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Visualization of Enzyme-catalyzed Reactions using pH Indicators: Rapid Screening of Hydrolase Libraries and Estimation of the Enantioselectivity

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Abstract—The use of pH indicators to monitor hydrolase-catalyzed reactions is described. The formation of acid following an enzyme-mediated hydrolysis causes a drop in the pH that can be visualized by a change in the color of the indicator-containing solution. The best indicators are those showing a color transition within the operational pH range of the hydrolases, like bromothymol blue and phenol red. The enantioselectivity of lipases and esterases can be estimated using single isomers under the same conditions and comparing the color turnover for each one. The method has been tested to quickly evaluate the enantioselectivity of a lipase towards a set of ester substrates and applied to the hierarchical screening of a library of thermophilic esterases.

Introduction

The synthesis of enantiomerically pure compounds (EPC) with one or more chiral centers is one of the most challenging tasks in modern organic chemistry. Enzymes are able to contribute significantly to this challenge and have been increasingly considered as a useful class of catalysts for organic synthesis.[1–4] Among these biocatalysts, hydrolases are well established as valuable tools for the food, pharmaceuticals and fine chemicals industry.[5]

The importance of biocatalysis has led to the search for novel enzymes with singular activities. Recently, extremophilic microorganisms have been investigated as a source of these novel activities.[6–10] Scientists at ThermoGen have developed a set of tools to obtain libraries of thermophilic enzymes by genetic engineering. ThermoCat® esterases consist of a set of 20 stable hydrolases, capable of working well at room or high temperature. It is of high interest to study the selectivity of each enzyme in the library, especially their enantiodiscrimination when exposed to racemic substrates. Time is the limiting factor in carrying out the work when screening a library of enzymatic activities against an array of substrates for either enzyme discovery, enzyme engineering (such as directed evolution) or process optimization experiments. The methods typically employed for this purpose are not often high-throughput amenable (HPLC, TLC, GC) or use an analogue of the target molecule. Thus, a method to run the assays in parallel has to be developed.

One of the most convenient ways to assay an enzyme is through a method that allows the development of color and thus can be used in qualitative as well as quantitative measurements. A number of colorimetric methods to measure enzymatic activity have been described.[11] Hydrolytic enzymes can be rapidly screened with chromogenic (nitrophenyl), fluorogenic (4-methylumbelliferone) or indigogenic (indoxyl) substrates that yield colored products upon hydrolysis. The main limitation of this approach is the presence of the latent colorimetric functionality within the substrate, whose introduction is at least time-consuming and yields a structure for analysis essentially different from the actual target (usually a methyl or ethyl ester). New methods need to be developed in order to identify new biocatalysts using rapid broad screening techniques. This challenge can be successfully overcome by implementing general and

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rapid screening methods to obtain results with genuine substrates without interfering in the reaction.

In this report we describe a simple, colorimetric, pH-responsive method for the rapid screening of hydrolase libraries. This is based on the pH drop that happens as the reaction proceeds, and the carboxylic acid is released. This drop can be monitored by the change in color of a pH indicator, provided the color profile of the indicator falls into the pH range of the enzymatic activity. Several kinds of reactions have been described including enzyme-catalyzed processes with hexokinase[12–14] and cholinesterase,[15] and in enzyme-free studies of carbon dioxide hydration.[16] Since the 1970s, this strategy has been used in kinetic analysis of enzyme reactions. Examples of this include human carbonic anhydrase,[17] amino acid decarboxylases[18] and serine proteases.[19] The progress of the hydrolysis can be monitored by visual inspection of the solution color after the enzyme has been added or by using a microplate reader to get a quantitative reading. Recently, the use of pH indicators has been extended to monitor the directed evolution of an esterase on a plate assay using a whole cell system, rather than the isolated enzyme.[20]

With regards to the enantioselectivity of hydrolases, the idea is to compare the reactivity (in this case color change) of pairs of enantiomers corresponding to the same racemic mixture and thus estimate the enantioselectivity. A large turnover difference between isomers means there is a good chance of successful kinetic resolution if the racemic mixture is subjected to the enzyme displaying such time difference. The method involves the use of single isomers, so the kinetics obtained in this way do not reflect the competition that exists when hydrolyzing the racemic mixture, and therefore, E value is only approximate. Kazlauskas has solved the problem of competition by using a reference non-chiral additive in classical chromogenic substrate assays.[21] The same author developed a quantitative method for the evaluation of the enantioselectivity (without considering the competition factor) for actual substrates based on a pH indicator/buffer system (*p*-nitrophenol/BES) with equal pK_a so the linearity of the color transition allows the quantitation of the enantioselectivity.[22]

