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Tuesday, 30 July 19

Mr. Benjamin Werth
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Dear Mr. Werth,

Please find our manuscript for consideration at JoVE, as an invited manuscript entitled "Screening protocol for identification of *Thermotoga maritima* membrane-bound pyrophosphatase inhibitors". In this manuscript, we describe a screening method for membrane-bound pyrophosphatase (mPPase) inhibitors based on the molybdenum blue reaction in a 96-well plate format. This protocol is simple, cheap, and producing a consistent and robust result. mPPases are candidates for drug design target as they are not found in animals and humans but present in protist parasites causing diseases such as *Plasmodium, Toxoplasma*, *Leishmania*, and *Trypanosoma*.

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Yours sincerely,

Adrian Goldman, Ph. D.
Leadership Chair in Membrane Biology.

TITLE:

Screening for *Thermotoga Maritima* Membrane-Bound Pyrophosphatase Inhibitors

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KEYWORDS:

membrane-bound pyrophosphatase, *Thermotoga maritima*, inhibitor screening, molybdenum blue reaction, protist diseases, drug design

SUMMARY:

Here we present a screening method for membrane-bound pyrophosphatase (from *Thermotoga maritima*) inhibitors based on the molybdenum blue reaction in a 96 well plate format.

ABSTRACT:

Membrane-bound pyrophosphatases (mPPases) are dimeric enzymes that occur in bacteria, archaea, plants, and protist parasites. These proteins cleave pyrophosphate into two orthophosphate molecules, which is coupled with proton and/or sodium ion pumping across the membrane. Since no homologous proteins occur in animals and humans, mPPases are good candidates in the design of potential drug targets. Here we present a detailed protocol to screen for mPPase inhibitors utilizing the molybdenum blue reaction in a 96 well plate system. We use mPPase from the thermophilic bacterium *Thermotoga maritima* (TmPPase) as a model enzyme.

This protocol is simple and inexpensive, producing a consistent and robust result. It takes only about one hour to complete the activity assay protocol from the start of the assay until the absorbance measurement. Since the blue color produced in this assay is stable for a long period of time, subsequent assay(s) can be performed immediately after the previous batch, and the absorbance can be measured later for all batches at once. The drawback of this protocol is that it is done manually and thus can be exhausting as well as require good skills of pipetting and time keeping. Furthermore, the arsenite-citrate solution used in this assay contains sodium arsenite, which is toxic and should be handled with necessary precautions.

INTRODUCTION:

Approximately 25% of the total cellular proteins are membrane proteins and about 60% of them are drug targets[1,2]. One of the potential drug targets[3], membrane-bound pyrophosphatases (mPPases), are dimeric enzymes that pump proton and/or sodium ion across the membrane by hydrolysis of pyrophosphate into two orthophosphates[4]. mPPases can be found in various organisms[5] such as bacteria, archaea, plants, and protist parasites, with the exception of humans and animals[4]. In protist parasites, for example *Plasmodium falciparum*, *Toxoplasma gondii* and *Trypanosoma brucei*, mPPases are essential for the parasite virulence[6] and knockout of this expression in the parasites lead to failure in maintaining intracellular pH upon exposure to the external basic pH[7]. Due to their importance and lack of homologous protein present in vertebrates, mPPases can be considered as potential drug targets for protistal diseases[3].

The in vitro screening of mPPase inhibitors in this work is based on a TmPPase model system. TmPPase is a sodium ion pumping and potassium ion dependent mPPase from *T. maritima* and has its optimum activity at 71 °C[8]. Benefits of this enzyme are for example its ease in production and purification, good thermal stability and high specific activity. TmPPase shows both high similarity in addition to the complete conservation of the position as well as identity of all catalytic residues to the protist mPPases[3,9] and to the solved structure of *Vigna radiata*[10] mPPase. The available structures of TmPPase in different conformations are also useful for structure-based drug design experiment (as virtual screening and de novo design).

Here we report a detailed protocol for screening of TmPPase inhibitors in a 96 well plate format (**Figure 1**). The protocol is based on the colorimetric method of the molybdenum blue reaction, which was first developed by Fiske and Subbarow[11]. This method involves the formation of 12-phosphomolybdic acid from orthophosphate and molybdate under acidic conditions, which is then reduced to give characteristic blue-colored phosphomolybdenum species[12].

PROTOCOL:

1. Protein preparation

NOTE: The expression and purification of TmPPase has been described elsewhere[13].

1.1. Prepare 10 mL of the reactivation buffer solution containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5, 3.5% (v/v) glycerol, 2 mM dithiothreitol (DTT), and 0.05% dodecyl maltoside (DDM).

1.2. Prepare 10 mL of the reaction mixture containing 200 mM Tris-Cl pH 8.0, 8.0 mM $MgCl_2$, 333 mM KCl, and 67 mM NaCl.

NOTE: Mg^{2+} is required to chelate the pyrophosphate as the substrate of mPPase, K^+ is required to increase the enzyme activity as TmPPase is a potassium dependent mPPase, and Na^+ is needed for the enzyme activity during sodium ion translocation by TmPPase.

1.3. Prepare 30 mg/mL liposomes for enzyme reactivation.

1.3.1. Add 0.3 g of L-α-phosphatidylcholine from soybean to 10 mL of 20 mM Tris-HCl pH 8.0 with 1 mM DTT.

1.3.2. Sonicate the liposome with 1 s pulse interval for 1 min, pause for 1 min, and repeat until the solution becomes transparent yellow.

1.3.3. Aliquot the liposomes, freeze in liquid nitrogen and store at -80 °C until used.

1.4. Reactivate the enzyme.

1.4.1. Mix 40 µL of the liposomes solution with 22.5 µL of 20% DDM.

1.4.2. Heat the mixture at 55 °C for 15 min and allow it to cool to room temperature.

1.4.3. Add 36.5 µL of the reactivation buffer solution, mix, and add 1 µL of concentrated protein (13 mg/mL) to make a total concentration of 0.13 mg/mL.

NOTE: Protein is usually frozen in 10 µL aliquots after purification and thawed on ice before use.

1.5. Take 20 µL of the reactivated enzyme and add to 1,480 µL of the reaction mixture, then mix gently.

NOTE: The addition of the reactivated enzyme to the reaction mixture should be performed just before it is used.

2. Compound preparation

2.1. Dissolve the compounds in dimethyl sulfoxide (DMSO) to make stock solutions of 25−100 mM in 50−200 µL, based on the availability of the compounds.

NOTE: All compounds used here (**Figure 2A**) have been published previously[9]. If the compound solubility is low, the stock concentration can be adjusted accordingly.

