



Isolation of Human Antibody Repertoires with Preservation of the Natural Heavy and Light Chain Pairing

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The humoral immune system in higher vertebrates is unique in its ability to generate highly diverse antibody responses against most pathogens as well as against certain malignancies. Several technologies have been developed to exploit this vast source of potentially therapeutic antibodies, including hybridoma technology, phage display and yeast display. Here, we present a novel, high-throughput technology (the Symplex Technology) for rapid direct cloning and identification of human antigen-specific high-affinity antibodies from single antibody-producing cells of immune individuals. The utility of the technology was demonstrated by isolation of diverse sets of unique high-affinity antibodies against tetanus toxoid and influenza virus from immunized volunteers. Hence, the Symplex Technology is a new method for the rapid isolation of high-affinity antibodies directly from humans.

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Introduction

Several powerful technologies have enabled the isolation of human antibodies against a range of target antigens.^{1,2} Combinatorial phage and yeast display libraries of antibody heavy and light chain variable region-encoding genes (V_H and V_L) allow isolation of antibodies against virtually any antigen.^{3–5} However, these methods rely on random pairing of V_H and V_L domains, which excludes information about the cognate pairing of heavy and light chains. Alternative techniques allow for

isolation of cognate pairs of V_H and V_L domains from human B cells such as PCR cloning from a single B cell or an *in vitro* expanded B cell clones,⁶ isolation of single antigen-reactive B cells from antigen-embedded media and subsequent cloning of their V genes by PCR (the so-called selected lymphocyte antibody method, SLAM),⁷ and EBV transformation of human B cells.⁸ Although efficient, these systems are limited by the requirements for manipulation and propagation of viable B cells *in vitro*. Finally, in model systems Embleton *et al.* indicated the possibility of V gene assembly by RT-PCR within chemically fixed cells to ensure cloning of cognate V_H - V_L pairs.⁹ The feasibility of this method, however, has yet to be demonstrated on a genuine B cell repertoire.

The aim of the present study was to develop a method for efficient isolation of human antibody repertoires with preservation of the natural heavy and light chain pairing. We present the Symplex Technology, which consists of high-throughput single-cell sorting, single-cell RT-PCR and bulk cloning of the isolated natural V_H - V_L pairs to enable the isolation of diverse antibody repertoires directly from immune individuals. Such repertoires are subsequently expressed in bacteria and screened for antigen reactivity.

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Abbreviations used: V_H , antibody heavy chain variable region-encoding gene; V_L , light chain variable region-encoding gene; LC_{κ} , kappa light chain encoding gene; PC, plasma cells; TT, tetanus toxoid; MACS, magnetic-assisted cell sorting; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin.

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Results

Isolation of TT-specific antibody encoding genes

Healthy volunteers were immunized with tetanus toxoid (TT; donors TT-1 and TT-2) or the 2003 influenza virus vaccine (donor Flu-1). Peripheral blood mononuclear cells were isolated and the B cell fraction was recovered by CD19-specific magnetic-assisted cell sorting (MACS). Single plasma cells (PC) were isolated by fluorescence-activated cell sorting (FACS), based on high expression of CD19 and CD38, and intermediate expression of CD45. The gated population typically constituted 1–4% of the B lymphocytes and most likely corresponds to PC precursors rather than mature PC, which are located in the bone marrow.¹⁰ Such antibody-producing cells are generated in lymphoid tissues upon *in vivo* antigen stimulation and can be detected transiently in circulation.^{11–14} For convenience, these cells are referred to as PC here. The frequency of TT-specific IgG-producing PC in TT-1 and TT-2 was estimated by ELISPOT to be 17% and 14% of the sorted PC population, respectively, and 0.2% in the CD19 sorted B cell pool of TT-1 (data not shown). For each donor a total of 50 96-well plates, containing sorted single PC, were subjected to the Symplex PCR (Figure 1(a)). The linkage of a V_H and an LC_κ results in a fragment of approximately 1070 bp, depending on the length of the CDR3 in the amplified V gene. Approximately 400 Symplex PCR fragments were isolated from each donor, corresponding to a frequency of productive PCR reactions of 8%. This frequency has since then increased to approximately 40% with maturation of the technology (data not shown). Figure 1(c) shows an agarose gel electrophoresis analysis of 48 typical PCR samples resulting in 17 linked V_H - LC_κ fragments.

In order to verify the absence of shuffling caused by multiple cells per well, sample mixing or contamination, Symplex PCR products from 15 randomly selected wells were cloned individually and sequenced. Sequences from 3 to 17 sub-clones from each PCR were analyzed (118 in total) and in all cases the V_H and V_L sequences from a particular PCR product were identical, indicating that the genes linked were derived from the same cell.

Expression of antibody repertoires in *Escherichia coli*

The pool of V_H - LC_κ gene products was inserted into a Fab expression vector, in a head-to-head configuration, followed by the replacement of the V_H - LC_κ linker with a bidirectional promoter and leader cassette enabling Fab expression (Figure 1(b)). Clones from TT-1 and TT-2 were screened for specificity against TT and clones from Flu-1 were screened for reactivity with influenza virus. On average, 7% of the bacterial colonies from

the libraries of donors TT-1 and TT-2 were shown to produce antigen-specific Fab-fragments (Table 1). Similarly, 9% of clones from the Flu-1 donor were specific for influenza virus (results not shown).

