Check for updates

the FEBS Journal

REVIEW ARTICLE

Reporter-based screening and selection of enzymes

Teunke van Rossum, Servé W. M. Kengen and John van der Oost

Laboratory of Microbiology, Wageningen University, The Netherlands

Keywords
enzyme; high-throughput; *in vivo*; library; post-translational modification; reporter; riboswitch; screening; selection; transcriptional regulator

Correspondence
T. van Rossum, Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands
Fax: +31 317 483829
Tel: +31 317 481066
E-mail: teunke.vanrossum@wur.nl

(Received 4 February 2013, revised 5 April 2013, accepted 9 April 2013)

doi:10.1111/febs.12281

The biotech industry is continuously seeking for new or improved biocatalysts. The success of these efforts is often hampered by the lack of an efficient screening assay. Thus, to be able to extend the number of enzymes available for industrial applications, high-throughput screening and selection methods are required. In the last few years an impressive range of screening and selection strategies has been developed. In this review, we will mainly focus on *in vivo* reporter systems in which the activity of a reporter is controlled by the activity of an enzyme of interest. Different mechanisms can be distinguished: (a) binding of the product of the enzymatic reaction to a transcriptional regulator and thereby turning on transcription of the reporter; (b) direct modification of a transcriptional regulator by the enzyme resulting in expression of the reporter; (c) binding of the product to a regulatory riboswitch or ribozyme, resulting in translation of the reporter; and (d) direct modification of the reporter by the enzyme, altering the reporter's activity. The choice for either a selection or a screening strategy depends on the type of reporter, e.g. providing antibiotic resistance (selection) or transmitting a fluorescent signal (screening). Although developing the specificity of each of these reporter-based selection or screening systems towards a certain enzymatic reaction is not yet straightforward, their adjustable modular design appears to be a promise for general applicability in the near future.

Introduction

Enzymes are unique because of their catalytic power as well as their extraordinary specificity and (enantio- and regio-) selectivity [1,2]. In addition, they can be employed under relatively mild temperatures and pH values with water as solvent, conditions that are energy efficient and environmentally friendly [2,3]. In theory, proteins can catalyse any thermodynamically feasible conversion. Hence, any product (natural or unnatural) can be generated with biocatalysts that possess the appropriate catalytic features. These characteristics make them very interesting for industrial processes. Examples of applications range from the production of pulp and paper, textiles and leather, to fine chemicals, food additives and pharmaceutical intermediates [1,2]. The fact that such enzymatic solutions are preferable over the traditional chemical ones has resulted in a shift towards biocatalysts in recent years [2]. The industrial enzyme market, comprising about 100 enzymes (over half originating from fungi, over one-third from bacteria and the remainder from archaea, animals and plants), increased between 1998 and 2009 from $1.6 billion to $5.1 billion [2]. Still, biocatalysts are often not efficient enough, too costly or just not available [2,4]. Industrial processes are often operated under rather extreme conditions, such as high temperature or pressure, non-neutral pH and non-aqueous solutions. Although enzymes are faster and more environmentally friendly than traditional

Abbreviations
FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein.

Petitioner Merck, Ex. 1147, p. 2979

chemical catalysts [5], these harsh conditions are demanding, especially on the enzyme stability; this implies that the performance on an industrial scale is often insufficient. So, novel or improved biocatalysts are urgently required [5,6].

New enzymes can be obtained (a) by making use of natural evolution (enriching (micro)organisms with desired bioconversion activity, generating (meta)genomic libraries, and subsequent screening and selection), (b) by performing laboratory evolution (screening and selection of libraries of randomly generated enzyme variants) or (c) by conducting computational evolution (*in silico* variation, followed by *in silico* screening, and eventually experimental verification of a few selected variants) [7]. Nature is an excellent resource for novel biocatalysts as it has had billions of years to evolve enzymes for a range of reactions. When searching for enzymes with certain characteristics, one could explore those environments that most probably host microorganisms that require those enzymes. For example, for novel lignin-degrading enzymes one could isolate lignin-degrading microorganisms from rain forest soils [8], and for enzymes stable at extreme conditions such as high temperature or high salt concentrations one could look in extreme environments like hot springs or salt marshes [5]. Unfortunately, it is estimated that <1% of all microorganisms are culturable [4,9,10]. Metagenomic libraries are therefore particularly valuable, but the number of available libraries exceeds the possibilities of investigating them [11]. Moreover, sequence-based *in silico* library screening may run into problems due to functional mis-annotation and a bias for previously gained available information, actually preventing discovery of novel enzymes [12]. On the other hand, the probability of identifying a certain gene in experimental screens or selections depends on several practical features: a gene's abundance in the generated metagenomic library, the size of the target gene, the presence of a full-length sequence, the selected heterologous host, the expression system, and last but not least the assay method [10,11].

Although nature is a very good source for enzymes, these enzymes generally perform optimally in the context of a living cell (moderate activity, narrow specificity, moderate stability and short life span), but they are often less suited for desired performance in an industrial setting (high activity, broad specificity, high stability and long life span). Laboratory evolution (often referred to as directed evolution) is a powerful approach to alter enzyme characteristics, such as substrate specificity, enantioselectivity and stability. This iterative process involves the generation of random genetic diversity by introducing point mutations or by recombination, followed by high-throughput screening or selection for desirable variants [13]. Remarkable progress has been made in this field, and after the initial harvest of low-hanging fruit Goldsmith and Tawfik [14] stated that 'directed evolution is now ready to tackle high-hanging fruit'. The major advantage is that significant changes in enzyme characteristics are possible in the absence of the enzyme's structure or detailed knowledge on the catalytic mechanism. This may work when relatively small changes (e.g. single amino acid substitutions) already contribute to the improvement of an enzyme for a certain feature or when multiple changes are cumulative, e.g. thermostability [15–17]. However, when more complicated adjustments are desired (e.g. adaptations that require introducing > 10 amino acid substitutions at once), sampling of sequence space without any prior knowledge is an impossible task, because the size of the library one needs for such an experiment is just too big to synthesize, let alone to screen [18]. In *in vivo* screening or selection the library size is limited by the transformation efficiency, which in practice implies a value of 10^9 for *Escherichia coli*. Enlarged capacities can be obtained by adjusting the overall procedure, either by carrying out library creation and screening both *in vitro* or by performing development of library diversity and screening both *in vivo* [19]. In addition, increasing the manageable library size and making the handling of large libraries more straightforward may contribute to solving high-throughput problems in screening (likewise applicable for metagenomic libraries). Interestingly, a recent trend directs towards smaller but smarter libraries, for which information on sequence, structure, function and evolution is integrated, and sometimes even combined with computational design [6,14].

Computational design by itself is also an interesting approach to extend the number of available enzymes for industrial applications, ranging from relatively simple enzyme improvements to the more challenging design of biocatalysts for completely new reactions [4,18,20]. Depending on the computer power, astronomical numbers of variants can be efficiently screened *in silico*. Despite impressive advances in computational enzyme design (both *de novo* and by re-designing existing systems), the actual improvements of enzyme performance obtained by the designed systems are rather poor, and still far from that of analogous systems resulting from natural evolution [18,20,21]. Unexpected behaviour or inactivity of designed enzymes in wet-lab experiments generally relates to insufficient insight in an enzyme's overall structure (including poorly structured elements), in an enzyme's active site and in the

Petitioner Merck, Ex. 1147, p. 2980

catalytic mechanism [4,18,22]. A recent development in this field is the movement towards *in silico* directed evolution, including *in silico* screening of variants [18,23]. This and other computational design methods are a very important step towards creating smart libraries for directed evolution. The combination of computational design and directed evolution potentially is a very powerful approach in enzyme engineering, certainly when combined with enzymological insights [3,4,14,18,20].

The search for novel and improved biocatalysts benefits from a wide range of recently developed approaches. Although many hurdles are still faced, the main obstacle remains the screening of large mutant libraries or metagenomic libraries for variants with the desired functionality. This can be a complicated and time-consuming effort, especially in the absence of a high-throughput screening or selection assay. In recent years various *in vitro* and *in vivo* screening and selection systems have been developed, which have been covered in some excellent reviews [3,11,13]. These reviews focused on *in vitro* and *in vivo* systems for screening of directed evolution or metagenomic libraries. *In vivo* methods involving reporter-based strategies were only briefly discussed. The current review will focus on the different *in vivo* screening and selection strategies as well, but with special emphasis on reporter-based strategies.

Together with the development of novel screening methods, there is also a growing awareness that smaller, more focused libraries are needed. However, as the focus of this overview is on screening/selection aspects, practical issues on library size and formation are only briefly mentioned. For a more elaborate discussion on this topic, the reader is referred to other reviews [3,14,19].

General overview of *in vivo* screening and selection strategies

In vivo systems are defined as replicating cellular entities, in most cases bacterial cells that produce a library of protein variants of interest. As in all functional screening and selection systems, the phenotype and the genotype of the protein(s) of interest are linked in *in vivo* strategies. A major advantage of *in vivo* systems may be the functional production of the protein(s) of interest, for instance correctly folded and with the incorporation of a cofactor; in the case of heterologous expression, functional enzyme production may depend on the choice of the production host. In addition, by changing the screening or selection conditions of an *in vivo* system, one can tune the properties of the desired biocatalysts [24], e.g. expression in a thermophilic bacterium at elevated temperatures for obtaining variants with enhanced stability [15,16]. However, *in vivo* strategies are limited by (a) the transformation efficiency of the host, (b) functional expression of the protein of interest, (c) difficulties in substrate uptake, (d) less sensitive product detection because of complex intracellular background [9,25] and (e) the growth rate of the microbial host.

