Rapid high-throughput cloning and stable expression of antibodies in HEK293 cells

Jared L. Spidel *, Benjamin Vaessen, Yin Yin Chan, Luigi Grasso, J. Bradford Kline

Morphotek Inc., 210 Welsh Pool Road, Exton, PA, USA

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**A B S T R A C T**

Single-cell based amplification of immunoglobulin variable regions is a rapid and powerful technique for cloning antigen-specific monoclonal antibodies (mAbs) for purposes ranging from general laboratory reagents to therapeutic drugs. From the initial screening process involving small quantities of hundreds or thousands of mAbs through in vitro characterization and subsequent in vivo experiments requiring large quantities of only a few, having a robust system for generating mAbs from cloning through stable cell line generation is essential. A protocol was developed to decrease the time, cost, and effort required by traditional cloning and expression methods by eliminating bottlenecks in these processes. Removing the clonal selection steps from the cloning process using a highly efficient ligation-independent protocol and from the stable cell line process by utilizing bicistronic plasmids to generate stable semi-clonal cell pools facilitated an increased throughput of the entire process from plasmid assembly through transient transfections and selection of stable semi-clonal cell pools. Furthermore, the time required by a single individual to clone, express, and select stable cell pools in a high-throughput format was reduced from 4 to 6 months to only 4 to 6 weeks.

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1. Introduction

The rapid production of monoclonal antibodies (mAbs) isolated from an immunized organism is essential for the development of therapeutic and diagnostic reagents and critical in the support of vaccine studies. Traditional methods for the production and screening of mAbs involving hybridomas or EBV transduction (Aman et al., 1984; Beerli and Rader, 2010; Kohler and Milstein, 1975; Kozbor et al., 1982; Redmond et al., 1986; Stahl et al., 1980; Steinitz et al., 1977; Stiebig et al., 1990; Strohl, 2014) are inefficient due to genomic instability and low mAb expression of the resulting cell lines. To circumvent these limitations, technologies have been developed to clone variable domain genes into a phage, yeast, ribosomal, or mammalian display platform (Beerli and Rader, 2010; Lim et al., 2014; Saggy et al., 2012; Strohl, 2014). PCR amplified variable heavy (VH) and light (VL) regions from an immunized organism are randomly paired in a library that is screened for antigen binding, and, subsequently, the top binding VH-VL region pairs are subcloned into mammalian expression plasmids for large-scale production. While antibody display technologies are powerful techniques, generating a quality library is limited by the efficiencies of the random VH-VL pairing (not all pairs result in optimal antigen binding) and cloning process and poor expression/enrichment of many relevant antigen specific V genes (Saggy et al., 2012).

To overcome the limitations associated with hybridoma/immortal cell lines and combinatorial display platforms, new techniques have been developed to isolate and clone variable region cDNAs from antigen-specific single B cells into antibody expression vectors (Scheid et al., 2009; Smith et al., 2009; Tiller et al., 2009; Tiller et al., 2008; Tiller et al., 2007; Wardemann et al., 2003; Wrammert et al., 2008). Since the heavy (HC) and light chains (LC) are already paired, there would be no need to screen large libraries for correctly paired mAbs. Single B cell cloning simplifies the process to a single cloning and expression step, bypasses the multiple rounds of panning/screening and the subsequent subcloning into mammalian expression vectors required by display techniques. Only a single round of screening is required to select the highest affinity mAbs whose recombinant production will be then accomplished by establishing high-producing stable cell lines. However, the number of antibodies capable of being cloned and expressed using this method is limited by traditional time- and effort-consuming methods.

Cloning PCR amplified variable domain genes into an expression vector typically requires growth on agar plates and screening of multiple clones that contain the desired insert. Plating hundreds of bacterial transformations on agar plates and screening multiple colonies from each plate is extremely time consuming and low throughput. Further, generating stable cell lines or performing large-scale or multiple small-scale transient transfections to serve as a continuous source of
recombinant mAb for screening and preclinical development is impractical for dozens or hundreds of lead mAbs. An ideal mAb cloning and expression platform would contain a single high throughput cloning step followed by rapid generation of a continuous source of recombinant mAbs.

