Overcome Complex Protein Challenges With Optimized Expression Technologies

By: Dr. Joachim Klein,
Head of Microbial Strain Development and Cell Banking
and
Dr. Rebecca Michael,
Principal Group Leader of the Cell and Molecular Biology Group, Lonza

While monoclonal antibodies (mAbs) have historically been a dominant force in the biopharmaceutical industry, over half of today's global biologics pipeline includes a diverse range of recombinant proteins, new molecular formats, and bioconjugates. Moving forward, the global presence of these therapies is expected to grow at a rate two times faster than standard antibodies. This is due to their ability to be more precisely targeted and more potent, and potentially open up access to new therapeutic targets and biological mechanisms previously not accessible with mAbs. Nevertheless, realizing the potential of these next-generation molecules means finding appropriate expression technologies and development processes that can facilitate their path to market.

At Lonza, we have more than 35 years of experience in developing and manufacturing novel molecular formats, with 40% of the large molecules our teams support falling in this array of modalities from preclinical to commercial-stage development. This is achieved by leveraging extensive expertise and a breadth of innovative technologies for mammalian- and microbial-derived molecules, allowing us to meet the challenges of complex protein expression necessary in today's changing industry.

GS XCEED® MAMMALIAN EXPRESSION TECHNOLOGIES

Lonza's suite of expression technology platforms begins with our GS Xceed® Mammalian Expression system, which is used for the optimal expression and production of monoclonal antibodies and next-generation recombinant proteins. This system offers a toolbox from which we can select the best vector system and expression host for the molecule type. This includes the GS® base vector system; the GS® multi-gene vector system for the expression of multi-chain molecules; the GS piggyBac® technology for difficult-to-express molecules; and the vector system for IgG site-specific conjugation (Figure 1).

For mammalian expression hosts, Lonza offers a standard GS knockout cell line, its POTELLIGENT® cell line

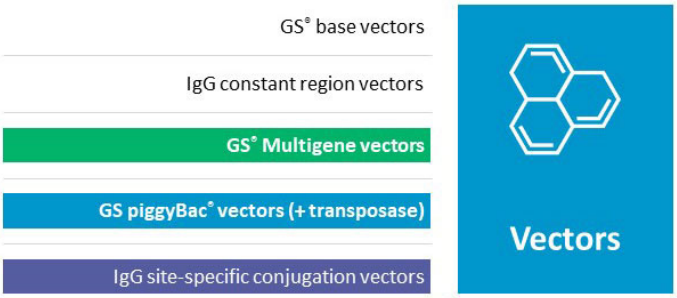


Figure 1: *Suite of vector systems for GS® expression technology*

for enhanced effector function, and the NSO cell line for biosimilars (Figure 2).

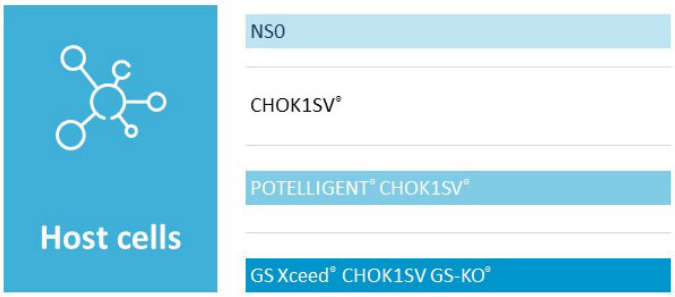


Figure 2: *Host cell options for GS® expression technology*

Lonza's wide range of experience with diverse protein types is outlined in the chart on the left in Figure 3 below. The chart on the right indicates the titer range our team observes for different molecule formats.

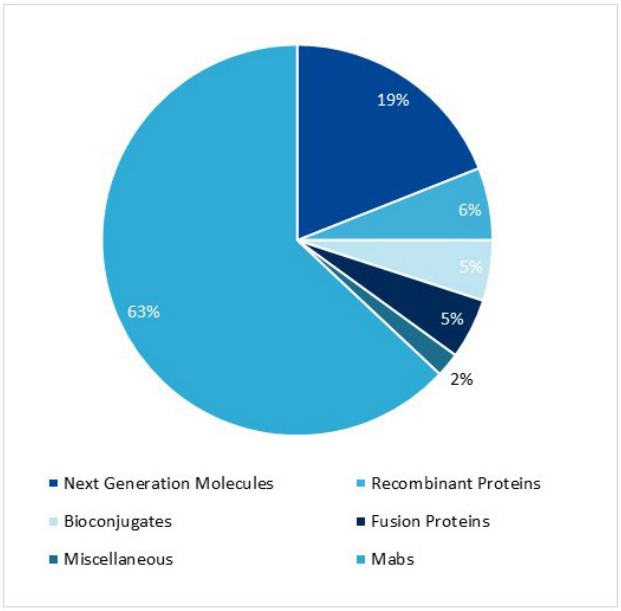
Lonza's team can obtain titers of around 6.5 g/L for standard antibodies and titers of multiple g/L for other diverse protein types.

ASSEMBLING A MULTI-GENE VECTOR

As recombinant protein therapeutics increase in complexity, improvements in gene expression and early-stage screening are necessary, especially for multi-chain products, which are an extremely diverse group of proteins. They include fusion proteins, various antibody formats, such as IgA or IgM, as well as multi-specific molecules containing three or four chains, resulting in molecules with two or more binding sites. Multi-chain proteins require a vector system that allows the expression of multiple genes in one single vector. Optimizing vector design early allows the development team to determine whether gene order and number within a single vector can influence the titer and product assembly. It also allows for product quality screening to de-risk process development.

Lonza's approach for multi-chain molecules is our multi-gene vector system. The assembly of multi-chain vectors starts with the cloning of each individual cassette into a part vector (labeled in Figure 4 as pXC-Part A, B, C, and D).

The part vectors are then combined into a donor vector, which can be a triple gene or a quadruple gene vector, and



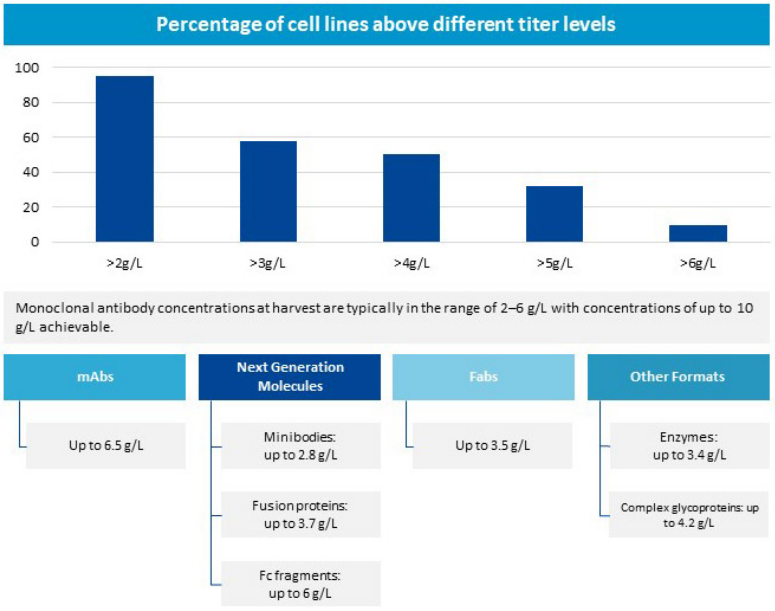


Figure 3: *Lonza's experience and titer range for diverse protein types*

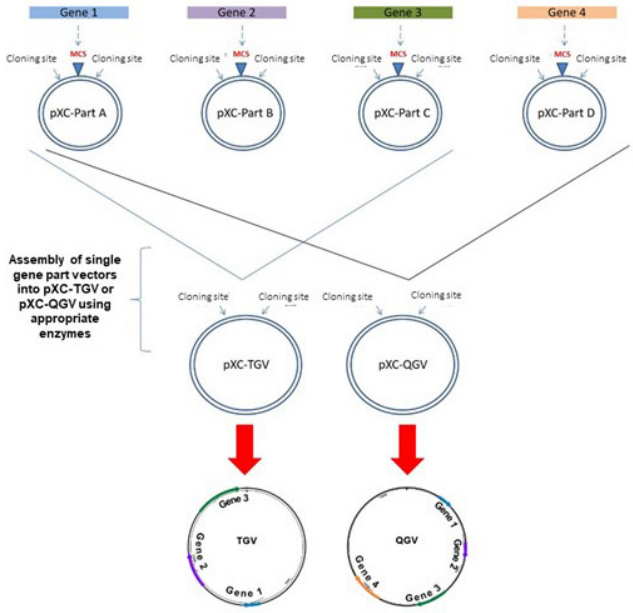


Figure 4: *Simple, one-vessel assembly reaction*

then assembled into a one-part assembly reaction. To produce a triple gene vector (TGV), the constituent product genes are inserted individually into pXC-Part A to C to create single gene vectors (SGVs) using methods familiar to GS Xceed® users. SGVs are then combined into pXC-TGV in a one-vessel assembly reaction. To produce a quadruple gene vector (QGV), the constituent product genes are inserted individually into pXC-Part A to D and then combined into pXC-QGV in a one-vessel assembly reaction.