Before describing the details of the various strategies, it is important to indicate the difference between 'selection' and 'screening'. In selection approaches, negative clones are not present in the final pool, e.g. because they do not survive (Fig. 1A). The main advantage is that usually a much smaller number of variants has to be screened. Of course, one should realize that some false positives may arise as well (see below). In contrast, in screening approaches, all clones, negative and positive, are maintained, meaning that all library variants need to be screened, which makes this approach significantly less efficient [13]. However, screening may also have some advantages, like a better dynamic range, precision of activity measurements, tailored reaction conditions and the possibility to monitor multiple parameters [14]. The screening step is of course followed by selection, i.e. picking the positive clones. This can be done either manually with toothpicks or automatically with for example fluorescence-activated cell sorting (FACS). In FACS, individual cells, emulsions of cells in aqueous droplets in oil or emulsions of aqueous droplets in oil (containing sophisticated *in vitro* expression systems with some colorimetric product detection) are separated in narrow channels, where illumination of cells or droplets occurs one by one by a focused laser beam. When the desired fluorescence is detected, a charge will be applied to the cell/droplet, resulting in deflection of the positive clone by an electrostatic field into a collection tube [26].

Thus compared to screening, the selection method allows for analysing much larger libraries, namely $\sim 10^9$ versus $\sim 10^5$ [3,13,27]. It should be mentioned that more recent screening techniques such as cells-in-droplet screens coupled to FACS are also used to screen relatively big libraries ($\sim 10^9$ [3,19]). In the next paragraphs a general overview will be given of the different *in vivo* screening and selection strategies, discussing only non-reporter-based approaches. The reporter-based strategies will be discussed separately in a later section: Reporter-based *in vivo* screening and selection strategies.

Petitioner Merck, Ex. 1147, p. 2981

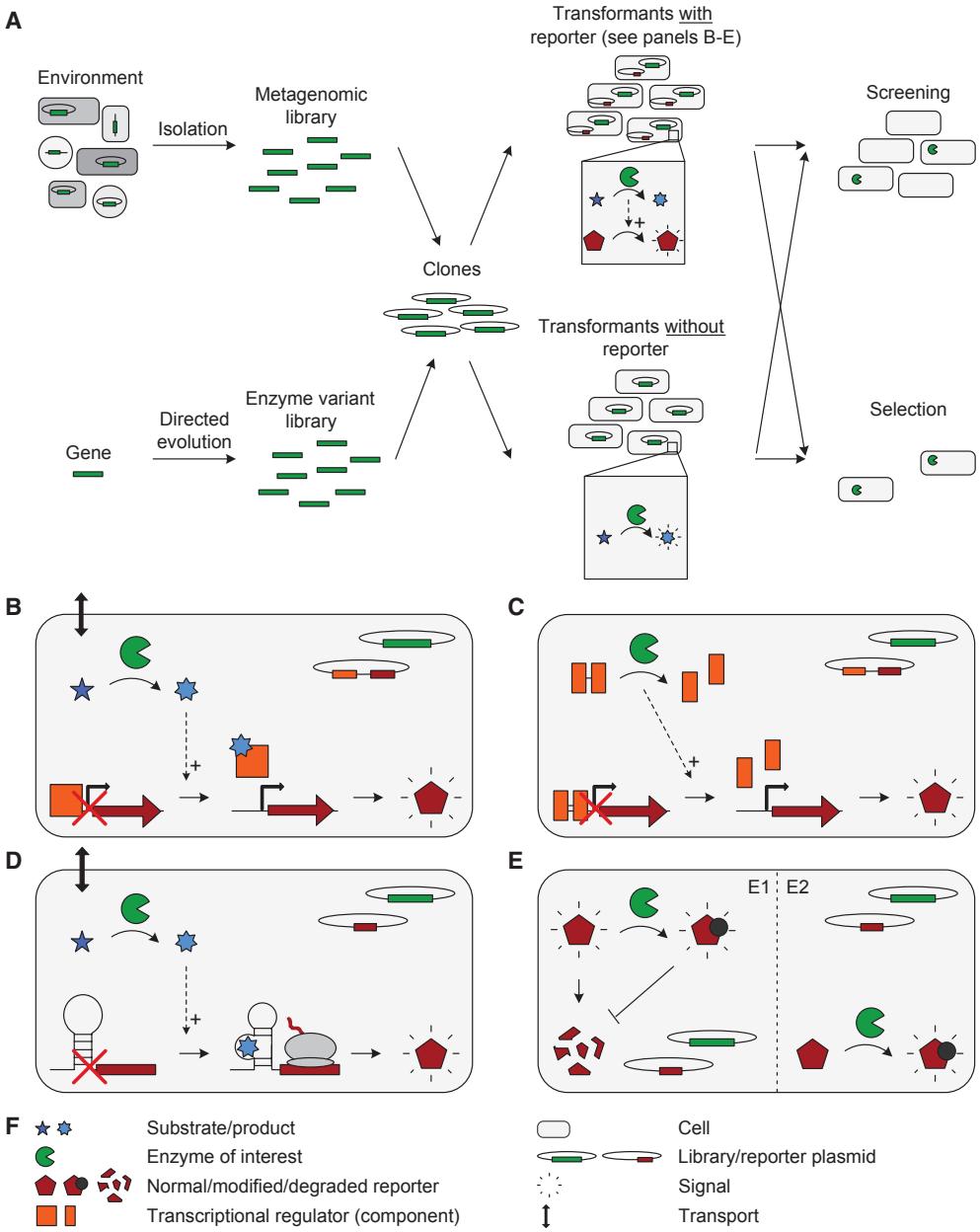

Fig. 1. Reporter-based *in vivo* screening and selection strategies. (A) Metagenomic libraries or enzyme variant libraries are created from DNA isolated from the environment or by mutating a gene through directed evolution, respectively. The libraries are cloned into a vector and are subsequently transformed to cells either with or without a plasmid, encoding a reporter. In reporter-based strategies it is not the product or the enzymatic reaction itself that directly results in a measurable characteristic, but rather a genetically encoded reporter that gives a conditional phenotype. The activity of the reporter is dependent on the activity of the enzyme of interest via interference at several regulatory levels as shown schematically in (B)–(E). These strategies are defined as 'selection' or 'screening' depending on the type of reporter. The 'signal' the reporter gives can thus vary from growth to fluorescence when the reporter is an antibiotic resistant protein or GFP, respectively. Non-reporter-based strategies are defined as 'selection' or 'screening' depending on the enzyme activity and/or product. The latter strategies are displayed in more detail by Leemhuis *et al.*[3]. (B) Natural transcriptional regulator based: binding of the product alters the conformation of the regulator, resulting in dissociation from the DNA and activation of transcription. (C) Synthetic transcriptional regulator based: the enzyme directly acts on the regulator, resulting in dissociation of its components and activation of transcription. (D) Riboswitch based: binding of the product to the riboswitch changes its secondary structure and activates translation. (E) Post-translational modification based: the enzyme directly acts on the reporter and modifies it, preventing degradation (E1) or resulting in detectability of the reporter (E2). (F) Legend.

Petitioner Merck, Ex. 1147, p. 2982

Selection

The selection strategies employed vary with respect to the selective principle they are based on, but often the desired enzymatic activity is coupled to cell survival and growth. This makes selection efficient (libraries of $\sim 10^9$ [3]) and applicable to both metagenomic and enzyme variant libraries. However, selection on growth appears to result in more false positives compared with screening. The higher selective pressure may select for variant cells that circumvent the coupling of the enzyme activity to growth and are able to survive by a different mechanism.

Three approaches have been described in which enzyme activity is coupled to growth: development of enzymes that complement auxotrophy, development of enzymes that neutralize lethal conditions, and use of a specific enantioselective counter selection. In genetic complementation, microbial strains are used which are auxotrophic for the product of the enzyme of interest [13,19]. Hence, this approach is limited to enzymes that catalyze the synthesis of an essential product and for which an auxotrophic host is available or can be constructed by deleting or mutating the corresponding gene. Otten *et al.* [28] applied auxotrophy complementation to evolve the glutaryl acylase of *Pseudomonas* SY-77 into an adipyl acylase with an improved activity towards adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA). The β-lactam component of the substrate is replaced by leucine to enable selection. Leucine auxotroph *E. coli* cells expressing an error-prone PCR library of the glutaryl acylase are grown in the presence of adipyl-leucine as sole leucine source. In this way, only enzymatic hydrolysis of adipyl-leucine allows for growth on minimal medium. Activity of the selected variants towards the desired substrate adipyl-7-ADCA is confirmed in a biochemical assay.

A second approach is the neutralization of increasing concentrations of toxic compounds (e.g. antibiotic resistance markers) or other lethal conditions (e.g. cold shock) [11,13]. This is of course restricted to the subset of enzymes which have such neutralizing activity. The third manner to couple enzyme activity to growth is used to select for enantioselective enzymes. Formation of the desired enantiomer enhances growth, whereas the wrong enantiomer is toxic [3,13]. Fernández-Álvaro *et al.* [29] applied this principle to select for enantioselective esterases. Two substrates are added to the medium, namely (*R*)-3-phenyl butaric acid that is covalently coupled to glycerol and (*S*)-3-phenyl butaric acid coupled to 2,3-dibromopropanol. Depending on the enantioselectivity of the esterase, either the growth-supporting glycerol or the toxic 2,3-dibromopropanol is released. Only *E. coli* cells with an esterase selective for the R enantiomer survive and are selected based on live/dead staining and FACS.

