Non-viral adeno-associated virus-based platform for stable expression of antibody combination therapeutics

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Antibody combination therapeutics (ACTs) are polyvalent biopharmaceuticals that are uniquely suited for the control of complex diseases, including antibiotic resistant infectious diseases, autoimmune disorders and cancers. However, ACTs also represent a distinct manufacturing challenge because the independent manufacture and subsequent mixing of monoclonal antibodies quickly becomes cost prohibitive as more complex mixtures are envisioned. We have developed a virus-free recombinant protein expression platform based on adeno-associated viral (AAV) elements that is capable of rapid and consistent production of complex antibody mixtures in a single batch format. Using both multiplexed immunoassays and cation exchange (CIEX) chromatography, cell culture supernatants generated using our system were assessed for stability of expression and ratios of the component antibodies over time. Cultures expressing combinations of three to ten antibodies maintained consistent expression levels and stable ratios of component antibodies for at least 60 days. Cultures showed remarkable reproducibility following cell banking, and AAV-based cultures showed higher stability and productivity than non-AAV based cultures. Therefore, this non-viral AAV-based expression platform represents a predictable, reproducible, quick and cost effective method to manufacture or quickly produce for preclinical testing recombinant antibody combination therapies and other recombinant protein mixtures.

Introduction

The global market for monoclonal antibody (mAb) therapies is currently ~$50 billion a year, and the biopharmaceutical industry has many powerful novel mAbs in the clinical pipeline. However, for many indications a mAb alone is insufficient to achieve a significant and durable response. Therefore, several companies have initiated clinical trials utilizing biclonal and triclonal combinations of antibodies for diseases against which mAb therapy has been ineffective. Recent clinical trials on mixed mAb products include treatments for cancer, and for infectious diseases such as rabies, C. difficile, and botulism. Preclinical studies suggest that three or more antibodies are often necessary to achieve the optimal therapeutic effect. Increasing the complexity and diversity of the antibody combination can improve the therapeutic benefit by many different mechanisms of action. Effective treatment may require blockade or modulation of multiple pathways, e.g., as in the case of infection with a bacterium such as Staphylococcus aureus expressing numerous virulence factors, or development of autoimmune disorders or cancer, where redundant pathways promote pathology. Similarly, some viruses, such as HIV, SARS, and influenza, show large degrees of antigenic diversity that require the combination of broadly neutralizing antibodies to effectively protect against different strains and prevent escape mutants. Indeed, a recent preclinical study comparing mono-, tri-, and penta-therapy with broadly neutralizing antibodies to different HIV epitopes in a humanized mouse model found that only the five antibody mix could prevent escape mutants and control viremia for the entire course of treatment.
In many cases, combinations of multiple antibodies have been shown to function synergistically, not additively, to achieve results that are not possible with mAbs alone, while allowing for a lower minimal effective dose. For example, antibodies that bind multiple epitopes can act synergistically to crosslink the signaling molecule epidermal growth factor receptor in cancer cells, or bind the highly potent botulinum toxin with sufficiently high affinity for neutralization. Some diseases are still treated with hyperimmune products, such as human or equine immunoglobulin products for rabies and botulism. These biological products are often effective, but can be difficult to obtain, inconsistent, and lead to serum sickness. Mounting data confirms that antibody therapeutics are most efficacious when mAbs are strategically combined, mimicking the polyclonal response of the human immune system.

Although there is growing acceptance of the effectiveness of the antibody combination therapeutic (ACT) approach, the cost of producing such complex antibody products using standard antibody manufacturing technology remains a substantial barrier. Because many of the current clinical trials are focused on combinations of mAbs previously approved for therapeutic use, these products are being manufactured using traditional methodology. Although manageable for small mixtures of antibodies that have already been independently developed, this approach is cost-prohibitive for larger mixtures, as the costs for cell line development and Chemistry, Manufacturing, and Control (CMC) for each antibody are substantial and additive. There are few alternatives for developing and producing larger mixtures of antibodies. Merus’ Oligoclonic™ system allows expression of multiple antibodies in a single cell line, but they must all share a common light chain, which limits the flexibility of the system. Many companies have attempted to address this issue by producing single antibody-like molecules that recognize multiple antigenic targets, such as bispecific or trispecific antibodies. Although these systems allow the cost-effective manufacture of multi-specific antibody-like molecules, these products may lack the stability of natural human antibodies. Symphogen has attempted to address these issues by developing single batch expression systems involving site specific integration (Sympress I) or random integration (Sympress II) that can produce polyclonal antibody mixtures in a single polyclonal cell bank. Using the Sympress I system, Symphogen has brought a 25 antibody mix to Phase 2 clinical trials, thereby establishing a regulatory path for complex combinations of human antibodies.

