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REPORT

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A platform-agnostic, function first-based antibody discovery strategy using plasmid-free mammalian expression of antibodies

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ABSTRACT

Hybridoma technology has been valuable in the development of therapeutic antibodies. More recently, antigen-specific B-cell selection and display technologies are also gaining importance. A major limitation of these approaches used for antibody discovery is the extensive process of cloning and expression involved in transitioning from antibody identification to validating the function, which compromises the throughput of antibody discovery. In this study, we describe a process to identify and rapidly re-format and express antibodies for functional characterization. We used two different approaches to isolate antibodies to five different targets: 1) flow cytometry to identify antigen-specific single B cells from the spleen of immunized human immunoglobulin transgenic mice; and 2) panning of phage libraries. PCR amplification allowed recovery of paired V_H and V_L sequences from 79% to 96% of antigen-specific B cells. All cognate V_H and V_L transcripts were formatted into transcription and translation compatible linear DNA expression cassettes (LEC) encoding whole IgG or Fab. Between 92% and 100% of paired V_H and V_L transcripts could be converted to LECs, and nearly 100% of them expressed as antibodies when transfected into Expi293F cells. The concentration of IgG in the cell culture supernatants ranged from 0.05 µg/ml to 145.8 µg/ml (mean = 18.4 µg/ml). Antigen-specific binding was displayed by 78–100% of antibodies. High throughput functional screening allowed the rapid identification of several functional antibodies. In summary, we describe a plasmid-free system for cloning and expressing antibodies isolated by different approaches, in any format of choice for deep functional screening that can be applied in any research setting during antibody discovery.

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Introduction

Therapeutic monoclonal antibodies (mAbs) have become the predominant treatment modality for various diseases, and currently more than 100 mAbs have been approved for marketing, with 19 antibody therapeutics in regulatory review and over 600 mAbs in various clinical phases.[1–4] Fully human mAbs have largely driven the rapid growth of this biopharmaceutical segment in recent years.[5] The various approaches now capable of deriving fully human antibodies include: 1) human donors who are vaccinated or convalescent patients,[6] 2) display of human antibodies from naïve, synthetic or immunized repertoires, and 3) immunization of human immunoglobulin (Ig) transgenic mice followed by conventional hybridoma.[2] In all these approaches, the development of therapeutic agents is a resource intensive multistep process that requires identification of antigen-specific antibodies, amplification of the relevant Ig genes, large-scale sequencing, plasmid generation, and expression of antibody for the assessment of specificity to the target, and, finally, verification of functional activity.

Hybridoma technology is the classic and most popular method of generating mAbs from wild type or Ig transgenic mice.[5] However, the low fusion efficiency of B cells with myeloma cells (<0.1%)[7] and time-consuming procedure pose challenges for screening, capture of large repertoire, and deep mining for rare and diverse candidate therapeutic antibodies. Fluorescence-activated cell sorting (FACS)-based single B-cell cloning (SBC) technology is becoming increasingly popular and has been developed for studying the B-cell repertoires of different species.[8–14] A major advantage of a flow-based platform is the ability to survey a large proportion of the B-cell immune repertoire and isolate dominant, as well as rare, antigen-specific clones[10] for generating a diverse array of antibodies. In addition, SBC allows the recovery of the natural cognate pairing of the antigen-specific heavy and light-chain variable regions.[15] This technology has been successfully used for the identification of broadly neutralizing influenza[9] and HIV[10] antibodies from sorted human plasma cells or antigen-specific memory B cells. In these studies, Ig variable regions were amplified and then cloned into plasmids for expression as antibodies to be screened for activity.[10,16,17] Also, the efficiency of recovery of V_H/V_L transcripts from single B cell ranged from less than 20% to about 55%.[14,16,17] Therefore, given the huge

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diversity of human Ig repertoire, the success and efficiency of recovery of Ig transcripts and reconstruction of the antibody from single B cell is critically dependent on efficiency of polymerase chain reaction (PCR) and expression strategies.

In-vitro display technologies are additional powerful strategies to discover fully human mAbs.[18] These display libraries are constructed from combinatorial pairing of heavy and light-chain variable genes isolated from human donors, immunized animals or vaccinated humans, or antibodies containing synthetic complementarity-determining regions (CDRs).[19,20] Although the antibodies isolated using display methods may sometimes require affinity maturation,[19] these libraries are a rich source of diverse therapeutic antibodies.[2]

All these platforms have been successful and complement each other in identifying therapeutic antibodies, but they also have inherent limitations. Hybridomas secrete antibodies into culture medium, which can be used for binding and activity screening.[21] However, most hybridomas have murine constant domains,[22] and therefore any functional screening needing human Fc contribution requires V-gene cloning and recombinant plasmid construction for human IgG production. If different formats or IgG isotypes are required, separate plasmids for each type need to be constructed. The SBC and the display platforms share a common limitation, requiring time-consuming reformatting, cloning and expression prior to activity screening. Both platforms rely heavily on bioinformatic analysis of the Ig sequences for triaging and cloning antibody genes into plasmids for transfection and expression.[16,17,23,24] The plasmid-based generation of recombinant mAbs (rmAbs) is labor-intensive, which limits the value of applying these technologies for rapid antibody discovery campaigns.

For the SBC platform, efforts to generate plasmid-free systems to express recombinant IgG using mammalian cells have been reported. Liao et al.[25] described a strategy for expressing antigen-specific antibodies from single B cells without cloning by using linear Ig heavy and light-chain gene expression cassettes. However, they used an adherent 293 T cell line for expressing rmAbs in a 6-well plate format,[25] which severely curtailed the throughput of antibody generation. In addition, the methodology for the Ig transcript recovery was inefficient and time-consuming, requiring multiple PCR reactions using different pooled primer sets to identify heavy and light-chain families for each individual B cell.[25] Starkie et al.[26] reported a similar strategy using the suspension Expi293F cell line for expressing recombinant mAbs in a 96-well format. Kato et al.[27] reported generation of antigen-binding fragments (Fabs) in cell free protein production systems for quick screening to assess binding to antigen. However, the expression level of the recombinant IgG was low in those studies (1 ng to 2 µg/ml), which may be sufficient for binding assays, but not for functional screening.[25,26] Similar to SBC, in phage display approaches, while the phenotype-genotype linkage helps in high throughput panning and binding-based screening, functional screening often requires reformatting of single-chain variable fragment (scFv) and Fabs into IgGs, creating a bottleneck and prolonging the time to lead antibody selection.[24,28] These studies highlight the shortage of workflows designed for simple, rapid and high throughput systems that can replace the labor intensive and time-consuming reformatting and cloning into plasmids for the expression of antibodies before assessing the specificity and function.

Here, we used flow cytometry to identify antibodies from the spleen of human Ig transgenic mice to three different antigens and used a phage display approach to isolate antibodies specific to two additional antigens. The heavy chain (V_H) and light chain (V_L) products from single B cells and phage colonies were recovered by PCR. The cognate V_H and V_L pairs were used in an overlapping PCR to construct individual linear expression cassettes (LEC), each of which contained the required elements for mRNA transcription and translation for IgG or Fab antibody expression in mammalian cells.[25] Co-transfection of heavy (H-LEC) and light (L-LEC) chain genes into Expi293F cells generated antibodies in the concentration range of 0.05 µg/ml to 145.8 µg/ml (mean = 18.4 µg/ml). The antibody concentrations in culture supernatants allowed not only for assessment of binding but also functional screening using high throughput reporter systems. The process described here provides significant advantage and impact on antibody discovery workflows and enables expression and functional evaluation of antibodies immediately after V_H/V_L isolation in about 10 days, irrespective of the platform used to derive them. We expect this new platform to have a significant impact on antibody discovery and its wide use within the field.

Results

Identification of antigen-specific cells by flow cytometry and phage clones by panning display libraries

In order to demonstrate that the utility of the LEC system is independent of the approach used to discover antigen-specific V_H/V_L pairs, we used SBC and phage display technologies to isolate antibodies to five different antigens. Antibodies specific to three different antigens, A, B and C, were derived using SBC from immunized human Ig transgenic Trianni mice.[29] The antibodies to antigens D and E were isolated in-vitro from human antibody phage display libraries. Following immunization of human Ig transgenic mice, B cells were enriched by negative selection using a Pan B-cell isolation Kit (Stem cell technologies) from single-cell suspension of spleen. Purified B cells were stained with a pool of chemically conjugated fluorescent labeled or biotinylated antigens along with a panel of B-cell specific fluorescent antibodies described in the methods section and summarized in Table 1. A systematic gating strategy was used to identify the antigen-specific B cells; Figure 1 shows the isolation of specific B cells for antigen A as an example. First, B cells were identified as $CD19^+$ population (Figure 1c) from the singlet population (Figure 1b) after excluding dead cells with 7AAD (Figure 1c). The IgM^- IgD^- B-cell population was then selected after excluding the IgM^+IgD^+ B cells (Figure 1d). As the mice used in the study were transgenic for only human V_H and V_κ (HHKK) and not V_λ, we used anti-mouse Igλ-BV711 antibody along with IgG-PE on IgM^-IgD^- cells to exclude mouse λ BCR^+ B cells and select the $IgG^+Ig\lambda^-$ B cells (Figure 1e). After ensuring that

Table 1. Panel for B cell staining.

Reagents	Fluorophore	Catalog number	Vendor
CD19	BV605	115540	Biolegend
IgD	BV650	405721	Biolegend
IgM	APC-EF780	47-5790-82	Thermo Fisher Sci
Igλ	BV711	744527	BD Bioscience
IgG	PE	ab98742	Abcam
Antigen	AF488		Home-made
Antigen	AF647		Home-made
Irrelevant Ag	AF700		Home-made
Ag_Tetramer	BV421		Home-made
Ag_Tetramer	AF647		Home-made
7AAD		00-6993-50	Thermo Fisher Sci

$IgG^+Ig\lambda^-$ B cells did not show any nonspecific binding to the AF700-conjugated irrelevant protein (Figure 1f), the antigen-specific B cells were identified as $IgG^+Ag\_AF488^+Ag\_AF647^+iAg\_AF700^-$ (Figure 1g). A very conservative gate was made to select the antigen-specific cell population bright for both fluorochromes and sort single cells into each well of a 96-well plate containing sorting buffer (Table 2). Based on our conservative gating strategy, we found that 0.09%, 0.02%, and 0.05% of all B cells from splenocytes were specific for antigens A, B, and C, respectively (Table 3).