Agar plate screening

Agar plate screening is the simplest format of screening: incubating colonies with a chromogenic substrate [3], which leads to colouring of the colonies themselves and/or the surrounding agar upon product formation (e.g. release of *o*-nitrophenol), or a coloured substrate that results in clearing halos around the colony upon substrate hydrolysis (e.g. disappearing Congo Red stained carbohydrates). Also indicators that react with the product can be added to the agar, e.g. Schiff's reagent that reacts with aldehydes [11]. In general, it is easy to operate and straightforward to identify active variants. However, low dynamic ranges of these screening approaches generally do not allow for accurately distinguishing differences in catalytic rates. Besides, only libraries of up to $\sim 10^5$ can be screened [3,19] and potential intracellular accumulation decreases the screening's sensitivity. The latter can be solved by coupling the initial *in vivo* screening to a second *in vitro* round after cell lysis [9]. For example, Böttcher *et al.* [30] aimed at obtaining enantioselective esterase variants. To do so, overlaid replicated agar plates were covered with soft agar that contained the substrate 1-naphthyl-acetate and the staining salt Fast Red TR. Esterase-positive clones were coloured brownish/red by coupling of Fast Red TR with the esterase product 1-naphthol (product 1); the corresponding cells were lysed and tested in an enzyme cascade assay. Acetyl-CoA was formed from CoA and acetic acid (product 2) by acetyl-CoA synthetase, and CoA was regenerated by citrate synthase, converting oxaloacetate to citrate. The required oxaloacetate was derived from L-malate by L-malate dehydrogenase, meanwhile reducing NAD^+ to NADH. NADH generation was measured in a microtiter plate spectrophotometer. It was possible to do an enantioselectivity screen by comparing the hydrolysis of two pure enantiomers in separate wells [30].

Enantioselectivity can also be established already during the initial screening on plates via coupling to some downstream product-converting reaction(s). In an enzyme variant library (alanine racemase) expressed in *E. coli*, Willies *et al.* [31] detected active variants that catalysed the racemization of L- to D-alanine. Cells were grown on Hybond-N membranes laid on top of agar plates. D-alanine-producing clones were detected by using a coupling assay, based on a D-amino acid oxidase generating H_2O_2 that is

Petitioner Merck, Ex. 1147, p. 2983

monitored in a horseradish-peroxidase-catalysed colour reaction. Two subsequent screens were performed to remove false positives. Plasmid-encoded D-amino acid oxidase localized colour change to the cell, whereas an oxidase overlay led to colour diffusion over the plate [31]. In general, agar plate screening has successfully been employed to screen for multiple enzyme classes, such as cellulases, lipases/esterase, proteases, laccases and oxidoreductases [9]. Moreover, both enzyme variant and metagenomic libraries can be screened.

Microtiter plate screening

The most commonly applied screening strategy is based on microtiter plates. Single transformants are grown in standard 96-well microtiter plates [3]. Deep-well plates may result in elevated yields (2 mL wells, typically with 0.2 mL culture volume), whereas more wells per plate (384, 1536) enable a higher throughput. An interesting alternative is the micro-Petri dish designed by Ingham *et al.* [32]. This microbial culture chip of 36×8 mm has up to 1×10^6 wells with 10-μm-high laminate side walls on top of porous aluminium oxide strips; for supply of nutrients the chips can be placed on agar-like matrices. This design allows for rapid changes in cellular environment by simply transferring the chip from one medium to another. As a proof of principle *E. coli* cells were screened for expression of *lacZ*.

In general a wide range of analytical methods can be employed for microtiter plate screening, such as colorimetry, liquid/gas chromatography, NMR or mass spectrometry. In addition, the dynamic range of this strategy is excellent [3,19] and both enzyme variant and metagenomic libraries can be screened. Although often cells are lysed in order to enhance sensitivity or to make substrate available to the enzyme, it is not a strict requirement, as shown by Wagschal and Lee [33]. They developed a screen in which a 4-methylumbelliferyl (mu) labelled substrate is added to a ~ 50–100 μL *E. coli* culture, expressing variants of a biomass-degrading enzyme (wild-type or error-prone PCR library). The change in fluorescence is measured with a top-reading microplate spectrophotometer (no interference by turbidity). A particularly attractive feature in this case is the fact that a single microplate is used for cultivating picked colonies, enzyme expression, the fluorescence assay, and finally storage of the library in a freezer after dispensing a cryoprotectant such as glycerol. Besides, if cell lysis is required, a simple cell permeabilization protocol has been developed in which only a single reagent, polymyxin-B-sulfate, has to be added prior to a direct assay, with no further manipulation in the same microplate well [33]. So, for this permeabilization protocol it is not necessary to lyse the cells, centrifuge them and transfer the cell-free extracts to a second plate. The main limitations of this type of assay for the activities tested relate to the reaction rate with mu-tagged substrates and background substrate hydrolysis. Moreover, general applicability of this method to other hydrolytic activities is limited by the commercial availability of appropriate mu-labelled substrates [33]. Other drawbacks of microtiter plate screening are that only libraries of up to ~ 10^4 can be screened [3,19] and that the method is limited to reactions of which the product has some measurable characteristic. Artificial substrates can increase the applicability, but as they differ from the substrate in the desired application other enzymes than the one demanded can be found. After all, 'you get what you screen for' [34].

Cell-in-droplets

A more recently developed screening strategy involves single cells in water-in-oil emulsions or cells in water-in-oil-in-water double emulsions. In a hydrophobic environment, water droplet compartments contain cells with enzyme variants, as well as substrates and products. This greatly enhances the screening capacity compared with microtiter plate screening (factor 100 000), since the reactor volume is much smaller, typically in pico- and femto-litre scale [3,35]. This strategy requires a flow-cytometry-based screening that allows for high-throughput analysis of the library. Further developments couple the droplet compartmentalization strategy with microfluidics. This nanotechnology approach enables exact control over the micro-reactor droplets' lifetime and allows for the addition of substrates and quenchers at a desired time point. In addition, small improvements in enzyme activity may become detectable [3,35].

A proof of principle study using this approach concerns the detection of cellulase activity in *Saccharomyces cerevisiae* cells by Ostafe *et al.* [36]. Positive cells (5%), expressing the endo-cellulase Cel5A from *Trichoderma reesei*, were mixed with negative cells (95%). After emulsification of the mixed population with assay components in water-in-oil-in-water double emulsions, cellulase activity was detected by coupling the release of reducing sugars to the formation of a fluorescent product in a coupled enzyme assay involving a hexose oxidase and a vanadium bromoperoxidase. One round of sorting with FACS enriched the positive cell population 12-fold, as was demonstrated by a subsequent screening on carboxymethyl cellulose containing agar plates and a Congo Red assay.

Petitioner Merck, Ex. 1147, p. 2984

Another good example of the cell-in-droplet approach is the work of Kintses *et al.* [35] on the improvement of a promiscuous activity of arylsulfatase from *Pseudomonas aerigunosa* (PAS) towards the non-native substrate phosphonate. Single *E. coli* cells expressing a PAS variant library are compartmentalized with substrate and cell lysis agents in water-in-oil droplets. These are incubated to generate a fluorescent product and hits are sorted after detection by laser-induced fluorescence. Plasmid DNA is isolated and transformed to *E. coli*. After seven rounds a six-fold improvement in both activity and expression was achieved.

A drawback of the cell-in-droplets strategy is that the substrates should readily enter the cell and that the (fluorescent) hydrophilic products should remain inside the droplets. Although the quality of the screens is comparable to microtiter plate screening, measurements at single cell level are influenced by cell-to-cell variation in expression levels. The signal variance is therefore slightly increased compared with microtiter plate screening [35]. In addition, this technique does not allow the identification of variants with improved properties, generated by for example directed evolution [29], unless coupled to microfluidics [35].

Cells as micro-reactors

Instead of using microtiter plate wells or cells in droplets as small reactors, one can also use a single microbial cell as micro-reactor, reducing the reactor volume even to the femto-litre scale. Also with this strategy libraries of $\sim 10^9$ can be screened, either enzyme variant or metagenomic libraries. Similar to the cell-in-droplets approach, the method is limited by the fact that the substrate should be able to enter the cell, but in contrast to cell-in-droplets the product should now also remain within the cell [3]. Besides, the product itself should be detectable, e.g. by fluorescence. Coupling to for example FACS makes this method high-throughput and since cells can be used straightaway without compartmentalization it is less laborious.

A good example of this strategy, showing the entrapment and the additional required washing away of substrate, is the work of Yang *et al.* [37]. They developed a screen to improve a β-1,3-galactosyltransferase (CgtB) which transfers galactose from UDP-galactose to oligosaccharides, thereby demonstrating that cellular entrapment is not restricted to charged products. Two acceptor substrates, bearing the same sugar but chemically distinct fluorophores, are imported into the *E. coli* cell by a sugar transporter (permease). After an incubation period during which the acceptor may have been modified by a CgtB variant, unreacted acceptor is washed out of the cells with LB medium and phosphate-buffered saline. Cells containing catalytically-active glycosyltransferases retain the fluorescent product inside the cell as it is no longer a substrate for the permease. The use of two distinct fluorophores minimizes the chance of selecting for improved fluorophore binding (you get what you screen for [34]). Tailored alterations in substrate specificity may be possible by using also two different sugar moieties, enabling positive and negative screening. A library of 2×10^7 was created by random mutagenesis and catalytically active enzymes were identified and isolated by three rounds of FACS. Subsequently, a second library (5×10^6) was created by gene shuffling, combining the parent gene and the best hits from the first step. After screening an improved enzyme was obtained with a higher substrate tolerance and a 300-fold increased catalytic activity compared with the parent enzyme [37].

