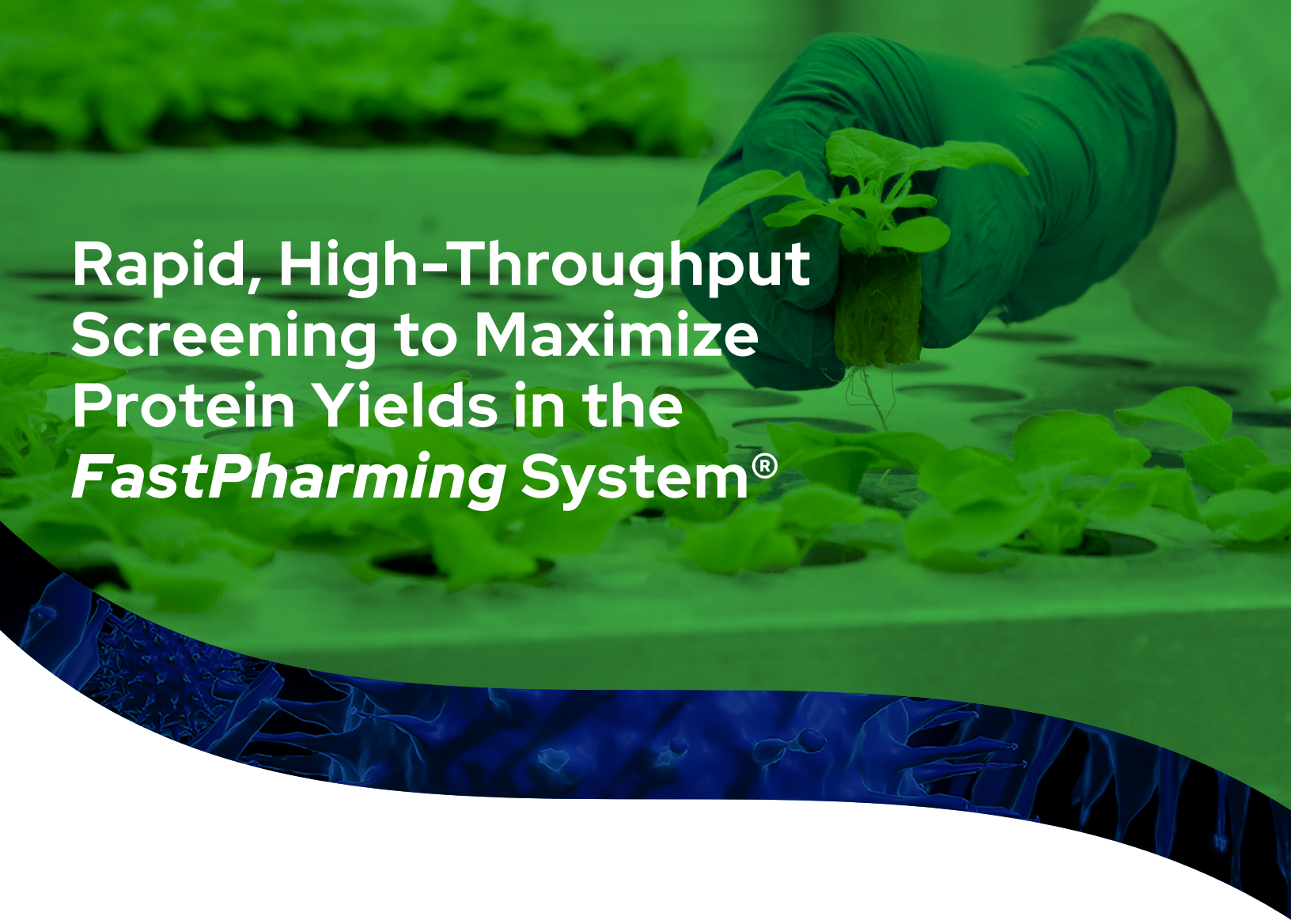


Rapid, High-Throughput Screening to Maximize Protein Yields in the *FastPharming* System®

Evaluating a large number of candidate variants using traditional cell culture systems such as CHO can be a significant bottleneck in recombinant protein development work. Using a plant-based platform methodology, iBio has demonstrated the ability to rapidly screen candidate variants for protein production. The identification of lead candidates for scale-up through the *FastPharming* System® enabled iBio's client, Safi Biosolutions, to identify six lead candidates from a pool of 36 variants in just eight weeks. Concurrently, iBio identified two lead candidates from an additional 12 variants.