Despite its impressive accuracy and sensitivity, the method demands special instrumentation (microplate reader) since the color transition cannot be visualized and involves data management that could be avoided by using a suitable indicator that effectively turns color so the monitoring could be simplified. In the case described below in this paper, the linearity of the assay (and consequently its accuracy) is compromised by the difficulty of choosing a pair buffer/indicator with the same pK_a and yielding color change. The bromothymol blue/potassium phosphate system falls within 0.1 units of pK_a (making it suitable for quantitation) allows a nice blue–yellow color transition and uses a common buffer for hydrolase-catalyzed biotransformations. Our goal is to use the method not for the quantitation but for the screening of large amounts of enzymes and/or substrates in parallel, allowing a quick identification of the highest enantioselectivities, discarding the poor-to-moderate enantioselectivities that will not be acceptable for the development of a biocatalytic resolution. This fits the strategy of a hierarchical screen for the identification of the best catalyst as one of the earliest steps, and eliminates the weakest candidates for a more streamlined process-viability study.[23]

Results and Discussion

The idea of combining the pH drop of hydrolase-catalyzed reactions with the change of color of an indicator dye associated with a pH transition is depicted in Figure 1.

The use of phosphate buffer at a pH equal to its pK_a (7.20)[24] is a standard situation that is compatible with the estability of the majority of lipases and esterases and their activity profile, and also satisfies the need for mild conditions (neutral pH) if sensitive substrates are to be hydrolyzed. It is also usual to use a pH slightly above or below neutral if the enzyme selected shows better activity under these conditions. Table 1 shows some of the pH indicator dyes suitable to follow the hydrolytic reaction by the color change in the range typically used for this class of biotransformations.[25]

We focused our efforts on the study of bromothymol blue (BTB) and phenol red, since their pK_a values are the closest to that of the buffer we were interested in using and their color transitions show high contrast (as opposed to neutral red, whose pK_a is also very close but displays poor distinction between red and amber). One can imagine that if a different operating pH is desired due to enzyme characteristics or process advantages, a different indicator can be used. Preliminary experiments showed that the concentration of indicator dye in the reaction had no effect on the reaction rate, suggesting that the indicator was not acting as an inhibitor of enzyme activity (data not shown). Control experiments using BSA as the protein source caused no change in indicator color and established that pH changes in solution were the result of enzyme catalyzed hydrolysis. Further tests of reaction solutions containing enzymes and indicators without substrates established that color changes in the solutions were not the result of buffer salts or the enzymes themselves.

In order to prove the concept of monitoring enantioselectivity using this method, we chose an array of

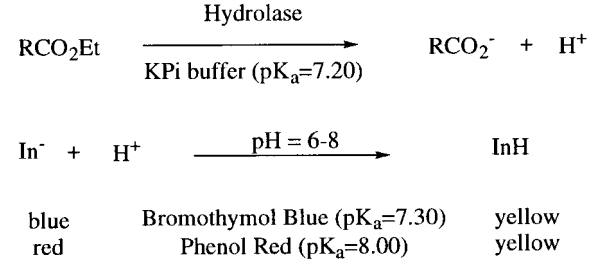

Figure 1.

Table 1. Indicators within the useful range for hydrolase-catalyzed reactions

Name	pK_a	pH range	Color change
Bromothymol blue	7.30	6.0–7.6	Yellow–blue
Neutral red	7.40	6.8–8.0	Red–amber
Phenol red	8.00	6.6–8.0	Yellow–red
Brilliant yellow	–	6.6–7.8	Yellow–red
Cresol red	8.46	7.0–8.8	Yellow–red
Turmaric (curcumin)	–	7.4–8.6	Yellow–red
Metacresol purple	8.3	7.4–9.0	Yellow–purple
2-(2,4-Dinitrophenylazo)-1-naphthol-3,6-disulphonic acid, disodium salt	–	6.0–7.0	Yellow–blue
6,8-Dinitro-2,4-(1H)quinazolinedione	–	6.4–8.0	Colorless–yellow