Case Study 1: Investigating Gene Order

This case study examines vector assembly for a triple chain molecule that has a knob-in-hole, heavy chain pairing and a common light chain. To demonstrate the efficacy of the multi-gene vector (MGV) system in constructing an expression vector for a relevant triple-chain protein, two variants of a triple gene vector (TGV) were assembled with the cassettes in different orders in the final triple gene vector (Figure 5).

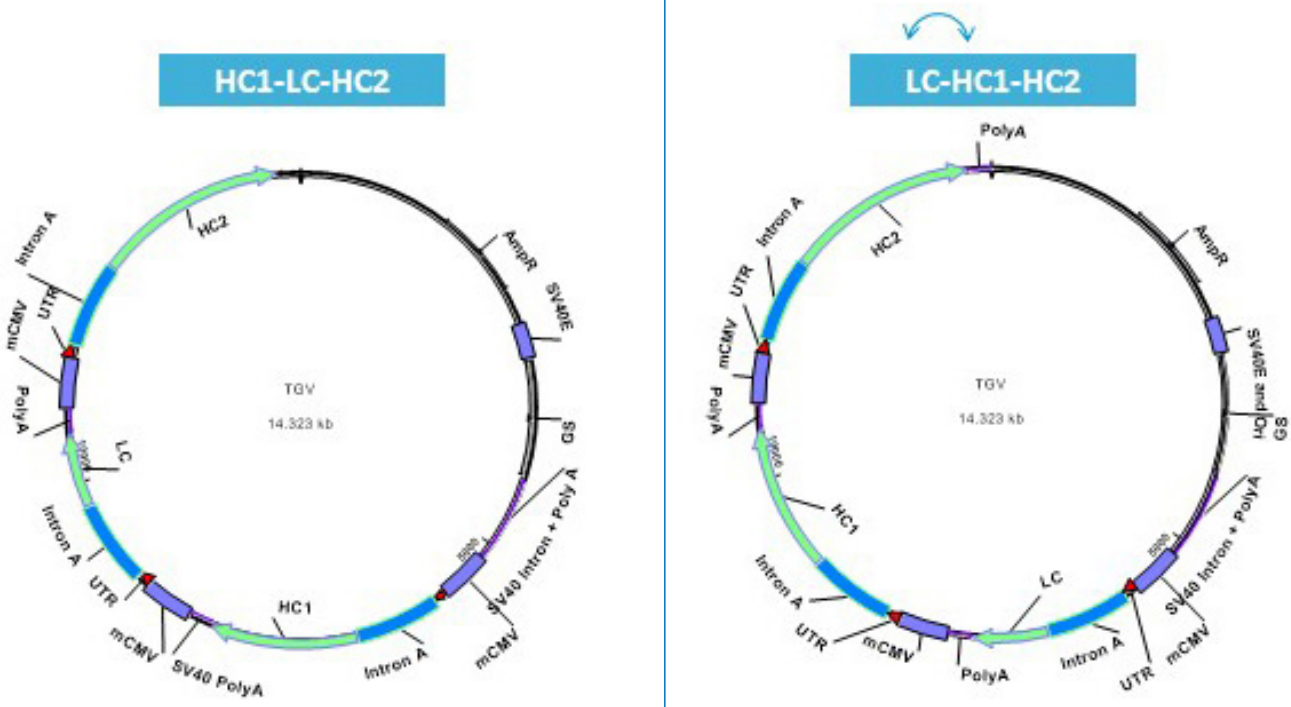


Figure 5: *Two variants of a TGV with the cassettes in different orders*

As calculated from a miniprep screen of ampicillin-positive bacterial colonies, Lonza achieved a success rate of 89% to 100% for this product using these vectors (Figure 6).

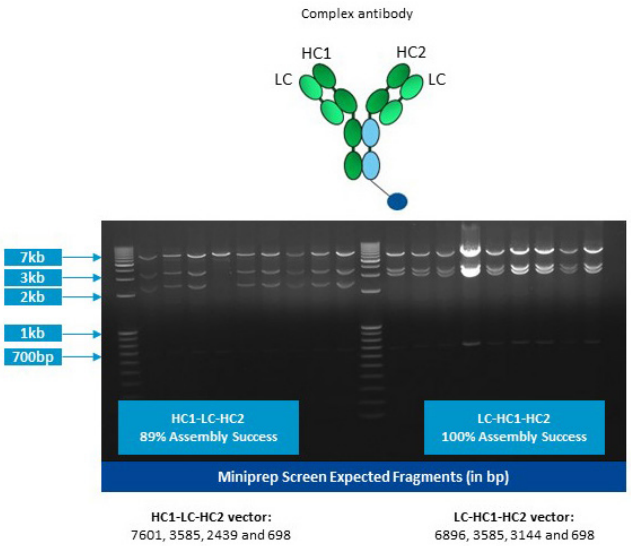


Figure 6: *Screening for correct vector assembly by Restriction Enzyme mapping*

To monitor for correctly assembled heterodimer, cell culture supernatant obtained from the stable pool expression was purified by Protein A and analyzed by mass spectroscopy. The correctly assembled heterodimer was the predominant species with a low level of mis-paired homodimers (Figure 7).

Titer was also monitored in an eight-day batch culture. The supernatant concentration was determined by octet and Protein A biosensors. For this experiment, titers of 193 mg/L were achieved for one of the vector designs (LC-HC1-HC2) in our GS Xceed® stable pools.

The data in Figure 8 demonstrates that the gene order in the multi-gene vector can influence expression titer.

GS PIGGYBAC® TECHNOLOGY

In addition to our standard GS vectors, Lonza also developed its GS piggyBac® technology for difficult-to-express and more complex proteins. This technology is based on a cut-and-paste system to insert DNA into the cell genome (Figure 9).

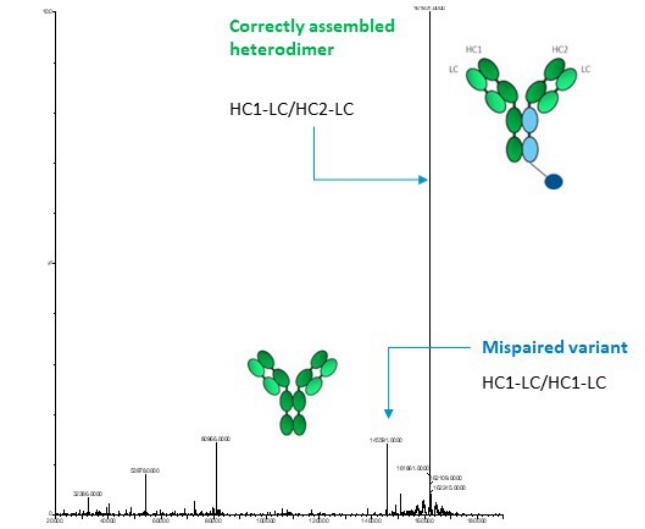


Figure 7: *Deconvoluted spectra*

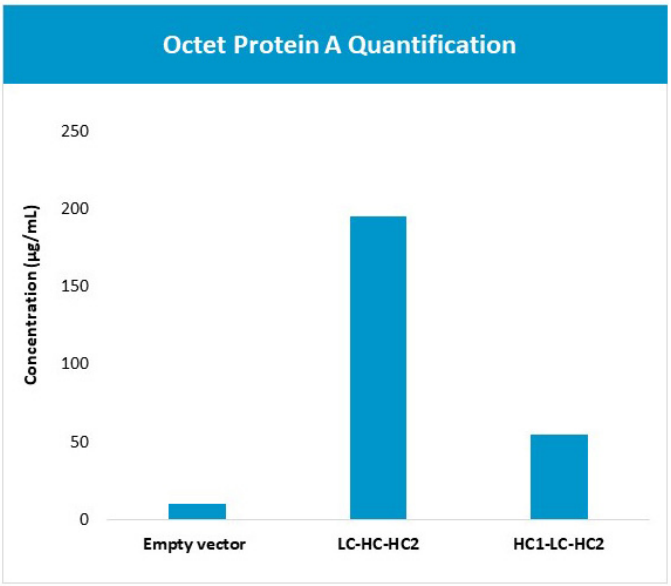


Figure 8: *Increased yield from LC-HC-HC2 in agreement with anecdotal data on influence of cassette order on expression*