In addition to the SBC-derived antigen-specific B cells, proprietary V_κ and V_λ Fab and scFv phage display libraries were panned to identify antibodies to two additional antigens, D and E. After three rounds of selection, 5000 colonies were screened by phage ELISA for binding to antigen D and 4000 colonies were screened by flow cytometry for binding to antigen E. These screenings resulted in a total of 1158 (23%) and 1124 (27%) human and cynomolgus monkey antigen cross-reactive clones for antigen D and E, respectively. Following sequencing, 211 and 221 sequence unique clones for antigens D and E, respectively, (Table 3) were progressed for V_H/V_L amplification and LEC generation.

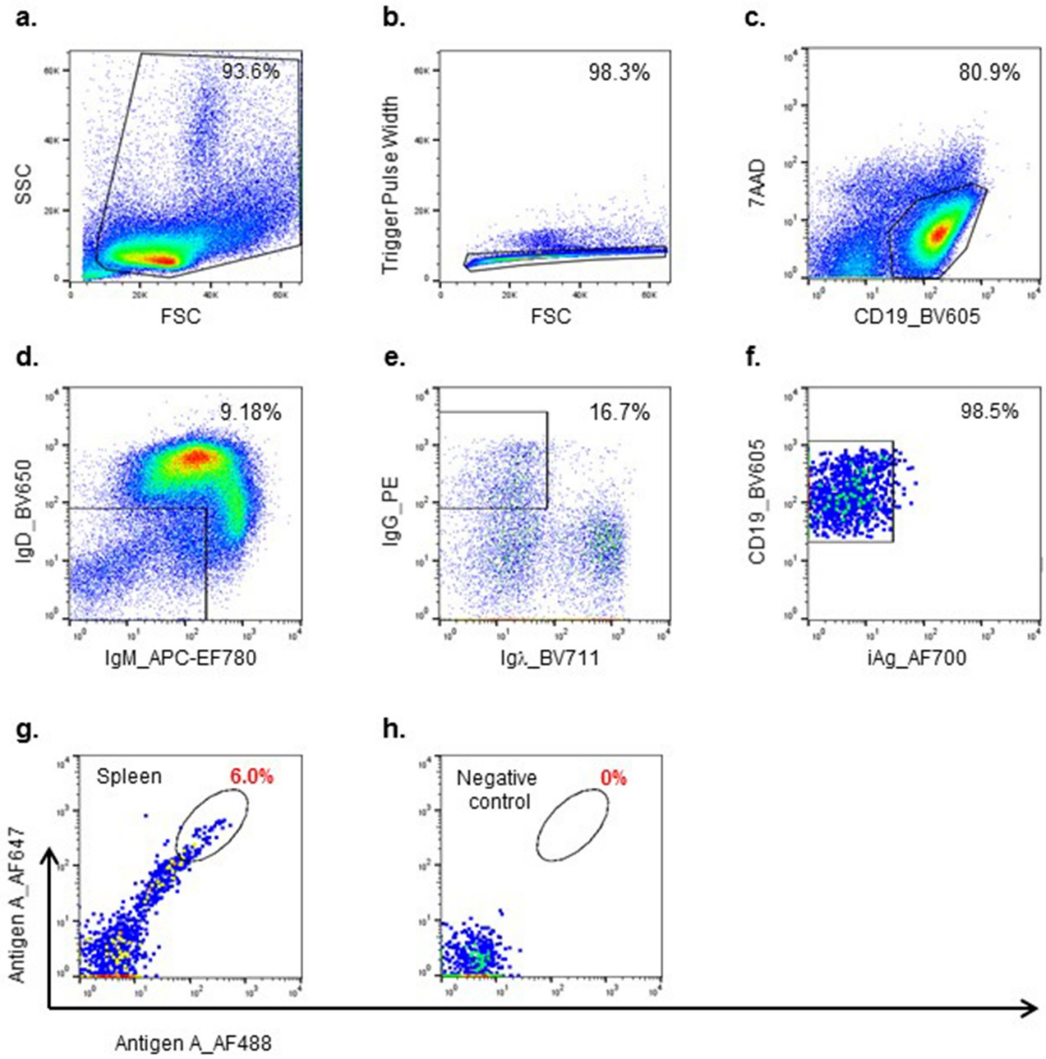

Figure 1. Polychromatic flow cytometry for sorting antigen A-specific B cells. The enriched B cells from antigen A-immunized Trianni mouse were stained with fluorophore-labeled antigens and the antibody panel (Table 1) as described in method section. Singlet cells (b) were gated from forward and side scatter (a) of all cells. From singlets gate, $7AAD^-CD19^+$ B cells (c) were selected and IgM^-IgD^- B cells were then gated (d). $IgG^+Ig\lambda^-$ B cells (e) were selected from IgM^-IgD^- B cells and after excluding cells with nonspecific binding to AF700-conjugated irrelevant antigen (iAg_AF700) (f). Antigen A-specific B cells were defined as the population of Antigen A_AF488^{bright} Antigen A_AF647^{bright} (g). The single cells were sorted into 96-well PCR plates. Splenocytes from Trianni mouse never exposed to antigen A stained with the same B-cell antibody panel and fluorophore-labeled antigens served as a control (h).

Table 2. Composition of sorting buffer.

Reagents	1X (µL)	Catalog number	Vendor
GeneLink Random Hexamer (150ng/µl)	1	26-4000-03	Gene Link
10 nM dNTP	0.6	N0447L	New England Bio
RNaseOUT (40 U/µl)	0.14	10777019	ThermoFisher Sci
DTT (100 mM)	0.6	18080085	ThermoFisher Sci
5x First-Strand Buffer	1.2	18080085	ThermoFisher Sci
Igepal (5%)	0.5	I8896-50ML	Millipore SIGMA
BSA (50 mg/ml, RNA)	0.012	AM2618	ThermoFisher Sci
DEPC treated H2O	1.748	AM9915G	ThermoFisher Sci
Single Cell	0		
Super Script III (200 U/µl)	0.2	18080085	ThermoFisher Sci
Total	6		

Table 3. Number and percentage of antigen-specific B cells and phage clones isolated.

Target	Technique	No. (%) of Antigen-specific clones	Antigen Class
Antigen A	SBC	376 (0.09%)[#]	Membrane protein
Antigen B	SBC	99(0.02%)[#]	Membrane protein
Antigen C	SBC	378 (0.05%)[#]	Secreted protein
Antigen D	Phage Display	211(19%)*	Secreted protein
Antigen E	Phage Display	221(18%)*	Membrane protein

[#]Number of antigen-specific cells sorted and percent frequency of antigen-specific B cells among all CD19+B cells

* Represents the number and percent of unique antigen-specific clones among all the phage clones binding to the target

Isolation and expression of paired V_H and V_L genes from SBC and phage panning

The antigen-specific V_H/V_L was amplified using two rounds of nested PCR from cDNA of sorted single B cells. To achieve efficient recovery of Ig V_H/V_L cognate pairs from single B cells by PCR, all primers were designed by referring to the V_H and V_κ germline sequences inserted in the Trianni transgenic mouse.[29] For the first-round PCR, the primers consisted of seven forward and two reverse primers for the heavy chain and eight forward and one reverse primer for kappa chain (Table 4). The forward primers were specific to the leader region and reverse primers to the mouse constant regions (C_H1 or C_κ). For the second-round PCR, a second set of primers, internal to the first set, consisted of nine forward primers and four reverse primers for heavy chain and a set of eight forward and one reverse primer for V_κ. Forward primers were complementary to the framework 1 regions of human V_H or V_κ and reverse primers were complementary to mouse C_H1 or Cκ (Table 5). Lambda specific primers were not used because only kappa positive antigen-specific B cells were isolated by flow cytometry (Figure 1e). Using these primer sets and the two-step PCR, we recovered V_H/V_L cognate pairs from 310 of 376 for antigen A (82%), 78 of 99 for antigen B (79%), and 361 of 378 (96%) for antigen C-specific single B cells (Table 6).

Unlike SBC, phage-derived clones required only a single round of PCR amplification because the starting template is a plasmid and not cDNA. For PCR amplification of phage clones, the second set of forward primers as described above for SBC were used for the V_H and V_κ amplification. The reverse primers, however, were different, with four primers for each of the heavy and kappa chains (Table 5). These primers annealed to the framework 4 regions. Additional 22 lambda-chain primers consisting of 16 forward primers complementary to the framework 1 region and 6 reverse primers complementary to the framework region 4 were designed for recovery of V_λ genes of antigen-specific colonies isolated from phage libraries (Table 5). Using this set of primer pools, all 211 V_H/V_L paired, unique phage clones specific to antigen D and 221 to antigen E (Table 6) were successfully amplified from the phagemids in a single PCR.

