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A novel helper phage for HaloTag-mediated co-display of enzyme and substrate on phage

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ABSTRACT

Phage display is an established technique for the molecular evolution of peptides and proteins. For the selection of enzymes based on catalytic activity however, simultaneous coupling of an enzyme and its substrate to the phage surface is required. To facilitate this process of co-display, we developed a new helper phage displaying HaloTag, a modified haloalkane dehalogenase that binds specifically and covalently to functionalized haloalkane ligands. The display of functional HaloTag was demonstrated by capture on streptavidin-coated magnetic beads, after coupling a biotinylated haloalkane ligand, or after on-phage extension of a DNA oligonucleotide primer with a biotinylated nucleotide by phi29 DNA polymerase. We also achieved co-display of HaloTag and phi29 DNA polymerase, thereby opening perspectives for the molecular evolution of this enzyme (and others) towards new substrate specificities.

1. Introduction

Molecular evolution is a powerful technique to improve the properties of enzymes by screening or selecting from a library of variants. Phage display, although originally developed for the selection of short peptide binders [1] and antibodies [2,3], has also shown its potential in enzyme evolution by the development of, e.g., catalytic antibodies [4] and DNA polymerases with altered specificities [5]. However, selection for catalytic activity is not so straightforward. Researchers have achieved varying success by using transition state analogs or suicide inhibitors as a ligand [6,7], but this is always an approximation to the enzymatic reaction one is selecting for. Ideally, to select for catalytic turnover, phage displaying active enzymes should be directly selected on product formation. This can be realized if displayed enzymes tag the phage itself by converting a co-displayed substrate into product. Affinity purification based on product binding can then be used to recover active variants.

Several approaches have been described to link a substrate to a phage particle, either before or after conversion to product. If the substrate is a peptide, it can simply be produced as a translational fusion to one of the phage coat proteins [8,9]. A more general solution is proximity coupling, proposed by Winter and co-workers [10]. They used a maleimide-linked DNA primer that would cross-link to the phage main coat protein while being extended with a biotinylated nucleotide by a displayed polymerase. The primer, however, randomly cross-linked with the long filamentous phage particle, may be beyond reach of the co-displayed polymerase, hence favoring cross-reactivity and loss of the genotype–phenotype association. It would therefore be preferable to have both enzyme and substrate located in close proximity at the same end of the phage particle.

Schultz and co-workers achieved co-display of staphylococcal nuclease next to a DNA oligonucleotide by coupling it to a synthetic peptide capable of forming an artificially designed coiled coil [11]. One half of this coil is an acidic peptide encoded as an N-terminal fusion with phage coat protein g3p in the helper phage genome; the other half is a basic peptide, chemically synthesized and conjugated to the substrate. Both peptides associate in a leucine-zipper-like fashion and form a disulfide bond tying them together covalently. However, the synthesis of the basic peptide is challenging, considering its length (46 amino acids) and the requirement of a non-standard chemical modification. In addition, the conjugation to the substrate and coupling to phage is a complicated procedure. A similar approach is the display of enzyme-calmodulin fusion proteins to which substrate-conjugated calmodulin-binding peptide can be attached [12]. The main difference here is the

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display of enzyme and substrate-coupling protein as a double fusion to a phagemid-encoded g3p, instead of encoding the substrate-coupling domain in the helper phage. Walker and co-workers developed yet another method for the evolution of glycosyltransferases, in which a selenocysteine residue is incorporated at the g3p N-terminus, exploiting its higher nucleophilicity and reactivity at lower pH in comparison with cysteine. Phage could be selectively derivatized with substrates functionalized with an α-iodoacetamide group [13]. A last example of enzyme and substrate co-display was described by Sunbul et al. [14]. They fused helper phage g3p to an 11-residue peptide, ybbR, which can be modified with small molecules conjugated to coenzyme A by Sfp, a phosphopantetheinyl transferase.

