**Investigating "Difficult-to-Express" Monoclonal Antibody Frameworks in Transient and Site-Specific Integration-Based Chinese Hamster Ovary Cell Expression Systems**

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The success of a monoclonal antibody (mAb) product is dependent on both its clinical performance and its "developability" profile, a set of characteristics that defines its ability to be manufactured at large-scale using current practices in the biopharmaceutical industry. Failure to identify low developability mAb candidates prior to process development can necessitate significant time and resource investments to mitigate these phenotypes. mAb expression in Chinese hamster ovary (CHO) cells, the production host cell of choice, is a crucial but poorly understood developability characteristic that can be significantly impacted by amino acid sequence, leading to orders of magnitude changes in titer driven by post-transcriptional bottlenecks. Previous work with "difficult-to-express" (DTE) mAbs has been limited by small study sizes and difficulties in translating findings across cell lines and products.

To better understand the relationship between mAb primary sequence and expression, we have leveraged both transient and site-specific integration-based (SSI) expression platforms to study DTE mAb variants at higher throughput in industrially relevant mAb formats, cell lines, and culture conditions. The rational selection and transient expression of 178 single amino acid mutations across 43 positions in a model IgG1k mAb revealed 91 novel mutations that decreased productivity. Mutations at buried residues that decreased residue hydrophobicity were particularly unfavorable for high expression. This work represents the largest mAb variant expression dataset as measured by secreted titers in CHO cells published to date.

To translate these findings into a stable expression system, we developed a hybrid SSI system in CHO that allows for rapid, efficient, and reproducible expression of mAbs from a consistent transcriptional and clonal background. We demonstrate the flexibility of this system by investigating the influence of two novel genetic regulatory elements on mAb expression at two genomic loci for two therapeutically relevant mAbs. The inclusion of these regulatory elements in mAb expression cassettes increased productivity up to 7 – 11-fold across conditions by simultaneously increasing transcription and modulating the ratio of heavy chain and light chain transcripts. Finally, we used this optimized SSI system to systematically compare the expression of ten mAb variants at two genomic loci under batch and fed-batch conditions. We identified eight variants with reduced expression in batch cultures, and three variants that maintained this low-expressing phenotype in fed-batch cultures. Characterization of biophysical properties and intracellular responses revealed variant-specific patterns.

Taken together, our work demonstrates the application of novel tools for assessing mAb expression at higher throughput under conditions similar to those used for large-scale mAb production. Continued collection of mAb expression data could support early identification of DTE mAb candidates, improve *de novo* design of well-expressed mAbs, and de-risk cell line and process development efforts, all of which can accelerate timelines for bringing novel antibody-based therapies into the clinic.