Despite the progress made in this area to date, the relative stability of expression levels of each component antibody in a single-batch, mixed antibody expression system remains a primary concern. Random integration of the antibody expression cassette into the host cell genome can have deleterious effects on host cells, either slowing or accelerating growth rates, and leading to silencing of protein expression over time. Clonal selection of lines exhibiting exceptional protein expression levels can exacerbate this effect. In contrast, single site integration yields only one copy of the expression cassette per cell resulting in low expression levels, as in Sympress I. This can be overcome by amplifying the integrated cassette, e.g., amplification of dihydrofolate reductase in Chinese hamster ovary cells (CHO), but this leads to instability through genomic rearrangements of the repeats. Techniques to screen for specific sites in the genome that lead to high stable expression, such as screening FLP recombinase sites, or fluorescence activated cell sorting (FACS) of the highest expressing cells may also help to isolate lines that are both stable and high expressing. The process of selecting clonal cell lines with matched doubling times and sufficient stability of expression for single batch mixed antibody production, particularly those derived by random integration, complicates and extends the cell line development process, resulting in longer timelines and higher costs.

To address the challenge of manufacturing ACTs, we developed an adeno-associated virus (AAV)-based methodology that utilizes viral inverted terminal repeats (ITRs) and the Rep78 integrase, but not the virus itself. Adeno-associated virus (AAV) is a non-pathogenic virus that, in the absence of a helper virus, establishes latency by integrating into sequence-specific genomic sites. Originally, only one integration site on chromosome 19, termed AAVS1, was identified. However, while AAVS1 integrants can make up between 10 and 45% of the total integration events, studies have since shown that AAV integrates at a number of “hotspots” throughout the genome, and that these spots correlate with regions of open chromatin, active genes, CpG islands, and high levels of gene expression. Integration sites throughout the genome correlate with binding sites for the viral integrase, Rep78 (or the shorter splice variant Rep68). The minimal requirements for integration are the viral ITR elements and one of the Rep proteins. An element from the p5 promoter (termed the p5 integration efficiency element or p5IEE) that drives Rep expression in the virus has also been reported to increase integration efficiency in some cases.

Using a non-viral AAV system, we developed a single batch expression platform for producing consistent and reproducible ACTs. Stable pools of cells, each expressing a single antibody, are generated and mixed, without a clonal selection step. We have found that multi-antibody cell cultures from these mixed stable pools of cells exhibit extremely stable expression ratios, with no more than eight percent average variability from the mean per antibody over eight weeks in culture. The batch-to-batch variability from different vials and between replicate cultures is even lower. These non-viral AAV based antibody combination cultures are four times more stable and four times more productive than mixed cultures made by random integration.

Results

Non-viral AAV based system for generation of stable pools of antibody expressing cells

We produced stable pools of antibody-expressing cells by co-transfecting HEK 293 cells with a heavy and light chain antibody expression plasmid, flanked by viral ITRs and the p5IEE (pExcel ITRp5), and a Rep78 expressing plasmid (pRep78low) (Fig. 1A). This is a non-viral system and, therefore, there are no packaging constraints on the size of the DNA cassettes between the ITRs. The Rep78 plasmid (pRep78low) has been optimized to express at
low levels through mutation of the promoter and Kozak sequences. This allows for the correct balance of maximal Rep78 integrase activity per cell while preventing the toxicity caused by high levels of Rep78 expression.30 The ratio of pExcel ITRp5 and pRep78low were titrated and a 1:1 ratio resulted in the highest percentage of stably transfected cells (data not shown). To determine the percentage of cells that stably maintained the antibody cassette, we replaced the heavy chain with green fluorescent protein (GFP), co-transfected the plasmids, and followed GFP expression by FACS until expression had levelled off and was stable for at least three time-points. Creation of stable pools of transfected cells was determined to be dependent both on the viral ITR sequences and on the presence of Rep78 (Fig. 1B). Stable expression occurred in 10% of the cells. In contrast, if no viral elements are included on the GFP/antibody expressing plasmid or if no Rep78 is co-transfected, only a small background percentage of cells, ~1%, stably express GFP.

