High-Throughput Assay for Enantiomeric Excess Determination of chiral Diols, Amino Alcohols and Amines and Direct Asymmetric Reaction Screening

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Determination of enantiomeric excess (*ee*) in chiral compounds is a key step in the development of chiral catalyst auxiliaries and chiral drugs. Here, we describe a sensitive and robust fluorescence-based assay for the determination of *ee* in mixtures of enantiomers of 1,2- and 1,3-diols, chiral amines, amino alcohols, and amino acid-esters. The method is based on the dynamic self-assembly of commercially available chiral amines, 2-formylphenylboronic acid, and chiral diols in acetonitrile to form diastereomeric fluorescent complexes. Each analyte enantiomer gives rise to a different diastereomer with a distinct fluorescence wavelength and intensity originating from the enantiopure fluorescent ligands. In this assay, enantiomers of amines and amine derivatives assemble with diol-type ligands containing bi-naphthol moiety (BINOL and VANOL), while diol enantiomers form complex with the enantiopure amine-type fluorescent ligand tryptophanol. This differential fluorescence can be utilized to determine the amount of each enantiomer in the mixture with errors below 1% *ee*. This method allows for the real-time evaluation of enantiomeric/diastereomeric excess (*ee*/*de*) and product yield of crude asymmetric reaction products in a high-throughput fashion. The only processes involved in

this protocol are high-throughput liquid dispensing of three components into 384-well plates and recording the fluorescence using automatic plate reader. Emergence of such high throughput approaches allows scaling up the screening of combinatorial libraries, and together with parallel synthesis creates a robust platform for discovering chiral catalysts or auxiliaries for asymmetric transformations and chiral drug development. The procedure takes 3-4 hours to run and requires 10–20 ng of the substrate per well. Our fluorescence-based assay offers distinct advantages over existing traditional methods as it is not sensitive to the presence of common additives or impurities as well as unreacted/incomplete utilized reagents and catalysts.

INTRODUCTION

Separation of enantiomers has become a significant research area in analytical chemistry owing to its impact on chemical, pharmaceutical, industries, and biotechnology. Traditional methods for determination of enantiomeric purity such as ^{1}H- and ^{19}F- NMR spectroscopy,[1–4] circular dichroism (CD),[5–7] and chiral separation techniques such as chiral-phase high performance liquid chromatography (HPLC),[8] HPLC coupled with circular dichroism (HPLC-CD),[9–11] or chiral gas chromatography (GC)[12,13] and capillary electrophoresis (CE),[14] are the reliable standard methods that are most used for the separation and determination of enantiomers.[14] However, these techniques are better suited for serial analyses. This is in contrast to the needs of combinatorial chemistry and parallel synthesis which require samples to be simultaneously analyzed in a real-time on a large scale, which require high-throughput platform for analysis.[15–17]

Kubo *et al.* were among the pioneers in the development of enantioselective optical sensors for *ee*; in their initial work, they described screening of amines using derivatized calixarenes in ethanol.[18,19] Later a number of research groups, such as Anslyn *et al*,[20–23] Wolf *et al*,[24–27] James *et al*,[28–30] among other groups have developed high-throughput screening (HTS) methods for real-time analysis of enantiomers, contributing to the development and optimization efforts in the discovery of efficient catalysts and conditions for asymmetric reactions.[14]

Our research team has developed a versatile fluorescence-based assay for determination of *ee* in mixtures of enantiomers of 1,2- and 1,3-diols, amines, amino acid esters, and amino alcohols (Figure 1).[31–33] The method is based on dynamic self-assembly of commercially available, enantiopure, yet inexpensive fluorescent ligands, linked by 2-formylphenylboronic acid, with chiral analytes to form diastereomeric complexes (Figure 2). Specifically, fluorescent (*S*)-(+)-1,1′-bi(2-naphthol) (BINOL) and (*S*)-3,3′-diphenyl-2,2′-bi(1-naphthol)(VANOL) ligands were utilized as a reporters with amine-type analytes, and enantiopure L-tryptophanol was used in a complex assembly for 1,2 and 1,3 chiral diol analytes. Each analyte enantiomer self-assembles through the 2-formylphenylboronic acid linker with the associated fluorescence reporter and creates a highly geometry-sensitive diastereomeric complex with unique fluorescence signatures. In case of diol and amino alcohol enantiomers, their diastereomeric complexes can be tracked by not only different fluorescence intensity (“turn-on” mode), but also by different shift of fluorescence maxima.

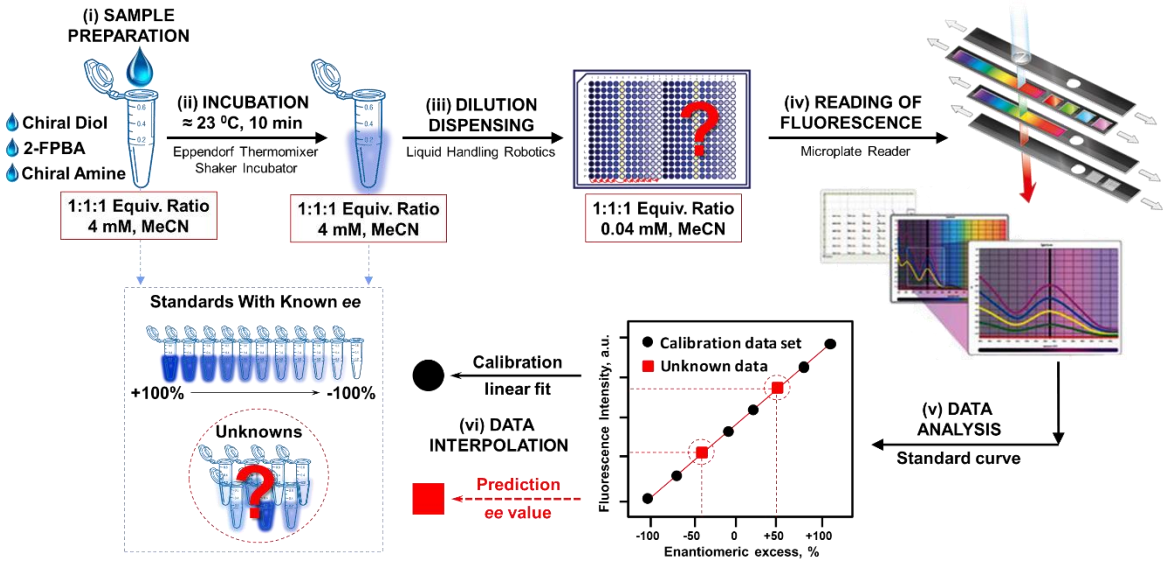

Figure 1. Schematic of the enantiomeric excess determination process and experimental workflow. All components of the self-assembly are commercially available and are used without further purification. (i) Three-component derivatization reaction between 1 equiv. of the chiral diol, 1 equiv. of 2-formylphenylboronic acid, 1 equiv. of chiral amine (ii) results in quantitative complexation that yields a mixture of structurally rigid fluorescent diastereomeric imino/oxazolidine-boronate esters. (iii) The assembly fluorescence is clearly observable upon 100 times dilution in the microplate well. (iv) The adaptation of the three-component self-assembly derivatization to a 384-well microplate format and use of a plate-reader to detect the fluorescence emission, allows to minimize volume and molar amount analyte required for a measurable fluorescence signal. (v) The fluorescence response pattern is acquired in a form of X *vs* Y data matrix. The linear/nonlinear regression enables finding the coefficients of polynomial equation that are a best linear fit to a set of X,Y data corresponding to *ee* and FI of the standards. (vi) Interpolation of a standard curve enables to simultaneously predict *ee* values (X) for measured FI (Y) of multiple unknowns.

In this protocol a signal output is used to determine the enantiomeric excess (*ee*) in the mixture with the errors below 1% *ee* (Figure 3, Procedure 1). This method can be extended to determining enantiomeric/diastereomeric excess (*ee*/*de*) and product concentration (yield) of chiral products in real time using a high-throughput platform (Procedure 2). Moreover, selection of different diol-type ligands allows for use of the same method for determination of *ee* in the mixtures of chiral

amines, amino alcohols, amino acid-esters, and amine-type ligands for determination of enantiomeric purity in the mixtures of chiral diols and sugars. The only techniques involved in the method are high-throughput liquid dispensing of three components into 384-well plate and recording the fluorescence using an automatic plate reader. Our fluorescence-based assay offers some distinct advantages over existing traditional methods such as cHPLC or NMR since it is not sensitive to the presence of any common additives or impurities or to unreacted/incomplete utilized reagents and catalysts. Furthermore, the proposed method is uniquely suitable for high-throughput analysis using simple fluorescence readers and microplates and is applicable to combinatorial chemistry and parallel synthesis that require simultaneous analysis of a large number of samples.

Assay Principle

This protocol is an extension of the simple three-component chiral derivatization protocol for determining the enantiopurity of chiral primary amines[34,35], and diols[2,3] by ^{1}H NMR spectroscopic analysis that was described first by T. D. James and S. D. Bull. The method is based on condensation of the chiral amines with 2-formylphenylboronic acid (FPBA) and 1,2-1,3- and 1,4-diols.

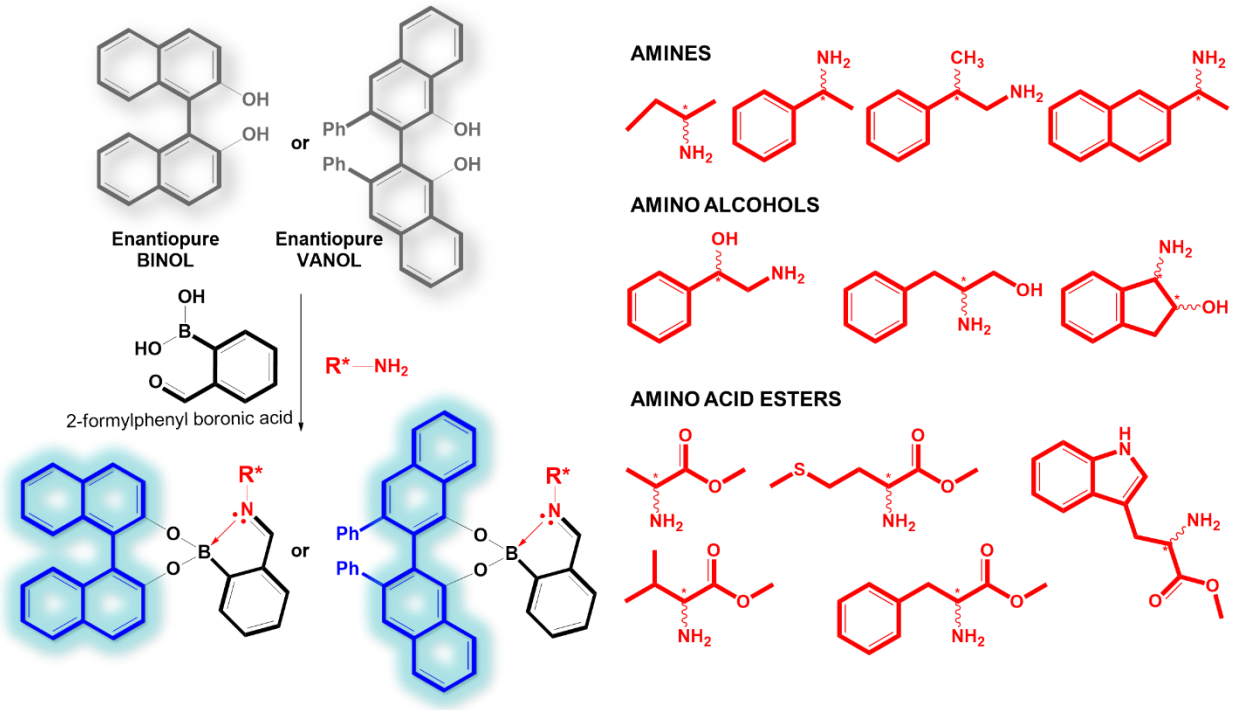

Figure 2. **Components and self-assembly mechanisms use for *ee* determination in chiral amines and amine-derivatives.** Left: Chiral derivatization protocol utilizing the self-assembly of chiral diol-type fluorophores BINOL and VANOL, with 2-FPBA, and a chiral amine or amine derivative.[31,32,40] Depending on the size and structure of the chiral amine/amine derivative, the interaction between the chiral ligand and amine, and an architecture of the stereocontrolled sites results in either recovery of partly quenched or further quenching of the fluorescence. Right: Range of chiral amines, amino alcohols, and amino acid esters successfully derivatized to afford fluorescent diastereomeric imino/oxazolidine-boronate esters that show highly variable enantiomer-induced fluorescence.