Construction of LECs

LECs encoding either the full-length IgG or the Fab portion of human antibody genes were generated by modification of a previously described method.[25] Each LEC was constructed by overlapping PCR to assemble three DNA fragments (Table 7), two were isolated from the pTT5 plasmid as described in methods, in addition to the V_H/V_L PCR products either from a single B cell or phage clone. The three fragments required for assembly were as follows: 1) a P_{CMV} fragment containing the cytomegalovirus promoter region and the human Ig κ leader sequence for the expression of Ig protein. Previous studies used mouse Ig leader sequence;[25] 2) the V_H/V_L fragment amplicons from a single B cell or phage colony as described above; and 3) the C_H or C_L fragments. The C_H fragment contained either: 1) the complete mouse or a human IgG1 constant region; or 2) only the human C_H1 region for Fabs (H-LEC). The C_L fragment contained either the C_κ (SBC and phage clones with V_κ) or C_λ (phage clones with V_λ) chain constant region genes (L-LEC). The C_H and C_L fragments also contained a rabbit beta globin (rbGlob) poly adenylation (polyA) signal sequence (Figure 2) instead of bovine poly A signal sequence as described previously.[25] Following the overlap extension PCR, H/L_LECs contain six functional elements (promoter, leader sequence, human Ig V_H/V_L gene, mouse or human Igγ/κ/λ chain constant region, transcription and translation terminators, and polyA signal sequence), all of

Table 4. Primers for the first round PCR of SBC.

Group	Primer name	Primer sequence	Tm(°C)
Heavy-chain primers	SBCVHEXF1	catggactgsacctggag	59.9
	SBCVHEXF2	catggagttkgggctgag	60.7
	SBCVHEXF3	gctgggttttccttgttgct	65.6
	SBCVHEXF4	gaatttgggctgagctgggt	67.8
	SBCVHEXF5	tcttggtgggagcagcgaca	72
	SBCVHEXF6	gagttgggactgagctgg	61.6
	SBCVHEXF7	gccaccatggacayactttg	65.1
	SBCVHEXR1	cggctcagggaagtagcc	65.3
	SBCVHEXR2	cccttgaccaggcatcc	64.8
Trianni Kappa-chain primers	SBCVKEXF1	gctcagctcctggggct	66.4
	SBCVKEXF2	tcttcctcctgctactctggc	65.2
	SBCVKEXF3	atggtgtccccgttgca	66.9
	SBCVKEXF4	gctgctaatgctctgggtc	63
	SBCVKEXF5	gctcctgctgctctggttc	65.8
	SBCVKEXF6	actcctgctgctctggctc	65.4
	SBCVKEXF7	ctcagcttcctcctcctttg	63.4
	SBCVKEXF8	ggtgttgcagacccaggt	64.3
	SBCVKEXR	gttcaggacgccattttgtc	64.8

k=g+t, s=c+g, y=c+t .

Table 5. Second set of primer for PCR of SBC and phage clones.

Group	Primer name	Primer sequence	Tm(°C)
Heavy chain forward primers	SBCVHINF1	**ctctggctccctgataccaccggt**gaggtkcagctggtgsagtctgg	72.6
	SBCVHINF2	**ctctggctccctgataccaccggt**gaggtgcagctggtggagtcygg	75.4
	SBCVHINF3	**ctctggctccctgataccaccggt**cagstgcagctgcaggagtcgg	75.7
	SBCVHINF4	**ctctggctccctgataccaccggt**cagrtcaccttgarggagtctgg	68.4
	SBCVHINF5	**ctctggctccctgataccaccggt**caratgcagctggtgcagtctgg	73.4
	SBCVHINF6	**ctctggctccctgataccaccggt**caggtccagctggtacagtctgg	69.4
	SBCVHINF7	**ctctggctccctgataccaccggt**gaggtgcagctgttggagtctgg	71.7
	SBCVHINF8	**ctctggctccctgataccaccggt**caggtacagctgcagcagtcagg	70.6
	SBCVHINF9	**ctctggctccctgataccaccggt**caggtgcagctacagcagtgggg	74.1
Heavy chain reverse primers for mouse Ig backbone	SBCVHINR1	**gagttagtttgggcagcagatc**caggggccagtggatagacagatg	71.7
	SBCVHINR2	**gagttagtttgggcagcagatc**cagggaccaagggatagacagatg	68.9
	SBCVHINR3	**gagttagtttgggcagcagatc**caggggccagtggatagactgatg	71.7
	SBCVHINR4	**gagttagtttgggcagcagatc**caggggccagtggatagaccgatg	75
Heavy chain reverse primers for human Ig backbone	SBCHUVHINR1	**gaaagacagaaggtcccttggttgaag**ctgaggagacrgtgaccagggtgcc	76.6
	SBCHUVHINR2	**gaaagacagaaggtcccttggttgaag**ctgaagagacggtgaccattgtccc	72.7
	SBCHUVHINR3	**gaaagacagaaggtcccttggttgaag**ctgaggagacggtgaccagggttcc	75.5
	SBCHUVHINR4	**gaaagacagaaggtcccttggttgaag**ctgaggagacggtgaccgtggtccc	78.1
Kappa chain primers	SBCVKINF1	**ctctggctccctgataccaccggt**gacatcbdgatgacccagtctcc	68.6
	SBCVKINF2	**ctctggctccctgataccaccggt**gacatccagwtgayccagtctcc	67.3
	SBCVKINF3	**ctctggctccctgataccaccggt**gaaattgtgytgacdcagtctcc	65.2
	SBCVKINF4	**ctctggctccctgataccaccggt**gatrttgtgatgacwcagtctcca	64.4
	SBCVKINF5	**ctctggctccctgataccaccggt**gatattgtgatgacccagactcc	63.8
	SBCVKINF6	**ctctggctccctgataccaccggt**gaaattgtaatgacacagtctccs	63.3
	SBCVKINF7	**ctctggctccctgataccaccggt**gaaatagtgatgacgcagtctcc	64.1
	SBCVKINF8	**ctctggctccctgataccaccggt**gaaacgacactcacgcagtctcc	69.6
Kappa chain reverse primers for mouse Ig backbone	SBCVKINR	ggatggtgggaagatggatac	64.6
Kappa chain reverse primers for human Ig backbone	SBCHUVKINR1	**gataaacacagaaggggctgccacagtg**cgtttgatttccaccttggtccc	67.7
	SBCHUVKINR2	**gataaacacagaaggggctgccacagtg**cgtttgatctccascttggtccc	68.0
	SBCHUVKINR3	**gataaacacagaaggggctgccacagtg**cgtttgatatccactttggtccc	63.5
	SBCHUVKINR4	**gataaacacagaaggggctgccacagtg**cgtttaatctccagtcgtgtccc	63.9
Human Lambda chain forward primers	SBCVLINF1	**ctctggctccctgataccaccggt**tcctatgwgctgacwcagccac	66.8
	SBCVLINF2	**ctctggctccctgataccaccggt**cagcttgtgctgactcaatcgc	69.6
	SBCVLINF3	**ctctggctccctgataccaccggt**cagcctgtgctgactcaatcat	66.9
	SBCVLINF4	**ctctggctccctgataccaccggt**aattttatgctgactcagcccc	65.4
	SBCVLINF5	**ctctggctccctgataccaccggt**ctgcctgtgctgactcagcccc	74.8
	SBCVLINF6	**ctctggctccctgataccaccggt**tcctatgagctgacacagccat	66.3
	SBCVLINF7	**ctctggctccctgataccaccggt**caggcagggctgactcagccac	74.8
	SBCVLINF8	**ctctggctccctgataccaccggt**cagcccgtgctgactcagccgc	79.2
	SBCVLINF9	**ctctggctccctgataccaccggt**cartctgccctgactcagccts	70.4
	SBCVLINF10	**ctctggctccctgataccaccggt**cagyctgtgctgacycagccrc	73.8
	SBCVLINF11	**ctctggctccctgataccaccggt**cagcctgtgctgactcagccaa	72.4
	SBCVLINF12	**ctctggctccctgataccaccggt**cagcctgtgctgactcagccrt	72.4
	SBCVLINF13	**ctctggctccctgataccaccggt**tcctctgggccaactcaggtgc	74
	SBCVLINF14	**ctctggctccctgataccaccggt**caggctgtggtgacycaggagc	72.8
	SBCVLINF15	**ctctggctccctgataccaccggt**tcctatgagctgacacagctac	61.5
	SBCVLINF16	**ctctggctccctgataccaccggt**tcttctgagctgactcaggacc	65.4
Lambda reverse primers for human Ig backbone	SBCHUVLINR1	**ggtgctgctttgggctggcctgcag**ctaggacggtgaccttggtccc	70.1
	SBCHUVLINR2	**ggtgctgctttgggctggcctgcag**ctaggacggtcagcttggtccc	70.2
	SBCHUVLINR3	**ggtgctgctttgggctggcctgcag**ctaggacagtgaccttggttcc	64.1
	SBCHUVLINR4	**ggtgctgctttgggctggcctgcag**ctaggacagtcagtttggttcc	62
	SBCHUVLINR5	**ggtgctgctttgggctggcctgcag**cgaggacggtcaccttggtgcc	76
	SBCHUVLINR6	**ggtgctgctttgggctggcctgcag**cgagggcggtcagctgggtgcc	81.2

(1) b=t+c+g, d=a+t+g, k=g+t, r=a+g, s=c+g, w=a+t, y=c+t.
(2) Overlapping tags are highlighted in bold.

which are necessary for antibody transcription and translation in mammalian cells. From the overlapping PCR, we generated 749 mouse IgG and Igκ LEC constructs from V_H/V_L cognate pairs. These LEC constructs consisted of 310 that were isolated from antigen A, 78 from antigen B and 361 from antigen C-specific B cells. From phage display libraries, 432 LECs of human IgG1 with either Igκ or Igλ light chains were constructed. These consisted of 211 clones for antigen D and 221 clones for antigen E (Table 3). In addition, we selected 76 V_H/V_L cognate pairs of Antigen C specific-B cells that showed antigen-specific reactivity (described below) and successfully generated Fab LEC constructs to demonstrate the adaptability of the platform (Table 8) for Fab protein expression.

Expression of rmAb from LEC DNA

All the cognate H_LEC and L_LEC pairs were co-transfected into Expi293F cells to express rmAbs. Five days post transfection, the concentration of recombinant IgG in cell culture supernatants for antigens A, D and E and the concentration of the 76 recombinant Fabs for antigen C were quantitated by biolayer interferometry (Octet). The recombinant whole IgG in supernatants of antigens B and C were not quantified. The

Table 6. Percentage of cognate VH/VL pairs recovered by PCR from B cells and phagemids.

