An efficient method for variable region assembly in the construction of scFv phage display libraries using independent strand amplification

Pablo H. Sotelo,1 Noberto Collazo,1 Roberto Zuñiga,1,3 Matías Gutiérrez-González, Diego Catalán,1,2 Carolina Hager Ribeiro,1 Juan Carlos Aguillón1,2,* and María Carmen Molina1,3,*

1Centro de InmunobioTecnología (IBT); ProgramaDisciplinario de Inmunología; Instituto de CienciasBiomédicas (ICBM); Facultad de Medicina; Universidad de Chile; Santiago, Chile; 2Millennium Institute on Immunology and Immunotherapy P09-016-F; Santiago, Chile; 3Facultad de Química; Universidad de la República; Montevideo, Uruguay

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Abbreviations: PCR, polymerase chain reaction; Ig, immunoglobulin; scFv, single chain variable fragment; Fab, fragment, antigen binding region; SOE-PCR, splicing by overlap extension PCR; HAMA, human anti-murine antibody; CDR, complementary determining regions; VHR, immunoglobulin heavy-chain variable region; VLR, immunoglobulin light-chain variable region; PBMC, peripheral blood mononuclear cells; ISA-PCR, independent strand amplification method; BSA, bovine serum albumin; TNFα, human tumor necrosis factor alpha; MICA, human MHC class I-related chain A

Phage display library technology is a common method to produce human antibodies. In this technique, the immunoglobulin variable regions are displayed in a bacteriophage in a way that each filamentous virus displays the product of a single antibody gene on its surface. From the collection of different phages, it is possible to isolate the virus that recognizes specific targets. The most common form in which to display antibody variable regions in the phage is the single chain variable fragment format (scFv), which requires assembly of the heavy and light immunoglobulin variable regions in a single gene.

In this work, we describe a simple and efficient method for the assembly of immunoglobulin heavy and light chain variable regions in a scFv format. This procedure involves a two-step reaction: (1) DNA amplification to produce the single strand form of the heavy or light chain gene required for the fusion; and (2) mixture of both single strand products followed by an assembly reaction to construct a complete scFv gene. Using this method, we produced 6-fold more scFv encoding DNA than the commonly used splicing by overlap extension PCR (SOE-PCR) approach. The scFv gene produced by this method also proved to be efficient in generating a diverse scFv phage display library. From this scFv library, we obtained phages that bound several non-related antigens, including recombinant proteins and rotavirus particles.

Introduction

Antibodies are the most common biological reagents used in pharmaceutical and medical research, and they are used to treat human pathologies, including cancer, autoimmunity and infectious diseases.1-6 Mouse monoclonal antibodies (mAbs) were used as therapeutic agents, but the immune reaction against the murine protein,7,9 which causes rapid elimination of the mAb and the possibility of anaphylaxis reaction, encouraged scientists to reduce the most immunogenic murine regions of the antibody structure using recombinant DNA technology. Using this approach, murine immunoglobulin regions were replaced by the corresponding human sequences to produce first chimeric antibodies, and then humanized antibodies.1,10 More recently, transgenic mouse11 and phage display technologies have been used to obtain human mAbs.12,13 The phage display technology presents several advantages compared to the classical mAb production system, including (1) the avoidance of animal use; (2) the possibility to improve antibody properties using mutagenesis14-18 and (3) the combination of different immunoglobulin variable domains to produce engineered antibodies.19-21

Phage display technology is a highly flexible method for the isolation of a single gene product with specific binding properties.22 Therefore, it could be easily combined with antibody recombinant technology to produce a very effective method to isolate human antibodies against specific targets.23,24 Using this methodology, the immunoglobulin variable regions are fused to a structural phage protein in such a way that each filamentous virus displays the product of a single antibody coding sequence on its surface. This allows the production of a wide collection of phages that are able to interact with different antigens. Two types of these phage display libraries can be produced: (1) a synthetic human variable gene library in which complementary determining regions are produced synthetically25-27 and (2) a naïve library

*Correspondence to: Juan Carlos Aguillón and María Carmen Molina; Email: jaguillo@med.uchile.cl and mcmolina@med.uchile.cl
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in which VHR and VLR are amplified by PCR with primers that share an overlapping region to create a linker region by combining both variable region amplifications. Both PCR products are then mixed and incubated with external primers to produce a scFv gene.\textsuperscript{32,33}

We report here that we modified the SOE-PCR to produce a simple and highly efficient method to combine antibody heavy and light chain variable regions in a scFv format by using a single strand DNA amplification strategy. In the first step, each immunoglobulin variable region is amplified independently using a single primer to produce a single strand DNA. Next, both variable regions are mixed and cycled to produce a full-length scFv gene. We used this method to produce a diverse scFv phage display library. In addition, this library was used to obtain phages capable of interacting with different non-related antigens.

