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A Cell-Based Adrenaline Assay for Automated High-Throughput Activity Screening of Epoxide Hydrolases

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Dedicated to Professor Teruaki Mukaiyama on the occasion of his 80th birthday

Abstract: Herein we describe the development of a cell-based pretest for high-throughput screening of the activity of epoxide hydrolases (EHs) by using the chemistry of the known adrenaline test. It is a practical tool for the directed evolution of EHs as catalysts in synthetic organic chemistry and/or for assaying culture strains. The pretest can be applied to a wide variety of epoxide substrates, thereby circumventing the need for cell rupture and enzyme purification. In the present study we focus on parameters to improve assay specificity and sensitivity in order to obtain a robust and reproducible cell-based test. The introduction of threshold values allows a clear and reliable hit identification which is a prerequisite for industrial or academic prescreens probing thousands of samples in strain collections or in directed evolution libraries.

Keywords: adrenaline test · cell-based assay · directed evolution · epoxide hydrolase · high-throughput screening

Introduction

Interest in biocatalysis in industrial and academic settings continues to grow, as judged by an ever-increasing number of reports.[1] Epoxide hydrolases (EHs)[1,2] are beginning to gain industrial importance in the synthesis of enantiopure intermediates needed in pharmaceutical and agrochemicals. New representatives of these enzymes continue to become available by classical techniques of microbiology, metagenome panning, and directed evolution.[3] The latter two methods require high-throughput assays for screening the activity of thousands of (mutant) enzymes.[4] For example, in the emerging area of the directed evolution of stereoselective epoxide hydrolases,[5,6] it is useful to install an efficient pretest that assesses the activity of large numbers of clones. This eliminates unnecessary further consideration of inactive mutants (which may populate a given library by up to 90% or higher), thus decreasing the more-time-consuming *ee* determination[7] that follows the pretest. Several pretests[8,9] (including a plate test)[10] for evaluating the activity of EHs have been reported. Generally, these are quantitative UV and fluorescence-based systems that are characterized either by labeled surrogate substrates or indirectly by monitoring indicators. The first group comprises substrates that contain and liberate chromophoric or fluorogenic molecules such as *p*-nitrophenolate[8a] or umbelliferone.[8b] A drawback of this first system is that these substrates are generally not those of actual interest in applications, which means that an additional screening step is necessary to evaluate the real substrate. The credo in directed evolution "You get what you screen for"[3] dictates that methods are desired that allow direct catalyst optimization. From a practical point of view, this is a crucial issue in directed evolution. An important step forward was the introduction of the adrenaline test as an endpoint measurement by Reymond and co-workers which uncouples the EH-catalyzed reaction from the signaling step.[9] Accordingly, the enzymatic reaction of the substrate of interest is carried out in the first step followed by periodate-mediated product cleavage and final detection in the second step (Scheme 1). Thus, this assay can be applied to EHs, which are used in the production of a wide variety of chiral or achiral diols.

Although successful, the adrenaline test was described for systems involving purified enzymes. Indeed, other groups have applied it successfully, but also after purification of the EH.[11] However, a cell-based test for probing thousands of samples would be even more desirable, because it avoids

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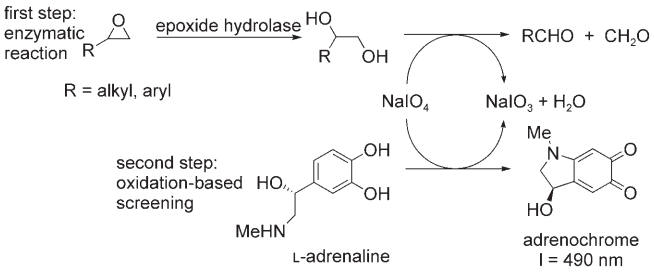