GS piggyBac® can accommodate large DNA cargoes of up to 300 kilobases, so it is highly suited to complex proteins. It preferentially targets stable regions of genomes associated with highly expressed genes. The

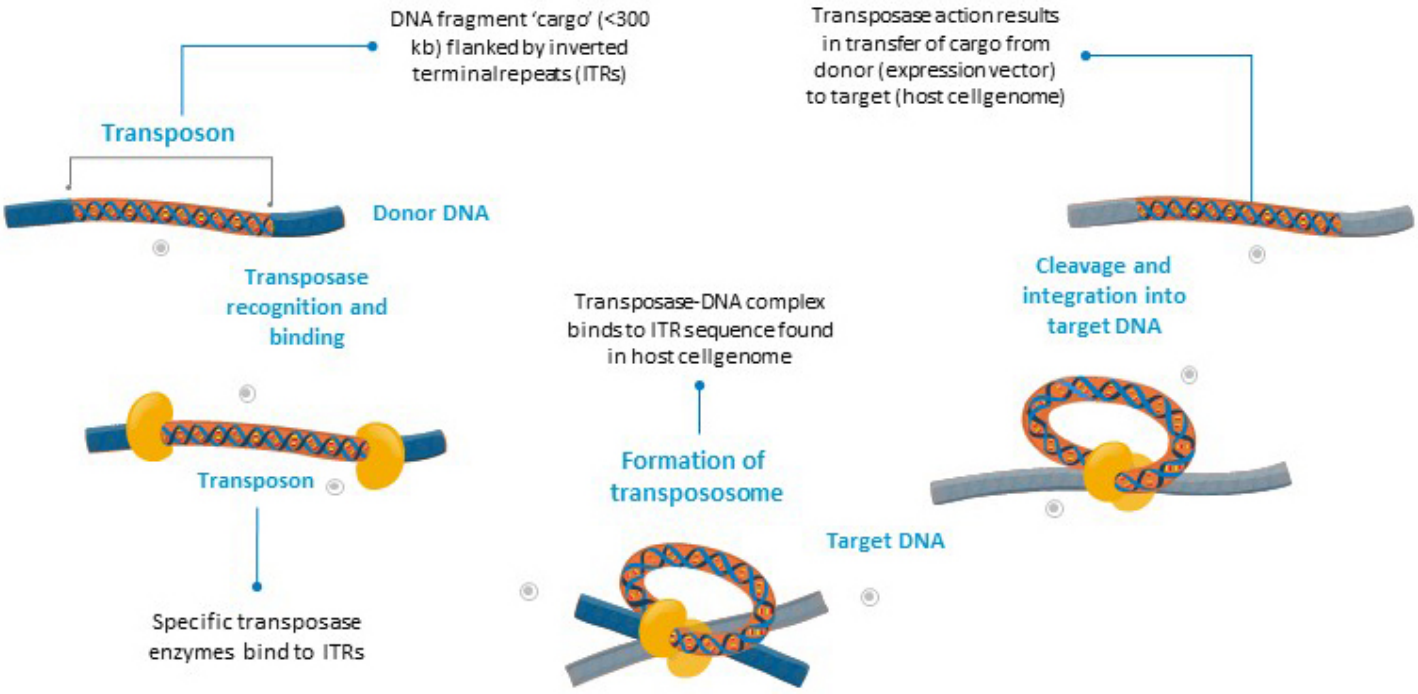


Figure 9: *GS piggyBac® cut-and-paste system*

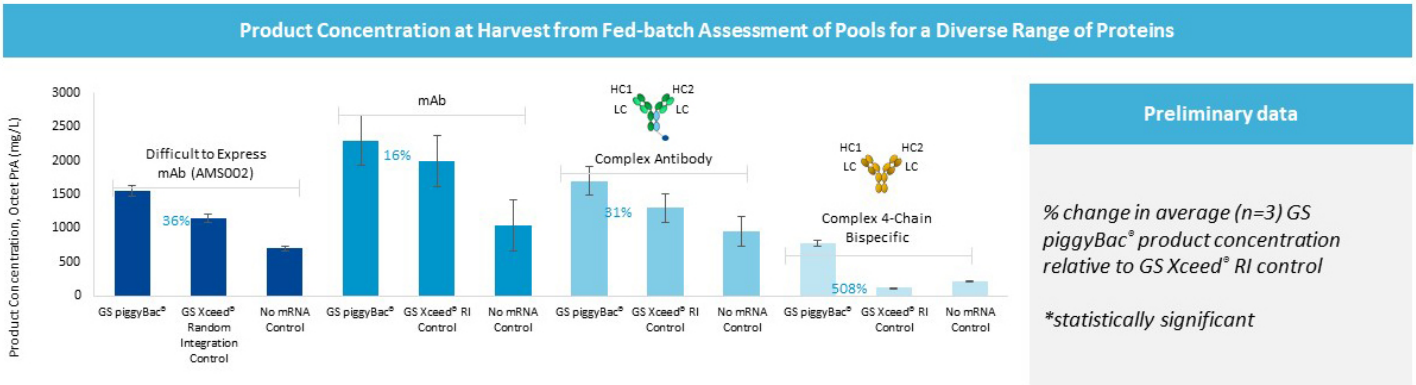


Figure 10: *GS piggyBac® increases productivity for a diverse range of molecules*

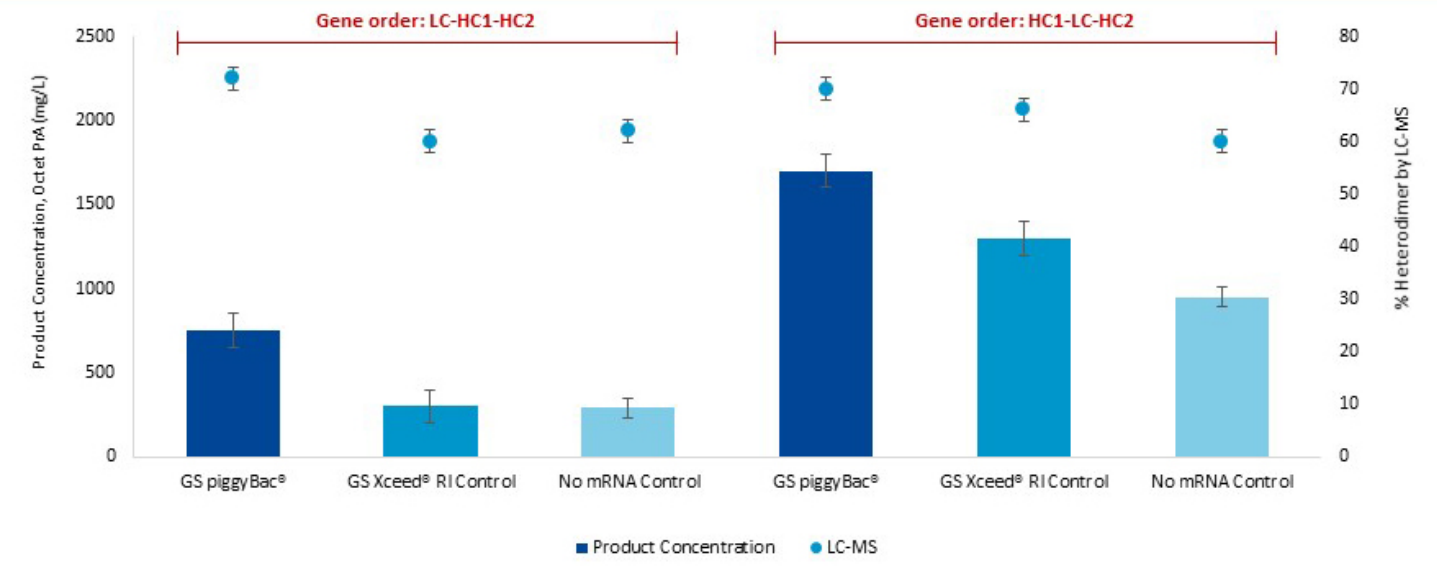


Figure 11: *Product concentration at harvest and percentage of heterodimer from fed-batch assessment of pools for a complex antibody*

donor DNA includes the DNA cargo called transposon, which is flanked by inverted terminal repeats. Specific transposase enzymes bind to the inverted terminal repeats and form a transposase DNA complex. This DNA complex then binds to the inverted terminal repeats found in the host cell genome, forming a transpososome. Finally, the donor DNA is cleaved and the gene of interest is integrated into the host cell genome. The GS piggyBac® technology can help address some of the key challenges in cell line development, such as manufacturing of difficult-to-express or complex proteins and cell line instability. It also has the potential to accelerate the speed to start toxicology and first-in-human studies. Figure 10 shows a comparison of the production concentration at harvest in cell lines using GS piggyBac® versus GS Xceed® vectors for various types of proteins. In the case of a difficult-to-express monoclonal antibody (mAb), a complex mAb, and a bispecific consisting of four separate chains, a substantial increase in product concentration was observed when using GS piggyBac®, particularly for the 4-chain bispecific. However, only a slight increase was observed for a standard mAb.
An increase in the percentage of heterodimer or correctly assembled molecule was also observed in conjunction with Lonza's multi-gene vector system as well as an increase of product concentration when using GS piggyBac® versus GS Xceed® (Figure 11).

