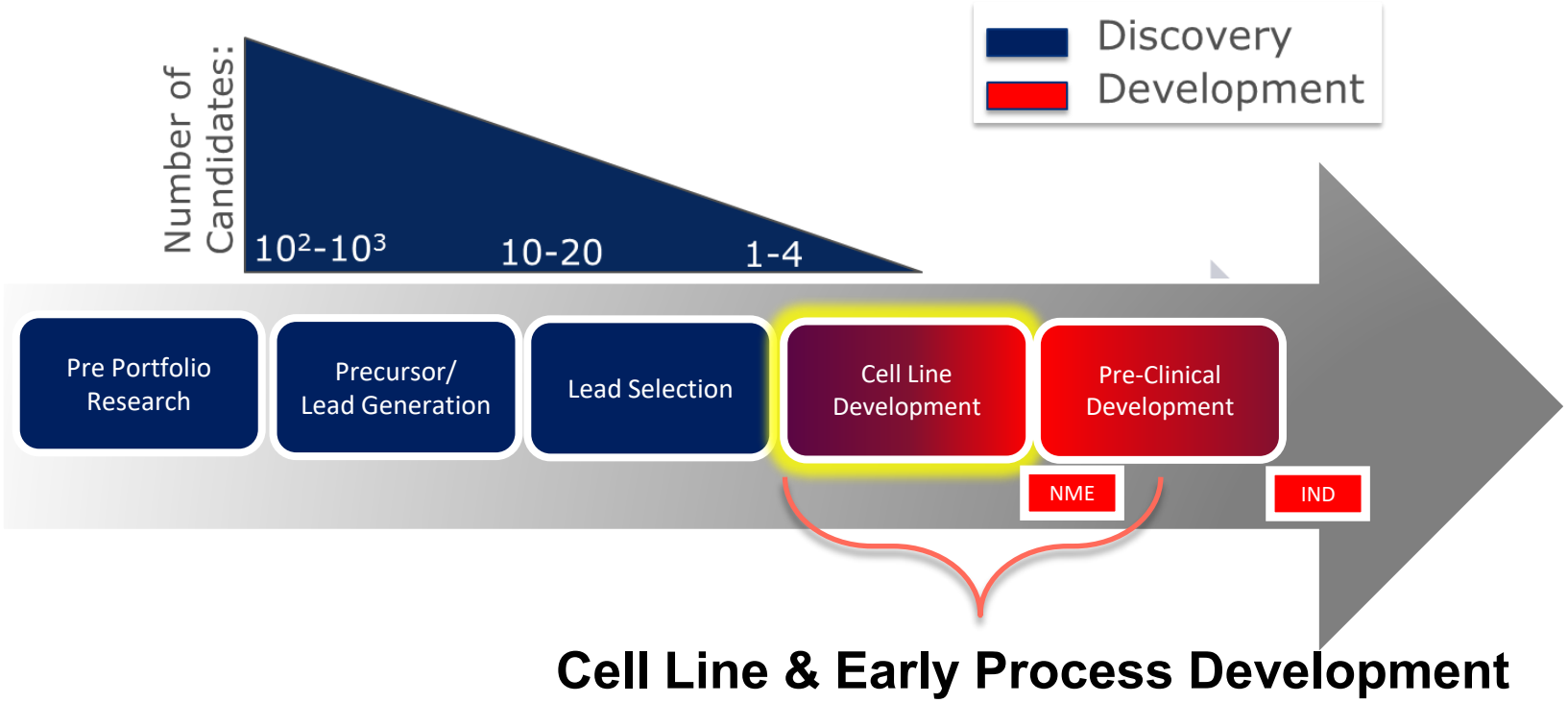
J&J

Outline

1. **Introduction to Cell Engineering & Analytical Sciences (CEAS)**
2. **Antibody Drug Conjugates & Site-Specific Conjugation**
3. **Transfection Pool – Confirmation of mAb Expression**
4. **Single Cell Cloning – Optimizing amber suppression**
5. **Reduced Scale Bioreactor – Optimizing for product quality**

Cell Engineering & Analytical Sciences (CEAS) Bridges Discovery and Development





- Build a Platform that Enables Selection of Clonal Manufacturing Cell Lines with High Expression & Product Quality
- Molecule knowledgebase enables transition to development work
- Fit for purpose analytical and process ensure the success of each program

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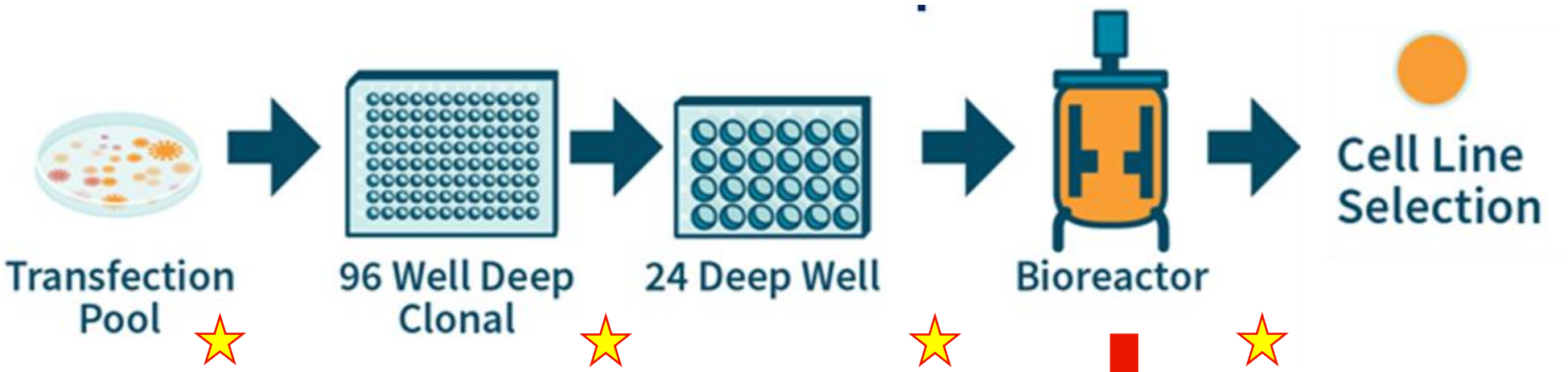
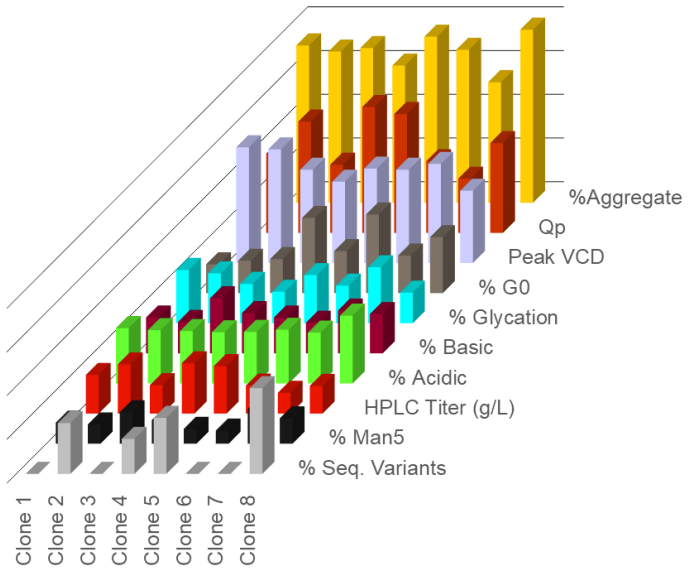
Cell Line Development Process



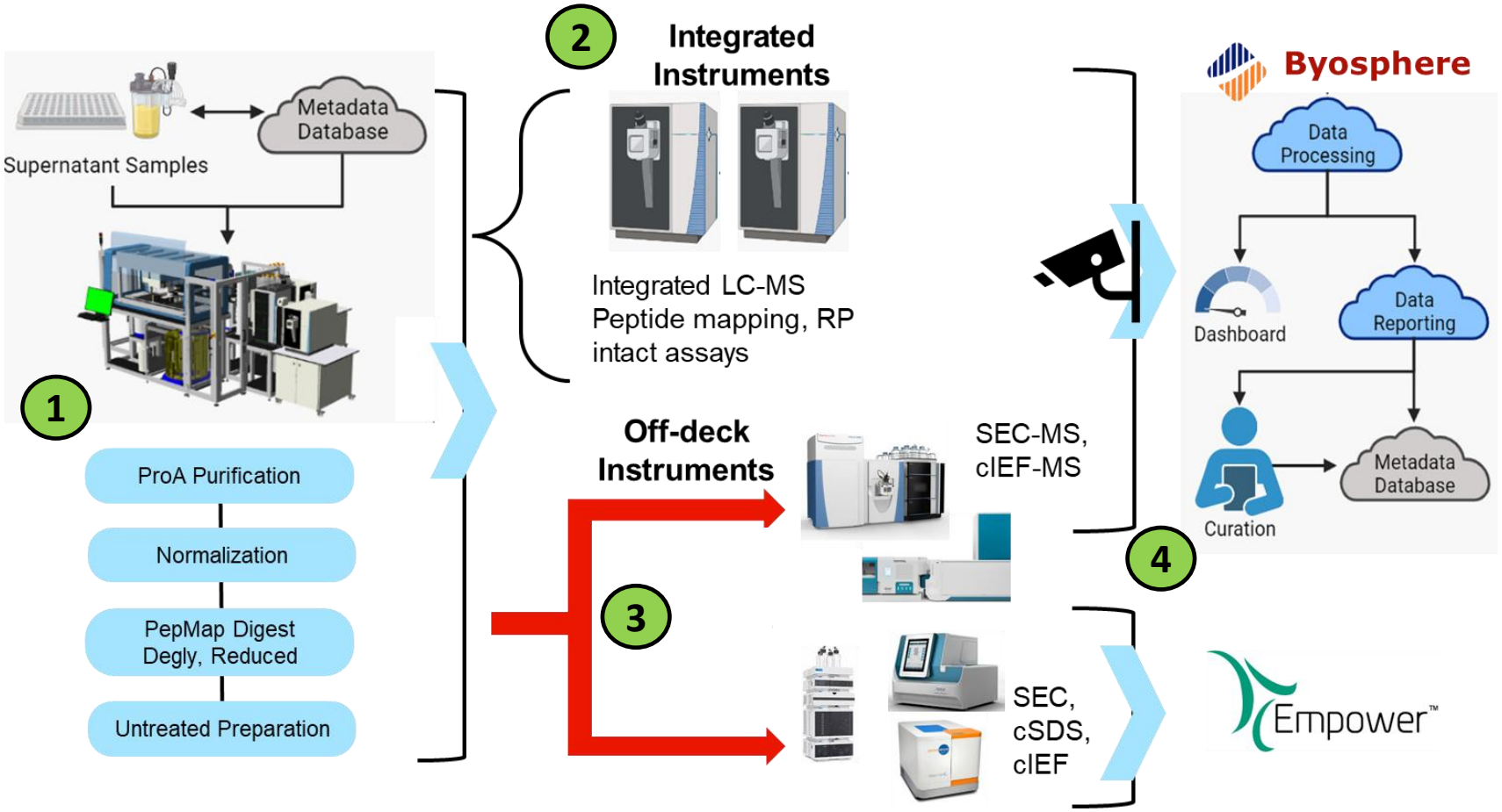
★ Points of analytical testing

- Screening 100's of clones in increasing representative cell culture conditions
- Integrated analytical testing at each stage used to cutdown clone list
- Top clones move into small scale bioreactors for final evaluation (titer and PQ)



In depth, multi-parametric characterization drives final clone decision

End to End Pipeline: for streamlined data processing & report generation assists the scientist in validating results and reduces turn-around-time



1. **Analytical Request & Sample Prep Automation**

2. **Integrated LC-MS enables automatic data acquisition.**

3. **Off-deck Instruments:** Sample plates automatically generated.