Stable pools of HEK 293 cells expressing four different antibodies were created using our AAV-based system or random integration. Varying degrees of intrinsic productivity were observed for the individual antibodies and are attributed to their different sequences. For the random integration condition, the viral ITRs and p51EE element were removed from the antibody expression vector. The cells were selected in the presence of neomycin until viability of the culture recovered to at least 95%. Cells were banked and thawed, and productivity was measured for two weeks. Antibody concentrations from cell culture supernatants were determined by Luminex® immunoassays, and specific productivities (pg/cell/day) were calculated as described in the Methods. Error bars represent the standard deviation between measurements at two time points.

Figure 1. Non-Viral AAV based single antibody stable pools. (A) Vectors for non-viral AAV expression system. Antibody expression cassettes and a neomycin selection cassette are flanked by AAV ITRs and the p51EE (pExcel ITRp5). This vector is co-transfected into mammalian cell lines with a plasmid expressing an optimized low level of Rep78 (pRep78low), and stable pools of cells are selected by culture with geneticin. See Methods section for details of vector elements and abbreviations. (B) Optimized AAV-based stable expression is ITR and Rep dependent. Freestyle 293-F cells were transfected with only the antibody expression plasmid with GFP in place of the heavy chain (-Rep) or co-transfected with pRep78low (+Rep). The antibody expression plasmids either contained viral ITR and p51EE elements (+ ITR and p51EE; pExcel ITRp5 GFP) or lacked the viral elements (- ITR and p51EE; pExcel GFP). Data shown are the average percent GFP positive cells as determined by FACS analysis from three time-points of duplicate cultures after transient expression was lost. Error bars represent the standard deviation between two cultures. (C) Single antibody stable pool cultures generated by non-viral AAV based integration express at higher levels than those generated by random integration. The non-viral AAV based pools were generated as described in the Methods section, and the random stable pools were generated identically except that the antibody plasmid did not contain the viral ITRs or p51EE sequences (pExcel) and was not co-transfected with pRep78low. Frozen vials of AAV-based or random stable pools were thawed and cultured for two weeks. Antibody concentrations from cell culture supernatants were determined by Luminex® immunoassays, and specific productivities (pg/cell/day) were calculated as described in the Methods. Error bars represent the standard deviation between measurements at two time points.
elements, ubiquitous chromatin opening elements, and stringent selection, can be introduced into this system to further enhance productivity. Individual antibody stable pools created with the ACT system were cultured for up to 17 wk with no decrease in productivity (data not shown).

**Production and stability of ACT cultures**

We tested the feasibility of batch-culturing antibody combinations using the ACT expression platform. Stable pools of cells expressing a single antibody were created using the non-viral AAV based system, as described in the methods, for five different antibodies with different antigen specificities, as measured by Luminex® immunoassays, and distinct cation exchange (CIEX) retention times and elution profiles, as measured by high performance liquid chromatography (HPLC). The individual stable pools were banked, thawed and equal numbers of cells from each individual stable pool were mixed together to create an ACT culture expressing all five antibodies, at levels relative to their intrinsic expression levels. This ACT culture was then frozen, thawed, and monitored in culture for nine weeks.

Antibody expression from these ACT cultures remained stable for the duration of the experiments. Total antibody expression remained stable for 10 independent parallel cultures (Fig. 2A). Ratios of the individual antibodies in the mix also remained constant. Data from three replicate cultures are shown in Figure 2B. The average variability from the mean for all of the antibodies in the mix was 7.3 ± 1.3% over the course of nine weeks (Table 1). The presence of each antibody in the mix was measured both by Luminex® immunoassays (Fig. 2B) and by quantitation based on CIEX HPLC antibody peak areas (Fig. 2C). Data from these two techniques were complementary.