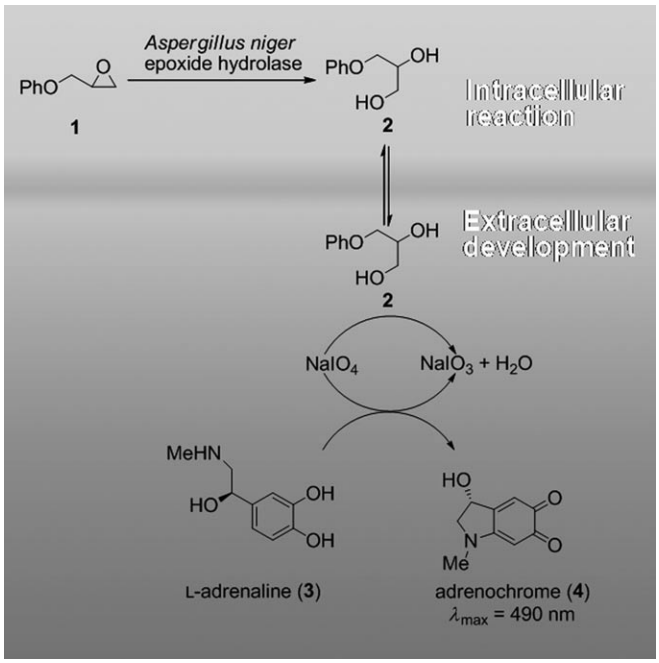
Scheme 1. Adrenaline test for assaying the activity of epoxide hydrolases (EHs).[9]

time- and labor-intensive cell rupture and purification of the enzymes. Unfortunately, adapting the adrenaline test to a cell-based arrangement is not trivial, because data-quality issues arise which are highly dependent on the quality and accuracy of each high-throughput screening (HTS) event. This problem is caused by low specificity and sensitivity in a cell-based environment leading to false-positive and -negative hits.[12] Herein we describe the development of a robust and reliable cell-based HTS assay as a primary screen for the activity of EHs by using the chemistry of the adrenaline test, thereby leading to the perspective of faster and more-efficient directed evolution of this class of enzymes.

Results and Discussion

Our endeavor entails a number of challenges. Random error reflects inevitable uncertainties in all measurements and includes biological, instrumental, and human-related influences. Inferential errors can also occur, however, caused by noise due to procedural factors that can be minimized by quality control. For this reason, we first directed our attention to factors leading to optimized signal specificity in order to obtain a robust and reproducible cell-based pretest for EHs. In a further step we considered signal sensitivity by evaluating threshold values, thereby ensuring an accurate hit identification. Generally, measured activity can be assigned to at least two factors: the true catalyst activity in a given reaction and unspecific activity. Several factors can be attributed to the latter aspect which we have evaluated in order to increase the precision of the cell-based test.

In principle, the adrenaline assay is based on a back-titration procedure, which simply indicates the quantity of periodate-sensitive products formed.[9,11] This indirect setting permits a separate enzymatic reaction and signaling step. In the present study we chose the hydrolytic kinetic resolution of glycidyl phenyl ether (**1**) as the model reaction because this substrate is known to be accepted by the epoxide hydrolase from A*spergillus niger* (ANEH) with formation of diol **2** (Scheme 2), albeit with low enantioselectivity.[5] With regard to whole-cell catalysis, which is the most convenient form in directed evolution, the epoxide **1** is enzymatically hydrolyzed in vivo to afford the corresponding diol **2**. After passing the cell membrane, the oxidizable product is cleaved



Scheme 2. General Scheme of the cell-based adrenaline assay. Spatial separation of in vivo product formation (**2**) from substrate (**1**) and in vitro product detection is featured.

by the equivalent amount of sodium periodate. The unreacted periodate reagent, in turn, is treated with L-adrenaline (**3**) to give adrenochrome (**4**), an orthoquinone dye with a specific red absorption in the visible spectrum at 490 nm. As this system is based on an indirect process, it must be emphasized that a product-containing sample will be indicated by a colorless tone, because all periodate is consumed and no chromophoric dye **4** can be formed. In directed evolution these are the active hits (EHs), which then have to be checked for enantioselectivity in a more-refined secondary *ee* assay.

To minimize false hits, we first considered factors that are expected to bias the assay specificity. A common source of artifacts that lead to a high background in cell-based assays is the oxidation-sensitive medium itself. Generally, highly dilute, oxidation-stable organic solvents are preferred, usually less than 10% in water. Other major sources of unspecific results are the growth media and supernatants, because carbohydrates and other polyols are highly sensitive to oxidation reactions. Even the highest dilution leads to inaccuracies. Therefore, we devised an automated cell-washing step, which can be performed by a multifunctional automated HTS system employing a cell-wash station.[13] Along these lines, the buffer itself should contain no alcohol functionalities. We therefore chose a phosphate saline buffer. Apart from either a fluorogenic or a chromophoric system necessary for detection, the removal of all oxidizable compounds is mandatory as long as periodate is involved in the quantitative back-titration.