We developed a fluorescence-based three-component chiral derivatization protocol that utilizes the self-assembly of 1 equiv. of a chiral diol, with 1 equiv. of 2-formylphenylboronic acid (2-FPBA), and 1 equiv. of a chiral amine or amine derivative. The assembled product is either an iminoboronate with BINOL or VANOL diol ligands as the fluorophore (Figure 2) or an oxazolidine boronate ester (Figure 4) with L-tryptophanol amine ligand as the fluorophore. Each diastereomer has a distinct analyte-enantiomer induced fluorescence signal. This differential

fluorescence signal is driven by different post-assembly geometries of the possible diastereomers, which are also related to the stability of the diastereomeric complexes in acetonitrile. The more stable diastereomer shows greater fluorescence intensity change and typically favors fluorescence amplification as opposed to quenching of the opposite less stable diastereomer. The complex stability with amino alcohols results in considerable shift of fluorescence maxima due to formation of oxazolidine ring (Figure 4) and overall increase of structural rigidity. Such changes and further equilibrium are reflected by differential fluorescence which enable the determination of the nature, absolute configuration, and enantiomeric purity of the chiral analytes (see Procedure 1).

Due to the high association constant of this self-assembly, maximum fluorescence change is observed when a 1:1:1 stoichiometry of the three components is reached followed by saturation of fluorescence signal (plateau). All fluorescence titrations a show well-resolved horizontal plateau region without any signal fluctuation after reaching 1 molar equivalent of analyte and were tested up to 5 molar equivalents. The use of excess of analyte would not have any effect on the protocol accuracy and can be used to simplify reactions sampling if there is no intention to determine the yield.

In experiments to determine the effects of additives such as water, alcohols, and other compounds to the mixture showed that the additives destabilize the less stable diastereomer resulting in an a higher difference between the diastereomeric iminoboronates and therefore better discrimination between the enantiomers.[32]

The most straightforward method of obtaining absolute configuration and *ee* of chiral analytes from fluorescence intensity (FI) acquired with high-throughput assay is by utilizing a standard curve method with linear/nonlinear data fitting (Figure 3 and Figure 5).[36] The developed

protocol allows for the accurate quantitative determination of the *ee* of chiral amines, amino acid esters, amino alcohols, or diols with errors of just 1%.

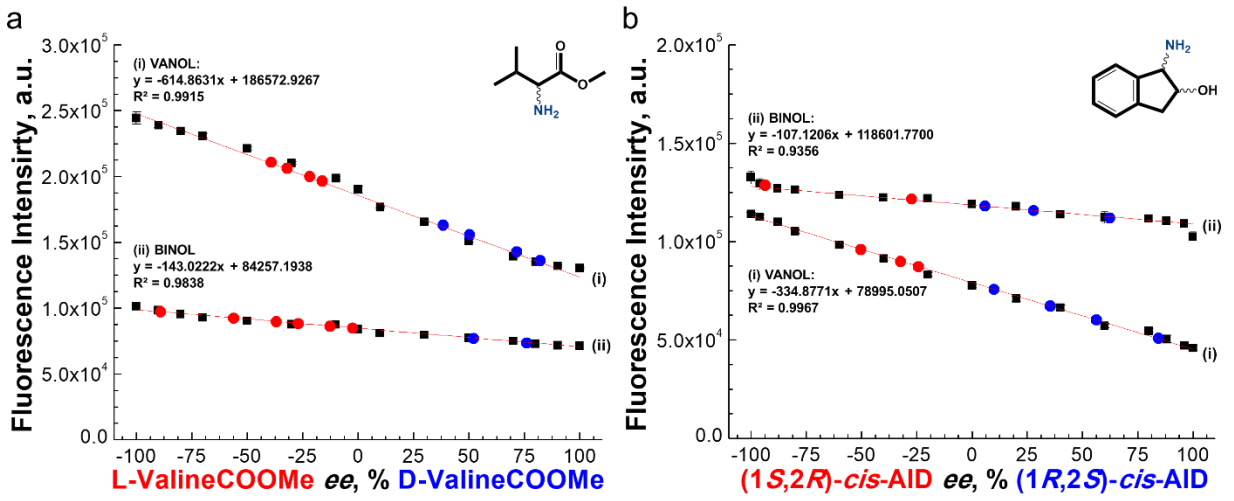

Figure 3. Standard curve from maximum fluorescence intensity of BINOL fluorescence at 355nm and VANOL at 375 nm. Monitoring enantiomeric purity of Valine methyl ester in **Figure 3a** and *cis*-1-amino-2-indanol (AID) **Figure 3b** using high-throughput fluorescence assay. All standards (black squares) and validation samples (red and blue circles) measured in a set of 24 repetitions. Student's T-test is performed in order to examine the difference (standard deviation) between data within the set of 24 repetitions for each sample and exclude 4 outlying data points. Each data point shown on a graph as an average value computed from 20 independent measurements (repetitions). Graphical representations of the variability of data shown on graphs as the error bars to indicate the standard deviation in the reported measurement. Due to coefficient of variation (CV) being below 4% for all performed experiments the error bar for most data points located within the corresponding mean point on the graph. Graphs of enantiomeric excess-induced fluorescence change are fitted with linear fit equation.

When the analyte concentration is unknown then an extended/training data set comprising range from -100 to +100 % *ee* should be prepared at several different total concentration of analyte for each receptor-premix (Figure 6, Procedure 2). All receptors (BINOL-FPBA, VANOL-FPBA, L-tryptophanol-FPBA, at 1:1 molar ratio) respond to both the

change in concentration and *ee* of the analyte of interest. This protocol may also be adapted for the simultaneous detection of yield, enantiomeric excess (*ee*), and diastereomeric excess (*de*) of crude chiral products using an artificial neural network (ANN) without the need of work-up and product isolation.[33] Fluorescence data for different concentrations and *ee* values of the analytes are collected in a 384-well plate (training plate) and the values obtained are used to train an ANN. The fluorescence data from unknowns/analytes are imported into a computer, analyzed with the trained ANN, and the values of *ee* and the total concentrations for the unknowns are exported. The comparison of the results obtained for crude and crystallized samples of Noyori asymmetric transfer hydrogenation of benzils shows that our method is not sensitive to traces of chiral catalysts or possible reaction additives and may be applied to the determination of *ee* in crude product mixtures. We have also tested sensitivity of the assay to the presence of meso-hydrobenzoin. It was found, that the method is not sensitive up to 7% of meso-form.

Assay for the Determination of Enantiomeric Purity of Amines, Amino Acid Esters, and Amino Alcohols

Differential amine enantiomers-induced fluorescence arises from the iminoboronates comprising 2-FPBA, the chiral amines under analysis, and different sized enantiopure fluorescent diol-type ligands (*S*)-(+)-1,1′-bi(2-naphthol) (BINOL) and (*S*)-3,3′-diphenyl-2,2′-bi(1-naphthol)(VANOL)[37–39] (Figure 2). Each analyte forms a complex with both BINOL and VANOL. The complexes formed between the chiral amine and BINOL are approximately equally stable. VANOL, however, is a lot bigger than BINOL and the steric effects mean that one of stereoisomers is going to be a lot more stable than the other. In choosing which fluorescent diol ligand to use, it is also worth bearing in mind that the size of the ligand also has an effect on the magnitude of fluorescence signal emitted.[31,32,40] Enantioselectivity for any potential chiral analyte can be

achieved by finding an appropriate size of enantiopure chiral receptor (Figure 3). Large variety of chiral fluorescent diols of different sizes are available for screening including, but not limited to (*S*)-3,3′-Di-9-anthracenyl-1,1′-bi-2-naphthol (CAS Number 361342-50-9), (*S*)-(–)-3-3′-Bis(3,5-bis(trifluoromethyl)phenyl)-1,1′-bi-2-naphthol (CAS Number 849939-13-5), (*S*)-(+)-3,3′-Bis(3,5-dimethylphenyl)-5,5′,6,6′,7,7′,8,8′-octahydro-1,1′-bi-2-naphthol, (*S*)-3,3′-Di-9-phenanthrenyl-1,1′-bi-2-naphthol, (*S*)-(–)-3,3′-Bis(triphenylsilyl)-1,1′-bi-2-naphthol. These commercially available chiral building blocks containing a 1,2- or 1,3-diol moiety can be bound to the 2-formylphenylboronic acid and form diastereomeric complexes with amine containing chiral analytes that have large optical differences for enantiomeric analytes, thereby allowing to make accurate assays. Such adaptive feature makes the protocols more amenable to adoption by other researchers.

User can find best suitable receptor and concentration conditions as preparatory work required before screening by the following procedure. These steps are quick because they involve well plate screening and lead to an analyte-specific assay:

1. By using traditional fluorescence titrations and standard binding isotherms or well plate screen, proper concentrations (recommended concentration is 40 μM) for combining the chiral fluorescent diol, 2-formylphenylboronic acid and chiral analyte must be determined and the 1:1:1 stoichiometry must be confirmed.

2. The chiral fluorescent receptors and enantiomers of chiral analytes are then screened in a well plate to reveal the best chiral receptor complex with largest differences in fluorescent signal for reporting the *ee* (Figure 7) of a chiral target diastereomers (largest Δ of diastereomers).There is substantial variation between the intensity/wavelength of fluorescence emitted by the different

diastereomeric boronate esters. Upon assembly with amines fluorescent ligands show a high degree of signal change compare to the free state (without being bound to 2-FPBA and amine-analyte) and high association constants ($K_a \approx 10^6$). The esters of amines and amino acids have a relatively low degree of fluorescence quenching, due to their small size and lower binding affinity ($K_a \approx 10^5$). Amino alcohols display ratiometric changes in fluorescence due to formation of oxazolidine boronate esters[41] which possess higher rigidity, compared to iminoboronates, and the introduction of another chiral center causes a shift and an amplification of the observed fluorescence signal.[32] All types of analytes show saturation of fluorescent ligand signal change at the 1:1:1 molar ratio. For our experiments we used an optimal concentration of 40 μM for fluorescent ligands BINOL/VANOL and the linker 2-FPBA. The quantity of chiral analyte can be simply determined by adding measured increments of it until the final concentration of 40 μM (or 1 molar equivalent) is reached followed by immediate saturation of fluorescence emitted by the bound ligand.

The formation of the iminoboronates requires dynamic covalent bond formation.[42] Destabilization of the formed diastereomeric iminoboronates improves the resolution of the enantiomers of amines, or amine derivatives. Addition of water <10 % or other common additives (carboxylic acids such as citric acid, or alcohols such as ethylene glycol) results in a change in the esterification equilibrium and destabilizes the formation of one of the two diastereomeric iminoboronates more than the other, effectively creating a significant difference in recognition of the amine enantiomers. Importantly, this method is not dramatically sensitive to potential impurities and can be applied to the analysis of chiral amines/amine derivatives in a complex environment including crude reaction products from asymmetric reactions.[33]

We performed the assay under ambient conditions using two diol-type fluorophores BINOL and VANOL (Figure 3, Supplementary Figure 1, Supplementary Figure 2). First, it is necessary to calibrate the system for up to the two extremes of *ee*-induced fluorescence by using mixtures of standards that range from -100% *ee* to +100% *ee*). Validation samples are prepared and measured in the same plate as the samples with unknown *ee*. The background fluorescence is generally 5-10% of the maximum fluorescence. The assay measures the total fluorescence as a function of enantiomeric composition (fluorescence intensity data for valine methyl ester and cis-1-amino-2-indanol are shown in Supplementary Data 1 and Supplementary Data 2 respectively). We obtained reliable results (Table1) using a set of 24 repetitions. In this context (Figure 3,a) a total of 23 independent Valine samples or observations including 15 different *ee* values (100, 90, 80, 70, 50, 30, 10, 0, -10, -30, -50, -70, -80, -90, -100, %ee) used for standard curve calibration and 8 unknown samples analyzed with BINOL-based system and 23 independent Valine observations including 15 different *ee* values (100, 90, 80, 70, 50, 30, 10, 0, -10, -30, -50, -70, -80, -90, -100, %*ee*) used for standard curve calibration and 8 unknown samples analyzed with VANOL-based system. Total number of observations is 46. For each sample, we also performed 24 technical replicates to prove the assay's reproducibility; and to minimize the effect of human error on the result. This means that a total amount of 1104 individual experiments are performed (46 observations X 24 technical repetitions). For our graphs we calculate the average between the technical replicates, standard deviation, and coefficient of variation.

For all tested analytes (Figure 2) (*S*)-BINOL gives a smaller magnitude of fluorescence change between diastereomers (the Δ of diastereomers) compared to the more fluorescent (*S*)-VANOL, therefore, the use of (*S*)-VANOL is recommended. We would recommend the use of (*S*)-VANOL for amines, amino alcohols, and amino acids because in most tested cases the use of

single VANOL-based system was enough to achieve sufficient discriminatory power of the assay. BINOL can be added as a second system to increase dimensionality of a data set and improve discriminatory potential of particular assay.