Peter Kipp, Brian Berquist, Sreenath Palle, Kevin Babilonia, Sopheap Sun, and **Rachel Jordan,** iBio

Screening Candidates for Productivity

In a drug development program, there are several steps to choosing the appropriate path to productivity. Once a candidate recombinant protein (or proteins) has been identified, the next step is to maximize expression of the protein of interest by selecting the best CHO cell line and clone. This process could take three to nine months,[1] unless expensive clone selection systems are used. Then a master cell bank will need to be created, taking several more months. Media development and identifying the best nutrient feed to optimize to a specific titer may take another three to six months. This whole process can be expedited by using iBio's ***FastPharming*** System. Identifying the variant(s) with the best performance at the start of a project can quickly impact the ultimate productivity of the process. There are many approaches to optimize production of proteins that

can be evaluated rapidly. These include screening sequence variants with different promoters or signal peptides, a variety of *cis*-acting elements, and codon optimization. This allows for genetic engineering to produce variants with synonymous codon changes (i.e., DNA sequence changes that do not alter the amino acid sequence of the protein).

Rapid Candidate Selection

This high-throughput screening approach starts with basic guiding concepts that support a rapid evaluation of candidate variants:

1. Genes of interest are cloned into existing iBio vectors using standard molecular biology protocols.
2. Variants with and without an affinity tag are expressed in parallel and compared to determine the effect an affinity tag has on protein accumulation, reducing the time to candidate selection.
3. Candidates with an affinity tag are recovered from protein extract, allowing rapid and inexpensive recovery of multiple candidates using a common capture/elution method.

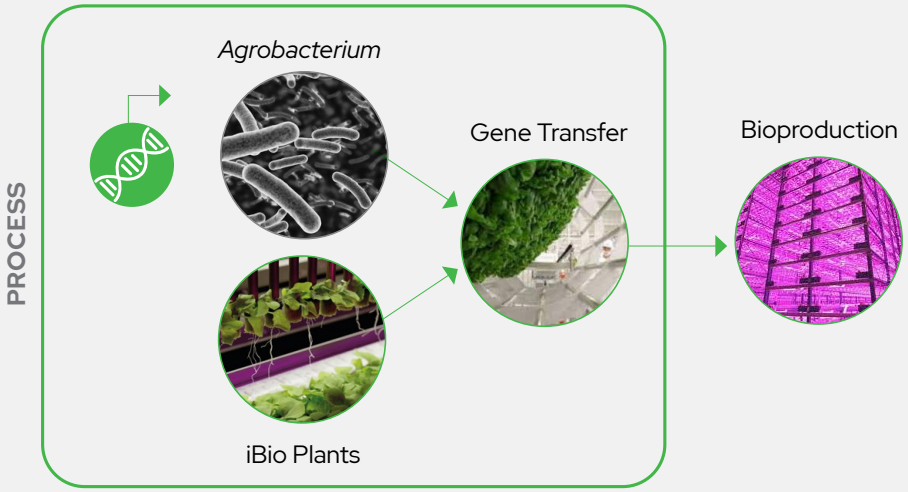
"When we came to iBio, we were optimistic that many of our cytokines — ideally, the most expensive ones — could be successfully produced leveraging the *FastPharming* System®. However, it was critically important to come to go or no-go decisions quickly."
– Doug McConnell, CEO and co-founder of Safi Biosolutions

FastPharming®: Scalable Plant-Based Expression of Recombinant Proteins

Founded in 2008, iBio has become a leader in plant-based biopharmaceutical production with the ***FastPharming*** System. The platform employs "transient transfection at scale" to provide a range of benefits over traditional mammalian cell culture, not the least of which is avoiding the time-consuming requirements associated with developing stable producer cell lines and master cell banks.

FastPharming uses a relative of the tobacco plant — *Nicotiana benthamiana* — as the bioreactor in the production process. *N. benthamiana's* weak immune system makes it well suited to infection by *Agrobacterium* species. That vulnerability is leveraged by using Agrobacteria as the "carrier" for the vector/gene that encodes the target recombinant protein that will ultimately be produced in the plant tissue. Following infiltration, performed by inverting plants into an Agrobacteria solution under a mild vacuum, the vector is transferred into the cytoplasm of leaf cells. Translation of these recombinant viral vector mRNAs can result in the accumulation of gram quantities of target protein in the plant leaves per batch of fresh plant tissue in less than a week.

