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High-Throughput Screening of Tissue-Nonspecific Alkaline Phosphatase for Identification of Effectors with Diverse Modes of Action

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Abstract

A protocol for the identification of effectors of tissue-nonspecific alkaline phosphatase (TNAP) is described. It is based on highly sensitive method for detecting TNAP activity. A dioxetane-based substrate after dephosphorylation by TNAP undergoes a series of chemical transformations resulting in light production. The light intensity serves as a quantitative measure of the velocity of the TNAP catalysed reaction in the steady state. The protocol includes guidelines for the optimization of the assay and execution of the high-throughput screening in multiwell plates. The assay is sensitive to the influence of diverse effectors of TNAP as long as the assay optimization steps are repeated for each new batch of the enzyme; full optimization is accomplished in under two days. Depending on the available equipment 10,000-100,000 compounds could be screened in 8-hour period. This protocol provides thousands-fold more sensitive and tenfold faster way of screening TNAP, when compared with a conventional colorimetric assay with p-nitrophenyl phosphate.

Keywords

alkaline phosphatase; chemiluminescent assay; enzyme assay; functional assay; high-throughput screening

INTRODUCTION

Alkaline phosphatases (E.C.3.1.3.1) (APs) are dimeric enzymes, present in most organisms¹, where they catalyze the hydrolysis of phosphomonoesters. In humans, three of the four isozymes are tissue-specific, i.e., the intestinal (IAP), placental (PLAP), and germ cell (GCAP) alkaline phosphatases; the fourth AP is tissue-nonspecific (TNAP) and is expressed in bone, liver and kidney². Recent studies have provided compelling evidence that a major role for TNAP in bone tissue is to hydrolyze extracellular inorganic pyrophosphate, PP_i, a powerful mineralization inhibitor, thus ensuring normal bone mineralization. These studies

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AUTHOR CONTRIBUTIONS

J.L.M. identified and validated TNAP as a potential therapeutic target. E.A.S. designed and optimized the assay and performed the screening. E.A.S. wrote the paper with guidance and discussion from J.L.M.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

suggest that TNAP may be an attractive therapeutic target for the treatment of calcification disorders.

Identification of small-molecule effectors of TNAP may not only lead to the therapeutic treatment of disorders where the involvement of TNAP is unambiguously documented, but would also provide invaluable research tools to study its role in diverse physiological and pathological conditions. Even though the enzyme has been known for many decades, its mechanisms of action in diverse biological processes is still unclear and is being actively investigated². Many phosphocompounds have been documented as substrates of TNAP, i.e., pNPP, α -naphthylphosphate, β -glycerophosphate, phosphoserine, PP_i, pyridoxal-5'-phosphate (PLP), phosphothreonine, phosphotyrosine, phosphatidates, polyphosphates, ATP, ADP, AMP, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and others². However, only two, PP_i and PLP, have been unequivocally confirmed as physiological substrates of TNAP as abnormalities in their metabolism explain the phenotypic changes of hypophosphatasia, a genetic disease resulting from TNAP deficiency³⁻⁵.

TNAP's *in vitro* reaction demonstrates significant rate acceleration in the presence of amino-containing alcohols acting as phosphate-acceptor substrates in transphosphorylation reactions. The physiological role of this reaction is unknown and it is unclear which if any metabolite could be acting as a natural transphosphorylation agent. Thus, there is an unmet need for chemical tools that could help in elucidating the physiological role of TNAP in normal and pathological states.

In order to make the assay sensitive to the influence of TNAP effectors with diverse modes of action, we based the assay optimization on the knowledge of the TNAP reaction mechanism (Figure 1). The molecular mechanism of AP catalysis is common to the enzyme from various species and tissues⁶. The initial AP (E in Figure 1) catalyzed reaction consists of a substrate (DO-P_i) binding step, phosphate-moiety transfer to the active site serine residue and product alcohol (DOH) release. In the second part of the reaction, phosphate is released through hydrolysis of the covalent intermediate (E-P_i) and dissociation of inorganic phosphate (P_i) from the non-covalent complex (E·P_i). In the presence of amino-containing alcohol molecules (AOH), P_i is also released via a faster transphosphorylation reaction branch.

AP assays commonly utilized in clinical practice⁷ (see also WHO Guidelines on Standard Operating Procedures for Clinical Chemistry, Section B http://www.searo.who.int/EN/Section10/Section17/Section53/Section481_1761.htm) are based on dephosphorylation of p-nitrophenol phosphate (pNPP, see its structure in Figure 2a) in the presence of high concentrations of an amino-containing buffer, such as 2-amino-2-methyl-1-propanol or diethanolamine (DEA). Besides maintaining alkaline pH, these buffers also serve as substrates for the AP transphosphorylation reaction present at their saturating levels. This type of assay is very easy and inexpensive to perform. However, detection of the product based on its absorbance at 405 nm results in significant optical interference from library collections. Many small molecules absorb in the range of wavelengths utilized in the assay. In addition, specific activity of TNAP in the colorimetric assay is low, the condition that translates in utilization of large quantities of the enzyme in the assay. This condition could be partially counteracted by extended incubations of the reaction mixture to promote accumulation of p-nitrophenol product. Although, high stability of TNAP in the assay conditions permits several days long incubations, a slow spontaneous hydrolysis of pNPP in aqueous solutions and evaporation of solutions should be taken into account when selecting the appropriate incubation time. After the incubation, absorbance in the plate could either be

assessed right away or after stopping the reaction. The latter approach also permits storing the plates for some time, e.g. overnight, prior to measuring the signal.