Table 1 The results of *ee* determination protocol with linear regression fitting function.

entry	BINOL				VANOL			
	Actual		Predicted		Actual		Predicted	
Valine	Abs. config.	*ee* (%)	Abs. config.	*ee* (%)	Abs. config.	*ee* (%)	Abs. config.	*ee* (%)
1	*R*	75	*R*	76	*R*	80	*R*	82
2	*R*	50	*R*	52	*R*	70	*R*	71
3	*rac*	0	*S*	-2	*R*	50	*R*	50
4	*S*	-15	*S*	-13	*R*	40	*R*	38
5	*S*	-25	*S*	-27	*S*	-15	*S*	-16
6	*S*	-35	*S*	-37	*S*	-20	*S*	-22
7	*S*	-55	*S*	-56	*S*	-30	*S*	-32
8	*S*	-90	*S*	-89	*S*	-40	*S*	-39
cis-AID	BINOL				VANOL			
9	*R*,*S*	60	*R*,*S*	62	*S*,*R*	-50	*S*,*R*	-50
10	*R*,*S*	25	*R*,*S*	28	*S*,*R*	-35	*S*,*R*	-32
11	*R*,*S*	5	*R*,*S*	6	*S*,*R*	-25	*S*,*R*	-24
12	*S*,*R*	-30	*S*,*R*	-27	*R*,*S*	10	*R*,*S*	10

13	*S*,*R*	-95	*S*,*R*	-94	*R*,*S*	35	*R*,*S*	35
14					*R*,*S*	55	*R*,*S*	56
15					*R*,*S*	85	*R*,*S*	84

Assay for the Determination of Enantiomeric Purity of 1,2 and 1,3-Diols

Stereodynamic self-assemblies for determination of the absolute configuration and enantiomeric composition of chiral 1,2- or 1,3-diol are described in the protocol (Figure 4). This approach utilizes a self-assembled oxazolidine ester formed as a product by dynamic covalent self-assembly of a chiral diol with an enantiopure fluorescent amino alcohol tryptophanol and 2-formylphenylboronic acid (Figure 4).[33] The tryptophanol possesses bright fluorescence and forms rigid oxazolidine boronate esters upon complexation with FPBA and a chiral diol. The resulting diastereomeric boronates display a distinctive enantiomer-induced fluorescence change allowing for the *ee* determination of molecules containing a 1,2- or 1,3-diol moiety.

Significant difference in the arrangement of the diastereomeric complexes is induced by the geometry/chirality of the diol and causes a dramatic change in the magnitude of the fluorescence signal. We evaluated the protocol using various enantiomeric diols clearly demonstrating that different enantiomers of diols always either quench or amplify the fluorescence albeit to different degrees (Figure 4).[33]

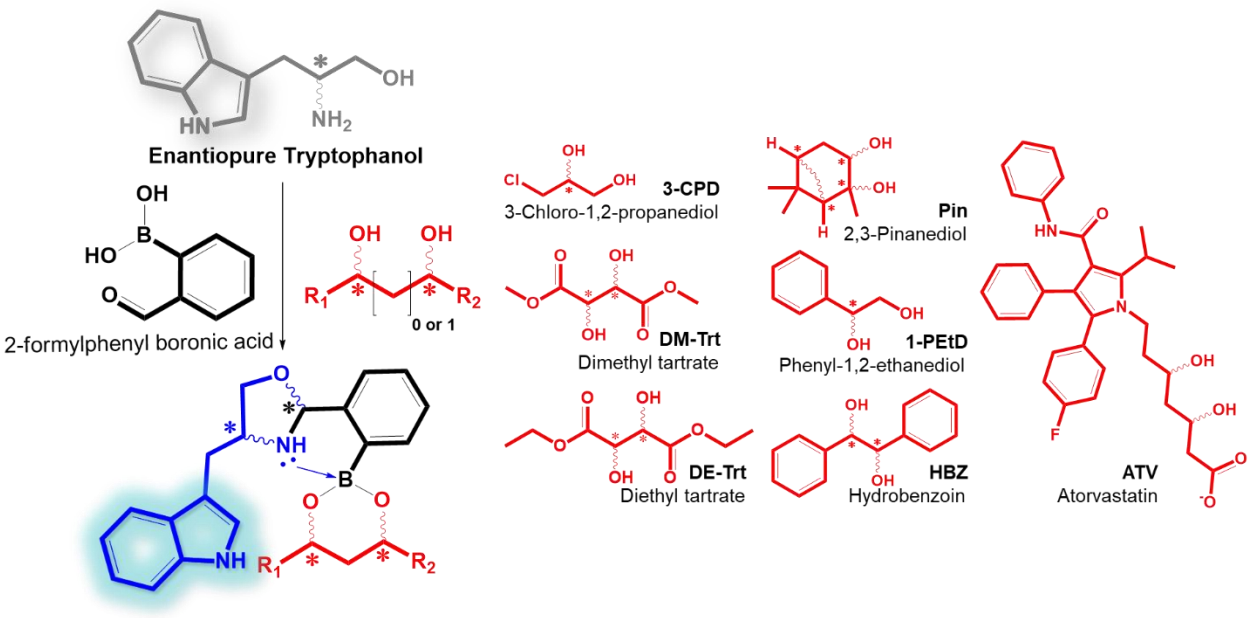

Figure 4. **Components and self-assembly mechanisms use for *ee* determination in chiral diols and sugars.** Left: The concept of dynamic covalent self-assembly for enantiomeric excess determination of chiral diols: 1,2- or 1,3-diols self-assemble with an enantiomerically pure fluorescent tryptophanol reporter and 2-formylphenylboronic acid. Right: Examples of chiral diols that display strong enantiomer-induced fluorescence signal upon formation of diastereomeric oxazolidine boronate esters.

We performed the protocol for the determination of enantiomeric purity of chiral diols by recording the fluorescence intensities utilizing a conventional microplate reader with total volume 120 µL in a 384-well density plate. The resulting standard curves are defined as a function of FI of L-tryptophanol (using just one channel: λ_{exc}=280 nm / λ_{em}=330) nm plotted on the Y-axis against various *ee* of standards along the X-axis (Figure 5).

The absolute configuration and *ee* of unknowns (i.e., the chiral analytes of interest or products of asymmetric reactions) were determined by interpolation of their fluorescence readings on the standard curve. Linear regression is well suited to fit the obtained standard curve with linear *ee*-dependency (See Supplementary Figure 3). To ensure reproducibility, the assay must be done

over a set of at least 3 technical replicates for each standard (mixtures with known *ee* composition used for standard curve calibration) and each unknown sample. Typical experiments include standards spanning -100 to +100% enantiomeric excess range to generate a standard curve. To maximize sensitivity, it is recommended to choose small *ee* step between standards (especially in the range of expected values as shown on Supplementary Figure 4). The number of points on the standard curve can be increased or decreased as desired, and as few as three points (-100% *ee*, *rac*-mix, +100% *ee*) can be used when the target asymmetric reaction or sample are well characterized (see fluorescence intensity data set for atorvastatin in Supplementary Data 3 and in Supplementary Data 4 for hydrobenzoin). If the concentration of analyte material is not known and must be determined, the extended protocol comprising set of standard curves of different total concentrations (Figure 6) must be applied and subjected to ANN for analysis. To ensure predictive ability of the generated models we always recommend to split created data sets for the "training data set" to create and calibrate the model and "validation data set" which would contain samples of known ee and concentration but used as an "artificial unknowns" and attest model as actual *vs* predictive values (See Supplementary Data 4).

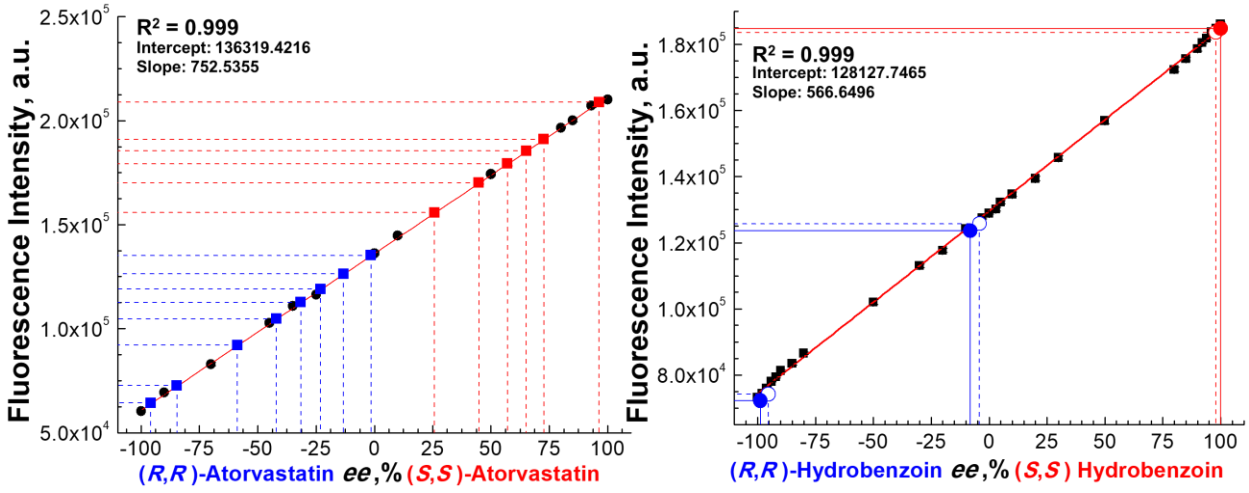

Figure 5. **Examples of the use of protocol for determination of enantiomeric purity of diols and their absolute configuration.** Left: Standard curve obtained from L-tryptophanol-2-FPBA assembly (1:1, 40 μM) with atorvastatin enantiomers at various *ee*. 14 samples of unknown enantiomeric composition of atorvastatin were simultaneously correctly interpolated from the curve using linear regression model (Supplementary Figure 3). Right: Standard curve generated from hydrobenzoin samples with various enantiomeric composition (*ee* range from 100% (*R*,*R*)-hydrobenzoin to 100% (*S*,*S*)-hydrobenzoin). Six unknown asymmetric reaction samples, their absolute configuration and *ee* were simultaneously correctly interpolated from the graph using linear regression model (Supplementary Figure 4). Each assay solution contains a 1:1:1 equiv. ratio and 40 μM total concentration of L-tryptophanol reporter, 2-formylphenylboronic acid, and enantiomeric mixture of diols under analysis. We employ measurements of 24 repetitions in each standard and unknowns to construct these curves. However, the number of repetitions can be substantially reduced to 8 or 4 as proposed in the procedure section. Student's T-test is performed in order to examine the difference (standard deviation) between data within the set of 24 repetitions for each sample and exclude 4 outlying data points. Each data point shown on a graph as an average value computed from 20 independent measurements (repetitions). Graphical representations of the variability of data shown on graphs as the error bars to indicate the standard deviation in the reported measurement. Due to coefficient of variation (CV) being below 4% for all performed experiments, the error bar for most data points is located within the corresponding mean point on the graph. Graphs are fitted with a linear fit equation

We assessed the protocol for the systematic examination of crude products of asymmetric reactions in real time. The products of a Noyori asymmetric transfer hydrogenation of benzils catalyzed by RuCl[(*R*,*R*)-Tsdpen](*p*-cymene) or RuCl[(*S*,*S*)- Tsdpen](*p*-cymene) catalysts with a formic acid/triethylamine mixture were evaluated. The reaction quantitatively afforded *S*,*S*- and *R*,*R*-hydrobenzoin with various enantiomeric purity allowing for perfect absolute configuration and *ee* determination of small amounts (<1 mg) in the crude form (without product crystallization). We generated a calibration dataset (Supplementary Data 4) using pure enantiomers of hydrobenzoin (Fig. 5, right). The resulting multivariate data set was analyzed by Support Vector

Machine and 29 data points from the calibration set were used to develop/calibrate the linear regression model (black on Figure 5, right).

Next, the validated model was used to simultaneously determine the *ee* in the six entries of unknown *ee* which were obtained from the parallel Noyori reactions. First two samples were prepared using RuCl[(*S*,*S*)-Tsdpen](p-cymene) catalysts, while next two samples using RuCl[(*R*,*R*)-Tsdpen](p-cymene). As expected, *S*,*S*-catalyst gave a pure (*R*,*R*)-hydrobenzoin and the *R*,*R*-catalyst gave (*S*,*S*)-hydrobenzoin respectively. The *ee* was determined as 95.4% *ee* for the crude product and 99.5% *ee* for the recrystallized sample. Result for the second pair of samples show 98.3% *ee* for the crude mixture and 99.7% ee for the recrystallized sample, which is in perfect agreement with the ^{1}H NMR results and literature values. Third pair of unknowns was prepared using a 1:1 mixture of RuCl[(*S*,*S*)-Tsdpen](p-cymene) catalysts and RuCl[(*R*,*R*)-Tsdpen](p-cymene) catalysts, respectively, and gave a small excess of (*R*,*R*)-hydrobenzoin. The corresponding *ee* values were predicted as 3.7% *ee* for the crude product and 2.8% *ee* for the recrystallized part of the reaction mixture. To perform the *ee* assignment using the present method in 20 technical replicates per entry takes < 1 min. Each well\experiment requires only nanograms of the substrate. The comparison of the results obtained for crude and crystallized samples shows that this method is not sensitive to traces of catalysts or reaction additives and may be applied for the high-throughput screening of *ee* in crude product mixtures of parallel asymmetric synthesis.