The increase in product concentration for GS piggyBac® compared to GS Xceed® random integration control represents assembled heterodimer rather than just an increase in Protein A binding due to the presence of homodimers.

CUSTOMIZED CELL LINE DEVELOPMENT

As well as considering vector choice and design for next-generation biologics, Lonza also offers a flexible, tailored cell line development program to suit customer requirements. Figure 12 outlines the information our team can collect at each stage of development.

The example below shows an accelerated timeline for the cell line development of a bispecific antibody (Figure 13). Starting the program with a screening of the different vector constructs and early cell line development will highlight issues, if any, early in the program. It will also ensure the selection of the vector that will give the optimal product assembly and titer.

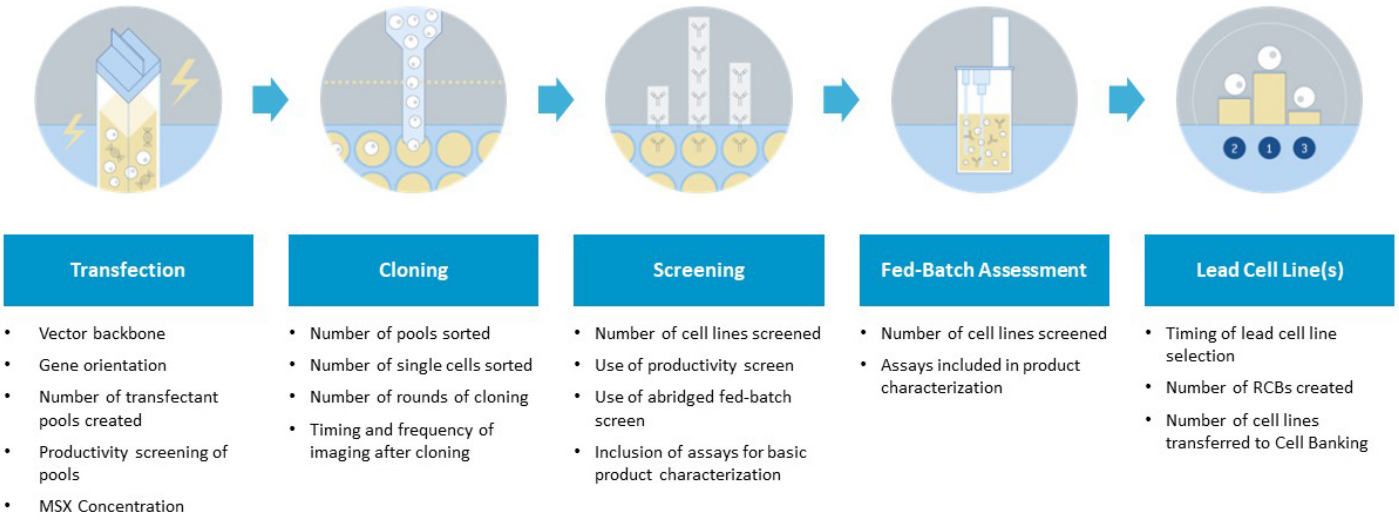


Figure 12: *Flexible cell line development bespoke to suit customer requirements*

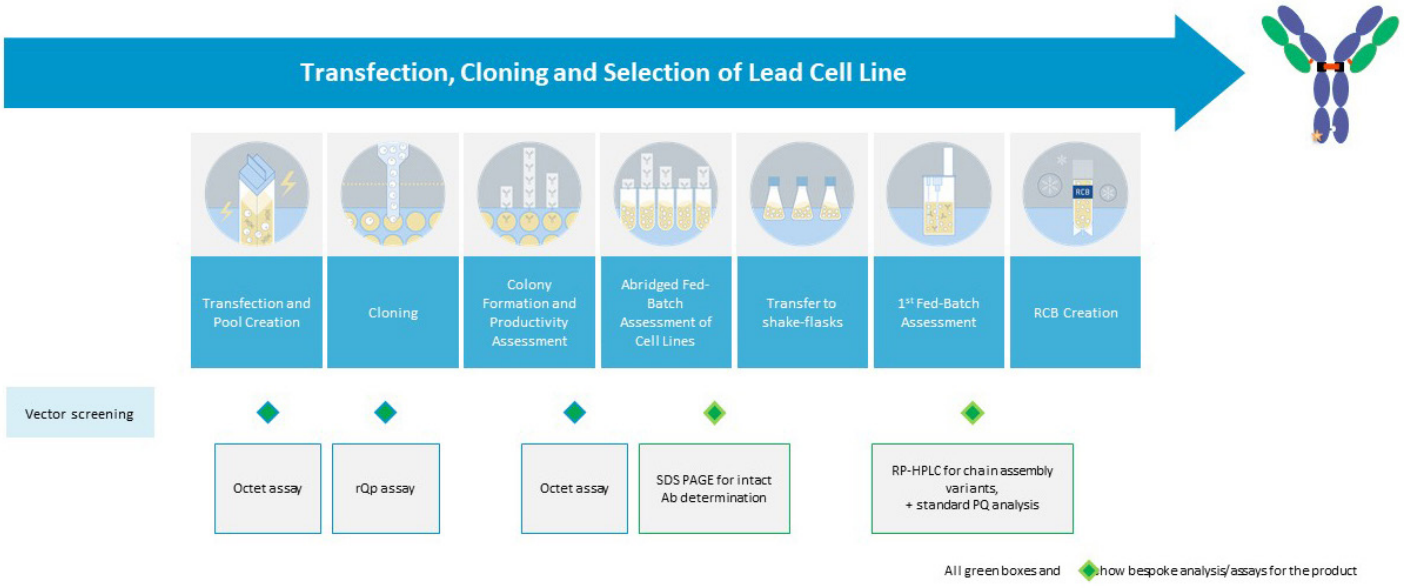


Figure 13: *Rapid development of a bispecific (14 months)*

In addition to product expression assays during the initial screening rounds, product-specific quality assays can also be included for cell line selection. This allows for early dismissal of cell lines that are not expressing the correctly assembled product. For these types of programs, it is essential to screen for product assembly in addition to expression levels during the cell line development.

Figure 14 shows the cell line development process for a complex, non-Protein A binding protein. For this type of molecule, Lonza's standard platform assays may not be suitable and assay development is often required ahead of the cell line construction start. This does add time to the overall timeline but is based on a right-first-time (RFT) approach to ensure that no rework or changes to the planned work scope will be required at a later date.

In this specific example, an octet assay, which is a high throughput method used for screening a large number of cell lines for product expression, is developed prior to the start of the program and used in the early screening stages. It is followed by the development of titer and product-specific methods that are developed in parallel with the cell line development and used in the later screening rounds.

Case Study 2: Bispecific Antibodies

As already discussed, it is important to rapidly develop analytical methods early to support cell line development using a toolbox approach. In order to support development and make the best decisions about achieving the right cell line with the right balance between product quality and titer, the rapid development of adequate analytical methods is essential. Structural variability between different bispecific molecule formats means a single analytical method is not readily available.