To-date, all the inhibitors available for TNAP, as for all other mammalian APs, are uncompetitive in nature. They include L-homoarginine⁸, as well as some non-related compounds, such as levamisole⁹ and theophylline¹⁰. These known inhibitors of TNAP are not entirely specific for this AP isozyme, have low affinity and are not particularly good at inhibiting the pyrophosphatase activity of TNAP.

The protocol described here allows for the identification of TNAP effectors with diverse modes of action (MOA). It has been successfully utilized for a high-throughput screening (HTS) campaign¹¹⁻¹². In the scope of this campaign the novel TNAP assay was implemented and optimized. The assay employs the luminescent detection method based on CDP-star, a 1,2-dioxetane-based substrate (see its structure in Figure 2b) that was invented and commercialized by Tropix, Inc. for detection of APs in blotting applications¹³⁻¹⁴. The overall chemical reaction represents a cascade of consecutive steps triggered by AP-catalyzed dephosphorylation of the substrate (Figure 3a). This initial step leads to the generation of an unstable product that decomposes to a stable product with concomitant light production in the lower portion of visible spectrum (Figure 3b). At the San Diego Center for Chemical Genomics (SDCCG) (that was recently renamed into Burnham Center for Chemical Genomics, then Conrad Prebys Center for Chemical Genomics) this detection method was successfully implemented as a homogeneous HTS assay for AP activity [11]. This approach is very robust and fast, as the exact determination of AP steady-state velocity, that is proportional to the light intensity at any given moment, is possible within minutes for an entire 384- or 1536-well plate.

In the scope of the project this assay was applied and optimized for TNAP. The concentrations of the enzyme, substrates and buffer components were adjusted to achieve maximal assay sensitivity. Subsequent HTS and follow-up studies led to the identification of several potent and selective chemical series targeting TNAP and exhibiting diverse MOA properties¹¹.

The luminescent TNAP assay is ~1000-fold more sensitive than the colorimetric assay, and thus, requires much lower concentration of TNAP protein in the assay. High sensitivity of the assay enabled SAR studies performed at the SDCCG that led to identification of 5-nM inhibitor of TNAP¹². In addition to superior sensitivity, the luminescent assay has 100-fold wider dynamic range comparing with the colorimetric assay, making the assay applicable for detection of inhibitors and activators of TNAP in a single screening campaign. A minor drawback of the luminescent assay is caused by a transient nature of the luminescence. The photons could not be “stored” or accumulated and must be detected at the time of their generation. Stability of the signal output in the luminescent assay over a period of more than 5 hours neutralizes this shortcoming, since the luminescence could be measured at any point during this time without loss of signal.

EXPERIMENTAL DESIGN

Sensitivity of a functional assay is defined by the degree of enzyme's saturation with its substrates. It is common knowledge that competitive inhibitors exhibit diminished potency in the presence of high substrate concentrations, whereas uncompetitive inhibitors have maximal potency at the saturating level of substrate. In our experiments, we set the substrate concentrations to match their respective K_m -values; these conditions are optimally suited for identification of various classes of inhibitors. The kinetic parameters of the luminescent TNAP reaction were determined for both CDP-star and DEA substrates. Experimental data

for CDP-star and DEA substrates concentration optimization are shown in Figures 4 and 5, respectively. The data were analyzed by nonlinear regression using Michaelis-Menten equation (Equation 1):

$$v = \frac{V_{\max} \cdot S}{K_m + S} \quad (\text{Equation 1})$$

where v is the steady-state rate of the enzymatic reaction, S is the concentration of the corresponding substrate, $V_{\max}$ is maximal velocity of the reaction and K_m is its Michaelis constant in respect to the studied substrate S .