In an attempt to simplify the process of displaying both an enzyme and its substrate on phage and make it more generically applicable, we present an alternative approach using a new helper phage displaying HaloTag, an engineered haloalkane dehalogenase (Fig. 1). Natural dehalogenases convert halogenated hydrocarbons into their corresponding alcohols by a nucleophilic displacement mechanism. In HaloTag, an essential histidine residue is mutated preventing the base-catalyzed hydrolysis of the alkyl-enzyme intermediate, resulting in a stable covalent adduct [15]. In this way, HaloTag can be linked to a plethora of useful molecules like fluorescent dyes, solid surfaces or small molecules. The protein tag was originally developed for cell imaging and isolation of protein complexes, but can in principle be used in any application, in vivo or in vitro, where a certain molecule needs to be linked to a protein. The reaction of HaloTag with its ligands is highly specific, fast and essentially irreversible under physiological conditions. Additionally, a number of commercially available ligand 'building blocks' facilitate the coupling of a large variety of molecules.

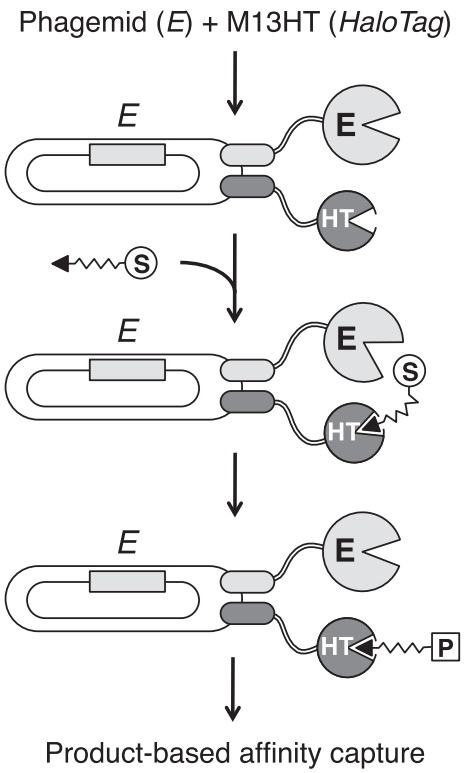

Fig. 1. Enzyme selection by HaloTag-mediated substrate co-display. Phagemid particles co-displaying HaloTag (HT) and enzyme (E) are produced by infecting phagemid-containing cells (encoding enzyme *E*) with M13HT helper phage (encoding HaloTag). The substrate (S) is covalently attached to HaloTag by means of the conjugated haloalkane ligand (represented by a black arrow). After conversion into product (P) by the enzyme, product-based affinity capture is used to capture phage displaying active enzymes.

As an example, we demonstrate the covalent coupling of a DNA oligonucleotide to phage particles through the HaloTag fusion. We show that the attached oligonucleotide, annealed with a template molecule, can be extended by phi29 DNA polymerase [16], a mesophilic enzyme that is used in numerous biotechnological applications [17–19]. Also, the simultaneous display of functional HaloTag and phi29 DNA polymerase is achieved, indicating the potential of this phage display system for the molecular evolution of this polymerase towards, e.g., enhanced incorporation of non-natural nucleotides.

2. Materials and methods

2.1. Construction of M13HT helper phage

The helper phage displaying HaloTag protein was derived from M13KO7 (New England Biolabs). First, we engineered an EagI restriction site just after the secretion signal in the g3p coding sequence using QuickChange mutagenesis (Agilent Technologies) with primers KO7-EagI-F (CAACAGTTTCGGCCGAGTGAGAATAGAAAGG) and KO7-EagI-R (CCTTTCTATTCTCACTCGGCCGAAACTGTTG), and M13KO7 replicative form (RF) DNA as a template. The product was transformed into *Escherichia coli* DH5α and positive clones were identified by PCR screening and digestion of the PCR product with EagI (Thermo Scientific). Plasmid DNA was prepared and sequence-verified to yield M13KO7-EagI.

The HaloTag-coding sequence was isolated from plasmid pH6HTN (Promega) by PCR with primers HT-F (TTTCTATTCTCACTCGGCGGCCGCAGAAATCGGTACTGGCTTTCC) and HT-R (AACAGTTTCTGCGGCCGCGTTATCGCTCTGAAAGTACAGATCC), thereby adding flanking NotI restriction sites to generate EagI-compatible overhangs. The PCR product was digested with NotI (Thermo Scientific), purified and ligated with M13KO7-EagI RF DNA digested and dephosphorylated with EagI and FastAP (Thermo Scientific). After transformation in DH5α and PCR screening, RF DNA was isolated and fully sequenced. RF DNA was then transformed into XL1-Blue MRF' (Agilent Technologies) yielding plaques from which phage, termed M13HT, was prepared using standard protocols. The M13HT genome sequence was deposited into Genbank under accession number KM505149.