4. **Automated data processing & report generation**:
   - Byosphere server for MS assays
   - Empower server for separation / purity assays

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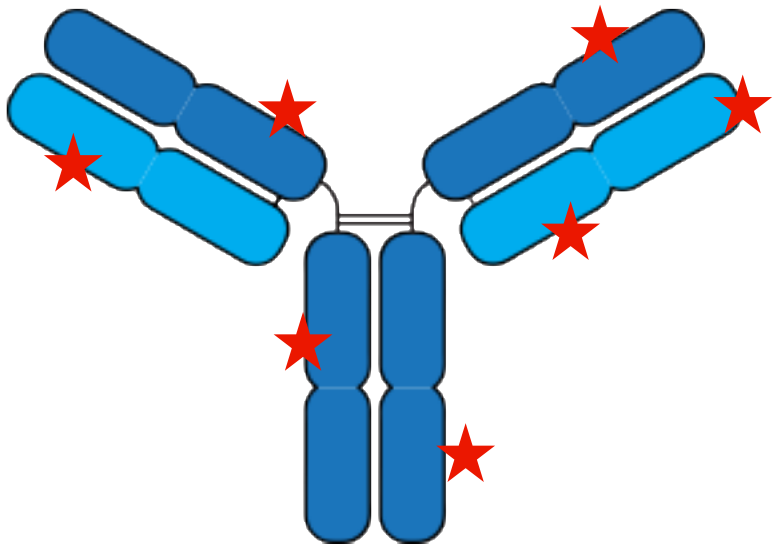
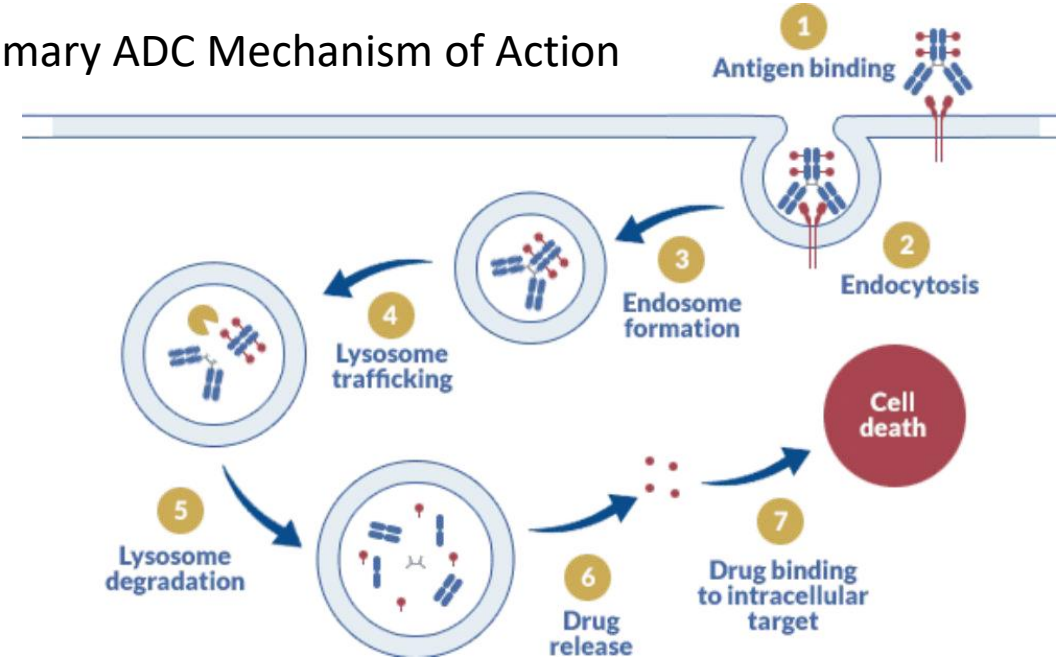
Antibody Drug Conjugates (ADC) Targeted Therapies for Cytotoxic Payloads



Lys conjugated (ADC)

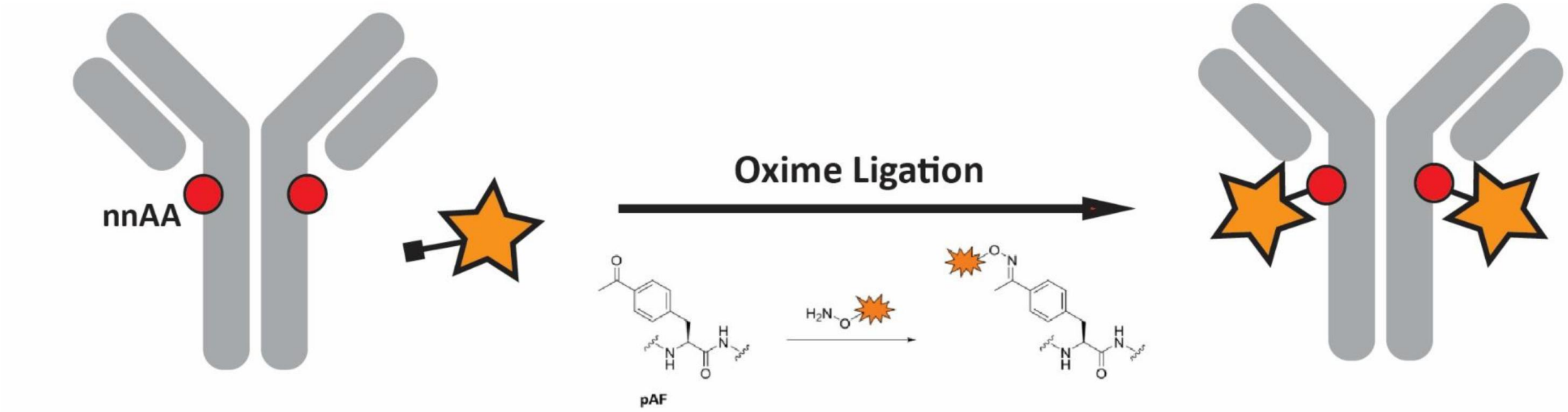
Primary ADC Mechanism of Action



- Typical linker/payload conjugated to Lys or Cys sites
- Result in heterogenous distribution across the molecule
- Difficult to control drug antibody ratio (DAR)
- Potential impact to: Molecule stability, Binding & pK (safety & efficacy)

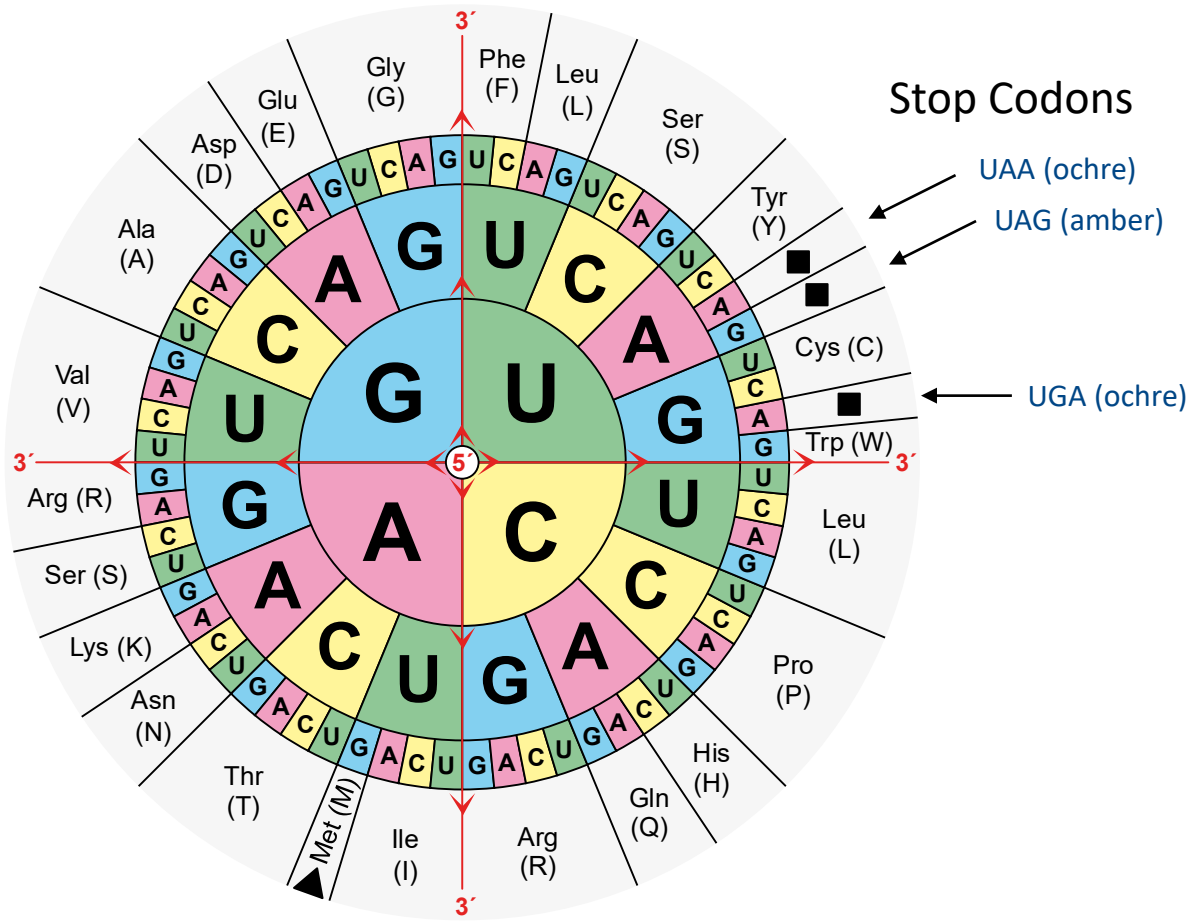
Achieving More Consistent ADC with Site Specific Conjugation

Antibody-Drug Conjugates (ADCs) by nnAA Insertion

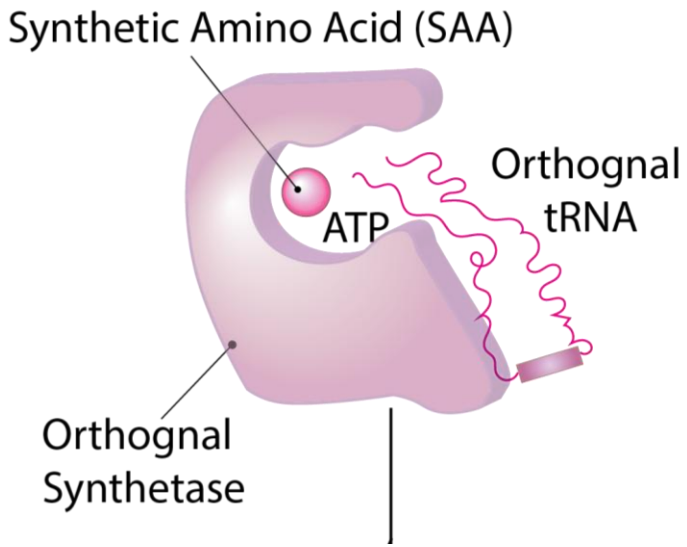


- Non-natural amino acid (para-acetyl phenylalanine) pAF incorporated into mAb sequence
- Genetic code-extension enables site specific placement of pAF
- Bio-orthogonal ligation chemistry – no off-site reactions