Antibody repertoire analysis

The diversity of antigen-specific antibodies obtained with the Symplex Technology was examined by DNA sequencing of the V_H and V_L of 169 and 102 antigen-specific clones from TT-1 and TT-2, respectively. The V_H and V_L encoding sequences of Symplex derived clones have been deposited in GenBank with accession no. AY866545–AY866817 and AY866983–AY867255, respectively. In total, 32 (TT-1) and 41 (TT-2) unique V_H and V_L rearrangements were identified (Supplementary Data, Table 4). Sequencing of 71 influenza virus-specific clones led to the identification of 17 unique V_H gene rearrangements paired with distinct V_L genes (data not shown). Most germline families (IGHV1 to IGHV5 and IGHJ2 to IGHJ6; IGKV1 to IGKV3 and IGKJ1 to IGKJ5) were represented in the TT-specific repertoires of TT-1 and TT-2, indicating an absence of V or J gene amplification biases.

Hypermutated variants of homologous pairs of V_H and V_L sequences were found in all repertoires, indicating isolation of cognate pairs. However, in two cases in the TT-1 repertoire, a particular V_H rearrangement was paired with more than one V_L sequence. One similar observation was made in the TT-2 repertoire. Rare shuffling events could be expected in these first repertoires, which were based on distribution of single cells into microtiter wells by limiting dilution (see Materials and Methods). No shuffling has been detected in any subsequent repertoires generated from cells that have been single-cell sorted by FACS.

In order to compare the clonal diversity of the TT-1 and TT-2 repertoires further, the V_H and V_L diversity and the cognate pairing were visualized using phylogenetic trees in combination with dot matrix plots indicating the V gene pairing (Figure 2(a) and (b)). The phylogenetic trees illustrate the significant genetic diversity found within both the V_H and V_L sequences. Furthermore, the pairing of V_H and V_L sequences appear in clusters in the dot matrix plot demonstrating the evolutionary relationship between the V_H and V_L sequences, i.e. lack of light chain shuffling, as it would be expected for cognate pairs subjected to somatic diversification.

Binding kinetics of isolated antibodies

In order to examine the affinities of antibodies identified by the Symplex Technology, the binding kinetics and affinities (K_D) of a subset of randomly selected TT-specific Fab fragments were determined by surface plasmon resonance (Figure 2(c)). The affinity constants of the antibody fragments were in the range of 250 nM to 0.3 nM with slow off-rates as expected for a hyperimmune response