2.2. Prepare three different concentrations of each compound in water.

2.2.1. Dilute the stock solution with water to 1 mL in microtubes to give 2 µM, 10 µM and 100 µM for soluble compounds, or alternatively 2 µM, 10 µM and 40 µM for sparingly soluble compounds.

2.2.2. Vortex the compound solution instantly after dilution of the stock solution for proper mixing.

2.3. Check the possible compound aggregation in the assay using a nephelometer.

NOTE: This was studied as triplicates in three concentrations (1 µM, 5 µM and 20 µM) and normalized to the blank in a 96 well plate.

2.3.1. Dispense 75 µL of the reaction mixture into each well using a multichannel pipette.

2.3.2. Add 75 µL of each compound (for the blank, use 75 µL of water instead) and mix by pipetting up and down 5x.

2.3.3. Measure the turbidity of each well at 300 V using a microplate nephelometer.

3. Reagents for the assay preparation

3.1. Prepare the arsenite-citrate solution.

3.1.1. Weigh 5 g of sodium arsenite and 5 g of trisodium citrate dihydrate.

CAUTION: Sodium arsenite is toxic, thus use proper protective equipment and handle with special care. As precaution, do not handle before all necessary safety precautions have been read and understood. Handle only in a fume hood in order not to inhale dust/vapors of the compound or its solution(s). If inhaled, move to fresh air and obtain medical attention. Wear appropriate chemical safety goggles, protective gloves and clothing to avoid ingestion and eye/skin contact. If swallowed, call immediately a poison center or doctor/physician. If it gets on the skin or in the eye(s), wash with plenty of water and obtain medical attention.

3.1.2. Dissolve into 100 mL of water.

3.1.3. Add 5 mL of glacial acetic acid, mix, and add water to 250 mL.

3.1.4. Store at room temperature protected from light.

NOTE: The solution is stable for more than a year.

3.2. Prepare solution A and solution B.

3.2.1. For solution A, add 10 mL of ice cold 0.5 M HCl to 0.3 g of ascorbic acid. Dissolve the ascorbic acid by vortexing.

3.2.2. For solution B, add 1 mL of ice cold water to 70 mg of ammonium heptamolybdate tetrahydrate and vortex to dissolve.

NOTE: Store both solutions on ice until use. For the consistency of the assay result, both solutions can be stored on ice for a maximum of a week.

3.3. Prepare the phosphate (P_i) standard with the concentration of 0 µM, 62.5 µM, 250 µM and 500 µM for calibration.

3.3.1. Add 0 µL, 25 µL, 50 µL, and 100 µL of 5 mM Na_2HPO_4 dihydrate to four microtubes containing 370 µL of the reaction mixture.

3.3.2. Top up to 1 mL with water.

4. Activity assay for one 96 well plate

NOTE: See **Figure 1** for the schematic workflow of the assay.

4.1. Add 1 mL of solution B to 10 mL of solution A, mix by vortexing and store the solution on ice.

NOTE: This solution should be transparent and yellow. Keep solution A + B on ice for at least 30 min prior to use. However, use the solution within 3 h as it will go bad after long-term storage.

4.2. Add 40 µL of 0 µM, 62.5 µM, 250 µM and 500 µM Pi standard to the tube strips in triplicate using a multichannel pipette.

NOTE: The reaction mixture with no Pi added will be used as a blank.

4.3. Add 25 µL of compound solution to the tube strips using a multichannel pipette.

NOTE: Each compound has three different concentrations in triplicate which is enough for initial estimation of the half maximal inhibitory concentration (IC_{50}). For a more accurate IC_{50} determination, eight different compound concentrations can be used. For the uninhibited enzyme the compound solution is replaced with equal amount of water. As positive controls 2.5 µM, 25 µM, and 250 µM of imidodiphosphate (IDP) sodium salt were used.

4.4. Add 15 µL of mPPase solution mixture to the tube strips (except to the tubes containing Pi standard) using a multichannel pipette.

4.5. Seal the tube strips with an adhesive sealing sheet. Cut the sealing sheet to separate each tube strip.

4.6. Pre-incubate the samples for 5 min at 71 °C. Place the samples on the heating block with 20 s interval between each strip in order to minimize the time consumption during the subsequent steps.

4.7. For each strip, open the adhesive sealing. Add 10 µL of 2 mM sodium pyrophosphate dibasic using a multichannel pipette and mix by pipetting up and down for 5x. Seal the tube strip again using the same sealing.

NOTE: This step might initially be difficult to accomplish in 20 s; however, it will become easier after some assays.

4.8. Incubate at 71 °C for 5 min.

4.9. Place the samples on the cooling apparatus with 20 s interval between each strip. Let them cool for 10 min but centrifuge each strip briefly after 5 min of cooling, to decant water drops under the sealing sheet, then put it back to the cooling apparatus and remove the sealing.

NOTE: The cooling apparatus can simply be made by placing a 96 well PCR plate on a polystyrene Petri dish (size 150 mm x 15 mm) filled with water and frozen for at least 1 h. The apparatus should be taken out from the freezer about 5 min prior to the beginning of the assay. Do not take out the cooling apparatus right before sample cooling as it will freeze the reaction mixture and hinder color development.

4.10. After 10 min of cooling, add 60 µL of solution A + B, mix by pipetting up and down for 5x and keep the tube strips on the cooling apparatus for 10 min.

4.11. Add 90 µL of the arsenite-citrate solution and keep at room temperature for at least 30 min to produce a stable blue color.

CAUTION: Due to its toxicity all solutions containing sodium arsenite should be handled with extra care at all time. Thus, the addition of arsenite-citrate solution should be done in a fume hood.

4.12. Dispense 180 µL of each reaction mixture into a clear 96 well polystyrene microplate.

4.13. Measure the absorbance of each well at 860 nm using a microplate spectrophotometer.

5. Result analysis

5.1. Average the triplicates of each sample and the P_i standards. Then subtract with the blank to eliminate the background signal.

5.2. Make a calibration curve by plotting the absorbance (A_{860}) values against the amount of P_i standard (nmol) and perform a linear regression to obtain the trendline function using the following formula:

$$A_{860} = mP_i + \mathrm{b}$$

5.3. Calculate the phosphate amount (nmol) released from the enzymatic reaction based on the linear regression formula above.

5.4. Calculate the specific activity using the following formula:

$$Specific\ activity\ (SA) = \frac{nP_i}{t \cdot m_{TmPPase}}$$

where nP_i is the amount of phosphate released from the reaction (nmol), t is the reaction time (min), and $m_{TmPPase}$ is the amount of the pure TmPPase used in the assay (mg).

5.5. Calculate the percent activity for each inhibitor concentration using the following formula:

$$\%\ Activity = \frac{SA_i}{SA_{un}} \times 100\%$$

where SA_i *i*s the specific activity of a sample with inhibitor and SA_{un} is the specific activity of the uninhibited sample.

