Sequence-independent construction of ordered combinatorial libraries with predefined crossover points

Laetitia Jézéquel, Jacqueline Loeper, and Denis Pompon

Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique,
Gif-sur-Yvette, France

BioTechniques 45:523-532 (November 2008)
doi 10.2144/000112954

Combinatorial libraries coding for mosaic enzymes with predefined crossover points constitute useful tools to address and model structure-function relationships and for functional optimization of enzymes based on multivariate statistics. The presented method, called sequence-independent generation of a chimera-ordered library (SIGNAL), allows easy shuffling of any predefined amino acid segment between two or more proteins. This method is particularly well adapted to the exchange of protein structural modules. The procedure could also be well suited to generate ordered combinatorial libraries independent of sequence similarities in a robotized manner. Sequence segments to be recombined are first extracted by PCR from a single-stranded template coding for an enzyme of interest using a biotin-avidin-based method. This technique allows the reduction of parental template contamination in the final library. Specific PCR primers allow amplification of two complementary mosaic DNA fragments, overlapping in the region to be exchanged. Fragments are finally reassembled using a fusion PCR. The process is illustrated via the construction of a set of mosaic CYP2B enzymes using this highly modular approach.

INTRODUCTION

Development of new in silico approaches for the prediction of protein structure-function relationships is of major interest for biotechnology (1–3). Classically, rational design of proteins with new properties of interest relies on X-ray or nuclear magnetic resonance (NMR)-based structures as well as modeling of substrate-protein interactions. However, structure-based predictions have limited power when multistep or complex mechanisms are involved. In contrast, in vitro approaches, like directed molecular evolution, have proven to be a powerful tool for function optimization, without prerequisite structural knowledge. But these methods are resource demanding, and these case-by-case approaches are generating very little reusable knowledge. Giving predictive value to combinatorial engineering remains a challenge. Classical directed evolution relies on the functional selection of sequence variants with a randomly recombined mutation set

giving improved property. Association of useful mutations into a single sequence is generally performed by DNA shuffling, also called molecular breeding (4–8). Although this optimization process is efficient, when high-throughput selection or screening systems are available, its applicability is limited when complex functional criteria are involved. An alternate semi-rational approach was proposed to accommodate the directed evolution to low-throughput selection systems. This is based on a strategy in which functional properties of fully ordered combinatorial libraries are determined and used to build predictive structure-function databases by statistical approaches. To make statistical calculation efficient, these combinatorial libraries must feature only predetermined recombination points. Similar requirements are found to apply the SCHEMA computational algorithm (9,10) which was proposed to reduce the probability to generate misfolded chimeric proteins that are frequently generated upon DNA shuffling.

SCHEMA method helps predicting recombination points between parents that can be tolerated or not, thus determining “structural modules” that can be successfully rearranged in a combinatorial manner. The described sequence-independent generation of a chimera-ordered library (SIGNAL) method offers a new efficient way to construct such libraries with predefined recombination points required both for SCHEMA algorithm-based library and for statistical approaches of structure-function relationship.

Alternate methods have been previously described to create such libraries, either based on common restriction sites (11–12) or on fusion-PCR (13), but are limited to high similarity sequences. Homology-independent approaches have been described too, but they do not allow control of crossover points and generate a high level of “out of alignment” fusion (14,15) or they require numerous steps (16). Although fully synthetic gene library also emerges as a promising approach, it remains resource demanding and highly expensive.

The developed SIGNAL method involves the partitioning of two protein sequences into sequence modules, chosen at will by the experimenter. For an easier description, one protein sequence will be defined as “donor” and the other as “acceptor.” Based on the rational design chosen for the library, any acceptor sequence block can be replaced by its donor counterpart in a sequence-independent manner. Crossover points can be freely chosen, and the approach would be easy to automate using robotic tools to generate full or defined subsets of combinatorial libraries. Furthermore, the role of donor and acceptor can be easily swapped. As a proof of principle, we used SIGNAL to create a set of defined chimeras between cytochromes P450 (CYP) 2B6 and 2B11. This library was designed to optimize the kinetic parameters for the activation of a chemotherapeutic prodrug, cyclophosphamide. In our case, the proteins have fairly high overall sequence similarity (78% identity), but this parameter has no influence on the construction efficiency of the desired chimeric sequences.