enantiomeric substrates described in the literature to be efficiently resolved by well-known enzymes. One of the most popular lipase biocatalysts is from *Pseudomonas cepacia* (PSL, Amano Pharmaceutical, Japan), that has been used both for enantioselective hydrolysis as well as transesterifications in organic solvents.[26] The substrates shown in Figure 2 display enantioselectivities in the desired range (from high to very high) for the development of a successful biocatalytic resolution in a cost-effective manner. Figure 2 shows the substrates ready to use in the hydrolytic assay, although many of them are available only as alcohols (both isomers) and have to be acetylated prior to the assay. The enantioselectivity (*E*) values are shown together with the fast-reacting isomer and the kind of reaction described in the literature. Since many of them are available as alcohols, the *E* values described are for the transesterification reaction, not for the hydrolysis, but this experiment will also prove valid (in an indirect way) to assess the enantioselective outcome of the reverse reaction. The substrates used are methyl mandelate acetate (**1**),[27] ethyl 3-hydroxy-3-phenylpropionate (**2**),[28] 1-indanol acetate (**3**),[29] 1-phenylethanol acetate (**4**),[30–37] mandelonitrile acetate (**5**),[38–41] and α-hydroxy-γ-butyrolactone acetate (**6**).[42,43] In the case of mandelonitrile, only the *R* isomer is available and it was compared to the racemic mixture. The comparison is valid if the single isomer (*R*) is the slow reacting one so the racemic mixture will change color faster.

The enantioselectivity is related to the time it takes each enantiomer to change the color. The larger this difference is, the higher the enantioselectivity for the fast-reacting enantiomer. It is important to note that this is only an estimation of the enatioselectivity, and we are seeking large enantioselectivity values, the only ones with practical application. The reactions were set up in a 96-well microplate and the total volume was 200 μL split as follows: 100 μL of PSL enzyme (1 mg, from a 10 mg/mL solution in buffer and spun off to avoid turbidity), 10 μL of substrate solution (60 mg/mL for a final concentration of 3 mg/mL) and 90 μL of 20 mM buffer solution pH = 7.20 containing 0.001% of indicator dye.

The amount of PSL enzyme was 1mg per well, which allowed a convenient time-window of no more than 48 h for the turnover of one of the isomers. If the reaction was slow with 1 mg, the alternatives were to use more enzyme or to raise the temperature (care had to be excercised since evaporation could dry the reaction in a few hours). In any case, the longer the substrates are exposed to the enzyme the better, since close monitoring is not required. Testing high enantioselectivities is an advantage, since the slow-reacting enantiomer is unlikely to react to a significant extent.

The concentration of the buffer employed was determined empirically and established at 20 mM, weak enough to be saturated by the carboxylic acid being produced and drive the pH to the turnover point of the indicator. We have tested also 5 mM and 10 mM KPi buffers, which turn color faster, since their buffer strength is lower. However, we do not advise the use of buffers at too low a concentration (5 mM), since the sensitivity of the system will become very high and minor pH shifts due to the background (after adding the enzyme or the substrate) can lead to inconsistent readings.

Regarding the amount of substrate, 10 μL of single isomers **1**–**4** and **6** (or *R*-isomer **5** and racemic **5**) solution were added which represents (at 60 mg/mL) 0.6 mg per 200 μL well (3 mg/mL final concentration). Since the molecular weight of the substrates is between 144 and 208, the molar concentration of the substrates ranges from 14 to 20 mM. This low concentration allows use of very little pure enantiomer (not often available in large amounts), so 1 mL stock solution would last for 100 experiments, which would test the range of hydrolases commercially available for laboratory testing.

As for the indicators used, Bromothymol Blue was the most extensively employed, although phenol red displayed good contrast, and two of the time points obtained are shown for comparison. Figure 3 depicts the results obtained with both indicators and PSL enzyme.