5.6. Calculate the $logIC_{50}$ (estimate) and IC_{50} (estimate) with a nonlinear regression fit from the four-parameter dose-response curve using the following formula:

$$Y = Bottom\ +\ (Top - Bottom)/(1 + 10^{((logIC_{50} - X)^{*}HillSlope)})$$

where X is log of concentration (µM), Y is activity (%), Top and Bottom are plateaus in the same unit as Y (100% and 0%, respectively), $logIC_{50}$ has the same log units as X, and HillSlope = slope factor or hill slope, which is unitless.

NOTE: Software (**Table of Materials**) is used for the fitting. Use the concentration of 0.01 µM (instead of 0.00 µM) for the sample without inhibitor as the logarithm of zero is not defined.

REPRESENTATIVE RESULTS:

In this protocol, eight compounds (1–8) were tested (**Figure 2A**) together with IDP, a common inhibitor of pyrophosphatases, as a positive control. Each compound was tested at three different

concentrations (1 µM, 5 µM and 20 µM) in triplicate. The workflow of the screening is depicted in **Figure 1**, starting from sample and reagent preparation until the absorbance measurement at 860 nm.

At the end of this protocol, after the addition of solution A + B and arsenite-citrate, the solutions develop a stable blue color with the maximum absorption at 709 nm and 860 nm[14] due to the complex formation of phosphate ions with molybdate that can be observed and shows the occurrence of the enzymatic reaction. For this experiment, we use the absorbance at 860 nm for the measurement of Pi amount released as it has better detection limit and sensitivity compared to the absorbance at 709 nm[15]. The blue color is fully developed in 30 min of incubation at room temperature and stable for at least 5 h[14]. The assay has the sensitivity down to Pi concentration of 10 µM and the absorbance is linear over a concentration range of 10−800 µM[14]. In the representative result here, wells E1−E3 (**Figure 2C)** contain the reaction mixture without inhibitor and the blue solution can be observed at the end of the assay. This can also be observed at low compound concentrations where complete inhibition has not been reached, as in wells F1−F3 for IDP and wells A4−A6 for compound **1** (ATC, a recently known uncompetitive inhibitor of TmPPase[9]) at the concentration of 2.5 µM and 1 µM, respectively. The higher concentration of IDP and compound **1**, the less to no blue color can be observed (G1−G3 and H1−H3 for IDP and B4−B6 and C4−C6 for compound **1**) indicating inhibition of the enzymatic activity. All three concentrations of non-inhibiting compounds (**2**, **3**, and **8**) displayed the same blue color intensity as wells E1−E3 without any inhibitor (**Figure 2C**).

After the absorbance measurement at 860 nm, the data can be processed and analyzed (see protocol section 5). **Figure 2D** shows the calibration plot of Pi standard with its linear fitting (y = 0.0576x + 0.0019; r^2 = 0.999). **Figure 3** shows the plot of enzymatic activity (%) against the concentration of each tested compound. For compounds with inhibition activity, a nonlinear curve fitting is also shown. IDP, used as a positive control, clearly shows a decrease in activity at higher concentration. The IC_{50} (estimate) calculated based on three different concentrations is 88.2 µM (**Table 1**), which is similar to the previous measurement (80.0 µM) with eight concentration points[14]. Compounds **1**, **4**, **5**, **6**, and **7** showed a similar trend as IDP since the concentration increased with the IC_{50} (estimate) of approximately 1.3 µM, 7.4 µM, 19.0 µM, 37.4 µM, and 156.1 µM, respectively (**Table 1**). For compounds **2**, **3**, and **8** no reduction in activity or inhibition can be observed at the assay concentrations. An additional assay with eight concentration points can be done to generate precise IC_{50}. **Figure 4** shows the inhibition curve for compounds **1**, **5**, **6**, **7** and **8** with an IC_{50} of 1.7 µM, 21.4 µM, 58.8 µM, 239.0 µM and >500 µM, respectively[9].

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic workflow of TmPPase inhibition assay in a 96 well plate format. The red numbering shows the steps of the assay according to the protocol and the blue arrows show the interval order.

Figure 2: Samples, their arrangement and color development in a 96 well plate. (**A**) The structures of compounds **1**–**8** used for the assay. The inhibition activity of these compounds has been reported in Vidilaseris et al.[9]. (**B**) Sample arrangement. (**C**) Color development, 30 min after the addition of arsenite-citrate solution. The concentrations of control inhibitor (IDP) and samples used, arranged from the top to the bottom, are 2.5 µM, 25 µM, and 250 µM concentration and 1 µM, 5 µM, and 20 µM concentration, respectively. The intensity of the blue color corresponds to the amount of P_i released due to the enzymatic reaction and the lack of color corresponds to no enzymatic reaction. (**D**) Calibration curve for P_i standard (nmol) against A_{860} with linear fitting ($y = 0.0576x + 0.0019$; $r^2 = 0.999$).

Figure 3: Curve of the TmPPase percent activity for three different inhibitor concentrations. The nonlinear regression curves to calculate the IC_{50} (estimate) are shown for IDP as well as for compounds **1**, **4**, **5**, **6** and **7** but not for compounds **2**, **3**, and **8** as they were not inhibiting TmPPase activity at the assay concentrations. The logIC_{50} and IC_{50} (estimate) of each compound is shown in **Table 1**. All data are shown as mean ± SD with three replicates.

Figure 4: Inhibition curve from eight concentration points of compounds 1, 5, 6, 7 and 8. This figure is taken from Vidilaseris et al.[9] with slight modification. All data are shown as mean ± SD with three replicates.

Table 1: LogIC_{50} and IC_{50} (estimate) of IDP and compounds 1–8 based on the data from Figure 3.

DISCUSSION:

Here we report a detailed protocol for simple screening of inhibitors for membrane-bound pyrophosphatase from *T. maritima* in a 96 well plate format based on Vidilaseris et al.[14]. This protocol is inexpensive and based on 12-phosphomolybdic acid, which is formed from orthophosphate and molybdate under acidic conditions and reduced to phosphomolybdenum species with a distinct blue color[12]. This method is preferred over other protocols, such as the more sensitive malachite green assay[16], because this method does not show interference in the presence of high phospholipid concentration which is required for TmPPase reactivation[14].

The workflow of the screening protocol is depicted in **Figure 1** and this process can be fully accomplished in 1 h. This protocol is optimized for TmPPase with the optimal working temperature at 71 °C and a 5 min reaction time. As water will evaporate at this temperature from the reaction mixture, an adhesive sealing sheet (sliced to fit and cover the strips) is applied to prevent evaporation[14] and the evaporated water is simply recollected with centrifugation. The 5 min incubation time is chosen as it is still in the linear range of the enzymatically released phosphate and sufficient for reliable screening[14]. In this protocol, the timing and pipetting skills are important factors to obtain a good and reliable result. Addition of reagents during the assay with 20 s interval between strips is an optimized timing option for ease of performing the subsequent steps.