MATERIALS AND METHODS

Reagents, Strains, and Material

Milli-Q water (Millipore, Billerica, MA, USA) with a resistance of 18.2 Ω was used for all the experiments. Platinum *Taq* DNA polymerase kit (Invitrogen, Cergy, France) was used for all amplifications. Magnetic carboxylic acid Masterbeads 500 nm and their recommended buffers were purchased from Ademtech (Pessac, France). Streptavidin was from Sigma-Aldrich (Saint Quentin Fallavier, France). Reverse phase-purified oligonucleotides (Eurogentec, Liège, Belgium) were resuspended in water and further purified using ion pairing reverse phase chromatography. CYP2B11 synthetic cassette was produced by GeneCust (Dudelange, Luxembourg).

All enzymes were purchased from New England Biolabs (Hitchin, UK).

Saccharomyces cerevisiae strain W303-1B (*MAT α* , *ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11, 15*, *ura3-1*) (17) was used for cloning by gap repair. *Escherichia coli* strains were DH5 α F'IQ competent cells (Invitrogen) for cloning into pM13mp19 vector and Top 10 chemically competent cells (Invitrogen) for DNA amplification for sequencing.

Construction of Template Plasmids

Single-stranded DNA of human CYP2B6 and of canine CYP2B11 templates was prepared by cloning the CYP open reading frames (ORFs) into a pM13mp19 plasmid (18). The CYP2B6 ORF was extracted from p2B6/pYeDP60 (19) by *Bam*HI digestion and ligated into *Bam*HI-

digested pM13mp19, giving p2B6/M13. Clones with the CYP2B6 ORF into the pM13mp19 positive strand were selected. A synthetic CYP2B11 expression cassette was designed based on GenBank CYP2B11 cDNA coding sequence (accession no. NM_001006652) and was recoded using a yeast codon bias to optimize expression (GenBank accession no. EU736326). The CYP2B11 ORF was flanked by 100 bps of the *GAL10-CYC1* promoter (delimited by *Bss*SI and *Pac*I sites) and the *PGK* terminator (delimited by *Nhe*I and *Asc*I sites) sequences, respectively. The synthetic gene cloned into pPCR Script Amp (Stratagene, Amsterdam, The Netherlands) was digested either with *Bss*SI and *Nhe*I or by *Pac*I and *Asc*I, and resulting fragments were made blunt-end by treatment with DNA polymerase I Klenow fragment before ligation into *Hinc*II-digested pM13mp19 to yield p2B11p/M13 and p2B11t/M13, respectively. The constructs were selected to have either the CYP2B11 ORF in the p2B11p/M13 negative strand or the CYP2B11 ORF in the p2B11t/M13 positive strand.

Preparation of Streptavidin-Covered Magnetic Beads

Three milligrams of magnetic Masterbeads were functionalized following the manufacturer's protocol. Washing steps were performed with 300 μ L manufacturer's recommended buffers, and protein immobilization was performed using 30 μ g streptavidin. Following bovine serum albumin (BSA) saturation, beads were washed three times and suspended in immobilization buffer for immediate use.

Modeling

CYP2B6 and CYP2B11 protein sequences were aligned, and their three-dimensional (3-D) structures were modeled using rabbit CYP2B4 (PDB reference 1suo) as template and the Modeller program (available at www.salilab.org/modeller). 3-D models were used to optimally partition P450 sequences into 15 fragments (A to O) as described (see Figure 2).

Primer Design

All primers used in this study are listed in Table 1. Names of primers A to O match names of constructed chimera. Forward primers A1 to O1 include 12- to 15-bp oligonucleotides complementary to the CYP2B11 ORF followed by 20–24 bp complementary to CYP2B6 ORF at their 3' end. Reverse primers A2 to O2 comprise 12–17 bp identical to CYP2B11 ORF followed by 19–22 bp identical to CYP2B6 ORF (see Figure 2 for details). Oligonucleotide 5'-biot

features a 5' Biotin-TEG structure that was purchased from Eurogentec.