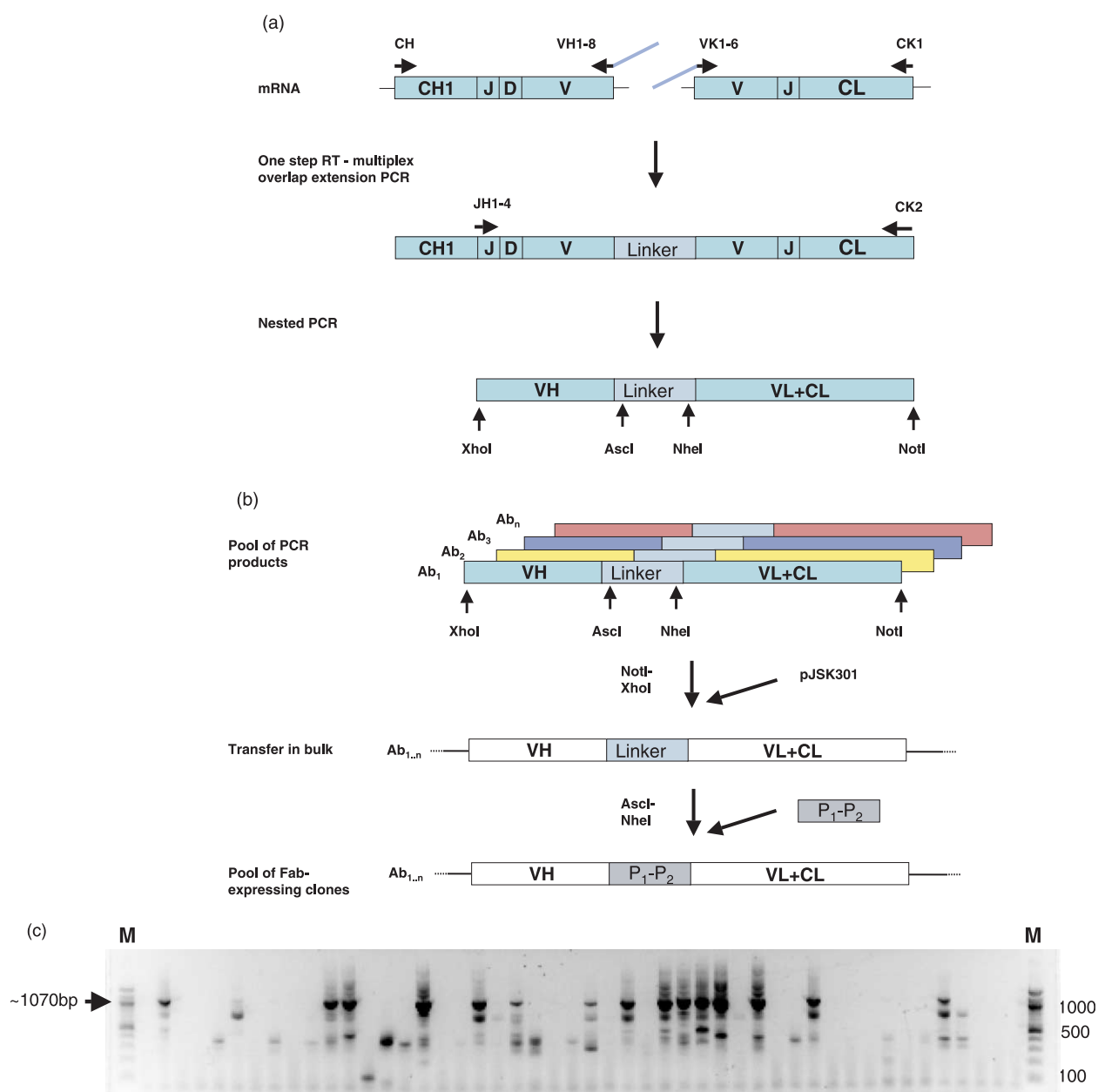


Figure 1. (a) Single-cell multiplex overlap extension RT-PCR. (b) Transfer of isolated and linked V_H -LC $_{\kappa}$ fragments. PCR primers used are indicated by horizontal arrows. CH1, constant domain 1, heavy chain; CL, constant domain, light chain; Ab, antibody; P₁-P₂, prokaryotic promoters. (c) Agarose gel (1%) electrophoresis analysis of 48 Symplex PCR samples each derived from single PC: 1070 bp, the average size of linked V_H and LC $_{\kappa}$ products of the Symplex PCR. M, 100 bp ladder (New England Biolabs).

and in support of their native origin and preservation of cognate pairing.

Verification of cognate pairing

To further illustrate the cognate pairing of the Symplex Technology, as well as to examine potential light chain promiscuity of isolated V_H genes, a combinatorial phage display library (TT-2-comb.) was constructed from the TT-2 Symplex repertoire. The V_H and LC $_{\kappa}$ gene fragments were inserted sequentially into a phage display vector and the resulting phage library was subjected to one round

of selection on TT. Sequencing of 102 TT-specific clones revealed 19 unique V_H rearrangements, six of which had not previously been found with the Symplex Technology (Supplementary Data, Table 4). The four V_H rearrangements most frequently recovered with phage display constituted 65% of the total number of clones analyzed and showed very promiscuous light chain usage. Moreover, only two of the V_H rearrangements were found in combination with the cognate V_L , as identified by Symplex Technology. These V_H constituted only 8% of the total number of clones analyzed and were characterized by little or no light

Table 1. Screening of antibody Fab fragments generated by Symplex Technology and phage display

Donor	TT-1	TT-2	TT-2-comb.	TT-2VH-comb.
Method	Symplex	Symplex	Combinatorial + phage display	Combinatorial + phage display
Total no. of clones	3×10^3	1.6×10^4	5×10^6	7×10^5
Fab expressing clones(5)	35	28	68	68
Repertoire size (V_H/V_L)	400/400	400/400	400/400	$400/2 \times 10^5$
Rounds of selection	N/A	N/A	One	One
Total no. of clones screened	3600	3400	170 ^a	170 ^a
Antigen-specific clones (%)	9.4	5.5	66	85
Unique V_H sequences ^b	74 (169)	76 (102)	43 (102)	40 (110)
Unique V_L sequences ^b	83 (169)	68 (102)	85 (102)	75 (110)
Unique V_H rearrangements ^c	32	41	19 (6)	13 (2)

^a After one round of selection.^b Uniqueness in deduced amino acid sequence of the variable domains excluding regions of priming for the PCR oligonucleotides and numbers in parentheses indicate total number of clones sequenced.^c Numbers in parentheses indicate V_H rearrangements not found with Symplex, N/A, not applicable.

chain promiscuity. The reduced V_H diversity among phage display-derived clones thus seems to be caused at least in part by a dominance of certain promiscuous V_H rearrangements. Plotting the genetic relationship between V_H - V_L pairs of TT-2 isolated by the combinatorial phage display approach (Figure 3(a)) revealed extensive scattering, thereby demonstrating that the evolutionary relationship observed for V_H - V_L pairs isolated by Symplex is not caused by the small repertoire sizes of V_L and V_H , but rather is the result of the method's ability in preserving cognate pairing.

To further understand the observed bias towards a predominance of certain V_H rearrangements characterized by promiscuous V_L usage, the V_H repertoire of TT-2 was combined with an unrelated V_L repertoire (size 2×10^5) from donor TT-1 and subjected to phage display (TT-2VH-comb.), and one round of panning on TT. Sequencing of 110 TT-specific clones revealed 13 unique V_H gene rearrangements, two of which were not previously found (Table 1); 78% of the clones were the same four highly promiscuous V_H rearrangements identified previously by phage display whereas less frequently found clones occasionally used light chains, which were homologous to those isolated by Symplex Technology. Thus, the size and composition of the light chain repertoire only marginally altered the outcome of the phage display approach. Plotting of the genetic relationship between V_H - V_L pairs isolated by this combinatorial approach also revealed extensive scattering in agreement with a set of clones dominated by promiscuous V_H rearrangements (Figure 3(b)). The results of these combinatorial libraries provided further evidence for the cognate nature of the Symplex libraries.

