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The *In Situ* Enzymatic Screening (ISES) Approach to Reaction Discovery and Catalyst Identification

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Abstract

The importance of discovering new chemical transformations and/or optimizing catalytic combinations has led to a flurry of activity in reaction screening. The *in situ* enzymatic screening (ISES) approach described here utilizes biological tools (enzymes/cofactors) to advance chemistry. The protocol interfaces an organic reaction layer with an adjacent aqueous layer containing reporting enzymes that act upon the organic reaction product, giving rise to a spectroscopic signal. ISES allows the experimentalist to rapidly glean information on the relative rates of a set of parallel organic/organometallic reactions under investigation, without the need to quench the reactions or draw aliquots. In certain cases, the real-time enzymatic readout also provides information on sense and magnitude of enantioselectivity and substrate specificity. This unit contains protocols for single-well (relative rate) and double-well (relative rate/ee) ISES, in addition to a colorimetric ISES protocol and a miniaturized double-well procedure.

Keywords

enzymatic screening; reaction discovery; catalysis; metal-ligand combinations; UV/vis spectrophotometry

INTRODUCTION

Modern chemical biology has been described as “an area of research in which chemical and biological concepts and tools interact synergistically in the pursuit of new discoveries or technologies” (C. Bertozzi). In that same recent discussion, it was noted (D. Liu) with favor that the field has “expand(ed) to embrace such concepts as the use of biological principles to advance chemistry” (Bertozzi et al., 2015). The ISES protocol presented in this unit utilizes enzymatic chemistry to advance an organic chemical or organometallic reaction of interest. To be sure, the ISES approach is part of a multi-pronged effort in the field to develop new screening assays to accelerate the process of discovering new chemistry (Collins et al., 2014; Loch and Crabtree, 2001; Stambuli and Hartwig, 2003) and new asymmetric catalysts (Finn, 2002; Jo et al., 2014; Leung et al., 2012; Pfaltz, 2010; Reetz, 2002). More open-ended screens for ‘serendipitous chemistry’ have also drawn significant attention in recent years (McNally et al., 2011; Robbins and Hartwig, 2011). Among the analytical tools being

harnessed to provide information on the reactions being screened are included mass spectrometry (Ebner et al., 2011; Isenegger et al., 2016; Lichtor and Miller, 2011; Montavon et al., 2012; Mueller et al., 2009), IR thermography (Loskyl et al., 2012; Reetz et al., 2000; Taylor and Morken, 1998) and circular dichroism (Bentley et al., 2016a; De los Santos and Wolf, 2016; Lin et al., 2016) with microfluidic technologies and miniaturization (Buitrago Santanilla et al., 2015; Goodell et al., 2009; Treece et al., 2010) being explored to increase throughput. Generally, these methods are performed after taking aliquots or otherwise stopping or working up the reaction. There are exceptions, primarily examples in which sensors are placed in the matrix, effectively allowing for a real time readout of reaction progress. Examples include coated Au nanoparticles for iodide-sensing (C-C cross-couplings; Jung et al., 2011), an aminomethylantracene dye for acid-sensing (nucleophilic catalyst-promoted acylations (Copeland and Miller, 1999) or direct IR thermographic measurement of relative heats of reaction, in parallel (Loskyl et al., 2012; Reetz et al., 1998; Reetz et al., 2000; Taylor and Morken, 1998).

ISES takes advantage of the inherent ability of the enzymes to fold into flexible, but well-defined three-dimensional structures that both exquisitely recognize chirality and are capable of rapid catalytic turnover of small-molecule substrates. Other screening methods that take advantage of macromolecular recognition elements are discussed in the *Commentary* section below. The goal is to exploit the ability of enzymes to catalytically process small non-chromogenic molecules in reactions associated with cofactors that provide spectroscopic signatures. In this way, one obtains a specific readout on the formation of product or byproduct with time without the need to install a chromophore or mass tag into the substrate, and without the need to quench or even draw aliquots from the reaction under study. Moreover, if run in a parallel cuvette or well format, multiple reactions can be screened, in parallel, by UV/vis scanning or with the naked eye, depending upon the reporting enzyme/cofactor.

This unit contains protocols for each variant of the ISES protocol developed so far. The technique is performed in a biphasic system, with an organic layer containing the reaction of interest and an aqueous layer (above or below the organic layer) containing reporting enzyme(s) and attendant cofactor(s). The product or byproduct of the organic reaction diffuses into the reporting layer where it is acted upon by the enzyme(s), giving rise to a spectroscopic change. Provided that the spectrophotometer beam is positioned to pass through the aqueous reporting layer or each cuvette or well in a multicell parallel-processing UV/vis apparatus, one then obtains direct in situ readouts on the relative reaction rates of a set of reactions under investigation. The biphasic embodiment of this technique requires the reaction of interest to produce an aqueous-diffusible product or byproduct that is a substrate for the reporting enzyme(s).

The first iteration of ISES was developed to explore catalytic combinations for a transition metal-mediated intramolecular amination (Berkowitz et al., 2002; Berkowitz and Maiti, 2004; Berkowitz et al., 2004) reaction upon an allylic ethyl carbonate substrate (Figure 1, Basic Protocol 1). Turnover of such substrates is expected to proceed via formation of a π -allyl or σ -allyl-metal intermediate with release of the ethyl carbonate leaving group in each catalytic cycle. The ethyl carbonate leaving group is expected to decarboxylate to generate

an ethoxide equivalent that will pick up a proton from the neighboring aqueous layer. The ethanol byproduct undergoes four-electron oxidation by the sequential action of alcohol and aldehyde dehydrogenase enzymes in the reporting layer, leading to the formation of two equivalents of NAD(P)H, easily detected at 340 nm [$E_{340}(\text{NAD(P)H}) = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$]. The rate of NADH formation therefore corresponds to the reaction rate. This assay is applied to an array of candidate reactions and the relative rates observed provide information of the most promising catalytic combinations for further study.

In the example shown in Figure 1, the synthetic goal was to find non-palladium (Trost et al., 2000) metal/ligand/N-protecting group combinations that would permit for the catalytic synthesis of vinylglycinol via intramolecular allylic amination. This is an example of the use of ISES for air-sensitive chemistry, as can be seen in Figure 1B, the cuvettes have all been purged with Ar, and are held under inert atmosphere via septa. Indeed, ISES was used to identify the combination of p-methoxyphenyl (PMP) protection on the nucleophilic nitrogen, $\text{Ni}(\text{cod})_2$ and bidentate phosphine ligands were found to be optimal based solely on iterative ISES screening (Berkowitz et al., 2002). This ultimately led to the first examples of asymmetric $\text{Ni}(0)$ -mediated allylic amination chemistry and to the first asymmetric base metal-mediated synthesis of L-vinylglycine (Berkowitz and Maiti, 2004; Berkowitz et al., 2004).

Subsequent variants of ISES share the use of dehydrogenases, but use these enzymes to report on the chiral reaction products themselves, e.g. in the chiral metal-salen [Note: Salens are bis-imine ligands constructed by condensing two equivalents of a (substituted) salicylaldehyde (sal) with a 1,2-diamine (the simplest 1,2-diamine = ethylenediamine = en)] mediated hydrolytic kinetic resolution (HKR) of terminal epoxides, thereby providing information on sense and magnitude of enantioselectivity, as well as relative rate (Dey et al., 2005; Dey et al., 2007; Karukurichi et al., 2015). In this case, because there are two unknowns, total product formed with time, and enantiomeric ratio of that product, two equations are needed to provide sufficient information to estimate both relative rate and enantioselectivity. To achieve this, two reporting enzymes with distinct enantioselectivities are utilized, each in a separate well for each catalyst-substrate pair screened. This is termed 'double-cuvette ISES' or 'double-well ISES' and is depicted in Figure 3 and described in Basic Protocol 3 ('cassette-ISES'). This type of screen has been miniaturized, as well, to just 20 μL organic reaction layer volume in a micromulticell format (Basic Protocol 4; 'mini-ISES').

A colorimetric version of ISES has also been developed that utilizes a visible dye chromophore as redox cofactor for a peroxidase enzyme-based readout (Friest et al., 2011). This assay was deployed to increase throughput (1152 combinations) to identify new (pseudo)halometallation/carbocyclization transformations (Figure 2, Basic Protocol 2). Screening substrates here feature an alkyne tethered to allylic methyl carbonate to provide for a metal-binding site to initiate the (pseudo)halocarbocyclization. With each turnover, a methyl carbonate leaving group is expected to be released, sending a molecule of methanol into the aqueous reporting layer, following decarboxylation. There, the methanol is oxidized to formaldehyde via an alcohol oxidase enzyme, generating hydrogen peroxide as a byproduct. A second enzyme, a peroxidase, then reduces H_2O_2 to H_2O , generating two

equivalents of the ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation with its vibrant green color serving as an indicator of a 'hit reaction.'