For different mPPases, the optimum temperature and incubation time should be determined separately prior to use in the inhibition assay. The enzyme reactivation protocol above is optimized for TmPPase and other mPPases might need a different reactivation protocol. For example, DDM should not be added for reactivation of mPPase from *Pyrobaculum aerophilum* as it will decrease its enzymatic activity[17]. As the enzyme will become less active if prepared well in advance, the addition of reactivated enzyme should be added to the reaction mixture shortly before the assay is initiated. After addition of the arsenite-citrate solution the reaction product is stable for at least 5 h[14]. Therefore, the next batch of the assay can be performed immediately, and the absorbance measurement can be done later to all batches at once.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 Figure 1

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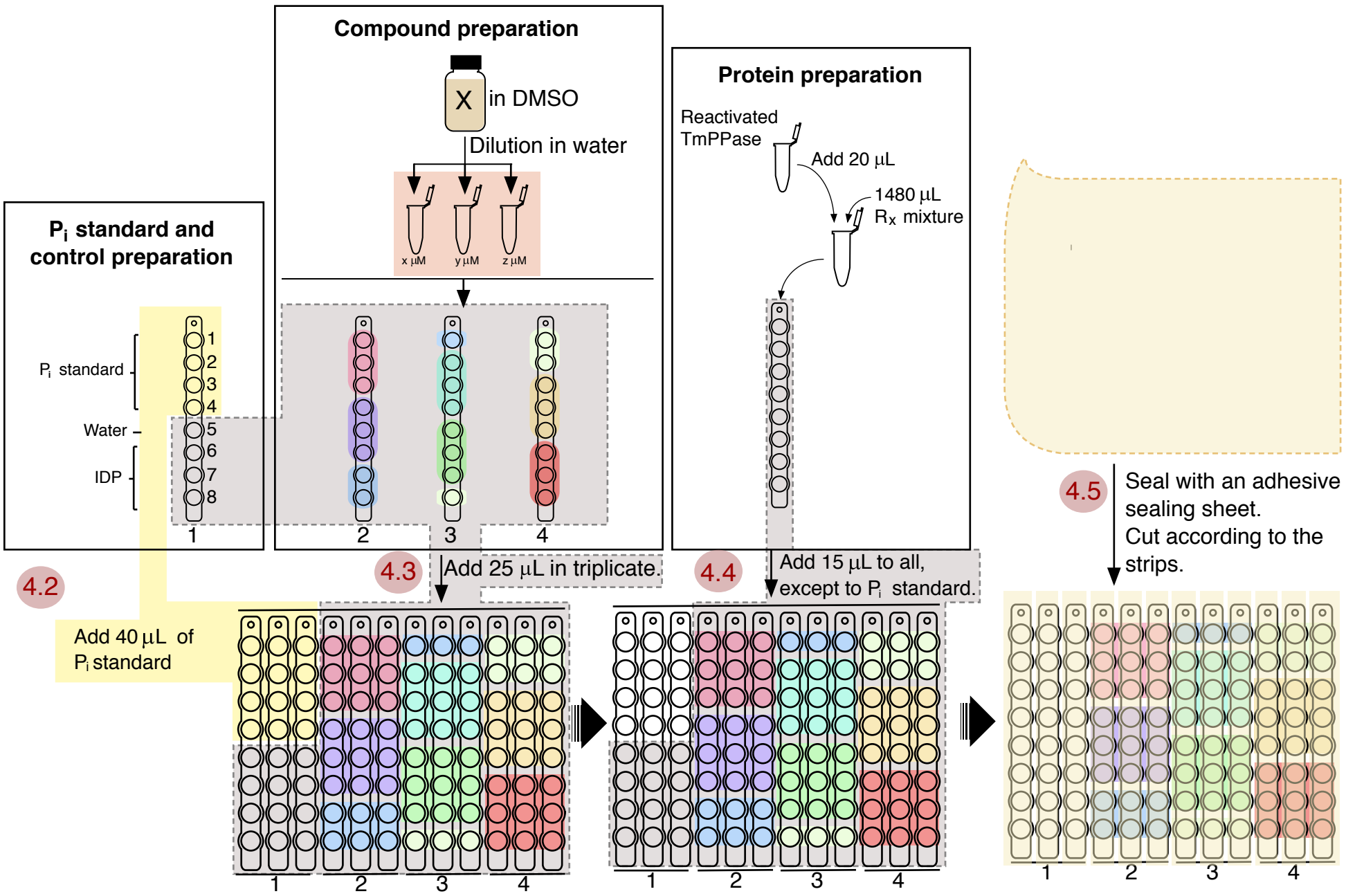
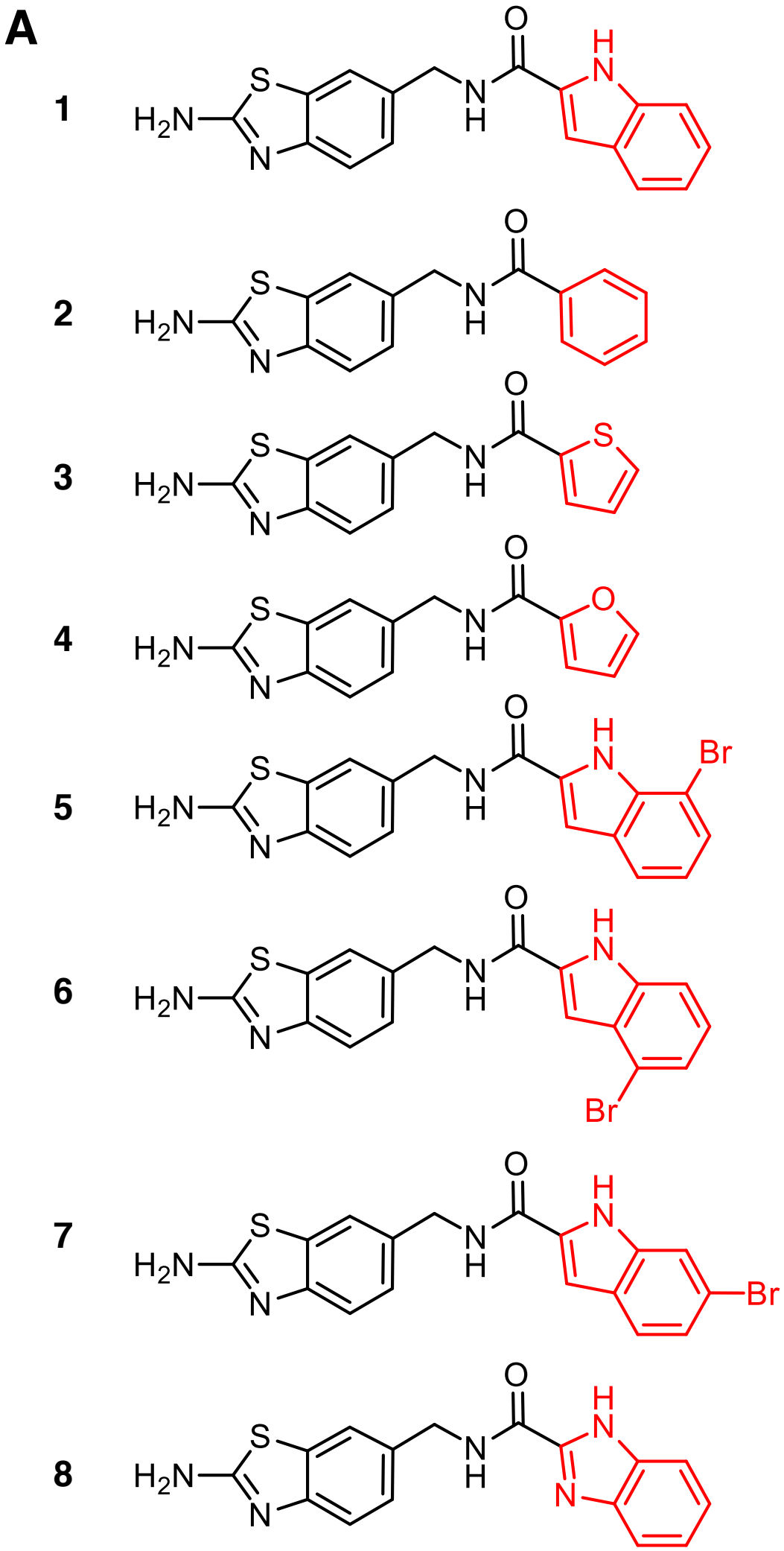
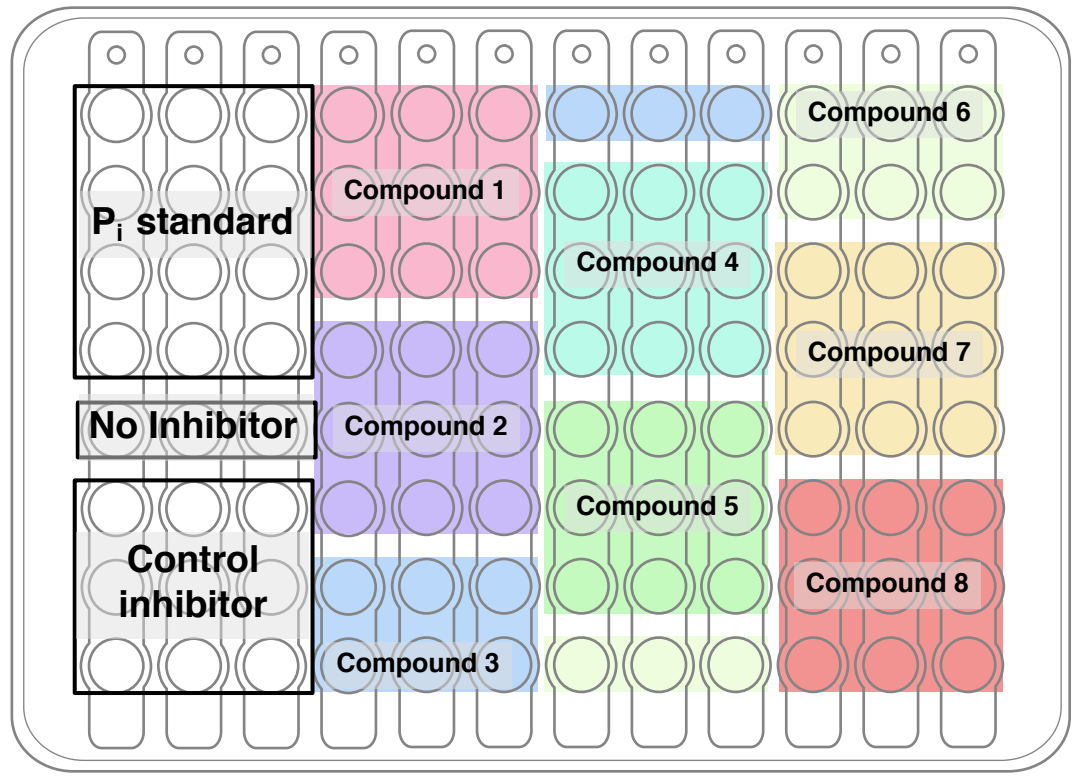
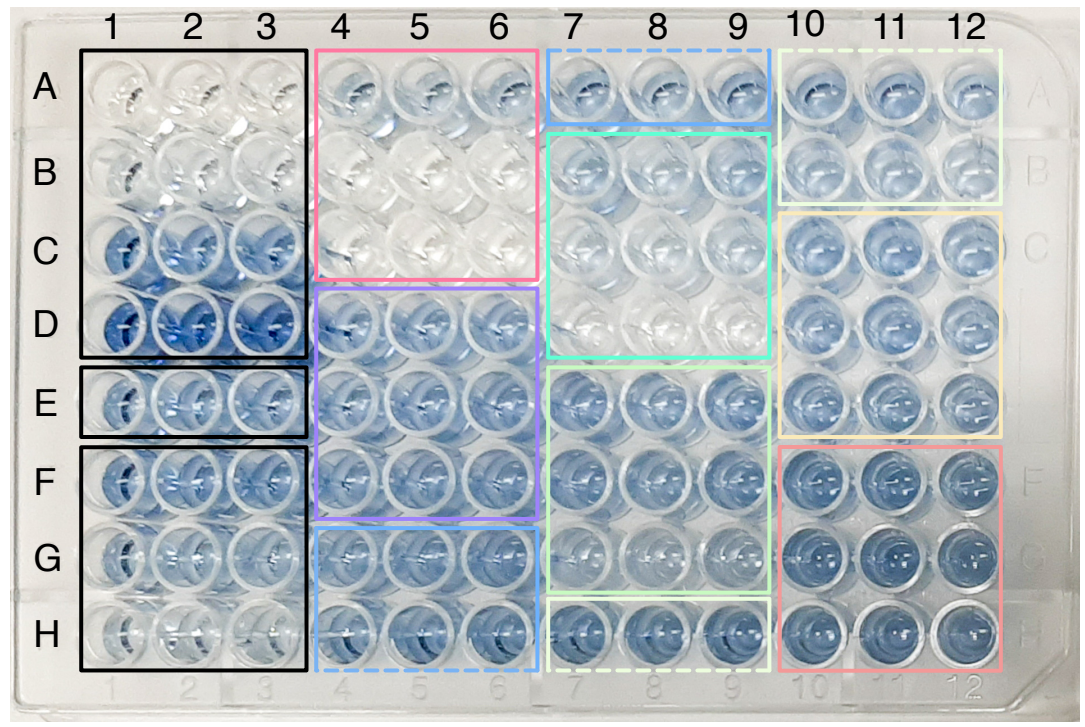