Chimera Construction

Fifteen chimeras were generated by replacing each sequence fragment from A to O into the CYP2B11 ORF by the corresponding CYP2B6 sequence fragments based on indicated alignment (see Figure 2). Each variant was given a unique name (capital letters A to O indicating the swapped CYP2B6 fragment). As an example, the following

protocol was carried out to create chimera A and was found effective for the construction of all chimeric structures. PCR amplifications were performed using Platinum *Taq* DNA polymerase according to the manufacturer's protocol and Table 2 parameters. Products were purified using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany), and then DNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer (Labtech, Palaiseau, France).

Step 1. PCR amplification described in Figure 1, step 1, which contained primers and templates described in Table 2, was carried out with following PCR cycling conditions: one cycle of 94°C for 2 min, then 25 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 100 s, and then 68°C for 10 min. After purification, the product was eluted in 50 µL recommended immobilization buffer (Ademtech).

Step 2. Ten microliters 1% streptavidin-covered bead solution were added to the totality of the purified amplification product from step 1 and incubated for 1 h at room temperature under gentle shaking. Beads were washed two times with 300 µL immobilization buffer and one time with water and suspended into 50 µL water. Suspension was heated for 5 min at 95°C, before ice cooling, allowing the recovery by magnetic separation of a supernatant containing only the nonbiotinylated strand. The single-stranded product solution was transferred into a clean tube.

Step 3. PCR amplification described in Figure 1, step 3, in accordance with Table 2 parameters, was performed with the same cycling conditions as in step 1 except for an elongation time of 10 s and the use of water to elute the purified product.

Step 4a and 4b. One nanogram of purified product from step 3 was added to PCRs 4a and 4b mixes, as described in Table 2. PCR cycles were the same as before except for an elongation time of 15 s for PCR 4a and 105 s for PCR 4b and the use of water to elute the purified product.

Step 5. Equimolar concentrations (corresponding to 1 ng of the larger product) from steps 4a and 4b were mixed together and with all PCR components except the enzyme (see

Table 1. Oligonucleotides Used in This Study

A1	5'- CTAAATTAATTAAT GGAAGTCAAGCTCCTCCTC-3'
A2	5'- GATGGG CAGCGGCCGGGGCCCTGGTGGGAGGCGG-3'
B1	5'- CACCAGGCCCCC GCCCTCTGCCCTTTTGGGAAAC-3'
B2	5'- GTACACCGTGAAG ACGTCCCATATTTCTCTCG-3'
C1	5'- GAAATATGGGGACGT CTTCACGGTACACCTGGGAC-3'
C2	5'- GAAAGGTGGAAGACC CTTCGGCGATTCTCTGTGAC-3'
D1	5'- GAAAGGTGGAAGACC CTTCGGCGATTCTCTGTGAC-3'
D2	5'- CTCTTCCACCAG ACACTGAGCCTCCTCTGAATC-3'
E1	5'- GAGGCTCAGTGT CTGATAGAGGAGCTTCGGAAATC-3'
E2	5'- GTAGCCAAAACG TTTTCCAAAGACGATGGAGC-3'
F1	5'- GTCTTTGGAAAAC GATTCCACTACCAAGATCAAG-3'
F2	5'- GGAGAAACTCG AGATGAGTGAAAAAGTCTGGTAG-3'
G1	5'- CTTCGCACTCAT CAGCTCTGTATTGGCCAGCTG-3'
G2	5'- GTGCCAGGAAAG TATTCAAGAAGCCAGAGAAG-3'
H1	5'- GCTTCTTGAAGT ACTTTCTGGGGCACACAGG-3'
H2	5'- GAGCGCTGGGAT CCAGGGTTTCACGGTGCTTCTC-3'
I1	5'- CAGGGAAACCCT GGACCCAGCGCCCCAAGGAC-3'
I2	5'- GTCTCCGTGCCG CAAAGAAGAGCGAGAGCGTG-3'
J1	5'- CGCTCTTCTT CGCTGGCACTGAGACCACCAGC-3'
J2	5'- CATTTTGGCTCG GTCTATGAAGCTCTGGAGGGC-3'
K1	5'- CTTCCCTTGACG ATCGAGCCAAATGCCATACAC-3'
K2	5'- GTGACCATATGG GCACACCCATGGGGAGAAGG-3'
L1	5'- CCATTGGGGTAC CCACATTGTACCCAACAC-3'
L2	5'- GCAGAGTGCAAT ATGAGAAATACTTCTGTGTC-3'
M1	5'- GAAGTATTCCAAT CCTGAGCACTGCTCTCCATG-3'
M2	5'- CATTCTTCTT CAGTGCCCCATTGGCATCCAGAAAG-3'
N1	5'- GCCAATGGAGCT CTGAAAAAGACTGAAGCTTTATC-3'
N2	5'- CACAGAGAAGTT CTGGAGGATGGTGGTGAAGAAG-3'
O1	5'- CACCATCCTGC AGAACTTCTCCATGGCCAGCC-3'
O2	5'- CTAGCTCAGCAC CTCAGCGGGGCAGGAAGCGGATC-3'
5'-biot	5'-biotin-CCAGCTGGCGAAAGGGGGATGTGCTGC-3' Complementary to pM13 mp19 positive strand, located about 100 bp downstream of the polylinker
pGAL	5'-CCTTATACATTAGGTCCTTTGTAGC-3' Specific of GAL10-CYC1 promoter
tPGK	5'-CTGGCAATTCCTTACCTTCC-3' Specific of PGK terminator