BASIC PROTOCOL 1

IN SITU ENZYMATIC SCREENING (ISES) METHOD (UV/VIS READOUT; SINGLE WELL PER CATALYST SCREENED)

This protocol provides a stepwise guide for a sample single well/cuvette ISES protocol. The example given is to rank catalytic combinations in terms of relative rate for a metal-catalyzed allylic amination reaction with an ethyl carbonate leaving group. The two reporting enzymes [(alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AIDH))] must first be characterized and their respective solutions titrated for activity using standard assays, prior to their application in the ISES protocol. In this example, turnover of an allylic ethyl carbonate substrate is expected to generate of a molecule of CO₂ and of ethanol with each catalytic cycle. Ethanol diffusing into the aqueous buffer reporting layer is oxidized to acetic acid by the sequential action of ADH and AIDH, thereby leading to the formation of two molecules of NADH, conveniently monitored at 340 nm. The traces shown in panel D of Figure 1 demonstrate the relative rate readout one obtains over a 12 minute window using a Shimadzu 6-cell changer for the six catalytic combinations shown in Figure 1B. This same screening procedure should apply to other organic or organometallic transformation of interest in which ethanol or ethyl carbonate is produced as a byproduct. A similar ISES procedure should also apply to reactions in which primary alcohol byproducts are released so long as these are diffusible and serve as ADH/AIDH substrates.

Materials

UV spectrophotometer [6–12 multi-cell positioner useful but not necessary]
Shimadzu UV-2101PC or Shimadzu UV-2401PC used for this protocol; Cary has a 12-cell positioner.

Quartz cuvettes (1 mL each)

Yeast alcohol dehydrogenase (ADH) - lyophilized powder (EC 1.1.1.1)- Sigma-Aldrich

Yeast aldehyde dehydrogenase (AIDH)- lyophilized powder (EC 1.2.1.5)- Boehringer-Mannheim

β-NAD⁺ (free acid) – Sigma-Aldrich

β-NADH (disodium salt) – Sigma-Aldrich

Ethanol

Acetaldehyde

Sodium phosphate, monobasic and dibasic salts

Sodium pyrophosphate

THF/hexane/toluene (2:1:1) - solvent for upper organic phase

Components for organic reaction of interest (example reaction- intramolecular allylic amination; specific conditions for the Ni cuvette in Figure 1 are given here)

Ni(cod)₂

Triphenyl phosphine

Substrate of interest (allylic carbonate bearing an appropriately positioned nucleophile – carbamate nitrogen here -see Figure 1)

LiHMDS (lithium hexamethyldisilazide)

Rubber septa (13 mm OD, with neck trimmed off; these fit 1 mL cuvettes snugly)

NOTE: This procedure describes ISES with an upper organic phase, subsequent protocols make use of a lower organic phase, generally generated by utilizing a significant percentage of a chlorinated solvent. See the protocols that follow and the troubleshooting section for more insight into organic layer selection. At the outset, care should always be taken to position the phase partition in the cuvette so that the UV beam passes squarely through the aqueous reporting layer.

Protocol steps—Step annotations

Enzyme Standardization: Solutions of both dehydrogenase enzymes are calibrated in terms of U/mL. One S.I. unit is taken as the amount of enzyme catalyzing the formation of one μmol of NADH per minute under standard assay conditions. In a 1 mL final cuvette volume and a 1 cm light path, this amounts to an absorbance change at 340 nm of 6.22 min^{-1} .

For Alcohol Dehydrogenase (ADH):

- 1a** Dissolve 1.5 mg of commercial ADH lyophilisate in 660 μL of 25 mM NaPO_4 , pH7.
- 2a** Prepare 1 mL cuvette: (final volume either 999.5 μL or 995 μL depending on step 3) 100 mM EtOH, 7.4 mM NAD^+ , 15 mM sodium pyrophosphate, pH 8.8. (Final pH ~ 7.7)
- 3a** Add 0.5 μL (or 5 μL of 1:10 dilution) of ADH solution from step 1 to assay cuvette from step 2 and monitor absorbance change at 340 nm; e.g. an absorbance change of 0.44 min^{-1} at 340 nm corresponds to a standard ADH solution of 0.14 U/ μL [$\text{NADH } \epsilon_{340} = 6.22 \text{ M}^{-1} \text{ cm}^{-1}$].

For Aldehyde Dehydrogenase (AIDH):

- 1b** Dissolve 5.2 mg of commercial AIDH lyophilisate in 500 μL of 25 mM NaPO_4 , pH7.
- 2b** Prepare 1 mL cuvette: (final volume 990 μL) 400 μM acetaldehyde, 7.4 mM NAD^+ , 15 mM sodium pyrophosphate pH 8.8. (Final pH ~ 7.7)

- 3b** Add 10 μL of AIDH solution from step 1 to assay cuvette from step 2 and monitor absorbance change at 340 nm.; e.g an absorbance change of 0.13 min^{-1} at 340 nm corresponds to a standard AIDH solution of $0.0021 \text{ U}/\mu\text{L}$.

ISES Screening Procedure: Quartz cuvettes with a 1 cm light path (nominal volume = 1 mL, actual filled volume = 1.6 mL) are used. The spectrophotometer used for this protocol has a beam height @ approximately 650 μL in such standard cuvettes. An acceptable layer interface height can be determined for a given spectrophotometer by running the standard assay conditions at various heights and examining the signal. These biphasic assays were run with a “tall” aqueous reporting layer (900 μL total volume) to insure that the UV/vis beam passes squarely through this reporting layer.

- 1** Prepare the following stock solutions:
 - a.** 37 mM NAD^+ in 25 mM NaPO_4 , pH 7
 - b.** Yeast ADH solution [prepared, as above, was $0.14 \text{ U}/\mu\text{L}$ in 25 mM NaPO_4 , pH 7]
 - c.** Yeast AIDH solution [prepared, as above, was $0.021 \text{ U}/\mu\text{L}$ in 25 mM NaPO_4 , pH 7]
 - d.** 15 mM sodium pyrophosphate, pH 8.8
- 2** Prepare reporting aq. phase in 1 mL quartz cuvette containing 7.4 mM NAD^+ , 1.3 U of ADH, and 0.12 U of AIDH; add sodium pyrophosphate to bring final volume to 900 μL .
 - a.** For this example, solution volumes (from step 1) are: stock soln. a = 180 μL , stock soln. b = 9 μL , stock soln. c = 55 μL , and stock soln. d = 656 μL .
- 3** Cover cuvette with truncated septum if screening an air-sensitive reaction and/or catalyst.
- 4** Prepare organic layer for reaction to be screened and add via syringe, piercing the septa.

Representative organic layer preparation for an air-sensitive, intramolecular allylic amination trial cuvette follows (use similar procedure for the screening of related reactions).

- 5** Dissolve substrate (110 μmol) in distilled THF (100 μL) in dry vessel under inert atmosphere.
- 6** Prepare active catalyst (11 μmol , 10 mol %) in a dry vessel under inert atmosphere as follows:
 - a.** Add PPh_3 (6–12 mg, 2–4 eq.) to a dry vial under inert atmosphere and dissolve in THF (100 μL)
 - b.** To the above add $\text{Ni}(\text{cod})_2$ (3 mg ; 11 μmol) weighed out under Ar in a glove bag/box
 - c.** Add toluene (100 μL) to the reaction mixture prepared in step b.

- 7 If external base is desired, LiHMDS (110 μ L of a 1.0 M solution in hexane; 1.0 eq. relative to substrate) is recommended as this base has been used effectively in this protocol. Otherwise, add hexane (100 μ L) to the above catalyst solution, via syringe.
- 8 Combine the substrate and catalyst solutions and immediately layer above the aqueous phase in the previously prepared cuvette.
- 9 For 6–12 parallel cuvettes, it is best to prepare the metal-ligand solutions first, then add the common reporting layers. This can be done via syringe, or more rapidly with a multichannel pipetter, particularly for upper reporting layer configurations (see protocols 2 and 3). Finally the reactions may be initiated by the addition of substrate, proceeding from the first cuvette to the last, so that the time course of the reactions being followed is similar as they are scanned via the automated cell changer.
- 10 Make sure that the UV/vis instrument is thermostatted at the desired temperature (25 °C here) and monitor absorbance at 340 nm vs. time. Choose an instrument with multi-cell scanning ability (i.e. with an automated cell changer) to monitor 6–12 organic/organometallic reactions, in parallel.
- 11 Compare relative rates to optimize reaction parameters, catalyst selection, or other variables of interest.

In this case, ISES screens were used to probe new metal/ligand N-protecting group combinations for an intramolecular allylic amination reaction leading to the natural product, *L*-vinylglycine (Berkowitz et al., 2000; Berkowitz et al., 1996b; Berkowitz et al., 1994; Berkowitz and Smith, 1996) (See Figure 1), a mechanism-based inhibitor for PLP enzymes (Berkowitz et al., 1996a; Karukurichi et al., 2007).