To demonstrate reproducibility for reliable manufacturing, ten different vials of the combined antibody cell bank were thawed at two different times. The average expression of each antibody in the mix over nine weeks was similar for all thawed

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**Figure 2.** Long-term stability of ACT cultures. Stable pools of cells expressing five different antibodies were mixed and co-cultured. Antibody expression was measured from the cell culture supernatants by Luminex® immunoassays and CIEX chromatography. (A) Fc production is stable for ACT cultures. Data represent ten replicate vials of an ACT composed of five antibodies. Specific productivities (pg/cell/day) were calculated as described in the Methods. Error bars represent the standard deviation from the mean of the 10 cultures. (B) Stability over nine weeks of three replicate vials. Data shown are the percent of each antibody reactivity relative to the sum of all antibody reactivities as measured by Luminex® immunoassays. Error bars represent the standard deviation from the mean of the three cultures. (C) Representative CIEX chromatograms from the beginning, middle, and end of the time course. The chromatogram peaks were assigned by comparison to single antibody expressing cultures analyzed using the same HPLC conditions and method. (D) Stability of 10 replicate cultures over nine weeks. Data shown are the percent of each antibody reactivity relative to the sum of all antibody reactivities as measured by Luminex® immunoassays, where each bar represents a single culture. The error bars represent the standard deviation of Luminex® measurements of the percent of each antibody in the mixture over a nine week period.
vials, and the average deviation from the vial mean for all of the antibodies in the mix was 4.9 ± 0.3% (Fig. 2D; Table 1).

**Production of different and complex ACT cultures**

We tested our system with a more complex mix consisting of 10 different antibodies. Four vials of the ACT cell bank, created as described in the methods, were thawed and cultured for eight weeks. Because antibody-specific immunoassays were not available for all of the antibodies in the mix, most of the antibodies were quantified by CIEX chromatography (representative peaks shown in Figure 3A). Nine of the 10 antibodies could be distinguished clearly in the HPLC analysis, and quantified based on antibody peak areas, as shown in Figure 3B. The tenth antibody (antibody J) did not form a unique peak on the CIEIX-HPLC chromatogram, but was monitored by reactivity against a specific antigen using Luminex® immunoassays (Fig. 3C). Individual antibody expression levels varied from the mean by 5.9 ± 0.9% over the course of the experiment, showing that even complex mixtures of antibodies can be maintained in this system (Table 1). In this experiment, one antibody was observed to begin to outgrow the culture, resulting in ratio skewing beyond 9 wk. We believe this to be a rare phenomenon in this system, given that we observed stability of antibody ratios in all subsequent experiments over culture periods ranging from 10–12 wk (Figs. 4 and 5).

**Reproducibility of ACT cultures**

To further investigate whether the system is reproducible enough to allow for scale up and manufacturing, we generated another three antibody ACT from two independent sets of transfections. Two stock vials of each ACT were thawed and cultured in duplicate, leading to eight cultures that were followed for ten weeks (Fig. 4A). The two independently generated ACTs have similar ratios of the three antibodies and the ratios remain consistent over the course of the ten weeks (Fig. 4B and 4C). Both replicate vials and replicate cultures also contained similar ratios of the three antibodies with only 1.2 ± 0.4% variability from the mean between replicate vials (Fig. 4D; Table 1).

**Comparison of non-viral AAV-based system to random integration**

We compared the stability of antibody combinations from AAV-based stable pools and stable pools generated by random integration. Antibody banks from stable pools, each containing three different antibodies, were created by either the non-viral AAV based method, using the optimized plasmid pRep78low, or by random integration. Random integration was achieved using identical vectors lacking the viral elements in the absence of co-transfection with Rep78low. Two independent antibody combination banks were made for both the random and AAV-based methods. Two stock vials of each of the mixes were thawed and cultured for 12 wk. The stability of the antibodies in the mixtures was measured by multiplexed immunoassays (Fig. 5) and CIEX HPLC (data not shown). The antibody ratios of the AAV-based cultures remained consistent over the 12 wk time-course, with less than 2% variability from the mean per antibody (Fig. 5A). In contrast, the randomly generated cell banks were less consistent, with up to 9% variability from the mean of each antibody in the identical mix over the same time period (Fig. 5B). To confirm the reproducibility of the stability of these AAV-based ACT cultures, we thawed four more stock vials at two different times and measured the stability for 12 wk and six weeks, respectively. The remarkable stability of each antibody over time (an average deviation of 3.9 ± 0.7% from the culture mean), and the remarkable reproducibility from vial to vial (an average deviation of 2.0 ± 0.6% from the vial mean) for these cultures is shown in Figure 5C and Table 1.