Cell surface display

A completely different screening strategy is cell surface display, in which the enzyme variant is displayed on the outside of the cell by fusing it to an anchor motif, making it freely accessible for the substrate. Also here, relatively large libraries of $\sim 10^9$ can be used [3]. However, the required fusion of the gene of interest to the anchoring motif limits the applicability to enzyme variant libraries. Both the enzyme variants and the products are displayed on the surface of the cells [3]. The choice for the anchoring motif to display the enzyme of interest as well as the choice for the host organism should be carefully made. The enzyme anchor needs to have an efficient signal sequence, a relatively stable structure, it should be compatible with the enzyme of interest, and it should be resistant to protease attacks. Also the host organism should be compatible with the enzyme of interest. In addition, the host should be easy to cultivate and possess as few as possible cell-wall-associated and extracellular proteases. Gram-positive bacteria, e.g. *Bacillus* or *Staphyloccocus*, are preferred over Gram-negative bacteria because they lack an outer membrane and they have a more rigid cell wall. However, *E. coli* is often employed because of its high transformation efficiency [13]. Enantioselectivity can be obtained by using *S*- and *R*-enantiomeric substrates, either simultaneously via coupling to different coloured fluorescent labels or separately [3]. The disadvantage of cell surface display in combination with FACS is that display may lead to loss of the enzyme's activity and that fluorescent substrates/products are required that remain bound to the cell surface

Petitioner Merck, Ex. 1147, p. 2985

[3]. Another drawback is the potential unspecific labelling of negative cells in the vicinity of a positive cell. A recent investigation by Prodanovic *et al.* [38] combines this selection strategy with *in vitro* compartmentalization in order to solve this problem. *S. cerevisiae* cells, expressing a library of glucose oxidase variants, were encapsulated in water-in-oil emulsions. Glucose oxidase entrapped within the cell wall matrix converts glucose to gluconolactone, releasing H_2O_2. The H_2O_2 is used by a displayed horseradish peroxidase to activate extracellular tyramide fluorescein, which can subsequently form a covalent link with tyrosine residues on the cell surface. After removal of the oil phase, FACS is applied and positive cells are further tested by microtiter plate screening [38]. This is a nice example of exploiting the best of several strategies.

Reporter-based *in vivo* screening and selection strategies

In reporter-based strategies, it is not the product of an enzymatic reaction or the enzymatic conversion itself that results in a measurable property, but rather a genetically encoded reporter that gives a discriminating phenotype (Fig. 1A). As the enzymatic activity is thus monitored indirectly, these strategies can in theory be applied for each enzymatic reaction and are therefore regarded as reaction independent. Both 'selection' and 'screening' is possible, depending on the type of reporter gene chosen by the researcher [19]. Some reporter types are colorimetric (e.g. LacZ), fluorescent (e.g. green fluorescent protein, GFP), bioluminescent (e.g. LuxCDABE) or they result in conditional survival (e.g. CAT), cell motility (e.g. CheZ), acidification (e.g. AraBAD), ice nucleation (e.g. InaZ) and cell display (e.g. LamB).

The activity of the reporter is dependent on the activity of the enzyme of interest via interference at the transcription, translation, post-translational modification, degradation or solubility level. Based on general signal transduction systems one could think of the following strategies (examples will be described in detail below): (a) binding of the product to a transcriptional regulator and thereby turning on transcription of a reporter; (b) direct modification of a transcriptional regulator by the enzyme and thereby turning on transcription of a reporter; (c) binding of the product to a riboswitch or ribozyme, resulting in translation of a reporter; and (d) direct modification of the reporter by the enzyme, altering the reporter's activity. Alternatively, transcription repression, reporter inactivation and translation inhibition upon enzyme activity is also possible; however, a stimulated reporter is preferred because an appearing signal is more readily detectable than a disappearing signal.

Such reporter-based strategies have been exploited for a range of applications, such as (a) making signal-responsive genetic parts with transcriptional regulators and riboswitches in synthetic biology [39], (b) small molecule detection in metabolic engineering [19], (c) pathway optimization in metabolic engineering [40], (d) drug discovery [41], (e) tracing explosives in soil [42] or (f) pollutant detection either alone [43] or coupled to activation of bioremediation pathways [44]. Until recently, only a limited number of studies that aim for identification of certain enzyme variants have used a reporter-based approach. Some proof of principle studies have been performed, e.g. changing the specificity of the transcriptional regulator HbpR from 2-hydroxybiphenyl to 2-chlorobiphenyl [45]. The potential of different reporter-based strategies employed in other applications can be very useful for the rapidly developing field of reporter-based strategies in enzyme discovery and optimization. Here an overview will be given of the strategies that are already employed in this field.

Although general and reporter-based strategies are treated separately here, the two are in fact integrated, depending on the reporter that is used. For example, a method in which a transcriptional regulator turns on *gfp* followed by FACS screening is described as 'cells as micro-reactor', but if the same regulator turns on the gene encoding an antibiotic resistance marker the method is referred to as 'selection'.

Transcriptional-regulator-based strategy

The most employed *in vivo* screening or selection strategy involving a reporter is the transcriptional-regulator-based approach. The activity of the enzyme is transduced through product-dependent activation or de-repression of the transcription of a reporter gene by the transcriptional regulator. For detailed information about the response profiles, describing the relation between small molecule and reporter, the reader is referred to Dietrich *et al.* [19]. The transcriptional regulators used can be divided into two subgroups: (a) a natural transcriptional regulator (its ligand specificity can potentially be adapted by laboratory evolution; Fig. 1B) or (b) a synthetic transcriptional regulator, composed of multiple components that are either designed or derived from natural systems, the association or dissociation of which is triggered by the enzyme activity (Fig. 1C).

The natural regulators generally depend on an allosteric event: binding of the enzyme's substrate or

Petitioner Merck, Ex. 1147, p. 2986

product in one domain alters the conformation and switches the DNA binding capacity of the regulator. Although this strategy is generally product-based, there are examples of substrate-based systems. In substrate-induced gene expression screening (SIGEX), for example, metagenomic fragments are cloned in front of *gfp* and the resulting library is screened with FACS, after addition of a substrate [46,47]. The rationale behind SIGEX is the possibility that catabolic operons are substrate or intermediate induced and that regulatory elements are often situated in close proximity to catabolic genes. However, as this rationale is not a rule and regulatory systems may even evolve separately from the metabolic genes they control [48], SIGEX does not always prove useful [49]. Besides, SIGEX is only suitable for metagenomic screening, not for enzyme optimization.

The strength, but also the limitation, of this natural transcriptional-regulator-based strategy is the specificity towards the product (or substrate) of the enzymatic reaction. This specificity makes it possible to obtain only the enzyme which produces the product of interest without false positives, caused for example by binding of the substrate instead of the product to the regulator. When choosing a proper transcriptional regulator for a specific screen or selection, it is recommended to look first at already described natural regulators. For example, Uchiyama and Miyazaki [50] screened metagenomic libraries in *E. coli* for benzamidases with the natural benzoate-responsive transcription activator BenR controlling expression of the reporter *gfp*. With this product-induced gene expression (PIGEX), they obtained 11 hits, of which three were genes with low sequence similarity towards known amidases. For all 11 hits benzamidase activity was confirmed. In contrast to most transcriptional-regulator-based studies, this group used two sets of cells, one with the metagenomic library and one with the sensor (*benR* and *gfp*), because they believed that, with single-cell intracellular screens, cross-talk between negative and positive clones might occur. The two sets of cells were grown separately and later combined in wells on ten 96-well plates, each well containing 100 fosmid library clones. So, several sequential screening steps were necessary. GFP fluorescence was only observed when the sensor cells were in log phase [50].

However, not for every enzyme substrate/product-of-interest is a fitting regulator known, and the ones which are known may be promiscuous. Probably this problem exists for most regulators and further engineering of the regulator is often required to obtain the desired functionality of specific binding of the product of interest and meanwhile preserve its DNA binding properties. A first step towards the required drastic adaptation of a regulator's ligand binding site might be accomplished by computational design. Unfortunately, efforts in this direction are not yet as sophisticated as one would like. For transcriptional regulators, no examples are known yet. Although ligand specificity has been changed for periplasmic binding proteins [51,52], problems in verifying these designs experimentally show that many hurdles still need to be taken [53]. The difficulties lie amongst others in loss of protein stability upon mutation and limitations in the description of molecular interactions between protein, ligand and water, such as long-range electrostatics and dynamics [52–54].

Alternatively, one could also strike the golden mean by using a less drastic, stepwise approach such as directed evolution. Here the specificity of a known regulator is changed by mutagenesis methods like saturation mutagenesis or gene shuffling. A few examples will be given here.

van Sint Fiet *et al.* [55] employed a previously described mutant of the transcriptional activator NahR to detect *E. coli* cells with XylC activity. This enzyme forms benzoate or 2-hydroxybenzoate from the corresponding aldehydes. Binding of these products to a mutant NahR turned on the expression of *tetA*. The colony size was related to the product concentration, and the optimal ratio of true and false positives could be established by adapting the tetracycline concentration. This selection system was also turned into a screening system by replacing *tetA* with *lacZ*. Changing the specificity of NahR from benzoate to salicylate by PCR-based saturation made this detection possible [56].

Mohn *et al.* [57] evolved the toluene-responsive transcription activator XylR by gene shuffling plus mutagenic PCR to be optimally responsive to 1,2,4-trichlorobenzene, the major product of γ-hexachlorocyclohexane dehydrochlorination. With *lacZ* as reporter and *E. coli* as host, the activity of the dehydrochlorinase LinA was demonstrated. Selection was possible by introducing the lactose transporter LacY: only cells which were able to produce LacZ could grow on lactose.

With saturation mutagenesis, Tang and Cirino [58] changed the arabinose-responsive AraC to respond to mevalonate. Reporting mevalonate synthesis is a handy tool in metabolic engineering, e.g. to improve the mevalonate-dependent isoprenoid pathway enzymes. Also here *E. coli* was the host and *lacZ* or *gfp* the reporter.