### Results

Amplification of immunoglobulin variable regions and scFv gene assembly. Total RNA was obtained from peripheral blood mononuclear cells (PBMC) of 8 healthy non-immunized donors. cDNA was prepared using oligo-dT and employed as a template to amplify the immunoglobulin rearranged variable genes using the primers detailed in Table 1. These primers were designed to allow the amplification of the entire repertoire of immunoglobulin heavy-chain variable region genes described in VBASE.\textsuperscript{34} The VLR reverse primer and all VHR forward primers included an artificial region of overlap that allows the formation of a flexible segment corresponding to the linker (Gly,Ser). In order to avoid a biased amplification, each cDNA donor was amplified independently with each primer set. Figure 1 shows amplification products from a single donor using all different primer combinations. As expected, the PCR products using the VlgGHFor6 primer produced less DNA due to an interaction with only a subset of the IGHV 2 family. The PCR products were pooled and further used for scFv assembly assays.

We hypothesized that the presence of cDNA strands in the PCR products competed with the single stranded DNA required for the scFv assembly, thereby decreasing the efficiency of SOE-PCR. To overcome this problem we decided to separate this reaction into two steps. First, we enriched the DNA strand

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**Table 1. Primers used to amplify the immunoglobulin rearranged variable genes**

| Name               | Sequence                          | Chain | Ig variable family* |
|--------------------|-----------------------------------|-------|---------------------|
| VlgGHFor01         | CTCGTCCTCTGGGCGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 1, 3, 5 and 7  |
| VlgGHFor02         | CTCGTCCTCTGGGCGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 1, 3 and 5     |
| VlgGHFor03         | CTCGTCCTCGGGCAGGGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 1, 3 and 7     |
| VlgGHFor04         | CTCGTCCTCTGGGCGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 4              |
| VlgGHFor05         | CTCGTCCTCTGGGCGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 1, 4 and 6     |
| VlgGHFor06         | CTCGTCCTCTGGGCGGAGGGATGCGGAGSAGTGGG | Heavy | IGHV 2              |
| IgGrevNhel          | TTTTTTTCTGACAGCAGGAGGAGGGATGCGGAGSAGTGGG | Heavy | IgG                 |
| VlgKFor01          | TTTTTTGATCGGCGGAGGAGGAGGGATGCGGAGSAGTGGG | Light | All kappa           |
| ClgKRev01 linker   | CACCGCCACAGGAGGAGGAGGAGGGATGCGGAGSAGTGGG | Light | All kappa           |

*Source VBase (http://vbase.mrc-cpe.cam.ac.uk/).

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**Figure 1.** Heavy and Light chains variable region amplification. Total RNA of 8 healthy donors was extracted from peripheral blood mononuclear cells and cDNA was produced using oligo-dT. The cDNA was used to amplify heavy and light chains variable regions by PCR using the primers from Table 1. The figure shows representative PCR products from a single donor, resolved by electrophoresis on a 1% w/v agarose gel. MW: 100 bp DNA ladder.

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derived from natural non-immunized human in which the complementarity-determining regions (CDRs) are obtained from rearranged genes of B lymphocytes.\textsuperscript{28-30}

To display antibody variable regions in the phage, several forms have been described, with the antigen-binding fragment (Fab) and single-chain variable fragment (scFv) arrangement the most commonly used. The Fab form is composed of two chains, that must be assembled in the same phage, while scFv is a single chain in which the immunoglobulin variable heavy and light chain regions are fused using a flexible linker. The latter form has been widely used because it is more stable and better tolerated by bacteria.\textsuperscript{23}

A critical step in the scFv library production process is the assembly of heavy and light chain variable regions in the same molecule. For this purpose, different strategies have been applied, including laborious methods such as independent cloning of immunoglobulin variable regions followed by the assembly of the scFv gene by cloning methods or the use of recombination systems involving bacteria carrying the immunoglobulin heavy-chain variable region (VHR) and a phage carrying the immunoglobulin light-chain variable region (VLR).\textsuperscript{31} The most common technique is the splicing by overlap extension PCR (SOE-PCR),
required for scFv fusion by a separate single strand amplification using external primers for the heavy and light chain regions. Then both single strand DNA products were mixed and the assembly of the scFv gene took place by repeated cycles of denaturation annealing and extension. The different strategies used in this work for the assembly of immunoglobulin variable regions are schematically shown in Figure 2.