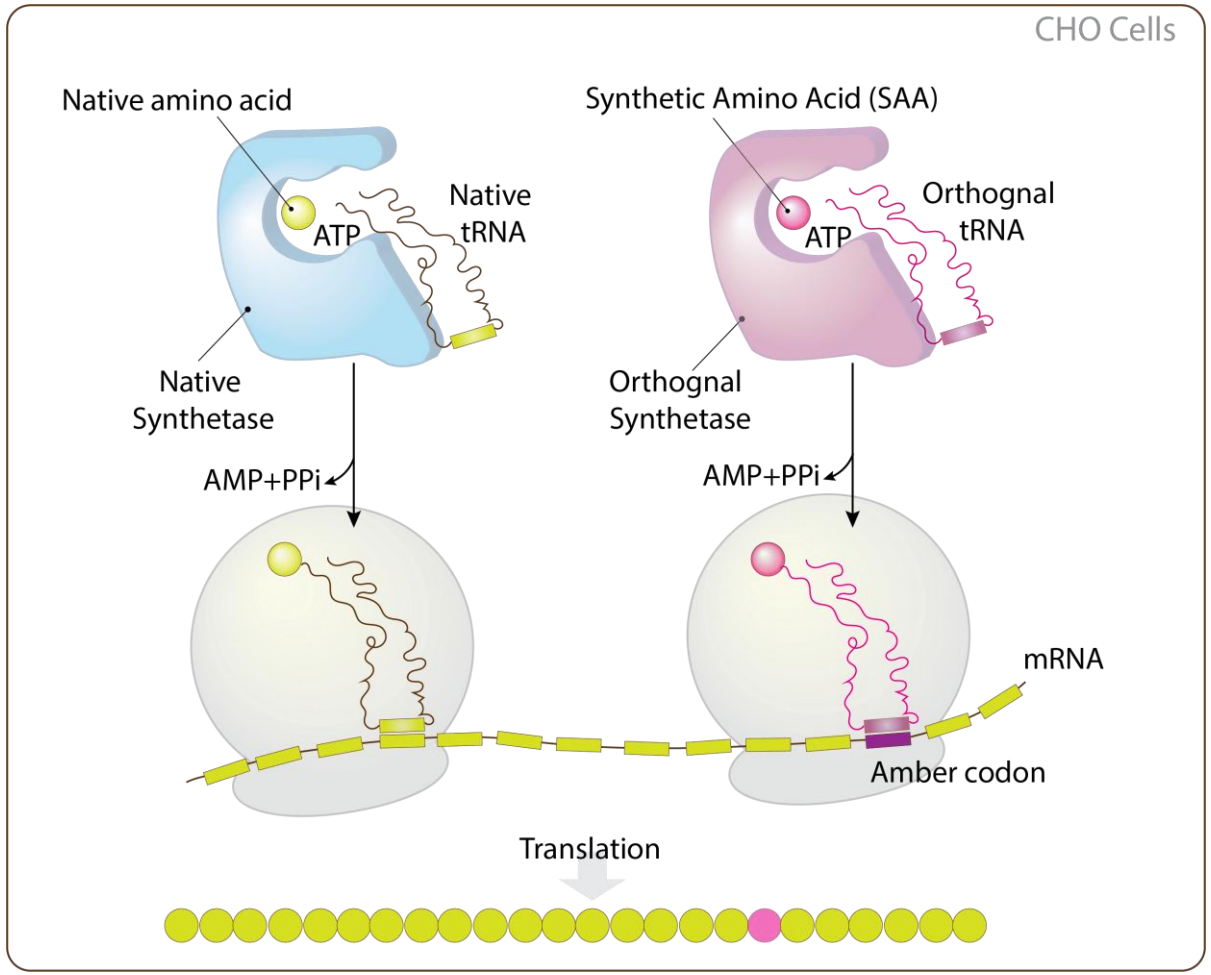
Re-Engineering the Genetic Code



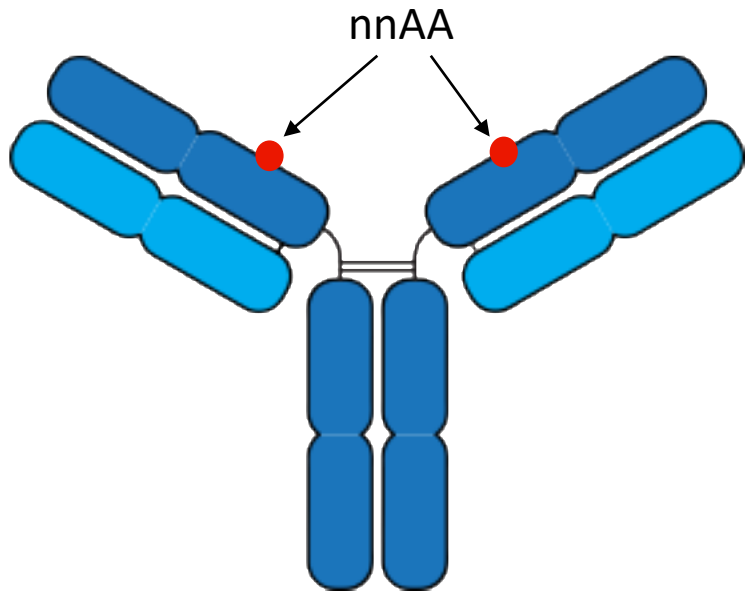
- Typically stop codons serve to terminate translation
- Re-program the **amber** stop codon (**UAG**) to code for the nnAA
- Introduce orthogonal translation elements to the CHO Cell



Translation Machinery Over-writes amber stop codon with nnAA



Full length mAb with site specific nnAA incorporated into the heavy chain



In the absence of the nnAA amber still functions as a stop codon. Resulting in N-term truncated HC.

Considerations in Developing Cell Lines with nnAA Expression

- Is the molecule forming correctly?
- How efficient is amber suppression?
- Product quality comparison between clones?
- How does engineering the genetic code impact cell function?

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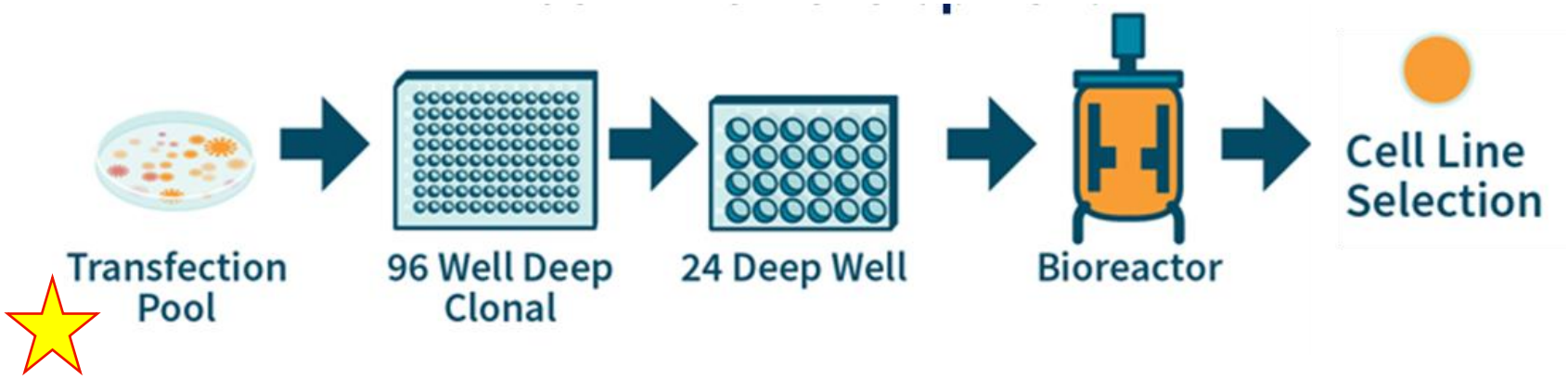
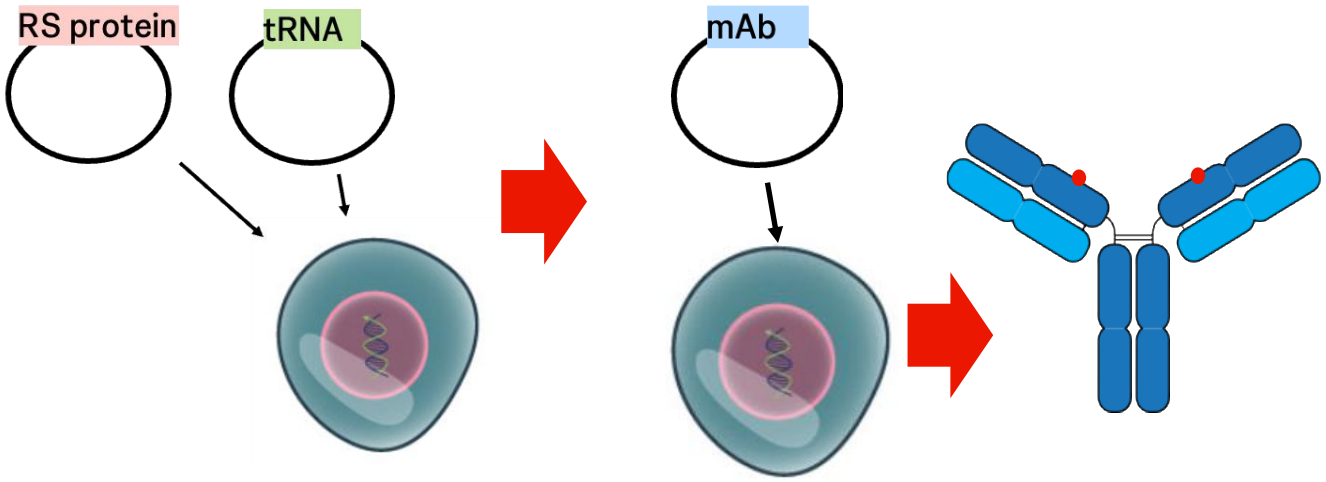
Cell Line Development Process



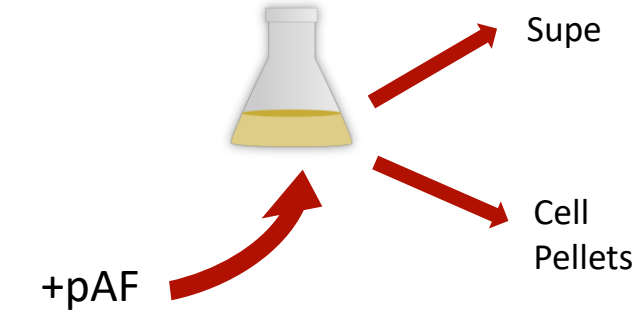


Transfection pool generation:

- Generate a parent cell line with successfully integrated RS gene & tRNA
- Transfect mAb of interest
- Analytical: confirm proper molecule expression

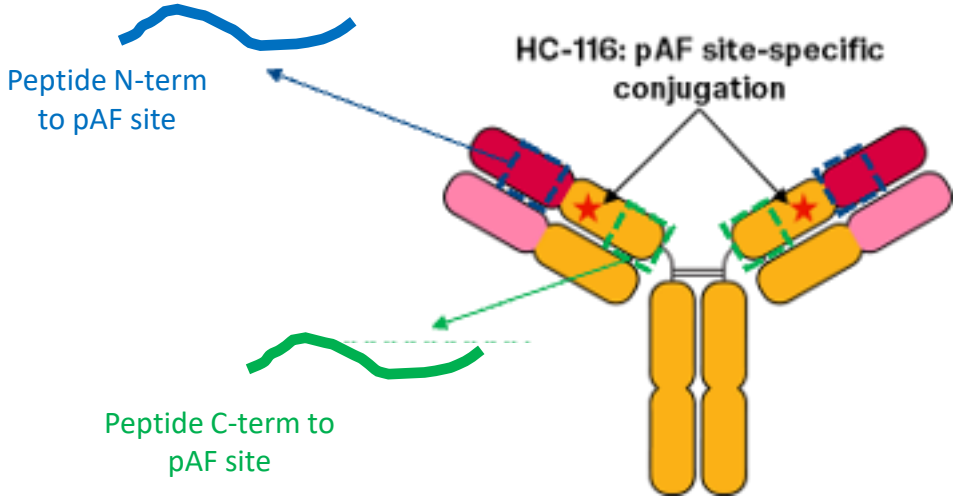
Monitoring cell culture dynamics and mAb expression after pAf is added

6 Day Cell Culture

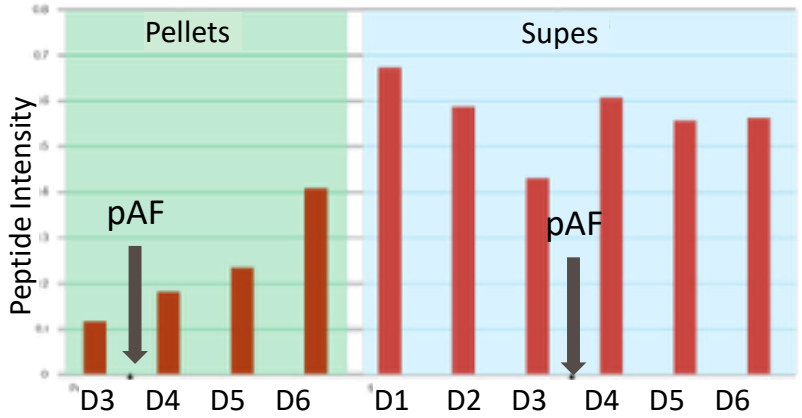


Added at day 3

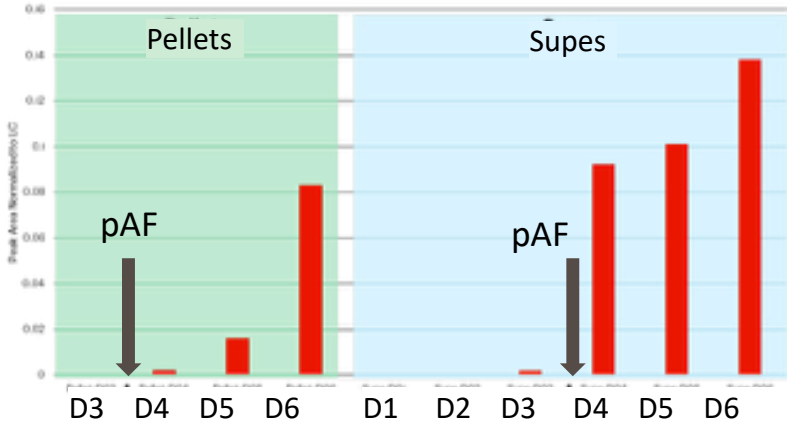
Samples collected daily from cell culture