In this report, we describe a robust high-throughput mAb discovery and production system that integrates the advantages of high-efficiency cloning and stable pool selection methods, reducing the time and effort required to clone and express large numbers of amplified V genes.

2. Materials and methods

2.1. Plasmid construction

Monocistronic plasmids were generated from a customized pcDNA-3.1-based plasmid where the HC or LC gene was cloned into a multiple cloning site between a CMV promoter and SV40 polyA signal. The HC plasmids contained the 5′ end of the CDR3 region, and the LC plasmids contained the last 100 nucleotides of the CDR3 region.

Bicistronic plasmids were generated from a customized pcDNA-3.1-based plasmid. A modified encephalomyocarditis virus (EMCV) IRES with point mutations G375A and A484G to remove Apal and HindIII restriction sites, respectively, was fused at the 5′ end of a cDNA segment (encoding either human gamma or kappa constant region) with the appropriate pair of selection drugs (5 μg/mL blasticidin, 100 μg/mL hygromycin, 400 μg/mL zeocin [ThermoFisher], 400 μg/mL G418 [Calbiochem]) one to four days after transfection. Antibody-expressing stable pools were selected by adding 3 mL of transfecants to 12 mL dMEM (complete DMEM supplemented with 10% FBS, ThermoFisher) in a 125-mL shake flask. Cultures were incubated at 37 °C in 8% CO2 with shaking at 125 rpm for 8 to 10 days. Point mutations were introduced in heavy chain plasmids using a QuikChange Lightning kit (Agilent) according to the manufacturer’s protocol.

2.2. Cell culture

Human embryonic kidney (HEK) FreeStyle 293-F cultures were maintained in FreeStyle 293 expression medium (ThermoFisher) according to the manufacturer’s instructions. Cells were transfected by seeding 1.8 × 10^7 cells in 30 mL medium in a 125-mL shake flask one day prior to transfection. On the day of transfection, 5 μg of each HC and LC plasmids were combined with 65 μg PEI (linear, MW 25000; Polysciences) in 1.5 mL OptiPro (Life Technologies). After incubation at room temperature for 15 min, the PEI-DNA mixture was added to 3 × 10^7 cells in 30 mL medium in a 125-mL shake flask. Cultures were incubated at 37 °C in 8% CO2 with shaking at 125 rpm for 8 to 10 days. Cells were physically dislodged by tapping the flask (trypsinization resulted in low viability, data not shown) and were then seeded at 6 × 10^5 cells/mL in 30 mL FreeStyle 293 expression medium in a 125-mL shake flask. Cultures were incubated at 37 °C in 8% CO2 with shaking at 125 rpm. After passaging the cells three times, terminal cultures were set up by seeding cells at 1 × 10^9 cells/mL in 30 mL FreeStyle 293 expression medium in a 125-mL shake flask. Cultures were incubated at 37 °C in 8% CO2 with shaking at 125 rpm for 10 days. It was found that when performing the drug selection in a serum-containing static adherent culture, cells became drug resistant much faster than when selected for resistance in a serum-free suspension culture. There was no difference in mAb productivity between the two selection methods (data not shown).

Clonal cell lines were obtained by seeding 96-well plates with an average of 0.5 cells per well in cDMEM. After 3 to 4 days, conditioned cDMEM from untransfected 293-F cells and drug selection to a final

![Fig. 1. Plasmid schematics for pCIx-leader-C region vectors.](image-url)
A.

3’ Homologous Region 5’ Homologous Region

\[ --\text{AfeI}--\text{PmeI}--\text{EcoRV}-- \]

...GCCACGGCGTGCACAGC gctgttaaacgatATCCACAAAGGGCC... nucleotide sequence

ACCGGCGTGCACAGC............gccATCCACAAAGGGCC PCR insert

leader VH insert human gamma

... A T G V H S | x x x |A S T K G P ... amino acid sequence

3’ Homologous Region 5’ Homologous Region

\[ --\text{AfeI} \text{ EcoRV} - \text{PmeI}--- \]

restriction sites

GCCACGGCGTGCACAGC gcttagatatctgttaAAACGAACCTGTGGCT... nucleotide sequence

ACCGGCGTGCACAGC............AAACGAACCTGTGGCT PCR insert

leader VK insert human kappa

... A T G V H S | x x x x x K |R T V A ... amino acid sequence

B.