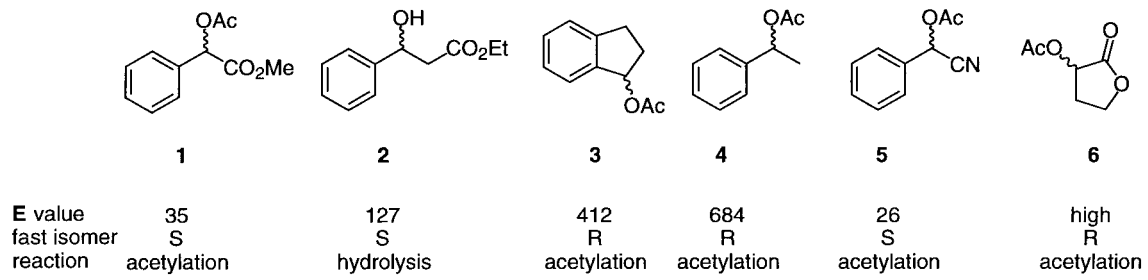

Figure 2.

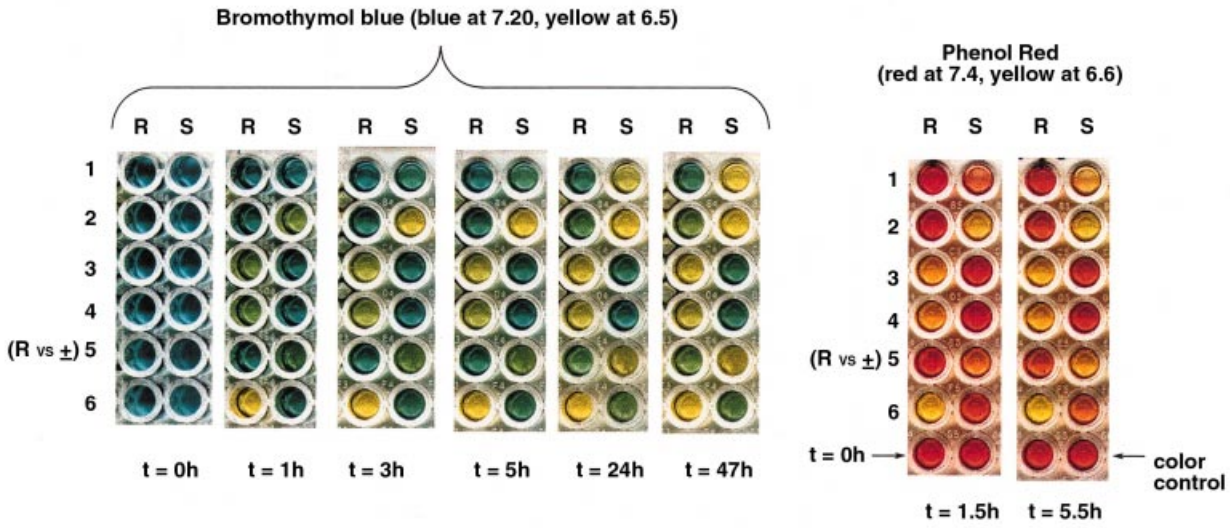

Figure 3.

As can be seen, the enantioselectivity described in Figure 2 corresponds with the faster turnover (development of yellow color) of the preferred isomer in all cases studied. Since the hydrolysis proceeds slowly, the time for monitoring the progress of the reaction is large. While substrates **2**, **3**, **4** and **6** are hydrolyzed in 1–3 h, substrates **1** and **5** required longer reaction times. In this case, the same biocatalyst is used to evaluate a library of substrates, therefore the nature of these will dictate the time frame of the assay. Although the actual biocatalytic transformation will take place under different conditions (especially with respect to stirring) this method estimates initial rates; this could serve as an indication of the enzyme:substrate ratio required for the biotransformation of one substrate versus another. An advantage of the method is noted in the case of mandelonitrile acetate (**5**), with only one of the isomers available. This has to be compared to the racemic mixture, and still yields a valid conclusion: the racemate turns color much faster than the *R* isomer. This is most likely due to the faster hydrolysis of the *S* isomer present in the racemate, so one can conclude that the enzyme is *S*-selective. For cases in which only one of the isomers is available, the method works if the available isomer is the slower reacting one, otherwise both wells will turn color almost simultaneously and no conclusion can be obtained from the experiment. It is not necessary to count on both isomers to run the screen, although success is not guaranteed unless this is the case.

