

# ISOLATION OF RECOMBINANT SINGLE-CHAIN ANTIBODIES (scFvs) WITH DESIRED SPECIFICITY FROM A 'SINGLE-SCAFFOLD' PHAGE DISPLAY LIBRARY.

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**Abstract:** Antibody (Ab) fragments produced in bacteria may provide high specific, low cost reagents for immunodiagnosis. An antibody library, encoding a diverse array of synthetic Ab fragments, each displayed on the surface of filamentous bacteriophage, was obtained by randomisation of residues involved in the binding site of a recombinant single-chain variable fragment (scFv) antibody with intrinsic high stability. By using site-specific mutagenesis we have generated a library of mutant scFvs molecules with different specificity. We report the isolation of scFv antibodies specific to the plant virus cucumber mosaic (CMV). After four rounds of selection and enrichment ('biopanning') on the immobilised virus antigen, a panel of different phage clones were obtained. Fully active soluble scFvs were produced in *Escherichia coli* at high yields. These engineered Ab fragments represent a valuable tool for inexpensive diagnosis of CMV in plant. Antibodies against virtually any antigen, including haptens, may be quickly derived from this library, thus representing a repertoire of proteins with different binding activity for the development of new biosensors.

*Keywords:* phage display, scFv, diagnostics, biosensor

## INTRODUCTION

Molecular repertoires (represented by phage libraries, synthetic peptide libraries, nucleic acid libraries and organic chemical libraries), comprising a high degree of diversity, are currently being used for the isolation of specific ligands, for diagnostic and therapeutic applications.

In phage display libraries, peptides or proteins are expressed on the surface of phage as fusion protein with the normal phage proteins. The coupling of protein on the surface of the phage with the gene encoding it inside the phage, allows a rapid selection of proteins with desired properties by a series of recursive cycles of phage binding, elution and amplification. One of the most successful applications of phage display has been the relatively easy derivation of recombinant monoclonal antibodies. In fact, functional fragments of antibody molecules can be expressed on the surface of filamentous phage, as a fusion product with the viral coat proteins (1,2,3). The most versatile antibody derivative comprises the heavy and light chain variable domains (VH and VL) of the Ab molecules, joined by a flexible peptide linker, usually the 15 aminoacid linker (Gly<sub>4</sub>Ser)<sub>3</sub>, to form a single polypeptide chain, the single chain antibody (scFv) (Fig. 1) (4,5). This molecule maintains antigen-binding properties. Antibody phage display libraries can be produced from different sources like hybridoma (6), human, mouse, chicken, rabbit cells or as synthetic libraries (for a review see ref. 2). From libraries containing more than 10<sup>8</sup> different clones it has been shown that, after several cycles of selection and amplification (Fig. 2), genetically pure populations of phages can be eluted with specificity toward several different antigens or haptens (7, 8, 9, 10). Subsequently, soluble recombinant antibodies may be derived at high yields without the need to immunise animals or to use hybridoma

technology. Moreover, mutation and further selection can improve of the affinity of the selected antibodies.

Here we describe the construction of a synthetic phage display library, starting from a scFv molecule endowed with peculiar thermodynamic properties such as thermal stability and functionality in a reducing environment. The natural scaffold provided by this scFv has been used for the construction of a molecular repertoire displayed on phage by random mutagenesis of four aminoacids in the complementarity determining region 3 (CDR3) of both VH and VL. This library represents a useful pool of diversity from which specific and stable recombinant antibodies can be isolated.

As a first example, we describe the isolation of scFvs which are specific for cucumber mosaic virus (CMV), a pandemic plant virus. CMV-specific scFvs can be used as classic diagnostics, and possibly for the development of new sensing devices.

