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Automated pipeline for rapid production & screening of HIV-specific monoclonal antibodies using *Pichia pastoris*

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Abstract

Monoclonal antibodies (mAbs) that bind and neutralize human pathogens have great therapeutic potential. Advances in automated screening and liquid handling have resulted in the ability to discover antigen-specific antibodies either directly from human blood or from various combinatorial libraries (phage, bacteria or yeast). There remain, however, bottlenecks in the cloning, expression and evaluation of such lead antibodies identified in primary screens that hinder high-throughput screening. As such, ‘hit-to-lead identification’ remains both expensive and time-consuming. By combining the advantages of overlap extension PCR (OE-PCR) and a genetically stable yet easily manipulatable microbial expression host *Pichia pastoris*, we have developed an automated pipeline for the rapid production and screening of full-length antigen-specific mAbs. Here, we demonstrate the speed, feasibility and cost-effectiveness of our approach by generating several broadly neutralizing antibodies against human immunodeficiency virus (HIV).

Keywords

OE-PCR; *Pichia pastoris*; monoclonal antibody; HIV; automation

Monoclonal antibodies (mAbs) are high-value biologics, an important class of therapeutic agents used to treat a wide range of diseases, generating ~ \$20 billion in revenue annually (Aggarwal, 2014). High-throughput tools now exist to identify antibodies against key human pathogens including HIV, influenza and dengue virus (Wilson and Andrews, 2012). The initial identification of antibody candidates can be carried out using binding kinetics of smaller fragments (including single-chain variable fragments (scFv) or antigen-binding fragments (Fab)), but complete functional characterization, including bioactivity, neutralization potential and antibody-dependent cell-mediated cytotoxicity (ADCC) etc.,

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requires moderate quantities (~ 0.1 – 1 mg) of the expressed full-length material (Hoogenboom, 2005).

Historically, the screening and production of antibodies has involved the creation of hybridomas or Epstein-Barr virus (EBV) transformed human B cells, followed by limiting serial dilution to recover antigen-specific clones (Wang, 2011). Generating full-length antibodies using these techniques is not only time-consuming, but often results in low material yields from the initial clonal stock (Frame and Hu, 1990). The ability to amplify the variable regions of an antibody (V_H and V_L) by PCR and clone them directly into expression plasmids has preempted the need for traditional hybridoma technology, allowing researchers to recombinantly produce mAbs from various host organisms, most commonly HEK 293T or CHO cell lines.

Recently, methods for the direct amplification of variable regions from single cells *via* reverse transcription-PCR (RT-PCR) have been developed (Tiller et al., 2008; Wang and Stollar, 2000). These advances have greatly expanded access to the human antibody repertoire, but bottlenecks in the expression of antibodies persist, hindering rapid ‘hit-to-lead’ identification. In particular, cloning of antibody variable regions through bacterial propagation and transfection of two separate expression vectors (containing heavy and light chain genes respectively) are time-consuming steps that require separate aseptic workspaces, expensive reagents (such as lipofectamine) as well as skilled personnel. The use of linear expression vectors constructed through overlap-extension PCR (OE-PCR) (Liao et al., 2009) has precluded the need for bacterial propagation, but still requires transient co-transfection.

There has been a strong interest in developing microbial expression systems for the purposes of antibody production (Spadiut et al., 2014). The methylotropic yeast *Pichia pastoris* has emerged as a promising eukaryotic host in this area, owing to its strong but tightly regulated alcohol oxidase I promoter (P_{AOX1}), rapid growth on inexpensive media and limited risk for viral contamination. Additionally, GlycoFi and GlycoSwitch technologies have led to the humanization of *Pichia*’s glycosylation pathway, allowing for the production of mAbs with human-like glycoforms that are comparable in potency and efficacy CHO-derived products (Meehl and Stadheim, 2014; Spadiut et al., 2014). Transformation of *Pichia* results in stable chromosomal integration of the expression vector, further facilitating strain generation and screening in a high-throughput and/or automated fashion (Barnard et al., 2010; Jiang et al., 2010).