N-term Peptide



C-term Peptide

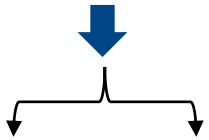


- C-term peptide is only detected after nnAA is added to the feed (Day 3)

Confirming Molecule Expression: Intact Mass & Subunit Analysis

Transfection pool

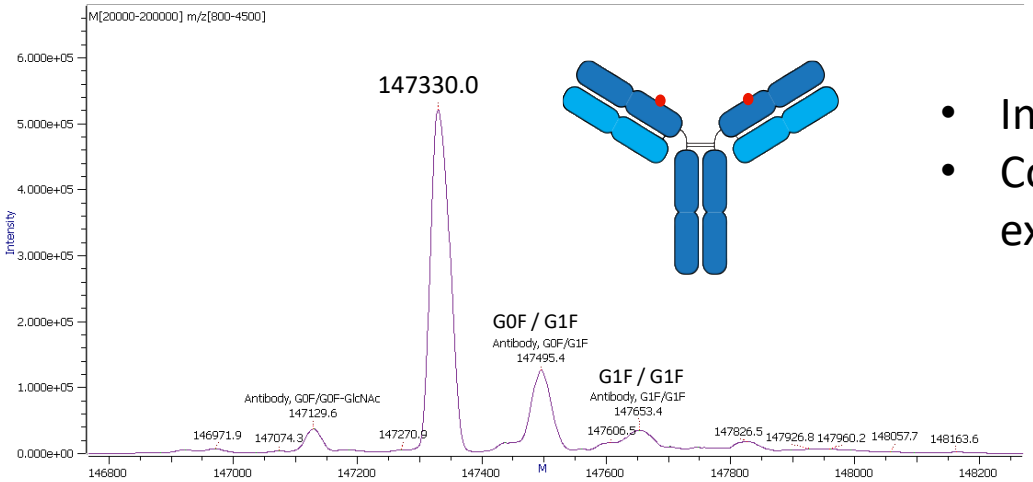
Protein A Capture



Reduced &. Non-reduced

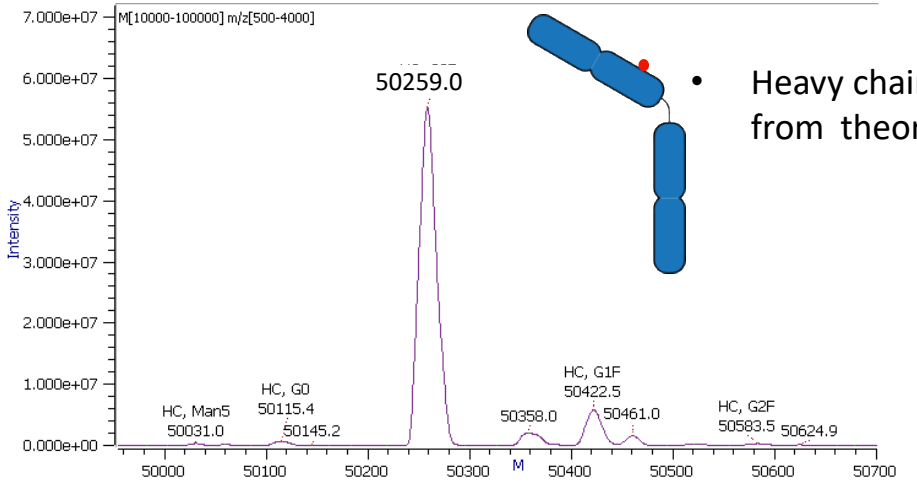
Intact Mass Analysis

Intact mAb



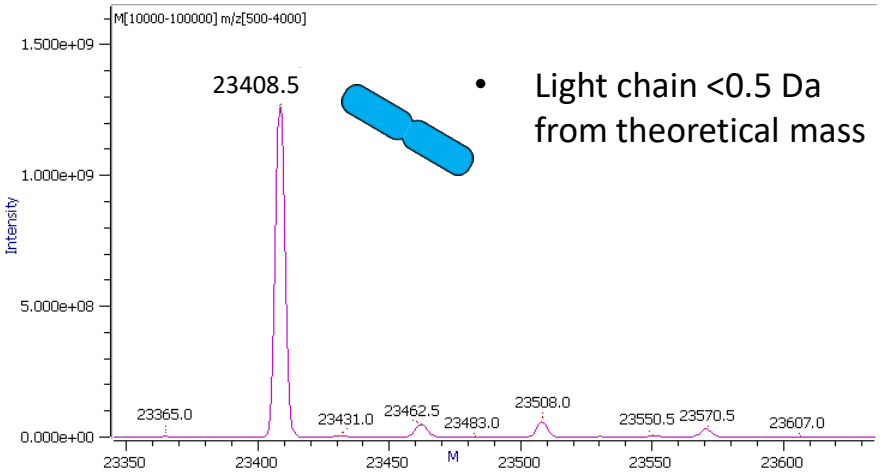
- Intact antibody <0.4Da from theoretical mass
- Confirm amber readthrough and full length mAb expression

Reduced HC



- Heavy chain < 1.2Da from theoretical mass

Reduced LC



- Light chain <0.5 Da from theoretical mass

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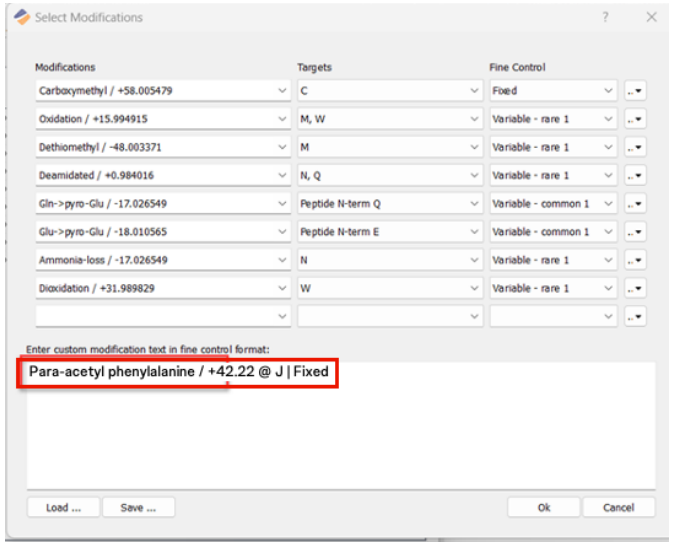
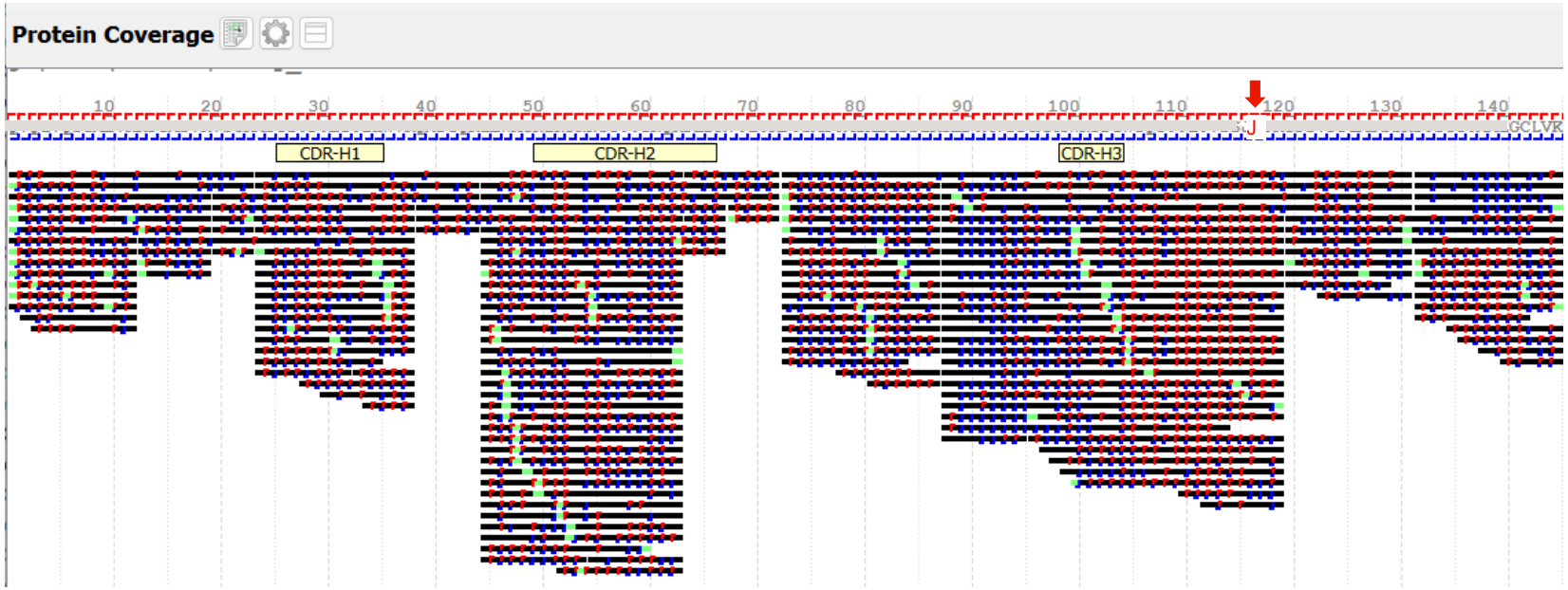
Confirming Molecule Expression: Site Specific nnAA Insertion

Creating a method that recognizes 21st aa



- Fixed modification in Byosphere to residue ‘J’



- nnAA inserted in framework after CDR regions
- Confirmed by MS2 fragmentation of entire antibody sequence

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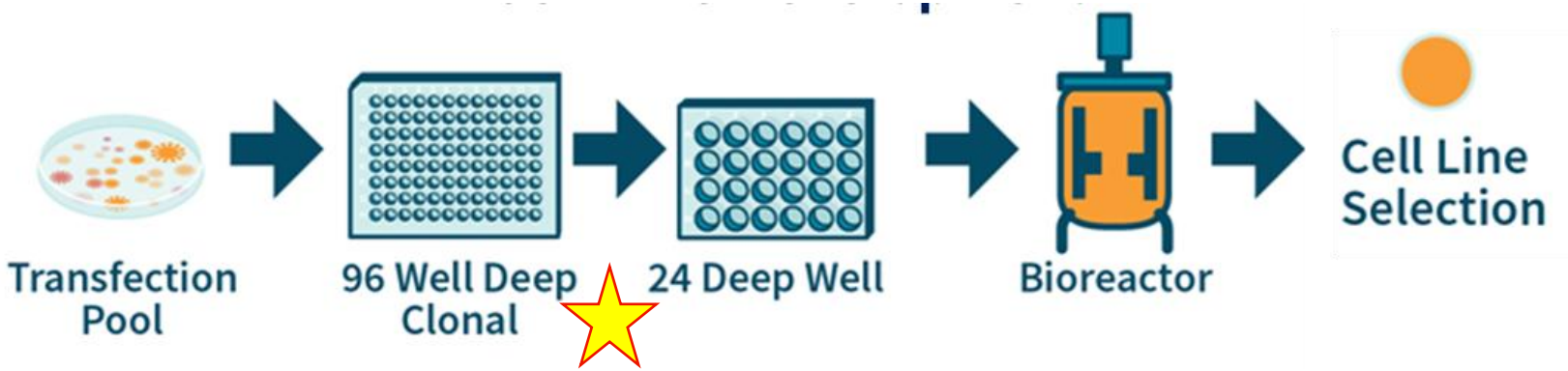
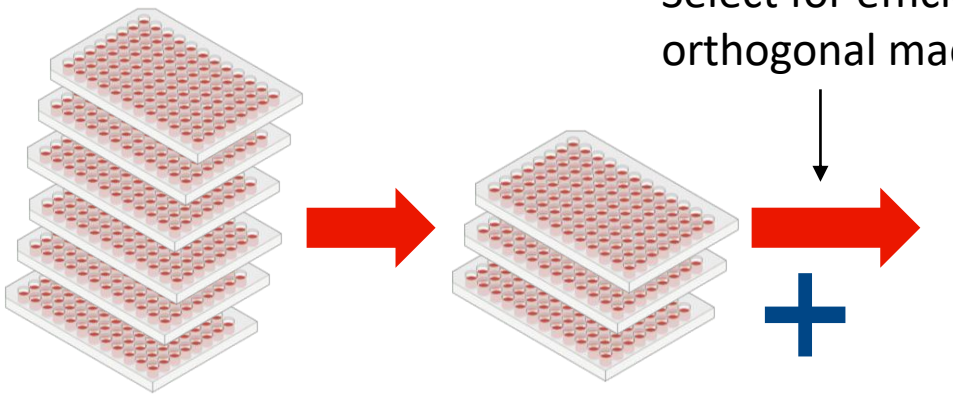
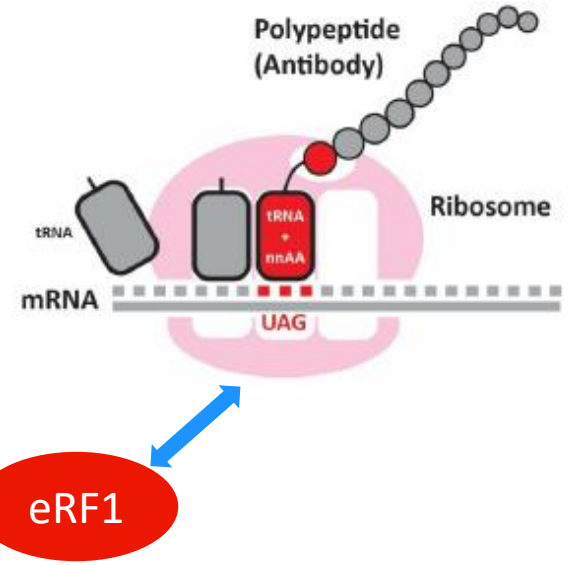
Cell Line Development Process