The use of whole cells in UV/Vis-based tests creates further problems. Accurate measurements can be prevented by

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either turbidity or background absorption. Therefore, the noise effect caused by whole cells was examined systematically, thereby making visible the influence of unspecific absorption at 490 nm (Figure 1). As described above, cells

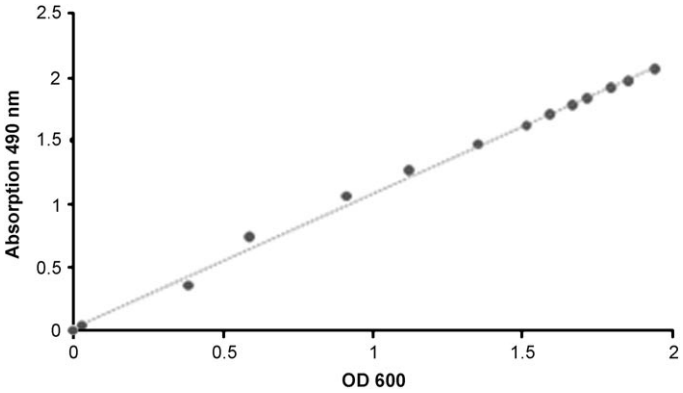


Figure 1. Background absorption caused by whole cells.

were subjected to a washing step before the developer solution was added. By increasing the cell density, a linear trend was observed which clearly demonstrates that the whole cells are, indeed, another source of background signals. The challenge herein consists in finding the balance between reasonable cell density for a detectable conversion and sufficient chromophore absorption for a reliable detection. With respect to fluctuating cell expression and therefore well-to-well variable background noise, a normalization of absorption data was applied. Furthermore, to increase the accuracy, an individual correction of the cell background of each well was considered, which can be performed automatically by using data software (e.g. Excel). Thus, to test the viability of the system, we assumed for a sufficient turnover a notably high cell density of $OD_{600}=1$, which in turn affords product signal intensities higher than 1.

In a second step, the concentration range of L-adrenaline was investigated (Figure 2). Whole cells were resuspended in sodium phosphate buffer (pH 7.2, 180 μL, $OD_{600}=1$) and subjected to the corresponding periodate solution. For a further normalization, the individual whole-cell background at 490 nm was measured. After adding adrenaline, the suspension was developed for 1 min at room temperature before measuring the final absorption. As expected, a linear increase in intensity was observed up to a developer concentration of 10 mM followed by saturation behavior (Figure 2, green line). This can be circumvented by further dilution. As a notable signal is obtained for low concentrations, which can be related to unspecific absorption (red line), an individual correction of values is necessary to obtain the specific product absorption (blue line).

Owing to variations caused by autohydrolysis of the specific substrate, variable protein-expression levels, and non-specific EH activity from *E. coli*, an exact determination of the enzymatic activity is not possible. Such data would be misleading for further analysis. For primary screens in cell-

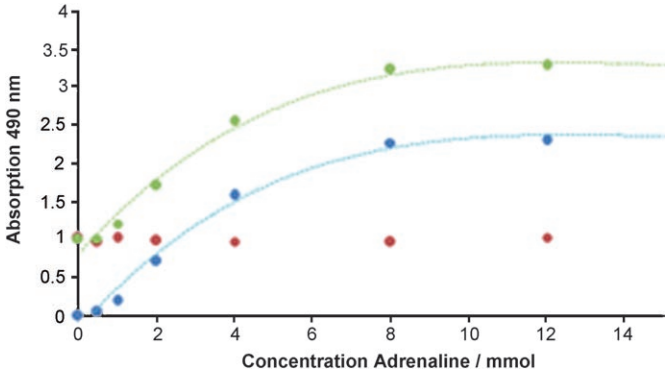


Figure 2. Absorption range and specificity of cell-based adrenaline assay. Absorption (green) was evaluated by various developer concentrations. Considering unspecific cell absorption (red) led to corrected and product-specific intensities (blue).