Here, we have combined the advantages of overlap extension PCR (OE-PCR) and the *Pichia pastoris* expression system to develop an integrated and automated pipeline for the generation of full-length mAbs. We demonstrate our approach through the rapid generation of stable mAb-producing *Pichia* strains for the expression and screening of broadly neutralizing antibodies (bnAbs) against HIV. Overall, our strategy enables rapid and cost-effective hit-to-lead identification for antibody development and could be adapted, in principle, to screen for specific mAbs against any infectious agent for which an antigen is available.

Currently, mAb production in *Pichia* is carried out using expression plasmids containing two separate promoter elements for expression of heavy and light chain genes cloned through the conventional bacterial propagation process (Zha, 2013). We used the foot-and-mouth disease virus (FMDV) 2A sequence to eliminate one promoter and enable bicistronic expression of heavy and light chains from a single open reading frame (ORF). Our strategy also minimizes the size of the expression construct (~ 6 Kb), thereby promoting PCR assembly as well as genetic stability at the integration loci (Zhu et al., 2009). The 2A sequence has been previously used for the production of full-length antibodies from mammalian cells (Fang et al., 2005) and has been effective in the coexpression of digestive enzymes in *Pichia* (Roongsawang et al., 2010). Figure 1 presents a schematic outlining the key features and steps for assembly of our expression construct. Heavy and light chains are expressed using the P_{AOX1} promoter and secreted using the α -factor secretion signal at their 5' ends (Fig. S1). The light chain is placed in front of the heavy chain; previous observations have shown that an excess of light chain can promote folding and increase antibody stability (Schlatter et al., 2008). We used the Kanamycin marker for G418-based selection of transformed strains (Scorer et al., 1994), as opposed to Zeocin-based selection, due to its relative cost-effectiveness, potential for automation and the sensitivity of Zeocin to light. Variable region fragments (V_L and V_H) originating either directly from RT-PCR of antibody-secreting cells or from synthesized genes are amplified using primers containing complimentary flanking sequences and incorporated into the light and heavy chain segments by OE-PCR. These two DNA segments, along with a segment that contains the selectable marker, are then assembled together to generate the full construct (Fig. 1A). Transformation of this vector into *Pichia* by electroporation (Fig. 1B) results in stable integration at the AOX1 locus with nearly 100% correct integration frequency (as verified by PCR).

We found two design features were essential to facilitate robust assembly of the expression construct: (1) large regions of overlap between parts and (2) carefully designed primers with high annealing temperatures (60°C – 65°C). By incorporating these considerations, we generated clean PCR products without non-specific amplification during each stage of assembly and proceed to subsequent steps without the need for any gel or column purification of assembled intermediates. As a representative example, Figure 2 shows PCR assembly of a construct for the expression of aglycosylated b12 (N297Q), a broadly neutralizing antibody against HIV. Our strategy allows such expression constructs to be generated routinely in less than 2 days without the need for any bacterial propagation. The modular nature of this assembly process also enables facile swapping of constituent parts (Fig. S1), including exchanging of the native Fc backbone for Fc domain variants in order to generate antibodies with diverse effector functions (Sazinsky et al., 2008). Finally, the entire process for assembling expression constructs has been designed in a manner amenable to high-throughput automation.

Next, we developed an automated pipeline (Fig. 3A) for expression and functional screening that includes (1) outgrowth and induction of mAb-producing *Pichia* strains in 24 or 96-well deep-well plates, (2) clarification of culture media through centrifugation, (3) purification of full-length antibodies from the culture supernatant using Protein A-resin tips and (4) binding assays to determine expression titer and binding affinities of purified full-length mAbs using

a liquid handling system. To test this platform, we used our OE-PCR assembly to generate constructs for the evaluation of three more aglycosylated mAbs against HIV: one described previously in the literature (479, (Scheid et al., 2009; Scheid et al., 2011)) and one discovered using a microengraving-based approach (MIT-41, (Ogunniyi et al., 2014)). Owing to the modular nature of our assembly process, we also simultaneously generated constructs for two additional variants of b12 and MIT-41 antibodies containing modified Fc regions – (1) G236A/S239D/A330L/I332E [GASDALIE], which enhances activating Fc γ R binding and (2) G236R/L328R [GRLR], which abolishes Fc γ R binding (Bournazos et al., 2014). These expression constructs were electroporated into competent *Pichia* cells and 6 – 8 transformants for each antibody construct were selected for our automated platform for mAb expression and evaluation. To test the fidelity of the OE-PCR assembly process, we also isolated genomic DNA from all 60 clones selected for mAb expression and verified the sequence of the integrated construct using Sanger sequencing. In all cases, we found a 100% homology between the designed and integrated construct, suggesting that the error rate of our OE-PCR assembly is likely determined by the fidelity of the DNA polymerase used.