## **MATERIALS AND METHODS**

### *Preparation of a phage display library*

CDR3 mutagenesis of the original scFv was performed by the polymerase chain reaction (PCR) using degenerated primers (Table 1; Fig.3). The primers VH(NcoI)back (a) and VHCDR3 for (b) were used for VH amplification ; VKLINKback (c) and VKCDR3 for (d) were used for the amplification of the peptide linker together with the VL. PCR products were run on a 1.5% agarose gel, purified using the 'QIAquick gel extraction kit' (QIAGEN) and scFv assembly was obtained by 'gene splicing for overlap extension' (SOE)-PCR. Primers VH4(NcoI)back and VKJ(NotI)for (e) were used to amplify the assembled scFv fragment.

After purification and digestion with the restriction enzymes Nco I and Not I, the scFv inserts were cloned in the phagemid pDN332 (10)

(Fig. 4). Here, the scFv gene is inserted between the bacterial PelB signal peptide and the FLAG tag sequence, (followed by an amber translation stop codon before the phage minor coat protein gene III, g3p). SupE *E. coli* TG1 cells (which allow the translation through the amber stop codon to produce the scFv-g3p fusion protein displayed on the phage tip), made competent for electroporation, were transformed and selected on 2xYT plates containing 2% glucose and 100 µg/ml ampicillin. Colonies were scraped by flooding the plates with 2xYT medium and the phage library was rescued by superinfection with  $2 \times 10^9$  cfu/ml of M13 K07 helper phage (Pharmacia). Phages were PEG-precipitated from the supernatant and used for panning.

#### *Affinity panning*

An immunotube (Nunc, Maxisorp) was coated with 10 µg/ml of purified cucumber mosaic virus (CMV, strain CMV-F100, serogroup I). Coating was performed in PBS and incubation overnight (O/N) at 4°C. A polyclonal rabbit antiserum against the same CMV strain was used to check immobilisation of CMV. After washing three times with PBS and blocking for 2 hours in 2% skimmed milk powder in PBS (PBSM) at 37°C, panning was performed with approximately  $10^{12}$  phages diluted in 4 ml PBSM. Unbound phages were removed by washing the tubes with PBS containing 0.1% Tween-20, (PBST, 20 times) and PBS (20 times) and the antigen-bound phages were eluted by adding 1 ml of 1 M triethylamine for 5 min and then immediately neutralised by adding 0.5 ml 1.0 M Tris-HCl, pH 7.4. Exponentially growing TG1 cells were infected by eluted phage particles, spread on 2xYT plates, containing 2% glucose and 100 µg/ml ampicillin, and an aliquot was used for titration. After helper phage infection, phage supernatants were precipitated and re-used for another cycle of panning on the same antigen. In total, four rounds of panning were repeated. At the

last round of panning *E. coli* non-suppressor HB2151 cells (which allow scFv soluble expression) were infected. Individual colonies were then grown in a microtitre plate, induced for soluble scFv expression by shaking O/N at 30°C after addition of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG, final concentration 1 mM). Cells were pelleted and the supernatants were used in an ELISA test, as described below.

Periplasmic extracts were obtained as already described (11). ScFv purification was performed by using rProtein L<sup>TM</sup>-agarose, (ACTigen), following the manufacturers instructions.

#### *Binding activity of scFv*

Binding activity was checked by enzyme-linked immunosorbent assay (ELISA). Plates (Nunc, Maxisorp) were coated with 10  $\mu$ g/ml of CMV diluted in PBS (O/N at 4°C). For analysis of reactivity on infected plant extracts, a triple antibody sandwich (TAS)-ELISA was performed where virus particles were captured by the polyclonal rabbit antiserum from plant sap at different dilutions. After blocking, induced culture supernatants, periplasmic extracts or purified scFvs, were applied to the plates (2 hours, 37°C). Bound scFv was detected by adding Anti-Flag M2 monoclonal antibody (Sigma, 2.5  $\mu$ g/ml, 2 hours), followed by one hour incubation with HRP-conjugated goat anti-mouse IgG antibodies (Kirkegaard and Perry Laboratories, KPL). Each step was followed by 3 times washing with PBST and PBS. Enzymatic activity was measured colorimetrically after 1 hour using 2,2-azino-di-3-ethylbenz-thiazoline sulphonate (ABTS, KPL) and read at 405 nm on an ELISA microtitre plate reader.