Figure 2







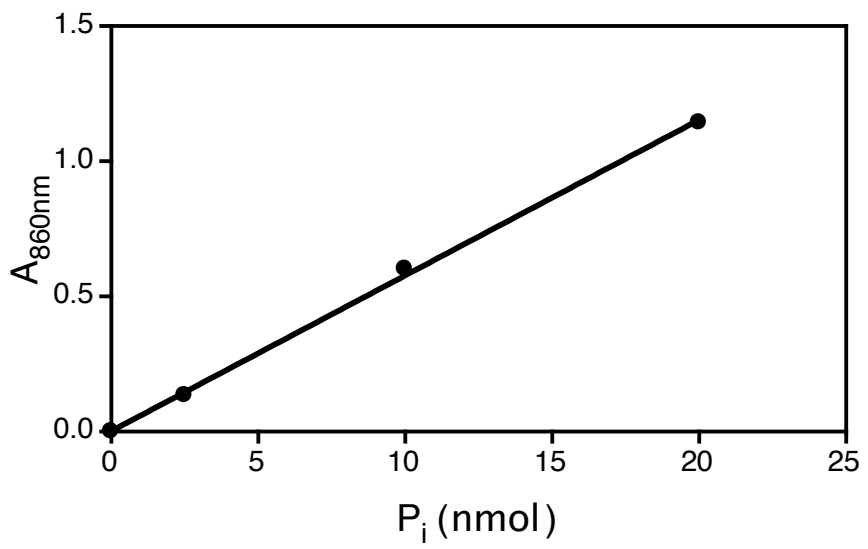


Figure 3

Click here to access/download;Figure;Fig3_IC50 estimate.pdf

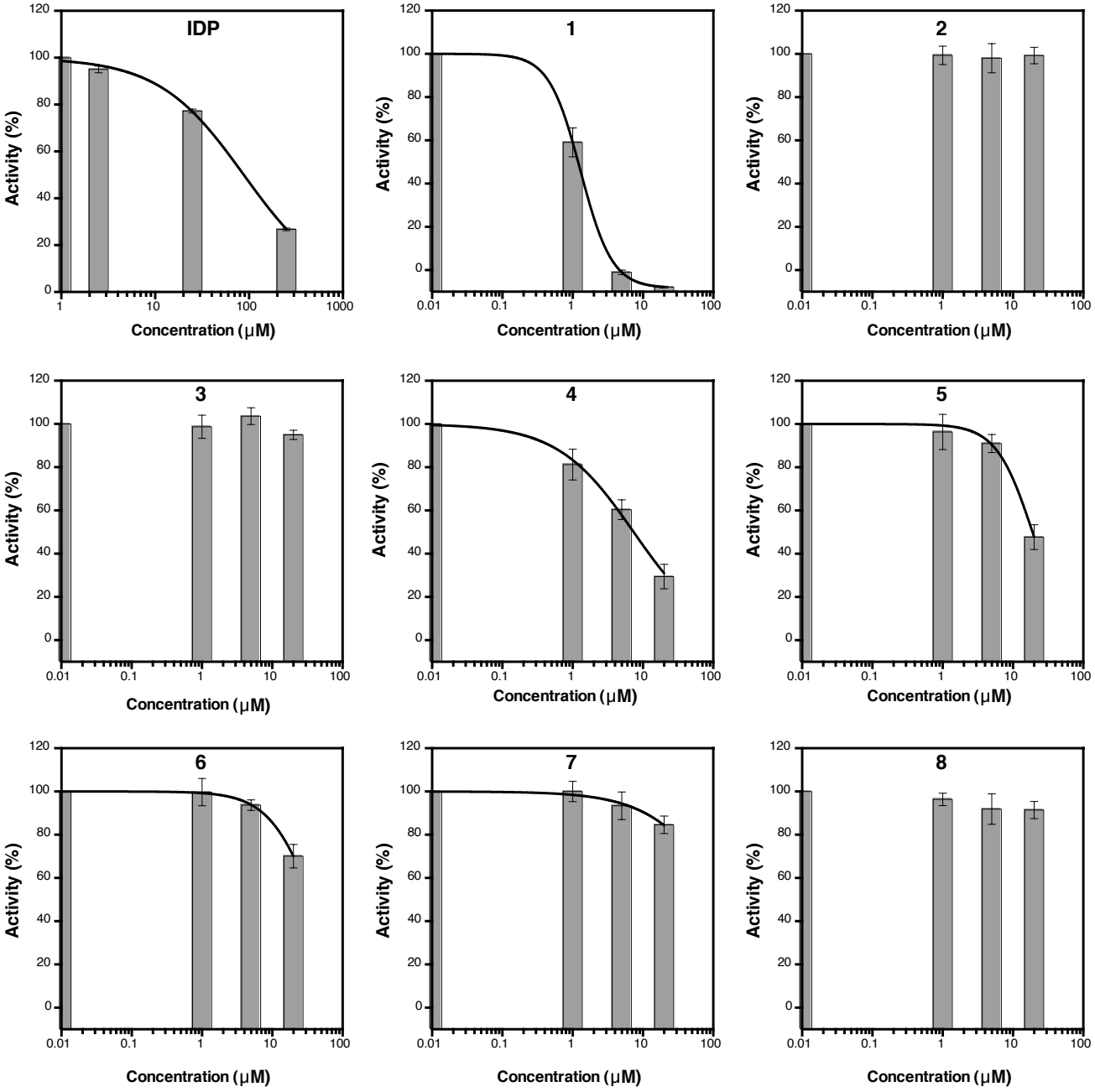
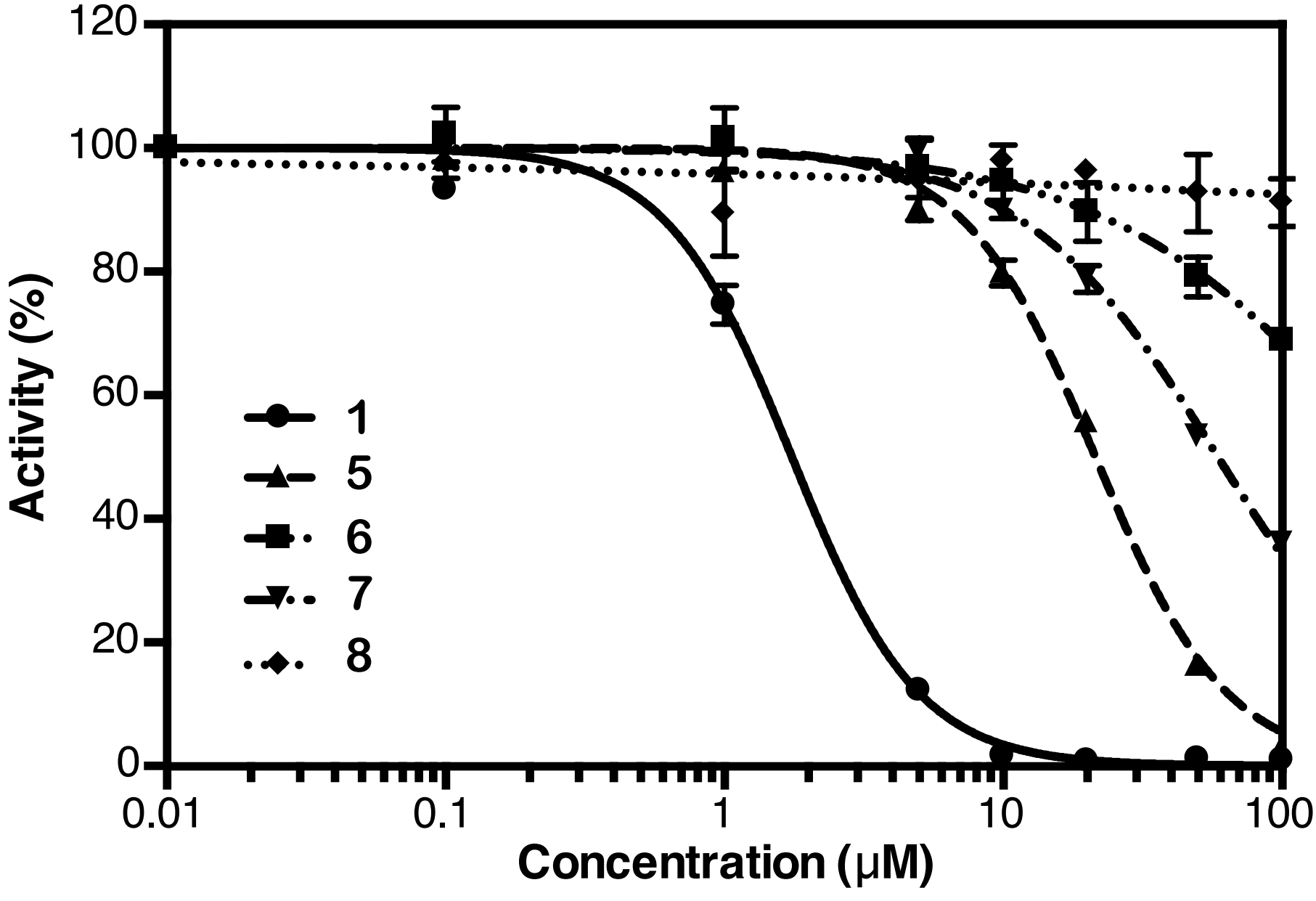