**ACT Expression System**

The single antibody stable pool cultures created by co-transfection of pRep78low and pExcel ITRp5 antibody-containing plasmids are the first step in our ACT Expression system (described in Figure 6). Single antibody stable cell banks are then created and a representative vial of each cell bank is thawed for productivity assessment. Although the mixtures shown in Figures 2–5 were created by mixing equal cell numbers, the productivity measurement allows the creation of ACT cultures with specific ratios of the component antibodies to be generated by varying the cell numbers added to the starting culture (data not shown). ACT cultures are expanded and master cell banks are created. For commercial production, ACT technology or cell banks would be transferred to a cGMP facility for the creation of master and working cell banks and scale up to production bioreactors.

**Discussion**

As the pharmaceutical industry begins to embrace the idea of creating more effective therapeutics through combinations of antibodies, the problem of how to develop and cost-effectively produce these mixtures becomes increasingly urgent. The
traditional method of combining separately produced mAbs will be cost-prohibitive for mixtures larger than a few antibodies and will likely substantially hinder the development of this class of therapeutics. We propose that a single batch expression system with the flexibility and reproducibility described here will make the cost of developing such complex biologic products competitive with that of other biopharmaceuticals. To test this hypothesis, we modeled the cost of developing mixtures consisting of two to ten antibodies, both as monoclonals mixed after separate production and purification, which requires a number of expensive processes to be performed multiple times, and as an ACT produced in our single batch process. Assuming comparable levels of expression, we found that while the cost of developing an ACT is higher than a single mAb due to the need for more complex analytical testing, the cost of an ACT increases only slightly for each additional antibody, such that even a two antibody ACT is less expensive than two mAbs developed in parallel (data not shown). Other analyses have come to similar conclusions. These early cost savings will be particularly important for the development of larger complex mixtures of antibodies and will also allow for testing of multiple preclinical combinations of antibodies, opening the door for the widespread application of complex antibody-based therapeutics to a diverse range of disease processes.

Stability and reproducibility of antibody composition is a major consideration when creating antibody combinations in single batch culture. First, the proportion of each antibody in the mixture must remain constant throughout the culture process to ensure flexibility and predictability in manufacturing. The composition of the mixture must also be reproducible between different cell bank vials to ensure production of a consistent and predictable therapeutic. The acceptable limits for variation of single antibodies within a multi-antibody mixture have yet to be established. The Sympress I system used by Symphogen for the production of a 25 antibody mix that completed a Phase 2 clinical trial has a published batch-to-batch variation of up to 20% for most antibodies in the mixture. Newer generations of the Symphogen technology show improved reproducibility of the final product between batches of the same size, but little quantitative data are available on the stability of expression of individual antibodies in the mixture over time. Reproducibility of the final product will also depend on the development of downstream processing methodologies that allow for the co-purification and co-formulation of all of the antibodies in the ACT in a reproducible manner. Others have found that complex antibody mixtures can be purified using standard techniques with minimal disruption of the relative antibody ratios.

The ACT expression system described here maintains highly consistent antibody ratios in culture, which is required for manufacture of a highly reproducible antibody combination product. More than 28 independent ACT cultures of 18 different antibodies in four different combinations have been cultured with an average percent deviation from the mean of 4.5 ± 1.6% over 60 doublings (see Table 1 for more details). This number of generations is within the range necessary to bank the ACT cell pools, and expand a seed train into a 10,000 L production bioreactor, and some cultures were monitored for up to 85 doublings with no sign of trending changes in the composition (Fig. 5). In addition, separate thaws from banked cells produced nearly identical expression profiles, with an average of 2.8 ± 1.5% variability from the mean expression of each antibody in replicate vials, thereby demonstrating the reproducibility of ACT antibody ratios from batch to batch. These observations suggest that a consistent