The concentration of the enzyme is another important parameter of the assay that contributes to its sensitivity. Initial velocity of the enzymatic reaction is directly proportional to the enzyme concentration. Departure from a steady state, such as rapid consumption of the substrate in the presence of high enzyme concentration, results in a deviation from the linearity of the reaction (as seen Figure 6). The lower limit of the enzyme concentration is determined by non-enzymatic processes responsible for the assay background. The separation between the highest and lowest enzyme concentrations detectable in the assay defines its dynamic range. The data for the optimization of TNAP concentration in the assay is shown in Figure 6. Limit of detection (LOD) of the assay is calculated using the following formula:

$$LOD = \frac{3 \cdot \sigma}{Slope} \quad (\text{Equation 2})$$

where σ is the standard deviation of the blank samples without TNAP and Slope is the slope of the corresponding calibration curve activity vs. enzyme concentration. The LOD value provides a quantitative measure of the assay sensitivity and is equal to the minimal concentration of the enzyme that could be reliably distinguished from the background. The actual LOD values and thus the concentration of the enzyme necessary for screening depend on the sensitivity of a plate reader available at a particular lab. The selection of the enzyme concentration with respect to the LOD value determines the assay robustness and its performance in HTS. The concentration of enzyme that is at least 20-fold over the LOD is normally selected for the screening; higher concentrations of the enzyme are preferred since they usually provide better assay performance.

The assay performance is quantified using a Z' -factor that is calculated according to the formula published by Zhang *et al.*¹⁵:

$$Z' = 1 - \left| \frac{3 \cdot \sigma_+ + 3 \cdot \sigma_-}{\mu_+ - \mu_-} \right| \quad (\text{Equation 3})$$

where μ_+ and μ_- are mean values and σ_+ and σ_- are standard deviations of the positive (fully inhibited enzyme) and negative (uninhibited enzyme) control wells of the plate, respectively. The Z' -factor is linked to the concentration of the enzyme in the assay. For the TNAP concentration optimization experiment (Figure 6), the Z' -factor has values above 0.5 corresponding to $[TNAP]/LOD > 20$ and reaches its maximal value at $[TNAP] \approx 150 \times LOD$.

The knowledge of the optimal time point for the data acquisition and DMSO tolerance of the assay is also required for a successful HTS campaign. TNAP is insensitive to the DMSO concentration present in the assay at or below 2%. We determined that the luminescent signal in the TNAP reaction reaches its steady-state output 15 min after initiation of the reaction and is stable over at least 5 hours. Extended life of the luminescent signal is an

important characteristic of the assay since it allows performing the assay on a luminescent plate reader with any reading speed.

To validate the sensitivity of the assay after completing its optimization we tested the effect of levamisole, a known inhibitor of TNAP, on its activity (Figure 7). Levamisole inhibited TNAP with an IC_{50} value equal 20 μ M. This value is almost identical to the one previously obtained in the colorimetric pNPP-based assay¹⁶. Applications of the screening to other mammalian AP isozymes would require the substitution of levamisole for other, more appropriate, isozyme-selective inhibitors as controls, such as L-Phenylalanine for IAP and PLAP, L-Leucine for Germ Cell-type AP (GCAP), and others¹⁻². In the final validation stage, the assay performance is tested in the desired plate format and using the same reagents, materials, equipment, and liquid handling techniques as would be employed during the HTS stage (Figure 8). The Z' -factor equal to 0.76 attests that the assay is robust and HTS-ready.

The final concentrations of the components in the fully optimized TNAP assay at SDCCG were as follows: 100 mM DEA, pH 9.8, 1.0 mM $MgCl_2$, 0.02 mM $ZnCl_2$, 50 μ M CDP-star and 2% DMSO. Positive control wells contained 1 mM levamisole; negative control wells contained 2% DMSO. Compounds were added to the appropriate wells at 20 μ M concentration. Compound efficacy is estimated after data conversion to percent inhibition (Equation 4):

$$\% \text{ inhibition} = \frac{(NC - Y)}{(NC - PC)} \cdot 100\% \quad (\text{Equation 4})$$

where NC and PC represent the average values of negative and positive control wells respectively, Y is the value observed in a well with the tested compound.

The protocol described in this paper can be easily adapted to utilize different plate densities (1536-, 384- or 96-wells) and diverse liquid handling and screening equipment. In a low throughput mode, the assay could be performed using 8- or 12-channel pipettes. For high throughput screening specialized liquid handling equipment is required. The intermediate-dilution compound plates are sometimes necessary to ensure that the DMSO concentration in the assay is kept below its tolerance limit. The library compounds are dissolved in 100% DMSO and thus keeping its concentration in the assay at 1-2% requires nanoliter to low-microliter dispense volumes attainable only with specialized screening instrumentation. Utilization of intermediate-dilution plates allows compound dispense with common liquid handling equipment without surpassing the DMSO tolerance limit. Extended stability of the assay reagents enabled us to run the assay in batches of multiple plates. The throughput of the primary screening assay could be easily adjusted and, depending on the laboratory-specific equipment and resources, may vary from several compounds to >100,000 compounds a day.

The same HTS assay is utilized for confirmation and characterization of the hits identified in the primary screening. Compounds are either cherry-picked from the source plates or from the “daughter” (intermediate-dilution) plates containing partial DMSO- aqueous compound solutions. If the intermediate-dilution plates are utilized then in the light of lower compound stability in aqueous solutions, it might be beneficial to perform the reconfirmation step on the day of compound dilution and screening. Low hit rates observed in the TNAP screening (described in the **ANTICIPATED RESULTS**) makes this a feasible arrangement. In addition, the HTS reagents could be prepared in excess and utilized for the hit confirmation on the same or next day without loss of performance.