BASIC PROTOCOL 2

COLORIMETRIC IN SITU ENZYMATIC SCREENING (ISES) METHOD (VISIBLE COLOR CHANGE; SINGLE WELL PER CATALYST SCREENED)

This embodiment of ISES eliminates the need for a spectrophotometer as the readout is in the visible range of the spectrum. However, to obtain better estimates of relative rate the assay can be performed in a cuvette with a spectrophotometer. In the example given here, for 'hit reactions', a deep green color develops over time in the reporting layer. This signal is easily discerned by the naked eye and so colorimetric ISES naturally allows for a significant array of reaction conditions to be screened in parallel. The protocol described herewith 96 reactions per experimental run, conducted in a 96 well tray.

Materials

UV/vis spectrophotometer Shimadzu UV-2101PC or Shimadzu UV-2401PC used for this protocol

Quartz cuvettes (1 mL)

Multichannel pipetter [e.g. Eppendorf (Thermo-Fischer) or Rainin (Mettler-Toledo)]

Alcohol oxidase [EC 1.1.3.13; from *Hansenula* sp. lyophilized powder-Bradford

Peroxidase [EC 1.11.1.7; from horseradish (HRP), Type VI, lyophilized powder] – Sigma-Aldrich

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) – Sigma-Aldrich

Plastic or plexiglass 96 well tray – Thermo-Fisher Scientific, or custom-fabricated, as needed, depending upon culture tube dimensions [plexiglass = poly(methyl methacrylate) is easily machined to make custom trays].

Disposable cell culture tubes – (6 × 50 mm utilized here; Thermo-Fisher)

Methanol

Hydrogen peroxide (aqueous, 30% w/w)

Potassium phosphate, monobasic and dibasic salts

1,1,2-Trichloroethane (TCE)

Components for the organic reaction of interest [example reaction: (pseudo)halometallation-carbocyclization-see Figure 2]

Lithium halide (LiF, LiCl, LiBr) or pseudohalide (e.g. LiSCN, LiOCN, LiCN), for this example.

Transition metal (TM) catalyst candidates: 64 TM complexes screened here with $\text{Rh}(\text{O}_2\text{CCF}_3)_2]_2$ and $\text{Cl}_2\text{Pd}(\text{NCPH})_2$ being among the most effective catalysts, depending upon substrate and halide/pseudohalide (Friest et al., 2011; Ginotra et al., 2012).

Substrates of interest: In this example, 5- and 6-*exo-trig* ester and 5-*exo-trig* ether substrates **1–3**, were used. All are outfitted with an allylic methyl carbonate functionality (Figure 2).

Enzyme Standardization—Enzyme units were calculated by measuring the rate of formation of the ABTS radical cation at 405 nm (extinction coefficient $36.8 \text{ mM}^{-1} \text{ cm}^{-1}$). For AO, one S.I. unit is taken as the amount of enzyme catalyzing the oxidation of 1 μmol of methanol per min, with concomitant formation of 1 μmol of H_2O_2 per min. This leads to the oxidation of 2 μmol of ABTS to the corresponding radical cation, in a coupled assay with HRP. For HRP, one S.I. unit is taken as the amount of enzyme that will catalyze the reduction of 1.0 μmol of H_2O_2 per min, again resulting in the formation of 2 μmol of the ABTS radical cation per min.

1. Prepare Stock solutions

- a. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; 2 mM in 100 mM potassium phosphate buffer, pH 7.5)
- b. Alcohol oxidase (AO; from *Hansenula* sp. in 100 mM potassium phosphate buffer pH 7.5; sample solution here determined to be 0.62 mU/ μL after standardization)

- c. Peroxidase [Type VI from horseradish (HRP) in 100 mM potassium phosphate buffer, pH 7.5; sample solution here determined to be 1.46 mU/ μ L after standardization]
 - d. MeOH (aq; 1 mM)
 - e. H_2O_2 [aq. 0.3% (w/w)]
2. HRP standardization: To 1 mL cuvette, add ABTS stock solution (950 μ L) and HRP stock solution (17 μ L).
3. Initiate by adding H_2O_2 stock solution (33.3 μ L) and measure Abs₄₀₅/min at 25 °C. In this example, a rate of 1.83 Abs/min was observed, corresponding to 1.46 mU HRP per μ L of stock solution.
4. AO standardization: To 1 mL cuvette, add ABTS stock solution (950 μ L), standardized HRP stock solution (20 μ L; 29.2 mU) and AO stock solution (20 μ L).
5. Initiate by adding MeOH stock solution (10 μ L) and measure Abs₄₀₅/min at 25 °C. In this case, a rate of 0.92 Abs/min was observed, corresponding to 0.62 mU AO per μ L of stock solution.

ISES Screening Procedure—In this example, colorimetric ISES is carried out in disposable culture tubes (6 × 50 mm) pressed into Plexiglas 96-well trays. For each tray, a single substrate is probed across a 16 (TM) × 6 [(pseudo)halide] array of candidate catalytic combinations. This means that with four trays, all 64 TM catalyst candidates in this array could be screened across all (pseudo)halides for a given substrate. Twelve trays then cover all three substrates here, accounting for the 1152 reaction configurations sampled. These screens were carried out with an upper aqueous enzymatic reporting layer, and a lower organic reaction layer in TCE/THF (95:5) solvent. Successful reactions are indicated by the appearance of a green color (ABTS radical cation formation) in the reporting layer (see Figure 2B).

NOTE: All stock solutions from the standardization step above will also be used here. Volumes are representative for the sample stock solutions.

1. Prepare Catalyst, substrate, and nucleophile solutions as follows:
 - a. Substrate: 300 mM stock solutions in TCE (3 total here, 1–3)
 - b. Catalyst candidates: 6.4 mM solutions in TCE (64 total here)
 - c. Nucleophile candidates: 2.0 M stock solutions in THF (6 total here)
2. Make a stock reporting solution containing ABTS stock solution (9.6 mL), AO stock solution (200 μ L, 140 mU) and HRP stock solution (200 μ L, 300 mU) – 10 mL total – enough for a complete 96 well plate @ 100 μ L per well.
3. To each culture tube column add substrate in TCE (8 tubes at a time, via syringe – 8 × 33 μ L of 300 mM stock solution; 50 mM final concentration) and

nucleophile in THF ($8 \times 10 \mu\text{L}$ of 2.0 M stock solution; 100 mM final concentration).

4. Initiate all reactions by addition of catalyst (6 tubes at a time, via syringe – $6 \times 157 \mu\text{L}$ of a 6.4 mM catalyst solution in TCE; 5 mM final concentration). To expedite this step to better synchronize the reaction set, two chemists can load the 16 catalysts in parallel (8 each).
5. Rapidly layer the aqueous reporting layer (from step 3) one row of 12 tubes at a time ($12 \times 100 \mu\text{L}$) using a multichannel pipetter (12 channels). Eight loadings will cover the 96 well setup.
6. Visualize with the naked eye and the appearance of a green color (ABTS radical cation) indicates a hit. Generally, greater catalyst rates correlate with more intense green color signals (Figure 2B).

BASIC PROTOCOL 3

IN SITU ENZYMATIC SCREENING (ISES) ASSAY DETECTING CHIRAL REACTION PRODUCTS (UV/VIS READOUT; TWO WELLS PER CATALYST SCREENED)

This ISES protocol utilizes two reporting enzymes per reaction to gain information about both sense and magnitude of enantioselectivity and relative rate, for a series of chiral catalysts being screened in parallel. The chiral metal-salen-mediated hydrolytic kinetic resolution (HKR) of a racemic terminal epoxide to an enantioenriched 1,2-diol and to the enantioenriched, antipodal remaining epoxide, pioneered by Jacobsen (Ford et al., 2013; Schaus et al., 2002) was chosen as the initial transformation to launch double-well ISES. This also serves as a useful platform to explore the development of novel chiral salen ligands for asymmetric catalysis. The HKR generates a chiral diol product that diffuses into the adjacent, aqueous reporting layer, where it is oxidized by an alcohol dehydrogenase (ADH) enzyme. Two different reporting ADHs (each in a separate well) with distinct enantioselectivities are used to provide enough information to extract both enantioselectivity (i.e. (*R*)-product:(*S*)-product ratio) and relative rate (i.e. total [(*R*) + (*S*)-product] with time) data from ISES. The enantioselectivities estimated by this double-well ISES methodology under biphasic conditions generally correspond well to those observed under conventional HKR conditions [neat epoxide or concentrated epoxide in organic solvent and < 1 equivalent water (Dey et al., 2005; Karukurichi et al., 2015)]. The following example is for the HKR of propylene oxide; however, a similar procedure has already been demonstrated for hexene oxide (Dey et al., 2007) and the process should be generalizable to a wide range of reactions that release alcohol or diol products.