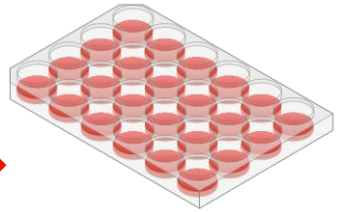




Select for efficient orthogonal machinery

pAF





24 Top Clones Move Forward

Cherry pick: confluence & viability

Stop codon release factor in competition with o-tRNA during translation

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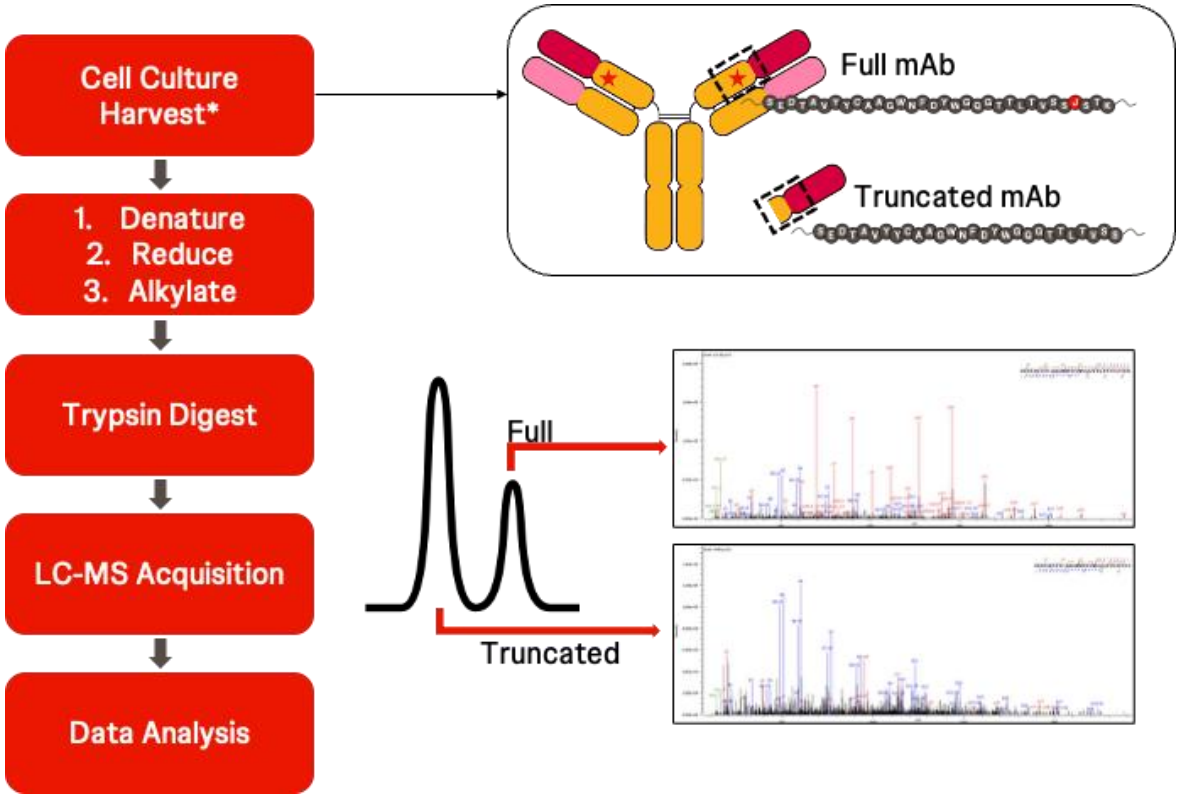
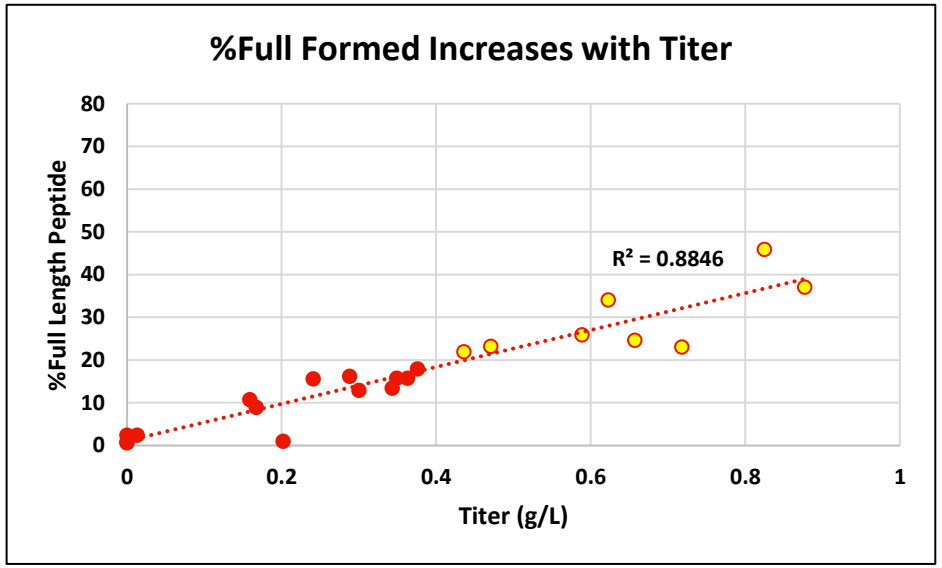
Screening for Efficient Amber Suppression will Improve Overall Titer & Yield



Directly Monitor the Truncated Peptide



- Wide variation in amber suppression between clones
- Choose clones with high nnAA incorporation for further evaluation

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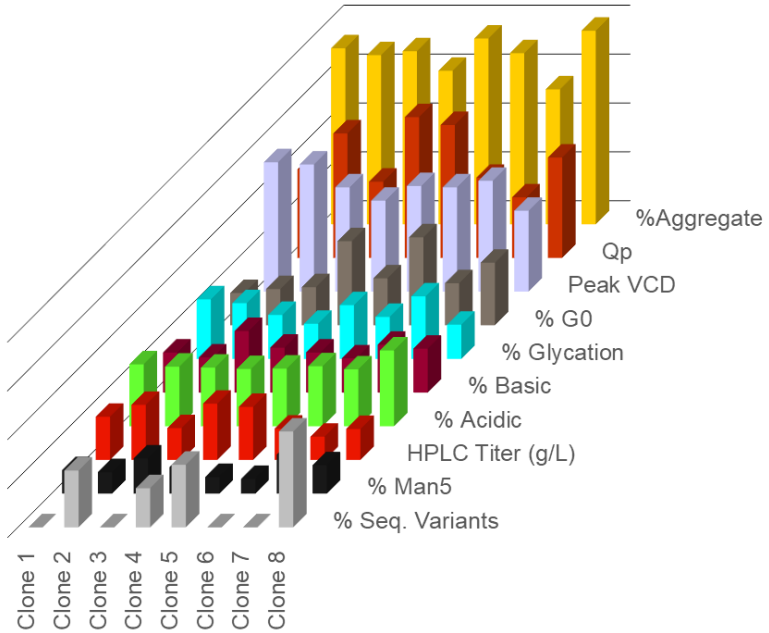
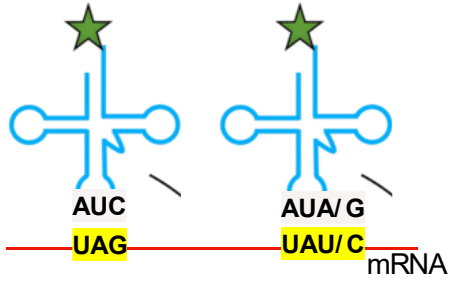
Cell Line Development Process

In-depth PQ Characterization

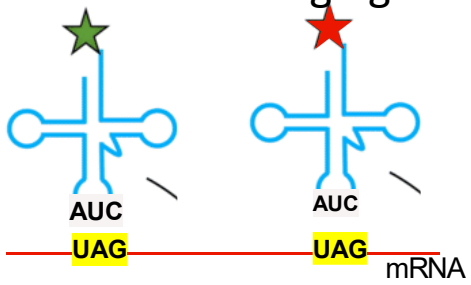


Screening Clones for Propensity of Mistranslation Events

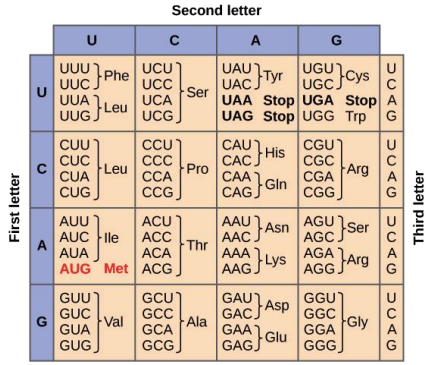
Wobble Pairing



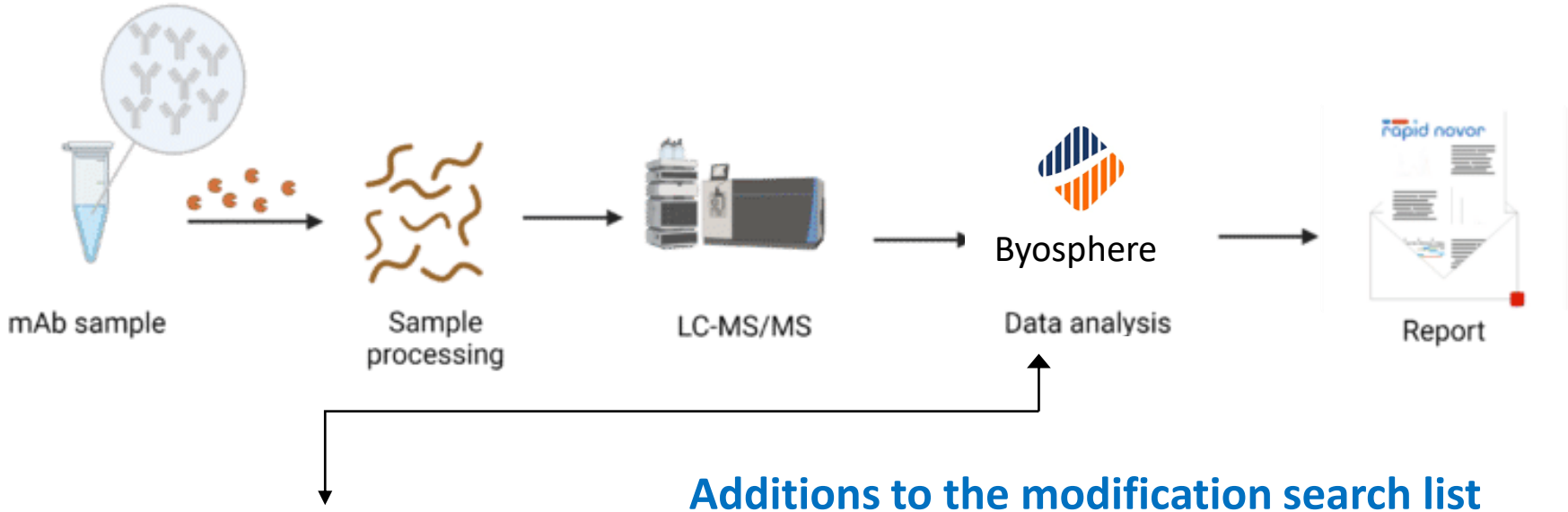
tRNA Mischarging



Misincorporation among the 20 amino acids often related to codon similarity



Extending our Misincorporation Analysis for the Expanded Genetic Code



pAF (UAG)