#### *SDS-PAGE analysis and immunoblotting*

Samples were denatured for 3 min at 100°C in SDS and  $\beta$ -mercaptoethanol and proteins were separated through SDS-

polyacrylamide gel electrophoresis (SDS-PAGE, 12%), then electrotransferred to nitrocellulose membrane (Hybond-C Super, Amersham). Apparent molecular weights were estimated by Rainbow markers (Amersham). Western blots were blocked O/N with 10% skimmed milk in PBS before adding purified scFv (5 µg/ml, 2 hours), and then Anti-Flag M2 monoclonal antibody (4 µg/ml, 1 hour) followed by 1 hour incubation with HRP-conjugated goat anti-mouse IgG antibodies (KPL) or HRP-conjugated streptavidin. Proteins were detected by enhanced chemiluminescence (ECL, Amersham).

#### *Recombinant DNA techniques and sequencing*

DNA sequencing was performed using dye terminator technology combined with a Perkin Elmer 373A Stretch Automated Sequencer. Molecular analysis were performed by standard methods (12).

## **RESULTS**

The VH-CDR3 and VL-CDR3 of the original scFv were PCR-mutagenized (Fig. 3) by using degenerated primers (Table 1).

The scFv was further modified in the VH-CDR3 by removing 9 aminoacids. After mutagenesis, VHs and VLs were assembled and cloned in the phagemid vector and, upon electroporation of *E. coli* cells, a library of about  $0.5 \times 10^8$  different clones was obtained. Phage supernatant was used for panning on immobilised CMV. After each round of panning, titres were estimated in order to evaluate the course of CMV-specific phages enrichment. Phage titres were:  $3 \times 10^6$  cfu/ml (1<sup>st</sup> panning),  $6.5 \times 10^6$  cfu/ml (2<sup>nd</sup> panning),  $8 \times 10^7$  cfu/ml (3<sup>rd</sup> panning) and  $2 \times 10^8$  cfu/ml (4<sup>th</sup> panning). An aliquot (100 µl) of eluted phages from the last panning was used to infect *E. coli* strain HB2151. Seventy clones were analysed for soluble expression of scFv in the culture supernatant and for reactivity with CMV. About forty clones proved to be positive

and they were classified into two groups according to the ELISA OD<sub>405nm</sub> readings after one hour (++ => 1.0; +=> 0.5). Twenty-four clones were classified ++ and fifteen clones +. Eight clones were chosen for DNA sequencing (Table 2). Three of them (clones A5, D5 and G4) showed to be identical. Three of them (clones F7, G3 and G10) proved to be identical only in the VH-CDR3 but different in the VL-CDR3 of VL. Clones G2 and B4 were different.

Upon induction, culture supernatants or periplasmic extracts were assayed for binding specificity by ELISA using the following unrelated antigens: bovine serum albumin (BSA), actin, lysozyme, superoxide dismutase (SOD), artichoke mottled crinkle virus (AMCV) coat protein and tomato spotted wilt virus nucleoprotein (TSWV-N). No binding was observed with these antigens for scFvs derived from clones G4 (and consequently A5 and D5), B4 and G2 (Table 2). Soluble scFvs deriving from clones G4, B4 and G2 were also used to detect CMV in plant extracts, at various dilutions, in comparison with the monoclonal antibodies (mAbs) 185 and 176, routinely used for CMV detection (Tab. 3). ELISA-OD<sub>405nm</sub> readings showed that all of scFvs detect CMV in infected plant extracts and do not cross-react with healthy plant extracts.

ScFv from clone G4 was affinity purified from periplasmic extracts. Approximately 2 mg of pure scFv could be obtained from 1.5 litre of bacterial culture. It was tested in Western blot, showing specific binding to CMV-CP protein (data not shown).