Bolded part of primers A1 to O2 correspond to CYP2B11 sequences, while the remaining part corresponds to CYP2B6 sequences.

Short Technical Reports

Figure 2. Amino acids alignment of CYP2B11 and CYP2B6 sequences. Arrows and letters above the sequence represent limits and labels of exchanged sequence fragments, respectively. Sequence extent and crossover points of oligonucleotides designed for chimera generation are illustrated by arrows under the first sequence line. Plain arrows indicate forward primers, and dotted arrows indicate reverse primers. This construction is identical for all the sequence fragments.

Table 2). The solution was incubated for 3 min at 94°C followed by 25 min at 60°C. Polymerase was then added, and the mixture was kept at 68°C during 105 s. Then, 25 cycles at 94°C for 15 s, 60°C for 90 s, and 68°C for 105 s were performed, followed by 10 min at 68°C.

Chimera Cloning

Generated products for chimeric P450s were cloned by homologous recombination with the expression vector pYeDP1–8-2 using gap repair in yeast as described in Reference 20. Expression cassette for this vector involves the *GAL10-CYC1* promoter and the *PGK*

terminator sequence. Two marker genes, *URA3* for yeast and *ampR* for bacteria, were used for selection. Recombination occurred between the promoter and terminator flanking sequences of the chimera and the corresponding vector sequences. Cells were transformed with 500 ng final PCR amplification product and 100 ng *Bam*HI/*Eco*RI digested pYeDP1–8-2. After selection for uracile prototrophy of transformed yeast cells, total DNA of yeast clones was extracted (21) and used to transform *E. coli* using an ampicillin resistance selection. For each construction, plasmidic DNAs of six randomly picked *E. coli* clones were

sequenced (GATC Biotech, Konstanz, Germany), and their structures checked.

RESULTS AND DISCUSSION

Chimera Construction

The developed chimeragenesis technique is illustrated in Figure 1, defining X as the donor and Y as the acceptor sequences, respectively. SIGNAL uses a first amplification to add an acceptor part upstream of the crossover point of the targeted chimera (step 1). The product is purified from parental template using a biotin-avidin system (step 2). The donor segment to be exchanged is flanked by short stretches of acceptor sequences using PCR (step 3). Resulting product is used to generate the two moieties of the targeted chimera (step 4). Finally, these two parts are assembled using a fusion PCR (step 5). A proof-of-principle is given for generation of mosaic structures between CYP2B11 (acceptor) and CYP2B6 (donor) ORFs. Used sequence fragments and primers are presented in Figure 2.