Fig. 2. In-Fusion cloning of variable region inserts. (A) HC and LC constant-region vectors were designed to contain a multiple cloning site between the leader sequence and the constant region. An in-frame stop codon was included in the multiple cloning site (underlined) to mitigate translation of proteins lacking a variable region insert. The HC constant-region vectors were cut with AfeI, PmeI, and EcoRV and LC vectors were cut with AfeI, EcoRV, and PmeI for linearization. Variable regions were PCR amplified with 15-bp 5’ and 3’ ends homologous to the vector. The variable region PCR fragments were fused to the vector by In-Fusion cloning. (B) Miniprepped pools of transformed E. coli were digested with restriction enzymes to determine the size of the antibody cDNA in the plasmid. Heavy chain plasmids with a variable region insert produced a ~1.4 kb fragment. Light chain plasmids that contain a variable region insert produced a ~700 bp fragment. Heavy and light chain plasmids without inserts produce 1 kb and 250 bp fragments, respectively. The wells alternate between HC and LC, except the last 8 samples on the bottom row are all LC plasmids. Arrows indicate fragments with no insert. Asterisks indicate a mixture of fragments with and without inserts.

concentration of 200 μg/mL zeocin and 2.5 μg/mL blasticidin was added. After further incubation for several more days or weeks, wells containing a single colony were grown in 24-well or 6-well plates and eventually expanded to T75 flasks. Once confluent, mAb expression of the clones was determined by seeding 1 x 10^6 cells in 3 mL of cDMEM supplemented with low-IgG FBS (ThermoFisher) for 7 days. The amount of mAb in the medium was then quantitated. Clones were then adapted to FreeStyle293 medium and the amount of mAb expressed was quantitated as above.

2.3. Antibody quantitation

Antibody titer yields were quantified using an Octet QK with Dip and Read Protein A Biosensors (ForteBio) according to the manufacturer’s protocol.

3. Results

3.1. High throughput, semi-clonal platform for constructing recombinant mAb expression plasmids

The biggest bottleneck in the cloning process of individual HCs and LCs is the selection of recombinant transformants on antibiotic agar plates and subsequent colony screening. To streamline the cloning process, these steps were eliminated by utilizing the highly efficient ligase-independent In-Fusion HD cloning kit. In the In-Fusion HD reaction, reactions rely on homologous recombination between the vector and insert to circularize the vector, and, therefore, there is little to no background from religated vector. Furthermore, it was found that there was little to no background colonies resulting from undigested plasmid
when the vector was digested with three restriction enzymes that cut between the leader and C region sequences (Fig. 2A; data not shown), thereby making it possible to grow and miniprep transformations as a semi-clonal pool.

To validate and quantify this method, 44 VH genes and 51 VL genes were RT-PCR amplified from clonal B cell populations and cloned into C region vectors. Minipreps of semi-clonal DNA pools were digested with restriction enzymes that cut 5’ and 3’ to the leader and C region, respectively, and analyzed on a 1% agarose gel. As predicted, HC plasmids with or without an insert yielded a ~1.4 kb or ~1 kb fragment, respectively, and LC plasmids with or without an insert yielded a fragment at ~700 bp or 250 bp, respectively. The great majority of LC plasmids (98%) and HC plasmids (95.4%) contained the expected insert (Fig. 2B), while only few plasmids did not (arrows). Some of the HC minipreps (6.8%) contained a mixture of plasmids with and without inserts (asterisks).

The percentage of plasmids with inserts in each transformation was further quantitated by streaking out a small portion of the transformation on agar plates and screening individual colonies by PCR for the presence of an insert. The percentage of clones with inserts varied from 31% to 100% per transformation with an average of 71.6% for HC plasmids and 81.1% for LC plasmids (Table 1). The wide range of positive clones was likely due to incomplete digestion of the vector by the restriction enzymes as subsequent experiments utilizing a different preparation of vectors resulted in much higher percentage of positive clones (data not shown).