Materials

β -NAD⁺ - Sigma-Aldrich

β -NADP⁺ - Sigma-Aldrich

Horse liver alcohol dehydrogenase (HLADH; EC 1.1.1.1)- Sigma-Aldrich

Thermoanaerobium brockii alcohol dehydrogenase (TBADH; EC 1.1.1.2)- Sigma-Aldrich

Sodium phosphate, monobasic and dibasic salts

1,2-Propanediol (*R*)-, (*S*)-, and racemic]

Sodium pyrophosphate

UV/vis spectrophotometer

1 mL quartz cuvettes

Components for organic reaction of interest (example reaction: hydrolytic kinetic resolution of epoxides employing Co(III)-salen catalysts designed/synthesized in the Berkowitz Lab)

Chloroform or dichloromethane for organic layer

Epoxide substrate (e.g. ($\pm$)-propylene oxide, ($\pm$)-hexene oxide)

Co(III)-salen catalyst array

Enzyme Standardization and Enantioselectivity Determination—Enzyme units are calculated by measuring the rate of formation of NAD(P)H at 340 nm (*vide infra*). In each case, one S.I. unit is taken as the amount of enzyme catalyzing the formation of one μmol of NAD(P)H per minute.

1. Prepare the following stock solutions:
 - a. 220 mM β -NAD⁺ in 25 mM sodium phosphate, pH 7.0
 - b. 220 mM β -NADP⁺ in 25 mM sodium phosphate, pH 7.0
 - c. HLADH solution (nominal 0.036 EtOH units/ μL) in 25 mM sodium phosphate, pH 7.0
 - d. TBADH solution (nominal 0.147 iPrOH units/ μL) in 25 mM sodium phosphate, pH 7.0
 - e. 2 M (*R*)-1,2-propanediol in H₂O (an equal number of (*R*)-1,2-propanediol units is of each reporting enzyme is used)
2. TBADH standardization: To 1 mL cuvette add NADP⁺ solution (10 μL), TBADH solution (2 μL), and reaction buffer (888 μL of 50 mM sodium pyrophosphate pH 8.8. Initiate reaction with 100 μL of (*R*)-1,2-propanediol solution. Measure absorbance in UV- vis spectrophotometer. The typical rate of 0.275 Abs/min at 340 nm indicates 0.022 (*R*)-1,2-propanediol U/ μL of TBADH solution.
3. HLADH standardization: To 1 mL cuvette add 33 μL NAD⁺ solution, 2 μL HLADH solution, 865 μL of 50 mM sodium pyrophosphate pH 8.8, and initiate reaction with 100 μL of (*R*)-1,2-propanediol solution. Measure absorbance in UV- vis soectrophotometer. The typical rate of 0.25 Abs/min at 340 nm indicates 0.020 (*R*)-1,2-propanediol U/ μL of HLADH solution.

ISES Screening Procedure—This protocol requires two cuvettes per reaction condition, one containing the (*R*)-selective enzyme (TBADH) and the other requires the (*S*)-selective enzyme (HLADH) for the 1,2-propanediol product (Dey et al., 2005). Equal numbers of (*R*)-1,2-propanediol units of individual reporting enzyme are added to the respective reporting cuvettes.

- 1 For each catalyst to be screened, two reporting cuvettes or wells are employed. In the first reporting cuvette, add 50 mM sodium pyrophosphate buffer, pH 8.8 (479.5 μ L), NADP⁺ stock solution (5 μ L) and 0.35 (*R*)-1,2-propanediol U TBADH (15.8 μ L of example stock solution).
- 2 In the second cuvette, add 50 mM sodium pyrophosphate buffer, pH 8.8 (465.8 μ L), NAD⁺ stock solution (16.5 μ L) and 0.35 (*R*)-1,2-propanediol U HLADH (17.6 μ L of example stock solution. The final aqueous volume of both reporting layers is 500 μ L, and the final pH is 8.6.
- 3 Prepare common organic phase for reaction of interest for HKR: ($\pm$)-propylene oxide (300 μ L; 2.15 mmol), CHCl₃ (300 μ L) and 0.25 mol% Co(III) salen catalyst. Total organic volume is 300 μ L per cuvette; the above solution is used for two reporting cuvettes.
- 4 Immediately after addition of the Co(III)-salen catalyst to solution in step 3, syringe 150 μ L thereof below the aqueous phase for each reporting cuvette. [It is also possible to add organic layers first and then expeditiously layer the aqueous reporting layers over the top of these]
- 5 Observe Abs/min at 340 nm

Enantioselectivity Estimation via ISES

$$\text{Catalyst Enantioselectivity} = \frac{[R]}{[S]} = \frac{\left[\left(\frac{Sel_{E2}}{Sel_{E1}} \right) \bullet V_{E2} - V_{E1} \right]}{Sel_{E2} \bullet (V_{E1} - V_{E2})} \quad [\text{Eqn. 1}]$$

- 6 The estimated enantiomeric excess (% ee = % major enantiomer minus % minor enantiomer) can be obtained from the above equation where Sel_{E1} is the *R:S*-selectivity for reporting enzyme-1 (HLADH for the first iteration of double-cuvette-ISES with the 1,2-propanediol product) and Sel_{E2} is the *R:S*-selectivity for enzyme-2 (TBADH for the first iteration of double-cuvette-ISES). The *Sel* parameter in this case was set as the enantioselectivity at 40 mM substrate concentration as obtained from enantioselectivity characterization (HLADH = 0.34, TBADH = 8.4) while *v* is the initial velocity measured (Abs₃₄₀/time) by either enzyme-1 or enzyme-2 in the ISES screen. For complete details of the reporting enzyme characterization, and of the derivation of the expression for enantioselectivity, see the SI for the initial report on double cuvette-ISES (Dey et al., 2005)

EXPANSION OF PROTOCOL 3 TO SCREEN TWO SUBSTRATE CANDIDATES IN PARALLEL (FOUR WELLS PER CATALYST SCREENED)

Parallel Multiple Substrate % ee Determination—It is also possible to perform double-cuvette ISES on multiple substrates utilizing two cuvettes per substrate. The procedure is the same as Basic Protocol 3, provided that two specific reporting enzymes with different enantioselectivities for the reaction product (a 1,2-diol here) are available. This screen is performed in the same way as in Basic Protocol 3: simply screen the initial enzyme rates for an array of dehydrogenases against the (*R*)- and (*S*)-enantiomer of product and identify a pair of enzymes with different enantioselectivities for these two antipodes. Note that while the two reporting enzymes must have distinct enantioselectivities for the reaction product, these need not be opposite. Thus, while HLADH is modestly (*S*)-selective and TBADH is (*R*)-selective for the 1,2-propanediol product from the HKR of propylene oxide (Dey et al., 2005), *Lactobacillus kefir* ADH (LKADH) is highly (*S*)-selective and HLADH modestly (*S*)-selective for the 1,2-hexanediol product from the HKR of hexene oxide (Dey et al., 2007). The miniaturized ISES example described in Basic Protocol 4 also employs this technique across several wells of a micromulticell.

BASIC PROTOCOL 4

MINIATURE-ISES ASSAY DETECTING CHIRAL REACTION PRODUCTS (TWO WELLS PER SUBSTRATE; FOUR WELLS PER CATALYST)

This protocol is a miniaturized version of Basic Protocol 3. The overall procedure is very similar, but is performed in a quartz micromulticell greatly reducing the volumes and quantities of catalyst, substrate and reporting enzymes needed for each set of conditions screened. The micromulticell is a single UV/vis cell (length = 7.5 cm; height = 0.9 cm; width = 1.2 cm; actual path length = 1 cm) available from Shimadzu. It features 16 wells each at 150 μ L nominal volume each; these are filled only to 110 μ L in this procedure. Use of the micromulticell allows for a reduction in the organic reaction layer volume from 300 to 20 μ L and in the aqueous reporting layer volume from 500 to 90 μ L; hence this protocol is referred to as “miniature-ISES” A schematic of this ISES protocol and a photo of the actual micromulticell are provided in Figure 3.