To investigate if the predominating V_H genes of the antigen-specific clones from the phage libraries were selected because of higher affinity, the off-rates were determined for a comparable number of randomly selected clones from each repertoire (Figure 3(c)). Both phage libraries showed a narrow clustering of clones of intermediate binding strength with average off-rates of $7.4 \times 10^{-4} \text{ s}^{-1}$ and $6.0 \times 10^{-4} \text{ s}^{-1}$, respectively. The off-rates of the

cognate TT-2 Symplex repertoire were of a similar average ($6.3 \times 10^{-4} \text{ s}^{-1}$). In contrast, the average off-rate of the TT-1 repertoire was $2.4 \times 10^{-4} \text{ s}^{-1}$ indicative of a more affinity-matured response. Together, these results demonstrate that the observed V_H bias of the phage display clones was not mediated by enhanced binding strength compared with the cognate repertoires. Since growth and expression characteristics were similar for the different sets of clones (data not shown), the V_H bias is thus most likely caused by a high frequency of promiscuous V_H domains existing as antigen-binding V_H - V_L pairs in combinatorial libraries.

Discussion

Here, we describe a novel method, the Symplex Technology, for the isolation of human IgG_k antibodies repertoires. The key feature of the technology is the ability to directly capture the immune antibody repertoire from human blood while preserving both the original pairing of V_H and V_L , and the natural diversity in the resulting antibody repertoire. Also, the method is fast, with less than 15 days from the time of single-cell FACS to the established cloned repertoire ready for screening.

From PC of three immunized healthy human volunteers, 90 unique antigen-specific antibodies were isolated and characterized. These antibodies displayed extensive genetic and functional diversity as well as high affinity. The preservation of the original pairing of heavy and light chain variable regions from the donor lymphocytes was confirmed by detailed sequence analysis. Any particular heavy chain, identified multiple times, from randomly selected clones was generally found to be paired with the same light chain. By maintaining the original pairing, any isolated genes will encode human antibodies that have been selected for high affinity and relevant functional properties in a human background.

The isolation of TT-specific antibodies from the two human donors TT-1 and TT-2 resulted in 32 and 41 uniquely rearranged antibodies in addition to

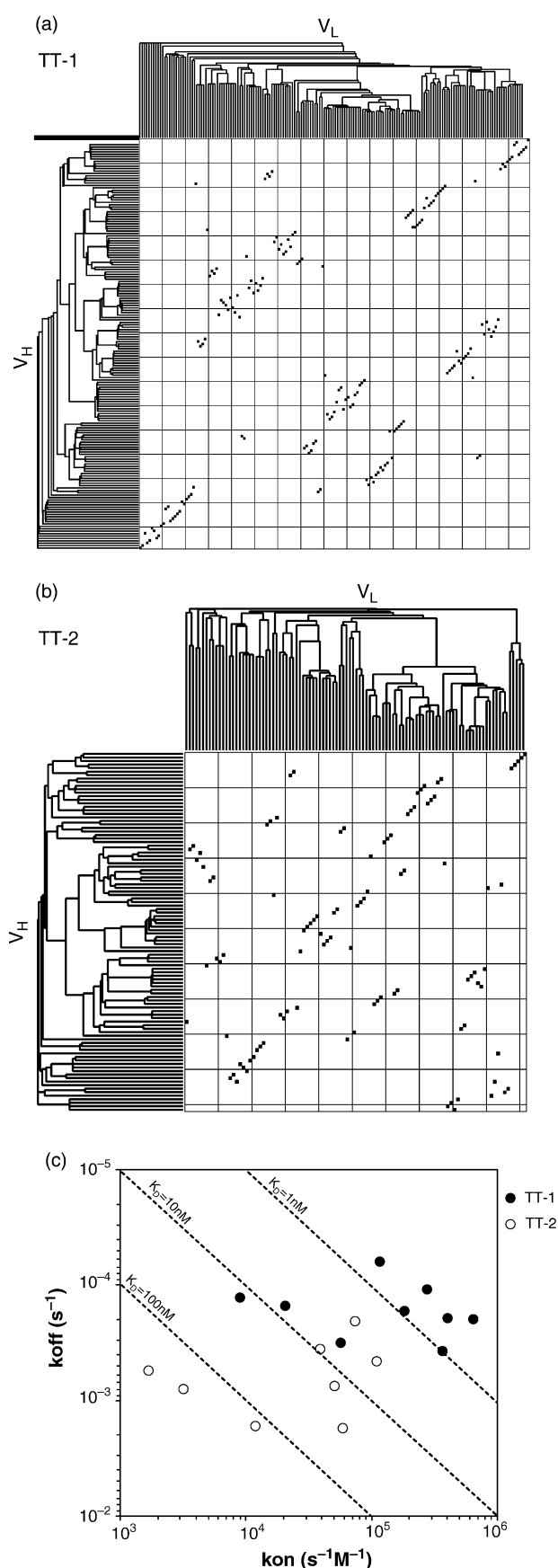


Figure 2. (a) and (b) Phylogenetic analysis of sets of V_H (vertical trees) and V_L (horizontal trees) genes isolated by Symplex Technology from (a) TT-1 and (b) TT-2. The dot

hypermuted variants of these, leading to a total of 146 and 81 unique pairs of antibody variable gene sequences, respectively. Both repertoires showed extensive diversity in their use of germline V and J genes, and in their use of CDR3 sequences, which was likely a result of the considerable structural complexity of the TT antigen. The TT-1 repertoire was somewhat less diverse, but of an approximately threefold higher affinity relative to the TT-2 repertoire, indicative of a more mature immune response. Furthermore, the observed evolutionary relationship between heavy and light chain sequences in combination with the exclusively high affinity further indicated efficient capture of the endogenous antibody response from the donors and preservation of the cognate heavy and light chain pairing.