Cell lines expressing knob-in-hold bispecific molecules can produce homodimer variants as well as the intended heterodimer product, and selection on titer alone does not guarantee a cell line producing high volumes of the desired product. To ensure we manufacture product with the required chain assembly, we need an analytical method that can support:

- identification of the product and homodimer variants present in the test sample
- relative quantification of the desired product variants
- testing up to 30 samples per analysis
- reporting data within three days of samples being available

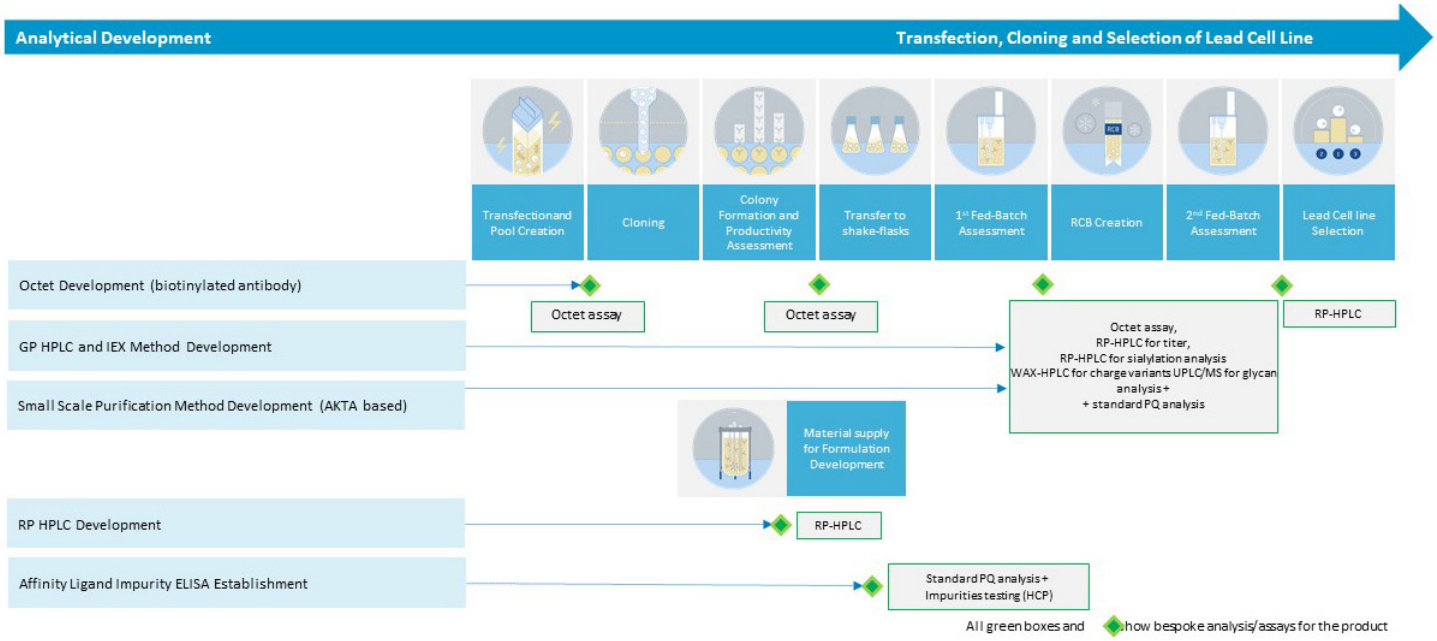


Figure 14: *Development of a complex protein (20 months); a focus on RFT approach, eliminating rework, and a cyclical approach*

As a result, Lonza has established a workflow and diverse analytical toolbox that allows a quick and simple method development process for bispecific antibody products.

In this case study, Lonza developed a reverse phase, high-performance liquid chromatography method in two weeks using partially purified material from early development stages. The assay was used to screen cell lines for relative levels of homodimer formation. Up to 30 samples could be tested per analysis with process data being available within three days of testing initiation. This method was successfully incorporated into our cell line development testing panel. This reverse-phase testing method — along with affinity-based titer, size-exclusion chromatography, chip electrophoresis, and glycan profiling methods — allowed our team to identify cell lines that met both titer and product quality requirements for the program.

In summary, the variety of solutions from our GS Xceed® toolbox allows us to de-risk, develop, and manufacture complex proteins. The key is to conduct the experimental work early in the process and select the right vector and right cell line and have the right analytical methods to make informed decisions.

XS® MICROBIAL EXPRESSION TECHNOLOGIES

In response to the increasing diversification of the biologics pipeline, Lonza's E. coli expression platform has been complemented by the development of a yeast, Pichia pastoris, to support the production of more complex proteins. Both systems contain several genetic elements to increase the productivity of the generated strains and clones. They include:

- more than 10 promoters and different means of induction to modulate the growth and induction conditions
- more than 10 different host backgrounds for better product quality and quantity
- five different proprietary signal sequences for periplasmic production and secretion into the culture supernatant
- several chromosomal integration sites for Pichia pastoris
- more than 10 helper factors to selectively increase the titer of the protein of interest

To mitigate the lack of predictability for quantity and quality of the protein of interest, a combinatorial screening approach was developed for both E. coli and Pichia pastoris. The combinatorial screening uses a toolbox of strains, promoters, signal sequences, and helper factors to assess the optimal combination of elements for the target protein. Analytical data is used to assess quantity and quality attributes of the protein of interest and to develop the right fermentation process. As a basis for the ideal process, a scientist must always keep the quantity and quality of the protein of interest in mind when aligning with the right production facility.

Figure 15 outlines the combinatorial screening workflow to identify a viable and scalable production strain for the protein of interest as fast as possible, which serves as a perfect starting point for process development.

The host pre-screening is used to select the best expression platform, either E. coli or Pichia pastoris. The full combinatorial screening where all relevant genetic elements can be used to select the best production clone out of a broader screening set. Overall, the more you screen, the better the performance of the selected clone. It is important to always use a selected number of analytical methods, addressing quantity and quality. From the primary screening, the workflow moves into secondary screening to verify the performance of the selected clones. We use a miniature bioreactor system, from which data can be translated into larger scale. If required, our team generates proof of concept material for functional studies. Finally, the generation of the research cell bank is properly documented and can be used to generate a cGMP master cell bank.

Case Study 3: XS® E. coli

This case study in E. coli compares the data from an IPTG inducible expression system and Lonza's rham-

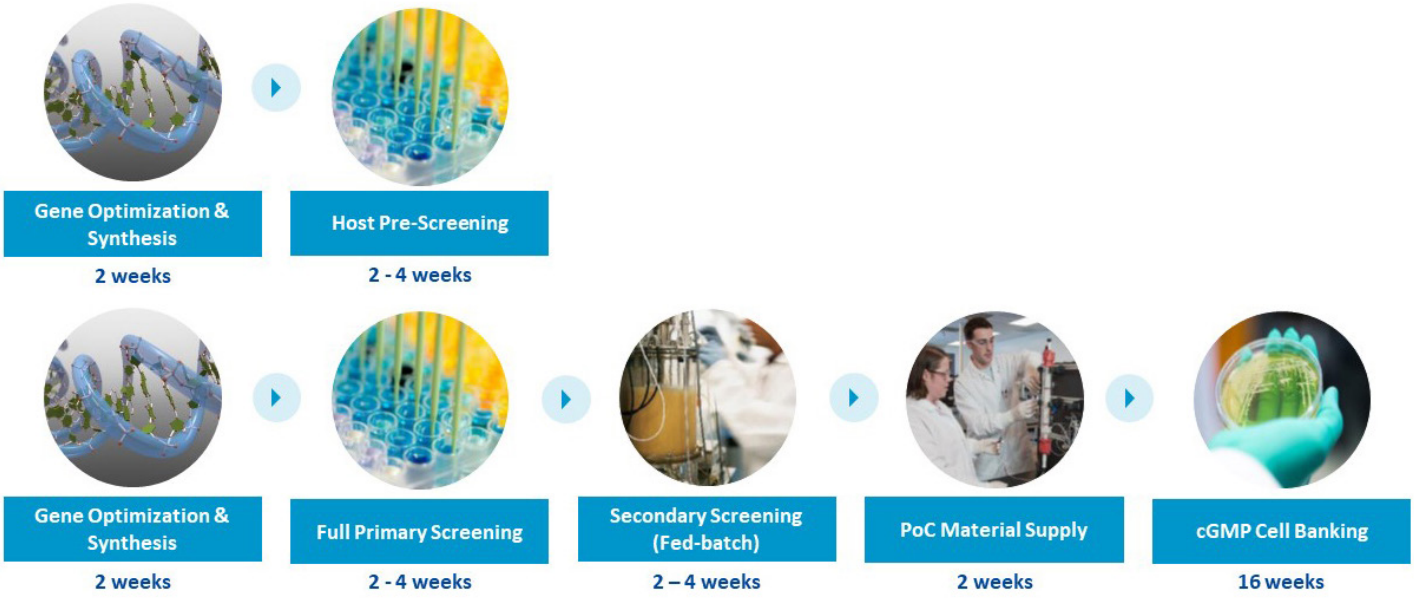


Figure 15: *Workflow in microbial strain development*

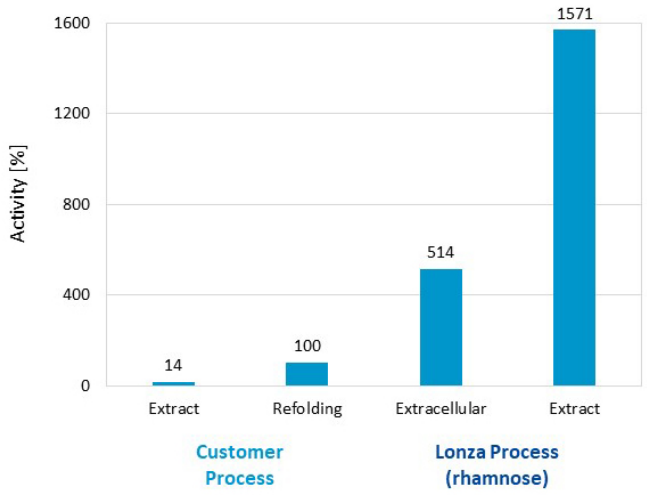


Figure 16: *Improved solubility using Lonza process compared to the customer process*