Note that, the product of incomplete reaction comprising only one reduced carbonyl, is unable to interfere with the complexation between boronic acid and tryptophanol and such sample was used as a control sample. We concluded that the method is not dramatically sensitive up to ≈7% of *meso*-form in the sample of an analyzed chiral diol. Beyond this value, new calibration

sets comprising different amount of *meso*-form must be developed, and consequently, the method could be used for the simultaneous determination of *ee* and total concentration.[33]

Because the tryptophanol based self-assembly reflects both the diol concentration and the change in *ee* value of the respective enantiomers, both can be calculated in conjunction with a supervised pattern recognition method Artificial Neural Networks (ANNs) to analyze the data. [23,43] A general approach for creating HTS assays for the determination of *ee* and total concentration (yield) should meet certain basic criteria. First, the receptor components and enantiopure reporters are commercially available in both enantiomeric forms. This criterion is the strength of our fluorescence-based approach, which is modular and thereby allows one to match commercially available enantiopure reporters such as BINOL, VANOL, or tryptophanol with 2-formylphenylboronic acid and the chiral analyte enantiomers. Second, HTS platforms are widely available and fast and allow for the generation of a large dataset to train ANN and analyze unknowns for *ee* and total concentration if needed. Here, we used a 384-well plate for data collection, this plate format is significantly more rapid than in a standard bench-top fluorimeter cuvette measurement. Fluorescence measurements for each well in a 384 well plate is generated in 5 min in endpoint mode, and fluorescence data are imported into a computer containing software able to perform linear/nonlinear regression, LDA, or ANN resulting in simultaneous output of the *ee* and total concentration values with overall assay time of 3 hour.

ANN utilizes a more complex and expanded calibration set that covers different total concentration values along with various *ee* (Figure 6). As a validation/"unknown" data set we used different concentrations of products from the Noyori asymmetric transfer hydrogenation reaction in our assay. ANN was employed to calculate the total concentrations and *ee* of the hydrobenzoin in the Noyori reaction mixtures. We created an expanded training set containing 15 different

scalemic mixtures (100, 96, 92, 80, 50, 20, 5, −5, −20, −50, −80, −92, −96, −100 % *ee*) and *rac*-mixture, using 5 total concentrations of hydrobenzoin (5, 10, 20, 30, and 40 µM). The entire data set comprised 75 cases with 20 repetitions in each case (1500 experiments). Simple LDA was used for graphical visualization of the resulting data. The semi-quantitative LDA analysis (Figure 6) yielded smooth *ee* and concentration-specific trends in the data, clear separation of the clusters, and 100% correct classification of scalemic mixtures as well as total concentrations of these analytes. Figure 6 shows the 2D representation of the LDA output defined by the first factor (F1) *vs*. *ee* values and comprised 100% of total variance. Five clearly resolved *ee*-dependent trends are very well distributed in the response space of LDA, and concentration dependence encoded by the magnitude of the F1 score for each trend. As expected, the amplitude of distribution for *ee*-calibration plot with the total concentration of 40µM (green in Figure 6) is 13 times larger than the amplitude for the set with 5µM total concentration (red in Figure 6).

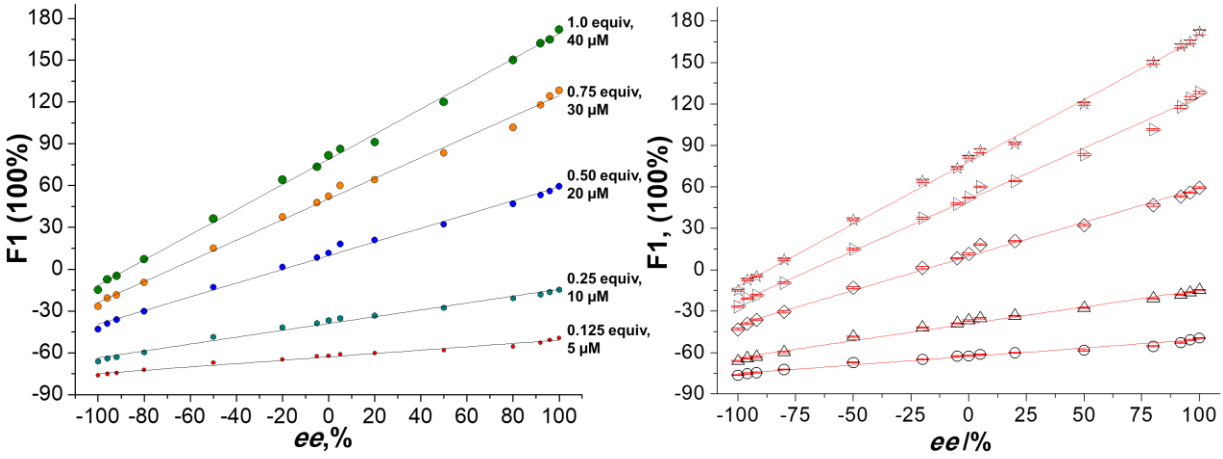

Figure 6. **LDA output representation of the expanded calibration data set using L-TrpOH/FPBA sensors assembly. Left:** Calibration plot comprised various scalemic mixtures of hydrobenzoin at five different total concentrations (5 µM, 10 µM, 20 µM, 30 µM, and 40 µM). The response space is defined by the first factor (F1) of LDA vs. *ee* values comprising 100% of total variances. Data represented as canonical scores of group means.

Measurements were made at room temperature with 20 repetitions for each mixture. Each data point is shown on the graph as an average value computed from 20 independent measurements (repetitions). Graphical representations of the variability of data shown on graphs as the error bars to indicate the standard deviation in the reported measurement. Right: Due to coefficient of variation (CV) being below 4% for all performed experiments, the error bar for most data points is located within the corresponding mean point on the graph. Graphs are fitted with a linear fit equation.

Fluorescence responses for different concentrations and *ee* values of the hydrobenzoin were obtained in the form of guest concentration and specific *ee* (X) × variable (Y) matrix, and first used as a training set to train an ANN. An Artificial Neural Network program automatically generates several neural networks that give in outputs the total guest concentration and *ee* of the hydrobenzoin for Noyori asymmetric hydrogenation reaction mixtures.

LIMITATIONS OF THE METHOD

All high-throughput fluorescence-based assays have fundamental limitations. They require high-precision liquid handling robotics. Also, fluorescence intensity changes may be environmentally sensitive (solvents, additives), potentially limiting the use of this methods. We have addressed this in part by suggesting that the user increase concentration of tested sample solution up to 80 mM (if possible) if interfering compounds such as solvents, inactive catalysts, crude reaction mixture components or any other possible additives are present (see Procedure 1, Step 2). This will decrease a volume of an analyte added to the receptor-premix and also decrease an effect of possible interfering impurities. Having said this, we have found that for all reactions tested so far, the inactive catalyst, formic acid, triethylamine, and benzils have not had any effect on complex formation and fluorescence.

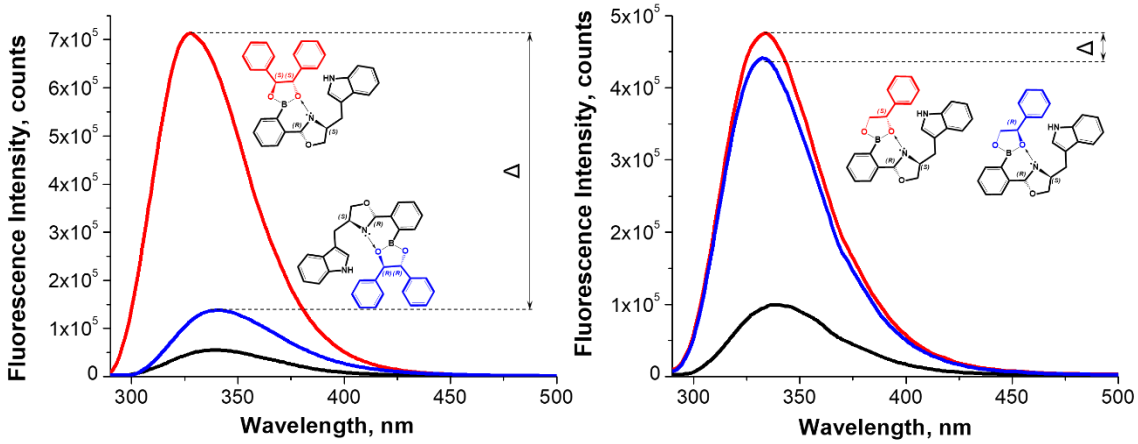

Figure 7. **Example of small and big Δ between diastereomeric complexes assembled with opposite enantiomers of chiral analyte at the same final concentration 40 μM.** Fluorescence spectra of L-tryptophanol - FPBA (1:1, 40 μM) with Right: Hydrobenzoin enantiomers (40 μM) in MeCN. λ_{ex} = 280 nm. Left: Phenylethylene glycol enantiomers. Δ diastereomers for hydrobenzoin is bigger than Δ diastereomers for phenylethylene glycol, which suggests simpler *ee* determination of hydrobenzoin, in comparison to phenylethylene glycol.

Calibrating a standard curve for products of asymmetric reaction can also be challenging, due to possible formation of *meso* form of chiral analytes which is also able to facilitate the complex assembly and contribute in overall system fluorescence response (false positive response). Another concern relevant to fluorescence-based assays is the small Δ (difference in emission intensity shown on Figure 7) of diastereomers at 1:1:1 stoichiometric ratio of assembly components (Figure 7). In other words, this occurs when the diastereomeric complexes assembled with opposite enantiomers of the analyte have a similar fluorescence intensity (Δ is small) at the same concentration (Figure 7, right), making it difficult to accurately measure the effect of *ee* on the fluorescence change. This potential issue can be overcome by combining several ligands that each have a different degree of sensitivity for each analyte to expand the information density of the array-protocol and improve its discriminatory power. This can lead to the generation of a large

multi-dimensional response space and improved resolution of the array but will require using statistical methods such as linear discriminant analysis (**LDA**) or support vector machine regression analysis (**SVM**).

Biocatalysts are widely used in industry in the production of enantiopure drugs and as well for the synthesis of complex chiral compounds in academic research.[44] The development of a biocatalyst involves iterative screening of potential enzymes and it would be very tempting to use the protocol as written here for this type of high throughput screening. . Unfortunately, it is necessary to use different reagents. This is because key classes of biocatalysts including many enzymes such as isomerases, hydrolases, lyases, oxidases, and reductases will absorb in the same region as tryptophanol (280-285 nm) due to the presence of tryptophan. Also, their emission (330-360 nm) can overlap with the emission of BINOL and VANOL. To overcome this issue, for enzymatic reactions screening the reporter (BINOL, VANOL, or tryptophanol) should be replaced with fluorescent amino acids (FlAAs) or diol ligands which will have red shift of absorption/emission spectra. Several amino acid derivatives of pyrene (λ_{em}=375 nm), 6-(2-dimethylaminonaphthoyl) alanine (DANA) (390 \ 450-550 nm)[45], as well as commercially available α-Dansyl-L-arginine hydrochloride or BINOL derivative (*R*)\(*S*)-3,3′-Di-9-phenanthrenyl-1,1′-bi-2-naphthol have absorption\emission profiles that don't overlap with enzymes. When adapting this procedure such that the alternative reagents (different fluorophores) are used, it is necessary to perform complex optimization to confirm optimum conditions (reagent equivalent ratio, concentration, and equilibration time) for assembly formation. Reagents solubility and specific assembly-enzyme interactions are another key factor which must be determined during optimization. Optimization step involves a fair amount of work, yet ultimately can be done in an automated way using 384 well plates, robotic liquid handler, and microplate reader suitable for fluorescence measurement.

Acquiring this information would require several fluorescence titrations or perform an initial screening directly in a 96-well screening plate as now described. A screening plate can be designed to determine which chiral fluorescent host and complex concentration shows the best discrimination between the 2 enantiomers of desired chiral analyte.