## **DISCUSSION**

The development of biosensors based on genetically engineered molecules may provide several potential advantages in comparison with sensors that rely on natural proteins only. In particular antibody derived

fragments, like the scFv, offer the necessary sensitivity and specificity required for a sensing element. These molecules can be produced in bacteria at unlimited quantities and, by genetically fusion with other proteins or peptides (13) they can be easily oriented and immobilised on biosensor chips (14). Moreover, the small size of scFvs (28 kDa vs 150 kDa of a full-length antibody) may be exploited for designing 'multifunctional' biosensors, allowing simultaneous analysis of several analytes.

Antibody phage display libraries (by linking together the phenotypic positive selection and the relevant coding sequence) can speed up the search for, and isolation of functional recombinant antibodies from molecular repertoires. Thus, antibody phage display libraries provide a useful pool of diversity from which specific molecules with different binding activity (but similar structure) can be isolated each time.

Stability is another important requirement in the design of a biosensor. By choosing the right protein scaffold, this requisite may be satisfied. Antibody stability varies as a consequence of sequence variations within both frameworks (FR) and complementarity determining regions (CDR) and the amino acid primary sequence determines the percentage of correctly folded molecules (15, 16). Antibody-derived fragments are generally enough stable molecules, although they need an intra-chain disulphide bridge to be functional.

Starting point of our work was a scFv, shown to be stable and functional in the reducing environment of both eukaryotic and prokaryotic cytoplasm, accumulating as a soluble cysteine-free scFv, with an *in vivo* half-life longer than 16 hours. This antibody also showed a remarkable stability *in vitro*, as demonstrated by guanidinium chloride denaturation/renaturation studies, indicating that the refolding occurs even in a reducing environment. Hence, this molecule does not rely on

disulphide bonds formation and has the required stability to fold correctly even in harsh conditions.

By using principles of protein design, from this scaffold we generated a repertoire of different scFvs by parsimonious mutagenesis, randomising only four aminoacids in the CDR3 (the hypervariable regions, those mainly involved in interaction with the antigen) of both VH and VL. We choose to mutate only CDR3 loops in our repertoire was dictated by the consideration that they are centrally located in the antigen-binding site and by the high diversity of CDR3 sequences observed in natural antibody repertoires. Moreover, since the majority of high affinity antibodies described has a short CDR3 in the heavy chain, we decided to reduce the length of VH-CDR3. The use of short CDR3s in VH and VL immunoglobulin domains is also generally associated with better antibody stability to proteolysis, improved binding and bacterial expression (10). Such a repertoire has been expressed as a fusion product on the surface of a filamentous phage. By repeated cycles of enrichment on the immobilised antigen we were able to rapidly isolate several scFvs specific to CMV. These recombinant antibodies represent simple and low-cost reagents for CMV diagnosis.

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**Table 1: Oligonucleotide Primers used for VH and VL mutagenesis and amplification.**

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- a) VH(NcoI)back (5')AAT CCA TGC CAT GGC CCA GGT GCA GCT GCA GGA GTC TGG G(3')
- b) VHCDR3 for (5')GGT CCC TTG GCC CCA GTA GTC AAA MNN MNN MNN MNN TCT TGC ACA GTA ATA CAT GGC TG(3')
- c) VKLINKback (5')TTT GAC TAC TGG GGC CAA GGG ACC(3')
- d) VKCDR3 for (5')GAG CTT GGT GCC TCC ACC GAA CGT CCA CGG MNN MNN MNN MNN TTG CTG ACA GTA ATA GGT TGC(3')
- e) VKJ(NotI)for (5') TTC TCG ACT TGC GGC CGC CCG TTT GAT CTC GAG CTT GGT GCC TCC ACC GAA CG(3')
- (N= each nucleotide; M= dATP or dCTP)