Figure 4



Table

Sample	LogIC_{50}	IC_{50} (estimate) (μM)
IDP	1.95 ± 0.0142	87.9 ± 2.46
1	0.112 ± 0.0274	1.29 ± 0.0816
2	–	no inhibition
3	–	no inhibition
4	0.870 ± 0.0447	7.39 ± 0.760
5	1.28 ± 0.0296	19.0 ± 1.29
6	1.57 ± 0.0846	37.4 ± 7.29
7	2.19 ± 0.366	156 ± 131
8	–	no inhibition

Table of Materials

Click here to access/download;Table of Materials;KV_JoVE_Materials_revision_v2.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adhesive sealing sheet	Thermo Scientific	AB0558	
Ammonium heptamolybdate tetrahydrate	Merck	F1412481 636	
Ascorbic acid	Sigma-Aldrich	95212-250G	
BioLite 96Well Multidish	Thermo Scientific	130188	
Dimethyl sulfoxide (DMSO)	Merck	1167431000	
8-well PCR Tube Strips 0.2 ml without caps (120)	Nippon genetics	FG-028	
Dodecyl maltoside (DDM)	Melford	B2010-100G	
Ethanol	Merck	1009901001	
Glacial acetic acid	Merck	1000631011	
Hydrochloric acid	Sigma-Aldrich	258148-500ML	
Imidodiphosphate sodium salt	Sigma-Aldrich	I0631-1G	
L-α-Phosphatidyl choline from soybean lecithin	Sigma	429415-100GM	
Magnesium chloride	Sigma-Aldrich	8147330500	
Multiplate 96-Well PCR Plates	Bio-Rad	MLL9651	
MultiSkan Go	Thermo Scientific	10680879	
Nepheloskan Ascent (Type 750)	Labsystems		
Polystyrene Petri dish (size 150 mm x 15 mm)	Sigma-Aldrich	P5981-100EA	
Potassium chloride	Merck	104936	
Prism 6 software	GraphPad		
QBT2 Heating block	Grant Instruments		
Sodium meta-arsenite	Fisher Chemical	12897692	
Sodium phosphate dibasic (P_i)	Sigma	S0876-1KG	
Sodium pyrophosphate dibasic	Fluka	71501-100G	
Trisodium citrate dihydrate	Fluka	71404-1KG	

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Title of Article:	Screening protocol for identification of Thermotoga maritima membrane-bound pyrophosphatase inhibitors
Author(s):	*Keni Vidilaseris*, Niklas G. Johansson*, Ainoleena Turku, Alexandros Kiriazis, Gustav Boije af Gennäs, Jari Yli-Kauhaluoma, Henri Xhaard, Adrian Goldman*

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CORRESPONDING AUTHOR

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Institution:	University of Helsinki, Helsinki, Finland
Title:	Dr.
Signature:	
Date:	30 July 2019

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August 26th, 2019

Ref: Ms. No. JoVE60619
Vidilaseris *et al.*
Title: Screening protocol for identification of *Thermotoga maritima* membrane-bound pyrophosphatase inhibitors

Dear Editor,

Please find our revised version of the manuscript. We are of course pleased that the reviewers thought the manuscript interesting and worthy of publication, and thank them for their constructive criticism. We have modified the manuscript according to their suggestions and hope that it is now suitable for publication in JoVE.