Fluorescence titrations can be performed to explore different chiral host scaffolds, such as described above, and 2-formylphenylboronic acid. Fluorescence titration profiles can be created with one equivalent of FlAAs or fluorescent chiral diol ligands self-assembled with one equivalent of 2-formylphenylboronic acid at the suggested starting concentration of 40 µM and upon the addition of incremental amounts of chiral analyte. It is recommended to reach a plateau of the 1:1 binding isotherm in order determined correct complex stoichiometry. For fluorescence titration the reagent that generates the fluorescent signal should be kept at a constant concentration throughout the experiment to avoid signal changes due to changes in the concentration. The bandwidth settings on the fluorescence spectrometer depend strongly on the quantum yield of the fluorophore but recommended to keep as narrow as possible to attain enough signal. If the observed fluorescence intensity is low the emission bandwidth must be increased. Also, the excitation bandwidth can be reduced If fluorescence bleaching is observed during the titration experiment. The concentration can be adjusted according to the sensitivity of the spectrometer and the Ka value of the interaction that is being analyzed.

STATISTICAL ANALYSIS

The response patterns associated with fluorescence-based assays (using several fluorescence channels, or combined data from BINOL and VANOL-receptor complexes) are acquired in a form of multivariate data set. Hence, chemometric pattern recognition techniques such as supervised

methods including linear discriminant analysis (LDA) and support vector machine (SVM) regression analysis can be used to identify the samples and generate a classification model that can later be used to assign the unknown samples

Data pre-treatment. The first steps prior to the application of multivariate data analysis techniques are variable selection/reduction and data pre-treatment.[46,] For this purpose, conventional manual analyses as well as robust statistical methods such as Student's T-test can be utilized in order to examine the difference (standard deviation) between data within the set of repetitions for each sample and exclude outlying data points from variables containing noisy information.

Linear Discriminant Analysis (LDA). LDA is the most frequently used supervised pattern recognition method for reduction of dimensionality and classification of the multivariate data. LDA is based on the determination of linear discriminant functions (DF) that aim to maximize the ratio of between-class variance while minimizing the ratio of within-class variance. First, LDA develops a model with part of data set (training set) which describes the relationship between the observed variables and their known classes. After that, the model is tested for its recognition (classification) ability using another part of the data (test set). Both, training and test sets contain data representative of each class. This procedure, consisting of a model development and model testing, is repeated several times. The latter process is called cross-validation.[47] The most common cross-validation approach is the so-called leave-one-out cross-validation which removes only one sample at a time from the training set and considers it as a test set.[48] The procedure is repeated until all samples have been left out and classified. This way, the data of unknown identity can be later assigned to one of the classes based on the similarity of its responses to the responses of the samples in the training set. Thus, the predictive power of an array can be determined.

LDA reduces the dimensionality of the data set by calculating the eigenvalues which are correlated with factors and factor scores (coordinates of the observations in the new space of reduced dimensionality). The magnitude of eigenvalues represents the variance in the data. The first factor (F1) accounts for the most variance in the data, and the other factors follow in the order of decreasing variance.

Support Vector Machine (SVM). For nonlinear quantitative analysis of the enantiomeric mixtures with small Δ, a supervised method such as support vector machine (SVM) is a very reliable method, especially when dealing with data sets containing several distinct classes that cannot be easily separated by linear boundaries.[49] The goal of SVM is to obtain the "optimal" boundaries which separates the analyte classes. This is achieved by using kernel functions, which consist of transformation of the nonlinear data set into an *n*-dimensional vector space where the classes can be separated linearly.[50]

Then, the recognition ability of the model is usually tested using *n*-fold cross-validation approach. Thus, each instance of the whole training set is predicted once and the cross-validation accuracy is the percentage of the data which were correctly classified. Based on kernel functions, the SVM generates the regression (calibration) model. First, the training set is used to calibrate the model producing the root mean square error of calibration (RMSEC). Then, the model is validated using *n*-fold cross-validation approach producing the root mean square error of cross-validation (RMSECV).[51] Finally, the validity and predictive ability of the developed model is tested using independent data set (unknown samples). The predictive accuracy of a model can be evaluated by the value of the root mean square error of prediction (RMSEP).

Artificial Neural Network (ANN). Fluorescence-based protocol for HTS of *ee* and total concentration (reaction yield) of chiral analytes (amines or diols) relies on using ANN as the data analysis tool for simultaneous prediction of both parameters and can be used in the case when Δ is small. Most of Neural Networks software have an embedded intelligent problem solver (IPS) function, which automatically generates several neural networks that are suitable for the selected problem. The ability to self-teach and to model complex nonlinear data are the main advantages of ANN-based protocol. In the literature there are many types of artificial neural networks available for different applications. The simplest and the most frequently used type of neural network is the multilayered perceptron (MLP) with a feed-forward topology. The most important part of the MLP-ANN is the training step. The training (ANN learning step) is a search procedure for a set of weight values with the main task of minimizing the squared errors of prediction (experimental vs estimated data). Among variety of learning algorithms for MLP (conjugate gradient descent, quasi-Newton, etc.,) we recommend using the most employed one - the back-propagation algorithm. The back-propagation algorithm uses the output or prediction layer's error values to adjust the weight of layer connections. Thus, his algorithm provides a guarantee of minimum (local or global) convergence. The main challenge of MLP-ANN is the choice of the most suitable architecture, because the speed and the performance of the MLP-ANN learning are strongly depending on the number of layers. The number of layers in MLP algorithm is directly proportional to the complexity of each specific problem. The higher the number of hidden layers, the higher the complexity of the pattern recognition of the neural network. Simple *n*-layered MLP network ($n \approx 3$) is usually suited for application in the described protocol.[23,43,52]

MATERIALS

REAGENTS.

CAUTION Inhalation of vapors or mists of toxic or corrosive chemicals can cause severe bronchial irritation. Many amines are highly toxic and corrosive and may causes serious eye damage and skin irritation if handled with standard precautions. Do not work alone when handling toxic chemicals. Use a fume hood to ensure proper ventilation or wear appropriate respiratory protection if a fume hood is not available.

Δ CRITICAL All the reagents can be purchased from commercial suppliers and used without further purification. Purchase enantiopure ligands and chiral standards with enantiomeric purity preferably ≥ 99.0% (for chiral derivatization).

- Enantiopure chiral diols:

(*R*)-(+)-1,1′-Bi(2-naphthol) (*ee* ≥99% by HPLC, Aldrich, cat. no. 246948)

(*S*)-(−)-1,1′-Bi(2-naphthol) (*ee* ≥99% by HPLC, Aldrich, cat. no. 246956)

(*S*)-3,3′-Diphenyl-2,2′-bi(1-naphthol) (*ee* ≥97% by HPLC, Aldrich, cat. no. 675334)

(*R*)-3,3′-Diphenyl-2,2′-bi(1-naphthol) (*ee* ≥97% by HPLC, Aldrich, cat. no. 675156)

(*S*)-(+)-3-chloro-1,2-propanediol (*ee* ≥98%, TCI, cat. no. C1542) ! CAUTION

(*R*)-(−)-3-chloro-1,2-propanediol (*ee* ≥98% (GLC), Aldrich, cat. no. 540056) ! CAUTION

(*S*)-(+)-1-phenyl-1,2-ethanediol (*ee* ≥98%, Aldrich, cat. no. 302155)

(*R*)-(−)-1-phenyl-1,2-ethanediol (*ee* ≥99%, Aldrich, cat. no. 302163)

(+)-dimethyl L-tartrate (*ee* ≥ 99% (GLC), Aldrich, cat. no. 163457)

(–)-dimethyl D-tartrate(*ee* ≥99% (GLC), Aldrich, cat. no. 242942)

(+)-diethyl L-tartrate (*ee* ≥99% (GLC), Aldrich, cat. no. 156841)

(–)-diethyl D-tartrate (*ee* ≥99% (GLC), Aldrich, cat. no. 213969)

(1*S*,2*S*,3*R*,5*S*)-(+)-pinanediol (*ee* 99% (GLC), Aldrich, cat. no. 282367)

(1*R*,2*R*,3*S*,5*R*)-(–)-pinanediol (*ee* 99%, Alfa Aesar, cat. no. H56580)

(*S*,*S*)-(–)-hydrobenzoin (*ee* 99% (GLC), Aldrich, cat. no. 256269)

meso-hydrobenzoin (Aldrich, cat. no. 294535)

(*R*,*R*)-(+)-hydrobenzoin (*ee* 99% (GLC), Aldrich, cat. no. 256277)

(3*S*,5*S*)-atorvastatin calcium (*ee* ≥99%, USP, cat. no. 1044571)

(3*R*,5*R*)-atorvastatin sodium (*ee* ≥99%, TRC, cat. no. A791730)

- 2-Formylphenylboronic acid (Aldrich, cat. no. 431958)
- Enantiopure chiral amines:

(*S*)-(−)-*α*-Methylbenzylamine (*ee* ≥ 99.0%, Sigma-Aldrich, cat. no. 77869) ! CAUTION

(*R*)-(+)-*α*-Methylbenzylamine (*ee* ≥ 99.0%, Sigma-Aldrich, cat. no. 77879) ! CAUTION

(*R*)-(+)-*β*-Methylphenethylamine (*ee* 99.0%, Aldrich, cat. no. 461385) ! CAUTION

(*S*)-(−)-*β*-Methylphenethylamine (*ee* 99.0%, Aldrich, cat. no. 461393) ! CAUTION

(*S*)-(−)-1-(2-Naphthyl)ethylamine (*ee* ≥ 99.0%, Aldrich, cat. no. 70942) ! CAUTION

(*R*)-(+)-1-(2-Naphthyl)ethylamine (*ee* ≥ 99.5%, Sigma-Aldrich, cat. no. 70710) ! CAUTION

(*S*)-(+)-2-Aminobutane (*ee* 99.0%, Aldrich, cat. no. 296651) ! CAUTION

(*R*)-(−)-2-Aminobutane (*ee* 99.0%, Aldrich, cat. no. 296643) ! CAUTION

- Chiral amino alcohols:

(*S*)-(−)-2-Amino-3-phenyl-1-propanol (*ee* 99.0% (HPLC), Aldrich, cat. no. 190438) ! CAUTION

(*R*)-(+)-2-Amino-3-phenyl-1-propanol (*ee* 99.0%, Aldrich, cat. no. 284491) ! CAUTION (1*S*,2*R*)-(−)-*cis*-1-Amino-2-indanol (*ee*: 99% by GLC, Aldrich, cat. no. 440833) ! CAUTION

(1*R*,2*S*)-(+)-*cis*-1-Amino-2-indanol (*ee*: 99% by GLC, Aldrich, cat. no. 440841) ! CAUTION

L-Tryptophanol (Aldrich, cat. no. 469971)

D-Tryptophanol (Aldrich, cat. no. 470031)

- Chiral amino acid esters.

L-Valine methyl ester hydrochloride (*ee* 99% Aldrich, cat. no. 860271)

D-Valine methyl ester hydrochloride (*ee* ≥99.0% (AT), Aldrich, cat. no. 94665)

L-Phenylalanine methyl ester hydrochloride (*ee* ≥99.0%, Alfa Aesar, cat. no. A11455)

D-Phenylalanine methyl ester hydrochloride (*ee* ≥99.0%, Alfa Aesar, cat. no. H25920).

L-Methionine methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 03105)

D-Methionine methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 03106)

L-Alanine methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 02890)

D-Alanine methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 03016).

L-Tryptophan methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 03152)

D-Tryptophan methyl ester hydrochloride (*ee* ≥99.0%, Chem-Impex International, Inc. cat. no. 04454)

- Solvents.

Propionitrile (optional, Aldrich, cat. no. 185590)

Acetonitrile (anhydrous, 99.8%, Sigma-Aldrich, cat. no. 271004)

Phosphorus pentoxide (optional, Sigma-Aldrich, cat. no. 298220)

- Cesium carbonate (optional, Aldrich, cat. no. 441902)
- Sodium bicarbonate (optional, Sigma-Aldrich, anhydrous, free-flowing, Redi-Dri™, ACS reagent, ≥99.7%, cat. no. 792519)
- Chloroform (optional, EMD MILLIPORE, cat. no. CX1050)

- Magnesium sulfate (optional, Sigma-Aldrich, anhydrous, ReagentPlus®, ≥99.5%, cat. no. M7506)
- Molecular sieves, 4 Å (beads, 4-8 mesh, Sigma-Aldrich, cat. no. 208590)
- Sodium citrate (optional, USP, cat. no. 1613859)

EQUIPMENT

- Rotavapor (R-100 System, Buchi, cat. no. 11100V2112)
- Glass separatory funnel, 50 mL (Aldrich, cat. no. Z548014)
- Glass round-bottom flask, 50 mL (Aldrich, cat. no. Z510416)
- Microplate reader (CLARIOstar multi-mode microplate reader (BMG LABTECH))
- Benchtop Centrifuges (Beckman Coulter Allegra X-30 Series, S6096 microplate rotor)
- Adjustable Volume Pipette, 0.1-1.0 µl (Accuris™ NextPette™, LPS, cat. no. P7700-1)
- Adjustable Volume Pipette, 0.5-10 µl (Accuris™ NextPette™, LPS, cat. no. P7700-10)
- Adjustable Volume Pipette, 10-100 µl (Accuris™ NextPette™, LPS, cat. no. P7700-100)
- Adjustable Volume Pipette, 100-1000 µl (Accuris™ NextPette™, LPS, cat. no. P7700-1000)
- Non-filtered pipette tips, 12.5 µL (Thermo Scientific™, cat. no. 94410050)
- Filter pipette tips, 200 µL (Thermo Scientific™, cat. no. 94300130)
- Filter pipette tips, 1000 µL (Thermo Scientific™, cat. no. 94300220)
- Amber Borosilicate Screw Thread Vials with Green PP Hole Cap & PTFE/Silicone Septa, 20 mL (VWR, cat. no. 36319-408)
- 384 well density microplates (Aurora Microplates, part# ABC010100A)
- Liquid handling system (16-channel Nanodrop Express, BioNex Solution Inc.)