Targets	Approach	Number of SBC*/ phagemids	% (number) of Ig cognate pairs recovered
Antigen A	Single B-cell sorting	376	82.4% (310/376)
Antigen B	Single B-cell sorting	99	78.8% (78/99)
Antigen C	Single B-cell sorting	378	95.5% (361/378)
Antigen D	Phage display	211	100% (211/211)
Antigen E	Phage display	221	100% (221/221)

*Single B cells

appropriate biosensors for quantification were selected according to antibody format. Antibodies against antigen A required the use of anti-murine IgG biosensors, while those targeting antigens D and E required the use of anti-human IgG or protein G biosensors. Quantitation of Fabs was achieved using anti C_H1 specific biosensors. To accurately monitor the antibody expression levels, the culture supernatants of cells transfected with the standard IgG plasmid were used as positive control and those of mock transfections as negative control. Additionally, the standard curves were generated using isotype matched IgG (murine or human IgG1) for each assay (data not shown).

The results of IgG and Fab expression levels in cell supernatants are shown in Figure 3. More than 90% of all LECs tested, expressed detectable levels of antibody, irrespective of the target specificity in the range of 0.05–145 μg/ml (Figure 3 and Table 8). Of 310 culture supernatants of mouse IgG LECs against antigen A, 307 (99%) expressed detectable levels of antibody with an average concentration of 17 ± 11 μg/ml (range: 0.05–54.9 μg/ml); for antigen D, 207 of 211 (98%) LEC constructs expressed human IgG and showed an average concentration of 4 ± 3 μg/ml ranging from 0.36 to 13.1 μg/ml. For antigen E, all 221 (100%) LEC constructs expressed antibodies, and the average concentration was 34 ± 23 μg/ml with a range of 0.05 – 145.8 μg/ml. Among the Fab clones against antigen C, 70 of 76 (92%) LECs expressed antibody, with an average concentration of 19 ± 5 μg/ml and a range of 9–31.8 μg/ml. While there is a wide range in the levels of antibody expression, the antibody concentrations observed in the present study are significantly higher than 1.9 ± 0.7 μg/ml[25] or 0.08–5 μg/ml[26] reported in previous studies.

The supernatants for each antigen were examined for binding specificity to the antigens A and E by flow cytometry and antigens B, C and D by ELISA. Although the recombinant whole IgG concentration of antibodies in cell culture supernatants from LECs of antigens B and C was not quantified, an aliquot of the supernatant was used to assess binding specificity to target antigens. As shown in Table 8, 298/307 (97%) IgGs against antigen A, 77/78 (99%) IgGs against antigen B, 280/361 (78%) IgGs against antigen C, 207/211 (98%) IgGs against antigen D and 221/221 (100%) IgGs against antigen E demonstrated specific binding to their respective antigens. These data illustrate that the current approach not only resulted in a very efficient recovery of Ig transcripts from SBC but also a large percentage of LECs expressed antibodies that exhibited antigen-specific binding. These results demonstrate a very significant improvement in the percentage of recovery of antigen-specific clones compared to a previous study.[26] In the majority of samples, the amount of antibodies in cell culture supernatants was sufficient to measure not only binding activity but also to assess function in high-throughput, cell-based, dose–response assays (described below). This entire process of LEC construction, expression and antibody characterization was very efficient and can be completed within a short time following V_H/V_L amplification (Figure 2). To the best of our knowledge, this study is the first report on the implementation of a plasmid-free expression system to express human IgGs and Fabs for performing deep functional screening of clones from SBC and phage panning.

High throughput functional screening of rmAbs against antigen A and D

We further determined the functional activity of antibodies against antigen A and D in high throughput cell-based reporter assays. At the time of these studies, a high throughput functional assay was not available for the remaining antigens. The functional activity of the rmAbs in transfected cell culture supernatants against antigens A and D were evaluated using Promega GloResponse™ NFkB-Luc2P/U2OS reporter

Table 7. Primers for DNA fragments and overlapping PCR.

DNA Fragments	Primers	Primer Sequences	50bp sequence of the fragment in the plasmid
PCMV fragment	F_CMV	gaccgagcgcagcgagtc	gaccgagcgcagcgagtcagtgagcgaggaagcgtacatttatattggct
	R_CMV	cggtggtatcagggagcc	cccagcgcagcttctcttcctcctgctactctggctccctgataccaccg
mulgG1_CH fragment	mulgHC_F	ggatctgctgcccaaactaactc	ggatctgctgcccaaactaactccatggtgacactgggatgcctggtcaa
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
mulgCk fragment	mulgkC_F	caactgtatccatcttcccaccatcc	caactgtatccatcttcccaccatccagtgagcagttaacatctggaggt
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
hulgG1_CH fragment	hulgHC_F	cttcaaccaagggaccttctg	cttcaaccaagggaccttctgtctttcctctggccccttcaagcaagagc
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
hulgG1_Fab fragment	hulgHC_F	cttcaaccaagggaccttctg	cttcaaccaagggaccttctgtctttcctctggccccttcaagcaagagc
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
hulgCk fragment	hulgkC_F	gcactgtggcagccccttctg	gcactgtggcagccccttctgtgtttatcttcccaccctccgacgagcag
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
hulgCl fragment	hulgIC_F	ctgcaggccagcccaaagcag	ctgcaggccagcccaaagcagcaccgtctgtgactctgttccctccgtca
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga
LECs	F_CMV	gaccgagcgcagcgagtc	gaccgagcgcagcgagtcagtgagcgaggaagcgtacatttatattggct
	IgHLC_R	tctccgagggatctcgacc	tcggaaggacatatgggagggcaaatcatttggtcgagatccctcggaga

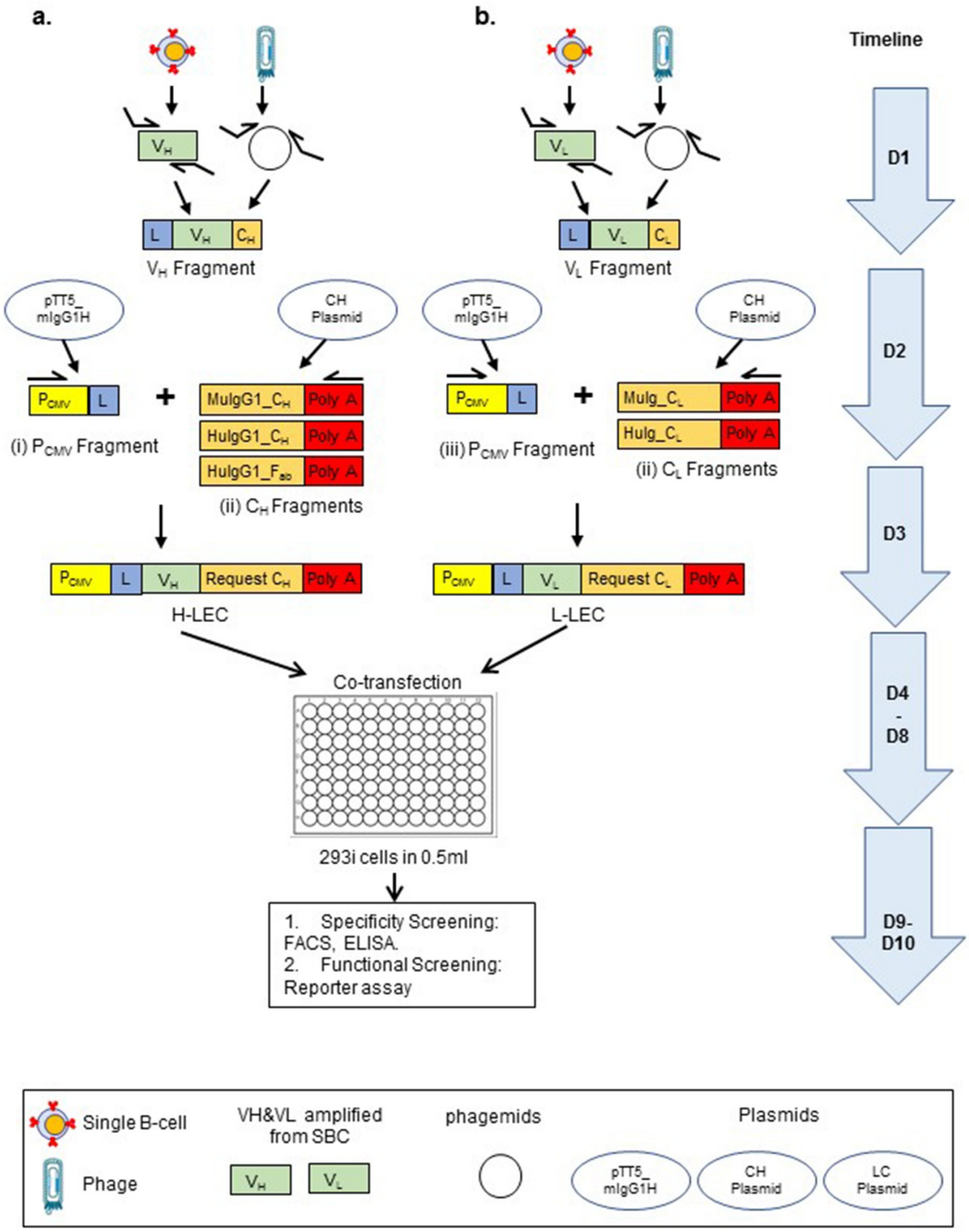

Figure 2. Schematic diagram for the construction of Ig heavy and light chain linear expression cassettes (H/L_LECs). H_LECs (a) and L_LECs (b) were constructed by assembly of three DNA fragments by overlapping PCR: 1) P_{CMV} fragment consisting of CMV promoter, enhancer and Ig leader, which was amplified from the plasmid of pTT5-mIgG; 2) C_H fragments or C_L fragments containing the Ig constant region, stop codon, and rabbit beta globin (rbGlob) polyA tail amplified from the plasmids of pTT5-mIgG1, pTT5-mIg kappa, pTT5-huIgG1, pTT5-huIgG1_Fab, pTT5-huIg kappa, or pTT5-huIg lambda; 3) V_H or V_L PCR products from sorted single B cells or monoclonal phagemids. The forward primer at the start of P_{CMV} fragment and the reverse primer at the end of rbGlob polyA in C_H/C_L fragments were used for constructing H/L_LECs by overlapping PCR. The arrows indicate the time (days) at each stage starting from PCR amplification of V_H/V_L fragments till the functional screening.