The presented repertoires have been isolated using IgG_{κ} specific primers but there are no technical restrictions to include primers with other specificity such as, e.g. IgA , IgE or any other subtype. Recently, the Symplex PCR has been developed to also encompass primers allowing for the cloning of IgA_{κ} (unpublished results), which could provide a means to investigate the antibody repertoires in diseases where IgA is the predominant subclass, such as in the respiratory tract or other mucosal surfaces.

To further verify the ability of Symplex Technology to preserve cognate pairing, a combinatorial antibody gene library was created directly from the cloned TT-2 repertoire, and screened for expression of TT-specific antibodies. The diversity among V_H -encoding sequences recovered with phage display was about half of that found using Symplex Technology. Further, the light chain usage in combinatorial clones was completely random as expected; heavy chains were paired with a multitude of different light chains and no evolutionary relationship between V_H and V_L -encoding sequences was evident (compare Figure 3(a) with Figure 2(b)). Consequently, the V_H genes represented in the TT-2 repertoire isolated by Symplex Technology can, if given the opportunity, pair with a multitude of different V_L genes and still retain their TT-specificity. Thus, the absence of V_H gene pairing with more than one V_L gene strongly indicates that Symplex Technology preserves cognate pairing.

Although phage display technology also gave rise to high-affinity antibodies, much diversity was lost in the selection process and by the use of non-cognate light chains. Most of the isolated phage display clones were of the same few highly promiscuous V_H rearrangements, indicating that phage display has a bias towards promiscuous V_H genes. Potentially, the bias could be a result of the relatively small V_L repertoire of the shuffled TT-2-comb repertoire. To address this, we combined

matrix indicates the observed pairing of V_H and V_L sequences. (c) Binding constants of Fab fragments encoded by genes isolated with the Symplex Technology of TT-1 and TT-2.

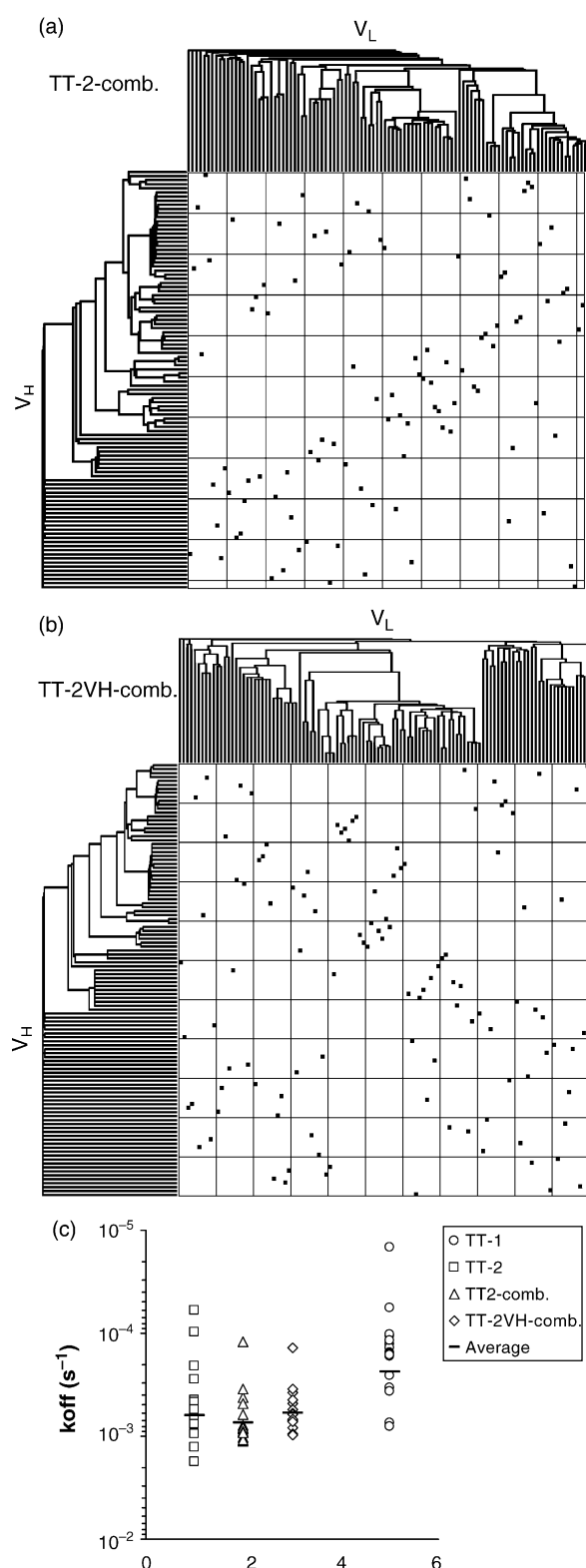


Figure 3. (a) and (b) Phylogenetic analysis of sets of V_H (vertical trees) and V_L (horizontal trees) genes isolated by phage display (a) from the scrambled TT-2 phage display library and (b) from the TT-2 V_H repertoire combined with an unrelated V_L repertoire from donor TT-1. The dot matrix plot indicates the observed pairing of V_H and V_L sequences. (c) Off-rates of Fab fragments encoded by clones isolated with Symplex Technology (TT-1 and TT-2) and of clones isolated with phage display (TT-2-comb. and TT-2VH-comb.).

the V_H genes from TT-2 repertoire with a large and unrelated V_L repertoire. However, the introduction of a larger V_L diversity did not increase the diversity of the isolated TT-specific clones, confirming the bias towards promiscuous V_H genes in phage display (Figure 3(c)).