Although cases have been described in which the ligand specificity of transcriptional regulators are

Petitioner Merck, Ex. 1147, p. 2987

successfully engineered, obtaining the proper regulator for the enzyme of interest is certainly not simple. Another approach to acquire such a regulator is to design one from multiple components. Strategies based on these synthetic transcriptional regulators rely on the fact that the binding of the multiple components of the regulator to one another is dependent on the enzyme activity. A few examples are described here.

In the QUEST system (QUerying for EnzymeS using the Three-hybrid System), catalysis is detected by coupling substrate turnover to a transcriptional event. The DNA binding domain of the transcriptional regulator AraC is fused to a domain that can bind either a substrate or a chemical inducer of dimerization (CID). If the substrate (the scytalone analogue 2,3-dihydro-2, 5-dihydroxy-4H-benzopyran-4-one) is present, it competes with the CID (although chemically different from substrate/product) for binding to the domain, resulting in monomerization of the activating regulator, dissociation from the DNA and downregulation of the *ara-BAD* operon. Conversion of the substrate to the product by the enzyme shifts the equilibrium towards CID binding and activated expression of *araBAD*, enabling the bacteria to grow on arabinose and thus acidifying the medium. Fungal scytalone dehydratase was detected using pH indicators on plates. A second screen was done to eliminate false positives. In theory this system could be tailor-made for other enzymes as well, but general applicability is limited as protein–CID pairs for the substrate of interest might not be available [59].

Baker *et al.* [60] developed a yeast–three hybrid system to detect enzyme catalysis. The transcriptional regulator is composed of two fusion proteins: a LexA DNA binding domain fused to a dihydrofolate reductase (LexA-DHFR), and a B42 activation domain fused to a glucocorticoid receptor (B42-GR). These two fusions are linked via the substrate, which consists of three parts, namely Mtx bound at one side to LexA-DHFR, Dex bound at the other side to B42-GR, and the substrate of interest positioned between Mtx and Dex. Breaking or formation of the bond by the enzyme monomerizes or dimerizes the transcriptional activator, resulting in repression or derepression of transcription, respectively. So, this system is limited to bond breaking or bond forming reactions. It has been applied for selecting glycosynthase activity from a Glu197 saturation library of the endoglucanase Cel7B, using a gene involved in leucine biogenesis (*LEU2*) as reporter to complement a leucine auxotroph. This resulted in a five-fold increase in glycosynthase activity [61]. Furthermore, a variant with six-fold increased catalytic efficiency (k_{cat}/K_M) was selected from a cellulase library created by DNA family shuffling of genes encoding Cel7B variants. *URA3* was the reporter, converting the substrate 5-fluoroorotic acid to the toxic product 5-fluorouracil (5-FU). So, upon cleavage of the Mtx-Cel-Dex substrate, dimerization of the transcriptional activator was disrupted and toxic 5-FU was no longer produced [62].

Also Verhoeven *et al.* [63] developed a system in which the enzyme disrupts dimerization of a transcriptional regulator. In this case two DNA binding domains of the repressor cI of bacteriophage 434 were linked with a flexible region. An active protease could cleave the linker of this single chain repressor, releasing repression of the reporter gene by losing DNA binding. Three reporters were used, *HIS3*, *kanR* and *lacZ*, of which the first two enabled growth of *E. coli* and the last was for quantitation. With this system a variant of the tobacco etch virus protease with changed substrate specificity was selected from an error-prone PCR library.

Riboswitch/ribozyme-based strategy

Riboswitch- or ribozyme-based approaches are not yet widely applied for finding novel and improved biocatalysts. Therefore additional information from other fields is included to get a more complete story. Several forms of regulation of the reporter by the aptamer are possible, on either transcriptional or translational level. They can be divided into riboswitches and ribozymes. In riboswitches an aptamer is often located in the 5′-UTR of the transcribed gene (often encoding an enzyme in natural cases or a reporter in synthetic constructs), in such a way that binding of a specific ligand to the aptamer triggers a change in its tertiary structure, switching (on or off) the translation of the reporter (Fig. 1D). Natural examples are ligand-dependent accessibility of the ribosomal binding site, ligand-dependent change from intrinsic transcription terminator to anti-terminator, or ligand-dependent accessibility of mRNA [25]. In the last case a conformational change in trans-acting RNA results in its inability to block the mRNA via its antisense aptamer sequence. In contrast, ribozymes have a catalytic activity, such as hydrolysis of the nucleic acid phosphodiester bonds in the backbone, usually of the aptamer-gene fusion transcript. When the self-cleaving hammerhead ribozyme, for example, is coupled to an aptamer and together inserted in the mRNA, ligand binding to the aptamer results in a structure switch, preventing self-cleavage of the ribozyme. Thus, the mRNA stays intact, allowing translation to occur [25].

Petitioner Merck, Ex. 1147, p. 2988

Also for choosing a proper aptamer for a certain screen/selection it is convenient to look at already described aptamers of natural origin. Although new aptamers/riboswitches are frequently identified, the repertoire is still quite limited. The most common method to obtain new aptamers is by systematic evolution of ligands by exponential enrichment (SELEX). In this *in vitro* approach a random RNA library goes through several rounds of selection, with or without the ligand [25,64,65]. However, aptamer binding *in vitro* does not ensure activity *in vivo*. To overcome this problem a combination of initial *in vitro* selection, to reduce the library size, and subsequent *in vivo* screening or selection, to find the proper *in vivo* activity, is possible [25].

Although not yet applied in finding or improving new biocatalysts, Desai and Gallivan [66] strongly suggest that *in vivo* screening or selection based on riboswitches is a good strategy for this purpose. They describe a theophylline-responsive riboswitch in the 5′-UTR of either *lacZ* or *cat*. The expression level is dependent on the distance between the ribosomal binding site and the aptamer. They demonstrate that synthetic riboswitches can be used to perform either genetic screening or genetic selection experiments to detect the presence of a specific, non-endogenous small molecule in *E. coli*, which in theory could be the product of an enzymatic reaction. In addition, they used the system to select for riboswitches with different ligand specificity. Moreover, it is demonstrated that a cell harbouring a synthetic riboswitch with a particular ligand specificity can be selectively amplified from a million-fold larger pool of cells containing mutant riboswitches that respond to a closely related ligand. This indicates the promise of successfully using this approach for selecting riboswitches with desired ligand specificities.

A recent study demonstrates the actual applicability of this strategy for biocatalyst improvement. Michener and Smolke [24] describe a theophylline-responsive ribozyme incorporated in the 3′-UTR of *gfp*, which couples product concentration to reporter expression in *S. cerevisiae*. The ribozyme consists of an input or product binding domain and an output or self-cleaving domain. The connection between the two domains is such that they cannot be folded properly simultaneously. When the output domain folds correctly it cleaves itself, resulting in removal of the poly-A tail and rapid degradation of the mRNA. Ligand binding favours the conformation with a properly folded input domain and an incorrect folded output domain. So, less cleavage and more gene expression occur. Coupled to flow cytometry (measuring the average fluorescence of the culture) or FACS (sorting the cells based on the fluorescence per cell), libraries of $\sim 10^3$ or $\sim 10^7$ respectively can be screened. Even small changes in fluorescence or enzyme activity are measurable with flow cytometry, whereas FACS measurements are less precise. Iterative application of this FACS screen to libraries of caffeine demethylase in yeast led to a series of beneficial mutations that ultimately increased enzyme activity *in vivo* 33-fold and product selectivity 22-fold.

A big advantage of this strategy is that multiple signals originating from different enzymes can be implemented by using for instance different GFP variants. This can be valuable when not one enzyme but a whole metabolic pathway needs improvement [24].

Post-translational modified reporter-based strategy

As screening or selection strategy, not only interference by the enzymatic reaction on the level of reporter transcription or translation is used, but also post-translational modification and degradation approaches have been developed (Fig. 1E). In contrast to transcriptional regulator- and riboswitch/ribozyme-based strategies, this strategy is less generally applicable. It is not the binding of the product but the actual activity of the enzyme which lies at the basis of the screening or selection. This limits each strategy to certain groups of enzymes. One enzyme group, the proteases, is very well represented. Kostallas *et al.* [67] designed a GFP with a protease substrate peptide and an ssrA-tag, co-expressed with the protease of interest. Proteases which can process the substrate peptide and remove the ssrA-tag rescue GFP from degradation by the protease ClpXP, increasing the fluorescence of the *E. coli* cell. In this case the system was used to find new substrates for the tobacco etch virus protease with FACS, but it could also be employed for directed evolution of proteases by changing the substrate peptide for the activity of interest [67].

Another approach was taken by O'Loughlin *et al.* [68]. Negative and positive selection are combined to alter the substrate specificity of HIV protease. In the first step, protease variants created by error-prone PCR are induced during the mid-logarithmic phase of growing *E. coli* cells. Based on the idea that HIV protease with broad specificity is cytotoxic by cleaving essential *E. coli* proteins, selection already reduced the pool from 60 000 to 15 000 cells. In the second step, HIV variants that could cleave β-galactosidase or an altered β-galactosidase with the protease substrate peptide embedded decreased the blue colouring of *E. coli*

Petitioner Merck, Ex. 1147, p. 2989

cells grown in the presence of the β-galactosidase artificial substrate X-Gal. Combination of these two steps more rapidly alters the protease specificity.

Another class of enzymes, 4′-phosphopantetheinyl transferase (PPTase), is of interest for finding new secondary metabolite biosynthetic clusters via association, because they are needed for the activation of nonribosomal peptide synthetases (NRPS) and polyketide synthase (PKS) enzymes, both members of such a cluster. PPTases catalyse the post-translational attachment of a 4′-phosphopantetheine group to a conserved serine residue in the carrier protein domains of NRPS and PKS enzymes, a modification that is essential for activity. Owen *et al.* [69] employed the NRPS enzyme BpsA as reporter, as it generates a coloured product, indigoidine, through cyclization of two L-glutamines upon activation by a PPTase. By selecting the indigoidine-positive clones, the number of clones is reduced such that lower throughput secondary screening methods, like *in vitro* activity measurements, become feasible. Although *E. coli* is used here, also applying this approach for screening other hosts such as *Pseudomonas* is possible. In addition, the carrier protein domain of BpsA can be modified in order to screen for a wider range of PPTases.