The dose-response confirmation performed at the SDCCG is based on a 2-fold serial dilution of compounds in 100% DMSO. We utilize 10-point dose response curves in duplicate for each compound tested. In 384-well format, columns 1-2 and 23-24 are reserved for positive and negative controls, respectively. Data is analyzed using nonlinear regression and fitted to the Hill equation (Equation 5):

$$\% \text{ inhibition} = \frac{100\% \cdot [C]^{n_H}}{IC_{50}^{n_H} + [C]^{n_H}} \quad (\text{Equation 5})$$

where [C] is compound concentration in micromolar units, IC_{50} is the concentration value of half-maximal inhibition also in micromolar units, n_H is the Hill coefficient of the curve.

The protocol described in this paper is amenable to and was successfully applied for screening of three isozymes of APs, one of them from two different sources, at the SDCCG (PubChem AID's 518, 690, 1017 and 1019; http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=518&loc=ea_ras, http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=690&loc=ea_ras, http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1017&loc=ea_ras, http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1019&loc=ea_ras). At different stages, the screening was performed using diverse instrumentation ranging from bulk dispensers (as described in this paper) to a fully automated ultra-high throughput screening system. The protocol's application to TNAP screening is described in more detail in the ANTICIPATED RESULTS.

MATERIALS

REAGENTS

- CDP-star substrate (2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo [3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate disodium salt; New England Biolabs (240 County Road, Ipswich, MA 01938-2723) cat. no. N7001S.
- DEA (diethanolamine): Sigma-Aldrich, USA (3050 Spruce St., St. Louis, MO 63103), cat. No 31590.
- $MgCl_2$ (magnesium chloride solution): Sigma-Aldrich, USA, cat. No M1028
- $ZnCl_2$ (zinc chloride): Sigma-Aldrich, USA, cat. No 229997
- Levamisole hydrochloride [(-)-Tetramisole hydrochloride]: Sigma-Aldrich, USA, cat. No L9756
- White small volume plates: Greiner Bio-One North America (4238 Capital Drive, Monroe, NC 28110), cat no 784075

REAGENT SETUP

▲ **CRITICAL** Prepare enough reagents for the screening of 1.5-fold the number of actual plates. This excess is necessary to offset the unusable ("dead") volumes associated with the operation of liquid dispensers. In addition, the remaining solutions will be utilized to repeat the plates with suboptimal performance and could be utilized for confirmation of the primary hits the same or next day. All the working solutions, including TNAP, CDP-star, levamisole and 10% DMSO, are stable at ambient temperatures (18-25°C) over several days with no significant loss of performance. However, one should exercise caution and avoid unnecessary use of aged solutions, since prolonged storage at ambient temperatures could affect the enzyme stability and thus its response to compounds.

Buffers and solutions Prepare the following solutions:

- High-DEA buffer containing 1M DEA, pH 9.8, 1 mM MgCl₂, 0.02 mM ZnCl₂.
- No-DEA buffer containing 25 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid), pH 9.8, 1 mM MgCl₂, 0.02 mM ZnCl₂.
- Assay buffer containing DEA concentration equal to $2.5 \times K_{m(DEA)}$, pH 9.8, 2.5 mM MgCl₂, and 0.05 mM ZnCl₂.
- TNAP working solution that contains 2.5-fold its desired concentration in the assay buffer. Fresh solution is prepared on the day of the screening.
- CDP-star working solution that contains its concentration equal $2.5 \times K_{m(CDP-star)}$ in Milli-Q water.
- Levamisole hydrochloride working solution is 5 mM in 10% DMSO. The solution is prepared on the day of screening from a 50 mM levamisole solution in DMSO stored at -80°C .

Compound library The compounds to be tested are dissolved in DMSO to a concentration of 1 mM. Compounds that do not readily dissolve could be sonicated during the solvation stage.

EQUIPMENT

▲ **CRITICAL** Utilization of plate readers and liquid dispensers equipped with the stackers decreases the manual handling of the plates and increases the throughput of the HTS. Besides eliminating plate-handling operator-induced mistakes it also allows multitasking of the steps of the screening process leading to significant time saving.

EnVision plate 2103 multilabel reader with stackers (PerkinElmer <http://las.perkinelmer.com/Catalog/ProductInfoPage.htm?ProductID=2104-0010>)

WellMate microplate dispenser (Thermo Scientific Matrix <http://www.matrixtechcorp.com/automated/pipetting.aspx?id=11>) with stacker unit (<http://www.matrixtechcorp.com/automated/pipetting.aspx?id=57>)

Multidrop Combi microplate dispenser (Thermo Scientific, <http://www.thermo.com/com/cda/product/detail/0,1055,12440,00.html>)

Beckman Coulter Dual Bridge Biomek FX liquid handler (span 8/384 well format) (http://www.beckmancoulter.com/products/instrument/automatedsolutions/Biomek/biomekfxp_inst_dcr.asp)

Eppendorf bench-top centrifuge 5810 (<http://www.eppendorfna.com/int/index.php?l=131&pb=84e52976b687ffa7&action=products&contentid=1&catalognode=10019&productpage=1>) with swing-bucket rotor A-4-62 and microplate adapters (<http://www.eppendorfna.com/int/index.php?l=131&pb=825e8d174d3f883d&action=products&contentid=1&catalognode=10045>).