In step 1, specific primer hybridization was facilitated by the use of

Advancing Aptabody™ Applications

	Antibody	Aptabody™
Amino acids	✓	✓
Fatty acids		✓
Carbohydrates		✓
Organics & Metals		✓

Industrial

Therapeutics

Diagnostics

Platform	Products & Tools
Animal	drug targeting and Imaging
Tissue	biomarkers
In Vitro	ligands & artificial enzymes
Material	nanobiotechnology

www.aptagen.com Toll Free: 877.278.2463

» RELIABLE SOLUTION FOR YOUR miRNA RESEARCH

a single-stranded template (using pM13mp19) for the donor sequence. The hybridization temperature was set to the lowest melting temperature (T_m) of all the used primers. A forward bipartite (crossover defining) primer and the biotinylated common reverse primer were used to amplify a biotinylated segment extending from the left-side crossover point to the end of the donor sequence template (see Figure 1). Typical amplification patterns for this step are presented (Figure 1A) and exhibit the expected size. Some nonspecific side products were observed but at a very low concentration compared with desired products. In step 2, each biotinylated product from step 1 was bound to streptavidin-covered magnetic beads. Single-stranded donor template, which is not biotinylated, was removed during this step, thus reducing the potential parental contamination of the targeted chimera. Furthermore, this process permitted to keep only the useful product strand. This single strand was used for step 3, in which the exchanged donor fragment itself, flanked by a dozen of acceptor base pairs, is amplified (see Figure 1). In steps 4a and 4b, the double-stranded amplification product from step 3 was used as a template. It was mixed with single-stranded templates consisting in common sequences placed either upstream (4a) or downstream (4b) of the acceptor sequence. This step was designed to avoid the possibility of parental sequence amplification in the following PCR where the two outermost primers were used simultaneously. The use of single-stranded templates improved the specific hybridization of primers and templates together. These steps led separately to the amplification of both chimera moieties (see Figure 1, C and D, for steps 4a and 4b, respectively). In step 5, PCR was carried out to fuse both amplification products from steps 4 through hybridization of their complementary sequences at the position of the exchanged fragment. For this fusion PCR, it was critical to use mole-to-mole ratios of templates. The PCR mixture without enzyme was heated at a denaturing temperature and then slowly cooled to hybridization temperature to ensure an efficient long fragment hybridization. All the resulting PCR amplification products exhibited the same and expected size, corresponding to the full-length chimera (Figure 1E).

This method represents the best compromise between efficiency, reduction of parental contamination, and number of PCR cycles. Some PCR steps could be fused together, but generally with detrimental effects on results.

The two outermost primers used for the fusion PCR included flanking sequences homologous to the promoter and terminator part of the yeast expression vector. This allowed an easy *in vivo* cloning of chimeras using homologous recombination in yeast. Alternatively, the Gateway system could also be used, as suitable restriction sites introduced in primers for classical cloning, giving high flexibility to the approach.

Cloning and Sequencing

Following yeast transformation and selection, DNA extraction and subcloning were performed in *E. coli*. For each construct, sequencing of five to six clones was found sufficient to secure the isolation of at least one fully correct sequence. A very low rate of parental contamination was observed in our approach compared with the sequence-independent site-directed chimeragenesis (SISDC) method (10). Contamination was never detected for the

febit's miRNA Profiling Service

febit offers an expert miRNA profiling service including consulting, raw data generation and bioinformatic analysis.

■ FAST

from data submission to bioinformatic report in 2–3 weeks

■ UP-TO-DATE

content covering the latest miRBase version

■ EASY

simply send us your samples and we will provide you with reliable results

■ CUSTOMIZATION

including use of predicted, mutated, or proprietary miRNAs free of charge

■ AFFORDABLE

competitively priced, cost-effective means to obtain miRNA profiles

GENIOM BIOCHIP FACTS

- light-activated probe synthesis within microfluidic channels
- variable probe length 18–60 mer
- 8 arrays per biochip
- up to 15,624 features per array
- enables parallel hybridization of up to 8 different samples

» PLEASE CONTACT US FOR DETAILS

READ, WRITE, UNDERSTAND THE CODE OF LIFE

Europe: phone +49 6221 6510-300 • www.febit.eu • info@febit.eu
Americas: phone +1 781 391 4360 • www.febit.com • info@febit.com