This approach cannot only be applied to ligation-independent cloning, but also to whole-plasmid amplification-based mutagenesis of heavy and light chain plasmids using methods such as the QuikChange Lightning Site-Directed Mutagenesis kit. Several hundred site-directed mutagenized HC plasmid mutants were generated and the transformants were miniprepped as semi-clonal pools as above. The semi-clonal DNA preps were each sequenced for the presence of the point mutation(s). All semi-clonal plasmid pools contained only the desired mutation(s) (data not shown).

3.2. Bicistronic plasmid design and expression

Even though a large number of mAbs can be rapidly produced via small-scale transient transfections for screening purposes, scaling up selected mAbs post-screening to use for in vitro/in vivo studies can be more challenging. Although several techniques to enhance sufficient production for biological studies have been described over the last several years (Backliwal et al., 2008; Pham et al., 2006; Pham et al., 2003; Sun et al., 2006), these methods do not yield a process for stable cell line production to provide the continuous source of material required for support of longer term development of candidate therapeutic or diagnostic mAbs. Similar to high-throughput cloning, the major bottleneck for generating stable mammalian cell lines is screening for high-titer clones. This method typically selects for plasmid integration in transfected cells by performing limiting dilution of the cells in growth medium containing drug selection (Jostock and Knopf, 2012), a process that is labor-intensive and requires between 4 and 6 months.

An alternative method utilizes selection of stable pools of cells. Here, transfected cells are not subcloned and drug selection is applied to the pool of transfected cells (Ye, 2012). Selection of a pool of mAb-expressing cells overcomes these hurdles since the expressing cell line is not subjected to multiple rounds of screening. To avoid generating stable cell pools with low antibody titers due to the abundance of non- or low-expressing antibiotic resistant cells, as seen in traditional approaches that utilize monocistronic expression plasmids (Ye et al., 2010), bicistronic plasmids were designed to transcriptionally link expression of a drug resistance protein for blasticidin (blasticidin S deaminase, BSD), G418 (neomycin phosphotransferase, NPT), hygromycin (hygromycin B phosphotransferase, HPH), puromycin (puromycin N-acetyltransferase, PAC), or zeocin (bleomycin resistance protein, BRP) to the expression of a HC or LC by an EMCV IRES on a single mRNA transcript (Fig. 1). The HC and LC for human (mAb1) and chimeric rabbitV-humanC (mAb2) mAbs were cloned into both bicistronic pCIZ and pCIB vectors, respectively, as well as monocistronic zeocin-resistance and blasticidin-resistance vectors, respectively. Transient mAb expression was reduced only 20–30% when the HC and LC were cotranslated with antibiotic resistance genes from bicistronic mRNA when compared to monocistronic mRNA (Fig. 3A). However, mAb expression from stable pools was 2- to 3-fold higher for the bicistronic plasmids.

Single cell clones were subsequently isolated from the stable pools via limiting dilution and tested for mAb titer yields. Up to forty clones were analyzed for mAb expression in static culture (Fig. 3B). The average mAb titers for bicistronic clones were 1.5- to 2.5-fold higher than monocistronic clones. The highest titer clones producing mAb1 and mAb2 were converted to serum-free medium, and analyzed for production yields in shaking cultures. Titers from clones using bicistronic expression plasmids were 2.2- to 2.4-fold higher than from monocistronic expression plasmids (Fig. 3C).

### Table 1

<table>
<thead>
<tr>
<th>No. InFusion reactions</th>
<th>No. colonies screened/reaction</th>
<th>Average % positive</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>42</td>
<td>16</td>
<td>71.6% ± 3.2%</td>
</tr>
<tr>
<td>LC</td>
<td>42</td>
<td>16</td>
<td>81.1% ± 3.7%</td>
</tr>
</tbody>
</table>