- Tyr (UAU, UAC)
- Leu (UUG)
- Ser (UCG)
- Trp (UGG)
- Gln (CAG)
- Lys (AAG)
- Glu (GAG)
- Phe (mischarge)

Additions to the modification search list

- Include all single base pair substitutions - potential codon wobble
- Phe – structural similarity – potential tRNA mischarging
- pAF substitutions could lead to DAR lower than target or off-site conjugations

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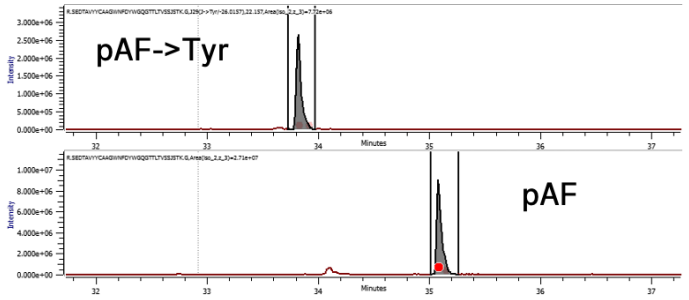
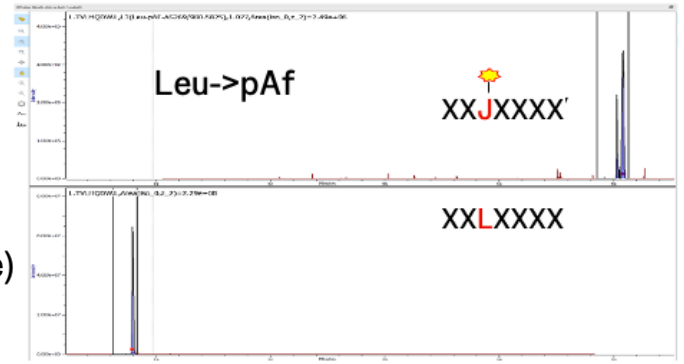
Extending our Misincorporation Analysis for the Expanded Genetic Code

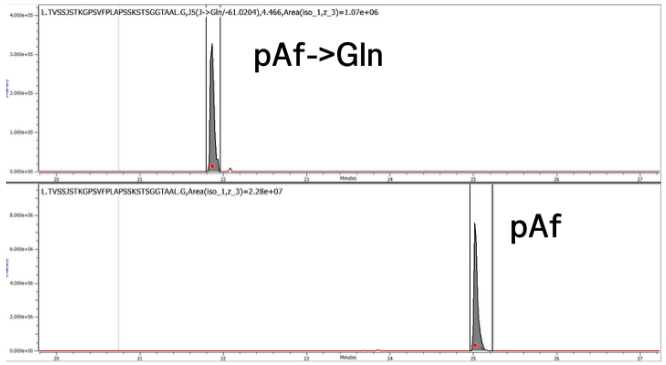
pAF (UAG)

- Tyr (UAU, UAC)
- Leu (UUG)
- Ser (UCG)
- Trp (UGG)
- Gln (CAG)
- Lys (AAG)
- Glu (GAG)

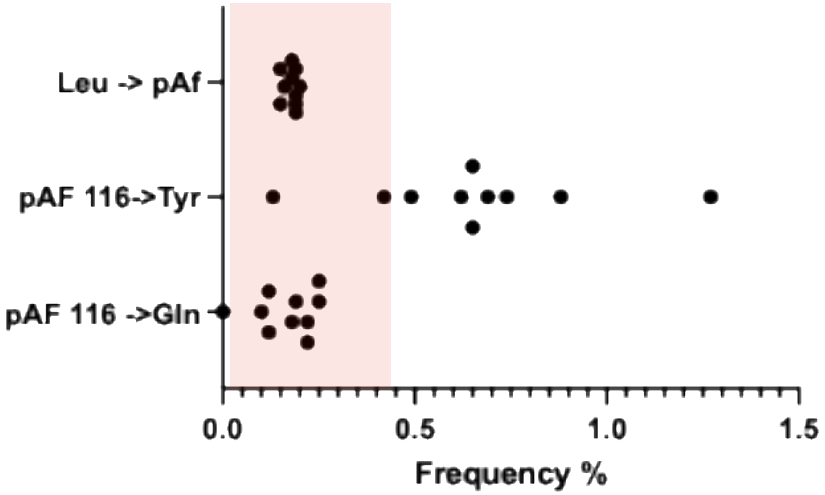
- Phe (mischarge)







Distribution by Clone



- Only a few misincorporated species involving the pAF site are identified
- AA substitutions are minimized by choosing the optimal clone from the ambr bioreactor run

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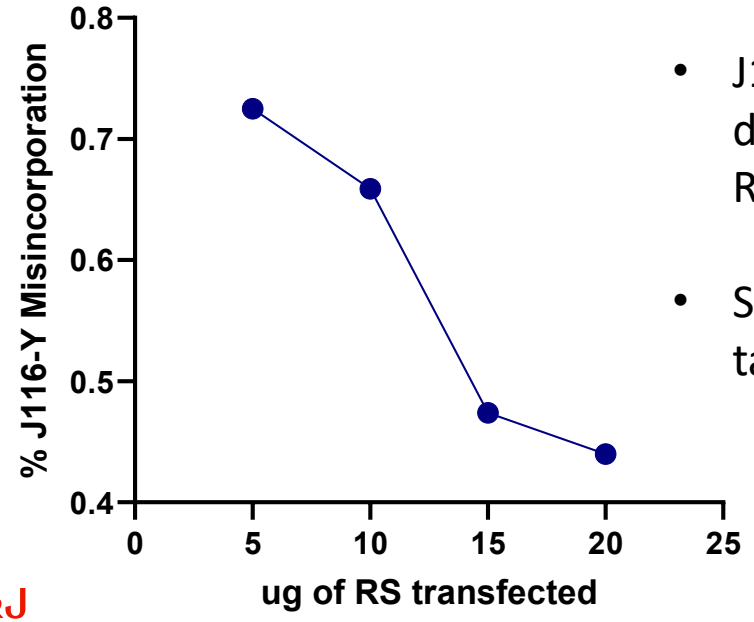
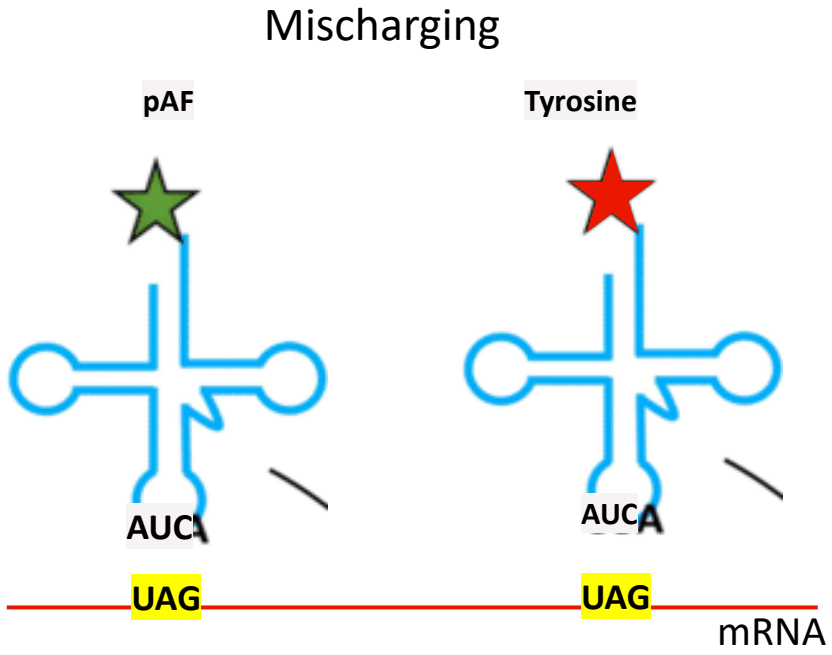
Mechanism for Misincorporation Events with Tyr

Sample Name	J116->Tyr (-26.0157Da)
Clones with increasing levels of RS expression	0.725
	0.659
	0.474
	0.440



- J116->Tyr misincoporation decreases in clones with higher RS expression
- Suggesting that a mischarging is taking effect.



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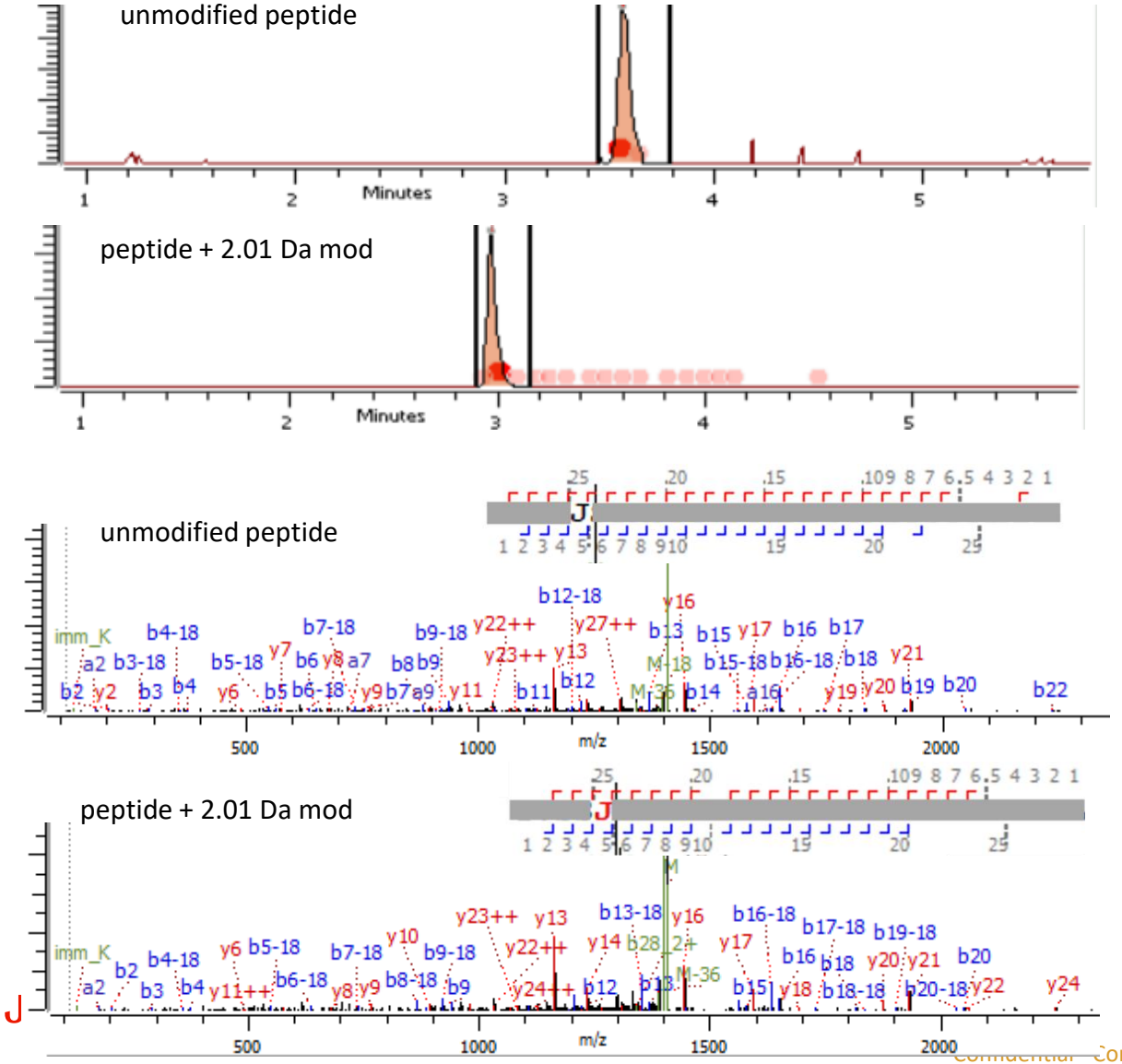
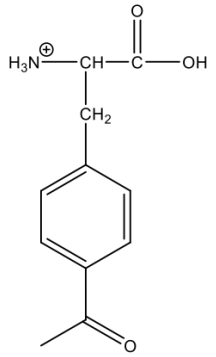
Searching for pAF modifications and/or potential AA byproducts