Figure 3. Stability of complex mixtures composed of 10 antibodies. (A) Sample CIEX chromatograms used for antibody quantitation. The antibody peaks were labeled by comparison with cell culture supernatants from cultures expressing single antibodies using the same HPLC conditions and method. (B) Stability of nine antibodies from the 10 antibody ACT over an eight week period. Data represent percent of each antibody relative to the sum of all antibodies as determined by integration of the area under the curve from the CIEX profiles. Error bars represent the standard deviation from four different thawed vials. (C) The tenth antibody (antibody J) was undetectable by CIEX chromatography, but was measurable by antigen-specific Luminex® immunoassay. The percent of the total Fc in the culture is graphed, and the error bars represent the average of four replicate cultures.
composition of antibodies can be maintained through scale-up and manufacture of an ACT product.

A crucial component of the stability of the ACT expression platform is the non-viral AAV-based integration of the antibody heavy and light chains. This system biases integration toward sites of open chromatin that favor high expression and genomic stability compared with transfection techniques that integrate randomly. As a result, antibody mixtures generated using the AAV-based system are both more highly expressed and more stable in co-culture than mixtures generated using random integration methods (Figs. 1 and 5). Although Rep78 is currently provided as plasmid DNA, Rep78 could also be provided as RNA or protein, if integration of the DNA and the potential for sustained, low level expression of Rep78 were of concern. Additionally, the non-viral system allows more flexibility than comparable viral systems, as the lack of packaging restrictions means that a construct of virtually any size can be inserted within the viral ITRs. As AAV has evolved to integrate into the human genome, the studies presented here have utilized a HEK 293 cell line, but preliminary data suggests that this system will also achieve an acceptable percentage of stable integration in CHO cells.

Another unique feature of the ACT expression system is the use of stable pools rather than clonal cell lines. The use of stable pools reduces the overall time from transfection to production by eliminating the time-consuming (3 to 12 mo) and expensive clonal selection process. Clonal cell lines have historically been developed for mAb production in order to achieve commercially viable expression levels. However, the highest expressing clonal lines are usually unstable and their expression level typically drops throughout the course of production. While this level of instability may be tolerated when producing a mAb, it is not suitable for batch production of antibody mixtures where stability of the antibody ratios is critical. We have found that the use of stable pools leads to a highly predictable final combination antibody product, which has previously been much more challenging with mixed clonal cell lines. Though perhaps counterintuitive, this effect is likely achieved by virtue of the complex population dynamics of a culture composed of mixed stable pools. A population of cells that is sufficiently large will have a distribution of growth rates and antibody production levels, each tending toward a mean, as a consequence of having multiple integration sites and copy numbers. Any instability created by the integration of the expression cassette into a specific site in a single cell, which leads to a change in expression or growth rate in that cell, will be buffered by the rest of the population, such that overall antibody composition will not be affected. In contrast, in clonally selected lines, composed entirely of cells having the same integration site, copy number and growth characteristics, the consequences of such genomic instability on antibody expression or growth properties of the cells will be amplified greatly, as this instability will
be present in all cells in the culture. Indeed, this is often observed in clonally derived antibody-producing cell lines reported in the literature.\textsuperscript{20,21} In the absence of extensive screening, others have found that mixing clonal cell lines leads to a highly unstable final antibody product with antibodies showing up to 80\% variability from the mean over five weeks in culture.\textsuperscript{17} We have found that mixing AAV-generated stable pools together leads to a consistent final product, with antibodies showing an average of 4.5 ± 1.6\% variability from the mean over eight weeks in culture (Table 1). The assumptions that led to the adoption of a clonal selection approach for the consistent manufacture of mAb therapeutic products are not necessarily applicable to more complex antibody combination products, where the relative ratios of the component antibodies is of primary importance. Our results suggest that it is time to revisit the idea that the best and least expensive way to manufacture a reproducible product is through the use of a clonal cell line. Adoption of a mixed stable pools approach will make possible reliable and cost-effective production of ACTs.

To summarize, the ACT expression system represents a novel approach to antibody production that provides a practical solution for the development of antibody combination products. Two critical innovations, a non-viral AAV-based integration system and the use of stable pools of cells rather than clonal cell lines, contribute to the robustness of this technology for consistent manufacturing of an antibody combination product. This system is characterized by remarkable stability over multiple cell doublings and high batch-to-batch reproducibility. We believe that this technology will make possible a new generation of superior antibody therapies.