2.2. Determination of functional HaloTag display

To 400 μl of TBS buffer (25 mM Tris–HCl pH 7.4, 140 mM NaCl, 2.5 mM KCl), 100 μl of M13HT phage suspension (10^{10}–10^{11} cfu/ml) and 1 μl of a 100-μM solution of HTBL (HaloTag® PEG-Biotin Ligand, Promega) were added, and the mixture was incubated for 1 h at room temperature. The phage were precipitated with polyethylene glycol (PEG) to remove unbound HTBL, and resuspended in 400 μl of TBS. Next, 50 μl of streptavidin-coated paramagnetic beads (Dynabeads® MyOne Streptavidin T1, Life Technologies) were washed three times in two volumes of TBST (TBS + 0.5% Tween-20), and added to the phage suspension. The mixture was incubated at room temperature for 15 min with gentle rotation to keep the beads suspended, followed by 5 washes with 200 μl of TBST. Bound helper phages were eluted by resuspending the beads in 500 μl of TEV protease reaction mix (AcTEV Protease, Life Technologies) and incubating at room temperature for 1 h with gentle rotation to cleave off bound phage. Helper phage concentrations were determined by spot titration.

2.3. Construction of phagemid pFABE-phi29

A synthetic gene coding for phi29 DNA polymerase was designed based on the best expressing sequence described by Welch et al. [20] and ordered from GeneArt (Life Technologies). The nucleotide sequence of this synthetic gene was deposited into Genbank under accession number KM505148. The gene was transferred to phagemid pFAB-SF [21] by digestion with SfiI (Thermo Scientific) and NotI, and ligation to give pFAB-phi29. An E-epitope tag (GAPVPYPDPLEPR, Pharmacia) was then inserted between the polymerase and g3p coding sequences by annealing two phosphorylated oligonucleotides, Etag-F (GGAGCGCCGGTGCCTTA TCCAGACCCGCTGGAACCGCGTGCA) and Etag-R (CGCGGTTCCAGC GGGTCTGGATAAGGCACCGGCGCTCCTGCA), followed by ligation in pFAB-phi29 cut with SdaI (Thermo Scientific). The resulting phagemid pFABE-phi29 was used to prepare phage particles displaying polymerase using a modified phagemid rescue protocol. Briefly, a 50-ml culture of *E. coli* TG1 cells containing the phagemid was grown at 37 °C until OD_{600} reached 0.5. After addition of ~10^{11} M13HT helper phage particles, the mixture was incubated for 30 min at 37 °C without shaking. Next, the cells were spun down and resuspended in 250 ml of 2xYT containing 50 μg/ml of spectinomycin, 50 μg/ml of kanamycin and 10 μM of IPTG, and grown for 8 h at 30 °C. Phage were recovered from the supernatant by two consecutive PEG precipitations and stored at 4 °C.

2.4. Determination of polymerase display efficiency by E-tag capture

A biotinylated anti-E tag antibody (Abcam ab87839) was allowed to interact with polymerase-displaying phage by mixing 50 μl of phage suspension (10^{10}–10^{11} cfu/ml) with 450 μl of TBS and 1 μl of antibody (1 μg/μl), followed by incubation for 30 min at room temperature. Next, 50 μl of washed streptavidin-coated paramagnetic beads were added to the mixture. After another 30 min of incubation at room temperature with gentle rotation, the beads were washed 5 times with 200 μl of TBST. Bound phages were eluted by incubating the beads for 5 min in 250 μl of 0.1 M glycine-HCl pH 3.0. After separation from the beads, the supernatant was neutralized with 250 μl of 0.5 M sodium phosphate buffer pH 7.8 and spot-titrated.