cells to identify potential antagonistic antibodies. We used an automated robotic GNF (Genomics Institute of the Novartis Research Foundation) screening system designed to load reporter cells and antibody supernatants into 384-well plates, incubate, apply reagents, read assay results, and record the data. The antagonistic activities of antibodies against antigens A and D, expressed as IC_{50} values, are shown in Figure 4. Results indicate that for antigen A (Figure 4a) the IC_{50} values of antibodies ranged from 450 pM to 1 pM and 44 antibodies showed either similar or 10–100-fold better antagonistic activity (range = 100 pM–1 pM) than the reference antibody (100 pM). Figure 4b shows at least one antibody for Antigen D with seven-fold improved (IC_{50} = 0.07 μM) and three antibodies with similar (IC_{50} = 0.46–0.54 μM) antagonistic activity compared with the reference antibody (IC_{50} = 0.5 μM). These results demonstrate that the plasmid-free expression can generate antibodies in ample quantities that enable dose-dependent functional screening for the

Table 8. Summary of LEC constructs and characterization of rmAbs for binding and function.

Antigen	Isotype	# samples	LEC construction efficiency^	% Ag specific rmAbs	% Functional rmAbs
Antigen A	mIgG1	310	99.0% (307/310)	97.0% (298/307)	77.5% (231/298)
Antigen B	mIgG1	78	100% (78/78)	98.7% (77/78)	NA¥
Antigen C	mIgG1	361		77.5% (280/361)	
Antigen C	huFab	76*	92% (70/76)	NA¥	NA¥
Antigen D	huIgG1	211	98% (207/211)	100% (207/207)	100% (207/207)
Antigen E	huIgG1	221	100% (221/221)	100% (221/221)	NA¥

*76/280 Ig pairs that showed reactivity to Antigen C were reformatted to Fabs
¥ No data available.
^Based on the number of LECs expressing antibodies exhibiting target-specific binding and measurable protein by Octet (Figure 3)

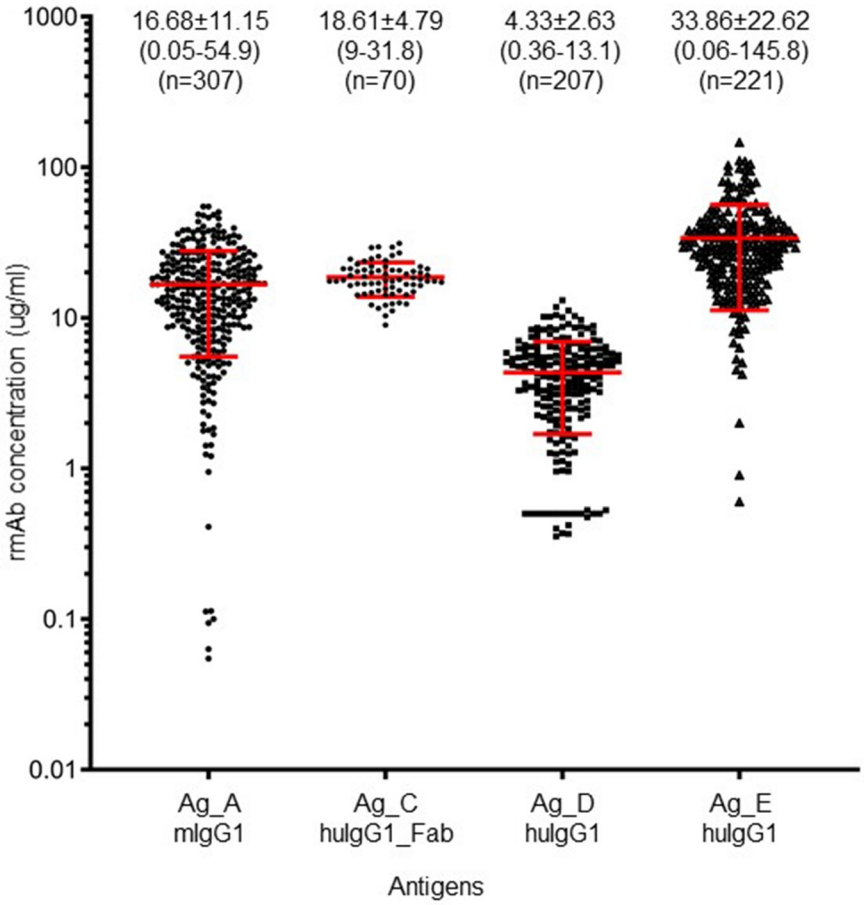

Figure 3. Protein concentration of rmAbs in the transient transfection supernatants. Antibody concentration in 450 µL culture supernatants of Expi293 cells transiently transfected with H/L-LEC pairs were quantitated by Octet. The concentrations of antigen A (Ag_A) with murine IgG1 backbone were measured with anti-murine IgG Quantitation Biosensors. The concentrations of antigen C (Ag_C) with human IgG1 Fab backbone, antigens D (Ag_D), and E (Ag_E) with human IgG1 backbone were detected using the appropriate biosensors. The red bars indicate mean and standard deviation in each group. The mean ± standard deviation along with the concentration range and the number of samples are presented on top of each group.

triaging of antibodies based on function rather than binding alone. This ability to triage the best antibodies to only a small number of antibodies for antigen A (44/387) and antigen D (4/211) for plasmid cloning and large-scale expression for additional analysis further highlights the value of the LEC platform in saving cost, time and resources for the overall process of antibody discovery.

Discussion

For antibody discovery, display methodologies and single B-cell selection approaches are widely used and complement the conventional hybridoma method.[1,2] Microfluidics is an evolving technology that is also gaining traction and being developed by investigators both in academia and the biopharmaceutical industries.[30] Despite these impressive advancements, a lingering problem that continues to plague the field is the inability to retrieve V_H/V_L cognate pairs efficiently[26,30] and the lack of strategies that enable identification of the desired hits in the final format from the beginning of the discovery process.[26,31] More importantly, the expression of the cognate V_H and V_L chains for activity screening is a bottleneck, requiring time-consuming, and sometimes cost-prohibitive, plasmid construction and high throughput functional

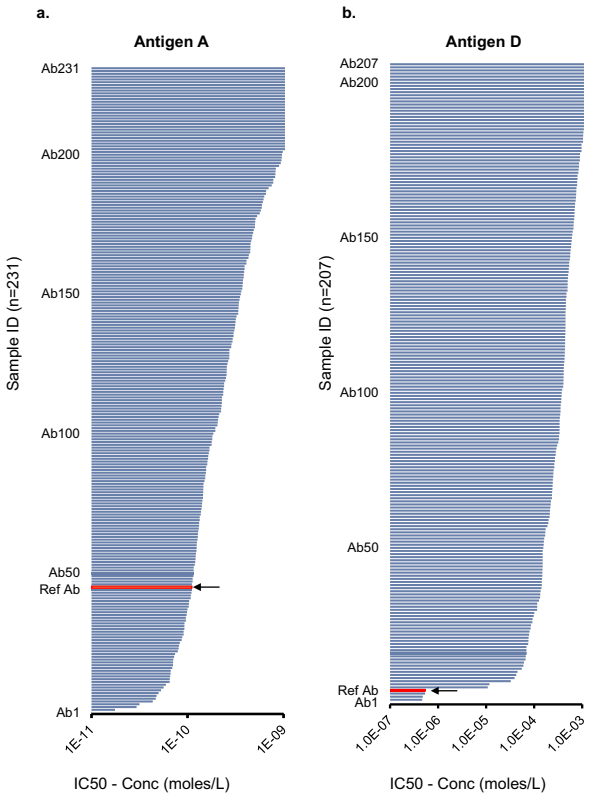

Figure 4. Antagonistic activity of rmAbs measured by high throughput reporter assay. IC_{50} values of rmAbs in culture supernatants of antigens A and D along with reference antibodies were determined using a Promega reporter cell assay with robotic automated high throughput GNF system. 44/231 antibodies against antigen A (panel a), and 4/207 antibodies against antigen D (panel b) show IC_{50} values better than the reference antibodies (red lines indicated by arrows).

screening. In this study, we addressed this important issue and created plasmid-free DNA expression cassettes (LECs) of antigen-specific cognate V_H/V_L gene fragments isolated by either single B-cell cloning or phage library panning. We expressed the LECs to obtain rmAbs in quantities sufficient to screen for binding and high-throughput functional screening. This entire process was relatively fast and could be completed within 10 days (Figure 2).