\textbf{Materials and Methods}

\textbf{Vector Construction}

The antibody expression vector pExcel ITRp5 (Fig. 1A) contains the inverted terminal repeats (ITRs) of AAV2 (derived from VPK-415-SER2; Cell Biolabs, Inc.), ColE1 bacterial origin (Ori), p5 Integration Efficiency Element (p5IEE)\textsuperscript{40} of AAV2, Neomycin/Kanamycin (Neo/Kan) gene, human cytomegalovirus immediate-early enhancer and human ferritin light chain promoter (CMV/hFerL prom), human \(\beta\) globin polyadenylation signal (\(\beta\) Globin pA), human (hEF1\(\alpha\) prom), and rat \(\beta\) globin polyadenylation signal (rEF1\(\alpha\) prom), bovine growth hormone polyadenylation sequence (BGH pA), and SV40 polyadenylation sequence (SV40 pA). Antibodies are preceded by secretory leader sequences Vh5–51 and Kappa 2–40*01 for heavy and light antibody chains, respectively. The vector contains the cDNA of the IGHG1*01 heavy chain constant region (IgG1 constant). pExcel (used for random integration in Figures 1C and 5B) is identical, but lacks the ITRs and p5IEE. For the experiments in Figure 1B, the heavy chains in the pExcel ITRp5 and pExcel vectors were replaced by GFP to make the vectors pExcel ITRp5 GFP and pExcel GFP.

For Rep78 expression, the pRep78 expression vector contains the Rep78 gene (derived from VPK-415-SER2; Cell Biolabs), Simian Virus 40 promoter (SV40 prom), SV40 late polyadenylation signal (SV40 pA) and the SV40 enhancer in a pUC19 backbone. To create pRep78low (Fig. 1A) from pRep78, the SV40 late polyadenylation signal was then replaced with early polyadenylation signal, the enhancer was removed, the Kozak sequence was mutated to TGTTCT (Mutated Kozak), and in the promoter a
21bp deletion of a repeat (tccgccccct aacctccgccc a) was made by PCR mutagenesis. Amp = Ampicillin resistance gene.

**Assay for Rep-dependent Stable Expression**

Freestyle™ 293-F cells (Life Technologies) were transfected according to manufacturer’s recommendations with varying ratios of pExcel ITRp5 GFP or pExcel GFP and pRep78low. Cells were subcultured in FreeStyle™ 293 Expression Media (Life Technologies) without a selective agent, and GFP expression was monitored biweekly by FACS analysis using a Guava easyCyte 8HT flow cytometer (EMD Millipore) until the percent of GFP positive cells remained consistent for three consecutive time points, indicating loss of non- or unstably integrated expression.

**Single Antibody Stable Pool Generation**

Freestyle™ 293-F suspension cells were co-transfected with an antibody expression vector (pExcel ITRp5) and a Rep78 expression vector [pRep78 (Figs. 2 and 3) or pRep78low (Figs. 4 and 5)] as per manufacturer’s instructions (Life Technologies). Stable pools of cells expressing the desired antibodies were selected by subculturing in FreeStyle™ 293 Expression Media (Life Technologies) containing the appropriate selective agent (500 μg/ml geneticin®; Life Technologies) for 25–30 d or until the cell viability was above 95%. Cells were passaged once in the absence of the selective agent prior to freezing aliquots of stable pools for each antibody of interest, and selective drugs were not included in any subsequent subcultures. One representative vial of cells was thawed for each antibody and cultured for two weeks to assess antibody expression and reactivity by Luminex® immunoassays using a FlexMap 3D (Luminex®) and antibody expression by CIEX chromatography using a Dionex Ultimate 3000 HPLC system (Thermo Scientific).

**ACT Culture Generation**

To generate ACT cultures, vials of single antibody stable pools were thawed and passaged twice prior to combining the single antibody stable pools at a desired ratio for a final viable cell density of $1.0 \times 10^5$ cells/ml. Total and viable cell counts were determined using a TC10™ automated cell counter (BioRad). After 24hrs, the ACT was subcultured to a viable cell density of $2.0 \times 10^5$ cells/ml. Vials of ACT cells were banked five days after combining.