Each independent single strand DNA amplification was performed using the VlGKFor01 and IgGrevNheI primers for the light and heavy chains, respectively, according to a PCR program as follows: 95°C for 5 min, 30 cycles at 95°C for 45 sec, 54°C for 45 sec, at 72°C for 45 sec, and an extension reaction at 72°C for 1 min. Immediately afterwards, both single strand DNA amplifications were mixed and the assembly reaction was performed by sequential rounds of annealing and extension (Fig. 2).

For comparison, we performed in parallel a SOE-PCR, with 30 cycles of amplification. This amplification included an assembly reaction of 5 cycles of denaturation annealing and extension in the absence of primers, which is reported to facilitate the assembly of chains, followed by amplification reaction where primers were present.

As shown in Figure 3A, the independent strand amplification method (ISA-PCR) produced 6-fold more scFv gene product than that obtained by SOE-PCR. Similar results were obtained when our method was compared with a variant of SOE-PCR that avoids the 5 cycles of denaturation annealing and extension in the absence of primers prior to PCR (data not shown). In addition, we found similar results to those described above using a VHR product amplified with an IgM-specific reverse primer (data not shown).

Since the second step of ISAV-PCR (the assembly reaction) did not involve an amplification step, we decided to analyze the effect of cycle step numbers on the scFv gene production. For this purpose, we performed a single strand DNA amplification reaction, as was previously described, and the production of scFv gene at different cycle numbers during the assembly reaction was analyzed, demonstrating an increase as the cycle numbers enhanced (Fig. 3B).

### scFv Library Construction and Phage Isolation

Despite the efficiency of the ISA-PCR method to produce high quantities of scFv library construction and phage isolation.
The production of functional scFv displayed in the phage, the library was infected with the helper phage M13KO7 to produce scFv display phages that were used to perform bio-panning against different antigens, including the recombinant human proteins ErbB3 ectodomain, tumor necrosis factor α (TNFα), vimentin and MHC class I-related chain A (MICA) ectodomain, as well as bovine serum albumin (BSA) and the viral particle of rotavirus. In parallel, bio-panning was performed using only blocking protein to quantify unspecific bound phages. The numbers of clones obtained after subtracting the unspecific bound phages from the first two rounds of selection are shown in Table 2.

After the second round of selection with a particular antigen, up to 15 clones were picked and their binding specificity determinate by phage ELISA. We were able to obtain at least one specific clone for each antigen. In the case of MICA, ErbB3 and TNFα, two or more different clones were isolated (Table 2). Figure 6 indicates the phage screening of scFv clones that interact with ErbB3 and TNFα.

Discussion

Phage display antibody technology is a potent tool to recover the recognition potential of the human humoral immune system. Although several protocols can be obtained from the literature, the use of this technology can be complex and not always successful, mostly due to the several steps it involves: (1) generation or recovery of the humoral diversity (synthetic or naïve library, respectively); (2) assembly of the binding domain into a single gene; and (3) isolation of phages against specific targets. The construction of a diverse antibody phage library able to recognize several antigens is strongly dependent on the first two factors.

Several methods have been described for the assembly of immunoglobulin variable regions in the scFv format. The assembly by cloning procedures involves a separate amplification of VHR and VLR by PCR and their cloning either into separate vectors or combining them by a cloning procedure. Another method involves a recombination system in which the VHR is cloned into a plasmid with lox sites flanking the gene, while the VLR is cloned into a phagemid with a dummy VHR flanked by lox sites. Bacteria containing the VHR plasmid and the Cre recombinase enzyme are infected with the phage containing the VLR to produce recombination. Despite their efficiency, these methods are laborious and time-consuming.