3.3. Pairing antibiotic resistance plasmids for maximal expression

To maximize mAb expression, the HC and LC genes are expressed using separate plasmids. Therefore, obtaining stable cell lines that consistently expressed both chains requires different resistant genes on each plasmid. To determine the best combination of drug resistance genes that may result in the highest mAb titers from both transient transfections and stable pools, the HC and LC of mAb1 were cloned into vectors that contained each drug resistance gene. Each HC plasmid consistently expressed both chains requires different resistant genes on each plasmid. To determine the best combination of drug resistance genes that may result in the highest mAb titers from both transient transfections and stable pools, the HC and LC of mAb1 were cloned into vectors that contained each drug resistance gene. Each HC plasmid was cotransfected in a matrix format into FreeStyle 293-F cells with each LC plasmid. The media from transient transfections were harvested after ten days, and the amount of secreted mAb was analyzed (Fig. 4A). Transient expression of the mAb was found to be influenced by the drug resistance gene in the LC plasmid as clones exhibiting the lowest titers (17–45 μg/mL) expressed NPT (pCIN) and HPH (pCIZ) while those with the highest-titers (66–75 μg/mL) expressed PAC (pCIP). These data were observed in other studies as well (see below). There was no correlation between mAb expression and the drug resistance gene expressed by the HC plasmid.

The effect of antibiotic selection on the expression of mAb1 in stable pools was determined by quantitating the amount of secreted mAb ten days after seeding terminal cultures. In contrast to transient expression, mAb production did not correlate to the drug resistance gene in the LC plasmid. However, linking expression of BRP (pCIZ) to either the HC or the LC resulted in the highest mAb titers (Fig. 4A).

To determine whether zeocin resistance results in the best universal expression, the HC and LC plasmids of two additional mAbs (mAb2 and ratV-mouseC chimeric, mAb3) were co-transfected as pCIB and pCIZ, pCIZ and pCIN, or pCIP and pCIZ combinations. Transient expression of mAb2 and mAb3 was similar to mAb1 in that the lowest titers were observed with NPT- and HPH-expressing LC plasmids (Fig. 4B and C). There was little difference in transient expression
between the other plasmid combinations (40–60 μg/mL and 36–51 μg/mL, respectively). Stable pools were selected, and the trends in expression of mAb2 and mAb3 were similar to mAb1 (Fig. 4B and C). Although the puromycin-resistant plasmids yielded the highest producing cell lines, a stable pool could not be obtained from the mAb3 HC-puromycin/LC-zeocin combination. Repeated attempts to generate a stable cell line from the mAb3 HC-puromycin/LC-zeocin combination were not successful even when the concentration of puromycin was reduced several fold. It was also observed that all mAb1, mAb2, and mAb3 stable cell lines made from any PAC-based plasmid took longer to grow to confluency following drug selection due to extreme sensitivity of 293-F cells to puromycin (data not shown). Given the inconsistency of obtaining a stable cell line from puromycin-resistant plasmids, these plasmids were deemed least useful and not used for any further development.

For all three mAbs listed above, the stable pool generated from the HC-blasticidin/LC-zeocin combination yielded the lowest-titers. Although stable mAb expression from HC plasmids containing hygromycin or neomycin was relatively high, the transient expression was rather low, limiting the usefulness of these plasmids. In addition, viable mAb3 stable pools were not obtained for the HC-hygromycin/LC-zeocin nor HC-zeocin/LC-neomycin combinations (Fig. 4B). Based on these results we focused our efforts on the HC-zeocin/LC-blasticidin plasmid combination platform since this format consistently yielded high mAb titers from both transient transfections and stable pools.

3.4. Effects of increased or decreased selection pressure on mAb expression

Since mAb expression was linked to drug resistance, it was possible that increasing the amount of antibiotics during selection could select for cells that express higher amounts of the antibiotic resistance proteins, and thereby higher levels of mAb. Varying concentrations of blasticidin and zeocin were added during the mAb1 selection process to evaluate this hypothesis. Unfortunately, increasing the concentration of zeocin up to 1200 μg/mL or blasticidin up to 15 μg/mL did not yield higher titer clones (Fig. 5A).
The stability of mAb expression by stable pools was analyzed by monitoring the expression of mAb1 over time. After the eighth passage in 400 μg/mL zeocin and 5 μg/mL blasticidin, the pooled culture was split into three subcultures and cultured using decreasing amounts of drug selection. Decreasing the concentration of antibiotics by one-half or one-quarter did not affect mAb expression. Furthermore, removal of drug selection resulted in little decrease in mAb production for at least 56 days (15 passages, Fig. 5B). By day 84 (passage 29 for 5/400; passage 22 for the remainder) production began to decrease for all pools, though cells cultured without selection decreased more than the others.