In summary, we describe a novel method, Symplex Technology, for the isolation of large repertoires of human antibodies. The key feature of the technology is the ability to capture immune antibody repertoires while preserving the diversity and the original pairing of V_H and V_L coding regions. Using model antigens, we were able to isolate a plethora of unique antibodies, which are likely to closely represent the genetic and functional diversity of the natural humoral immune response of the donors. We have recently applied the technology on PC from donors vaccinated with an attenuated strain of Vaccinia virus and donors exposed to Rous sarcoma virus. In both cases, a large number of specific antibodies were isolated, demonstrating that the method can be successfully extended to infectious disease targets (unpublished results). Thus, Symplex Technology offers a new method for the rapid isolation of high-affinity and potentially therapeutic antibodies directly from human PC. Additionally, the technology can provide detailed insight into the nature and diversity of the natural human antibody response.

Materials and Methods

Donors and vaccination

Healthy volunteers were boosted with a tetanus vaccine (Statens Serum Institut, Copenhagen, Denmark) or the 2003 influenza vaccine Vaxigrip (Aventis Pasteur) composed of A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Shangdong/7/97. Six days after the boost, blood (200 ml) was collected in anticoagulant. The project was approved by the Regional Ethical Review Board.

Isolation of plasma cells

PBMC were isolated using Lymphoprep (Axis-Shield PoC AS), stained with anti-CD19-FITC (Becton Dickinson) in MACS buffer (phosphate-buffered saline (PBS) (pH 7.2), 0.5% (w/v) bovine serum albumin (BSA), 2 mM EDTA), washed, and labeled with anti-FITC conjugated magnetic beads in order to enrich for B cells on a MACS LS column (Milenyi Biotec) according to the manufacturer's recommendations. Next, the CD19-enriched fraction was resuspended in FACS buffer (PBS (pH 7.2), 2% BSA), and incubated with appropriate dilutions of anti-CD19-FITC, anti-CD38-PE and anti-CD45-PerCP (Becton Dickinson) for 20 min at 4 °C in the dark, washed and resuspended in FACS buffer. Single-cell sorting was performed on a FACS Aria cell-sorting system (Becton Dickinson). Cells that stained positive for CD19, highly positive for CD38 and intermediately positive for CD45 were collected and designated plasma cells (PC). Additional gates on forward scatter/side scatter and side scatter width/side scatter height were included to

select live lymphocytes and singlets, respectively. Single cells were distributed directly into the wells of 96-well PCR plates (AB0800; ABgene), containing all the necessary Symplex PCR reagents in a volume of 10 μ l, except for reverse transcriptase, DNA polymerase, and dNTPs (see below), and frozen at -80°C for later processing. For cloning of the TT-1 and TT-2 repertoires, single PC were distributed into microtiter plates by limiting dilution based on manual pipetting whereas single PC of the Flu-1 repertoire were distributed using the automatic dispenser of the FACS Aria system.

Plasma cell ELISPOT

Nitrocellulose-bottomed 96-well (HA) plates (Millipore) were coated with 10 $\mu\text{g}/\text{ml}$ of purified TT (Statens Serum Institut) in 100 μl of PBS overnight at 4°C . After blocking of wells in complete medium (RPMI1640 (Invitrogen) with 10% (v/v) fetal calf serum), PC dilutions (500–1000 sorted cells) were added in complete medium and incubated at 37°C , 5% CO_2 overnight. Wells were washed three times in PBS with 0.01% (v/v) Tween-20 and three times in PBS. Zones of solid-phase bound antibodies, secreted by individual PC, were revealed as spots by adding 100 $\mu\text{l}/\text{well}$ of horse radish peroxidase (HRP) conjugated goat anti-hIgG (CalTag) diluted 1:3000 in PBS with 0.5% BSA, followed by six wash steps (as above) and addition of a chromogenic substrate consisting of 0.015% H_2O_2 and 0.3 mg/ml of 3-amino-9-ethylcarbazole (Sigma) in 0.1 M sodium acetate buffer (pH 5.0). After development for 4 min, positive cells were counted in a stereomicroscope.

V_H and LC_k gene amplification and linkage

Immunoglobulin G κ -specific primers were modified from de Haard¹⁵ by trimming the 3' ends to reduce non-specific interactions and addition of 5' tails containing restriction sites for cloning and complementary regions resulting in linkage through overlap extension¹⁶ (Figure 1(a); Table 2). Single-cell sorted PCs were distributed in 96-well plates containing twice the final