**Table 2: Sequences and reactivity of anti-CMV selected antibody clones**

Clone	Reactivity* ELISA – O.D. <sub>405nm</sub> readings after 1 hour: ++ > 1.0 + > 0.5	VH-CDR3 Original residues: RRNYPYYYGSRGY	VL-CDR3 Original residues: SNED	Cross-reactivity
<b>F7</b>	<b>+</b>	<b>RRNYPYYYGSRGY</b>	<b>GRYN</b>	<b>+</b>
<b>G3</b>	<b>+</b>	“	<b>GRRG</b>	“
<b>G10</b>	<b>+</b>	“	<b>GRRH</b>	“
<b>B4</b>	<b>++</b>	<b>NNYS</b>	<b>GRRR</b>	<b>None</b>
<b>G2</b>	<b>++</b>	<b>VTYN</b>	<b>SRRR</b>	“
<b>A5</b>	<b>++</b>	<b>NNWS</b>	<b>NNWS</b>	“
<b>D5</b>	<b>++</b>	“	“	“
<b>G4</b>	<b>++</b>	“	“	“

**Table 3: Reactivity\* of anti-CMV selected antibody clones with infected and healthy plant extracts**

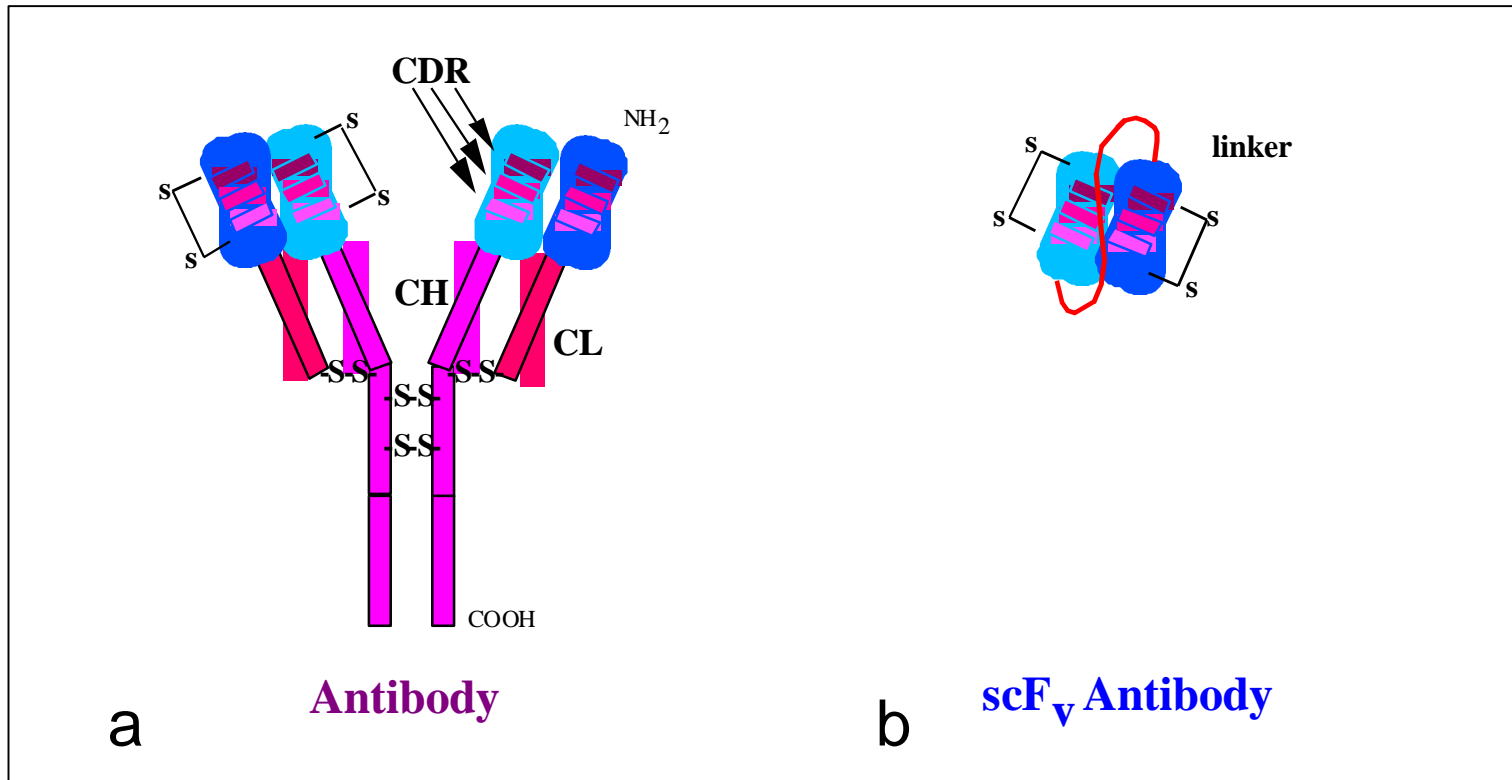
\* TAS-ELISA OD<sub>405nm</sub> readings after 1 hour