Upon putting the transformants through our automated expression platform, we found that titers of full-length mAbs in deep-well plates ranged between 100 – 500 ng/mL and were sufficient to carry out assays to (1) confirm production of full-length antibody, (2) determine the best-producing isolate and (3) identify binders to a specific antigen (in this case HIV coat protein gp120). Purified antibodies were analyzed by SDS-PAGE (Fig. 3B, non-reducing and Fig. 3C, reducing) and western blot analysis (Fig. S2A, non-reducing and Fig. S2B, reducing). Our results show the presence of fully assembled antibody (~ 150 kDa) along with several other fragments that are typically observed in unfractionated Protein A affinity-purified eluates. We also found the presence of fragmented heavy chain (Fig. S2), which is likely a result of residual proteolytic activity from host cell proteins (HCPs) - a well known issue and common occurrence in the production of therapeutic antibodies (Gao et al., 2010). When subjected to gp120 binding assays (native Fc, Fig. 3D and Fc variants, Fig. 3E), we found that the full-length *Pichia*-derived aglycosylated b12 mAb had a binding affinity for gp120 similar to standard CHO-derived glycosylated b12 mAb. Our results also showed that the MIT-41 mAb has a gp120 binding affinity similar to that of b12 mAb, suggesting it as a suitable candidate for further functional testing (virus neutralization, ADCC etc.).

In conclusion, our data show that we can produce full-length and functional antibodies using the OE-PCR strategy in a rapid and flexible manner. The use of the 2A peptide sequence simplifies vector design for production of mAbs in *Pichia*. Most importantly, the time required to translate a set of V_L and V_H from genes into antibody using our pipeline is ~ 12 days, and can be carried out in an automated and high-throughput fashion. While we have demonstrated production of aglycosylated mAbs from a wild-type *Pichia* strain, this strategy can be readily adapted for the expression and evaluation of glycosylated mAbs from glycoengineered *Pichia* strains (Meehl and Stadheim, 2014; Spadiut et al., 2014). Overall, we believe that our approach provides advantages over current methodologies with respect to time and costs (Table I and Table S2). Combining it with a production-ready/optimized host (i.e. glycoengineered and/or super-secreter strain) will further accelerate lead

optimization, as scale up from plate-based testing to fermentation in bioreactors does not require changing the expression host or re-cloning the constructs. Our approach should facilitate the characterization of many new mAbs in a shorter amount of time and with fewer material and personnel resources.

Materials and Methods

OE-PCR assembly

KOD Hot Start Xtreme polymerase (EMD Millipore, Cat. No. 71975) was used for all PCR reactions. Sequences of primers and steps used for OE-PCR assembly are described in Supplementary Material. Initial DNA fragments were synthesized using Invitrogen's gene synthesis service and amplified from their vectors using the following thermocycler conditions: 94°C for 2 min, followed by 30 – 35 cycles of 94°C for 20 s, 65°C for 15 s and 72°C for 1 min/kb (25 µL reaction volume, ~ 1 ng template). The three segments – (1) light chain, (2) heavy chain and (3) selectable marker were then assembled from these amplified fragments. For each segment, an extension step without primers was first carried out using the following conditions: 94°C for 2 min, followed by 12 cycles of 94°C for 20 s, 45°C for 15 s and 72°C for 1 min. Then, 2 µL of each piece was used as template to amplify the segments using the following conditions: 94°C for 2 min, followed by 32 cycles of 94°C for 20 s, 65°C for 15 s and 72°C for 1 min/kb. Finally, 2 µL template from each amplified segment was pooled together and used for full construct assembly using two-step PCR conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 20 s and 72°C for 6 min. Multiple reactions were carried out to generate 5 – 10 µg of DNA followed by isopropanol precipitation down to a volume of 10 µL for electroporation into *Pichia*.