- BNX 1536™ liquid handling system
- Eppendorf Safe-Lock tubes, 0.5 mL (SIGMA, cat. no. T8911)
- Eppendorf Thermomixer R
- A graphing program that enables to perform linear/nonlinear regression analysis. We have used Microsoft Excel, Origin (OriginLab), SOLO (Eigenvector Research, Inc.), STATISTICA Automated Neural Networks (SANN).

REAGENT SETUP

Parent amines In the case that amine is available only as their corresponding hydrochloride (HCl salt), in order to obtain the free amine in acetonitrile, 1.0 equivalent of anhydrous Cs_2CO_3 has to be added and stirred for 60 minutes. The solid suspension then has to be filtered through syringe filters (diameter: 4mm; membrane: nylon; pore size: 0.2µm).

Amino acid esters Prepare these using the liquid/liquid extraction procedure as described below.

1) Spike 1 ml of an aqueous sample with enantiopure amino acid ester hydrochloride at a concentration of 1g/ml, and 3 ml of sodium bicarbonate ($NaHCO_3$, saturated aqueous solution).
2) Add 5 ml of H_2O and 20 ml of chloroform in a 50 ml separation funnel. Collect the organic phase and repeat same procedure 2 more times.
3) Dry the collected organic phase over $MgSO_4$ and filter. Then transfer the organic layer into a clean round-bottom flask and evaporate chloroform to dryness on a rotary evaporator. Δ CRITICAL We recommend leaving the dry residue under vacuum at room temperature (≈ 23-25 °C) overnight to ensure a complete evaporation of all volatiles.

EQUIPMENT SETUP

Round-bottom flask Dry a 50 mL glass round-bottom flask by placing it for at least 1 hour in an oven set at 120 °C

Liquid-handling dispensing device Set up liquid-handling dispensing devices for a pipetting the 384 well density plates format as outlined in the manufacturer's guidelines for steps 10 of Procedure 1 and Step 13 of Procedure 2.

Plate reader Turn on the plate reader as outlined in the manufacturer's guidelines.

If you are using the BMG CLARIOstar, use these instrument settings:

Detection Mode	Fluorescence Intensity-Emission
Method	Top optics
Optic settings	Monochromator presets will depend on the enantiopure dye-receptor premix pair.
No. of flashes	40
Focus and Gain	Need to be adjusted for your particular instrument to reduce signal-to-noise ratio. Adjustment must be set by using standards well. Enantiopure L-Tryptophanol dye-receptor premix pair shows strong amplification of the fluorescence signal, so

	the adjustment must be set by using S1 and S16 (-100% *ee* and +100% *ee*) wells to avoid saturation of the detector.

Monochromator presets for the chiral diol dye-receptor BINOL/VANOL premix:

FI channel	Excitation wavelength [nm]	Emission wavelength [nm]	Excitation bandwidth [nm]	Emission bandwidth [nm]	Dichroic filter
BINOL	335	355	8	8	349.5
VANOL	335	375	8	10	363.8

Monochromator presets for the enantiopure L-Tryptophanol dye-receptor premix:

FI channel	Excitation wavelength [nm]	Emission wavelength [nm]	Excitation bandwidth [nm]	Emission bandwidth [nm]	Dichroic filter
L-Tryptophanol	280	330	8	8	320

PROCEDURE

Δ CRITICAL Each standard for the calibration curve should be prepared separately and in a same manner as analyzed/validation/unknown samples (i.e. according to the procedure described in Steps 2-6 of the Procedure 1 and Steps 3-5 of the Procedure 2). The number of standards strongly depends on Δ i.e. the signal difference between final diastereomers formed with opposite enantiomers of standard and sensitivity of the plate reader. If the signal Δ between final diastereomers of a standard is relatively low and gives small room for *ee* resolution, we recommend using two receptor-premixes (BINOL:2-FPBA and VANOL:2-FPBA) to add more read-out channels and increase informational density of the assay. We also recommend increasing the number of standards to improve the quality of the calibration model and increase its prediction capability.

Use 8.8 mM stock solution of enantiopure fluorescent diol-ligand to reach a final concentration of receptor-premix (4 mM BINOL : 2-FPBA) with a slight excess of chiral diol presented in the system, to ensure the completion of the complexation reaction for standards and analyzed/unknown samples,

The procedure is divided into 2 sections: Procedure 1) simple assay for determination of enantiomeric purity of chiral amines, amino alcohols, amino acid esters, diols and sugars (steps 1-14); Procedure 2) assay for simultaneous determination of *ee* and total concentration (yield) with ANN (steps 1-17).

PROCEDURE 1: simple assay for determination of enantiomeric purity of chiral amines, amino alcohols, amino acid esters, diols and sugars

Samples Preparation ● TIMING 3.5 hours

1| While the method for preparing the working solutions is very similar, there are a few minor differences depending on whether you are analyzing Chiral Amines, Amino Alcohols, and Amino acid esters (option A) or Chiral Diols and Sugars (option B).

A Solutions for Analyzing Chiral Amines, Amino Alcohols, and Amino acid esters ● TIMING 25-30 min

i. **Prepare 10 mL of 8.8 mM stock solution of BINOL.** Weigh out 25.2 mg (0.088 mmol) of (*S*)-BINOL (MW=286.32) and transfer to the amber borosilicate screw thread 20 mL vial.

ii. Transfer 10 mL of acetonitrile to the vial from previous step using a 100-1000 µl adjustable volume pipette with disposable tips.

iii. **Prepare 10 mL of 8.8 mM stock solution of VANOL.** Weigh out 38.6 mg (0.088 mmol) of (*S*)-VANOL (MW=438.52) and transfer this to the amber borosilicate screw thread 20 mL vial.

iv. Transfer 10 mL of acetonitrile to the vial from previous step using a 100-1000 µl adjustable volume pipette with disposable tips.

v. **Prepare 10 mL of 8.0 mM stock solution of 2-formylphenylboronic acid.** Weigh out 12.0 mg (0.080 mmol) of 2-formylphenylboronic acid (MW=149.94) and transfer to the suitable 20 mL vial.

vi. Transfer 10 mL of acetonitrile to the vial from previous step using a 100-1000 µl adjustable volume pipette with disposable tips.

vii. **Prepare the mixture.** Mix 10 mL of fluorescent chiral diol-dye and 10 mL of 2-formylphenylboronic acid stock solutions in the amber borosilicate screw thread 20 mL vial. Stir ≈ 10 min until a homogeneous 4mM receptor-premix solution is obtained. See step 2 to continue.

□ PAUSE POINT The resulting 4 mM receptor-premix solution is stable to moisture and can be handled without the need to operate in an inert atmosphere. At this step premix can be left overnight.

B Simple Assay for Chiral Diols and Sugars ● TIMING 25-30 min

<CRITICAL> To ensure the completion of the complexation reaction for standards and unknown samples, mix 1.1:1 equiv. ratio from 8.8 mM stock solutions of enantiopure L-tryptophanol-dye with 8.0 mM stock solutions of 2-formylphenylboronic acid (FPBA) to achieve final concentration of receptor-premix 4 mM in acetonitrile with a slight excess (1.1 equiv.) of L-tryptophanol-dye.

i. **Prepare 10 mL of 8.8 mM stock solution of L-tryptophanol.** Weigh out 16.74 mg (0.088 mmol) of L-tryptophanol (MW=190.24) and transfer to the amber borosilicate screw thread 20 mL vial.

ii. Transfer 10 mL of acetonitrile to the vial from previous step using a 100-1000 µl adjustable volume pipette with disposable tips.

iii. **Prepare 10 mL of 8.0 mM stock solution of 2-formylphenylboronic acid.** Weigh out 12.0 mg (0.080 mmol) of (MW=149.94) and transfer to the suitable 20 mL vial.

iv. Transfer 10 mL of acetonitrile to the vial from previous step using a 100-1000 µl adjustable volume pipette with disposable tips.

v. **Prepare the mixture.** Mix 10 mL of enantiopure L-tryptophanol-dye and 10 mL of 2-formylphenylboronic acid stock solutions in the amber borosilicate screw thread vial. Stir ≈ 10 min until a homogeneous 4mM receptor-premix solution is obtained.

□ PAUSE POINT The resulting 4 mM receptor-premix solution is stable to moisture and can be handled without the need to operate in an inert atmosphere. At this step premix can be left overnight.

Addition of the component under analysis. ● TIMING 2 hours

<CRITICAL> A three-component condensation reaction proceeds to completion only in a presence of 1.1 equiv. of the enantiopure fluorescent dye (S-BINOL/S-VANOL for amines, or L-tryptophanol for diols), 1 equiv. of 2-formylphenylboronic acid and 1 equiv. of amine/amine derivative, or diol to be tested. We recommend preparing 2 mL concentrated stock solutions of enantiopure analytes to ensure integrity of the assay.

2| Prepare concentrated stock solutions (2 mL) of enantiopure analytes in acetonitrile:

For amine/amine derivative assay	Two opposite enantiomers of the chiral amine/amine derivative to make suitable number of standards (16 in this procedure) for calibration.
	Amine/amine derivative samples to be analyzed.
For diol assay	Sufficient quantities of each enantiomer of the appropriate diols to make the number of standards needed for calibration (16 in this procedure).
	Diol samples to be analyzed

Δ CRITICAL STEP The concentration of the chiral analytes stock solutions strongly depends on the solubility and the purity of the tested system. We recommend using 40 mM stocks, but increase concentration up to 80 mM (if possible) if interfering compounds such as ethylene glycol, inactive catalysts, crude reaction mixture components or any other possible additives are present in the tested samples.

? TROUBLESHOOTING

3| Transfer in to 0.5 mL Eppendorf tubes appropriate amounts of receptor-premix solution (X, mL) of enantiopure dye with 2-formylphenylboronic acid using following equation:

$$X\ mL + Y_n\ mL = 0.2\ mL$$

$$Y_n = Y_R + Y_S, \text{ so that}$$

$$X\ mL+(Y_R+Y_S\ mL)_n = 0.2\ mL$$

Assuming that the reactions are being done with all the reagents at 4 mM, then:

X is the volume of the 4 mM receptor-premix solution of enantiopure dye and 2-formylphenylboronic acid, mL

Y_n – 4 mM, corresponding total volume of analyzed amine/amine derivative, or diol stock solution for all *n* number of measured standards tested samples, mL.

4| Prepare (*n*) number of standards at 1.1:1:1 ratio (1.1:1 equiv. is a receptor-premix, and 1 equiv. of tested chiral amine/amine derivative, or diol) which will cover desired range of enantiomeric compositions (See Table 2 for suggested standards *ee* range). Combine different volumes of stock solutions of enantiopure amine/amine derivative, or diol enantiomers (Y_R and Y_S, mL) in order to reach a total concentration 4 mM (1 equiv.) in a Y_n = 20 µL (for 40 mM stocks) total volume or 10 µL (for 80 mM stocks). Δ CRITICAL STEP We recommend covering range from -100% (pure *S*-enantiomer) to +100% (pure *R*-enantiomer).