2.5. Determination of phage-displayed polymerase activity

Functional display of phi29 DNA polymerase was assayed by measuring extension of a Cy5-labeled primer (IDT). Primer and template were annealed in a 200-μl reaction containing 1 μM of template and 0.5 μM of primer by heating to 75 °C for 3–5 min and cooling down slowly to room temperature. Next, 20-μl extension reactions were prepared containing 125 nM of primer:template complex, 50 μM of dNTPs and 2 μl of polymerase-carrying phage (about 10^9 phage particles) in 1x phi29 DNA polymerase buffer (33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1% Tween-20 and 1 mM dithiothreitol). Reactions were incubated at 30 °C and 5-μl samples were taken at different time intervals. These were quenched by adding 5 μl of quenching buffer (90% formamide containing 0.05% bromophenol blue and 50 mM EDTA) and incubating at 95 °C for 5 min. The products were separated on a 20% denaturing PAGE gel and visualized using an Ettan DIGE imager (GE Healthcare).

2.6. Synthesis of HaloTag ligand-primer conjugate

The primer oligonucleotide P1S (*TTATGTATGTATTTTCGACG TTTGCTAACAGACAGGAAACAGCTATGAC), modified at its 5′-end (*) with 5′ Thiol Modifier C6 S–S, was ordered from IDT. P1S was dissolved in 100 mM Tris buffer (pH 8) and reduced with tris-(2-carboxyethyl)phosphine (TCEP, Aldrich) in a 500-μl reaction (50 μl of 1 mM oligonucleotide, 100 μl of 10 mM TCEP, 50 μl of 1 M Tris–HCl pH 8 and 300 μl of water). After incubating for 2 h at room temperature, 50 μl of HaloTag® Iodoacetamide (O4) Ligand (Promega), dissolved in dimethylsulfoxide at 50 mM, was added together with 50 μl of 1 M Tris–HCl pH 8 and 150 μl of water. The mixture was again incubated for 2 h at room temperature, protected from light, followed by ethanol precipitation. The HaloTag ligand-conjugated primer was dissolved in 500 μl of 10 mM Tris–HCl pH 8.5 and stored at −20 °C.

2.7. Extension of phage-coupled primer and phage recovery

We evaluated on-phage primer extension by coupling a primer:template complex to HaloTag-displaying phage and incubating with dNTPs and free phi29 DNA polymerase. Therefore, equal amounts of 10 μM HaloTag ligand-conjugated primer and 20 μM template oligonucleotide (CCACAGGTTTTTTTGTCATAGCTGTTTCC TG*, sequence complementary to the primer underlined and 3′-end nucleotide modified (*) with a 3′ Inverted dT modification to avoid extension of the template) were mixed, heated quickly to 85 °C and cooled down slowly to allow annealing. The HaloTag ligand-conjugated primer:template complex was coupled to phage in a 200-μl reaction containing approximately 10^{10} phage particles and 100 nM of complex. The mixture was incubated for 1 h at 37 °C, followed by PEG precipitation and resuspension in 100 μl of TBS. Incorporation reactions were setup on ice by mixing 100 μl of primer:template-coupled phage in a 200-μl reaction containing 20 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 100 μM dATP and dCTP, and 10 μM Biotin-16-dUTP (TriLink BioTechnologies). After addition of 10 units of phi29 DNA Polymerase (Thermo Scientific), the reaction mixture was transferred to 30 °C for 15 min, followed by addition of 100 μl of 0.5 M EDTA pH 8 to quench the reaction. Phage were then PEG-precipitated and resuspended in 400 μl of TBS. Washed streptavidin-coated magnetic beads (50 μl) were added to the mixture, followed by rotation for 15 min at room temperature. The beads were washed 10 times with 500 μl of TBST and bound phages eluted by DNA cleavage with 500 μl of DNase solution (1 mg/ml DNase I, 10 mM $MgCl_2$ in TBS). After 1 h of incubation at room temperature under gentle rotation, the beads were separated from the supernatant, which was then titrated to determine recovered phage concentrations.

3. Results

3.1. Construction and characterization of HaloTag-displaying helper phage

Based on the widely used M13KO7, we constructed a new helper phage, termed M13HT, displaying HaloTag protein, to allow co-display of both enzyme and substrate on phage. The PCR-amplified HaloTag-coding sequence was inserted as an N-terminal fusion to the phage coat protein g3p. A recognition site for TEV protease was included between HaloTag and g3p to allow convenient removal of the fusion tag. Sequence verification of the complete M13HT genome (9602 nt) confirmed correct insertion of HaloTag and the absence of any additional mutations. We noticed that M13HT consistently yields considerably smaller plaques, compared to M13KO7, which makes it difficult to accurately determine phage titers by plaque counting. Therefore, all phage titers in this report were determined as colony-forming units (cfu) by spotting infected cells on selective medium. This showed that M13HT phage titers were comparable with titers of M13KO7 prepared under the same conditions. To verify if the small plaques were