The most common method used for the assembly of heavy and light variable regions is the SOE-PCR. In this work, we modified this technique to produce a fast and simple method for the assembly of scFv gene that produces six-fold more scFv gene than SOE-PCR (Fig. 3A). To achieve this, we separated the reaction into two steps. Initially, we enriched the single-stranded DNA required for scFv gene assembly by an independent DNA strand amplification using a single primer, which minimizes the
inhibitory effect produced by the presence of complementary chains in the assembly reaction. In the second step, we produced the assembly reaction by mixing both single strand products (Fig. 2). We observed that multiple cycling between denaturation; annealing and extension strongly increases scFv gene production (Fig. 3B). This process probably facilitates the annealing of both single strand products, but we cannot rule out possible amplification of the assembled scFv gene due to the presence of primers remaining from the single strand production reaction.

The diversity produced by the ISA-PCR method was analyzed by a restriction analysis using the enzyme MvaI (Fig. 4); this showed at least 12 different patterns in 14 clones analyzed, which is in agreement with previous observations. It is noteworthy that all clones possess an amplification for full-length scFv gene, showing no misassembly during the ISA-PCR and the absence of vector background in the library.

The DNA sequence analysis of 21 randomly selected scFv genes revealed that all clones were correctly assembled. We observed a bias in the variable gene usage with the over-representations of the VH3 and VLkappa2 families (Fig. 5). The VH3 family bias has been previously observed during the construction of different scFv libraries. In addition, this bias reflects the variable gene used in resting B cells repertoire. The bias in the use of VLkappa gene family is not as common as the VH gene. Sheets et al. identified the VLkappa1 as the most frequent family in library developed by sequential cloning, but Glaville et al. found the VLkappa3 as the most frequent. The identification of the VLkappa2 as the most frequent VL family in our library could be produced by the diversity in primer annealing for different families rather than a preferential assembly induced by ISA-PCR.

The scFv genes produced by our method were used to construct an antibody phage display library generated by the amplification of immunoglobulin variable regions from the blood of 8 healthy donors and obtained in a single transformation. Regardless of the limited number of donors and the limited library size (our library possesses 2.8 x 10⁷ clones, while other libraries are in the range 10⁸ to 10¹⁰ clones), we were able to isolate phages binders to all tested antigens (Table 2). These included different recombinant proteins and rotavirus particles. The frequency of positive clones observed in the second panning was in agreement with previous results using different assembly methods, including SOE-PCR. Moreover, we were able to identify different scFv sequences of binding phages obtained from the proteins TNFα, ErbB3 and MICA (Table 2 and Fig. 6).

The results obtained from random clone sequencing and phage binders isolations suggest that the ISA-PCR method is efficient in generating antibody diversity.

The success in the use of antibodies as therapeutic agents and the advent of antibodies in high-throughput research have dramatically increased their demand. Antibody phage display technology appears to be a suitable source of candidates. According to our experience, the use of ISA-PCR method would facilitate the development of antibody phage display libraries. It is noteworthy that the simplicity and the minimal equipment required for the ISA-PCR method could facilitate the construction of scFv libraries in all types of institutions, including small research facilities, making antibody phage display technology more accessible.

Considering the characteristics of this technique, the use of ISA-PCR is not restricted to the construction of scFv libraries because it could also be used in the construction of any fusion protein with DNA from diverse origin, including evolutionary studies, modular protein constructions and synthetic protein development.

In this study, we developed a fast, simple and highly efficient method to assemble the immunoglobulin light and heavy variable regions in scFv coding genes. The methodology is based on the specific enrichment of DNA strands required for the scFv gene assembly by independent single strand amplification. The ISA-PCR produced high amounts of scFv genes together to the production of sequence diversity and phages that interact with a wide range of different non-related antigens. Taking advantage of its efficiency, the method requires fewer assembly reactions to produce an antibody phage display library, minimizing time and resources, and could be used in the construction of fusion protein.