Table 2 Preparation of $Y_n = Y_R+Y_S$ (µL) for standards with different enantiomeric composition.

n	**ee%**	%R	%S	$Y_{R,}$ µL	$Y_{S,}$ µL	$Y_{n,}$ µL
S1	**100**	100	0	20.0	0.0	20.0
S2	**95**	97.5	2.5	19.5	0.5	20.0
S3	**90**	95	5	19.0	1.0	20.0

S4	**80**	90	10	18.0	2.0	20.0
S5	**60**	80	20	16.0	4.0	20.0
S6	**30**	65	35	13.0	7.0	20.0
S7	**10**	55	45	11.0	9.0	20.0
S8	**0**	50	50	10.0	10.0	20.0
S9	**-10**	45	55	9.0	11.0	20.0
S10	**-20**	40	60	8.0	12.0	20.0
S11	**-40**	30	70	6.0	14.0	20.0
S12	**-60**	20	80	4.0	16.0	20.0
S13	**-80**	10	90	2.0	18.0	20.0
S14	**-90**	5	95	1.0	19.0	20.0
S15	**-95**	2.5	97.5	0.5	19.5	20.0
S16	**-100**	0	100	0.0	20.0	20.0

Δ CRITICAL Total volume and concentration of added chiral amine/amine derivative $Y_n = Y_R + Y_S$ must be Y_n = 0.02 mL and 4 mM (1 equivalent) respectively for all *n* number of standards and analyzed unknown samples.

5| Add Y_n, mL of Y_R+Y_S into *n* number of 0.5 mL Eppendorf tubes previously filled with X mL of receptor-premix solution in an Eppendorf tube rack.

6| Incubate the reaction mixtures for 10 min at room temperature (23 °C) to ensure the complexation of diastereomeric imino/oxazolidine-boronate esters.

□ PAUSE POINT These reaction mixtures can be stored at room temperature overnight.

Distribution of samples

7| Design your pipetting plate layout. An example for one receptor-premix is shown in Figure 8. Each sample must be performed in at least 8 repetitions to generate acceptable (statistically meaningful) data and to verify the results.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A
B	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	B
C	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
D	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	D
E	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	E
F	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	F
G	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G
H	B	Ctr	S1	S3	S5	S7	S9	S11	S13	S15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H
I	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	I
J	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	J
K	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	K
L	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L
M	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	M
N	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	N
O	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	O
P	B	Ctr	S2	S4	S6	S8	S10	S12	S14	S16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	P
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	

Figure 8. Example of pipetting plate layout in a simple assay for determination of enantiomeric purity of chiral amines, amino alcohols, amino acid esters, diols and sugars using one receptor-premix. "B" is a blank (acetonitrile); "Ctr" is a control receptor-premix solution(fluorescent ligand : 2-FPBA at 1.1:1 molar ratio with total concentration 4 mM); "S1-S16" are standards 1-16; * - samples with unknown *ee*.

Δ CRITICAL STEP The first column (B) should be set for blanking. If you need to use two receptor-premix solutions (BINOL and VANOL-based) in order to get the required assay resolution, use the expanded layout shown at Figure 8 (Ctr and S1-S16). Do not pipet a second set of Blanks for expanded layout. We recommend designing the layout in a way that Ctr, standards, and samples under analysis with one receptor-premix placed and measured in a same plate for assay integrity.

Pipetting the reagents ● TIMING 5 min

8| The standards, samples with unknown *ee* and acetonitrile for control should be dispensed with high-precision pipetting system, and diluted 100 times with acetonitrile to 0.04 mM working concentration. ! CAUTION Acetonitrile is a volatile solvent. Δ CRITICAL STEP We recommend dispensing solvent first, due to low volume of dispensed samples and volatility of acetonitrile.

Solvent for dilution	Dispense 99 µL to each well, including Blank (B) wells
Control	dispense 1 µL of receptor-premix solution to Control (Ctr) wells
Standards	dispense 1 µL of each of the sixteen standards (S1-S16) in repetitions to appropriate wells

Chiral amines/amine derivatives, or diol samples to be analyzed	dispense 1 µL in repetitions to appropriate wells

Incubating the plate ● TIMING 2min

9| Cover the plate with a lid and centrifuge for 2 min at 2500 rpm (T = 294 K).

Δ CRITICAL STEP The plate must be analyzed immediately after centrifugation due to volatility of acetonitrile, to reduce the microplate edge effect. The "edge effect" is a discrepancy between the center and outer wells and caused by evaporation. It is a very real phenomenon and can greatly impact your assay results. Due to the extremely low volumes the 384- and 1536-well microplates may experience a more expressed "edge effect".

? TROUBLESHOOTING

Reading the Plate ● TIMING 5 min in the end point mode.

10| Transfer the plate prepared in previous step to the plate reader. Record the fluorescence intensity at the maximum wavelength. Δ CRITICAL STEP Make sure that your plate reader has subtracted the fluorescence of the blank wells (fluorescence of the plate and solvent) from the fluorescence readings of the rest of the plate. ? TROUBLESHOOTING

Data Analysis ● TIMING 30 min

<CRITICAL> Most plate readers are supplied with standard curve-fitting software protocol, capable of graphing the data (linear curve fit, data prediction). We recommend using this built in protocol otherwise refer to step 11-14 for future information. ? TROUBLESHOOTING

11| Calculate the average fluorescence for each control, standard, and unknown sample.

12| Plot the average fluorescence for each standard (Y-axis) versus the enantiomeric excess (X-axis), preferably using a graphing program that enables to perform both linear and nonlinear regression analysis. We have used Microsoft Excel, Origin (OriginLab), and SOLO (Eigenvector Research, Inc.) to obtain a good fit and data prediction.

13| Use a linear regression model to fit the data. Δ CRITICAL STEP We recommend to use the linear least squares method for finding the coefficients of polynomial equations that are a best linear fit to a set of X,Y data corresponding to *ee* and FI of the standards and unknown samples. A polynomial equation expresses the dependent variable Y (FI) as a first-order polynomial in the independent variable X (*ee*) generate a straight line:

$$Y=mX+b$$

Where Y is the fluorescence intensity reading; *m* is the slope of the line; X is the *ee*; *b* is the X-axis intercept.

14| Use coefficients (*m, b*) to predict values of enantiomeric excess (X) for each measured fluorescence intensity (Y) of tested samples. In most cases, Y is a linear function of the parameters *m* and *b*. ? TROUBLESHOOTING

PROCEDURE 2 - Assay for Simultaneous Determination of *ee* and Total Concentration (Yield) with ANN. (TIMING 4.5 hours)

Samples preparation ● TIMING 2 hours

1| To determine *ee* and total concentration of the chiral products perform Steps 1-6 of the Procedure 1 to prepare 80 Eppendorf tubes prefilled with the appropriate receptor-premix solution needed to make a training set of standards for ANN in a next step.

2| Prepare analyte enantiomers stocks at 5 different concentrations: 5 mM (for step 3a), 10 mM (for step 3b), 20 mM (for step 3c), 30 mM (for step 3d), 40 mM (for step e) and 8 validation samples of various *ee* and total concentration (for step 3v).

3| Prepare 80 standards (S1-80) to cover desired range of enantiomeric compositions. **Table 2** corresponds to S65-S80 in the table below. The other series have the same series of enantiomeric ratios, but at different analyte concentrations.

A	S1-S16	1.1:1:0.125 equiv. ratio, where 1.1:1 equiv. is a receptor-premix 4mM, and 0.125 equiv. of tested chiral amine/amine derivative, or diol with total concentration of chiral analyte is 0.5 mM
B	S17-S32	1.1:1:0.25 equiv. ratio, where 1.1:1 equiv. is a receptor-premix 4mM, and 0.25 equiv. of tested chiral amine/amine derivative, or diol with total concentration of chiral analyte is 1 mM

C	S33-S48	1.1:1:0.50 equiv. ratio, where 1.1:1 equiv. is a receptor-premix 4mM, and 0.5 equiv. of tested chiral amine/amine derivative, or diol with total concentration of chiral analyte is 2 mM
D	S49-S64	1.1:1:0.75 equiv. ratio, where 1.1:1 equiv. is a receptor-premix 4mM, and 0.75 equiv. of tested chiral amine/amine derivative, or diol with total concentration of chiral analyte is 3 mM
E	S65-S80	1.1:1:1 equiv. ratio, where 1.1:1 equiv. is a receptor-premix 4mM, and 1 equiv. of tested chiral amine/amine derivative, or diol with total concentration of chiral analyte is 4 mM
V	V_1-V_8	Validation samples (artificial "unknown" samples) for ANN model which can consist any known equivalent ratio and total concentration.

To prepare this sets of standards combine different volumes of stock solutions of enantiopure amine/amine derivative, or diol enantiomers (Y_R and Y_S, mL) in order to reach a desired total concentration in a Y_n = 20 µL

Δ CRITICAL STEP It is important to keep Y_n = 20 µL equal for entire set of training standards.

4| Add Y_n =20 µL of Y_R+Y_S into *n* number of 0.5 mL Eppendorf tubes prefilled with X mL of receptor-premix solution in an Eppendorf tube rack.

5| Incubate the reaction mixtures for 10 min at room temperature to ensure the complexation of diastereomeric imino/oxazolidine-boronate esters.

□ PAUSE POINT These reaction mixtures can be stored at room temperature overnight.

Distribution of samples

6| Design a pipetting "training plate" and "analysis plate" layout. Example layouts are suggested in Figure 9. Prepare 4 repetitions of each sample to generate statistically meaningful data and to verify the results.

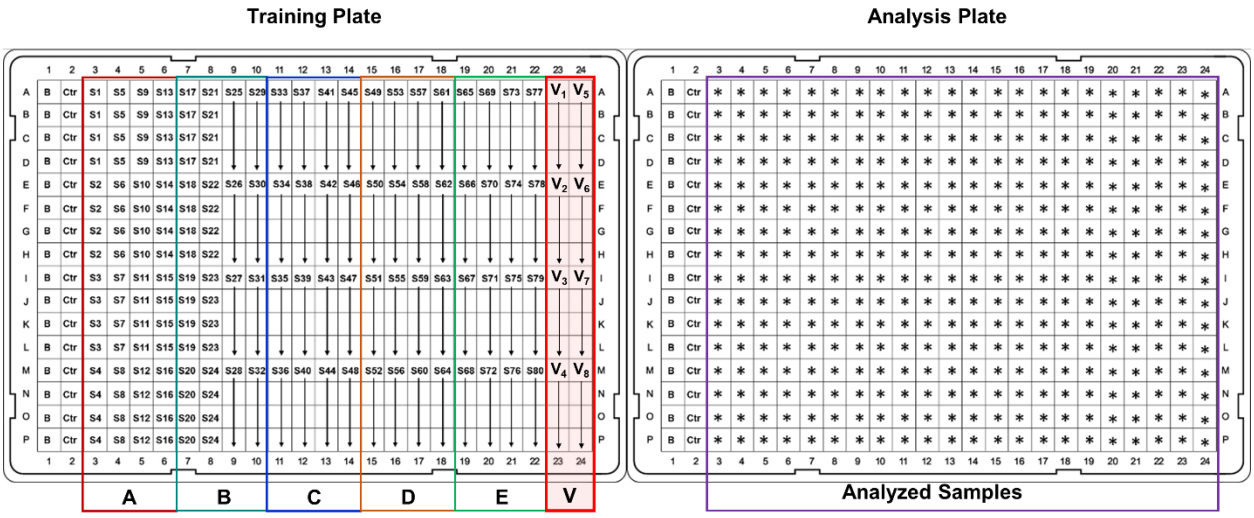

Figure 9. Example of pipetting plate layout in an assay for simultaneous determination of *ee* and total concentration (yield) with ANN using one receptor-premix. "B" is a blank (acetonitrile); "Ctr" is a control receptor-premix solution (fluorescent ligand : 2-FPBA at 1.1:1 molar ratio with total concentration 4 mM); S1-S80-standards 1-80; *- samples with unknown *ee*. The layout for the training plate design is such that the concentration of the final complex would vary along each row of the plate, whereas the ee of the solution varied from 100% to −100%.

Δ CRITICAL STEP The first column (B) should be set for blanking, and second column set for control (Ctr) in both "training" and "analysis" plates to ensure integrity of the assay.

Pipetting the reagents ● TIMING 20 min

<CRITICAL> In the following steps, dispense the standards, samples with unknown *ee* and concentration, and acetonitrile for negative control with high-precision pipetting system, and dilute 100 times with acetonitrile to 0.04 mM working concentration of fluorescence reporter and 2-formylphenylboronic acid. We recommend dispensing solvent first, due to low volume of dispensed samples and volatility of acetonitrile.

! CAUTION Acetonitrile is a volatile solvent.

7| **Dispensing of the training plate** Add the solvent for dilution: dispense 99 µL to each well, including Blank (B) wells.

8| Add the control solution: dispense 1 µL of receptor-premix solution to Control (Ctr) wells.

9| Dispense the standards: dispense 1 µL of each of standards (S1-S80) in repetitions to appropriate wells. The training plate should consist of a set of 16 *ee* values (100, 95, 90, 80, 60, 30, 10, 0, -10, -20, -40, -60, -80, -90, -95, and 100) %, created at 5 different total concentrations of analyte (5, 10, 20, 30, and 40 µM) generated a total of 80 cases.