Transformation and cultivation of *Pichia* strains

A wild-type *Pichia pastoris* strain (*Komagataella phaffii*, NRRL Y-11430, ATCC 76273) was used in this study. Competent cell preparation, electroporation and cultivation were carried out as described in the Invitrogen *Pichia* manual (Aitchison, 2009). Transformants were selected on YPD+Sorbitol media containing 500 µg/mL Geneticin (Invitrogen, Cat. No. 10131-035) and freezer stocks were made in YPD + 20% glycerol. Strains were outgrown and induced using standard BMGY and BMMY media (Teknova, Cat. No. B8000 and B8100 respectively) containing 0.1% Sigma A204 antifoam and supplemented with 1% methanol every 24 hours in BMMY media. After ~ 72 hours of induction, cells were spun down at 3000 rpm for 5 min and clarified supernatant was transferred to fresh plates for purification of mAbs.

Protein A purification

mAb-containing supernatants were purified on a Tecan Freedom Evo 150 liquid handling system with an MCA96 head using tips containing 20 µL of Protein A resin and according to manufacturers instructions. (PhyNexus, Cat. No. PTM 92-20-01).

SDS-PAGE and Western blot

Purified mAb samples were denatured at 90°C for 30 minutes before running on 12% precast gels (Bio-Rad, Cat. No. 456-1044). 50mM Dithiothreitol (DTT) was used as

reducing agent. For western blot analysis, mouse anti-human Fc specific IgG (Millipore, Cat. No. MAB1302) was used at a 1:1000 dilution to detect heavy chain and mouse anti-human kappa specific IgG (Abcam, Cat. No. ab1050) was used at a 1:1000 dilution to detect light chain. Goat anti-mouse IgG (Abcam, ab97040) was used as secondary antibody in both cases.