**ACT Subculturating and Sample Collection**

To assess antibody expression and stability, ACT cell banks were thawed and subcultured at a viable cell density of $2–3 \times 10^5$ cells/ml twice per week for 8–12 wk. For one subculture per week, samples of ACT culture supernatant were collected by removing 5 mls of cell culture, and pelleting cells at 100xg for 5 min at room temperature (RT) in a Sorvall Legend XTR (Thermo Scientific). ACT culture supernatants were transferred into 1.2 ml Sample Library Tubes (VWR) or 1.4 ml Matrix storage tubes and boxes (Thermo Scientific), snap frozen in liquid nitrogen and stored at $-80 \ ^\circ C$.

**Luminex® Immunoassays**

Antibodies or antigens were coupled to MagPlex beads (Luminex®) using the xMAP Antibody Coupling Kit (Luminex®) as per manufacturer’s instructions. For Fc quantitation, the antibody

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**Figure 6. ACT Expression System** Single antibody stable pools are created by co-transfection of pRep78low and pExcel ITRp5 antibody containing plasmids (Fig. 1). These cultures are frozen as single antibody stable pool cell banks, thawed, and productivity for each stable pool is measured. Using the productivity data, single antibody cultures are mixed into one ACT culture containing cells expressing all of the antibodies in the desired combination at the desired concentrations. Master cell banks are made from the ACT cultures and frozen. These cell banks will be transferred to a cGMP facility for the creation of working cell banks for scale up to production bioreactors to create the ACT product.
coupled to MagPlex beads was AffiniPure Goat Anti-Human IgG, Fc Fragment Specific (Jackson ImmunoResearch). For antigen-specific reactivity assessment, appropriate antigens were coupled for each antibody. For positive controls, antibodies were expressed in Freestyle™ 293-F suspension cells (Life Technologies) and purified using HiTrap MabSelect SuRe (GE Healthcare Life Sciences) columns on a BioLogic Duoflow-10 system (BioRad).

Luminex® immunoassays were performed according to manufacturer’s instructions with the following additional specifications. Standard curves were prepared by diluting Fc standard antibody (ChromoPure Human IgG, Jackson ImmunoResearch) or antigen-specific antibodies from 128 ng/ml to 0.5 ng/ml in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA; Sigma Aldrich). Cell culture supernatants were diluted in PBS containing 1% BSA to 1/200 and 1/2000 dilutions for Fc quantitation and antigen reactivity analysis. Biotin-SP-conjugated AffiniPure F(ab′)2 fragment goat anti-human IgG Fc specific antibody (Jackson ImmunoResearch) was used to detect human antibodies. For detection, MagPlex beads were incubated with Streptavidin, R-phycocerythrin conjugate (SAPE; Life Technologies) at a final concentration of 2 µg/ml. Beads were analyzed using a FlexMap 3D and Xponent software (Luminex®) using a 4P logistic analysis.

Specific Productivity Calculations

Specific productivity for ACT pools was calculated as described by Adams.41

Cation Exchange (CIEX) Chromatography

For analysis of the ten antibody combination (Fig. 3), antibodies were purified using PreDicator MabSelect 96-well plates (GE Healthcare Life Sciences). The antibodies were eluted using 100 mM Na-Citrate, pH 3.0 into collection plates containing 1 M Tris, pH 9.0. Other samples were not purified prior to analysis.

CIEX chromatography was performed on an Ultimate3000 HPLC system, and Chromatleon software was used for HPLC control and data analysis (both system and software from Thermo Scientific). The cation exchange column (TSKgel-CM STAT, 4.6 × 150 mm, TOSOH Bioscience, Japan) was equilibrated with 20 mM MES, 50 mM NaCl and antibodies were eluted by a linear gradient of NaCl from 50 to 500 mM. The column temperature was 35 °C and detection was at 280 nm.

Disclosure of Potential Conflicts of Interest

All authors were employees of Excllumine, Inc. during the research period. G.M.W., K.L.C., H.H.R., A.B.C., and E.E.R. own Excllumine stock and/or stock options. This research was funded internally as an Excllumine research project.

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