In the case of using phenol red as indicator, Figure 3 also shows essentially the same results. A control without enzyme or substrate is also shown. In this case the pH of the buffer is slightly higher (7.4) in order to get the red initial color. This could be the cause of the substrate *S*-**1** being hydrolyzed apparently faster than in the bromothymol blue case. Phenol red is a good choice if the reaction has to be carried out at slightly basic pH.

An application of the method is illustrated by the hierarchical screening of a ThermoCat® library of thermophilic esterases, against α-hydroxy-γ-butyrolactone (**2**). This compound is a useful synthon in the preparation of 4-substituted-2-hydroxy-butanoates and other optically and physiologically active compounds.[44,45] The resolution can be envisioned as a transesterification or as an hydrolysis of the previously prepared acetate. The latter is the only choice for the colorimetric assay we propose, and we believe the conclusions from the hydrolysis could be translated into the transesterification, at least in terms of enantioselectivity. However, the stability of the enzyme in organic solvent may jeopardize the resolution.

The commercial hydrolase kit from ThermoGen consists of 20 different esterases (E001 to E020) that are organized in a 5×4 microplate array using BTB-containing 20 mM KPi buffer at pH 7.2 and substrate at 3 mg/mL, as explained above. Total volume is 210 μL, and every one of kit enzymes (10 units each) were dissolved in 1 mL of buffer, from which 200 μL was added to the microplate wells (2 units per experiment). This first round of hierarchical screening involved all the enzymes in the kit and the goal was to identify those that react with the racemic substrate and select the fastest enzymes. In this way we did not need to use the precious pure isomers to screen out negatives. Figure 4 depicts the process, and in this first cut many enzymes turned out as potential candidates to the resolution of the hydroxylactone: E004, E009, E011, E013, E015, E017b and E018b were selected for the enantioselectivity assay shown at the bottom of Figure 4. In this case single isomers of the acetate were tested side by side using the same protocol as above. The control (substrate but no enzyme) and the PSL experiment are also shown in the same experiment. It is apparent that enzymes E004, E009, E011 and E013 are good candidates to follow up, since in 20 min they turn to the color corresponding to the *R* isomer, as does PS lipase. On the other hand, E017b appears to be slower and E018b does not seem selective according to the observations during the first 3 h of reaction.