PROCEDURE

Optimization of buffer conditions of TNAP luminescent reaction ● TIMING 1-2 days

1. Determine the activity of TNAP in high-DEA buffer in the presence of varied concentrations of CDP-star substrate, e.g. between 0 and 500 μM . Activity of TNAP is equal to the luminescent light intensity at the steady state of the overall reaction; due to a lag-phase of the detection step the steady-state is normally achieved after 15-20 min. Analyze the data and determine the $K_{m(CDP-star)}$ value

using nonlinear regression within an appropriate curve-fitting software, e.g. GraphPad Prism (GraphPad Software, Inc.), according to Equation 1 as seen in Figure 4.

2. Determine the activity of TNAP in No-DEA buffer containing CDP-star at the concentration equal to its K_m value in the presence of varied DEA substrate concentration. Analyze the data and determine the $K_{m(DEA)}$ value using nonlinear regression within an appropriate curve-fitting software, e.g. GraphPad Prism, according to the Equation 1 as seen in Figure 5.

▲ **CRITICAL STEP** It is imperative to carefully determine the K_m -values of the substrates. Triplicate or quadruplicate data points give more reliable results.

- 3 Determine TNAP concentration ([TNAP])-dependent activity (Figure 6) in the presence of CDP-star and DEA concentrations equal to their respective K_m values determined in steps 1-2. Calculate LOD using Equation 2.

▲ **CRITICAL STEP** It is important to carefully determine the LOD and the range of rate linearity for TNAP. The concentration of the enzyme relative to the LOD value of the assay determines the assay performance. The minimal concentration of TNAP in the assay is $20 \times \text{LOD}$.

- 4 Validate the assay performance using the conditions determined in steps 1-3. Obtain the activity of TNAP within positive and negative control conditions each replicated in multiple wells. Calculate the Z' -factor using Equation 3. The assay validation could be combined with an expanded version of the step 3 as shown in Figure 6. In this case, the plate is added with serially diluted TNAP in the working buffer such that each concentration of TNAP occupies 2-4 columns. Buffer without TNAP is also included as part of the experiment. The reaction is initiated by adding CDP-star working solution to the whole plate. This experiment allows simultaneous determination of the LOD for TNAP and Z' -factor values for different concentration of TNAP, making easy the selection of the appropriate TNAP concentration for HTS.

High throughput screening assay ● **TIMING** 0.5-1 day (per 20-50 plates)

- 5 Dilute the 1mM compound stocks 10-fold with Milli-Q water using a WellMate microplate dispenser. Prepare a batch of 20-50 plates according to the intended throughput.
- 6 Transfer 4 μL of 100 μM compounds in 10% DMSO in columns 3-24 of 384-well white small volume plates using a 384-well head-equipped liquid handler.
- 7 Prepare TNAP working solution by diluting TNAP in the assay buffer
- 8 Prepare CDP-star working solution by diluting CDP-star in Milli-Q water
- 9 Prepare levamisole working solution by dilution of 10X stock solution in Milli-Q water.
- 10 Test the assay performance by preparing quadruplicate wells for each of the following conditions:

	Negative control test wells	Positive control test wells	Background signal test wells
10% DMSO	4 μL	0 μL	4 μL

	Negative control test wells	Positive control test wells	Background signal test wells
Levamisole working solution	0 uL	4 uL	0 uL
TNAP working solution	8 uL	8 uL	0 uL
CDP-star working solution	8 uL	8 uL	8 uL
Assay buffer	0 uL	0 uL	8 uL

▲ **CRITICAL STEP** This step is necessary to confirm that working solutions are prepared correctly and ready for HTS. At least 20-fold difference in activity is expected between the DMSO- and levamisole samples.

? TROUBLESHOOTING

- 11 Add 4 μ L of 10% DMSO to column 2 (negative control) of each plate using a Multidrop Combi microplate dispenser.
- 12 Add 4 μ L of levamisole working solution (positive control) to column 1 of each plate using a Multidrop Combi microplate dispenser.
- 13 Add 8 μ L of CDP-star working solution to all the wells of each plate using a Thermo Matrix WellMate dispenser.
- 14 Add 8 μ L of TNAP working solution to all the wells of each plate using a Thermo Matrix WellMate bulk dispenser.
- 15 Briefly centrifuge the plates and incubate them for 30 min at room temperature.
- 16 Measure luminescence using an Envision plate reader (Perkin Elmer) in the whole batch of prepared plates.
- 17 Determine the Z'-factor of each plate using Equation 3
- 18 Reject the data for the plates with Z'-factors below 0.5, and repeat the screening of the plates
- 19 Calculate percent inhibition in each well using Equation 4
- 20 Identify hits that meet "cutoff" criteria, i.e., demonstrate 50% inhibition

■ **PAUSE POINT** Hit confirmation could be performed on the day of screening or the following day.