\*\* CMV-i= CMV-infected plants

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Clone	CMV-i**	CMV-i**	CMV-i**	Healthy plants	Healthy plants	Healthy plants
	1/100	1/1000	1/10000	1/100	1/1000	1/10000
G4	1.94	1.41	0.46	0.30	0.17	0.09
B4	1.33	0.93	0.29	0.22	0.09	0.08
G2	1.12	0.28	0.12	0.22	0.12	0.14
mAb185	1.25	0.93	0.31	0.07	0.08	0.08
mAb176	0.41	0.37	0.27	0.07	0.08	0.08

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**Fig. 1: Structure of: a) a full-length antibody; b) an antibody-derived (single-chain Fv) fragment (b).  
 CDR= complementarity determining regions; CH= heavy chain constant region; CL= light chain constant region; S-S= disulphide bridges.**

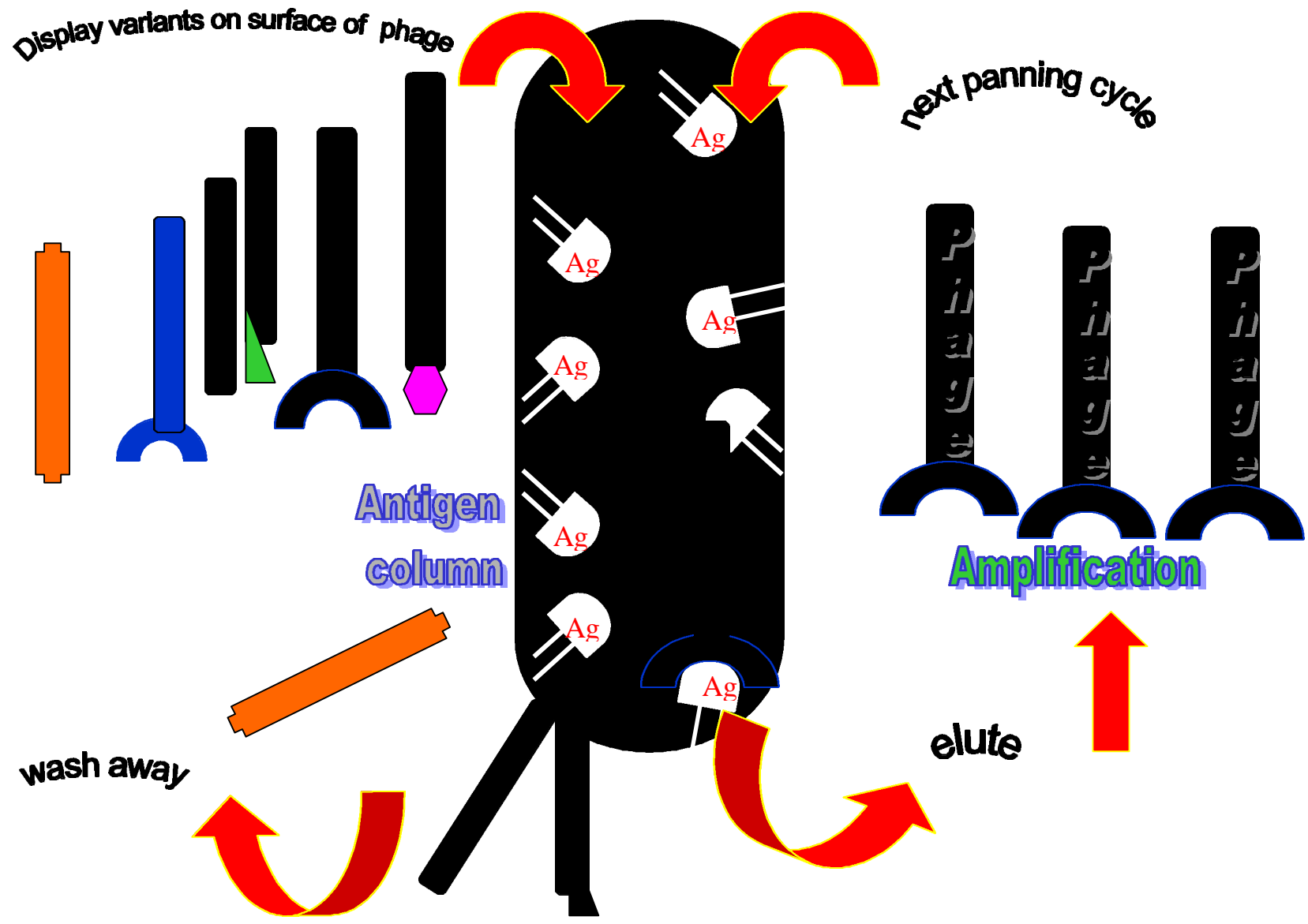