FIGURE 1. ***FastPharming*** **Schematic**



Following post-infiltration growth, the plant biomass is harvested, homogenized, and clarified to produce an extract containing the protein of interest. Proteins are further purified using conventional separation and chromatography steps. The ultimate output is consistent, high-quality, animal-free recombinant proteins, including cytokines, growth factors, antigens, and antibodies. The system addresses the growing demand for animal-free protein products that eliminate the risk of contamination by adventitious viral agents or other undesired pathogens.

Plant-based recombinant protein production in *N. benthamiana* offers the benefits of eukaryotic posttranslational processing with the rapid and simple manipulation of transient transfection at both laboratory and commercial scale, allowing expeditious scale-up from demonstration to production. Transient transfection is possible in many expression systems. However, scaled and cost-effective recombinant protein production requires the development and characterization of stable eukaryotic cell lines, a considerable upfront investment of time and cost. The ***FastPharming*** System does not require cell line development or commercial-scale cell culture infrastructure. Scale-up is simple — only involving growing more plants with the same process conditions, as compared with switching to and re-optimizing cell growth in larger bioreactors.

With a standardized approach and success criteria defined in advance of screening, evaluation of candidate variants is straightforward and efficient. Gene constructs can be rapidly cloned into vectors and incorporated into *Agrobacterium*, which are then used to transiently transfect the *N. benthamiana* plants. Vectors can be evaluated in parallel in large groups to identify the top candidates from the portfolio.

Case Study: Evaluating 48 Candidate Variants in 8 Weeks

Proof-of-Concept for the Production of High-Volume, On-Demand Blood Products

The benefits of this high-throughput candidate screening method are well illustrated in a project iBio completed for cell therapy company Safi Biosolutions. As a partner of the U.S. Department of Defense On-Demand Blood Program, Safi Biosolutions aims to manufacture high-quality blood products at a large scale. The challenge is exploring ways to produce all the components of whole blood, including red blood cells, neutrophils, and acellular components.

On-demand blood requires high quality reagents that must also be produced cost-effectively in extremely high quantities. Cytokines are needed to produce on-demand blood, and the economics of manufactured blood products depends in part on a cost-effective supply of these critical proteins. Safi initially engaged iBio to help address scale and cost-control challenges. The cell therapy field traditionally uses an adherent cell culture platform. While the cell culture process is proven, it is limited to small scales. The scalability of the ***FastPharming*** System® provided a solution that was faster and superior to cell culture methods.

It was crucial for Safi to evaluate cytokine candidates quickly to determine which would be most suitable for production in the ***FastPharming*** System. As a first step, the iBio and Safi teams reviewed existing scientific literature to determine which proteins would be the best fit for a plant-based protein expression system. With the understanding of volume requirements and costs, the team selected a slate of 10 candidates. Three additional candidates of interest to iBio were also included in the screening workflow. A total of 48 variants from 13 target proteins were evaluated in just eight weeks, and lead candidates were selected.

Evaluation batches of the selected lead candidates were produced to test proof of concept for further scale-up, allowing the combined Safi and iBio team to determine which proteins would meet the required criteria for scaled production.