the result of reduced infectivity caused by the 33.5-kDa fusion tag attached to g3p, phage were incubated with TEV protease before titration. Neither phage titers nor the size of the plaques were affected by removal of tag (data not shown), indicating that fusion of HaloTag to g3p does not compromise infectivity. Therefore, small plaques might rather be due to less efficient secretion of the g3p-HaloTag fusion and/or phage assembly causing a lower phage production rate and giving cells more time to overgrow the plate.

Successful display of functional HaloTag protein on phage was demonstrated by incubation of M13HT phage particles with HaloTag® PEG-Biotin Ligand (HTBL). Reaction of HaloTag with appropriate ligands is fast and highly specific [15], resulting in phage being covalently labeled with biotin, enabling capture on streptavidin-coated paramagnetic beads. After elution of bound phage with TEV protease, phage titers were determined and compared with input phage (Table 1). When compared to a control reaction in which no ligand was added, ~400 times more HTBL-treated phage were recovered. The recovery was virtually complete demonstrating that HaloTag display as well as ligand binding is highly efficient. When the beads were resuspended after elution and titered, phage titers were 10–100-fold lower (data not shown), indicating that phages were efficiently released from the beads. From the M13KO7 control, only 0.01% of input phages were recovered, whether treated with HTBL or not. Repetition of the experiment showed similar data. This clearly shows that phage recovery is resulting from the combination of displayed HaloTag protein and the reaction with an appropriate haloalkane ligand.

A number of HaloTag ligand 'building blocks' are commercially available to facilitate synthesis of customized HaloTag ligands. We used such a building block, consisting of a reactive iodoacetamide group connected to an alkyl chloride through a 12-atom spacer, to couple a primer:template complex to phage. The alkyl chloride reacts with HaloTag to form a stable covalent ester bond, while the iodoacetamide group was used to covalently link a 5′-sulfhydryl-modified oligonucleotide primer. A 3′-blocked template oligonucleotide was annealed to the HaloTag ligand-primer conjugate before attachment to M13HT phage particles. To verify attachment, the primer:template-linked phages were incubated with free phi29 DNA polymerase and dNTPs including a biotinylated nucleotide, enabling extension of the primer and recovery by capture with streptavidin-coated magnetic beads. Similar to the biotinylated HTBL ligand (see above), more than 300 times more phages were recovered when compared to a control reaction in which no polymerase was added (Table 1). However, the recovery itself of primer-extended phage is lower in comparison with the biotinylated ligand, which might be ascribed to multiple PEG precipitations and concomitant loss of phage particles.

Table 1
Recovery of phage particles after capture with different biotinylated ligands.

Phage	Conditions[a]	Input (cfu)[b]	Output (cfu)[b]	Recovery[c]	Ratio[d]
M13KO7	HTBL+	9.0×10^9	1.0×10^6	0.011%	0.85
	HTBL−	9.0×10^9	1.2×10^6	0.013%	
M13HT	HTBL+	8.8×10^9	1.0×10^{10}	114%	407
	HTBL−	8.8×10^9	2.5×10^7	0.28%	
	oligo+, pol+	4.3×10^9	5.5×10^8	13%	333
	oligo+, pol−	4.3×10^9	1.7×10^6	0.039%	
pFABE-phi29	HTBL+	5.0×10^{10}	4.3×10^{10}	85%	607
	HTBL−	5.0×10^{10}	7.0×10^7	0.14%	
	anti-E+	3.5×10^{10}	3.1×10^8	0.89%	918
	anti-E−	3.5×10^{10}	3.4×10^5	0.00097%	
	oligo+, pol+	4.8×10^{10}	1.3×10^{10}	26%	413
	oligo+, pol−	4.8×10^{10}	3.0×10^7	0.063%	

[a] Phage were incubated with HaloTag® PEG-Biotin Ligand (HTBL), biotinylated anti-E tag antibody (anti-E), or a HaloTag ligand-primer conjugate (oligo) that was extended by incubation with a biotinylated nucleotide and phi29 DNA polymerase (pol). For every condition, the recovery was compared to a negative control (−) in which the indicated component was omitted.