Materials

β -NAD⁺ (free acid) – Sigma-Aldrich

β -NADP⁺ - Sigma-Aldrich

Reporting enzymes [KRED 23, 107, 119 enzymes (all Codexis) and TBADH (Sigma) used here]

UV/vis spectrophotometer with micromulticell capability

16-Well quartz micromulticell (Shimadzu or other supplier)

(*R*)- and (*S*)-1,2-Propanediol (or product of interest)

(*R*)- and (*S*)-1,2-Hexanediol (or product of interest)

($\pm$)-1-Hexene oxide

(±)-Propylene oxide
Sodium pyrophosphate
Sodium phosphate, monobasic and dibasic salts
Chloroform

Enzyme Characterization—Useful reporting enzymes can be mined by screening arrays of dehydrogenase enzymes against racemic product (1,2-diols here) first to establish initial hits, followed by detailed kinetics evaluation with individual product enantiomers, across a range of concentration. For details regarding the determination of the selectivity parameters for the three new KRED enzymes utilized in this example of mini-ISES, see the Supplementary Material of the original publication (Karukurichi et al., 2015). Practically, these miniaturization studies showed that ISES protocols can be scaled down to 20 μL (organic reaction volume-lower layer)/90 μL (aq. reporting volume-upper layer):

1. Make stock solutions of:
 - a. 40 mM NADP^+
 - b. 130 mM NAD^+
 - c. 0.016 mg/ μL TBADH in 25 mM sodium phosphate, pH 7.0
 - d. 0.016 mg/ μL KRED 23 in 25 mM sodium phosphate, pH 7.0
 - e. 0.002 mg/ μL KRED 107 in 25 mM sodium phosphate, pH 7.0
 - f. 0.002 mg/ μL KRED 119 in 25mM sodium phosphate, pH 7.0
 - g. (*R*)-1,2-Propanediol (2 M in water)
 - h. (*R*)-1,2-Hexanediol (2 M in water)
 - i. 50 mM sodium pyrophosphate
2. (*R*)-1,2-Propanediol standardization (TBADH, KRED 23): To sodium pyrophosphate buffer (81 μL), add NADP^+ (for TBADH; 5 μL) or NAD^+ (for KRED 23, 5 μL) stock solution, enzyme solution (5 μL), and (*R*)-1,2-propanediol solution (9 μL).
3. Measure $\text{Abs}_{340}/\text{min}$ to obtain rate and calculate enzyme units, as described above.
4. (*R*)-1,2-Hexanediol standardization (KRED 107, KRED 119): To sodium pyrophosphate buffer (81 μL), add NADP^+ stock solution (5 μL), enzyme solution (5 μL) and (*R*)-1,2-hexanediol solution (9 μL).
5. Measure $\text{Abs}_{340}/\text{min}$ to obtain rate and calculate enzyme units, as described in Basic Protocol 1 step 3a.

ISES Procedure—Mini-ISES measurements were performed on a Shimadzu UV-2401PC spectrophotometer utilizing the Shimadzu MMC-1600C attachment and a 16 series micromulticell. This allows for temperature control via a water circulation system. The

micromulticell used contains 16 quartz wells each with a 100 μL volume, and a path length of 10 mm. The spectrophotometer moves the multicell to align each well with the UV beam; the residence time per cell varies with the number of cells used per assay. When all 16 cells are used the cycle time is 48 seconds in between readings, this time for two cells is 5 seconds, and for one cell readings can take place every 100 ms. This allows for a residence time between 2.5 and 3 seconds per cell. For every catalyst, a four-well “cassette screen” is performed over the two different substrates: propylene oxide and hexene oxide. For screening the HKR of ($\pm$)-propylene oxide, wells A & B contain respectively 0.10 (*R*)-1,2-propanediol units of TBADH [favors (*R*)-1,2-propanediol] and KRED 23 [favors (*S*)-1,2-propanediol], respectively. For screening the HKR of ($\pm$)-hexene oxide, wells C & D contain 0.037 (*R*)-1,2-hexanediol units of KRED 107 [strongly favors (*S*)-1,2-hexenediol], and KRED 119 [favors (*R*)-1,2-hexenediol], respectively.

1. Aqueous layer for well A: 0.10 (*R*)-1,2-propanediol units of TBADH reporting enzyme (3.6 μL TBADH stock solution) 2.2 mM NADP^+ (5 μL stock solution), and 50 mM sodium pyrophosphate, pH 8.8. (81.4 μL).
2. Aqueous layer for well B: 0.10 (*R*)-1,2-propanediol units of KRED 23 reporting enzyme (6.2 μL KRED 23 stock solution) 7.2 mM NAD^+ (5 μL stock solution) and 50 mM sodium pyrophosphate, pH 8.8. (78.8 μL).
3. Aqueous layer for well C: 0.037 (*R*)-1,2-hexanediol units of KRED 107 reporting enzyme (9.4 μL KRED 107 stock solution) 2.2 mM NADP^+ (5 μL stock solution) and 50 mM sodium pyrophosphate, pH 8.8 (75.6 μL).
4. Aqueous layer for well D: 0.037 (*R*)-1,2-hexanediol units of KRED 119 reporting enzyme (4 μL KRED 119 stock solution) 2.2 mM NADP^+ (5 μL stock solution) and 50 mM sodium pyrophosphate, pH 8.8 (81 μL).
5. Layer aqueous phase in microwell, subsequently prepare organic layer and use a syringe to deliver it to the bottom of well under the aqueous phase. The organic layers (next steps) are prepared and layered immediately thereafter to best synchronize start times for parallel reaction
6. Organic layer for wells A and B: 20 μL ($\pm$)-propylene oxide, 20 μL CHCl_3 and 0.25 mol% Co(III)-salen catalyst. Use a syringe to deliver a total of 20 μL into each reporting well [Replace with reaction conditions for a new reaction, if applicable]
7. Organic layer for wells B and C: 20 μL ($\pm$)-hexene oxide, 20 μL CHCl_3 and 0.25 mol % catalyst. Use a syringe to deliver a total of 20 μL into each reporting well [Replace with reaction conditions for a new reaction, if applicable]
8. Use initial rates taken from the first few minutes (see Figure 3B for an example) to predict % ee for each catalyst/substrate pair from the equation given in Basic Protocol 3.

COMMENTARY

Background information

As noted in the *Introduction*, both for the identification of fundamentally new transformations and for the optimization of existing reaction manifolds, screening techniques are invaluable. The same holds true for asymmetric induction; it is important to continue to explore chiral space in search of as yet unidentified “privileged chiral elements” (Karukurichi et al., 2015; Yoon and Jacobsen, 2003) for stereocontrolled synthesis. Such undertakings are inherently empirical; hence there has been a flurry of activity in the development of screening assays in addition to the ISES effort described herein.

Many of the more recent screens for chirality involve host-guest systems, often leading to pattern recognition, and giving rise to specific patterns that are either read spectrophotometrically or via circular dichroism (CD) or UV vis spectrometry to determine reaction selectivity. Here, the screening technology revolves around the development of a suitable array of sensors for each type of product analyte. There are many examples for each of these methods of detection useful for amines, alcohols, and carboxylates (Bentley et al., 2016a; Bentley et al., 2016b; De los Santos and Wolf, 2016; Jo et al., 2014; Leung and Anslyn, 2011; Leung et al., 2008; Leung et al., 2012; Mei and Wolf, 2006; Shabbir et al., 2009). These methods can often be carried out in 96-well plates leading to throughput comparable to the colorimetric ISES techniques described herein. Generality and throughput are advantages of these methods. However, such assays are generally not conducted *in situ*, but rather a product sample or crude reaction mixture is typically added to the screening solution (Collins et al., 2014).

The ISES method is part of an emerging class of screening approaches that use biology in service of chemistry. Specifically, biological macromolecules—antibodies, enzymes and nucleic acids—are deployed to glean information about relative reaction rate and enantioselectivity for an array of reactions. Recent advances in this field utilize DNA (Feagin et al., 2015; Kanan et al., 2004) and host-guest approaches, whereby enantiomers are differentiated by forming reporting complexes through hydrogen bonding (Jo et al., 2014; Mei and Wolf, 2006). The Feagin/Heemstra approach utilizes D- and L-DNA biosensors labeled with orthogonal fluorophores to determine ee. Utilizing a previously developed probe for L-tyrosinamide the authors were able to determine the % ee of mixtures of each enantiomer based on the fluorescence of the complementary fluorophores. This can be done with small aliquots taken from the reaction mixture. Liu cleverly employs DNA base-pairing to bring small quantities of reactants bound to DNA strands in close proximity to promote a reaction. If the reaction is successful the product is isolated and a coding region on the DNA strands is amplified to determine what two reactants produced the observed transformation. Recent reviews by Liu provide greater details and a sense of the scope of this technique (Chan et al., 2015; Kleiner et al., 2011).

Others have utilized the inherent chiral recognition properties of enzymes to report on reaction product chirality. Thus, Seto’s EMDee technique has been applied to the analysis of chiral alcohols (Abato and Seto, 2001), chiral esters (Onaran and Seto, 2003), and chiral sulfoxides (Sprout et al., 2005). This technique is performed post reaction, and so requires

the experimenter to work-up the reaction and analyze the reaction mixture in a separate enzymatic step. While this makes the screen more indirect, and inherently a time point assay, the isolation of the reaction product allows for greater control of the enzymatic reaction and a smaller uncertainty in product ee than the *in situ* method described here. The Moberg group also has exploited enzymes to estimate both extent of reaction and % ee for the TM-catalyzed formation of O-acetyl cyanohydrins (Hamberg et al., 2006). Both ADH and lipase enzymes are employed in this technique, the former to quantitate unreacted benzaldehyde to indicate extent of reaction; the latter to enantioselectively recognize product. The product recognition protocol here involves sequential enantioselective acetate ester cleavage (lipase), followed by cyanohydrin breakdown to cyanide and free benzaldehyde, with the latter being quantitated by ADH-mediated reduction (NAD(P)H cofactor).