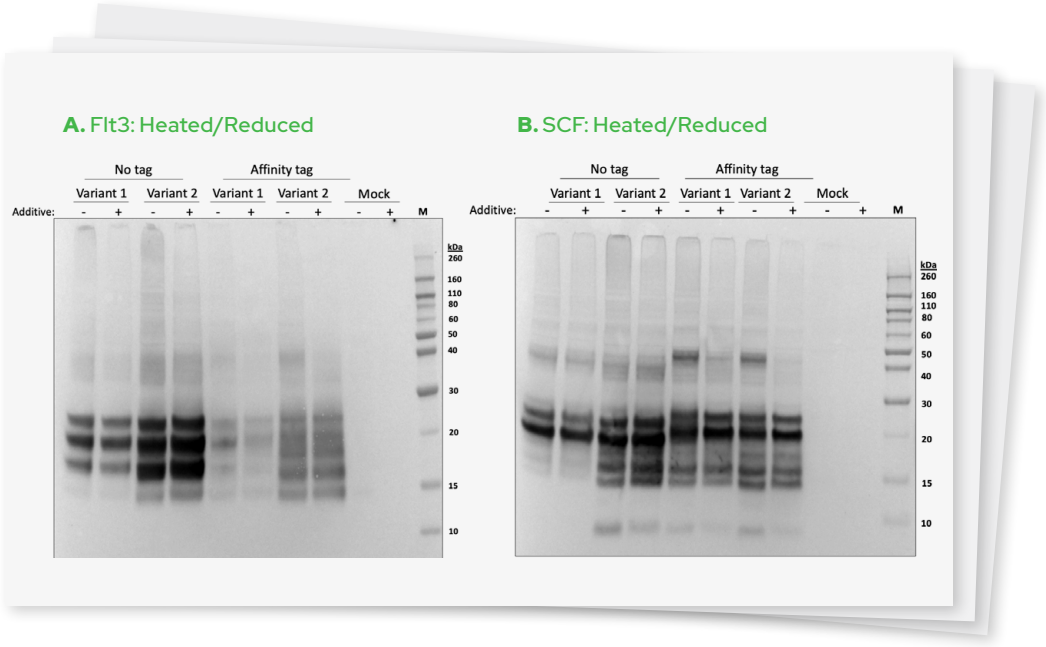
Representative Results

To illustrate the application of the high-throughput screening method in evaluating candidate variants for accumulation of protein, data are presented from two representative cytokines (FMS-like tyrosine kinase 3 ligand (Flt3L) and stem cell factor (SCF)). Two codon variants, each with and without affinity tag, of Flt3L and SCF are included in this example. **FIGURE 2** shows western blots evaluating the variants of Flt3L (A) and SCF (B). Each codon variant was expressed with and without an affinity tag, and each was extracted with and without additive in the buffer. Protein-specific antibody was used to visualize the target protein in crude protein extract. The western blot evaluating Flt3L demonstrates that codon variant 2 accumulated at a higher level than variant 1. For both variants, the untagged variant accumulated at a higher level than the affinity-tagged variant. This produced a more definitive lead candidate than the assessment of

FIGURE 2.

Evaluation of eight of the 48 candidates screened in eight weeks

Western blots evaluating accumulation of variant candidates of two cytokines (Flt3L and SCF), with and without an affinity tag, under two different extraction conditions (with and without an additive). Samples of crude protein extract were evaluated. (A) Flt3, heated/reduced conditions. (B) SCF, heated/reduced conditions. All blots were probed with protein-specific antibody. Mock, biomass not expressing the recombinant protein. M, molecular weight protein marker.



accumulation for the codon variants of SCF, in which variants were equally successful. The same vector was utilized for the expression of codon variants of both cytokines, but different expression vectors could also be evaluated in an analogous screening process workflow.

After the selection of a lead candidate for each protein, small-scale recovery using the affinity tag was performed to obtain protein for further evaluation. Peptide mapping confirmed the sequence identity of the recovered tagged proteins. **FIGURE 3** illustrates the peptide mapping results for one His-tagged variant of Flt3L, which demonstrated 90.8% coverage. The recovered proteins were also evaluated for the presence of endotoxins, which were found to be well below acceptable limits at <1.5 EU/ml.

Additionally, activity assays were conducted on affinity-purified protein to demonstrate that the tagged forms of the cytokines were capable of driving proliferation of relevant cell types. Each affinity-tagged cytokine was assessed for its ability to stimulate proliferation of appropriate target cells (AML5 cells for Flt3L and TF-1 cells for SCF). **FIGURE 4** illustrates these results, demonstrating that the affinity -tagged forms of both cytokines exhibit robust pro-proliferation activity in respective cell lines.