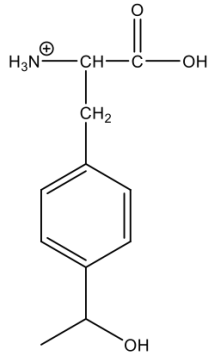
Peptide mapping with wild card search to look for modified forms of the nnAA



- Modified form of pAF (+2 Da) observed in wild-card search
- -16 Da mass shift also observed at same RT
- Approximate relative abundance of all species ~1.5%



Chemical Formula: $C_{11}H_{14}NO_3^+$
Exact Mass: 208.09682

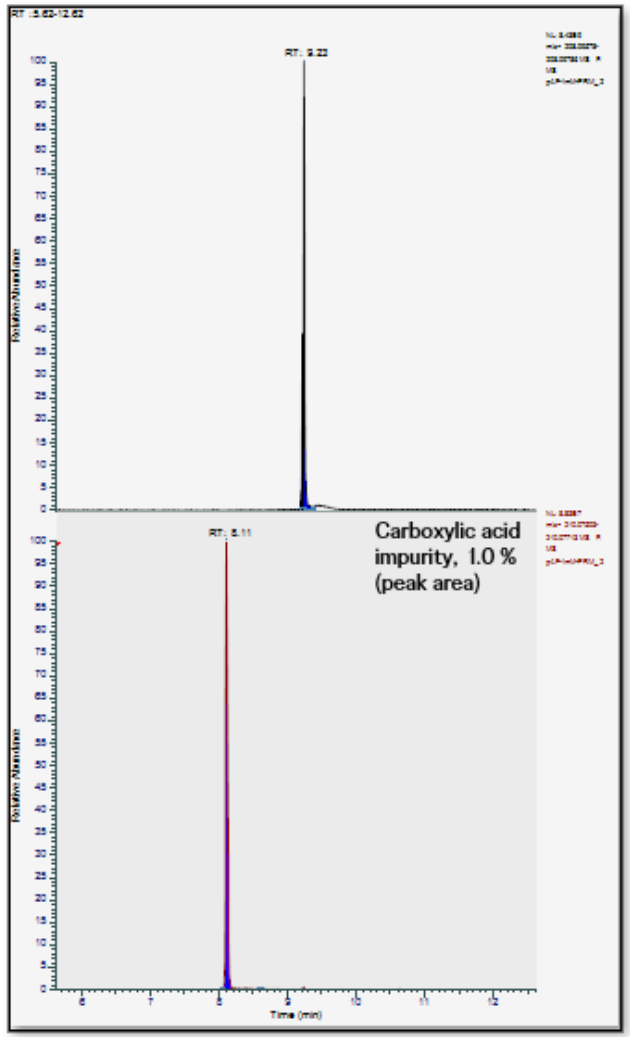


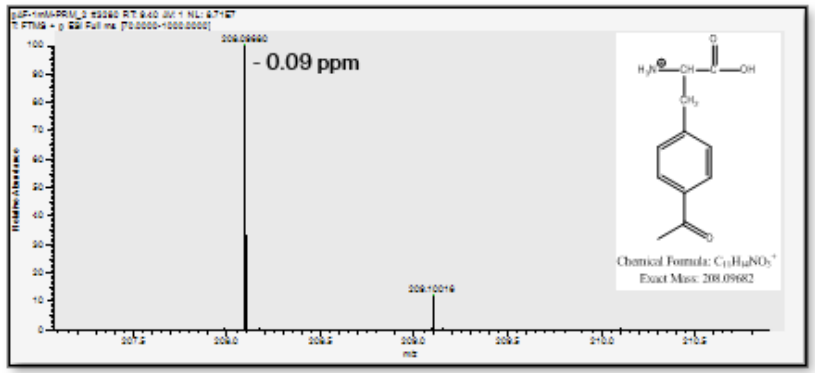
Chemical Formula: $C_{11}H_{16}NO_3^+$
Exact Mass: 210.11247

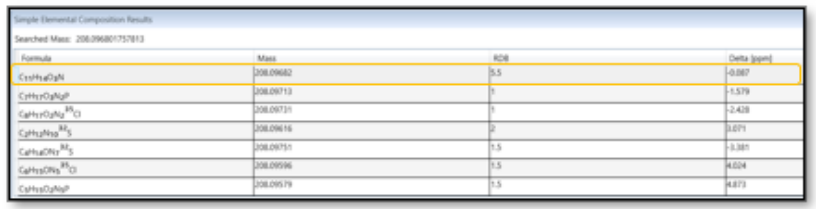
Potential hydroxy ethyl species. Impurity in pAF feed?

Accurate Mass and Fragment Ion Spectra

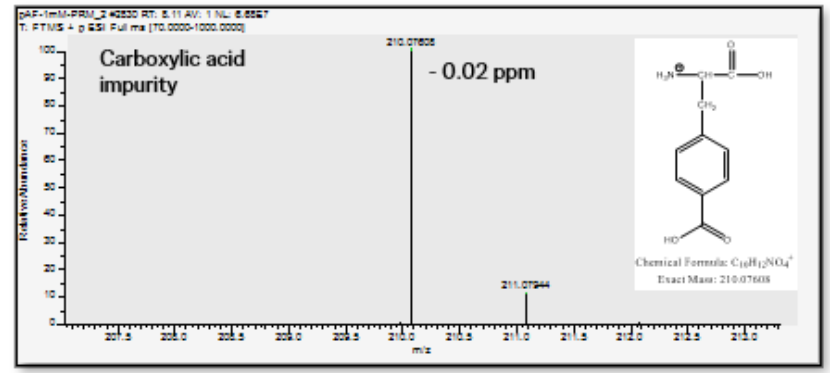
LC/MS Analysis of pAF Raw Material

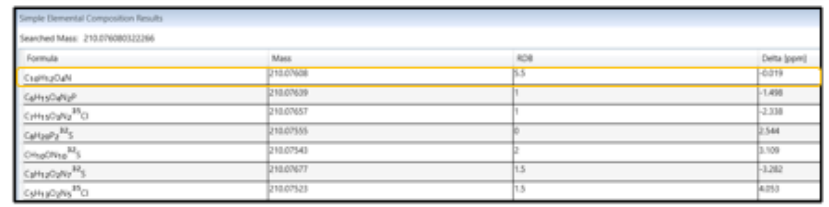




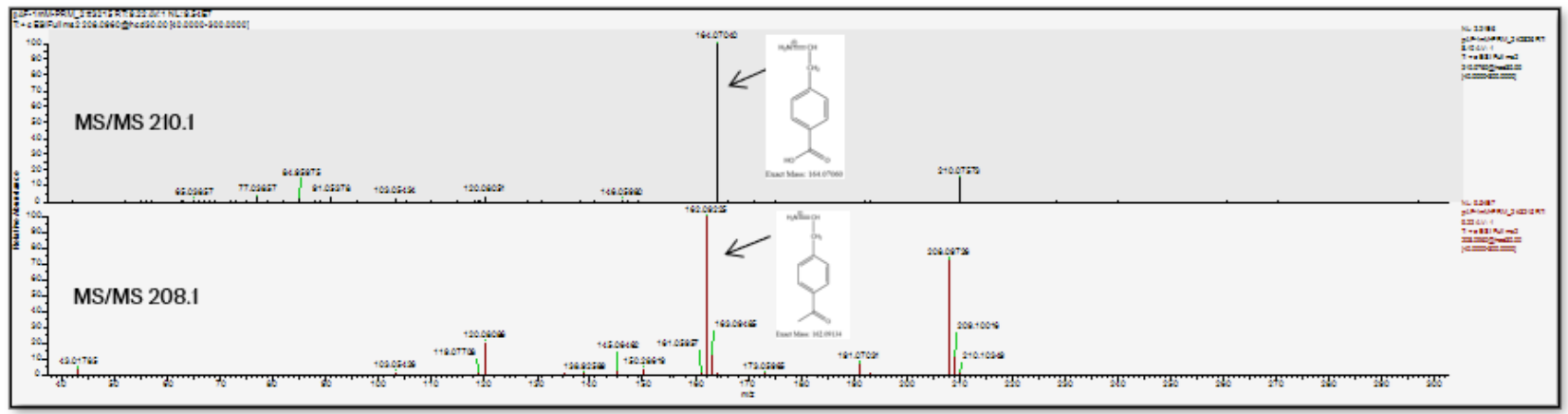


Formula	Mass	RDB	Delta (ppm)
C11H14O3N	208.09682	5.5	-0.087
C9H12O3N2P	208.05713	1	-1.579
C8H12O2N2 34S O	208.05731	1	-2.428
C9H12N2 34S	208.05616	2	3.071
C8H8ON2 34S	208.05751	1.5	-3.381
C8H12ON2 34S O	208.05596	1.5	4.024
C9H12O2N2P	208.05579	1.5	4.873





Formula	Mass	RDB	Delta (ppm)
C10H12O4N	210.07608	5.5	-0.019
C8H12O4N2P	210.07639	1	-1.498
C7H12O4N2 34S O	210.07657	1	-2.338
C8H12P2 34S2	210.07555	0	2.544
C7H8O4N2 34S2	210.07543	2	3.109
C8H12O4N2 34S2	210.07677	1.5	-3.282
C7H12O4N2 34S2 O	210.07523	1.5	4.053

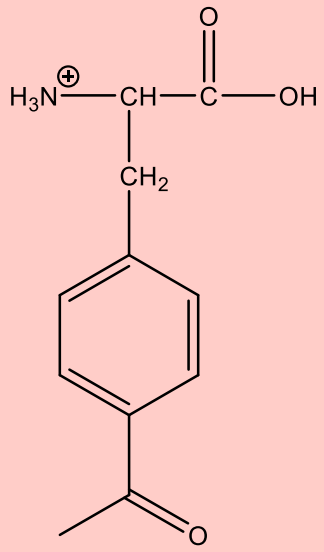


Note: MS accurate mass data provides elemental composition and MS/MS can support structure or indicate certain functional group information to propose structure above additional experiments are required to confirm structure of impurity

pAF species

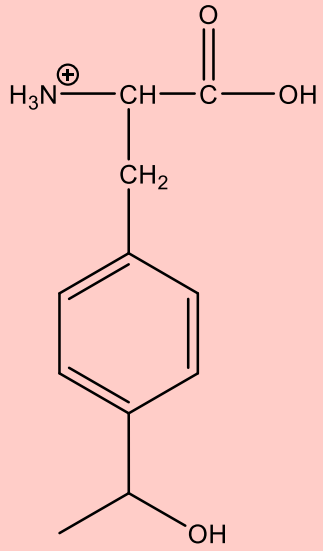
High Res -Accurate Mass experiments indicate a closer match to carboxylic acid pAF impurity

Para-acetyl Phenylalanine



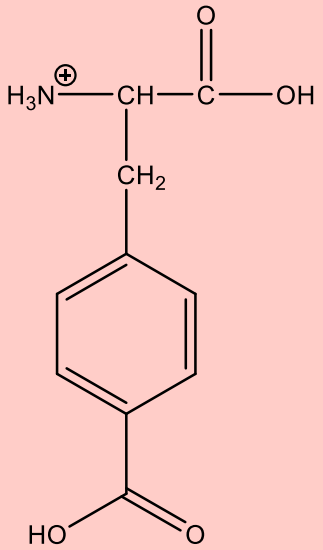
Chemical Formula: $C_{11}H_{14}NO_3^+$
Exact Mass: 208.09682

Suspected impurity
(from low-res MS)