TNAP hit confirmation ● TIMING 0.5-1 day

- 21 Cherry-pick hits and dilute them appropriately to obtain 100 μ M solution containing 10% DMSO

▲ **CRITICAL STEP** If the step is performed on the same day as the HTS, the intermediate-dilution plates from step 5 could be utilized.

- 22 Dispense 4 μ L of compound solution in replicate wells in columns 3-24 of Greiner 384-well white small volume plates.
- 23 Repeat steps 6-19
- 24 Identify hits, i.e., the compounds with 50% average inhibition value calculated from replicate wells

? TROUBLESHOOTING

TNAP dose-response confirmation screening protocol ● TIMING 1 day

- 25 Obtain re-supply of the hits and prepare 10-mM compound DMSO solutions
- 26 Dilute the hit compounds in DMSO solution to obtain 10-point curves with 2-fold serial dilution. Dilute levamisole in parallel with the compounds within each plate
- 27 Dilute compounds into intermediate (10% DMSO) plates to achieve 500 μ M as highest concentration
- 28 Transfer 4 μ L aliquots of compound solutions into assay plates
- 29 Repeat steps 6-19.
- 30 Analyze the data using Equation 5.

? TROUBLESHOOTING

● TIMING

Steps 1-4, Optimization of buffer conditions of TNAP luminescent reaction: 1-2 d

Steps 5-20, **High throughput screening assay**: 0.5-1 d (20-50 plates)

Steps 21-24, **TNAP hit confirmation**: 0.5-1 d

Steps 25-30, TNAP dose-response confirmation screening protocol: 1 d

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

This protocol describes the implementation of novel homogeneous assay for TNAP and its application in HTS. During the implementation stage, the screening conditions are optimized for a particular alkaline phosphatase preparation to identify the most sensitive and reliable screening conditions. The screening portion of the protocol efficiently captures TNAP effectors and separates them from rare false positives.

Optimization of the substrate and enzyme concentrations lays foundation to the assay sensitivity; thus, accurate determination of K_m values for the substrates is very important. Too high or too low concentration of TNAP would lead to erroneous results. For example, if the signal in the presence of saturating concentrations of CPD-star and/or DEA (see Figures 4 and 5) is less than 10-fold the signal obtained in the absence of the corresponding substrate then higher concentration of TNAP should be employed. On the other hand, too high a concentration of TNAP would result in premature substrate consumption leading to reduced activity and significant decline of signal over time.

In the absence of *a priori* knowledge of enzyme preparation activity and its affinity to substrates, going through a couple of cyclic re-iterations of the steps 1-3 would help to ensure that both enzyme and substrate concentrations are optimally adjusted. Alternatively, substrate-dependent activity might be measured in the presence of different concentrations of TNAP to provide enzyme concentration-independent values of K_m along with enzyme-dependent curves for each substrate concentration.

Results anticipated from enzyme-dependent experiments are shown in Table 2. Background activity inherent to the reaction in the absence of enzyme is utilized as a positive control of

the reaction. Average and standard deviation values determined for each TNAP concentration from all replicate wells are utilized to calculate Z'-factor values. Similarly to this experiment, Z'-factor is also determined at the assay validation stage (Figure 8) and during the HTS.

We found that the CDP-star-based reaction provided much higher sensitivity and required much less enzyme than the commonly utilized colorimetric assay. The luminescent assay also provided much broader dynamic range enabling us to simultaneously monitor both inhibition and activation of the enzyme.

Using the described protocol at SDCCG we successfully screened TNAP activity vs. 64K compound collection. The average Z'-factor for the full screen was equal to 0.82, ranging from 0.75 to 0.89. From the primary screen, 73 primary positives were identified resulting in 0.11% hit rate. The primary HTS hits were re-tested in single-concentration mode and 55 primary hits were re-confirmed. Further characterization of the hits led to the identification of 53 compounds with concentration-dependent inhibition and IC₅₀ values below 20 μ M. The results of the screening are summarized in Table 3. This high percentage of reconfirmation attests to the robustness and reliability of the assay.

As a result of the screening, multiple chemical series of both the inhibitors and activators were identified. Attesting to the assay sensitivity, among them we identified CDP-star-competitive and uncompetitive inhibitor classes^{12,17}. The former ones are discovered for APs the first time¹². The inhibitors of both classes were active in the HTS assay conditions, but demonstrated a characteristic effect of performing the assay at different concentrations of CPD-star on IC₅₀ values. The chemical series were also diverse with respect to the DEA substrate and contained representatives of both competitive and non-competitive classes^{12,17}. The fact that hits identified through the screening have such diverse mechanistic properties confirms that the assay was sensitive to compounds with diverse MOA. The full account of the TNAP HTS campaign and follow-up studies performed at SDCCG is provided elsewhere¹¹.