concentration of One-Step RT-PCR buffer (Qiagen), 20 units of RNasin (Promega) and twice the final concentration of the primer sets VH, VK, CH, and CK1 (Table 2) in a total volume of 10 μl , and prepared for multiplex overlap-extension RT-PCR by adding 10 μl containing 400 μM each dNTP, 20 units of RNasin, 0.8 μl of One-step RT-PCR enzyme mix (Qiagen). The RT-PCR reactions were incubated for 30 min at 55°C for the RT reaction followed by thermal cycling: 95°C , 15 min followed by 35 cycles of 94°C , 30 s; 60°C , 30 s; 72°C , 5 min and a final extension step at 72°C for 10 min, performed in a H₂OBIT thermal cycler (ABgene); 1 μl of each multiplex overlap-extension RT-PCR product was subjected to semi-nested PCR in a total volume of 20 μl , with a final concentration of $1\times$ BioTaq buffer, 200 μM each dNTP, primers set JH and primer CK2, 2 mM MgCl_2 , 0.025 unit/ μl of BioTaq DNA polymerase (Bioline). The reaction was performed in a H₂OBIT thermal cycler with the following cycling conditions: 30 cycles of 95°C , 30 s; 60°C , 30 s; 72°C , 90 s, and a final extension step at 72°C for 5 min.

Expression plasmid construction

Two microliters from each of the nested PCR reactions were, for each donor, combined in a single tube and digested with XhoI and NotI. The ~ 1070 bp linked V_H - κ light chain encoding gene (LC_k)³ products were subsequently purified by preparative 1% (w/v) agarose gel electrophoresis. Pooling of PCR reactions greatly simplifies the cloning procedure by reducing it to only one reaction in bulk. The pool of digested V_H - LC_k fragments were inserted into pJSK301, a Fab-expression vector modified from phh3¹⁷ by deletion of the pIII-encoding gene, insertion of AscI and NheI sites in the *pelB* leaders and XhoI-NotI to accommodate inserts (Figure 1(b)). *Escherichia coli* (TOP10) was transformed by electroporation and the transformants were selected on $2\times$ YT broth agar containing 100 $\mu\text{g}/\text{ml}$ of carbenicillin. Plasmid DNA was prepared from >2000 colonies directly off the agar plates. The plasmid was digested with AscI and NheI, and a prokaryotic promoter and leader cassette was inserted by ligation. *E. coli* (TG1) was

Table 2. Symplex PCR primers

Primer set	Concn (nM)	Primer name	Primer sequence ^a
VH	40 (each)	VH1	tattcccatggcgcgcccCAGRTGCAGCTGGTGCART
		VH2	tattcccatggcgcgcccSAGGTCCAGCTGGTRCAGT
		VH3	tattcccatggcgcgcccCAGRTCACTTGAAGGAGT
		VH4	tattcccatggcgcgcccSAGGTGCAGCTGGTGGAG
		VH5	tattcccatggcgcgcccCAGGTGCAGCTACAGCAGT
		VH6	tattcccatggcgcgcccCAGSTGCAGCTGAGGAGT
		VH7	tattcccatggcgcgcccGARGTGCAGCTGGTGCAGT
		VH8	tattcccatggcgcgcccCAGGTACAGCTGCAGCAGTC
CH	200	CH	GACSGATGGGCCCTTGGTGG
VK	40 (each)	VK1	ggcgcgccatgggaatagctagcccGACATCCAGWTGACCCAGTCT
		VK2	ggcgcgccatgggaatagctagcccGATGTTGTGATGACTCAGTCT
		VK3	ggcgcgccatgggaatagctagcccGAAATTGTGTGACRCAGTCT
		VK4	ggcgcgccatgggaatagctagcccGATATTGTGATGACCCACACT
		VK5	ggcgcgccatgggaatagctagcccGAAACGACACTCAGCAGT
		VK6	ggcgcgccatgggaatagctagcccGAAATTGTGCTGACTCAGTCT
CK1	200	CK1	atataatagcgccgcTTAACTCTCCCCTGTTGAA
JH	200 (each)	JH1	ggaggcgctcGAGACGGTGACCAAGGGTGCC
		JH2	ggaggcgctcGAGACGGTGACCATTTGCC
		JH3	ggaggcgctcGAGACGGTGACCAAGGGTGCC
		JH4	ggaggcgctcGAGACGGTGACCGTGGTCCC
CK2	200	CK2	accgctccaccggcgccgcttaTTAACTCTCCCCTGTT-GAAGCTCTT

^a Capital letters correspond to the gene-specific regions.

Table 3. Kappa light chain-specific primers

Primer set	Primer name	Primer sequence ^a
C _κ FWD		accgcctccaccggcgccgcttaTTAACACTCTCCCCTGTTGAAGCTCTT
V _κ REV	1B	caaccagcgctagccGACATCCAGWTGACCCAGTCTCC
	1B	caaccagcgctagccGACATCCAGWTGACCCAGTCTCC
	2	caaccagcgctagccGATGTTGTGATGACTCAGTCTCC
	3B	caaccagcgctagccGAAATTGTGWTGACRCAGTCTCC
	4B	caaccagcgctagccGATATTGTGATGACCCACTCTCC
	5	caaccagcgctagccGAAACGACACTCAGCAGTCTCC
	6	caaccagcgctagccGAAATTGTGCTGACTCAGTCTCC

^a Capital letters correspond to the gene-specific regions.

transformed by electroporation and selection was performed as described above. The frequency of V_H-LC_κ inserts in the libraries was tested by colony PCR and was found to be 70–95% (data not shown).