Amplifying the Ig V_H/V_L genes from single memory B cells can pose challenges because the numbers of mRNA copies in memory B cells are much lower than those of antibody-secreting cells (plasma cells or plasmablasts).[32,33] To increase the recovery of cognate V_H/V_L genes by PCR, we designed two sets of degenerate primers for both Ig V_H and V_L genes (Tables 4 and 5) by referring to the human germline gene sequences. These primers were applied to both antigen-specific single memory B cells isolated from human Ig transgenic mouse and the antibodies from human Fab and scFv phage display libraries. This strategy yielded a very high percentage of cognate Ig V_H, V_L pair recovery, especially for SBC, ranging from 79% to 96% (Table 6) and a majority (78–99%) exhibited antigen-specific binding (Table 8) after expression in mammalian cells. This contrasts with an earlier study where the PCR recovery of cognate V_H, V_L was only 33% and 53%, respectively, for two different antigens from B cells obtained from immunized wild-type mice. The overall specificity of recombinant IgGs was also low, with only 26% and 39% of the sorted antigen-specific B cells demonstrating antigen-specific binding, respectively.[26] In another study on immunized human Ig transgenic rats, Ouisse et al.,[23] using only a small number of antigen-specific B cells (7 to 72 cells) to five different antigens, rescued 20–86% of cognate V_H, V_L pairs, and expressed antibody using plasmid-based expression. However, the recombinant antibodies showing antigen-specific binding were very low, ranging in number from 1 to 27.[23] In a third study where an additional mRNA amplification step was introduced to increase the amount of mRNA by more than 80-fold, Franz et al. recovered only 50% of Ig pairs from memory B cells from human blood sample, all of which were antigen-specific, thereby giving an overall efficiency of only 50%.[34] In this study, we reproducibly achieved high efficiency of PCR recovery of cognate V_H and V_L and antigen-specific binding of recombinant IgGs from the sorted B cells for all the three targets.

Following amplification of cognate V-domain genes, H/L_LEC constructs were constructed in a way that contained all the necessary elements for mRNA transcription and corresponding protein expression. LECs, used with limited success, have been described previously.[25] Here, we used a similar strategy, but with substantial modifications that enable their successful application to antibody discovery process. We replaced the mouse Ig Vκ and the bovine growth hormone (BGH) polyA signal, as described in a previous report,[25] with human Ig Vκ leader sequence and the more efficient rbGlob polyA[35] to increase the yield of antibody. We show that LEC construction and expression provide the versatility and flexibility needed to adapt to a range of antibody isotypes from any species, formats (whole IgG or Fabs). It is conceivable that Fc mutants for effector and other functions can also be introduced during LEC construction and tested in functional screening. Since the LEC assembly offers flexibility to use V_H, V_L PCR products derived from any discovery platform and create antibody of any isotype of choice (Figure 2), we generated hundreds of expression-compatible linear DNA from cognate pairs of transcripts derived from single B-cell sorting and phage display libraries against five different target antigens. Some were human IgG1 Fab constructs, while others contained either a full-length mouse or human IgG1 constant regions (Table 8). The efficiency of LEC construction and expression was assessed based on the measurable levels of antibody in the culture supernatant by Octet quantitation, as well as target-specific binding by ELISA or flow cytometry.

The overall rmAb concentration in cell culture supernatants ranged from 0.05 to 145 μg/ml with an average range of 4.3–33.9 μg/ml (Figure 3), which is significantly higher (2– 17-fold) than the previous reports where the average expression level of transfection supernatants was 1.9 ± 0.7 μg/ml,[25] with concentrations ranging between 0.08 and 5 μg/ml.[26] Thus, in contrast to previous studies, this study used a much larger number of samples, using several different antigens and discovery platforms, such as flow cytometric isolation of antigen-specific single memory B cells and phage libraries. Nearly 100% of IgG1 LECs and 92% of Fab LECs successfully expressed antibody, with almost all showing binding specificity to their target antigens. For antigen C, only 78% of the antibodies were

specific (Table 8). The relatively low recovery of antigen-specific mAbs from antigen C was most likely a result of nonspecific staining of B cells during cell sorting. The nonspecific binding of streptavidin-fluorophore dyes may have resulted in some level of nonspecific selection of B cells,[34] leading to a relatively lower percentage of antigen-specific mAbs isolated by the tetramer staining compared with direct chemical labeling of antigens. Overall, after LEC transfection, the percentage of culture supernatants showing high level of IgG and antigen specificity are significantly higher in this study compared to an earlier report using similar technology.[25] We believe that our study is the first to report: 1) on the application of LEC to phage platform derived cognate V_H, V_L pairs for rapid high throughput expression of recombinant IgGs; and 2) the ability to perform deep functional screening of antibodies without having to perform resource-intensive plasmid construction. The overall expression levels and the recovery of antigen-specific mAbs reported here were substantially increased, demonstrating that our methodology provides significant improvement over previous methods.[23,26]

A major requirement in biologics drug development is the identification of several lead antibodies with appropriate functional characteristics. It is important to develop, establish and utilize systems that can deep screen for functional activity of a large number of clones. The ability to quickly generate whole IgGs, or their fragments, of any isotype and in sufficient quantities in a high throughput manner opens the way for deep functional screening rather than binding alone during the selection of appropriate antibody leads. It is not difficult to envisage that coupling the LEC platform with automated functional screening would transform the practice of functional antibody discovery. For the single B-cell selection approach using flow cytometry, the entire process starting from the preparation of single-cell suspension to the functional screening of antibodies can be completed in less than 2 weeks after immunization is completed (Figure 2). Our study also demonstrates that the LEC platform is also applicable to other antibody discovery platforms, including phage display, and can be completed on a similar timeline as SBC.

Taken together, our study demonstrates the utility of LEC to rapidly generate therapeutic antibodies and triage them based on function rather than binding, which is relevant in research and commercial settings. The ability to bypass generation of a large number of plasmids is a tremendous process improvement that offers cost and time savings in the identification of therapeutic antibodies. This method would be of great utility in the discovery of biologically active antibodies during pandemic situations, such as occurred with COVID-19.

Materials and methods

Mice and immunization

The creation of Trianni human Ig transgenic mice was previously described.[29] Trianni mice have the mouse immunoglobulin V_H, D_H and J_H, V_κ and J_κ gene segments replaced by their human counterparts (HHKK). The mouse endogenous V_λ and J_λ gene segments are retained along with the heavy and light chain constant regions. All experiments were approved and conducted in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) approved animal facility in accordance with the guidelines of Sanofi Genzyme Institutional Animal Care and Use Committee. Cohorts of 15 mice per group were immunized with 100 μg of antigen A, B, or C by intraperitoneal injection five to seven times in two-week intervals. Sera were collected after third and last immunization to assess the antibody titers. The mice were rested for 3 weeks and boosted 4 days before harvesting spleen samples for processing.

Preparation of fluorochrome conjugated antigens

To stain antigen-specific B cells for sorting, we prepared fluorochrome-labeled antigens that were either human Fc fused or biotinylated via avi-tag. The human Fc fused proteins were labeled either with Alexa Fluor (AF) 488 (Thermo Fisher Scientific, A20000) or Alexa Fluor (AF) 647 (Thermo Fisher Scientific, A20006), and an irrelevant antigen (iAg) labeled with Alexa Fluor (AF) 700 (Thermo Fisher Scientific, A20010) by chemical conjugation following the manufacturer's instructions. After the conjugation, the residual free dye was removed using 7K MWCO, Zeba Spin Desalting Column (Thermo Fisher Scientific, 89889). The concentration of the labeled protein and the degree of labeling were determined by measuring the absorbance of protein (A280) and dye at A495 for AF488, A650 for AF647, and A702 for AF700 with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, nd-2000). The labeled antigens could be stored for 3 months at 4°C in the dark. Avi-tag biotinylated antigens were tetramerized with streptavidin-fluorophores following a modification of a previously described procedure.[34,36] In brief, the avi-tag biotinylated proteins were mixed with Streptavidin-BV421 (Biolegend, 405225) or Streptavidin-AF647 (Biolegend, 405237) at a molar ratio of 4 biotinylated antigen molecules to 1 molecule of the dye-conjugated streptavidin. To maximize the yield of tetramers, streptavidin dyes were added to the biotinylated proteins over three 10-minute intervals and incubated for additional 15 minutes at 4°C in the dark. All tetramers were freshly prepared for each experiment.

Fluorescence-activated cell sorting

Antigen-specific single B-cell sorting was performed using splenocytes. Spleens were harvested from immunized transgenic mice 4 days post final boost. Total B cells from the single-cell suspension of splenocytes were enriched with EasySep™ Mouse Pan-B-cell Isolation Kit (STEMCELL Technologies, 19844) following the manufacturer's instructions. The enriched B cells were washed once and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). 20×10^6 enriched B cells were stained with predetermined concentrations of the fluorochrome conjugated antigens, and a 50-fold excess of irrelevant antigen_AF700, followed by the B-cell antibody cocktail (Table 1) containing rat anti-mouse CD19-BV605 (Biolegend, 115540), rat anti-mouse IgD-BV650 (Biolegend, 405721), rat anti-mouse IgM-APC-eFluor 780 (Thermo Fisher Scientific, 18080085), rat anti-mouse Igλ1, λ2, & γ3-BV711 (BD Bioscience, 744527), and goat $F(ab)_2$ anti-mouse IgG-PE (Abcam, ab98742). After staining, the cells were washed twice

with PBS containing 1% BSA and re-suspended into 0.5 ml of PBS containing 1% BSA. 5% v/v of 7AAD viability staining solution was added to the cell slurry just before loading the samples into the BD influx sorter for analysis and cell sorting. Antigen-specific B cells from the spleen, which were identified as $7AAD^{-}CD19^{+}IgM^{-}IgD^{-}IgG^{+}Ig\lambda^{-}iAg^{-}Ag\_dye1^{+}Ag\_dye2^{+}$, were individually sorted into wells of a 96-well PCR plate (Bio-Rad, HSP9601) containing 6 µl of sorting buffer, containing the reverse transcriptase reaction mixture (Table 2).