[b] Phage titers were measured as colony-forming units (cfu) by bacterial infection after capture on streptavidin-coated magnetic beads.

[c] The recovery rate is determined by dividing output titer by input titer.

[d] The ratio of recovery rates (+/− conditions) gives an indication of the background.

3.2. Co-display of phi29 DNA polymerase and HaloTag

Rescue of a phagemid expressing phi29 DNA polymerase was carried out with M13HT helper phage, yielding phage titers comparable with preparations obtained by infection with M13KO7. The presence of HaloTag and polymerase proteins on the phage surface was confirmed by capture on streptavidin-coated magnetic beads with HTBL and with a biotinylated anti-E tag antibody, respectively (the polymerase-g3p fusion was labeled with an E-epitope tag). The recovery of phagemid particles with HTBL was again nearly complete, as was the case for helper phage, with a 600-fold improvement over the negative control without HTBL. For capture based on E-tag binding, however, only 0.1–1% of phage were recovered, which was still 900 times more than the negative control without antibody (Table 1). This indicates that display of phi29 DNA polymerase on phagemid particles is rather inefficient, but nonetheless comparable to values obtained with regular helper phage M13KO7, or reported elsewhere for display of polymerases [10,21]. Polymerases are indeed no secretory proteins and large-sized, which may explain why the HaloTag-g3p fusion is preferentially incorporated into the phage coat. Nevertheless, phage-displayed polymerase activity was clearly demonstrated in an extension assay using added template and fluorescently labeled primer (Fig. 2), despite very low phage-displayed enzyme concentration (1–10 pM compared to 1 μM for a typical reaction with free enzyme).

On-phage primer extension and capture on magnetic beads by added free phi29 DNA polymerase resulted in 25–60% recovery rates (Table 1), showing efficient coupling to HaloTag and accessibility of primer:template complex on phagemid particles. On-phage primer extension by the co-displayed phi29 DNA polymerase on the other hand was insufficient to raise phage recovery rates above the background of aspecifically binding phage, which might be due to the low polymerase:HaloTag display ratio.

4. Discussion

We developed a new phage display system for the co-display of enzyme and substrate, which can be used for the selection of enzymes based on their catalytic properties (Fig. 1). The new helper phage M13HT displays HaloTag protein as a g3p fusion, thereby enabling the specific and covalent coupling of haloalkane-functionalized molecules. We demonstrated this by attaching (i) HaloTag® PEG-Biotin Ligand, or (ii) an oligonucleotide primer:-template complex. In both cases, biotin enabled efficient recovery of phage by capture with streptavidin-coated magnetic beads, either directly or after on-phage primer extension by incorporation of a biotinylated nucleotide. We also achieved co-display of HaloTag and phi29 DNA polymerase on phagemid particles, both in their active form but with different display efficiencies guaranteeing monovalent display of the polymerase.

This fulfills all prerequisites for the selective enrichment of active polymerases out of a pool of non- or less active variants, and presents an alternative to polymerase selection based on compartmentalized self-replication [22] or self-tagging [23,24]. The same setup might as well be used for selection and molecular evolution of other nucleic acid-modifying enzymes, e.g. DNA ligase by selecting for the joining of two separate DNA fragments, one of which is linked to HaloTag and the other one is biotinylated, or

Fig. 2. Primer-extension assay by phage-displayed phi29 DNA polymerase. A fluorescently labeled primer was incubated with nucleotides and phagemid particles displaying phi29 DNA polymerase. Full-length product is formed after 30 min of incubation. Only a small amount of primer is extended because of the very low effective concentration of enzyme.

modifying enzymes that label DNA at random or at sequence-defined sites along its sequence.

The convenience with which different molecules can be linked to HaloTag-phage particles implies that selection is not limited to nucleic acid-modifying enzymes. In principle any enzyme can be used, provided that (i) it can be displayed on phage in its active form, (ii) its substrate can be conjugated to a haloalkane (either by de novo synthesis or by conjugation to a HaloTag ligand 'building block') while still remaining accessible for conversion into product by the enzyme, and (iii) affinity selection for the product is possible or, in the case of substrate-cleaving enzymes, the substrate can be bound to a solid support after which active phage-enzymes can release themselves by substrate cleavage.

Conflict of interest

The authors declare that they have no conflict of interest.

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