Table 2. PCR Parameters

Step Number	1	3	4a	4b	5
Templates (ng)					
2B6/M13	1				
2B11p/M13			1		
2B11t/M13				1	
Product from previous PCR		1	1	1	Variable
Primers (pmol)					
A1	10	10		10	
A2		10	10		
5'-biot	10				
pGAL			10		10
tPGK				10	10

donor sequence and represents less than 15% of clones for the acceptor sequence. This illustrates that the use of the streptavidin-biotin system efficiently removes the donor template. Moreover, alternate approaches using double-stranded instead of single-stranded templates were generally found to generate a great number of side products due to unwanted initiation of amplification by partially extended sequences from the complementary strand. This leads to strong increase in parental contamination and in the formation of undesired sequences needing the sequencing of a large amount of clones to isolate expected recombinants. The observed residual contamination by parental acceptor sequence can result from steps 4a and 4b and could be avoided by adding a gel purification step between steps 4 and 5, although this could be problematic for automation. Concerning the observation of a low frequency of point mutations in our approach, it is classically attributed to polymerase-generated copy error and could be limited by use of higher fidelity enzymes.

Conclusion

We successfully applied SIGNAL for the generation of a library of chimera with predefined crossover points between two CYPs. SIGNAL presents

significant advantages over previously described chimeragenesis techniques used for structure-function relationship analysis. Indeed, compared with classical sequence-independent (14–16) or not (4–8) shuffling methods, our approach allows the design of chimeras with defined junction points. This greatly facilitates the identification by multivariate analysis (22) of structure blocks responsible for functional control. In contrast to previously described methods for sequence blocks swapping (11–13), SIGNAL is fully sequence-independent both at the protein and DNA level. Compared with the SCHEMA-related approaches (9,10), our method differs by the control of parental contamination through the use of single-stranded templates and magnetic beads-based purification. SIGNAL places itself between site-directed and random mutagenesis approaches and allows the generation of highly tuneable diversity that relies on the user's choice with very few constraints. Created libraries can be of various sizes, and their controlled and ordered combinatorial structure reduces requirement for high-throughput functional selection, significantly. At the same time, SIGNAL can be used to acquire information useful to target random mutagenesis approaches by pinpointing crucial structural modules being implicated in the protein activities.

This paper describes, as an example, the construction of 15 chimeras, but the technique could be adapted to create much larger libraries. Indeed, SIGNAL is relatively simple to carry out and is well-suited to be robotized. This promises to constitute a useful tool for the study of structure function relationship and for activity optimization.

ACKNOWLEDGMENTS

This work has been supported by a grant from the Association pour la Recherche sur le Cancer (ARC Grant 4011). We thank Gilles Truan for his help during the modeling phase.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. Roberts, R.J. 2004. Identifying protein function—a call for community action. *PLoS Biol.* 2:E42.
2. Meyer, A., R. Pellaux, and S. Panke. 2007. Bioengineering novel in vitro metabolic pathways using synthetic biology. *Curr. Opin. Microbiol.* 10:246–253.
3. Bloom, J.D., M.M. Meyer, P. Meinhold, C.R. Otey, D. MacMillan, and F.H. Arnold. 2005. Evolving strategies for enzyme engineering. *Curr. Opin. Struct. Biol.* 15:447–452.
4. Orr-Weaver, T.L. and J.W. Szostak. 1983. Yeast recombination: the association between double-strand gap repair and crossing-over. *Proc. Natl. Acad. Sci. USA* 80:4417–4421.
5. Nogami, T., T. Mizuno, and S. Mizushima. 1985. Construction of a series of ompF-ompC chimeric genes by in vivo homologous recombination in *Escherichia coli* and characterization of the translational products. *J. Bacteriol.* 164:797–801.
6. Cramer, A., S.A. Raillard, E. Bermudez, and W.P. Stemmer. 1998. DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* 391:288–291.
7. Abécassis, V., D. Pompon, and G. Truan. 2000. High efficiency family shuffling based on multi-step PCR and in vivo DNA recombination in yeast: statistical and functional analysis of a combinatorial library between human cytochrome P450 1A1 and 1A2. *Nucleic Acids Res.* 28:E88.
8. Sun, J., J.A. Katzenellenbogen, H. Zhao, and B.S. Katzenellenbogen. 2003. DNA