Phage panning for antigen-specific antibodies on human Ig libraries

Panning was performed against antigens D and E using two proprietary human Fab and scFv antibody libraries. Antigen immobilization on polystyrene beads, selections, and washing steps were modified from the previously published method[37] for automation on a Kingfisher 1 mL (Thermo Fisher Scientific, A31508) for three rounds of panning at room temperature. Dynabeads M-280 Streptavidin beads (Thermo Fisher Scientific, 11205D) captured the biotinylated antigens (constant at 200 nM) or the depletion reagents. The depletion reagents were irrelevant proteins containing identical purification tags as the target antigen of interest. In the first round of selection, 10^{12} phages from the phage libraries were depleted against the streptavidin beads and depletion reagents for 30 minutes. The depleted phage libraries (~10^{11}) were then incubated with the target antigen of interest for 60 minutes. Phage bound to the immobilized antigen on the streptavidin beads were washed six times with PBS containing 0.05% Tween20 pH 7.2 followed by 2 washes with PBS. The antigen-bound phages were eluted using 100 mM triethylamine (Millipore Sigma, 471283) for 8 minutes, immediately quenched with 1 M Tris-HCl pH 8.0 (Teknova, T5080) and used for infection. The panning outputs were amplified using M13KO7 helper phage for subsequent rounds of panning (~10^{12} phage per panning). The rounds 2 and 3 were titrated and single colonies were isolated and cultured in 96-well plates (Falcon) for small-scale phage rescue and culture (~150 µl) for use in binding assays. Monoclonal phages with specific binding to antigens D and E were sequenced. The phage clones were selected for quality and uniqueness using Sequencher v5.4.6 (Gene Codes Corporation) and a Sanofi proprietary sequence analysis software called Pipeline pilot and Microsoft Excel Macros, respectively. Unique antibody clones were selected for further characterization.

Isolation of Ig heavy and light chain variable genes from SBC and phage clones

Ig V_H and V_L genes were amplified from single B cells by reverse transcription (RT) reaction and nested PCR following a modification of a previously described method.[25] cDNA was synthesized in a 6 µl reaction mixture with Superscript III reverse transcriptase (Thermo Fisher Scientific, 18080093) primed with random hexamer (Gene Like, 26-4000-03) by incubating at 55°C for 5 minutes, 20°C for 10 minutes, 55°C for 30 minutes. The first-round of PCR (PCR1) of V_H and V_L were carried out in a 25 µl reaction mixture containing 2 µl of RT reaction products, 12.5 µl of AmpliTaq Gold 360 Mastermix (Thermo Fisher Scientific, 4398881), and a pool of primers, each at a concentration of 0.1 µM (Table 4). The first-round of PCRs for V_H and V_L were performed at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. One µl of the first-round PCR product was used as the template for the second-round PCR (PCR2) and was carried out in a 25 µl reaction mixture containing 12.5 µl of AmpliTaq Gold 360 Mastermix, and the nested primer pool each at a concentration of 0.1 µM (Table 5). The PCR was performed using the same conditions as PCR1 except the annealing temperature was 58°C.

For phage-derived clones, glycerol stocks containing TG1 bacteria harboring phagemids with unique Fab and scFv sequences with the reactivity of targets D and E were re-arrayed into 96-well suspension culture plate (Griener) containing 2xYT media (Teknova, Y2140) supplemented with 2% glucose (Teknova) and 100 µg/mL ampicillin (Sigma, A5354) using a QPix460 colony picker. These cultures were grown to confluence in Innova44 shakers at 37°C at 220 RPM. Cultures were diluted by 1:10 in UltraPure Water (Thermo Fisher Scientific, 10977023) and incubated at −80°C for 2 hours. The master mix of 10 µL/well for human VH/VL amplification contained 2 µL of the diluted bacterial culture, 5 µL of AmpliTaq mastermix, 2.8 µL UltraPure Water and the pool of primers for the human heavy and light-chain genes listed in Table 5, each at a concentration of 0.1 µM. The PCR was performed using the same conditions as PCR2 described above.

The PCR products were viewed using 2% agarose 96-well E-gel (Thermo Fisher Scientific, G720802) with a 100-base pair (bp) ladder and purified using the enzymatic method with ExoSAP-IT (Thermo Fisher Scientific, 78200) for 1 minute at 37°C and 15 minutes at 85°C prior to storage at 4°C or −20°C. Following PCRs, the excess primers were removed with ExoSAP-IT according to manufacturer's instructions. The DNA concentration of the cleaned PCR2 products was determined using nanodrop1000 (ThermoFisher Scientific) and adjusted to 10 ng/µl. The samples were used for Sanger sequencing and construction of LECs as described below.

The second set of forward and reverse primers used for PCR2 of SBC and phage contained sequence tags overlapping with the leader sequence at 3'-end of P_{CMV} fragment and 5' end of IgG1_HC or IgLC fragment (Figure 2 and Table 5), respectively, for generation of LEC. The details of the construction of P_{CMV} and IgG1/IgL constant fragments, sequence tags to the V_H/V_L PCR products from SBC and phage, and the final assembly of LECs are described in sections below.

Construction of linear expression cassettes

Primers and DNA fragments for construction of LECs

The plasmids of pTT5-mIgG1H, pTT5-mIg kappa, pTT5-HuIgG1H, pTT5-HuIg kappa, pTT5-HuIg lambda, and pTT5-HuIgG1Fab, constructed and kept by Sanofi US, were used as templates to prepare the DNA fragments for constructing heavy- and light-chain LEC (H_LECs/L_LECs) (Figure 2 and Table 7). The DNA fragment (P_{CMV} fragment) composed of CMV promoter and Ig leader sequence (human IgκV3

leader: MEAPAQLLFLLLLWLPDTTG) was amplified from the plasmid pTT5-mIgG1H with the primers F_CMV (5'-gaccgagcgcagcgagtc-3'), and R_CMV (5'-cggtggtatcagggagcc-3') by using AccuPrime™ Pfx DNA Polymerase (Thermo Fisher Scientific, 12344032). The P_{CMV} fragment is common for both H-LEC and L-LEC. The DNA fragment containing mouse IgG1 constant region, stop codon and rabbit beta globin (rbGlob) poly (A) tail (muIgG1_C_H fragment) was amplified from the plasmid, pTT5-mIgG1H, using the primers, muIgHC_F (5'-ggatctgctgcccaaactaactc-3'), and IgHLC_R (5'-tctccgagggatctcgacc-3'). The DNA fragment of mouse Igκ constant region and rbGlob poly (A) tail (muIg$C_κ$ fragment) was amplified from the plasmid of pTT5_mIg kappa using the primers muIgκC_F (5'-caactgtatccatcttcccaccatc-3'), and IgHLC_R (5'- tctccgagggatctcgacc-3'). The DNA fragment containing human IgG1 constant region or human IgG1 Fab constant region and rbGlob poly (A) tail (huIgG1_C_H fragment or huIgG1_F_{ab} fragment) were amplified from the plasmid of pTT5-huIgG1H or pTT5-huIgG1_Fab with the same primer pair, huIgHC_F (5'-cttcaaccaagggaccttctg-3'), and IgHLC_R (5'- tctccgagggatctcgacc-3'). The DNA fragment containing human Igκ constant region, stop codon and rbGlob poly (A) tail (huIg$C_κ$ fragment) was amplified from the plasmid of pTT5_HuIg kappa with the primers, huIgκC_F (5'-gcactgtggcagccccttctg-3'), and IgHL(k)C_R (5'- tctccgagggatctcgacc-3'). The DNA fragment containing human Igλ constant region, stop codon and rbGlob poly (A) tail (huIg$C_λ$ fragment) was amplified from the plasmid of pTT5_HuIg Lambda with the primers huIgλC_F (5'-ctgcaggccagcccaaagcag-3'), and IgHLC_R (5'- tctccgagggatctcgacc-3'). The PCR was carried out in a total volume of 50 µl with 1 unit of AccPrime Pfx DNA Polymerase, 5 µl of 10X Pfx AccuPrime PfxReaction mix, 1 ng of plasmid, 10 pmol of each primer at 95°C for 3 minutes followed by 27 cycles at 95°C for 17 seconds, 61°C for 30 seconds, 68°C for 80 seconds, and a final elongation step at 68°C for 2 minutes. The DNA fragments were purified by gel electrophoresis (ThermoFisher) and QIAquick Gel Extraction Kit (Qiagen). The primers F_CMV (5'-gaccgagcgcagcgagtc-3') and IgHLC_R (5'- tctccgagggatctcgacc-3') were also used for constructing H/L_LECs as described below.

Sequence tags for overlapping PCR to generate H_LECs/L_LECs

To construct H/L_LECs by overlapping PCR, heavy and light chains from SBC and phage were amplified with tagged primers to allow addition of short DNA tag sequences to 5' and 3' ends of Ig V_H/V_L PCR products. These sequence tags were complementary to P_{CMV} fragment or Ig constant region DNA fragments (Table 7) for creation of human IgG1/κ or IgG1/λ whole or Fab fragments, mouse IgG1/κ constructs. The sequence tag at 5' end of all V_H/V_L PCR products contained the same 24 nucleotides sequence (5'-ctctggctccctgataccaccggt-3') that was complementary with the 3' end of the P_{CMV} fragment. However, the sequence tag at the 3' end of V_H/V_L PCR products was different and contained one of the following five sequence tags depending on whether it was a mouse or human heavy/light chain LEC: **1)** a 22 nucleotide sequence (5'-gagttagtttgggcagcagatc-3') at the 3'end of V_H which was complementary with 5' end of muIgG1_C_H for creating H_LEC coding for mouse IgG1; **2)** a 27 nucleotides sequence tag (5'-gaaagacagaaggtcccttggttgaag-3') at the 3' end of Ig V_H PCR fragment complementary with 5' end of huIgG1_C_H/huIgG1_F_{ab} fragment for the construction of H_LEC coding either a full-length or Fab human IgG1 heavy chains; **3)** a 21 nucleotide sequence (5'-ggatggtgggaagatggatac-3') at the 3'end of VL PCR fragment complementary with 5' end of muIg$C_κ$.3' for making L_LEC coding for $C_κ$ chains; **4)** a 29 nucleotides sequence (5'- gataaacacagaaggggctgccacagtgc-3') at the 3' end of Ig $V_κ$ PCR fragment complementary with 5' end of huIgC for L_LEC coding for human $C_κ$; **5)** a 25 nucleotides sequence (5'-ggtgctgctttgggctggcctgcag-3') at the 3' end of Ig $V_λ$ PCR fragment complementary with 5' end of huIg$C_λ$ for building L_LEC coding $C_λ$.