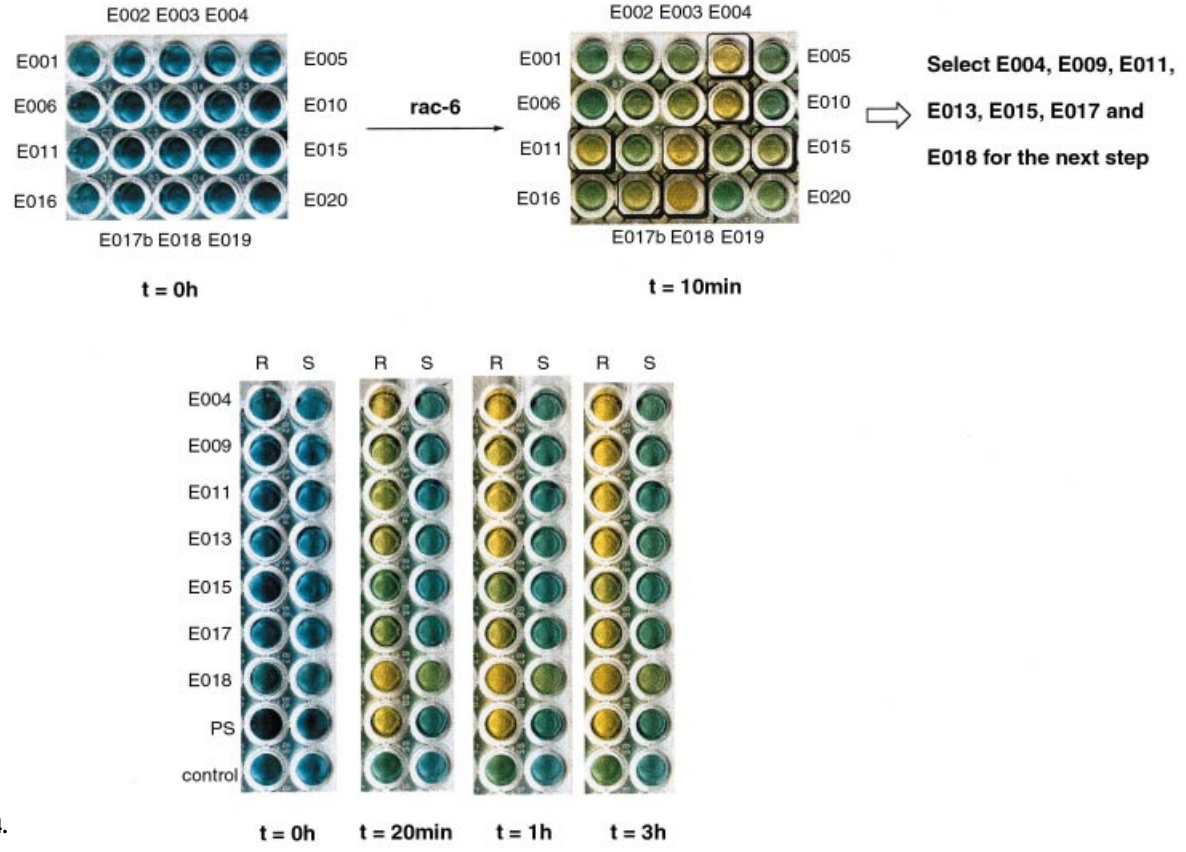

Figure 4.

E004, E009, E011 and E013 were selected for detailed studies involving low-throughput analytical techniques, like HPLC, NMR or GC. Our initial attempts to run the acetylation of the alcohol with vinyl acetate and crude E009 (not immobilized) rendered very low conversion rates. This does not imply that the enzyme is not selective: it is probably not active enough for the reverse reaction under the conditions tested. For PSL the transesterification approach works as described in the literature and the assay based on the hydrolysis provides consistent results.

Conclusion

There is an increasingly important need to develop new biocatalysis processes rapidly and inexpensively, especially for the development of novel pharmaceuticals where time is extremely valuable. The use of a powerful, analytical screening strategy is often the key to speeding up development time at several different levels of the process. In the discovery of novel enzymes, screening plays an important role in identifying which subset of candidates contain an enzyme of interest from a collection of organisms, clone banks, or enzyme libraries.[23] Directed evolution approaches to engineer custom biocatalysts requires powerful screening strategies to sift through large mutant pools to find enzymes with properties that have often been only slightly altered against a high activity background. Finally, process optimization and development can often take an excessive amount of time, especially to perform a comprehensive analysis of different reaction conditions including temperature, pH, cosolvent, reaction time, and other parameters, both individually and in combination. This type of analysis requires the implementation of a rapid, high throughput screen which is amenable to automation and use in a hierarchical strategy. By using a strategy such as the pH detection method described here as a tool to optimize reaction parameters (such as conditions for the stereoselective transformation of a molecule), only a small subset of reactions needs to be analyzed in more quantitative detail (HPLC), thus significantly increasing the number of samples and conditions which can be analyzed in a short period of time.

While twenty enzymes were until recently considered a large number to screen in the development of a new biocatalytic process, it is only a modest number by today's standards. Increasingly, enzyme libraries are becoming larger and larger, and an effective tool for screening and comparison is becoming a necessity. The pH-based approach demonstrated here is an extremely powerful tool that holds a great potential for applications which require extensive enzyme screening.

Materials and Methods

The enzymes used were obtained from Amano Pharmaceutical (Japan) and ThermoGen, Inc. (Chicago, USA). PSL was dissolved in buffer and centrifuged before use to remove insoluble material. Both single isomers (*R* and *S*) of methyl mandelate, 1-indanol, 1-phenylethanol,

and α-hydroxy-γ-butyrolactone were purchased from Aldrich (Milwaukee, USA) together with R-mandelonitrile acetate (*R*-5) and its corresponding racemic mixture. These were derivatized by acetylation to obtain both isomers of **1**, **3**, **4** and **6** together with the racemic **5**. *R*- and *S*-ethyl 3-hydroxy-3-phenylpropionate (**2**) were purchased from Fluka (Switzerland) and used as is. The phosphate salts and the indicators bromothymol blue and phenol red were obtained from Aldrich (Milwaukee, USA). Assay protocols are described throughout the paper for simplicity.

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