based HTS, a clear and reliable result or signal response is necessary to minimize false-hit indication. Rather than monitoring full conversion, a simple detection of low conversion is desired which goes along with improving assay sensitivity. This, in turn, can be realized in a more sensitive manner by introducing absorption scopes that cover turnovers less than 50%. We therefore aimed for the determination of threshold conversion values that allow a reliable detection of product formation in order to give a simple YES/NO response for hit detection (Figure 3). Thus, we propose to determine threshold conversion values that incorporate the aforementioned variations and the expected conversions that should be at least as high as these variations. Since the adrenaline assay is based on back-titration, periodate consumption indicates product formation, which means a low absorption for the adrenochrome chromophore. Thus, to monitor higher threshold conversions, the corresponding amount of periodate has to be added, which leads to a continuous increase in maximal signal intensity (SI). However, to indicate accurately a YES response to a set threshold

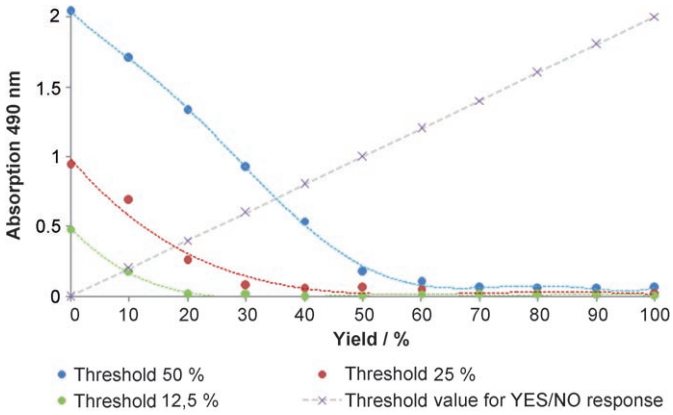


Figure 3. Evaluation of threshold values for YES/NO response. A threshold for detecting conversions between 12.5 and 50% (green, red, and blue) for a 4-mM substrate/product solution. A positive sample should give an absorption below SI_{50} line (gray), which corresponds to a YES response.

value, the measured absorption should be at least below 50% signal intensity (SI_{50}) of the given threshold (Figure 3, gray line), which now can be related to specific EH activity. To verify this concept, a cell density of $OD_{600}=1$ and a 4-mM product concentration range were chosen. Periodate and adrenaline concentrations were adjusted to monitor conversions higher than the set benchmark as a clearly positive sample (colorless).

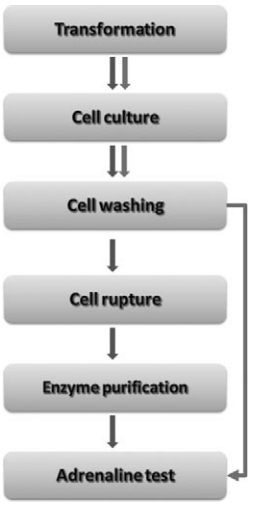
In doing so, we investigated a threshold range between 12.5 and 50% product conversion in order to evaluate the detection limits of the cell-based assay. We first tested the benchmark of 50% product formation and recorded the signal intensity with increasing product concentration (blue line). For low or no turnovers, a strong absorption could be observed, being related to a NO response. The complementary result was obtained at higher yields. They were correctly indicated either by a definite YES response (yields >50%) or by intensities below (yields 35–50%) which can be analyzed for further decision. In the same manner we investigated a 12.5 and 25% detection threshold. For the latter case, clear indications below and above 25% conversion were recorded as expected (red line).

It became clear that a 12.5% benchmark constitutes a problem because the specific chromophore absorption represents only a small fraction compared to the cell absorption (green line). As shown in Figure 3, the cell-corrected signal for a negative sample can be clearly distinguished from a positive reaction since its corrected value for 12.5% yield ranges below the SI_{50}. As we were aware of the variability at lower concentrations, this screening arrangement benefits even from slightly increasing yields and leads to a clearer and more-accurate response.

As a proof of principle for the preceding concept, we examined the cell-based assay under automated HTS conditions with a 25% threshold for a 4-mM substrate concentration. We chose once more the hydrolytic kinetic resolution of glycidyl phenyl ether (**1**) as model reaction, catalyzed by the ANEH with formation of diol **2** (Scheme 2). For a prescreen with the cell-based adrenaline-test, we followed a general procedure for preparing the cell cultures (Scheme 3, right arrow). It can be seen that the present pretest eliminates two work-intensive steps in the traditional approach (center arrows). When handling thousands of clones in directed evolution, this shortcut constitutes a significant increase in overall efficiency.