Screens have also been developed that utilize antibodies raised against the product of interest (Matsushita et al., 2003; Taran et al., 2002). While such approaches require much more protein work on the front end, they can in principle be generalized for most reaction products. A recent review on immunoassay-based approaches to screening nicely summarizes the advances made in this area (Créminon and Taran, 2015). Highlights include the development of fluorescent antibodies that allow for the estimation of alkene geometry and/or product ee via fluorescence, particularly for styrenyl systems (Debler et al., 2008; Matsushita et al., 2003), as well as the optimization and discovery of biocompatible 'click-like' transformations (Kolodych et al., 2013).

As noted, the ISES protocol described herein is attractive because it is performed *in situ*, eliminating the need to interrupt the reaction by drawing aliquots, quenching and working up the reaction. Where ISES is applicable, multiple time points are obtained, *in situ*, by scanning multiple cuvettes or wells in parallel, quantitatively with a spectrophotometer or qualitatively with the naked eye. This aids in combinatorial reaction screening as various reaction conditions can be easily screened once the ISES protocol is optimized for the desired substrate. In addition, only readily available instruments are required for the technique.

Critical Parameters

ISES does not allow determination of kinetic constants. It is conducted at early conversion time regimes whereby diffusing (by)product is present at concentrations below saturation for the reporting enzyme(s). There are too many variables to easily obtain precise information on kinetic constants. Rather the procedure is very good at ranking different potential catalytic combinations for the organic reaction of interest. Reliable rankings may be obtained for relative rate and sense and magnitude of enantioselection, depending upon the variant of ISES being employed. The most important parameter is probably reporting enzyme selection. There must be an appreciable rate for the product or byproduct to be analyzed. If performing a double-well experiment, the two reporting enzymes (dehydrogenases in our work so far) must have different selectivities for the (*R*)- and (*S*)-products. This information can be obtained by screening an array of enzymes against both the pure (*R*)- and (*S*)-product and observing the initial rates for these across a range of concentrations appropriate to the ISES experiment. This also allows the experimentalist to

establish the K_m values for each reporting enzyme, for each antipode of product. Dehydrogenases can be obtained from traditional commercial suppliers such as Sigma-Aldrich, but other companies sell kits of these enzymes (Codexis, Almac and others). They can also be obtained by purification from native sources, by heterologous expression, most commonly in *E. coli* (Applegate et al., 2011; Friest et al., 2010) or by directed evolution.

Troubleshooting

The reaction of interest must also be ISES-applicable. For the current biphasic ISES configuration, the organic reaction must be tolerant of some water diffusing into the organic layer, or utilize water as a reactant as in the HKR. A general guide to troubleshooting is presented in Table 1. For example, the product must diffuse into the aqueous reporting phase readily enough to obtain useful initial rate information. Adjusting the solvent composition may facilitate product diffusion and can also limit the rate of water diffusion into the organic layer (Berkowitz et al., 2002). Proper solvent choice also limits precipitation at the organic/aqueous interface, a significant issue when an upper organic layer is used. In the first iteration of ISES, it was found that addition of 25% toluene helps to hold the upper organic layer exhibiting better surface tension than hexane/THF alone. The inclusion of 25% hexane, on the other hand, makes for more efficient diffusion of the alcohol byproduct. Using a lower organic phase (DCM or chloroform) limits the exposure to air if performing an air-sensitive reaction and prevents any precipitates in the organic layer from passing through the light beam) causing a false positive due to light scattering. If an air-free reaction screen is desired the experimenter should also note that the colorimetric version of ISES requires oxygen for the signaling cascade and would likely not be a good choice for a reporting mechanism compared to a dehydrogenase-based screen (i.e. detection of NADH) for which the enzymatic chemistry can proceed efficiently in the absence of oxygen. If using an upper organic phase it is possible to exclude air by purging with an inert gas, and sealing the cuvette with an appropriate septum thereafter.

Anticipated Results

Successful reactions should have a positive slope that is easily read by a UV-vis spectrophotometer. Sensitivity can be increased or decreased as needed by increasing catalyst loading, or uniformly scaling up reporting enzyme loading in the aqueous layer. For stereochemistry-predicting methods, the *R/S*-selectivity parameters for the two reporting enzymes chosen should be significantly different, though the enzymes need not display opposite enantiopreferences for each enantiomer. All of this can be examined *a priori*, if desired, by performing model ISES experiments by making small infusions of product itself into the organic layer, in place of the reaction of interest, and recording the enzymatic readout. While we have provided examples of three specific types of reactions herein; namely (i) TM-mediated intramolecular allylic amination reactions (Tsuji-Trost type chemistry); (ii) Co(III)-salen mediated HKR of terminal epoxides and (iii) TM-mediated (pseudo)halometallation/carbocyclization transformations, many more reaction types will surely prove amenable to ISES screening.

Time Considerations

Typically an ISES screen can be performed on the time scale of one day to one week if new reporting enzymes are not needed, in the absence of robotics, and depending upon throughput desired. Finding appropriate reporting enzymes can be the bottleneck; thus far alcohol dehydrogenase (sometimes coupled with aldehyde dehydrogenase) and alcohol oxidase (coupled with peroxidase) enzymes have been employed as reporting enzymes. The former are particularly convenient reporters for double-well techniques, as libraries of these are commercially available. For such techniques, significant effort is required on the front end to mine such ADH arrays for appropriate reporting pairs for a chiral product of interest. Once this is set, a large number of catalytic combinations may be screened; experiments have shown that useful information can be obtained at early conversions, with time points over the 5 to 20 min normally sufficing for the ISES screening window.

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LITERATURE CITED

- Abato P, Seto CT. EMDee: an enzymatic method for determining enantiomeric excess. *J Am Chem Soc.* 2001; 123:9206–9207. [PubMed: 11552847]
- Applegate GA, Cheloha RW, Nelson DL, Berkowitz DB. A new dehydrogenase from *Clostridium acetobutylicum* for asymmetric synthesis: Dynamic reductive kinetic resolution entry into the Taxotère side chain. *Chem Commun.* 2011; 47:2420–2422.
- Bentley KW, Proano D, Wolf C. Chirality imprinting and direct asymmetric reaction screening using a stereodynamic Bronsted/Lewis acid receptor. *Nat Commun.* 2016a; 7:12539. [PubMed: 27549926]
- Bentley KW, Zhang P, Wolf C. Miniature high-throughput chemosensing of yield, ee, and absolute configuration from crude reaction mixtures. *Sci Adv.* 2016b; 2:e1501162. [PubMed: 26933684]
- Berkowitz DB, Bose M, Choi S. In situ enzymatic screening (ISES): a tool for catalyst discovery and reaction development. *Angew Chem Int Ed.* 2002; 41:1603–1607.
- Berkowitz DB, Jahng WJ, Pedersen ML. alpha-Vinyllysine and alpha-vinylarginine are time-dependent inhibitors of their cognate decarboxylases. *Bioorg Med Chem Lett.* 1996a; 6:2151–2156. [PubMed: 29123334]
- Berkowitz DB, Maiti G. Following an ISES lead: the first examples of asymmetric Ni(0)-mediated allylic amination. *Org Lett.* 2004; 6:2661–2664. [PubMed: 15281738]
- Berkowitz DB, McFadden JM, Chisowa E, Semerad CL. Organoselenium-based entry into versatile, alpha-(2-tributylstannyl)vinyl amino acids in scalemic form: A new route to vinyl stannanes. *J Am Chem Soc.* 2000; 122:11031–11032.
- Berkowitz DB, Pedersen ML, Jahng WJ. Synthesis of higher alpha-chlorovinyl and alpha-bromovinyl amino acids: the amino protecting group determines the reaction course. *Tetrahedron Lett.* 1996b; 37:4309–4312. [PubMed: 29200438]
- Berkowitz DB, Pumphrey JA, Shen Q. Enantiomerically enriched alpha-vinyl amino acids via lipase-mediated reverse transesterification. *Tetrahedron Lett.* 1994; 35:8743–8746.
- Berkowitz DB, Shen W, Maiti G. In situ enzymatic screening (ISES) of P, N-ligands for Ni(0)-mediated asymmetric intramolecular allylic amination. *Tetrahedron: Asymmetry.* 2004; 15:2845–2851.
- Berkowitz DB, Smith MK. A convenient synthesis of L-alpha-vinylglycine from L-homoserine lactone. *Synthesis.* 1996:39–41.