Fusion-based strategy

The fusion-based approach is not a screening or selection strategy by itself, but it can certainly improve the ratio of active versus inactive clones in a pool, making fewer high-throughput methods possible in further steps. Fusing GFP to the enzyme variant and measuring the fluorescence with FACS or a fluorometer is a way to remove the insoluble and therefore inactive clones from the pool, because GFP only gives a fluorescent signal when soluble. Gupta and Tawfik [70] improved the activity of serum paraoxonase PON1 towards several substrates via small and effective neutral drift libraries. In these libraries the protein function is maintained but mutations are accumulated leading to highly polymorphic, stable and evolvable variants, which can be used as the starting point for directed evolution. To reduce the pool size they fused PON1 to a certain variant of GFP, sfGFP-F12, to have a balance in stability of GFP and the fused enzyme. The fraction of positive clones was enhanced from ~ 15% to > 85% by sorting, indicating the loss of a significant number of inactive variants. The fraction of false positives, variants showing high fluorescence but no activity, was ~ 10%. The activity was measured with a chromogenic substrate after growth and lysis of the *E. coli* cells in 96-well plates.

Heterologous expression frequently results in low yields of functional protein due to incorrect folding of the polypeptide chain; a majority of the proteins may end up in insoluble aggregates, inclusion bodies. When that is the case, fusion-based strategies can also be used to improve the folding and increase the solubility of an enzyme. Japrung *et al.* [71] first selected active variants of pfDHFR–GFP that could rescue growth of DHFR-deficient mutants of *E. coli*. These variants were subsequently sorted with FACS based on their fluorescence. Only variants which exhibited the highest 10% fluorescence, and thus the highest solubility, were selected.

A drawback of this approach is that false positives may be obtained if the fusion proteins end up in inclusion bodies. Both enzyme and GFP can still be (partially) functional in inclusion bodies [72]. However, it is not guaranteed that the activity of the two goes hand in hand. GFP may still be fully functional without the enzyme of interest being soluble/active. In addition, it is important to test whether the fusion itself does not interfere with the activity of the enzyme. Testing both N-terminal and C-terminal fusions might help. Also the improved activity of the enzyme variants found with the screen should be verified without the fusion.

Comparison of the *in vivo* screening and selection strategies

First the general *in vivo* screening and selection strategies will be compared with respect to library type, library size, enantioselectivity and experimental requirements, followed by a comparison of reporter-based strategies. An overview of the characteristics of both strategy types is summarized in Tables 1 and 2, respectively.

A comparison of the general *in vivo* screening and selection strategies shows that most of them are suitable for both metagenomic (m) and enzyme variant (ev) libraries. Due to the required fusion between enzyme and anchoring motif, cell surface display is the only strategy that is not compatible with screening a metagenomic library.

All approaches, except for agar and microtiter plate screening, are high-throughput (frequently due to the use of FACS), although the library size is still limited by the transformation efficiency (~ 10^9). Concerning the expression host for *in vivo* screening, *E. coli* is used in the majority (~ 90%) of all directed evolution studies. However, other bacterial hosts such as *Bacillus subtilis* and *Thermus* species are also used, as well as eukaryotic hosts including *S. cerevisiae* (second host

Petitioner Merck, Ex. 1147, p. 2990

Table 1. Characteristics of general *in vivo* screening and selection strategies. It is the product of the enzymatic reaction or the enzymatic conversion itself that needs to be detected, making these strategies reaction dependent (in contrast to most reporter-based strategies). Requirements encompass everything except basic microbial techniques/reagents and substrate. m, metagenomic library; ev, enzyme variant library. Adapted from [3].

Strategy	Library type	Library size	Requirements	Advantages	Disadvantages
Selection	m/ev	~ 10^9	None	Only desired variants Simple to operate	Low activity variants might be missed Difficult to quantify Higher false positive risk
Agar plate screening	m/ev	~ 10^5	Reagents[a] Detection device[a]	Simple to operate	Low dynamic range Difficult to quantify
Microtiter plate screening	m/ev	~ 10^4	Detection device	Multiple analytical detection methods possible Increased sensitivity by cell lysis Large dynamic range	
Cell-in-droplets (screening)	m/ev	~ 10^9	Detection device Cell sorter	Pico/femto-litre scale (not much reagent needed)	Product should stay in the droplet Technically challenging
Cells as micro-reactors (screening)	m/ev	~ 10^9	Detection device Cell sorter	Femto-litre scale (not much reagent needed) No compartmentalization needed	Product should stay in the cell Technically challenging
Cell surface display (screening)	ev	~ 10^9	Detection device Cell sorter	Substrate can stay extracellular	Product needs to attach to cell surface Technically challenging Careful selection of host and anchoring motif needed Fusion can alter enzyme structure/activity

[a] In some cases.

in directed evolution studies, 9%) and even insect and mammalian cells [73]. No clear relationship exists between a specific host and a certain strategy. Nevertheless, bacterial hosts are often preferred over eukaryotic hosts because they have higher transformation efficiencies, have a faster growth rate and are easier to manipulate [74,75]. On the other hand, as discussed earlier, for cell surface display Gram-positive species like *Bacillus* are favoured because of their single cell membrane and rigid cell wall [13]. Another important aspect in choosing a proper host is the origin of the genes in the library. Since expression mechanisms (e.g. codon bias) vary between organisms, a host which is closely related to the organism from which the genes originate is preferred. However, with metagenomic libraries, covering genes from multiple organisms, this criterion is no longer feasible. Luckily, more hosts and improved hosts are still being developed [11,73].

All general strategies can be used to screen or select for enantioselective enzymes as long as it concerns reactions in which the enzyme is enantioselective with respect to the substrate. In that case two substrates with different chirality can be added. In some elegant cases this has been done in a single experiment where one of the two enantiomer products is toxic [29]. Another option is the use of blocking of those enzyme variants that have the unwanted enantioselectivity with suicide inhibitors of the corresponding chirality. A more laborious option is the performance of two subsequent screens, each with one substrate. Unfortunately, these approaches are useless when the enzyme converts a non-chiral substrate to either the L- or the D-product. Here only a coupled enzyme assay, such as coupling to the D-amino acid oxidase as described above [31], is able to determine the enantioselectivity of the enzyme.

The general strategies which are the simplest to operate and have minimum requirements are selection and agar plate screening. Selection is of course a special strategy, as in theory only desired variants are kept in the pool. However, in some cases a range of conversion rates is demanded, meaning that one tries

Petitioner Merck, Ex. 1147, p. 2991

Table 2. Characteristics of reporter-based *in vivo* screening and selection strategies. The actual screening or selection method is dependent on the reporter type and will fall within the general strategies described in Table 1. TR, transcriptional regulator; PTM, post-translational modification; m, metagenomic library; ev, enzyme variant library.

Strategy based on	Library type	Reporter types	Advantages	Disadvantages
Natural TR	m/ev	All	High specificity Adaptation TR possible (directed evolution) Reaction independent No artificial substrates required Extra possibilities for enantioselectivity Detection low activities Signal enhancement possible via transcription/translation	Proper TR needs to be found/designed: Number of natural TRs available limited Computational design TR to be developed
Synthetic TR	m/ev	All	Reaction independent[a] No artificial substrates required[a] Relative simple design TR due to multiple components Signal enhancement possible via transcription/translation Detection low activities	Reaction dependent[a]
Riboswitch/ ribozyme	m/ev	All	High specificity Initial *in vitro* selection of aptamer possible Reaction independent No artificial substrates required Extra possibilities for enantioselectivity Integration multiple signals possible Detection low activities Signal enhancement possible via transcription/translation	Proper aptamer needs to be found/ designed: Number of natural aptamers available limited No guaranteed translation *in vitro* to *in vivo* activity
PTM	m/ev	Modifiable by enzyme of interest	No artificial substrates required	Reaction dependent
Fusion	ev	Reporting solubility	Increase ratio active/inactive variants in pool possible Reaction independent No artificial substrates required	Fusion can alter enzyme structure/activity False positives when in inclusion bodies

[a] In some cases.

to find multiple enzymes performing the same conversion but having different conversion rates. Selection may not be the right choice in this case, because enzymes with a lower activity might not be kept in the pool and quantification is not always possible. Reporters with different efficiency might offer an alternative.

A major hurdle of the general *in vivo* strategies may be that the substrate should enter the cell, and in some cases (such as cells as micro-reactors) the product should also stay inside the cell. Therefore, not every enzymatic reaction can be screened or selected. Cell surface display circumvents this limitation partially by presenting the enzyme on the outside of the cell. Of course, the product now has to be contained at the outside of the cell as well, for otherwise the link between genotype and phenotype is lost. Thus the type of *in vivo* strategies that can be used are often reaction or at least product dependent, meaning that the enzymatic activity itself or the product needs to be detectable, e.g. as a fluorescent product or as a pH change. The use of artificial substrates which are converted into a measurable product can reduce this problem.

Petitioner Merck, Ex. 1147, p. 2992

However, another solution is the use of certain reporter-based strategies, as discussed above.