In the case of EHs, the present pretest coupled with a refined *ee* assay based on MS,[7] GC, or HPLC provides a viable screening platform. However, it is conceivable that our pretest can also be modified so as to indicate roughly enantioselective hits (see below).

The previously reported recombinant expression system (pQEEH) for *Aspergillus niger* epoxide hydrolase[5] was used as a positive control, whereas the same plasmid construct without EH-coding insert served as the negative control. Cells were prepared and harvested (see Experimental Section) as reported in a 96-well multi-titerplate, which affords variable cell densities and expression levels. The reaction was carried out in 96-test-plates as described above and allowed to proceed for 30 min at 37 °C. The visual results clearly identify active and non-active cultures as YES/NO signal responses (Figure 4). Whereas inactive cultures with a conversion definitely lower than 25% are shown in red (NO response, for example, in the series A–F1), samples with a conversion higher than 25% are depicted as colorless (YES response, for example, in the series G–H1).



Scheme 3. General procedure for a high-throughput activity prescreen for epoxide hydrolases by using the conventional adrenaline test (center arrows). Two steps—cell rupture and enzyme purification—can be circumvented when the cell-based adrenaline assay is employed (right arrow).

For more detailed information about quantity and quality in hit identification, analysis of processed absorption data is desired. Besides common absorption value analysis, we have also visualized the data in a grayscale color scheme, which is particularly easy and fast to read (Figure 5): According to the real sample, probes can be differentiated by gray tones proportional to their true conversion. Light squares indicate the most-active EHs. Owing to the higher degree of contrast, this visualization mode allows a more convenient iden-

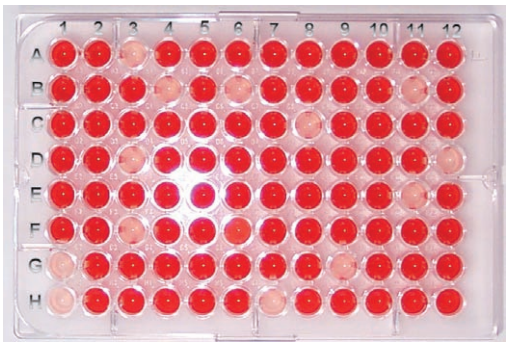


Figure 4. Whole-cell adrenaline assay for HTS. Conditions were optimized towards YES/NO response using 4 mM substrate and 25% threshold. A turnover higher than 25% is shown as a white field, which is encoded as a YES response, whereas a conversion less than 25% is identified as a red field.

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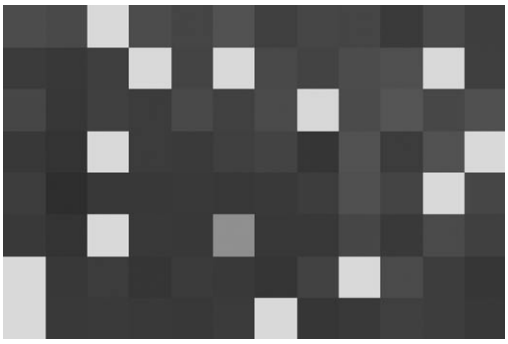

Figure 5. Gray-color-coded representation of the activity of EHs. Tones are proportional to the individual intensities of the wells of the 96-well plate. By applying this, data can be stored and processed for further analysis.

tification of samples with lower conversion than the threshold. Although the threshold was set for 25% conversion, sample F6 could be identified as a possible hit with low turnover. This was confirmed by a workup step and HPLC analysis, which yielded 10% product formation. This pattern can be simply obtained by converting the raw data into portable-picture-map format (.ppm) and visualized by a graphics software of choice (see Experimental Section). This cell-based procedure should be applicable to other back-titration-based tests that use periodate.[9g]

A very important application of directed evolution is to enhance enantioselectivity.[3h,i,4a] If both enantiomers of an epoxide substrate are used in separate wells and tested pairwise with a given EH clone, the assay would detect the optically pure products, which in turn would allow a rough assessment of the apparent enantioselectivity. Hereby, the adaptation of the threshold conversion values—according to the faster and the slower reacting enantiomer—would form the basis of an *ee* prescreen.