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**Fig. 4.** Antibody expression using IRES-linked antibiotic selection plasmids. Bicistronic IRES plasmids encoding various antibiotic resistant genes (B – blasticidin, H – hygromycin, N – neomycin, P – puromycin, Z – zeocin) downstream of immunoglobulin HC or LC genes were co-transfected into 293-F cells, e.g. B-H is a blasticidin-HC plasmid cotransfected with a hygromycin-LC plasmid. Various combinations of resistance genes and immunoglobulin HC and LCs were used including (A) human (mAb1), (B) rabbit-human chimeric (mAb2), or (C) rat-human chimeric (mAb3) mAbs. Antibody titers were analyzed using an Octet QK with protein A Biosensor tips 10–12 days post-transfection for transient transfections or 7–10 days after seeding the terminal cultures with stably selected cell lines.

**Fig. 5.** Blasticidin and zeocin concentrations have little effect on mAb production. (A) HC/LC cotransfected 293-F cells were dually selected with varying concentrations of blasticidin (5 or 15 μg/mL) and zeocin (400, 800, or 1200 μg/mL). Terminal cultures from stably selected cell pools were analyzed after 7–10 days for antibody titers using an Octet QK with protein A Biosensor tips. (B) The concentration of antibiotics was reduced to one-half (2.5/200), one-quarter (1.25/100), or none (0/0) of the original concentration (5/400) and assayed every 4 weeks for antibody production as in (A).
3.5. Summary of workflow

An exemplary workflow is detailed in Fig. 6. Herein the VH and VL regions are RT-PCR amplified from individual single B cell cultures and ligation-independently inserted into constant region vectors. The E. coli transformation for each reaction is grown in liquid medium, rather than plated for isolation of individual colonies. This shortens the cloning process to 2 to 4 days from 1 to 2 weeks. The semi-clonal HC and LC DNAs cloned from each B cell culture are cotransfected and a portion of each transfection is selected as a pool in static serum-containing culture or serum-free shaking culture. Bypassing the need to select and screen individual colonies, the semi-clonal transfectants are selected within 7 to 11 days, as opposed to 4 to 5 weeks, and eliminates the expansion process, thereby save an additional 4 to 5 weeks. During the selection process the transient cultures can be screened for antigen binding, and the top mAbs picked for serum-free adaptation and expansion. The adaptation of the semi-clonal culture to serum-free medium requires no subsequent rescreening for mAb production, thereby saving >3 weeks. In total, our semi-clonal method cuts the time from cloning through stable cell line development by 3 to 5 months.

4. Discussion

In this report, we have described a rapid, optimized high-throughput system for the cloning and expression of immunoglobulin VH and VL cDNAs by utilizing the same plasmid for both transient mAb production as well as large-scale production by stably selected lines. Several groups have demonstrated either high-throughput methods for cloning mAbs (Dodev et al., 2014; Jones et al., 2010; Kurosawa et al., 2011; Liao et al., 2009) or rapid generation of stable mAb-expressing cell lines, but...
to date there are no reports that couple these technologies into a single platform. Indeed, the methods and vectors used in high-throughput cloning methods are not amenable to rapid stable cell line production as they are based on transfection of PCR products (Liao et al., 2009), lack an antibiotic resistance gene (Dodev et al., 2014) or utilize monocistronic plasmids (Jones et al., 2010; Kurosawa et al., 2011). While, Ho et al. (Ho et al., 2012; Ho et al., 2013) demonstrated rapid generation of stable pools expressing monoclonal antibodies in CHO cells using a tricistronic plasmid where the HC, LC, and antibiotic resistance gene were translated from a single transcript, this method requires extra steps in the cloning process. Two rounds of cloning are required to insert both the HC and LC into the plasmid. Alternatively, a single cloning step can be used to insert a single PCR fragment containing an IRES sequence flanked by the HC and LC. However, this method requires an extra round of PCR to create the aforementioned PCR product. Therefore, cloning the HC and LC into separate bicistronic expression plasmids provides the simplest and quickest means to generate expression plasmids and stable cell lines.