### Material and Methods

**scFv gene production.** Immunoglobulin variable regions amplification. PBMC were obtained from 12.5 mL of blood from 8 healthy volunteers by separation with Ficoll-Hypaque (Axis-Shield). All volunteers signed an informed consent approved by the Ethical Committee of the Faculty of Medicine, University of Chile, in the report N°044-2009. Total RNA extraction was performed with Trizol as recommended by the manufacturer (Life Technologies). Full-length cDNAs were generated from 2 μg of total RNA using
strand amplification reaction. 50 ng of the PCR product of the heavy or light chain variable genes were incubated with 0.4 μM of the corresponding primer (VIgKFor01 in the case of VLC and IgGrevNheI for VHC) and Paq DNA polymerase (Stratagene) in 25 μL reaction. Heavy or light chain single strand amplification reactions were performed using the following program: 95°C for the initial denaturation; 30 cycles at 95°C for 45 sec, 54°C for 45 sec and 72°C for 45 s, and a final extension reaction of 1 min at 72°C. Both single strand amplification products were mixed and 10 μM of dNTPs and 5 units of Paq polymerase were added. The assembly reaction was performed by the following conditions; 95°C for the initial denaturation; different number of cycles (5, 10, 15 and 30) at 95°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, and a final extension reaction for 10 min at 72°C. The products were resolved by electrophoresis on 1% w/v agarose gels and purified as previously using QIAQuick gel extraction kit (QIAGEN).

To quantify scFv production, an aliquot of 10% of ISA-PCR product was resolved by electrophoresis on 1% w/v agarose gels. Digital images were captured and the signal intensity of each band was quantified. A region in the upper part of the lane was quantified and subtracted from the corresponding band to eliminate background. The total scFv gene produced was calculated using 200 U of Moloney Murine Leukemia Virus reverse transcriptase, according to the manufacturer’s instructions (Promega).

VHR genes were amplified using each of the 6 primers described in Table 1, as well as the reverse primer IgGrevNheI. Briefly, a 20 μL PCR reaction was performed containing 2 μL of cDNA, 0.4 μM of each primer and GoTaq Green Master Mix (Promega). The amplification was performed using the following program: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 sec, 56°C for 45 sec and 72°C for 45 s, and a final extension reaction for 10 min at 72°C. To amplify the kappa light variable gene, a PCR reaction was performed using the primers described in Table 1, along with the same reaction mixture previously used for the heavy variable gene amplification. The PCR reaction was performed by heating at 95°C for 2 min followed by 30 cycles at 95°C for 45 sec, 54°C for 45 sec and 72°C for 45 s, and a final extension reaction for 5 min at 72°C. An independent amplification reaction using each primer set was performed for each donor. After checking the amplification, all heavy or light chain PCR products were pooled, resolved by electrophoresis on a 1% w/v agarose gel and purified using QIAQuick gel extraction kit (QIAGEN).

**ISA-PCR assembly reaction.** The ISA-PCR method is composed by two independent reactions: The single strand DNA amplification and the assembly reaction (Fig. 2). For the single strand amplification reaction 50 ng of the PCR product of the heavy or light chain variable genes were incubated with 0.4 μM of the corresponding primer (VIgKFor01 in the case of VLC and IgGrevNheI for VHC) and Paq DNA polymerase (Stratagene) in 25 μL reaction. Heavy or light chain single strand amplification reactions were performed using the following program: 95°C for the initial denaturation; 30 cycles at 95°C for 45 sec, 54°C for 45 sec and 72°C for 45 s, and a final extension reaction of 1 min at 72°C. Both single strand amplifications products were mixed and 10 μM of dNTPs and 5 units of Paq polymerase were added. The assembly reaction was performed by the following conditions; 95°C for the initial denaturation; different number of cycles (5, 10, 15 and 30) at 95°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, and a final extension reaction for 10 min at 72°C. The products were resolved by electrophoresis on 1% w/v agarose gels and purified as previously using QIAQuick gel extraction kit (QIAGEN).

To quantify scFv production, an aliquot of 10% of ISA-PCR product was resolved by electrophoresis on 1% w/v agarose gels. Digital images were captured and the signal intensity of each band was quantified. A region in the upper part of the lane was quantified and subtracted from the corresponding band to eliminate background. The total scFv gene produced was calculated using