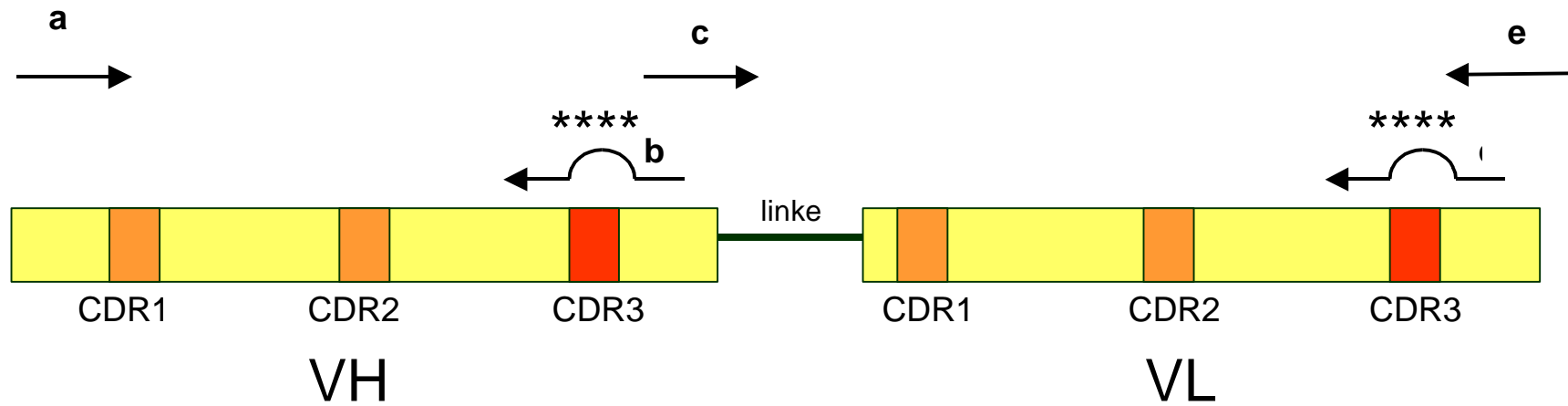
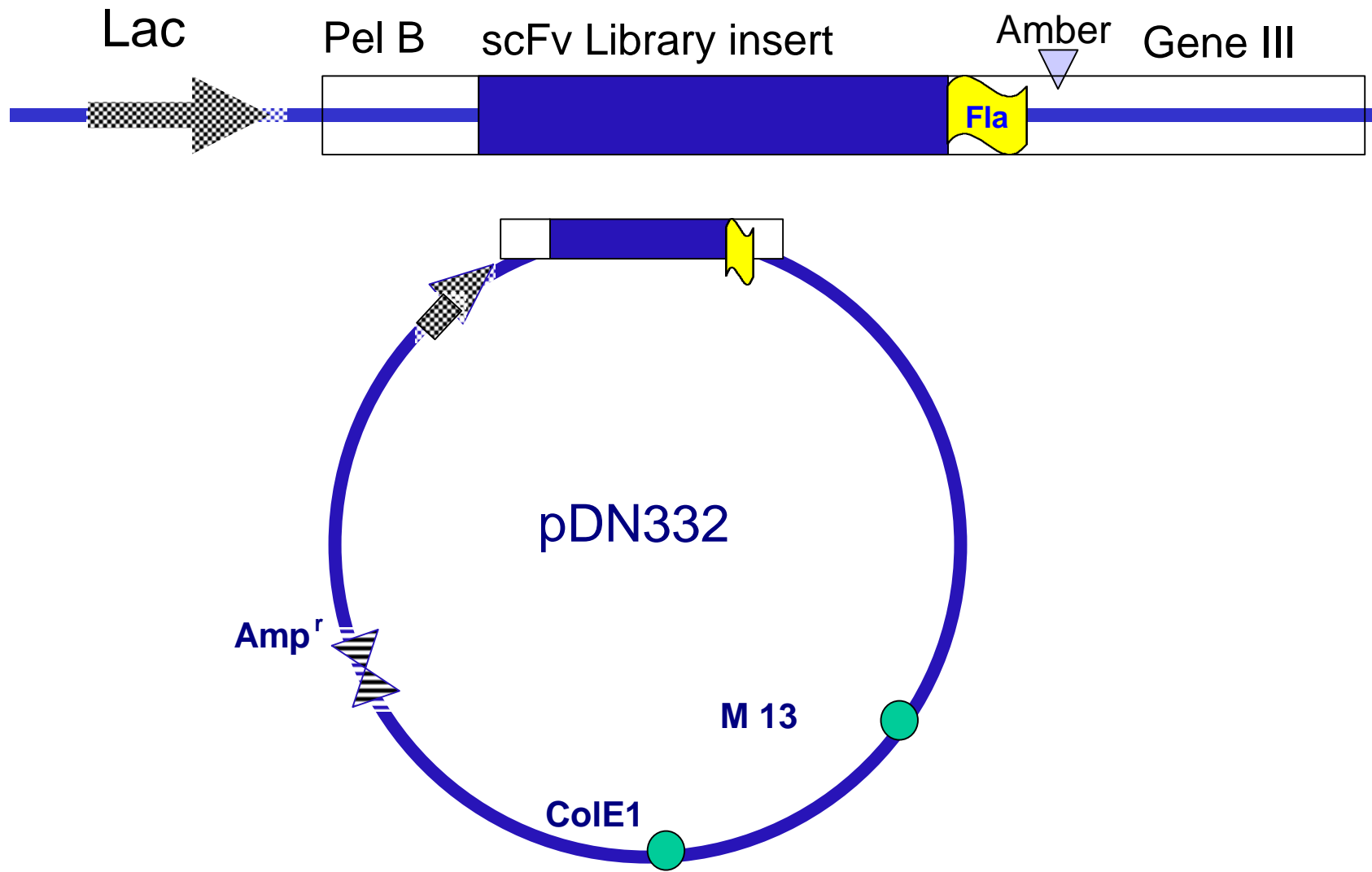


Fig. 2: scheme of the 'biopanning'



**Fig. 3: Randomisation of 4 aminoacidic residues in the CDR3 of both VH and VL for the construction of a 'single-scaffold' antibody library. Asterisks indicate mutated positions.**



**Fig. 4: scFv library cloning in the phagemid vector**