Immunoassays

Two sandwich ELISAs were validated for use on the Tecan liquid handling system. For the detection of full-length antibody and determination of mAb titers, goat anti-human Fc specific IgG (Jackson ImmuneR, Cat. No. 109-005-098) was used as capture antibody (5 µg/mL) and HRP-conjugated goat anti-human kappa chain specific IgG (Invivogen, Cat. No. hrp-igak) as the detection antibody (500 ng/mL). For determination of binding affinity to gp120, the same goat anti-human Fc specific IgG capture antibody was used (5 µg/mL), followed by addition of sample, gp120 (1 µg/mL) and HRP-conjugated goat anti-gp120 (Pierce, Cat. No. PA1-73097) as detection antibody (500 ng/mL). For both assays, signal was developed using the QuantaBlu Fluorogenic Peroxidase Substrate Kit (Thermo, Cat. No. 15169) and read with excitation and emission wavelengths set at 325 nm and 420 nm respectively. CHO-derived b12 (Cat. No. 2640), used as a standard throughout this work, was obtained through the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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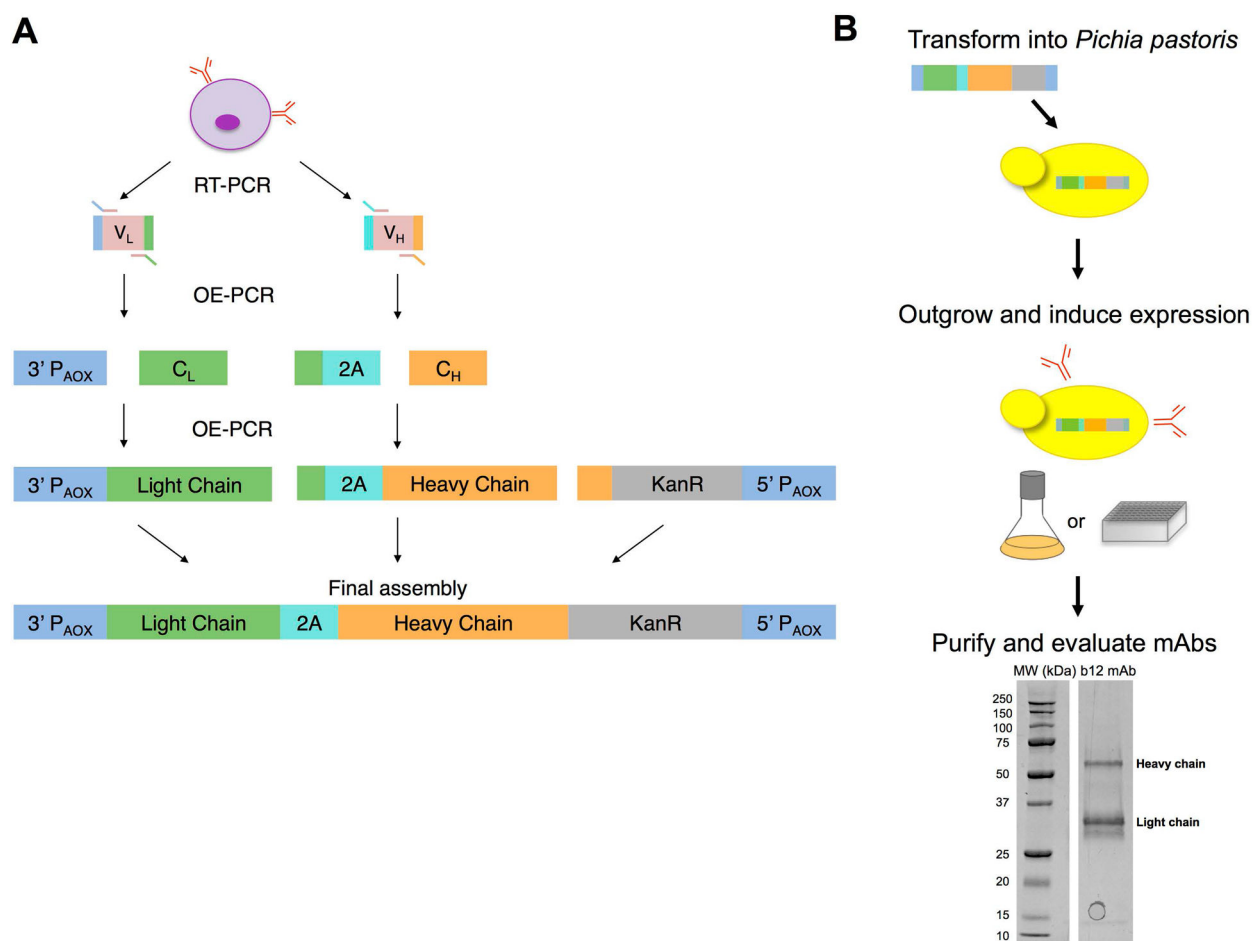
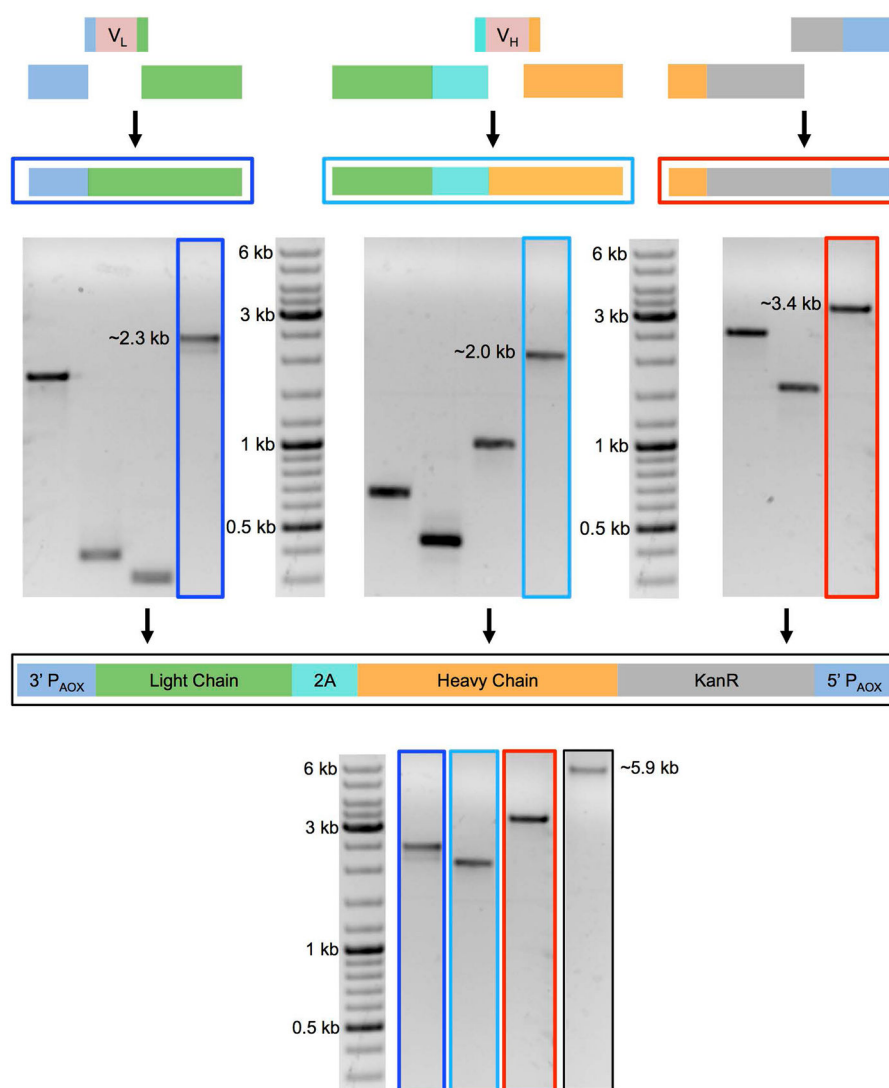
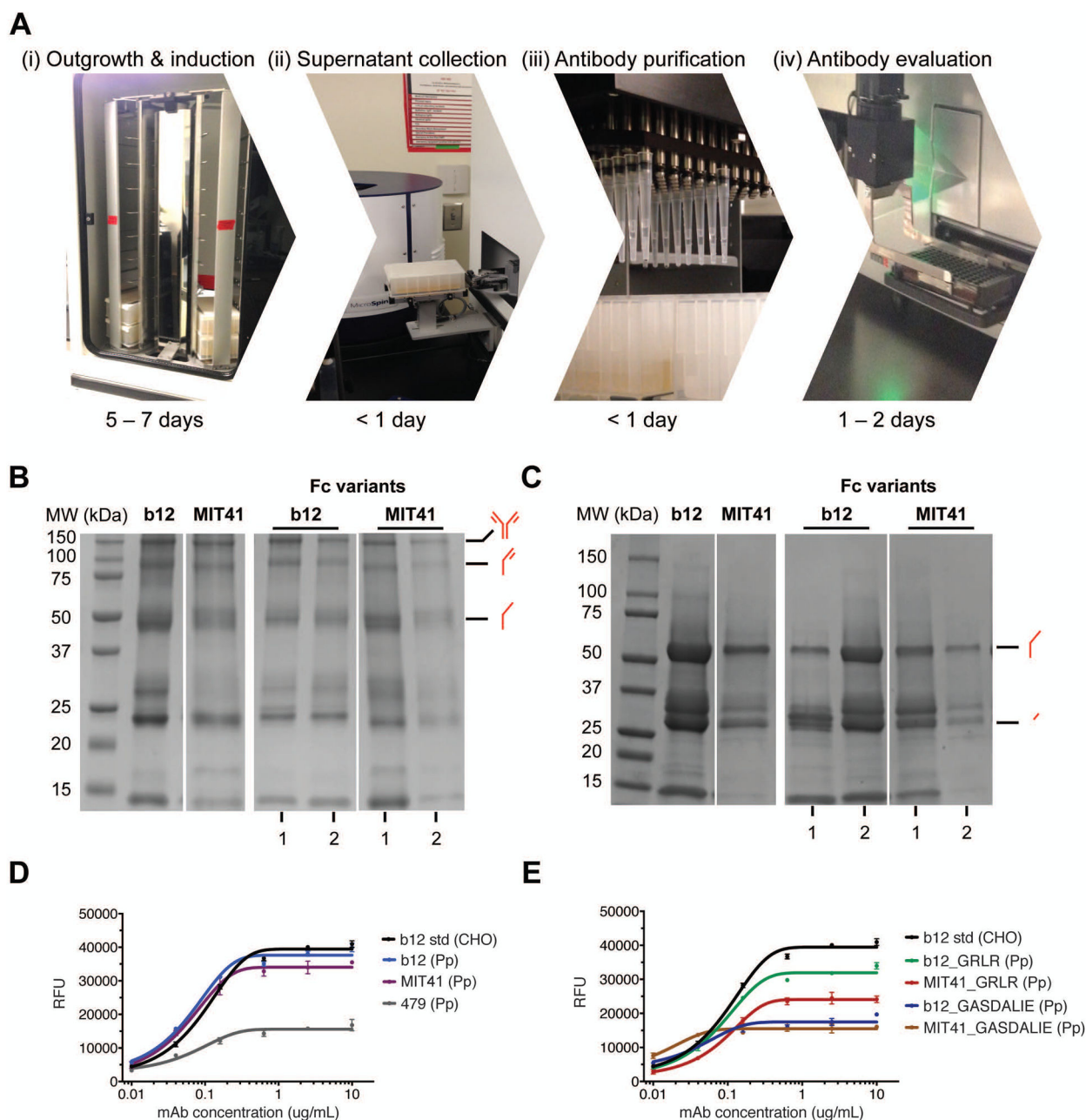