10| **Dispense an analysis plate where unknown samples of varying *ee* and total concentration values need to be tested.** Solvent for dilution: dispense 99 µL to each well, including Blank (B) wells.

11| Control: dispense 1 µL of receptor-premix solution to Control (Ctr) wells.

12| Chiral amines/amine derivatives, or diol samples to be analyzed: dispense 1 µL in repetitions to appropriate wells.

Plate incubation ● TIMING 15 min-1 hour.

13| Perform plate incubation and reading as described in Steps 8 – 9 of the Procedure 1. If plate reader allows for a spectral scanning, we recommend measuring range of 345-365nm for BINOL, 360-385 for VANOL, and 320-340 for tryptophanol with step of 2 nm. If only one read-out channel is used in the analysis of unknown samples, the error in ANN prediction may be higher.

ANN Data Analysis ● TIMING 2.5 hours

<CRITICAL> In this section, we describe how analyze unknown samples for concentration (reaction yield) and *ee*. We were using a multilayer perceptron (MLP) artificial neural network which is a class of feedforward ANN. MLP is multi-layer feed-forward neural networks where the data information flows only in the forward direction. Therefore, the created output of a layer is used as input for the next layer. MLP-ANN using multiple layers and non-linear activation and can distinguish data that is not linearly separable.

<CRITICAL> An MLP-ANN was generated by using the training data set generated in the step 13 of Procedure 2. We used TIBCO Statistica Automated Neural Networks software to perform this analysis, but any available software (MatLab artificial neural network can be used with the described parameters. Note: TIBCO Statistica ANN has an wizard-style Automated Network Search (ANS) which guides the user step-by-step through the procedure of creating different networks and

choosing the network with the best performance (minimizing the possibility of a lengthy "trial-and-error" process).

14| Design the input layer. Use the fluorescence readouts data obtained in a Step 13 to construct the training set consisted of the S1 -S80 fluorescent outcomes from [λ_{max}], nm from five different concentrations (0.5mM, 1mM, 2mM, 3mM, and 4 mm) of chiral fluorescent ligand along with 16 *ee* values for each concentration, giving 80 solutions as the training set. Constructed input layer should contain the fluorescence values of each *ee* for each used assembly. Consider using multiple emission wavelengths (if microplate reader is suitable for scanning mode or multiple end-point measurements can be performed) to increase the accuracy of the analysis. Based on our experience with MLP neural network and these assays only the 1 emission wavelength [λ_{max}] can give sufficient model for the analysis of unknown samples. Use this wavelength or wavelength ranges (described in step 13 of Procedure 2) to measure emission intensities for all used assemblies. In case of using 2 complexes (VANOL-based and BINOL-based assemblies) making a total of 2 (single end-point measurement) or number of used assemblies x number of emission wavelengths values for each of the 80 cases that differ in *ee* and total concentration. Δ CRITICAL STEP We recommend avoiding use of negative values because ANN does not work well with negative values, therefore % of single enantiomer (R-enantiomer or % of S-enantiomer) must be used instead of *ee* values. Convert the predicted values of %R or %S by ANN into *ee* in a separate step after the analysis using the following expression for recalculation *ee* = (%R-50)*2, [%].

15| Consider to use a multilayered perceptron (MLP) network with 80 inputs, 8 processing units in the hidden layer, and 2 outputs (was selected for most of our tested cases). Perform the training of MLP-ANN with the emission values found for the 80 samples of varying concentrations and *ee* value using back-propagation algorithm, which minimize the discrepancy between the input and the outputs. The resulting outputs of the analysis would be the total concentration and *ee*. Δ CRITICAL STEP The number of hidden layers can be varied until the MLP-ANN could accurately generate the outputs from the inputs of emission values. In most tested cases an MLP-ANN with 3 hidden layers gave the best results, but hidden layers number can be increased to 10 or even 11. Consider whether to assess the predictive ability of the model developed by the MLP-ANN, to do that the collected emissions from [λ_{max}] for "validation" samples (we used 8 validation samples shown as V_1-V_8 on Figure 9), independent of the training set can be subjected for the analysis. Comparison of actual *ee* values and concentrations *vs* predicted will give you a strong sense of the predictive potential of the developed model. We used these emission values as inputs in the MLP-ANN, and tested its ability to predict the accurate *ee* values and concentrations for all assays performed during development.

16| Next, subject true unknown samples (reaction outcomes), prepared completely independent of the training set (samples measured in "analysis plate") and treated with the same sensor array into the previously developed and validated MLP-ANN model (in the steps 14 and 15)to get an outcome layer with their predicted concentrations and %R values. Then, convert the obtained %R values predicted by the network to *ee* values using the following formula *ee* = (%R-50)*2, %. Δ

CRITICAL STEP The *ee* error can be high because of a single outlier that has an *ee* error high due to measurement or the pipetting issue. By excluding the outliers with the Student's T-test, the average absolute error in *ee* can be significantly dropped. The *ee* error can be reduced even further by using a larger training set containing multiple assemblies and multi-mode measurements.

17| The time required to perform the full analysis from measurement and training, to validation, and to analysis by ANN, will be approximately 3 hrs of work. Consider that the ANN network development is a limiting step and once it has been developed by using a training set for a particular analyte it is becoming a very rapid prediction tool. The concentration and *ee* values of 88 unknown samples (analysis plate) will require ≈ 1 hour to be determined , as it take 40 min for the liquid-handler to load a 384-well plate with the premix, and solutions of analytes, then 7 min for the 384-well plate measurement by the reader to record the single end-point emission value of 88 unknown samples with four technical replicates for each sample. After these steps the fluorescence data from the unknown samples can be simply load to the developed ANN, which will predict the concentration and *ee* of unknowns in real time.

18| THIS STEP IS OPTIONAL. Consider whether you data required pretreatment, it is recommended only in case of insufficient data analysis results. Use common methods for data classification, and more specifically supervised pattern recognition methods, which clean up and structuring data by finding mathematical relationships between a set of descriptive variables (chemical measurements) and a qualitative variable (sample identity). These methods resolve multivariate data by reducing their dimensionality into a number of correlated variables, called the principal components (or factors). Consider that any chemometrics software can be used to

run data pretreatment. Absolute majority of these software packages will contain a list of supervised methods including LDA (qualitative data analysis) and SWM (quantitative data analysis). We used the EigenVector Research Inc‘s SOLO software package because it provides point-and-click data-discovery tools including PLS, PCA coupled with SVM and many other multivariate and machine learning methods. SOLO offers integrated modeling guide and access to a large number of analysis and preprocessing techniques which we find very user friendly and suggest that this graphical user interface environment appropriate for novice as well as expert users. Note that to run these methods using any software package will request several sub-sets of data including: Calibration data set (observations only), Calibration data identities, Validation data set (observations only), Observation data identities. Dimensionality reduction outcome would be a new data set with a smaller number of dimensions without substantial information loss. Use this new structured data set to create the ANN model as described in a steps 14-17 of this procedure.

TROUBLESHOOTING

Procedure 1, Step 2 High concentration of the chiral analytes stock will decrease the value of Y_n, in µL which will also decrease the contribution of potential impurities from tested samples in a final mixture with receptor-premix (200 µL) and ensure correct and precise measurement in further steps.

Edge effect in Procedure 1, Step 9-10 Step 9 were the edge effect originated. Step 10 is the step where a plate edge effect becomes evident. Evaporation causes a change in the concentration of assay components in the assay media (acetonitrile) between the center

and outer wells of the microplate. This effect results in an increase in CV values, directly impacting assay robustness (Z-factor).[53]

To prevent edge effect:

- Reduce assay time (amount of time fluids are stored in the well, resulting an overall reduction of evaporation)
- Use a low evaporation lid and plates with evaporation barrier (a lid with condensation rings, and pipet the same media (acetonitrile) in a evaporation barrier wells)
- Use an optically transparent plate sealing or tape.
- In case of 1536 well plates used with working volume (≈10 μL) acetonitrile is deemed too volatile. We recommend using propionitrile distilled on phosphorus pentoxide.

Procedure 1, Step 14 The plot of the "residuals" (which are the differences between the Y values in the input data and the Y values computed by the fit equation) is the main indicator of the fitting function suitable for particular data set. Deviations from linearity will be much more evident in a plot of residuals than in the calibration curve plot. If the residuals are randomly scattered all along the best-fit line, then it means that the deviations are caused by random errors such as instrument noise or by random volumetric or procedural errors; in that case you can use a straight line (linear) fit. Non-linear curve fit, such as a quadratic or cubic fit should be used if the residuals have a smooth "U"-like shape (the calibration curve is curved). In cases when the residual plot has "S" shape, we recommend using a cubic fit.

To reduce the volumetric error (random errors on residuals plot), recalibrate the liquid-handling equipment. To reduce the signal reading error ($R^2 < 0.990$), adjust the instrument conditions (e.g. wavelength, focal height, gain, plate type to reduce scattering) for best signal-to-noise ratio and average several readings of each sample or standard.

When protocol is applied to complex asymmetric reaction samples measurement error can occur due to interferences that influence the measured signal (for example by producing their own fluorescence signal or by reducing or increasing the signal from the analyte-complex). One way to correct for interferences is to use "matched-matrix standards", standard solution that are prepared to contain everything that the reaction samples contain, except that they have known concentrations of chiral analyte.

● TIMING

ROCEDURE 1. Steps 1-14: 3 hours 30 min total for assay

Step 1A, preparation of receptor-premix for amine assay: 30 min

Steps 1B, preparation of receptor-premix for diol assay: 30 min

Step 2-6, preparation of standards: 2 hours

Steps 7–8, plate layout and pipetting: 5-10 min (depending on the number of used wells and plate density)

Steps 9, plate incubation: 2 min

Step 10, fluorescence reading: 5 min per plate using end point experiment mode.

Step 11-14, data analysis: 30 min

PROCEDURE 2. Steps 1-18: 4 hours 30 min total for assay

Step 1, preparation of receptor-premix for assay: 30 min

Steps 2, preparation of stocks: 30 min

Steps 3-5 preparation of standards: 1 hour

Steps 6-12, plates layout and pipetting: 20 min (for one full 384 wells “training” plate and one full 384-wells “analysis” plate)

Steps 13, plates incubation and reading 15 min. -1 hour.

Plates incubation: 2 min;

Fluorescence reading: 5 min per plate using end point experiment mode (10 min in total).

In case of spectral scanning ≈20 min per plate (40 min in total).

Step 14-18, ANN data analysis: 2 hour 30 min for 88 unknowns with four technical replicates in full 384 well “analysis plate”.

ANTICIPATED RESULTS

The anticipated results with the high-throughput fluorescence assay depend on several factors including the number and enantiomeric purity of calibration standards used to calibrate the model.

To ensure precise measurement, make sure that no additional fluorescent species are present in the samples, otherwise overlapping fluorescent signal may occur. In such case whether negative control (sample containing just analyzed material without assembly-premix) can be tested during prescreening step or added to the training plate. For large-scale screening validation of the model, prior prediction with artificial samples or the “testing set” would increase the quality of interpolation. Testing set is generated at the same time as a standards and consist a samples of known *ee* and concentration which used as “artificial unknowns” to validate the model (actual values *vs* predicted values). A successful procedure is dependent upon accuracy in administering the full dispensing volume to the microplate, in a rapid and reliable manner to avoid plate edge

effect. Accurate plate mapping procedure is essential to determine the exact well positions of a 384 well plate used.

Interpreting results of this assays in most cases can be done by linear regression model fitting, but in case of low signal-to-noise ratio chemometric pattern recognition technique such as supervised linear discriminant analysis (**LDA**) (to reduce the dimensionality of the data into a lower dimensional space), can been applied to structure and improve the data separation. For the large number of repetitions data can be subjected to the Student's T-test to exclude outlying data points from set containing noisy information in order to minimize the standard deviation between data within the set of repetitions for each sample. For quantitative nonlinear regression analyses a supervised algorithm such as support vector machine (**SVM**) coupled with principal component analysis (**PCA**) and partial least squares (**PLS**) can be applied (typical data set examples presented in Supplementary Information as Supplementary Data 1-4).

Finally, the high-throughput protocol described below is based on a 384-well plate format but could be easily adapted to a larger density format (1536-wells) using automated liquid handlers or a robotic platform. If these alternatives are pursued, the times required for preparation and completion of the assay may differ significantly from those indicated here.

CFI

The authors declare that they have no competing financial and non-financial interests.

Acknowledgements

We are thankful to our colleague Prof. T. Minami who provided expertise that greatly assisted the research. We are also grateful to Dr. V. Brega for her assistance with parallel asymmetric synthesis, and Prof. S. Gozem for his help with computational modeling of BINOL/VAPOL self-assemblies with analytes.

Author contributions statement

E.S. and P.A. designed the protocol. E.S. performed the experiments. P.A. and T.J. supervised the project. All authors contributed to the writing of the manuscript.

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