The protocol described in this paper is applicable for assay optimization and the HTS of various APs. At the SDCCG, the protocol was utilized for three APs and led to their successful HTS campaigns. Although, the protocol specifically describes the screening performed in 384-well plates, it could be easily adapted to screening in 96- or 1536-well plates by increasing the volume 2.5-fold or by reducing the volume 4-fold, respectively. At the SDCCG we successfully performed additional TNAP and IAP 200K-screening in 1536-well plates.

Although in the protocol we described utilization of some specific instrumentation, it could easily be substituted with analogous instruments without sacrificing the assay performance. Optimization of the assay with the instrumentation available in a screening lab would ensure its successful application for identification of TNAP and other AP effectors. Applications of the screening to other mammalian AP isozymes would require the substitution of levamisole for other, more appropriate, isozyme-selective inhibitors as controls, such as L-Phenylalanine for IAP and PLAP, L-Leucine for Germ Cell-type AP (GCAP), and others¹⁻².

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REFERENCES

1. McComb, RB.; Bowers, GN., Jr.; Posen, S. Alkaline phosphatase. Plenum Press; New York, USA: 1979.
2. Millán, JL. Mammalian alkaline phosphatases. From biology to applications in medicine and biotechnology. Wiley-VCH Verlag GmbH & Co; Weinheim, Germany: 2006. p. 1-322.
3. Millán JL, et al. Enzyme replacement therapy for murine hypophosphatasia. *J. Bone Miner. Res.* 2008; 23:777–787. [PubMed: 18086009]
4. Fedde KN, et al. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J. Bone Miner. Res.* 1999; 14:2015–2026. [PubMed: 10620060]
5. Whyte MP, et al. Perinatal hypophosphatasia: Tissue levels of vitamin B6 are unremarkable despite markedly increased circulating concentrations of pyridoxal-5/-phosphate. Evidence for an ectoenzyme role for tissue-nonspecific alkaline phosphatase. *J. Clin. Invest.* 1988; 81:1234–1239. [PubMed: 3350970]
6. Holtz KM, Stec B, Kantrowitz ER. A model of the transition state in the alkaline phosphatase reaction. *J. Biol. Chem.* 1999; 274:8351–8354. [PubMed: 10085061]
7. Stinson RA. Kinetic parameters for the cleaved substrate, and enzyme and substrate stability, vary with the phosphoacceptor in alkaline phosphatase catalysis. *Clin. Chem.* 1993; 39:2293–2297. [PubMed: 8222223]
8. Fishman WH, Sie H-G. Organ-specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine and placenta; L-phenylalanine, L-tryptophan and L-homoarginine. *Enzymologia.* 1971; 41:140–167.
9. Van Belle H. Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. *Clin. Chem.* 1976; 22:972–976. [PubMed: 6169]
10. Farley JR, Ivey JL, Baylink DJ. Human skeletal alkaline phosphatase - Kinetic studies including pH dependence and inhibition by theophylline. *J. Biol. Chem.* 1980; 255:4680–4686. [PubMed: 7372601]
11. Sergienko E, et al. Identification and characterization of novel tissue-nonspecific alkaline phosphatase inhibitors with diverse modes of action. *J. Biomol. Screen.* 2009; 14:824–837. [PubMed: 19556612]
12. Ardecky R, et al. Design and synthesis of pyrazole derivatives as potent and selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP). *Bioorg. Med. Chem. Lett.* 2009; 19:222–225. [PubMed: 19038545]
13. Brooks, E.; Voyta, JC. Purification of stable water-soluble dioxetanes.. 1990. U.S. Patent 4,931,569, filed 14 Sept. 1988, and issued 5 June 1990
14. Bronstein, I.; Brooks, E.; Juo, Rouh-Rong. Chemiluminescent 3-(substituted Adamant-2'-Ylidene) 1,2-dioxetanes.. 1994. U.S. Patent 5,326,882, filed 30 April, 1992, and issued 5 July, 1994
15. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* 1999; 4:67–73. [PubMed: 10838414]
16. Narisawa S, et al. High throughput screening and identification of novel alkaline phosphatase inhibitors - A druggable target for the treatment of vascular calcification. *J. Bone Miner. Res.* 2007; 22:1700–1710. [PubMed: 17638573]
17. Dahl R, et al. Discovery and validation of a series of aryl sulfonamides as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP). *J. Med. Chem.* 2009; 52:6919–6925. [PubMed: 19821572]

Figure 1.