Fab expression and screening

Single Fab-expressing *E. coli* colonies were picked into single wells of 96 deep well plates containing 2× YT broth supplemented with 100 µg/ml of carbenicillin and 0.1% (w/v) glucose and incubated for 3 h at 37 °C at 250 rpm. Fab expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM. The plates were sealed and incubated overnight at 30 °C, 250 rpm. Then 96-well or 384-well ELISA plates (Corning) were coated with 1 µg/ml of TT (Statens Serum Institut) in PBS or with goat anti-human kappa LC antibody (Caltag) diluted 1:1000 in 50 mM sodium carbonate buffer (pH 8.5). ELISA plates were blocked with M-PBS-T (2% (w/v) skim milk powder in PBS, 0.05% (v/v) Tween-20) and washed twice with PBS-T (PBS, 0.05% Tween20). Fab-containing bacterial supernatants were diluted 1:2 in M-PBS-T and added to the coated plates. After 1 h incubation, the plates were washed four times with PBS-T. HRP-conjugated goat anti-human Fab-specific IgG (A0293; Sigma-Aldrich), diluted 1:10,000 in M-PBS-T, was added and incubated for 1 h. After washing four times with PBS-T, the plates were developed with TMB Plus substrate for HRP (KemEnTec) and the A_{450nm} determined.

Phage display

The combinatorial TT-2 phage display library was generated by successive insertion of the V_H and LC_κ gene fragments isolated by Symplex Technology from donor TT-2 into a phage display vector Em351 modified from phh3¹⁷ as described above, except for the pIII-encoding gene not having been deleted. In a second phage library the Symplex V_H repertoire isolated from TT-2 was combined with a larger unrelated V_L repertoire prepared by amplifying the total V_κ repertoire of CD19⁺ B cells from donor TT-1. cDNA was synthesized from total RNA in an oligo(dT)-primed reaction using ThermoScript reverse transcriptase (Invitrogen) and the LC_κ genes were PCR amplified using HotMasterTaq DNA polymerase (Eppendorf) and primers essentially as described by de Haard *et al.*¹⁵ only modified with respect to restriction enzyme recognition sequences at the 5' end (Table 3). Phage display libraries were generated by using XhoI-AscI and NotI-NheI restriction enzymes for cloning V_H and LC_κ, respectively. Ligation, transformation, and phage display were done according to standard procedures.¹⁸ One round of panning was performed in MaxiSorp immunotubes (Nunc) coated with TT

(Statens Serum Institut) at 1 µg/ml in PBS. Briefly, polyethylene glycol (PEG)-precipitated library phage particles were diluted 1:40 in 4 ml of PBS containing 2% skimmed milk and left incubating in antigen-coated immunotubes for 1 h. After ten rounds of washing in PBS-T bound phage particles were eluted using 1 ml of 0.1 M glycine-HCl (pH 2.2), neutralized with 0.5 ml of 1 M Tris-HCl (pH 7.5) and used to infect exponentially growing *E. coli* TG1 cells. Infected cells were grown overnight on agar plates (2× YT broth, 100 µg/ml of ampicillin, 1% glucose) and colonies were picked directly for screening and sequencing.

Sequence analysis

DNA of antigen-positive clones was sequenced (AGOWA, Berlin, Germany) using appropriate DNA oligonucleotides to an extent that secured more than 100 pairs of V gene sequences per antibody repertoire. Sequences were trimmed using the Vector NTI package (Informax) and subsequently batch translated using Transeq at European Bioinformatics Institute†. Alignment performed in Vector NTI (AlignX) allowed for the grouping of clones according to sequence homology. For each group the V-D-J usage and location of somatic mutations were determined by alignment with germ line sequences using the IMGT sequence directory‡. The phylogenetic trees were generated using ClustalW§ and combined with dot matrix plots generated in Microsoft Excel.

Surface plasmon resonance

Fab fragments were expressed in culture volumes of 100 ml, 300 ml or 1000 ml dependent on expression levels. Bacteria were grown at 37 °C in 2× YT broth supplemented with 100 µg/ml of carbenicillin and 0.1% glucose until the A₆₀₀ reached 1.2 after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and the culture was continued overnight at 30 °C. Bacteria were pelleted by centrifugation and the periplasmic fraction extracted by dissolving the pellet in 1–3 ml of ice-cold PBS containing 0.94 M NaCl. Fab fragments were further purified on protein L spin columns according the manufacturer's instructions (Pierce, Rockford, IL). The concentration of Fab fragment was determined by an indirect immunoassay using purified Fab fragment as standard. Surface plasmon resonance analysis was performed using a Biacore 2000 (Biacore AB). TT was immobilized on a CM5 chip surface using standard amine coupling chemistry to a level

† www.ebi.ac.uk; <http://imgt.cines.fr>

‡ www.ebi.ac.uk

resulting in an RUmax of approximately 100 RU or less. Purified antibody Fab fragments were diluted serially in HBS-EP running-buffer (Biacore AB) and passed over the chip at 50 μ l/min. Rate constants (k_{on} and k_{off}) were determined using the Biaevaluation software (Biacore AB) by global fitting of four to six different concentrations passed over the same sensor surface. Global fits of k_{on} and k_{off} simultaneously (Figure 2(c)) or k_{off} (Figure 3(c)) gave average χ^2 values of 1.8 and 0.8, respectively.

Data Bank accession codes

The sequences reported here have been deposited in the GenBank database (V_{H} , accession no. AY866545–AY866817; V_{L} , accession no. AY866983–AY867255).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2006.02.040](https://doi.org/10.1016/j.jmb.2006.02.040)

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