Conclusions

We have developed a reliable cell-based pretest to assess the activity of epoxide hydrolases (EHs). It is based on the known adrenaline test, which had previously been developed by Reymond and co-workers for the assessment of EHs in pure form or in supernatants.[9,11] The present cell-based adaptation saves considerable time because cell rupture and purification of enzymes can be avoided. It delivers reliable and clear signals in combination with threshold values for relatively precise decision making by simple visualization or automatic indication during the screening process. This is a prerequisite for probing large numbers of samples. Indeed, we have applied our test to the directed evolution of enantioselective EHs, processing thousands of clones per day.[14] We believe that this new form of the adrenaline assay will become a practical and therefore valuable tool in the development of new EHs, especially in the evaluation of strain collections and in directed evolution of these useful enzymes. Synthetic organic chemistry can thus be expected to profit indirectly from our study.[15]

Experimental Section

General

All reagents were purchased from Sigma and used without purification. For the preparation of PBS buffer, deionized water was used and adjusted to pH 7.2. Absorption spectra were obtained on a Spectramax UV/Vis spectrophotometer from Molecular Devices Corp. Sodium periodate and L-adrenaline were dissolved in deionized water and 270 mM stock solutions were prepared for the development reagents used in the assay with deionized water. Aqueous HCl (10%) was added to solubilize L-adrenaline. DH5α competent cells were obtained from Invitrogen.

Protein Expression

The epoxide hydrolase ANEH-encoding plasmid pQEEH as positive control was used to transform the enzyme in DH5α-competent cells.[5] The same plasmid construct without insert served as negative control. Randomly single colonies were isolated from Lauria-Bertani (LB) agar plates, supplemented with carbenicillin (100 mg mL^{-1}) and inoculated in LB media (800 μL) supplemented with carbenicillin (100 mg mL^{-1}) using 96-deepwell plates. The cultures were incubated overnight at 30 °C, under shaking at 800 rpm. The cells were washed by using an automated cell-washing device. Alternatively, this step was carried out manually by centrifuging at 4000 rpm for 3 min at 4 °C. Supernatants were discarded and cells were resuspended in PBS buffer (800 μL).

Reaction Conditions and Cell-Based Assay Development

The reactions were performed as follows: Washed expression culture (20 μL) was added to PBS buffer (150 μL, 57 mmol, pH 7.2) and the substrate was dissolved in acetonitrile (10 μL). The reaction suspension was shaken at 800 rpm for 30 min at 37 °C. In order to monitor the reaction development, sodium periodate (20 μL) was added and the samples shaken at 800 rpm at 37 °C. The exact reaction time is dependent on the amount of diol formed. The reaction mixtures (with a 4-mmol substrate concentration) were allowed to develop for 5 min at 37 °C and 800 rpm. A blank measurement on a Spectramax spectroscope was carried out to obtain the cell background absorption $A_{490\ cells}$ [cells + buffer + solvent + substrate + sodium periodate] followed by the addition of L-adrenaline solution (20 μL). For a uniform detection, the reaction mixtures were allowed to proceed for 1 min at 37 °C and 800 rpm before the adrenochrome absorption was measured $A_{490\ Adren.}$ [cells + buffer + solvent + substrate + sodium periodate + L-adrenaline].

It should be noted that when working with other substrates, it is necessary to adjust the appropriate development time and temperature.

Evaluation of Concentration Range, Development Times, and Threshold Values

Optimal results can be obtained by determining concentration range, development times, and threshold values individually of the substrate by using the previous described protocol.

Data Processing and Visualization

Raw data were processed by correcting the absorption values as cell background absorption was subtracted: $A_{490\ Corrected} = A_{490\ Adren.} - A_{490\ cells}$ by using Microsoft Excel software. For visualization in gray, portable-gray-map (.pgm) or a portable-pixel-map (.ppm) formats were used to generate the gray-scale visualization proportional to the conversions in the 96-well array. Each grid position is first assigned to a whole number between 0 (full black, NO response) and 255 (white, YES response) by converting the corrected absorption data to a range from 0 to 255 which can be realized by Microsoft Excel. Each column was replicated and grouped as a triplet to obtain a RGB (red-green-blue) color model which was performed by using an Excel macro. The values in the first column (red) were set to 255, whereas the next columns (green, blue) contained twice

the proportional absorption value. The file was saved as a comma-separated-value (.csv) file and opened in a text editor (Wordpad, Notepad, Simpletext) for modifications in the first line as follows:

P3

(optional line with identifier)

X Y

255

X denotes the number of columns in the RGB value array, and Y refers to the number of lines—for a 96-well array usually 36 and 8, respectively. The file is then saved as portable-gray-map (.pgm) or portable-pixel-map (.ppm) files and opened with commercial graphics software (such as Photoshop, Paintshop, Coreldraw, etc.), resized to a width of 500 pixels, and saved in bitmap file format (.bmp). Color visualizations other than gray can be generated by editing the columns for either red, green, or blue in the Excel file.