Figure 1.

Schematic of OE-PCR based construct and steps for expression of monoclonal antibodies using *Pichia pastoris*. **A:** Heavy and light chain variable regions (V_H and V_L) are incorporated into the assembly process using primers containing adapter sequences. C_H and C_L – constant region sequences for heavy and light chain, 2A – FMD virus ribosome skip sequence, P_{AOX} – alcohol oxidase promoter, KanR – G418 resistance marker. The three segments (containing light chain, heavy chain and selectable marker) are assembled together to generate the full construct. **B:** The assembled full construct is electroporated into *Pichia* where it stably integrates into the genome. Upon methanol induction, antibodies are expressed and secreted by transformed cells into the culture media.

**Figure 2.**

Stepwise assembly of an expression construct. Heavy and light chain variable regions (V_H and V_L), originating either from RT-PCR of antigen-specific antibody-secreting cells or from synthesized genes, along with other pre-amplified pieces are incorporated into segments using OE-PCR. Each of the three amplified segments are then pooled and assembled together using the same method. 3' P_{AOX} and 5' P_{AOX} are the 3' and 5' portions of the whole promoter one would obtain when cleaving with the MssI restriction enzyme. This design facilitates integration into the AOX1 locus through a single crossover event.

**Figure 3.**

Automation of antibody production pipeline and confirmation of full-length and functional mAbs using the OE-PCR approach. **A:** (i) Transformed *Pichia* strains are outgrown and induced in 96 or 24-well deep-well plates. (ii) The plates are then spun down for clarification of culture media and (iii) mAbs are purified from the supernatant using Protein A resin-containing tips on a liquid handling system. (iv) Automated ELISAs confirm production of mAb, identifying best-producing isolate as well as determining antigen binding. **B:** and **C:** SDS-PAGE of *Pichia*-derived mAbs under non-reducing (**B**) and reducing conditions (**C**). Fc variants shown here are 1 - GASDALIE and 2 - GRLR. **D:** and

E: HIV-gp120 binding curves of *Pichia*-derived (Pp) mAbs generated using automated ELISA assays for antibodies with native Fc backbone (D) or Fc variant backbone (E). Figures plotted using mean fluorescence units (RFU) and error bars show standard deviation derived from triplicate measurements.

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Table I

Comparison between methods for cloning and expression of mAbs

	Cloning-based mammalian cell derived mAbs	OE-PCR-based <i>Pichia</i> derived mAbs
Steps during construction of expression vector	PCR, gel/column purification, ligation, bacterial propagation	PCR only
Host organisms required	<i>E. coli</i> and HEK or CHO	<i>Pichia pastoris</i>
Culture media	Animal-derived	Animal-free
Viral contamination risks	High	Low (no endogenous viruses)
Genetic stability of expression vector	Transient transfection	Stable chromosomal integration
Amenable to scale up	No	Yes
Automation-friendly	No	Yes
Time from V _L & V _H genes to antibody	30 – 60 days	12 – 14 days
Cost per 100 ug of 96 antibodies (raw materials only)	> \$ 3000	~ \$ 1500
FTE requirements	Multiple	Single