| Antigen                              | Number of clones after 1st round of panning | Number of clones after 2nd round of panning | Number of binder clones after 2nd round of panning | Number of different scFv |
|--------------------------------------|---------------------------------------------|---------------------------------------------|-----------------------------------------------------|--------------------------|
| Bovine serum albumin (BSA)           | 3.0 x 10^2                                  | 1.8 x 10^3                                  | 1/15                                                | 1/1                      |
| Human ErbB3 ectodomain               | 2.3 x 10^4                                  | 3.7 x 10^4                                  | 3/12                                                | 3/3                      |
| Human tumor necrosis factor (TNF)    | 3.0 x 10^2                                  | 3.0 x 10^3                                  | 2/15                                                | 2/2                      |
| Human vimentin                       | 6.0 x 10^2                                  | 2.4 x 10^2                                  | 3/15                                                | 1/3                      |
| Human MHC class I-related chain A (MICA) | 6.6 x 10^2                                  | 1.0 x 10^4                                  | 6/15                                                | 3/6                      |
| Rotavirus                            | 6.0 x 10^2                                  | 1.5 x 10^4                                  | 2/15                                                | 1/2                      |

*Phage title obtained after subtraction of the unspecific phage retrieved in controls.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Frequency of heavy and light chain families. Plasmids obtained from 21 random clones from the phage display library, constructed using the scFv gene produced by ISA-PCR, were sequenced and analyzed using IgBlast. (A) Heavy and (B) Kappa light family frequencies observed in the library.

Table 2. Results of binding selection using different targets

| Antigen                              | Number of clones after 1st round of panning | Number of clones after 2nd round of panning | Number of binder clones after 2nd round of panning | Number of different scFv |
|--------------------------------------|---------------------------------------------|---------------------------------------------|-----------------------------------------------------|--------------------------|
| Bovine serum albumin (BSA)           | 3.0 x 10^2                                  | 1.8 x 10^3                                  | 1/15                                                | 1/1                      |
| Human ErbB3 ectodomain               | 2.3 x 10^4                                  | 3.7 x 10^4                                  | 3/12                                                | 3/3                      |
| Human tumor necrosis factor (TNF)    | 3.0 x 10^2                                  | 3.0 x 10^3                                  | 2/15                                                | 2/2                      |
| Human vimentin                       | 6.0 x 10^2                                  | 2.4 x 10^2                                  | 3/15                                                | 1/3                      |
| Human MHC class I-related chain A (MICA) | 6.6 x 10^2                                  | 1.0 x 10^4                                  | 6/15                                                | 3/6                      |
| Rotavirus                            | 6.0 x 10^2                                  | 1.5 x 10^4                                  | 2/15                                                | 1/2                      |
the ladder band of 800 bp from the 100 bp ladder (Fermentas) as mass reference.

SOE-PCR assembly reaction. SOE-PCR was performed as previously described in reference 32 and 37. Briefly, 50 ng of VHC and VLC PCR products were mixed with dNTPs and Paq DNA polymerase (Stratagene) without primers. The reaction was cycled 5 times (94°C for 45 sec, 60°C for 50 sec and 72°C for 60 sec). Primers were added to a final concentration of 0.4 μM and amplification was performed by the subsequent program: An initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 45 sec, 54°C for 45 sec and 72°C for 1 min, and a final extension reaction for 10 min at 72°C. To quantify scFv production, an aliquot of 10% of SOE-PCR product was resolved by electrophoresis on a 1% w/v agarose gel and quantified using QIAQuick gel extraction kit (QIAGEN). A total of 1.2 μg of digested scFv gene was ligated with 600 ng of pUCH1 using T4 DNA ligase (NEB) and incubated overnight at 16°C. The ligation product was precipitated using glycogen (Fermentas) following the manufacturer’s instructions and used to electroporate E. coli ER2738 (NEB). Later, cells were resuspended in 6 mL of SOC and incubated 1 h at 37°C. To quantify the number of transformants, 10-fold serial dilutions were made and plated in LB ampicillin (100 μg/mL). The remaining bacteria were centrifuged for 5 min at 5,000 rpm, resuspended in LB/ampicillin (100 μg/mL) and infected with 1 x 10^10 helper phage M13KO7 (NEB) for 1 h at 37°C. After cells were transferred to a flask with 50 mL of LB containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) and incubated overnight at 37°C, phages were precipitated using PEG and stored at -80°C.

DNA fingerprinting. Isolated clones obtained from the library were grown overnight at 37°C and plasmids were purified using Wizard Plus SV Minipreps DNA Purification Systems (Promega). The 1:30 plasmid dilution was used as a template to amplify the ladder band of 800 bp from the 100 bp ladder (Fermentas) as mass reference.