Acknowledgements

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[1] a) *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook*, *2nd ed.*, *Vol. I–III* (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**; b) *Biotransformations in Organic Chemistry*, *4nd ed.* (Ed.: K. Faber), Springer, Berlin, **2000**; c) *Industrial Biotransformations* (Eds.: A. Liese, K. Seelbach, C. Wandrey), Wiley-VCH, Weinheim, **2006**; d) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258–268; e) K. M. Koeller, C.-H. Wong, *Nature* **2001**, *409*, 232–240; f) M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, *Angew. Chem.* **2004**, *116*, 806–843; *Angew. Chem. Int. Ed.* **2004**, *43*, 788–824; g) D. J. Pollard, J. M. Woodley, *Trends Biotechnol.* **2007**, *25*, 66–73.

[2] a) A. Steinreiber, S. F. Mayer, K. Faber, *Synthesis* **2001**, 2035–2039; b) A. Archelas, R. Furstoss, *Curr. Opin. Chem. Biol.* **2001**, *5*, 112–119.

[3] a) *Directed Enzyme Evolution: Screening and Selection Methods*, *Vol. 230* (Eds.: F. H. Arnold, G. Georgiou), Humana, Totowa, **2003**; b) *Directed Molecular Evolution of Proteins; or How to Improve Enzymes for Biocatalysis* (Eds.: S. Brakmann, K. Johnsson), Wiley-VCH, Weinheim, **2002**; c) *Evolutionary Methods in Biotechnology: Clever Tricks for Directed Evolution* (Eds.: S. Brakmann, A. Schwienhorst), Wiley-VCH, Weinheim, **2004**; d) S. V. Taylor, P. Kast, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3408–3436; *Angew. Chem. Int. Ed.* **2001**, *40*, 3310–3335; e) K. A. Powell, S. W. Ramer, S. B. del Cardayré, W. P. C. Stemmer, M. B. Tobin, P. F. Longchamp, G. W. Huisman, *Angew. Chem.* **2001**, *113*, 4068–4080; *Angew. Chem. Int. Ed.* **2001**, *40*, 3948–3959; f) S. B. Rubin-Pitel, H. Zhao, *Comb. Chem. High Throughput Screening* **2006**, *9*, 247–257; g) J. Kaur, R. Sharma, *Crit. Rev. Biotechnol.* **2006**, *26*, 165199; h) M. T. Reetz, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5716–5722; M. T. Reetz in *Advances in Catalysis*, *Vol. 49* (Eds.: B. C. Gates, H. Knözinger), Elsevier, San Diego, **2006**, pp. 1–69.

[4] For reviews of high-throughput-screening systems, see: a) M. T. Reetz, *Angew. Chem.* **2001**, *113*, 292–320; *Angew. Chem. Int. Ed.* **2001**, *40*, 284–310; b) *Enzyme Assays—High-throughput Screening, Genetic Selection and Fingerprinting* (Ed.: J.-L. Reymond), Wiley-VCH, Weinheim, **2005**; c) M. T. Reetz in *Evolutionary Methods in Biotechnology* (Eds.: S. Brakmann, A. Schwienhorst), Wiley-VCH, Weinheim, **2004**, pp. 113–141; d) D. Wahler, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2001**, *12*, 535–544; e) M. Schmidt, U. T. Bornscheuer, *Biomol. Eng.* **2005**, *22*, 51–56; f) G. Wu, S. K. Doberstein, *Drug Discovery Today* **2006**, *11*, 718–724; g) R. A. Kumar, D. S. Clark, *Curr. Opin. Chem. Biol.* **2006**, *10*, 162–168; h) S. Fox, S. Farr-Jones, L. Sopchak, A. Boggs, J. Comley, *J. Biomol. Screening* **2004**, *9*, 354–358.