"Various transfusion catalysts, like trauma, infection, or radiation exposure, can be best served with different 'recipes' of cells and acellular components that meet the transfusion needs of specific conditions. We're developing individual blood components and will ultimately have the capability to fulfill these recipes."
– Doug McConnell, CEO and co-founder of Safi Biosolutions

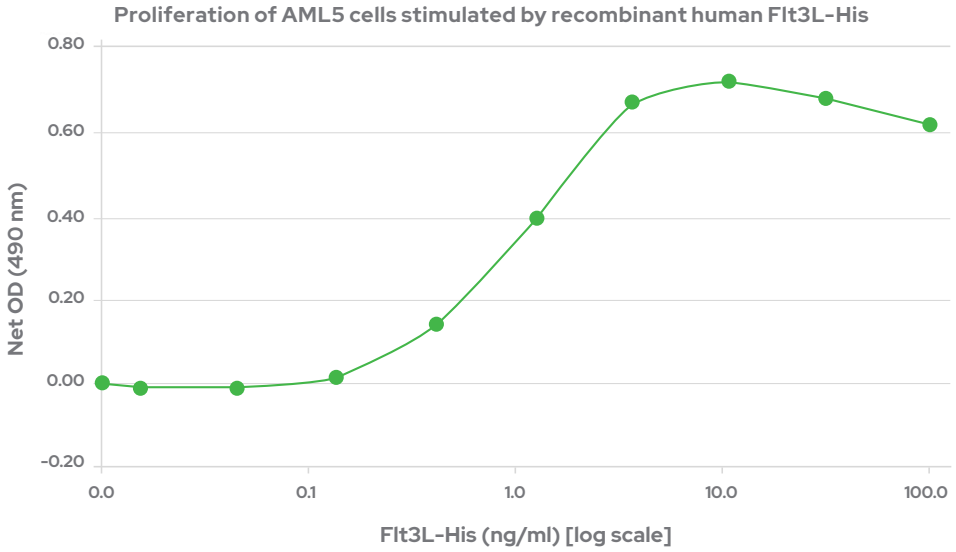
FIGURE 3. Flt3L-His peptide mapping

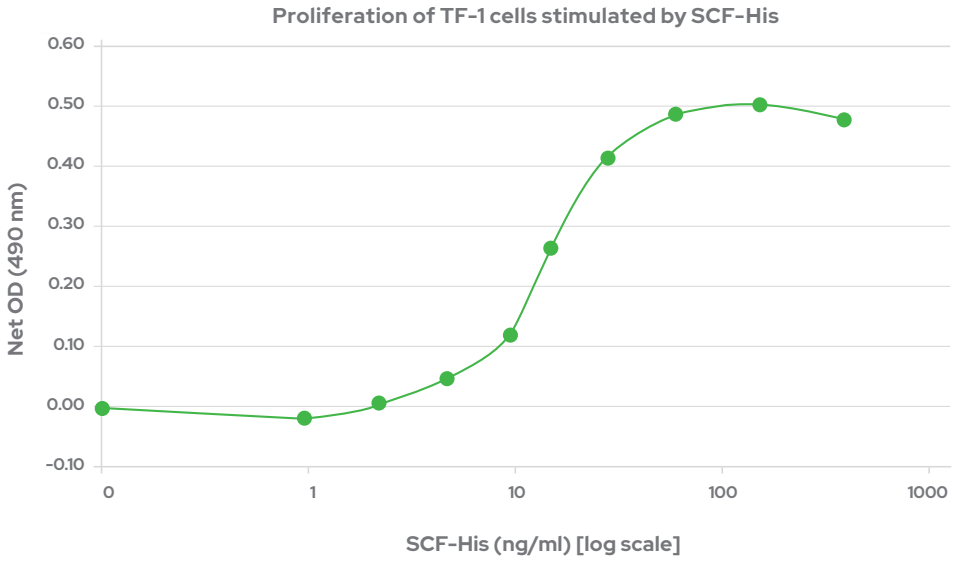
Sequences in bold were observed, non-bold (Roman) sequences were not observed. The peptide mapping analysis demonstrated 90.8% coverage.

TQDCSFQHSPISSDFAVKIRELSDYLLQDY
PVTVASNLQDEELCGGLWRLVLAQRWME
RLKTVAGSKMQGLLERVNTEIHFVT
KCAFQPPPSCLRFVQTNISRLLQETSE
QLVALKPWITRQNFSRCLELQCQP
DSSTLPPPWSPRPLEATAPTAHHHHHHHHH

FIGURE 4. Activity assays

Activity assays demonstrating pro-proliferation activity of the affinity-tagged variants of the two example cytokines in growth factor–dependent cell lines. (A) Recombinant Flt3L-His stimulates proliferation of acute myeloid leukemia 5 (AML5) cells. (B) SCF-His stimulates proliferation of hematopoietic progenitor TF-1 cells.