- Bertozzi CR, Chang CJ, Chattopadhyay A, Stockwell BR, Schultz C, Butcher RA, Kubicek S, Silver PA, Park SB, Gestwicki JE, Shoichet BK, Dickinson BC, Chiosis G, Flitsch SL, He C, Oddershede LB, Zhang J, Moroni A, Thiel G, Liu DR, Chang YT, Christopoulos A, Ohnishi Y, Waldmann H, Ehrlich H, Weerapana E, Williams AH, Bogoy M, Chen X, Prescher JA. Voices of chemical biology. *Nat Chem Biol*. 2015; 11:378–379. [PubMed: 25978987]
- Buitrago Santanilla A, Regalado EL, Pereira T, Shevlin M, Bateman K, Campeau LC, Schneeweis J, Berritt S, Shi ZC, Nantermet P, Liu Y, Helmy R, Welch CJ, Vachal P, Davies IW, Cernak T, Dreher SD. Nanomole-scale high-throughput chemistry for the synthesis of complex molecules. *Science*. 2015; 347:49–53. [PubMed: 25554781]
- Chan AI, McGregor LM, Liu DR. Novel selection methods for DNA-encoded chemical libraries. *Curr Opin Chem Biol*. 2015; 26:55–61. [PubMed: 25723146]
- Collins KD, Gensch T, Glorius F. Contemporary screening approaches to reaction discovery and development. *Nat Chem*. 2014; 6:859–871. [PubMed: 25242480]
- Copeland GT, Miller SJ. A chemosensor-based approach to catalyst discovery in solution and on solid support. *J Am Chem Soc*. 1999; 121:4306–4307.
- Créminon C, Taran F. Enzyme immunoassays as screening tools for catalysts and reaction discovery. *Chem Commun*. 2015; 51:7996–8009.
- De los Santos ZA, Wolf C. Chiroptical asymmetric reaction screening via multicomponent self-assembly. *J Am Chem Soc*. 2016; 138:13517–13520.
- Debler EW, Kaufmann GF, Meijler MM, Heine A, Mee JM, Pljevalj i G, Di Bilio AJ, Schultz PG, Millar DP, Janda KD. Deeply inverted electron-hole recombination in a luminescent antibody-stilbene complex. *Science*. 2008; 319:1232–1235. [PubMed: 18309081]
- Dey S, Karukurichi KR, Shen W, Berkowitz DB. Double-cuvette ISES: In situ estimation of enantioselectivity and relative rate for catalyst screening. *J Am Chem Soc*. 2005; 127:8610–8611. [PubMed: 15954763]
- Dey S, Powell DR, Hu C, Berkowitz DB. “Cassette” in situ enzymatic screening identifies complementary chiral scaffolds for hydrolytic kinetic resolution across a range of epoxides. *Angew Chem Int Ed*. 2007; 46:7010–7014.
- Ebner C, Muller CA, Markert C, Pfaltz A. Determining the enantioselectivity of chiral catalysts by mass spectrometric screening of their racemic forms. *J Am Chem Soc*. 2011; 133:4710–4713. [PubMed: 21401128]
- Feagin TA, Olsen DP, Headman ZC, Heemstra JM. High-throughput enantiopurity analysis using enantiomeric DNA-based sensors. *J Am Chem Soc*. 2015; 137:4198–4206. [PubMed: 25747268]
- Finn MG. Emerging methods for the rapid determination of enantiomeric excess. *Chirality*. 2002; 14:534–540. [PubMed: 12112324]
- Ford DD, Nielsen LPC, Zuend SJ, Jacobsen EN. Mechanistic basis for high stereoselectivity and broad substrate scope in the (salen)Co(III)-catalyzed hydrolytic kinetic resolution. *J Am Chem Soc*. 2013; 135:15595–15608. [PubMed: 24041239]
- Friest JA, Broussy S, Chung WJ, Berkowitz DB. Combinatorial catalysis employing a visible enzymatic beacon in real time: Synthetically versatile (pseudo)halometalation/carbocyclizations. *Angew Chem Int Ed*. 2011; 50:8895–8899.
- Friest JA, Maezato Y, Broussy S, Blum P, Berkowitz DB. Use of a robust dehydrogenase from an archaeal hyperthermophile in asymmetric catalysis-Dynamic reductive kinetic resolution entry into (*S*)-Profens. *J Am Chem Soc*. 2010; 132:5930–5931. [PubMed: 20377222]
- Ginotra SK, Friest JA, Berkowitz DB. Halocarbocyclization entry into the oxabicyclo[4.3.1]decyl exomethylene- δ -Lactone cores of Linearifolin and Zaluzanin A: exploiting combinatorial catalysis. *Org Lett*. 2012; 14:968–971. [PubMed: 22316136]
- Goodell JR, McMullen JP, Zaborenko N, Maloney JR, Ho CX, Jensen KF, Porco JA, Beeler AB. Development of an automated microfluidic reaction platform for multidimensional screening: Reaction discovery employing bicyclo[3.2.1]octanoid scaffolds. *J Org Chem*. 2009; 74:6169–6180. [PubMed: 20560568]
- Hamberg A, Lundgren S, Penhoat M, Moberg C, Hult K. High-throughput enzymatic method for enantiomeric excess determination of O-acetylated cyanohydrins. *J Am Chem Soc*. 2006; 128:2234–2235. [PubMed: 16478176]

- Isenegger PG, Baechle F, Pfaltz A. Asymmetric Morita-Baylis-Hillman reaction: catalyst development and mechanistic insights based on mass spectrometric back-reaction screening. *Chem Eur J*. 2016; 22:17595–17599. [PubMed: 27775188]
- Jo HH, Lin CY, Anslyn EV. Rapid optical methods for enantiomeric excess analysis: From enantioselective indicator displacement assays to exciton-coupled circular dichroism. *Acc Chem Res*. 2014; 47:2212–2221. [PubMed: 24892802]
- Jung E, Kim S, Kim Y, Seo SH, Lee SS, Han MS, Lee S. A colorimetric high-throughput screening method for palladium-catalyzed coupling reactions of aryl iodides using a gold nanoparticle-based iodide-selective probe. *Angew Chem Int Ed*. 2011; 50:4386–4389. S4386/4381–S4386/4316.
- Kanan MW, Rozenman MM, Sakurai K, Snyder TM, Liu DR. Reaction discovery enabled by DNA-templated synthesis and in vitro selection. *Nature*. 2004; 431:545–549. [PubMed: 15457254]
- Karukurichi KR, De la Salud-Bea R, Jahng WJ, Berkowitz DB. Examination of the new α -(2'-Z-fluoro)vinyl trigger with lysine decarboxylase: The absolute stereochemistry dictates the reaction course. *J Am Chem Soc*. 2007; 129:258–259. [PubMed: 17212389]
- Karukurichi KR, Fei X, Swyka RA, Broussy S, Shen W, Dey S, Roy SK, Berkowitz DB. Mini-ISES identifies promising carbafructopyranose-based salens for asymmetric catalysis: Tuning ligand shape via the anomeric effect. *Sci Adv*. 2015; 1:e1500066. [PubMed: 26501130]
- Kleiner RE, Dumelin CE, Liu DR. Small-molecule discovery from DNA-encoded chemical libraries. *Chem Soc Rev*. 2011; 40:5707–5717. [PubMed: 21674077]
- Kolodych S, Rasolofonjatovo E, Chaumontet M, Nevers MC, Crminon C, Taran F. Discovery of chemoselective and biocompatible reactions using a high-throughput immunoassay screening. *Angew Chem Int Ed*. 2013; 52:12056–12060.
- Leung D, Anslyn EV. Rapid determination of enantiomeric excess of α -chiral cyclohexanones using circular dichroism spectroscopy. *Org Lett*. 2011; 13:2298–2301. [PubMed: 21486023]
- Leung D, Folmer-Andersen JF, Lynch VM, Anslyn EV. Using enantioselective indicator displacement assays to determine the enantiomeric excess of α -amino acids. *J Am Chem Soc*. 2008; 130:12318–12327. [PubMed: 18714996]
- Leung D, Kang SO, Anslyn EV. Rapid determination of enantiomeric excess: a focus on optical approaches. *Chem Soc Rev*. 2012; 41:448–479. [PubMed: 21892514]
- Lichtor PA, Miller SJ. One-bead-one-catalyst approach to aspartic acid-based oxidation catalyst discovery. *ACS Comb Sci*. 2011; 13:321–326. [PubMed: 21417485]
- Lin CY, Giuliano MW, Ellis BD, Miller SJ, Anslyn EV. From substituent effects to applications: enhancing the optical response of a four-component assembly for reporting ee values. *Chem Sci*. 2016; 7:4085–4090. [PubMed: 27904740]
- Loch JA, Crabtree RH. Rapid screening and combinatorial methods in homogeneous organometallic catalysis. *Pure Appl Chem*. 2001; 73:119–128.
- Loskyl J, Stoeve K, Maier WF. Infrared thermography as a high-throughput tool in catalysis research. *ACS Comb Sci*. 2012; 14:295–303. [PubMed: 22432458]
- Matsushita M, Yoshida K, Yamamoto N, Wirsching P, Lerner RA, Janda KD. High-throughput screening by using a blue-fluorescent antibody sensor. *Angew Chem Int Ed*. 2003; 42:5984–5987.
- McNally A, Prier CK, MacMillan DWC. Discovery of an α -amino C-H arylation reaction using the strategy of accelerated serendipity. *Science*. 2011; 334:1114–1117. [PubMed: 22116882]
- Mei X, Wolf C. Determination of enantiomeric excess and concentration of unprotected amino acids, amines, amino alcohols, and carboxylic acids by competitive binding assays with a chiral scandium complex. *J Am Chem Soc*. 2006; 128:13326–13327. [PubMed: 17031923]
- Montavon TJ, Li J, Cabrera-Pardo JR, Mrksich M, Kozmin SA. Three-component reaction discovery enabled by mass spectrometry of self-assembled monolayers. *Nat Chem*. 2012; 4:45–51.
- Mueller CA, Markert C, Teichert AM, Pfaltz A. Mass spectrometric screening of chiral catalysts and catalyst mixtures. *Chem Commun*. 2009:1607–1618.
- Onaran MB, Seto CT. Using a lipase as a high-throughput screening method for measuring the enantiomeric excess of allylic acetates. *J Org Chem*. 2003; 68:8136–8141. [PubMed: 14535795]
- Pfaltz A. Design and screening of chiral catalysts. *Chimia*. 2010; 64:860–862.