[5] a) F. Cedrone, S. Niel, S. Roca, T. Bhatnagar, N. Ait-Abdelkader, C. Torre, H. Krumm, A. Maichele, M. T. Reetz, J. C. Baratti, *Biocatal. Biotransform.* **2003**, *21*, 357–364; b) M. T. Reetz, C. Torre, A. Eipper, R. Lohmer, M. Hermes, B. Brunner, A. Maichele, M. Bocola, M. Arand, A. Cronin, Y. Genzel, A. Archelas, R. Furstoss, *Org. Lett.* **2004**, *6*, 177–180; c) M. T. Reetz, L.-W. Wang, in part M. Bocola, *Angew. Chem.* **2006**, *118*, 1258–1263; *Angew. Chem. Int. Ed.* **2006**, *45*, 1236–1241; Corrigendum, M. T. Reetz, L.-W. Wang, M. Bocola, *Angew. Chem.* **2006**, *118*, 2556; *Angew. Chem. Int. Ed.* **2006**, *45*, 2494.

[6] B. van Loo, J. H. L. Spelberg, J. Kingma, T. Sonke, M. G. Wubbolts, D. B. Janssen, *Chem. Biol.* **2004**, *11*, 981–990.

[7] a) W. Schrader, A. Eipper, D. J. Pugh, M. T. Reetz, *Can. J. Chem.* **2002**, *80*, 626–632; b) M. T. Reetz, M. H. Becker, H.-W. Klein, D. Stöckigt, *Angew. Chem.* **1999**, *111*, 1872–1875; *Angew. Chem. Int. Ed.* **1999**, *38*, 1758–1761.

[8] a) J.-P. Goddard, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2004**, *15*, 314–322; b) J.-P. Goddard, J.-L. Reymond, *Trends Biotechnol.* **2004**, *22*, 363–370; c) F. Zocher, M. M. Enzelberger, U. T. Bornscheuer, B. Hauer, R. D. Schmid, *Anal. Chim. Acta* **1999**, *391*, 345–351; d) K. Doderer, S. Lutz-Wahl, B. Hauer, R. D. Schmid, *Anal. Biochem.* **2003**, *321*, 131–134; e) D. Wahler, F. Badalassi, P. Crotti, J.-L. Reymond, *Angew. Chem.* **2001**, *113*, 4589–4592; *Angew. Chem. Int. Ed.* **2001**, *40*, 4457–4460; f) K. Doderer, R. D. Schmid, *Biotechnol. Lett.* **2004**, *26*, 835–839; g) C. Mateo, A. Archelas, R. Furstoss, *Anal. Biochem.* **2003**, *314*, 135–141.

[9] a) D. Wahler, J.-L. Reymond, *Angew. Chem.* **2002**, *114*, 1277–1280; *Angew. Chem. Int. Ed.* **2002**, *41*, 1229–1232; b) D. Wahler, O. Boujard, F. Lefèvre, J.-L. Reymond, *Tetrahedron* **2004**, *60*, 703–710.

[10] a) In reference [6] an agar plate assay is described; b) for a preselection test based on bacterial growth advantages as a function of EH activity, see: M. T. Reetz, L.-W. Wang, *Comb. Chem. High Throughput Screening* **2006**, *9*, 295–299.

[11] F. Cedrone, T. Bhatnagar, J. C. Baratti, *Biotechnol. Lett.* **2005**, *27*, 1921–1927.

[12] a) N. Malo, J. A. Hanley, S. Cerquozzi, J. Pelletier, R. Nadon, *Nat. Biotechnol.* **2006**, *24*, 167–175; b) G. Harper, S. D. Pickett, *Drug Discovery Today* **2006**, *11*, 694–699.

[13] a) G. Wu, S. K. Doberstein, *Drug Discovery Today* **2006**, *11*, 718–724; b) A. S. Verkman, *Am. J. Physiol. Cell Physiol.* **2004**, *286*, C465–C474.

[14] M. T. Reetz, D. Kahakeaw, R. Lohmer, unpublished results.

[15] For a comparison of EHs and synthetic transition-metal catalysts, see: a) C. Schneider, *Synthesis* **2006**, 3919–3944; for a review of the Jacobsen catalysts, see: b) E. N. Jacobsen, *Acc. Chem. Res.* **2000**, *33*, 421–431.

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