One of the major advantages of this system is the ability to move quickly a lead mAb to large-scale production by creating stable cell pools using bicistronic expression plasmids that transcriptionally linking expression of the HC and LC with antibiotic resistance genes. In contrast to stable pools generated from monocistronic plasmids (Ye et al., 2010), there is no need to sort cells for enrichment (Ye, 2012; Ye et al., 2010) or subclone for high-producing cells (Jostock and Knopf, 2012) since all antibiotic resistant cells theoretically express the mAb (Gurtu et al., 1996; Rees et al., 1996). Furthermore, this system is amenable to the cloning and expression of a large number of other types of recombinant proteins (e.g., surface antigens for high-throughput target cell line generation).

Several steps have been eliminated to significantly enhance the cloning throughput while decreasing labor hours typically required for high-throughput mAb discovery. A few areas for improvement were experienced during this optimization process. Given that 20–30% of plasmids lacking an insert in the miniprep pool, it was possible that mAb expression could be affected by either expression of aberrant leader-/constant domain protein or by diluting out the overall amount of insert-containing plasmid. To minimize these issues, a stop codon was engineered in the cloning site between the signal sequence and C region (Fig. 2A). A V region insert replaces this stop codon so that only productive mAbs are translated. In addition, it has been shown that non-coding DNA can supplement more than half of the coding DNA in a PEI-based transfection with little effect on recombinant protein expression (Kichler et al., 2005; Rajendra et al., 2012; data not shown). Even though the presence of insert-null vector likely has little effect on overall mAb expression within a pooled culture, one could employ a zero-background cloning strategy to ensure a cloning efficiency closer to 100% (Bernard, 1995; Bernard et al., 1994; Lund et al., 2014; Miyazaki, 2010; Parr and Ball, 2003; Schefer et al., 2014; Wang et al., 2014).

For recombinant mAb production, careful consideration was given to the choice of cells used. While Chinese hamster ovary (CHO) cells are predominantly the cells of choice for the production of biopharmaceuticals, HEK cells are much more popular for transient transfection due to their inherent ease of transfection and high production from transient transfections (Butler and Meneses-Acosta, 2012). Despite recent improvements of the protocols to increase the expression levels of mAbs in transient CHO transfections, these methods tend to involve additional steps, including diluting cells several hours after transfection, adding feed several hours or days post-transfection, decreasing the incubation temperature, and/or performing multiple rounds of transfections (Chusainow et al., 2009; Codamo et al., 2011; Piclier et al., 2011; Rajendra et al., 2015; Rajendra et al., 2011; Reisinger et al., 2009; Wulhfard et al., 2008). As these additional manipulations increase effort and cost, we decided that the use of HEKs would be most ideal for at least transient expression. It can be argued that the cell type used during mAb characterization should be the same cell type used to manufacture the therapeutic mAb due to differences in post-translational modification differences (e.g. glycosylation). However, a recent report demonstrated that the mAbs produced by transient HEK transfections correlate with stable CHO cell lines in regards to mAb titers and the quality of the product (Dippenbruck et al., 2013). Given this observation and as the intended use of this system being the expression of mAbs for primary candidate selection, any differences between HEK-derived mAbs versus CHO-derived mAbs are negligible.

Interestingly, our findings that the choice of antibiotic resistance gene pairings influences the mAb expression offers future opportunities to test other selection markers for even higher titers than is achieved in our current optimal system. Importantly, while our findings that resistance to zeocin was essential in obtaining a high expressing pool were similar to Lanza et al. (2013), we found, in contrast to Lanza et al., no advantage in maintaining drug selection after primary selection for up to eight weeks. Therefore, drug selection can be removed while scaling up the culture for mAb production, providing a distinct advantage over stable pools selected from monocistronic plasmids which require maintenance of drug selection during scale-up (Ye, 2012).

In conclusion, we outline an antibody cloning and screening system that can facilitate the cloning, expression, and screening of up to a thousand mAbs, which may be expandable using automation. By removing the bottlenecks described here, the time from isolating V-region cDNA to producing large-scale mAbs from stable pools has been reduced from 4 to 6 months to only 4 to 6 weeks.

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