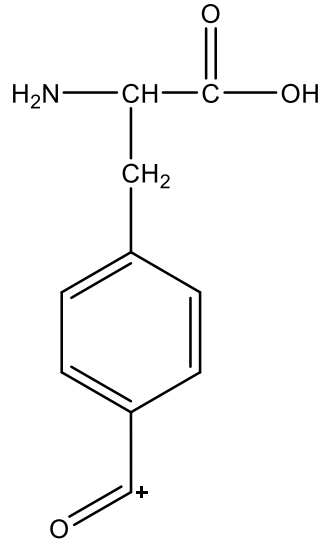
Chemical Formula: $C_{11}H_{16}NO_3^+$
Exact Mass: 210.11247

Revised impurity ID
(from high-res MS)



Chemical Formula: $C_{10}H_{12}NO_4^+$
Exact Mass: 210.07608

In source water loss
- 18.0 Da

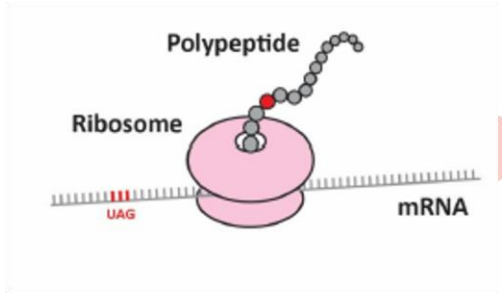


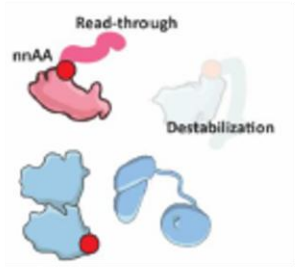
Exact Mass: 192.06552

Impurity was confirmed using a synthesized standard

Conclusions

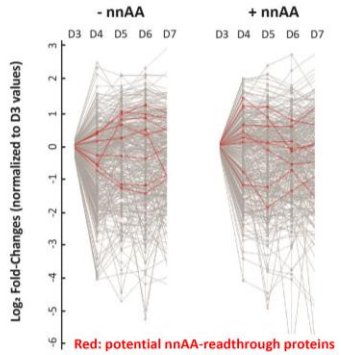
- Non-natural amino acids are a unique technology for enabling site-specific ADC
- Developing and leveraging the correct analytical tools can ensure the development of manufacturing ready cell lines
  - Ensure correct molecule expression and site insertion of the nnAA at trfx pool
  - Screen clones for efficient amber suppression machinery
  - Characterize reduced scale model bioreactor material to minimize amino acid substitutions and ensure purity of the raw materials
- Proteomic analysis can also enable profiling of the modified host cell to further ensure a robust cell line





Host CHO proteins are potentially affected by the amber stop codon suppression

Profiling Expression Dynamics



Ingenuity Pathway Analysis

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Annabel Torres
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Hillary Miller
Gargi Roy
… many others in CLD group

Analytical Development

Adam Evans

Ambrx team

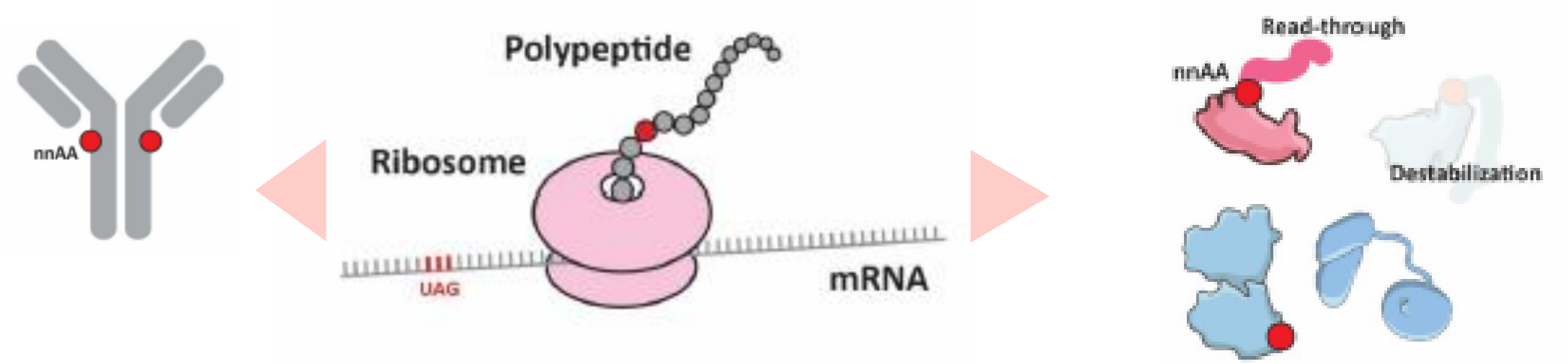
SCIEX

ThermoFisher SCIENTIFIC

Outline

1. **Introduction to Cell Engineering & Analytical Sciences (CEAS)**
2. **Antibody Drug Conjugates & Site-Specific Conjugation**
3. **Transfection Pool – Confirmation of mAb Expression**
4. **Single Cell Cloning – Optimizing amber suppression**
5. **Reduced Scale Bioreactor – Optimizing for product quality**
6. **Analyzing the host cell proteome**

Suppressing the Amber stop codon. Impact to CHO Proteins



Full-Length mAb
(Desired)

Host CHO proteins are potentially affected by the amber stop codon suppression

Changes to host cell proteome has been previously reported

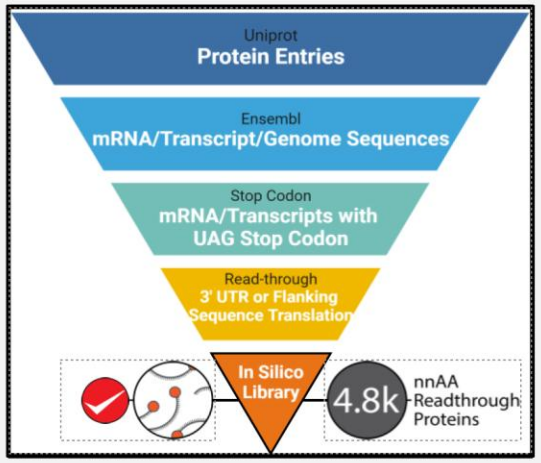
Chembiochem. 2014 Aug 18;15(12):1744-9

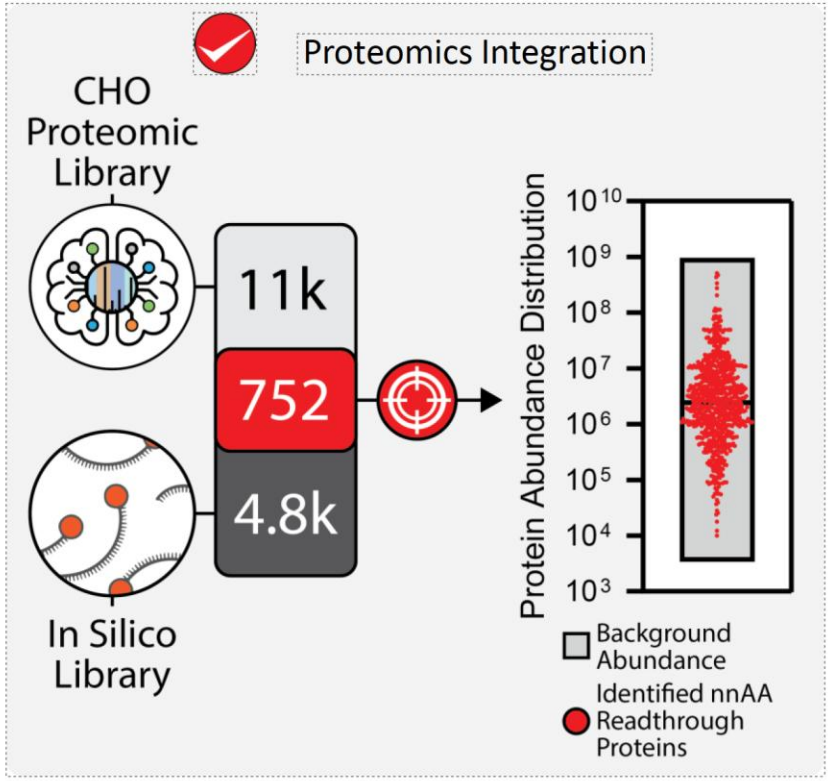
Nucleic Acids Res. 2021 Jun 21;49(11):e62

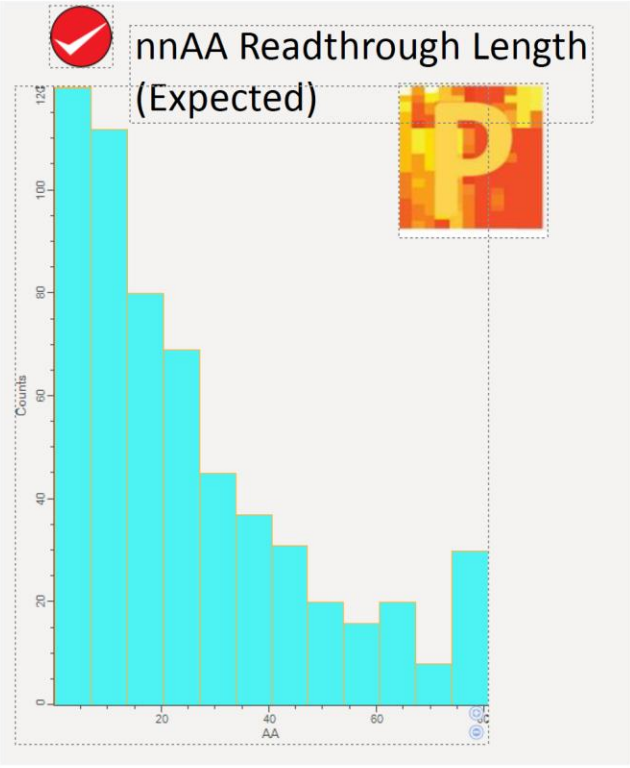
Life (Basel). 2015 Nov 12;5(4):1610-28

Bioinformatic Analysis Combined with Proteomic Profiling Identifies Potentially Modified Proteins

- Bioinformatic based ID-tion of TAG genes
- Creation of in silico read through library

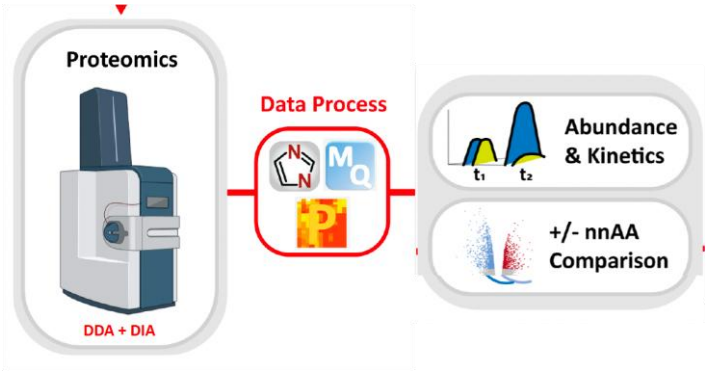




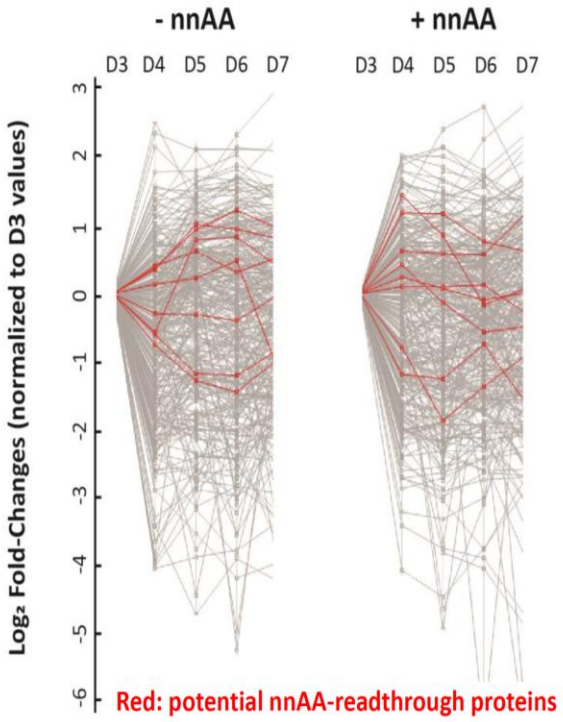


Expression Dynamics Identifies Proteins & Pathways Impacted by nnAA Induction

Proteomics Pipeline



Profiling Expression Dynamics



Ingenuity Pathway Analysis

- Differences observed in some cellular pathways: cell cycle, protein folding and stress response