Figure 6. Phages selected against ErbB3. (A) To analyze the diversity displayed by phages binding ErbB3 and TNFα, all obtained in the second panning assay, were isolated and analyzed by phage ELISA, as described in Materials and Methods. A helper phage was used as negative control. Solid-colored bars indicate positive clones. (B) Phagemids were obtained from positives clones, sequenced and analyzed using IgBlast.25

Table: DNA fingerprinting

| Clone | Heavy Chain | Light Chain |
|-------|-------------|-------------|
|       | V | D | J | V | J |
| Clone 3 | IGHV3-48*02 | IGHD3-9*01 | IGHU4*02 | IKGVAD-2*01 | ISKJ2*01 |
| Clone 4 | IGHV3-23*04 | IGHD4-17*01 | IGHU8*04 | IKGV4*1*01 | ISKJ2*04 |
| Clone 11 | IGHV4-30-2*01 | IGHD2-8*02 | IGHU3*02 | IKGV6-2*01 | ISKJ2*04 |

Figure 6. Phages selected against ErbB3. (A) To analyze the diversity displayed by phages binding ErbB3 and TNFα, 12 clones for ErbB3 and 15 clones for TNFα, all obtained in the second panning assay, were isolated and analyzed by phage ELISA, as described in Materials and Methods. A helper phage was used as negative control. Solid-colored bars indicate positive clones. (B) Phagemids were obtained from positives clones, sequenced and analyzed using IgBlast.
scFv gene by PCR. The 25 μL reactions were composed of 3 μL of the corresponding plasmid 0.4 μM of primers VlgKFor01 and IgGrevNheI and GoTaq Green Master Mix (Promega). The PCR was performed using the following program: 95°C for the initial denaturation; 30 cycles at 95°C for 45 sec, 54°C for 45 sec and 72°C for 45 sec, and a final extension reaction of 1 min at 72°C. Five units of MvaI were added to 10 μL PCR product and incubated at 37°C for 3 h. Digestion products were resolved by electrophoresis on 2% w/v agarose gels.

Selection of phages. Selection of phages was performed with immobilized antigens by ELISA. 96 NUNC Maxisorb flat wells (Nunc) were coated with antigens (2–5 μg/well) diluted in carbonate buffer (15 mM of NaCO₃, 35 mM of NaHCO₃, pH 9.6) for 2 h at 37°C and blocked with 1% BSA in TBS (Tris 20 mM, NaCl 150 mM, pH 7.6), except in the case of BSA panning assay, when 0.05% of soybean extract protein in TBS was used as a blocking agent. The following antigens were used: Human TNFα was purchased from R&D; BSA was purchased from Rockland, Rotavirus particles and human vimentin were kindly provided by Dr. Jonas Chnaiderman (University of Chile, Chile) and Dr. Diego Catalan (University of Chile, Chile), respectively. The ectodomains of human ErbB3 and human MICA were cloned in pETb15 (Novagen) transformed in E. coli BL21 DE3 and purified in agarose Ni-NTA columns (Invitrogen).

10⁸ phages from the library were added to the coated wells and incubated for 2 h at 37°C. Phages were also incubated with wells coated with the blocking protein, to identify unspecific binders. Unbound phages were removed by washing 10–20 times with TBS containing 0.5% Tween 20 and the bound phages were eluted incubating with 100 μL of 100 mM glycine-HCl pH 2.2 solution for 10 min at room temperature. Immediately afterwards, the solution was neutralized with 6 μL of 2 M Tris-Base, and used to infect exponentially growing E. coli ER2738 for 1 h at 37°C. To quantify phage binders, a 1:100 bacteria dilution was plated on LB-Agar containing ampicillin (100 μg/mL); TBS was used as a blocking agent. As a control, wells were coated only with the corresponding blocking protein. 50 μL of purified phage solution was added to coated plates and incubated for 2 h at 37°C. Plates were then washed with TBS 0.05% Tween20 and incubated with 1:5,000 dilution of a mouse anti-M13 peroxidase conjugated antibody (GE Healthcare) in 1% BSA in TBS for 2 h at room temperature. Plates were washed as previously described. ABTS substrate was added and the absorbance was read at 405 nm using a Sunrise absorbance reader (TECAN). Absorbance from the control wells were subtracted from test wells to obtain the total absorbance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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