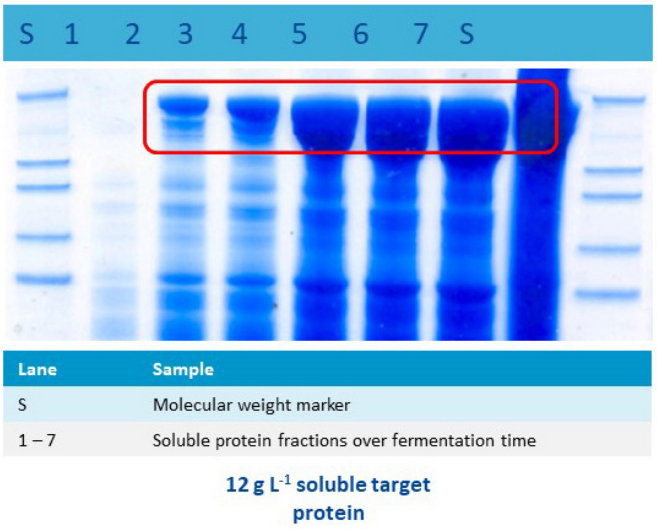


Figure 17: *SDS page of cell associated material*

nose-inducible expression platform. The protein of interest is a more than 200 kilodaltons complex protein produced in the periplasm and in the culture supernatant. The problem was that the protein could only be produced as an insoluble protein using the IPTG inducible expression system of the customer. To find a soluble route, Lonza completed a limited screening with three promoters, two rhamnose-negative hosts, two signal peptides, and intracellular production. With a rhamnose-inducible promoter, our team was able to produce soluble material that was 20 times more active than the material from the reference strain (Figure 16).

An SDS page of the cell associated material is shown in Figure 17. Lonza's strain produced 12.4 g/L of soluble enzyme, eliminating the need for a laborious large-scale refolding process. The key to success in this case study was high plasmid stability.

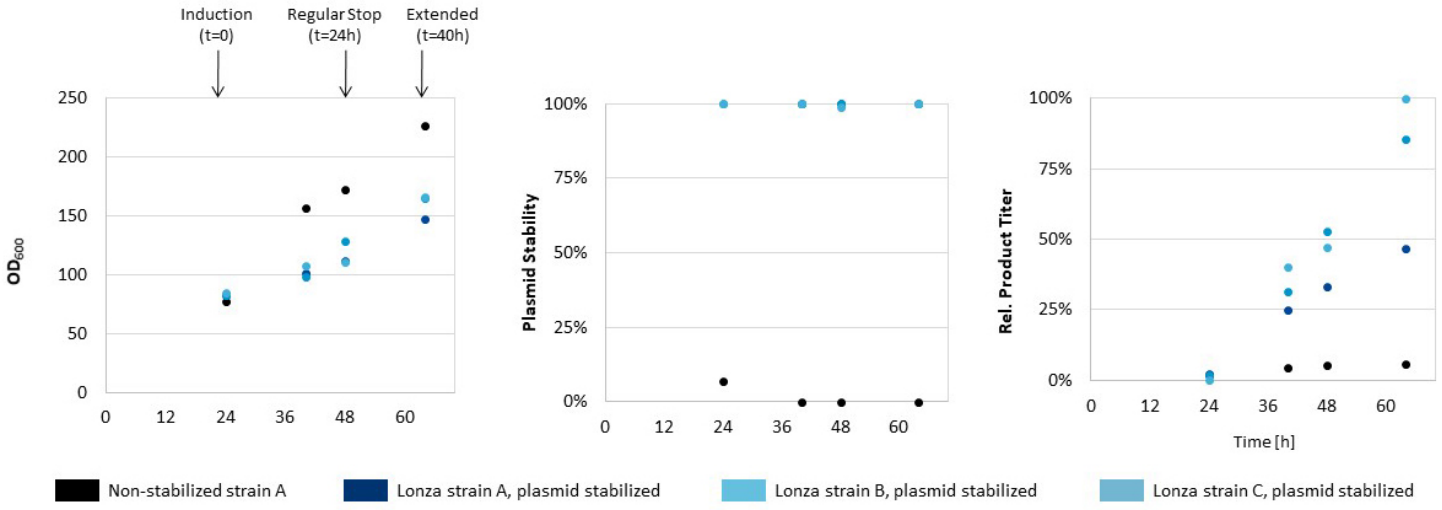


Figure 18: *Plasmid stability is essential for reasonable titers of difficult-to-express proteins*

The graph on the far left-hand side of Figure 18 shows the optical density of fed-batch fermentation runs of four strains (one strain without plasmid stabilization in black and three strains with stabilized plasmids in blue).

It is clear from this data that the strain with plasmid stabilization grows faster (middle graph), which is an indication of plasmid loss. The strain with a non-stabilized plasmid (far-right graph) loses the expression plasmid completely over the fermentation run and, as expected, produces only low amounts of the target protein. The key message here is that high plasmid stability is essential for reasonable titers of difficult-to-express proteins.

Figure 19 shows a box plot distribution of expression titers of over 30 different recombinant proteins using the E. coli toolbox.

The four effective expression modes include: insoluble as inclusion bodies (C1), soluble in the cytoplasm (C2), soluble in the cell free medium (C3), and soluble in the periplasm (C4). The proteins include novel molecule formats, fragmented antibodies, hormone fusion proteins, and peptides. The size varies from 2 to 220 kilodaltons. The highest titers are possible with soluble or insoluble product in the cytoplasm with a median of 11 and 8.8 g/L. As expected,

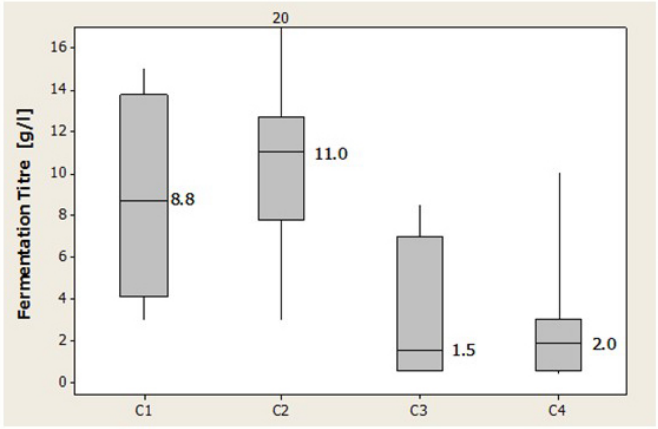
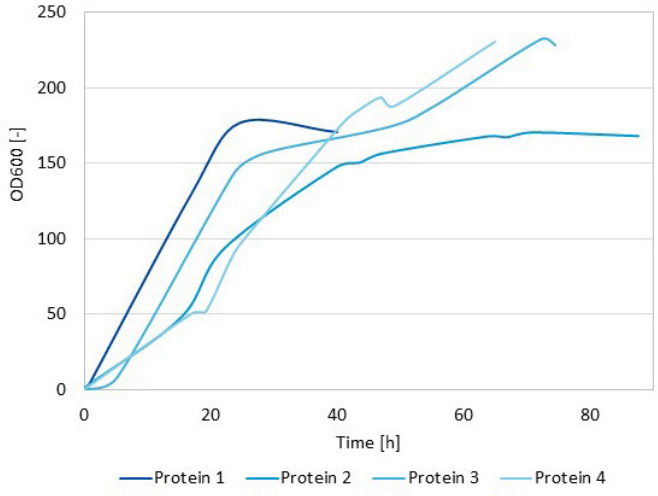
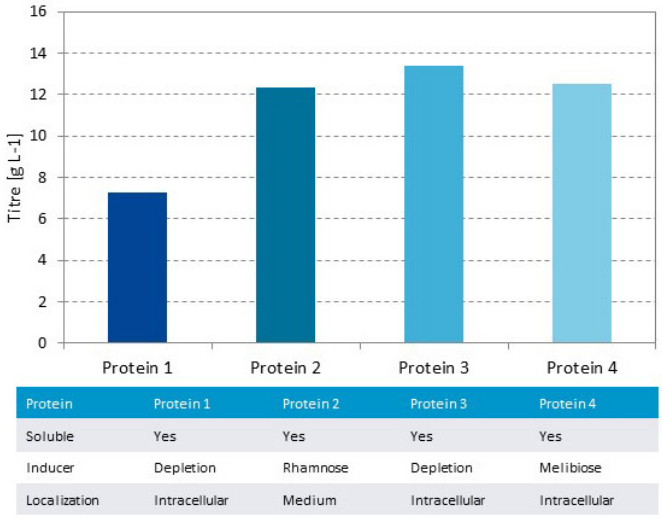