The type of reporter determines the general strategy a reporter-based method belongs to and thus its characteristics such as library size and requirements (Table 1). The unique characteristics of the reporter-based strategies are described in a separate table (Table 2). In cases where expression of the reporter is dependent on the enzyme activity, all available reporter types can be employed. However, for fusion-based and post-translational modification-based strategies, the reporters need to report on solubility and be modifiable by the enzyme of interest, respectively. Of course, the choice for a specific reporter is also determined by the available equipment, the necessary reagents and the complexity of the protocol that needs to be followed in the measurements, and the desired characteristics of the reporter–measurement combination with respect to, for example, sensitivity and dynamic range. Comparing the different reporter-based strategies shows that, except for the fusion-based approach, all are suitable for selection and screening of both metagenomic and enzyme variant libraries.

Involvement of reporters in screening or selection strategies has some advantages. (a) Extra selection or screening criteria can be implemented, e.g. solubility in the case of a fusion-based approach. (b) Artificial substrates are often not necessary because the product is not measured directly but via a reporter. This is not always the case for synthetic-transcriptional-regulator-based strategies, as in some approaches the substrate needs to be attached to parts of the regulator. (c) One has the ability to screen or select for enantioselective enzyme variants which convert a non-chiral substrate to either the L- or the D-product, provided the binding of the transcriptional regulator or the riboswitch/ribozyme to the product is enantioselective. (d) They are more widely applicable, because they are reaction independent and the screening or selection strategy is dependent on the reporter choice, as explained earlier. (e) The signal may be enhanced by coupling enzyme activity to reporter expression. Altering features such as promoter strength, ribosomal binding site and codon bias may allow for detection of weak signals. This can be very valuable for further laboratory evolution, as improving an existing activity is much more feasible than changing towards a new activity.

Despite these interesting features of reporter-based strategies, there are two major downsides. (a) Developing such systems requires time and effort. In particular, the development of transcriptional regulators and riboswitches/ribozymes to specifically bind to the product of interest is challenging and time consuming. The number of known natural regulators [11] and aptamers is limited. However, the possibility of doing an initial riboswitch/ribozyme selection *in vitro* may greatly simplify the development of riboswitches/ribozymes compared with transcriptional regulators. For modifying the transcriptional regulators the most successful method at the moment is directed evolution. (b) Coupling to selection on growth may result in more false positives compared with screening. The higher selective pressure may select for cells that circumvent the coupling of the enzyme activity to growth and are able to survive by a different mechanism. For example, false positives may result from spontaneous escape mutants, such as a variant transcriptional regulator that turns on expression independent of product binding.

Conclusions and future prospects

None of the *in vivo* selection and screening strategies described here is perfect, and none is able to target each possible enzyme. Introducing reporters certainly increases the general applicability of a strategy, but the fact that each strategy only targets a subset of enzymes still holds. For each study one has to choose the proper approach on the basis of preferred features. It is expected that this field of reporter-based strategies will increase in the coming years. Existing strategies will be further improved and new strategies will be developed. Detection of the product by binding to a cytoplasmic sugar binding protein with incorporated GFP and subsequent alteration of its fluorescence could be one of them [76]. Besides, research on reporters themselves is ongoing [77]. Since transcriptional-regulator- and riboswitch/ribozyme-based strategies are reaction independent, these will gain the most interest among reporter-based strategies.

In vivo methods have advantages such as enhanced success rates of functionally produced (correctly folded, cofactor incorporated, catalytically active) protein(s) of interest and the possibility of varying the properties of the desired biocatalysts by changing the screening or selection conditions. However, one should keep in mind that these methods are limited by problems associated with heterologous expression, transformation efficiency, transport of substrate and/or product over the cell boundaries, reduced sensitivity and host growth rate. It is therefore important not only to further develop and improve the selection and screening strategies themselves, but also to improve for example heterologous expression by changes in library construction (e.g. codon optimization in enzyme variant libraries) and increasing the number of expression hosts. In addition, progress at the level of computa-

Petitioner Merck, Ex. 1147, p. 2993

tional design and *in silico* and *in vitro* selection and screening strategies will be very valuable. Both smaller but smarter libraries as well as making the handling of large libraries more straightforward or increasing the manageable library size are interesting approaches. Also combining different strategies can help in the search for novel or improved biocatalysts: e.g. cells as micro-reactors screened with FACS, followed by microtiter plate screening.

Although there are still many problems to be solved, spectacular developments are initiated. Certainly the combination of computational design and laboratory evolution will have an enormous influence and will in time lead to more universal strategies.

References

1 Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M & Witholt B (2001) Industrial biocatalysis today and tomorrow. *Nature* **409**, 258–268.

2 Sanchez S & Demain AL (2011) Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance. *Org Process Res Dev* **15**, 224–230.

3 Leemhuis H, Kelly RM & Dijkhuizen L (2009) Directed evolution of enzymes: library screening strategies. *IUBMB Life* **61**, 222–228.

4 Kaul P & Asano Y (2012) Strategies for discovery and improvement of enzyme function: state of the art and opportunities. *Microb Biotechnol* **5**, 18–33.

5 Liszka MJ, Clark ME, Schneider E & Clark DS (2012) Nature versus nurture: developing enzymes that function under extreme conditions. *Annu Rev Chem Biomol Eng* **3**, 77–102.

6 Illanes A, Cauerhff A, Wilson L & Castro GR (2012) Recent trends in biocatalysis engineering. *Bioresour Technol* **115**, 48–57.

7 Davids T, Schmidt M, Böttcher D & Bornscheuer UT (2013) Strategies for the discovery and engineering of enzymes for biocatalysis. *Curr Opin Chem Biol*, in press.

8 Huang XF, Santhanam N, Badri DV, Hunter WJ, Manter DK, Decker SR, Vivanco JM & Reardon KF (2013) Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnol Bioeng*, in press.

9 Kennedy J, O'Leary ND, Kiran GS, Morrissey JP, O'Gara F, Selvin J & Dobson ADW (2011) Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems. *J Appl Microbiol* **111**, 787–799.

10 Uchiyama T & Miyazaki K (2009) Functional metagenomics for enzyme discovery: challenges to efficient screening. *Curr Opin Biotechnol* **20**, 616–622.

11 Timmis K, Carreño CA & de Lorenzo V (2010) Genetic traps for surveying new catalysts in (meta) genomic DNA. In Handbook of Hydrocarbon and Lipid Microbiology, pp. 4563–4579. Springer, Berlin.

12 Fernández-Arrojo L, Guazzaroni ME, López-Cortés N, Beloqui A & Ferrer M (2010) Metagenomic era for biocatalyst identification. *Curr Opin Biotechnol* **21**, 725–733.

13 Boersma YL, Dröge MJ & Quax WJ (2007) Selection strategies for improved biocatalysts. *FEBS J* **274**, 2181–2195.

14 Goldsmith M & Tawfik DS (2012) Directed enzyme evolution: beyond the low-hanging fruit. *Curr Opin Struct Biol* **22**, 406–412.

15 Brouns SJJ, Wu H, Akerboom J, Turnbull AP, de Vos WM & van der Oost J (2005) Engineering a selectable marker for hyperthermophiles. *J Biol Chem* **280**, 11422–11431.

16 Heselpoth RD & Nelson DC (2012) A new screening method for the directed evolution of thermostable bacteriolytic enzymes. *J Vis Exp*, **69**, e4216.

17 Acharya P, Rajakumara E, Sankaranarayanan R & Rao NM (2004) Structural basis of selection and thermostability of laboratory evolved *Bacillus subtilis* lipase. *J Mol Biol* **341**, 1271–1281.

18 Barrozo A, Borstnar R, Marloie G & Kamerlin SCL (2012) Computational protein engineering: bridging the gap between rational design and laboratory evolution. *Int J Mol Sci* **13**, 12428–12460.

19 Dietrich JA, McKee AE & Keasling JD (2010) High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu Rev Biochem* **79**, 563–590.

20 Kiss G, Çelebi-Ölçüm N, Moretti R, Baker D & Houk KN (2013) Computational enzyme design. *Angew Chem Int Ed Engl*, in press.

21 Kipnis Y & Baker D (2012) Comparison of designed and randomly generated catalysts for simple chemical reactions. *Protein Sci* **21**, 1388–1395.

22 Khare SD & Fleishman SJ (2012) Emerging themes in the computational design of novel enzymes and protein-protein interfaces. *FEBS Lett* **587**, 1147–1154.

23 Hediger MR, De Vico L, Svendsen A, Besenmatter W & Jensen JH (2012) A computational methodology to screen activities of enzyme variants. *PLoS One* **7**, e49849.

24 Michener JK & Smolke CD (2012) High-throughput enzyme evolution in *Saccharomyces cerevisiae* using a synthetic RNA switch. *Metab Eng* **14**, 306–316.

25 Gredell JA, Frei CS & Cirino PC (2012) Protein and RNA engineering to customize microbial molecular reporting. *Biotechnol J* **7**, 477–499.

26 Yang G & Withers SG (2009) Ultrahigh-throughput FACS-based screening for directed enzyme evolution. *ChemBioChem* **10**, 2704–2715.

Petitioner Merck, Ex. 1147, p. 2994

27 Konarzycka-Bessler M & Jaeger KE (2006) Select the best: novel biocatalysts for industrial applications. *Trends Biotechnol* **24**, 248–250.

28 Otten LG, Sio CF, Vrielink J, Cool RH & Quax WJ (2002) Altering the substrate specificity of cephalosporin acylase by directed evolution of the β-subunit. *J Biol Chem* **277**, 42121–42127.

29 Fernández-Álvaro E, Snajdrova R, Jochens H, Davids T, Böttcher D & Bornscheuer UT (2011) A combination of *in vivo* selection and cell sorting for the identification of enantioselective biocatalysts. *Angew Chem Int Ed Engl* **50**, 8584–8587.

30 Böttcher D, Schmidt M & Bornscheuer UT (2010) Screens for active and stereoselective hydrolytic enzymes. *Methods Mol Biol* **668**, 169–176.

31 Willies SC, White JL & Turner NJ (2012) Development of a high-throughput screening method for racemase activity and its application to the identification of alanine racemase variants with activity towards L-arginine. *Tetrahedron* **68**, 7564–7567.