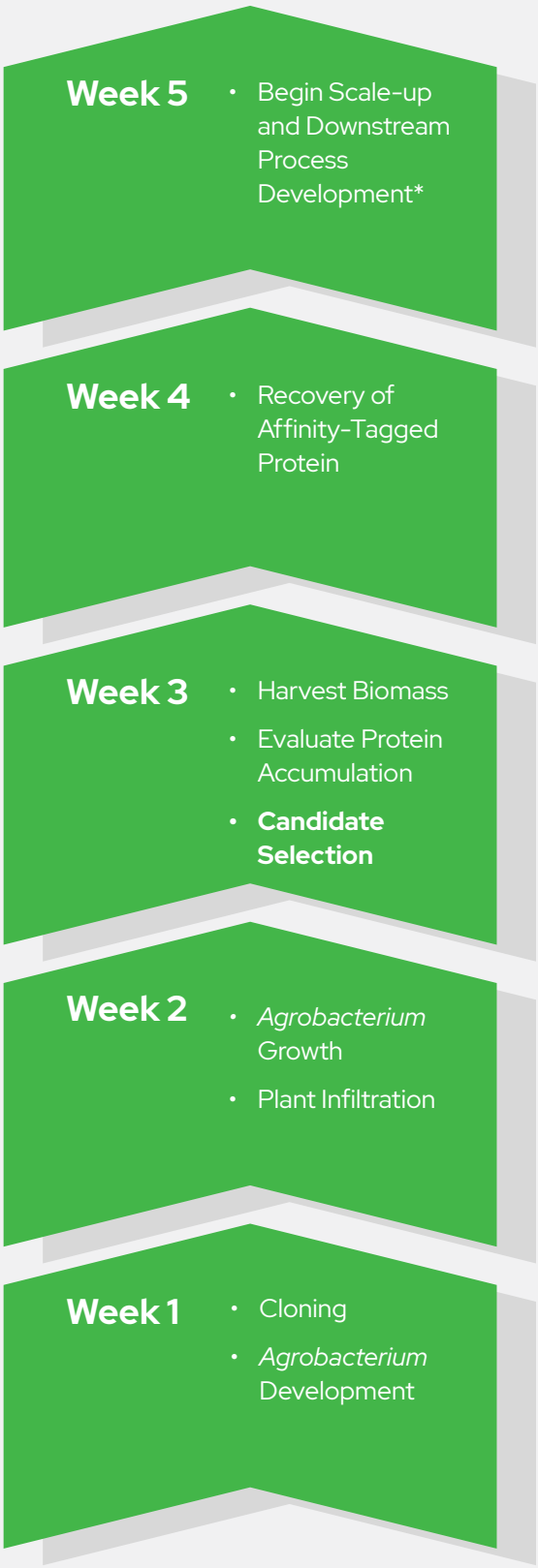
Conclusions

A summary of the timeline to complete a screening experiment is shown in **TABLE 1.** A candidate can be selected for process development and scale-up in as little as three weeks from inception, while affinity-tagged protein can be available one week or less after candidate selection. The timeline for the development of downstream processing depends on the protein target and the presence or absence of an affinity tag.

The representative examples illustrated in this case study are a subset of 48 variant candidates that were assessed in eight weeks, from the beginning of cloning to the evaluation of protein accumulation by western blot using protein-specific antibody. The 48 candidates were processed in two batches, with 27 candidates in the first batch and 21 candidates in the second batch. From this screen, affinity-tagged variants of 11 candidate proteins were selected for affinity recovery, eight of which were selected as lead candidates for further downstream processing.

This specific example focused on a portfolio of cytokines. However, the high-throughput screening approach is equally applicable to screening different protein types or other groups of *cis*-acting sequence variants. This includes libraries of promoter and signal peptide variants. Regardless of the details of the project and the specific goals, application of this rapid, high-throughput screening approach typically results in significant development time savings and an efficient path to a scalable process. This directly translates into cost savings for the client. ■

TABLE 1. Timeline to candidate selection, protein recovery, and process development



*Duration of scale-up and downstream process development timeline is protein-dependent

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