Figure 19: *High productivity of XS® E. coli, regardless of product class and molecular weight*

the periplasmic and the release titers are lower (between 2 and 1.5 g/L). This indicates XS E. coli delivers reasonable titers for a variety of simple to complex proteins.

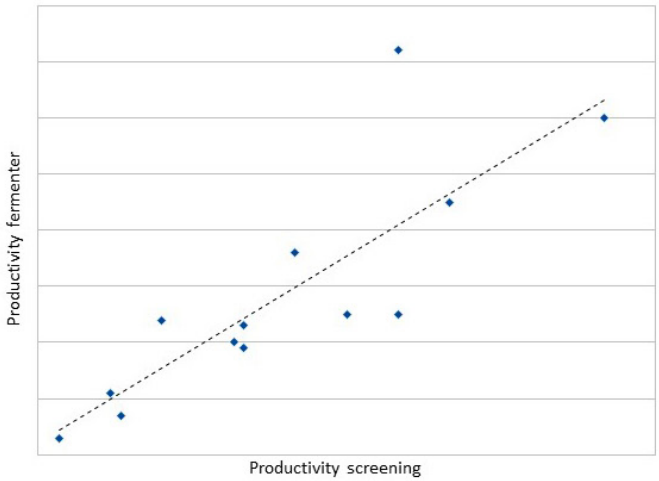
The need for combinatorial screening to maximize productivity is illustrated in Figure 20. The left graph shows fed-batch fermentation runs for four different E. coli strains, producing four different target proteins. The right graph shows the corresponding end-to-end of fermentation titers from 7 g/L to 13 g/L. This indicates the selected best strains vary in their genetic elements, like the best-performing promoter and means of induction.





Protein	Protein 1	Protein 2	Protein 3	Protein 4
Soluble	Yes	Yes	Yes	Yes
Inducer	Depletion	Rhamnose	Depletion	Melibiose
Localization	Intracellular	Medium	Intracellular	Intracellular

Figure 20: *High titer is dependent on selection of the right promoter/host/compartment*



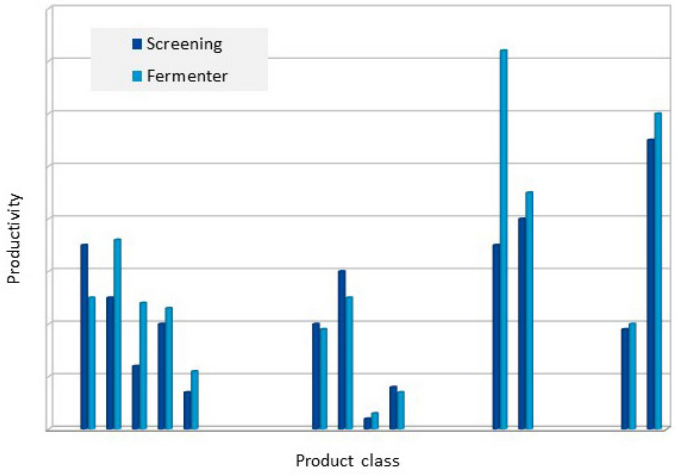


Figure 21: *Scalability from screening to small scale fermenters*

The standard vessel size for fed-batch fermentation runs in early development is between 250 milliliters and two liters. As shown on the left graph in Figure 21, there is a reasonable alignment between screening and fed-batch fermentation titers. The outlier may be an analytical issue, as most of the analytical methods during the feasibility phase are generic and not specifically adapted or optimized to the target protein; therefore, the variation might be higher.

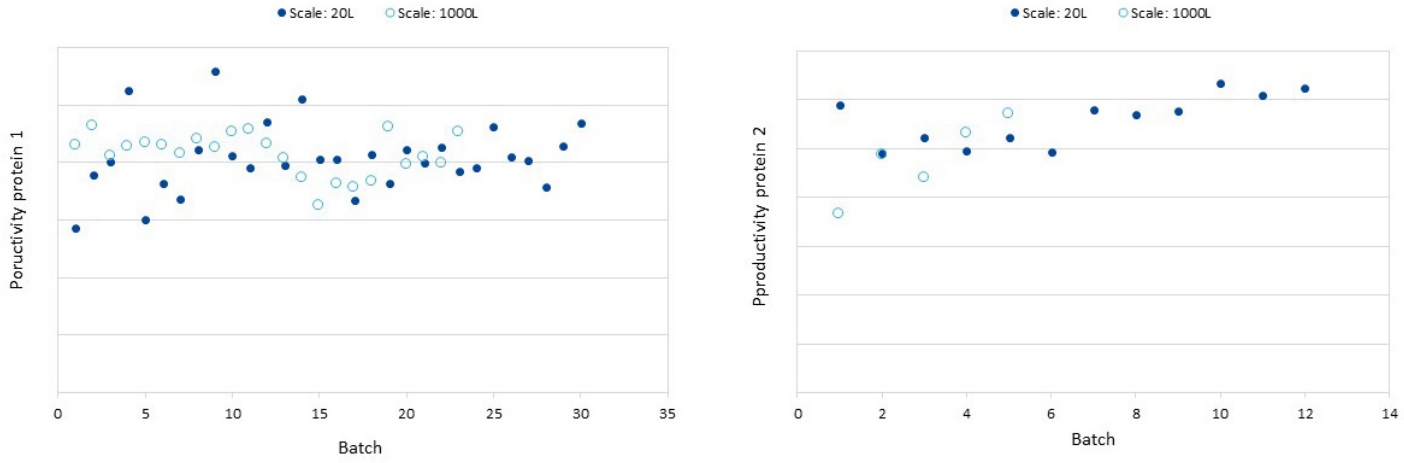


Figure 22: *Scalability from small-scale fermenter (20 liters) to at-scale process (1,000 liters)*

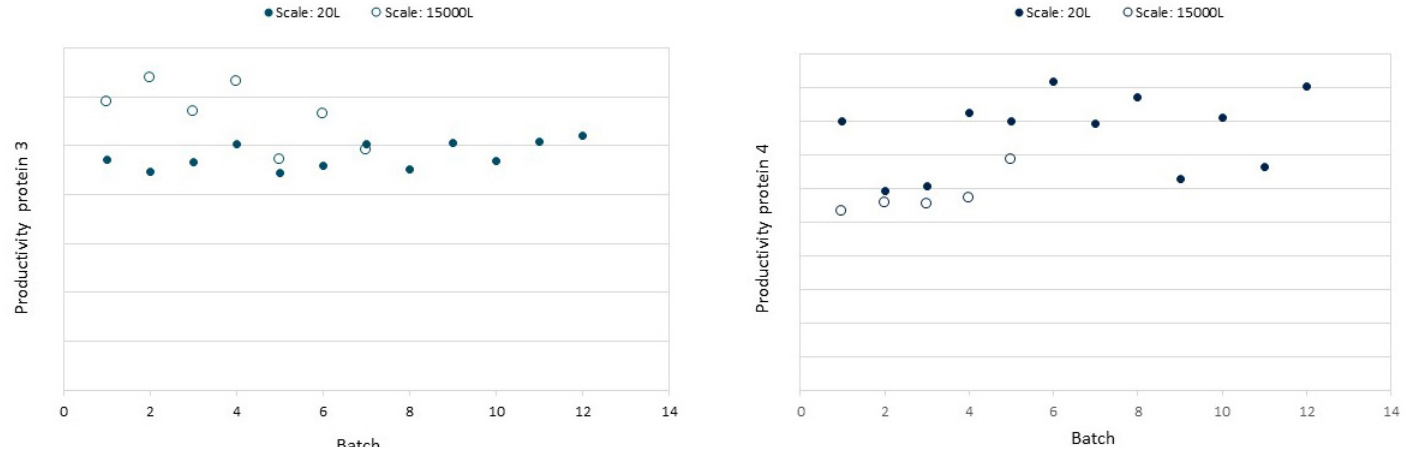


Figure 23: *Scalability from small-scale fermenter (20 liters) to at-scale process (15,000 liters)*

The correlation of screening and fermentation titer is suitable for all tested proteins, independent of the product class, as illustrated in the right chart in Figure 21.