Catalytic mechanism of alkaline phosphatase reaction¹¹. Abbreviations: E - alkaline phosphatase enzyme molecule; P_i – inorganic phosphate; DO-P_i - substrate molecule; E-P_i – phosphoenzyme (enzyme phosphorylated on Ser-93 in the TNAP sequence of its active site); DOH - product alcohol; E·P_i - non-covalent complex of inorganic phosphate in the active site; AOH – an alcohol molecule, acceptor substrate of transphosphorylation reaction; AO-P_i – product of transphosphorylation reaction.

Figure 2. Structures of two commonly utilized alkaline phosphatase substrates: (A) p-nitrophenyl phosphate (pNPP) is utilized in colorimetric homogeneous assay; (B) CDP-star utilized for luminescent detection of alkaline phosphatase in blotting applications and now applied for homogeneous TNAP assay.

Figure 3.

Luminescence-based assay for TNAP. (A) Reaction mechanism - dioxetane-phosphate is dephosphorylated by an alkaline phosphatase leading to generation of an unstable product that decomposes with concomitant light production. (B) Spectrum of light emitted in the CDP-star dephosphorylation reaction on nylon membrane. Y-axis is in relative units (Fundamentals of chemiluminescent detection. Application note No. 6, Fujifilm Life Sciences).

Figure 4. Optimization of CDP-star concentration. Experimental data (triplicate values) were fitted to Michaelis-Menten equation (Equation 1) to determine the K_m value using GraphPad Prism software. Error bars represent standard deviations for each series.

Figure 5. Optimization of DEA concentration. Experimental data (triplicate values) were fitted to Michaelis-Menten equation (Equation 1) to determine the K_m value using GraphPad Prism software. Error bars represent standard deviations for each series.

Figure 6.

Optimization of TNAP concentration in the luminescent assay. Activity (●) for each enzyme concentration is measured in 32 replicate wells. Error bars represent standard deviations for each series. The encircled point is not included in the linear regression analysis of the data and shown for illustration purposes. The concentration of TNAP is expressed in units relative to the value utilized in colorimetric assay. The limit of detection determined in the experiment is equal $9.2 \cdot 10^{-4}$ RU. Z'-factor (□) is calculated according to Equation 3. Arrows point out the Y-axis utilized for each curve.

Figure 7.

Inhibition of TNAP activity by levamisole. The assay was performed in the optimized assay, with the concentrations of CDP-star and DEA equal to their respective K_m values. Levamisole concentration corresponding to its half-maximal effect is equal to 20.1 uM.

Figure 8. Z'-factor study of the optimized TNAP luminescent assay

The assay was performed in 384-well small-volume white plates. Levamisole working solution (PC) and 10% DMSO (NC) were added to columns 1-12 and 13-24 of the plate, respectively, using a Multidrop Combi microplate dispenser. TNAP and CDP-star working solutions were added with a Matrix WellMate microplate dispenser. The luminescent signal was acquired on an EnVision plate reader after 30-min incubation. Z'-factor was calculated according to Equation 3.

Table 1

Troubleshooting table.

Step number	Problem	Possible reason	Solution
Step 10	Signal is comparable to background in both positive and negative control wells	TNAP is missing	Remake test points. If problem persists remake TNAP working solution.
Step 10	Signal is much higher than background yet of equal value in both positive and negative control wells	Levamisole is missing.	Remake test points. If problem persists remake levamisole working solution.
Step 24	No hits are confirmed	There is a logistics error in the identification or cherry picking of hits	Inspect the steps involved. If the problem is not resolved, repeat screening of a HTS plate containing the highest number of hits. Compare results with the HTS stage.
Step 30	Z'-factor failure	See troubleshooting for step 10	See troubleshooting for step 10
Step 30	Nether hits nor levamisole demonstrate dose-response	Compound dilution error	Inspect the calculations and steps involved
Step 30	Some dose-response curves have pronounced decrease in inhibition at higher concentration	Compound precipitation	Remove affected data points prior to curve-fitting. It is possible to improve the compound performance by avoiding intermediate compound dilution

Table 2

[TNAP]-dependent experiment (step 3).

[TNAP] (RU)	0.5	0.25	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001	0
Luminescence mean value, μ (RLU)	42174	22796	9290	3691	1752	872	416	260	131	97	37
Luminescence standard deviation, σ (RLU)	4106	1811	683	362	243	122	60	48	36	34	26
$Z' = 1 - \frac{3\sigma_{[TNAP]} + 3\sigma_{[TNAP]=0}}{\mu_{[TNAP]} - \mu_{[TNAP]=0}}$	0.71	0.76	0.77	0.68	0.53	0.47	0.31	-0.01	-0.99	-2.01	0

Table 3

Summary of HTS campaign for screening TNAP using luminescent assay.

	Compounds screened	Hits identified	Hit criteria	Hit rate
Primary HTS	64,394	73	>50% inhibition	0.11%
Single-concentration confirmation	73	55	>50% inhibition	75.34%
Dose-response confirmation	55	53	IC ₅₀ < 20 μ M	96.36%