Short Technical Reports

- shuffling method for generating estrogen receptor alpha and beta chimeras in yeast. *BioTechniques* 34:278-288.
9. Meyer, M.M., J.J. Silberg, C.A. Voigt, J.B. Endelman, S.L. Mayo, Z.G. Wang, and F.H. Arnold. 2003. Library analysis of SCHEMA-guided protein recombination. *Protein Sci.* 12:1686-1693.
 10. Hiraga, K. and F.H. Arnold. 2003. General method for sequence-independent site-directed chimeragenesis. *J. Mol. Biol.* 330:287-296.
 11. Rosic, N.N., W. Huang, W.A. Johnston, J.J. DeVoss, and E.M. Gillam. 2007. Extending the diversity of cytochrome P450 enzymes by DNA family shuffling. *Gene* 395:40-48.
 12. Johnston, W.A., W. Huang, J.J. De Voss, M.A. Hayes, and E.M. Gillam. 2007. A shuffled CYP1A library shows both structural integrity and functional diversity. *Drug Metab. Dispos.* 35:2177-2185.
 13. Cartwright, A.M., E.K. Lim, C. Kleanthous, and D.J. Bowles. 2008. A kinetic analysis of regiospecific glucosylation by two glycosyltransferases of *Arabidopsis thaliana*: domain swapping to introduce new activities. *J. Biol. Chem.* 283:15724-15731.
 14. Ostermeier, M., J.H. Shim, and S.J. Benkovic. 1999. A combinatorial approach to hybrid enzymes independent of DNA homology. *Nat. Biotechnol.* 17:1205-1209.
 15. Lutz, S., M. Ostermeier, G.L. Moore, C.D. Maranas, and S.J. Benkovic. 2001. Creating multiple-crossover DNA libraries independent of sequence identity. *Proc. Natl. Acad. Sci. USA* 98:11248-11253.
 16. Sieber, V., C.A. Martinez, and F.H. Arnold. 2001. Libraries of hybrid proteins from distantly related sequences. *Nat. Biotechnol.* 19:456-460.
 17. Truan, G., C. Cullin, P. Reisdorf, P. Urban, and D. Pompon. 1993. Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5. *Gene* 125:49-55.
 18. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning*, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
 19. Gervot, L., B. Rochat, J.C. Gautier, F. Bohnenstengel, H. Kroemer, V. de Berardinis, H. Martin, P. Beaune, and I. de Waziers. 1999. Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* 9:295-306.
 20. Pompon, D. and A. Nicolas. 1989. Protein engineering by cDNA recombination in yeasts: shuffling of mammalian cytochrome P-450 functions. *Gene* 83:15-24.
 21. Hoffman, C.S. and F. Winston. 1987. A ten-minute DNA preparation from yeast ef-

ficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* 57:267-272.

22. Urban, P., G. Truan, and D. Pompon. 2008. High-throughput enzymology and combinatorial mutagenesis for mining cytochrome P450 functions. *Expert Opin. Drug Metab. Toxicol.* 4:733-747.

Received 20 June 2008; accepted 4 August 2008.

Address correspondence to Denis Pompon, Laboratoire d'Ingénierie des Protéines Membranaires, CGM—CNRS, Bâtiment 23B, 1 avenue de la Terrasse, F91190 Gif-sur-Yvette, France. e-mail: pompon@cgm.cnrs-gif.fr

To purchase reprints of this article, contact: Reprints@BioTechniques.com

Step up to BrightLine® fluorescence filters

All BrightLine filters are hard-coated for superior performance and *no burn-out* reliability. And today BrightLine includes a wide selection that ships from stock for *all* your fluorescence needs, from basic microscopy to the most advanced laser-based imaging systems.

www.semrock.com

1-866-SEMROCK

Semrock
Quality Optical Filters