Assembly of VH (H_LECs) and VL (L_LECs) into linear expression cassettes

The cleaned amplicons of Ig V_H and V_L from single B cells or monoclonal phagemids were used to construct heavy chain and light chain LEC by overlapping PCR following the method previously described.[25] In brief, 5–20 ng of cleaned V_H or V_L PCR2 product was used in a 50 µl PCR reaction containing 1 unit of KOD Hot Start DNA polymerase (Millipore Sigma, 71086), 10 µmol of dNTP, 50 µmol of MgSO4, 10 pmol of each primer, 10 ng of P_{CMV} fragment, 10 ng of C_H fragment or C_L fragment (Figure 2). Relevant C_H fragments were used to assemble H_LECs for expression of mouse IgG1, huIgG1, or huIgG1_Fab (Figure 2). PCR cycles consisted of one cycle at 95°C for 2 minutes followed by 27 cycles of 95°C for 20 seconds, 62°C for 12 seconds, 70°C for 70 seconds, and a final elongation step at 70°C for 2 minutes. H-LEC and L-LEC amplicon generation was confirmed by running a sample of PCR products in agarose gels, and the LECs were purified using MinElute 96 UF PCR Purification Kit and eluted in 50 µl of ultrapure H_2O. DNA concentration was determining using nanodrop1000 (Thermo Fisher Scientific) and adjusted to 100 ng/µl

Expression of recombinant antibodies

Purified cognate pairs of H_LEC and L_LEC were co-transfected using a concentration of 250–500 ng of each into 0.5 ml of Expi293F cells at 3×10^6 cells/ml (Thermo Fisher Scientific, A14528) in 96 deep-well plate (USA Scientific, 1896–2110) with ExpiFectamine 293 Transfection Kit (Thermo Fisher Scientific, A14524) following the manufacturer's recommendation and incubated at 37°C, 8% CO_2 with shaking at 900 rpm. Five hundred nanogram of heavy-chain and light-chain plasmids (in the vector of pTT5) of the reference antibody was transfected into two wells as the positive control. Two mock transfection wells with transfection reagents only were set as negative control. After 5 days, the cell culture supernatants were harvested and immediately used for assessment of antibody quantification, binding to antigen, and functional screening.

The concentration of the antibodies in each LEC supernatant was determined using biolayer interferometry on an Octet RED96 or QK384. Anti-mouse IgG (AMQ), anti-human IgG (AHC), Protein A (Pro A) or anti CH1 (FAB2B) biosensors (ForteBio) were used to quantify murine IgG,

human IgG or human Fabs. The QK384 was used if more than 96 antibodies were investigated in a single assay run. The LEC supernatants were diluted 1:2 in Octet running buffer containing 1x PBS pH 7.4 supplemented with 0.1% BSA (Sigma, A3294) and 0.02% Tween-20 (Sigma, 655204) to a final volume of 200 µL in 96-well, polypropylene black flat-bottom plates (Grenier Bio-one). If the QK384 was used, the final volume was 100 µL of each supernatant in identical plates in a 384-well format. The biosensors were pre-incubated with 200 µL of Octet buffer for 30 minutes at room temperature in 96-well, polypropylene black flat-bottom plates prior to the assay run for proper biosensor hydration. The supernatants derived from mock and reference plasmid controls were used as negative and positive controls, respectively. Prior to data collection, both the antibody and biosensor plates were incubated for 10 minutes at 30°C while shaking at 1000 RPM. In the Octet Data Acquisition v11 software, the binding rate of each LEC supernatant to the biosensors was measured using the quantitation without regeneration method for a load time of 120 sec at 30°C and shaking at 1000 RPM. In addition, a standard curve of a 1:2 serial dilution from 300 to 0.5 µg/mL of a purified murine IgG, human IgG or human Fab reference antibody was included for each assay. The evaluation was performed in the Octet Data Analysis software v11.1 to determine the initial binding rates for all samples, including the standard curve. Antibody concentration was calculated based on the reference antibody standard curve prepared in Octet diluent.

ELISA screening of rmAbs for antigen-specificity

The rmAbs in cell culture supernatants were screened either by flow cytometry or ELISA to assess antigen-specificity. HEK293 cells stably expressing the antigens were cultured in a high glucose DMEM medium (Thermo Fisher Scientific, 11965092) supplemented with 10% of Fetal Bovine Serum (Thermo Fisher Scientific, 10100147). 1×10^5 cells were stained with 50 µl of culture supernatant containing mAbs and incubated for 30 minutes at 4°C. The cells were washed in PBS containing 1% BSA and stained with Alexa fluor 488-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific, A32728) or Alexa fluor 647-conjugated goat anti-human IgG (H + L) (Thermo Fisher Scientific, A21445) for an additional 30 minutes at 4°C. Following a final wash, flow cytometric acquisition was performed using BD FACS Canto II (BD Biosciences) and data analyzed using Flowjo 10.6 software (Becton, Dickinson and Company).

For screening by ELISA assay, 96-well ELISA plates were coated with 2 µg/ml of antigen in PBS overnight at 4°C. The following day, plates were washed three times with TPBS before being blocked with PBS containing 1% BSA. 50 µl of the culture supernatant was added to each well separately. One µg/ml of commercially available antigen-specific antibody was used as positive control and PBS as a negative control in each plate. The plates were incubated at 37°C for 4 hours and washed three times with TPBS. Horseradish peroxidase-conjugated goat anti-mouse IgG (1+2a+2b+3) Fcγ (Jackson ImmunoResearch, 115-035-164) or goat anti-human IgG Fc cross-adsorbed (Thermo Fisher Scientific, H10007) was used as a secondary antibody at a dilution of 1:2000 and incubated for 2 hours at 37°C. After washing for three times with TPBS, the plates were developed with TMB substrate (KPL, 50-76-00) for 5 minutes and the reaction was stopped by stop reagent (VWR, 100359-300). The plates were read at 450 nm on a Perkin EnVision microplate reader (PerkinElmer, 2105-0010). Samples that showed greater than 3-fold absorbance than the negative control at 450 nm were considered positive for antigen binding.

Functional screening using high throughput reporter assay

Promega GloResponse™ NFkB-Luc2P/U2OS reporter cells (Promega Corporation) were grown under standard cell culture conditions using McCoy's 5A medium (Thermo Fisher Scientific, 16600082) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, 10082147) and 200 mg/ml Hygromycin B (Thermo Fisher Scientific, 10687010). Prior to seeding into assay plates, reporter cells were resuspended in an assay medium (McCoy's 5A supplemented with 1% fetal bovine serum). Automated reporter screens were run on a GNF screening system, equipped with a Stäubli TX90L robotic arm (Stäubli Corporation) serving an integrated Envision plate reader (Perkin Elmer Inc.), a Bravo liquid handling platform (Agilent Technologies Inc.), two GNF Model I washers/dispensers, and two Thermo Fisher incubators (Forma Environmental Chamber, model 4933, Thermo Fisher Scientific).

Reporter cells for screening functional activity of antibodies against each antigen were seeded into 384-well assay plates (white, flat-bottom tissue-culture treated, Greiner Bio-One) at 40,000 cells per well in 12.5 µl of assay medium. Samples and positive control (Commercial Target A) were added manually to 384-well master plates (polypropylene, V-bottom, Greiner Bio-One). Assay medium was added to master plates and 9-point or 18-point serial dilutions were performed on the Agilent Bravo. Subsequently, 12.5 µl of sample solution was stamped from a master plate into each of three replicate assay plates. Assay plates were incubated for 4 hours at 37°C, and then equilibrated for 15 minutes at room temperature. In the meantime, the Bio-Glo detection reagent (Bio-Glo Luciferase Assay System, Promega Corporation) was prepared according to the manufacturer's instructions. 50 µl/well of Bio-Glo reagent was added to the assay plates via the GNF dispenser; after 5–10 minutes of incubation at room temperature, the luminescence output was measured on the Envision using the US luminescence aperture and 0.1 sec/well readout time. Raw data files from the Envision were imported into Genedata Screener (version 16.0.2, Genedata). Well metadata were added through a cmt file that was generated by a custom-written Knime pipeline (Knime AG). Data were normalized and scaled using negative and positive controls as central and scale reference, respectively. Plate-based RZ' factors were calculated in Screener and plates with RZ' <0.5 were masked and excluded from further analysis. Curve fitting was carried out using the

Screener SmartFit algorithm, and valid IC50 values were reported as qAC50 values.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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R.Z., P.S.C., S.P.R. designed research studies; R.Z., X.Y., B.C.M., E.B., J.H., J.S., J.S., H.C., H.S.M. performed the research; R.Z., X.Y., B.C.M and P.P. analyzed immunoglobulin sequences. R.Z., P.P., X.Y., B.C. M., E.B., J.H., J. S., J.S., H.C., H.S.M., D.H., A.P., P.S.C., and S.P.R. interpreted and discussed the data; R.Z., P.S.C. and S.P.R. wrote the paper and all authors participated in manuscript review and revisions.

Abbreviations

BV711	Brilliant Violet 711
BGH	bovine growth hormone
FR	Framework region
C_H	heavy-chain constant region
scFv	single-chain variable fragment
V_H	heavy-chain variable region
V_L	light-chain variable region
V_κ	kappa chain variable region
V_λ	kappa chain variable region
Fab	fragment antigen-binding
Fc	fragment crystallizable
CDR	complementarity-determining region
FcRn	the neonatal Fc receptor
FACS	fluorescence-activated cell sorting
ELISA	enzyme-linked immunosorbent assay
GNF	Genomics Institute of the Novartis Research Foundation
IC50	the half maximal inhibitory concentration
Ig	immunoglobulin
LEC	linear expression cassettes
mAb	monoclonal antibody
PCR	polymerase chain reaction
rbGlob	rabbit beta globin
RT	reverse transcription reaction
poly A	polyadenylic acid
rmAb	recombinant monoclonal antibody
SBC	single B-cell cloning
7AAD	7-Aminoactinomycin D

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