- Reetz MT. New methods for the high-throughput screening of enantioselective catalysts and biocatalysts. *Angew Chem Int Ed.* 2002; 41:1335–1338.
- Reetz MT, Becker MH, Kuhling KM, Holzwarth A. Time-resolved IR-thermographic detection and screening of enantioselectivity in catalytic reactions. *Angew Chem Int Ed.* 1998; 37:2647–2650.
- Reetz MT, Becker MH, Liebl M, Fürstner A. IR-thermographic screening of thermoneutral or endothermic transformations: the ring-closing olefin metathesis reaction. *Angew Chem Int Ed.* 2000; 39:1236–1239.
- Robbins DW, Hartwig JF. A simple, multidimensional approach to high-throughput discovery of catalytic reactions. *Science.* 2011; 333:1423–1427. [PubMed: 21903809]
- Schaus SE, Brandes BD, Larrow JF, Tokunaga M, Hansen KB, Gould AE, Furrow ME, Jacobsen EN. Highly selective hydrolytic kinetic resolution of terminal epoxides catalyzed by chiral (salen)Co(III) complexes. Practical synthesis of enantioenriched terminal epoxides and 1,2-diols. *J Am Chem Soc.* 2002; 124:1307–1315. [PubMed: 11841300]
- Shabbir SH, Joyce LA, da Cruz GM, Lynch VM, Sorey S, Anslyn EV. Pattern-based recognition for the rapid determination of identity, concentration, and enantiomeric excess of subtly different *threo*-diols. *J Am Chem Soc.* 2009; 131:13125–13131. [PubMed: 19691315]
- Sprout CM, Richmond ML, Seto CT. A positional scanning approach to the discovery of dipeptide-based catalysts for the enantioselective addition of vinylzinc reagents to aldehydes. *J Org Chem.* 2005; 70:7408–7417. [PubMed: 16122266]
- Stambuli JP, Hartwig JF. Recent advances in the discovery of organometallic catalysts using high-throughput screening assays. *Curr Opin Chem Biol.* 2003; 7:420–426. [PubMed: 12826131]
- Taran F, Gauchet C, Mohar B, Meunier S, Valleix A, Renard PY, Créminon C, Grassi J, Wagner A, Mioskowski C. High-throughput screening of enantioselective catalysts by immunoassay. *Angew Chem Int Ed.* 2002; 41:124–127.
- Taylor SJ, Morken JP. Thermographic selection of effective catalysts from an encoded polymer-bound library. *Science.* 1998; 280:267–270. [PubMed: 9535652]
- Treece JL, Goodell JR, Vander Velde D, Porco JA Jr, Aube J. Reaction discovery using microfluidic-based multidimensional screening of polycyclic iminium ethers. *J Org Chem.* 2010; 75:2028–2038. [PubMed: 20163127]
- Trost BM, Bunt RC, Lemoine RC, Calkins TL. Dynamic kinetic asymmetric transformation of diene monoepoxides: a practical asymmetric synthesis of vinylglycinol, vigabatrin, and ethambutol. *J Am Chem Soc.* 2000; 122:5968–5976.
- Yoon TP, Jacobsen EN. Privileged chiral catalysts. *Science.* 2003; 299:1691–1693. [PubMed: 12637734]

Catalyst	Slope [mAbs/min]
$\text{Ni(cod)}_2/4\text{PPh}_3$	58
$\text{Mo(CO)}_3(\text{C}_7\text{H}_8)/3\text{PPh}_3$ (50 mol%)	6.8
$\text{Mo(CO)}_3(\text{C}_7\text{H}_8)/4\text{PPh}_3$	0.6
$\text{Pt(PPh}_3)_4$	2.5
$\text{RhCl(PPh}_3)_3$	1.5
$\text{Co(Cl)}_2/4\text{PPh}_3$	0.7

Figure 1.

(A) Schematic of ISES for a transition metal-mediated intramolecular allylic amination reaction leading to a protected vinylglycinol product. (B) Cuvette array for the transition metal-ligand combinations being screened. (C) UV/vis spectral scans of the aqueous reporting layer (Ni cuvette). (D) UV traces (@ 340 nm) for the cuvette array shown in panel B. (E) Linear least squares fit rates of NADH formation for the catalytic combinations being sampled here. Elements of this figure were adapted with permission from *Angew. Chem. Int. Ed.* 2002, 41: 1603–1607. Copyright Wiley-VCH Verlag.

Substrates Screened

(Pseudo)halides Screened

LiF , LiCl , LiBr , LiCN , LiOCN , LiSCN

64 metal complexes
x 6 (pseudo)halides
x 3 substrates (1152 combinations)

Figure 2.

(A) Schematic of a colorimetric *in situ* enzymatic screen for new (pseudo)halometallation/carbocyclization transformations. (B) Actual 96-well array of reactions being screened for substrate **1** across the 16 transition metal complexes $\times$ 6 (pseudo)halides listed. (C) The structure of the three substrates screened for the $64 \times 6 \times 3 = 1152$ combinatorial array in this case. (D) The UV/vis spectral signature of the ABTS radical cation, as generated in the peroxidase reporting reaction. Adapted with permission from *Angew. Chem. Int. Ed.* 2011, 50: 8895–8899. Copyright Wiley-VCH Verlag.

Figure 3.

(A) Schematic of double well-ISES for two different substrates as performed in mini-ISES format. Note that for each substrate screened, two distinct reporting enzymes are employed, with different enantioselectivities for the organic reaction product. (B) Actual UV/vis traces from the four reporting enzymes for this Co(III)-salen HKR-catalyst candidate [KRED 23: (*S*)-selective-1,2-propanediol; TBADH: (*R*)-selective-1,2-propanediol; KRED 107: (*S*)-selective-1,2-hexanediol; KRED 119: (*R*)-selective-1,2-hexanediol] (C) Photograph of the completely loaded micromulticell (16 wells, each @ 20 μ L lower organic layer, 90 μ L upper aqueous enzymatic reporting layer).

Table 1

Troubleshooting Commonly Encountered Problems

Problem	Possible Cause	Solution
False positive due to light scattering	Precipitation at the organic/aqueous interface	Add a small percentage of a solubilizing agent (10% toluene, for example) Invert the layers; add a significant percent of a chlorinated co-solvent so that the organic layer is the lower layer
No signal observed	Light beam is not passing through the aqueous reporting layer	Reposition the organic/aqueous interface appropriately by adjusting the total volumes of each layer
UV/vis signal too weak	Insufficient catalyst to obtain signal in a 5–20 min window Insufficient loading of reporting enzyme(s)	Increase catalyst loading Increase reporting enzyme loading, by the same factor across all reporting wells/cuvettes
False negative due to failure of the product/byproduct to diffuse into the reporting layer	Product/byproduct does not sufficiently partition into the aqueous layer	Add non-polar cosolvent (e.g. hexane) to the organic layer to increase partitioning in the aqueous layer Reduce buffer salt concentration
Organic reaction oxygen-sensitive; fails under initial ISES conditions screened	Oxidation of a low valent transition metal catalyst or air-sensitive substrate	Purge cuvettes with Ar and cover cuvettes with septa
Organic reaction may be water-sensitive; inhibited by water diffusing into the organic layer	Catalytic complex may be hydrolyzed or chemically transformed by water or hydrolysis byproducts may be appearing	Check water concentration diffusing into the organic layer with time by means of Karl Fischer titration [see SI from (Berkowitz et al., 2002)]. Increase buffer salt concentration and/or decrease organic solvent polarity (include significant fraction of hexane or toluene, for example)