32 Ingham CJ, Sprenkels A, Bomer J, Molenaar D, van den Berg A, van Hylckama Vlieg JET & de Vos WM (2007) The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. *Proc Natl Acad Sci USA* **104**, 18217–18222.

33 Wagschal K & Lee CC (2012) Microplate-based active/inactive 1 degrees screen for biomass degrading enzyme library purification and gene discovery. *J Microbiol Methods* **89**, 83–85.

34 Schmidt-Dannert C & Arnold FH (1999) Directed evolution of industrial enzymes. *Trends Biotechnol* **17**, 135–136.

35 Kintses B, Hein C, Mohamed MF, Fischlechner M, Courtois F, Lainé C & Hollfelder F (2012) Picoliter cell lysate assays in microfluidic droplet compartments for directed enzyme evolution. *Chem Biol* **19**, 1001–1009.

36 Ostafe R, Prodanovic R, Commandeur U & Fischer R (2013) Flow cytometry-based ultra-high-throughput screening assay for cellulase activity. *Anal Biochem* **435**, 93–98.

37 Yang G, Rich JR, Gilbert M, Wakarchuk WW, Feng Y & Withers SG (2010) Fluorescence activated cell sorting as a general ultra-high-throughput screening method for directed evolution of glycosyltransferases. *J Am Chem Soc* **132**, 10570–10577.

38 Prodanovic R, Ostafe R, Scacioc A & Schwaneberg U (2011) Ultrahigh throughput screening system for directed glucose oxidase evolution in yeast cells. *Comb Chem High Throughput Screen* **14**, 55–60.

39 de Las Heras A, Carreño CA, Martínez-García E & de Lorenzo V (2010) Engineering input/output nodes in prokaryotic regulatory circuits. *FEMS Microbiol Rev* **34**, 842–865.

40 Michener JK, Thodey K, Liang JC & Smolke CD (2012) Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab Eng* **14**, 212–222.

41 Kelkar M & De A (2012) Bioluminescence based *in vivo* screening technologies. *Curr Opin Pharmacol* **12**, 592–600.

42 Garmendia J, de las Heras A, Galvão TC & de Lorenzo V (2008) Tracing explosives in soil with transcriptional regulators of *Pseudomonas putida* evolved for responding to nitrotoluenes. *Microb Biotechnol* **1**, 236–246.

43 de Las Heras A & de Lorenzo V (2012) Engineering whole-cell biosensors with no antibiotic markers for monitoring aromatic compounds in the environment. *Methods Mol Biol* **834**, 261–281.

44 Sinha J, Reyes SJ & Gallivan JP (2010) Reprogramming bacteria to seek and destroy an herbicide. *Nat Chem Biol* **6**, 464–470.

45 Beggah S, Vogne C, Zenaro E & Van Der Meer JR (2008) Mutant HbpR transcription activator isolation for 2-chlorobiphenyl via green fluorescent protein-based flow cytometry and cell sorting. *Microb Biotechnol* **1**, 68–78.

46 Uchiyama T, Abe T, Ikemura T & Watanabe K (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. *Nat Biotechnol* **23**, 88–93.

47 Uchiyama T & Watanabe K (2008) Substrate-induced gene expression (SIGEX) screening of metagenome libraries. *Nat Protoc* **3**, 1202–1212.

48 de Las Heras A, Chavarría M & de Lorenzo V (2011) Association of *dnt* genes of *Burkholderia* sp. DNT with the substrate-blind regulator DntR draws the evolutionary itinerary of 2,4-dinitrotoluene biodegradation. *Mol Microbiol* **82**, 287–299.

49 de Lorenzo V (2005) Problems with metagenomic screening. *Nat Biotechnol* **23**, 1045; author reply, 1045–1046.

50 Uchiyama T & Miyazaki K (2010) Product-induced gene expression, a product-responsive reporter assay used to screen metagenomic libraries for enzyme-encoding genes. *Appl Environ Microbiol* **76**, 7029–7035.

51 Allert M, Rizk SS, Looger LL & Hellinga HW (2004) Computational design of receptors for an organophosphate surrogate of the nerve agent soman. *Proc Natl Acad Sci USA* **101**, 7907–7912.

52 Looger LL, Dwyer MA, Smith JJ & Hellinga HW (2003) Computational design of receptor and sensor proteins with novel functions. *Nature* **423**, 185–190.

53 Schreier B, Stumpp C, Wiesner S & Höcker B (2009) Computational design of ligand binding is not a solved problem. *Proc Natl Acad Sci USA* **106**, 18491–18496.

54 Malisi C, Schumann M, Toussaint NC, Kageyama J, Kohlbacher O & Höcker B (2012) Binding pocket

Petitioner Merck, Ex. 1147, p. 2995

optimization by computational protein design. *PLoS One* **7**, e52505.
55 van Sint Fiet S, van Beilen JB & Witholt B (2006) Selection of biocatalysts for chemical synthesis. *Proc Natl Acad Sci USA* **103**, 1693–1698.
56 Cebolla A, Sousa C & de Lorenzo V (1997) Effector specificity mutants of the transcriptional activator NahR of naphthalene degrading *Pseudomonas* define protein sites involved in binding of aromatic inducers. *J Biol Chem* **272**, 3986–3992.
57 Mohn WW, Garmendia J, Galvao TC & de Lorenzo V (2006) Surveying biotransformations with *à la carte* genetic traps: translating dehydrochlorination of lindane (gamma-hexachlorocyclohexane) into *lacZ*-based phenotypes. *Environ Microbiol* **8**, 546–555.
58 Tang SY & Cirino PC (2011) Design and application of a mevalonate-responsive regulatory protein. *Angew Chem Int Ed Engl* **50**, 1084–1086.
59 Firestine SM, Salinas F, Nixon AE, Baker SJ & Benkovic SJ (2000) Using an AraC-based three-hybrid system to detect biocatalysts *in vivo*. *Nat Biotechnol* **18**, 544–547.
60 Baker K, Bleczinski C, Lin H, Salazar-Jimenez G, Sengupta D, Krane S & Cornish VW (2002) Chemical complementation: a reaction-independent genetic assay for enzyme catalysis. *Proc Natl Acad Sci USA* **99**, 16537–16542.
61 Lin H, Tao HY & Cornish VW (2004) Directed evolution of a glycosynthase via chemical complementation. *J Am Chem Soc* **126**, 15051–15059.
62 Peralta-Yahya P, Carter BT, Lin H, Tao H & Cornish VW (2008) High-throughput selection for cellulase catalysts using chemical complementation. *J Am Chem Soc* **130**, 17446–17452.
63 Verhoeven KD, Altstadt OC & Savinov SN (2012) Intracellular detection and evolution of site-specific proteases using a genetic selection system. *Appl Biochem Biotechnol* **166**, 1340–1354.
64 Ruigrok VJ, Levisson M, Eppink MHM, Smidt H & van der Oost J (2011) Alternative affinity tools: more attractive than antibodies? *Biochem J* **436**, 1–13.
65 Ruigrok VJ, van Duijn E, Barendregt A, Dyer K, Tainer JA, Stoltenburg R, Strehlitz B, Levisson M, Smidt H & van der Oost J (2012) Kinetic and stoichiometric characterisation of streptavidin-binding aptamers. *ChemBioChem* **13**, 829–836.
66 Desai SK & Gallivan JP (2004) Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation. *J Am Chem Soc* **126**, 13247–13254.
67 Kostallas G, Löfdahl PA & Samuelson P (2011) Substrate profiling of tobacco etch virus protease using a novel fluorescence-assisted whole-cell assay. *PLoS One* **6**, e16136.
68 O'Loughlin TL, Greene DN & Matsumura I (2006) Diversification and specialization of HIV protease function during *in vitro* evolution. *Mol Biol Evol* **23**, 764–778.
69 Owen JG, Robins KJ, Parachin NS & Ackerley DF (2012) A functional screen for recovery of 4′-phosphopantetheinyl transferase and associated natural product biosynthesis genes from metagenome libraries. *Environ Microbiol* **14**, 1198–1209.
70 Gupta RD & Tawfik DS (2008) Directed enzyme evolution via small and effective neutral drift libraries. *Nat Methods* **5**, 939–942.
71 Japrung D, Chusacultanachai S, Yuvaniyama J, Wilairat P & Yuthavong Y (2005) A simple dual selection for functionally active mutants of *Plasmodium falciparum* dihydrofolate reductase with improved solubility. *Protein Eng Des Sel* **18**, 457–464.
72 García-Fruitós E, González-Montalbán N, Morell M, Vera A, Ferraz RM, Arís A, Ventura S & Villaverde A (2005) Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. *Microb Cell Fact* **4**, 27.
73 Pourmir A & Johannes TW (2012) Directed evolution: selection of the host organism. *Comput Struct Biotechnol J* **2**, e201209012.
74 Hu JC, Kornacker MG & Hochschild A (2000) *Escherichia coli* one- and two-hybrid systems for the analysis and identification of protein–protein interactions. *Methods* **20**, 80–94.
75 Joung JK, Ramm EI & Pabo CO (2000) A bacterial two-hybrid selection system for studying protein–DNA and protein–protein interactions. *Proc Natl Acad Sci USA* **97**, 7382–7387.
76 Marvin JS, Schreiter ER, Echevarría IM & Looger LL (2011) A genetically encoded, high-signal-to-noise maltose sensor. *Proteins* **79**, 3025–3036.
77 Xiong AS, Peng RH, Zhuang J, Davies J, Zhang J & Yao QH (2012) Advances in directed molecular evolution of reporter genes. *Crit Rev Biotechnol* **32**, 133–142.

Petitioner Merck, Ex. 1147, p. 2996