Another mandatory aspect of development is scalability. Miniature bioreactors (250mL), 2L and 20L fermenters show similar performance data demonstrating the scalability of the expression system and process. The graphs in Figure 22 compare end of fermentation titers of 20-liter and a 1,000-liter fermentation batches for two target proteins.

The same is true for 20-liter end of fermentation titers compared to 15,000-liter batches.

Case Study 4: XS® Pichia Pastoris

This case study illustrates the benefits of Lonza's Pichia pastoris platform in a head-to-head comparison of Lonza's glucose-regulated Pichia pastoris expression system versus the state-of-the-art methanol-induced expression platform. The use of methanol creates several challenges, such as toxicity of methanol and its metabolites, the need for explosion-proof production

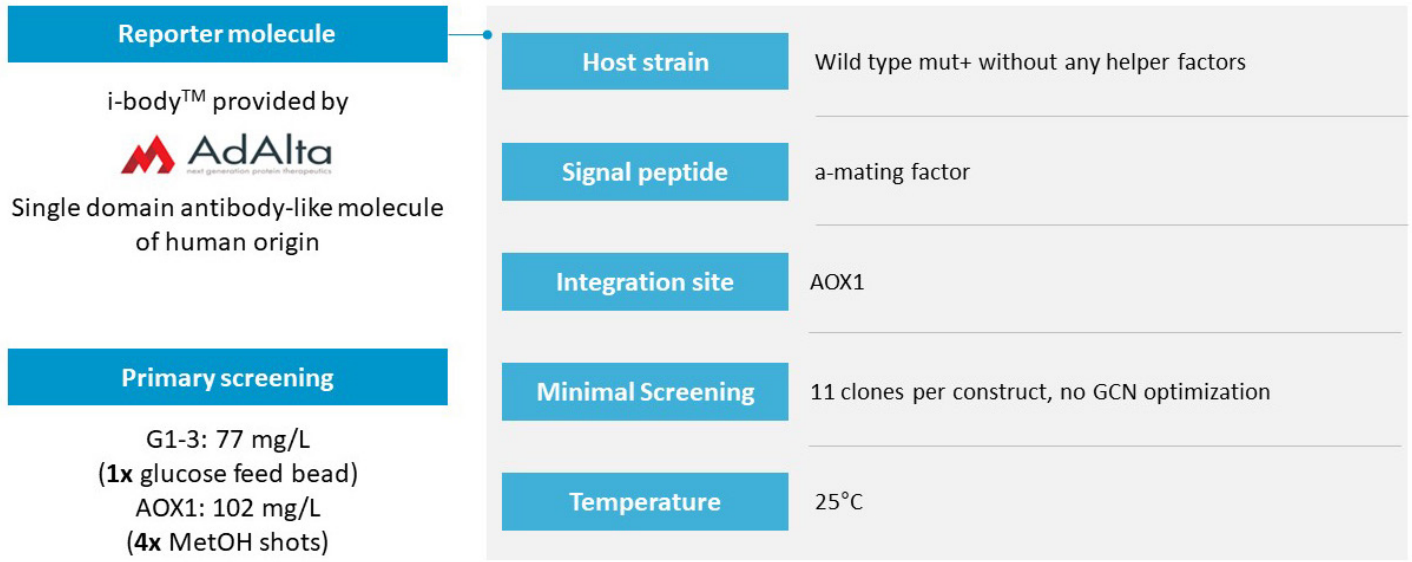


Figure 24: *G1-3 versus AOX1 case study*

facilities, long fermentation times, and low space-time yield. The details of the molecule and primary screening are outlined in Figure 24.

A comparison of the feed regimes for both glucose-regulated fermentation and methanol-based fermentation in Figure 25 shows the complexity of a typical methanol-induced fermentation strategy.

This process begins with a glycerol batch, followed by a glycerol fed batch to generate cell mass, and then a methanol shot is added to adapt the cells to the methanol. Once this is complete, you can begin the methanol fed batch. In comparison, the glucose regulated fermentation strategy requires only moving from glycerol batch to glucose fed batch to induce the G1 promoter.

For the case study, the G1-3 and AOX1 clones were used for fed-batch fermentation runs according to the illustrated feed regimes. In the same fermentation time, a slightly higher titer (4.3 g/L) for G1-3 was achieved compared to 3.6 grams per liter for AOX1 with a little lower dry-cell weight. To address the issue

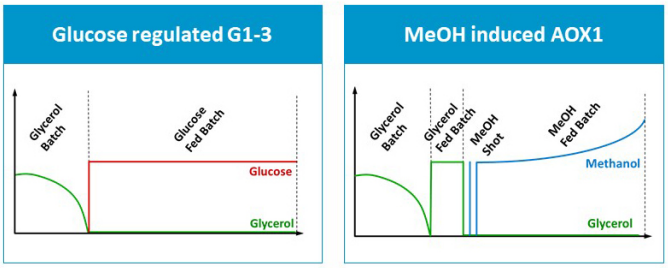
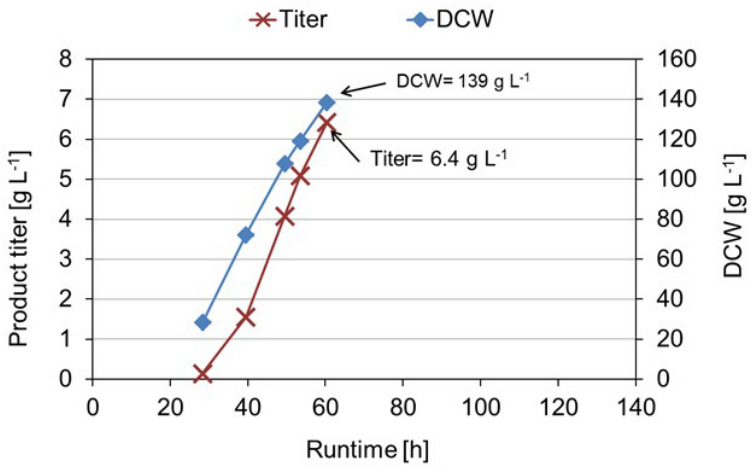


Figure 25: *Glucose-regulated fermentation versus methanol-induced fermentation*

of limited space-time yield during long fermentation time, Lonza developed a speed fermentation strategy with bacteria-like fermentation times. This is possible due to the induction properties of the G1-3 promoter with moderate promoter activity at high growth rates. This is not possible for the AOX1 promoter, as the promoter activity is negatively correlated to the growth rate. Therefore, longer fermentation times are needed for high productivity. The results of the speed fermentation are shown in Figure 26.

With the bacteria-like fermentation time, we could generate even higher titers compared to the standard fermentation process with 120 hours, meaning 6.4 grams



G1-3 Speed	result	AOX1
6.4	max. titer [g L^{-1}]	3.6
60 h	reached at	125 h
58	highest STY [mg L^{-1} h^{-1}]	21
2.3	highest q_P [mg g^{-1} h^{-1}]	0.6
139 g L^{-1}	cell density	109 g L^{-1}
80 x	increase of titer (vs. MTP)	35 x

Figure 26: *Speed fermentation maximizes space-time yield*

per liter compared to 3.6 grams per liter. The benefits of the strategy are obvious: bacteria-like fermentation time, high space-time yield, higher titer, and short product retention time in the supernatant, which means less risk for post translation modifications and a reasonable titer after two days of fermentation time.

SUMMARY

Lonza's mammalian and microbial expression technologies offer commercially viable systems that cover the diversity of complex proteins and are suitable for large-scale development. The combinatorial screening uses a broad range of genetic elements that mitigate the lack of predictability for both quantity and quality of your target protein. This, combined with extensive experience in establishing customized product-specific assay methods that aid in cell line decision, gives you the tools you need to be successful in a biopharmaceutical landscape that presents both new challenges as well as exciting opportunities.