Our detailed responses to the reviewers' comments follow:

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Yes, thank you for the reminder.

2. As one of the co-authors is affiliated with UK institutions, please check whether open access is required by your funding agencies.

The funding agencies indeed require open access publication. However, the 6-month embargo would be compliant and therefore the green route would be fine.

3. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We upload each Figure as required.

4. Figure 1: Please replace commercial language (MilliQ) with a generic term. Fix typo in panel G (Add). Please describe different panels in the figure legend and reference them in the protocol and ensure that details in the protocol and the figure match.

It has been done as requested.

5. Figure 3 and Figure 4: Please define error bars in the figure legend.

It has been done as requested.

6. Table 1: Please upload it to your Editorial Manager account as an .xlsx file. Avoid any coloring or formatting in the tables.

Yes, we will.

7. Authors and affiliations: Please provide an email address for each author in the manuscript.

Email address of each author has been added to the manuscript.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

It has been done as requested.

9. Line 91: Please specify the protein used here.

Now, the protein used is specified to 1 µL of 13 mg/mL to make a protein concentration of 0.13 mg/mL in 100 µL of reactivated enzyme.

10. Line 92: Does the reactivated enzyme here refer to the product from lines 90-91?

Yes. We now put this to a new step in protein preparation section, separated from the enzyme reactivation step.

11. Line 97: Please list an approximate volume of stock solutions to prepare.

The approximate volume of stock solution has been added to the text.

12. Line 109: Is a 96-well plate used here?

Yes, we added that to the text.

13. Line 112: How is the solubility evaluated by turbidity?

We are sorry for our mistake and for the confusion. We are not evaluating the solubility of the compounds, but evaluating for the potential aggregation. The aggregation potential is evaluated by nephelometry and should mainly be considered as an indicative screening to detect compounds that might result in the formation of insoluble particles/aggregates in the assay (compared to the reaction mixture). Sparingly soluble compounds give rise greater deviations in the measured activity, especially at high concentrations approximately >10 µM, whereas at lower concentration the results have been consistent for all compounds in the assay.

14. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

It has been done as requested.

15. Please include single line spacing between each numbered step or note in the protocol.

It has been done as requested.

16. Please number the figures/tables in the sequence in which you refer to them in the manuscript text.

It has been done as requested.

17. Line 236: Figure 8 does not exist. Do you mean Figure 4?

Yes, it should be Figure 4. Sorry for the mistake.

18. References: Please do not abbreviate journal titles; use full journal name.

It has been done as requested.

19. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

We removed all requested symbols and sort the materials alphabetically.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describes an assay procedure to manually measure effects of a series of compounds

on the hydrolytic activity of a thermostable bacterial membrane PPase. The procedure is simple and robust and is appropriate for the screening purposes. I recommend publication after a revision.

Major Concerns:
The description is OK for a technician but lacks some details allowing a deeper understanding of the assay. One would expect to find here (a) the value of the Michaelis constant in the conditions used; (b) the percentage of substrate conversion in the control lacking the compounds tested; (c) b and m values in the first equation (line 187), or a calibration plot. It remains unclear without these details if the activity is measured in the time (degree of substrate conversion) range where product amount is proportional to enzyme concentration. It would be also fine to mention the roles of Mg2+, Na+ and K+ included to the assay medium.

We do not put the Michaelis-Menten constant in the condition used, however we state that at the chosen incubation time is in the linear range of the enzymatically released phosphate and sufficient for a reliable screening as observed previously (DOI: 10.1039/C7AY02558K). We put the calibration plot in Figure 2D. We also mention the roles of Mg2+, Na+ and K+ in the text (line 96-99)

Minor Concerns:
Lines 49,50 - a reference would be appropriate here.
We added a reference there.

Line 80 - perhaps it would be reasonable to round up concentrations, unless odd values are dictated by some reasons.
We fix the concentrations. The final reaction concentrations of Tris-Cl pH 8.0, $MgCl_2$, KCl, and NaCl in the reaction mixture after substrate addition are 60 mM, 2.4 mM, 100 mM, and 20 mM, respectively.

Line 82 - "Dissolve" is not a proper word here, replace with "Add" or something alike.
We replaced the "Dissolve" word with “Add”.

Line 84 - "with 1 s pulse for 1 min" is unclear.

The phrase has been changed to “1 s pulse interval for 1 min” for clarity.

Line 112 - "300 V" means "300 Volts"? Should be clarified.
Yes, the text has been replaced with “300 volts”.

Line 126 - it would be reasonable to indicate the allowed duration of storage for consistency.
We add a sentence to indicate a maximum storage duration of a week on ice for consistency of the assay result.

Line 132 - analysts prefer to use anhydrous K3PO4 as phosphate standard because other salts easily loose or gain water on storage.
We thank reviewer for the input. Indeed, anhydrous K3PO4 is probably the best option for a phosphate standard. However, we never see any inconsistency in the standard curve generated for the assay using Na2HPO4 dihydrate solution as the phosphate standard. Therefore, we keep with that.

Line 231, Table 1 and elsewhere - parameter values obtained by fitting should be rounded up to

significant digits and provided with standard errors.
Lines 260 and 263 - replace "compound" with "compounds".
The words have been replaced.

Line 273 - the authors should be aware that the malachite green assay is much more sensitive and consequently requires much less enzyme and accompanying phospholipids to be added.
Yes, we aware of that. A phrase to mention that malachite green assay is more sensitive that our assay has been added to the text.

Line 275 - the statement may be misleading as there is a plethora of procedures for phosphate determination, some being more sensitive.
Thank you for the comment, the sentence has been removed to avoid misunderstanding.

Reference 16 - improper use of capital letters.
The reference has been fixed.
Figure 1E - "2 uM Na2PPi" should be replaced with "2 mM Na2PPi".
Thank you. We replaced "2 uM Na2PPi" should be replaced with "2 mM Na2PPi".

"Materials" table:
Source of IDP is not indicated
Source of IDP has been added to the list.

from soybean
Typo has been corrected

Sodium phosphate…
Typo has been corrected

Some materials are trivial, like pipets, pipet tips and Kimwipes
We removed the trivial materials from the table.

Three types of 96-well plates are mentioned, but it remains unclear at which steps each was used
We removed 96-well BRANDplates® (pureGrade™) as it has the same purpose as BioLite 96Well Multidish. We keep Multiplate 96-Well PCR Plates as this is for making the cooling apparatus.

Reviewer #2:
Manuscript Summary:
In this article, the authors describe a straightforward protocol for measuring pyrophosphatase activity in microplate format and for screening for novel pyrophosphatase inhibitors. With easy to follow graphics, a detailed step-by-step procedure, and clear example outputs, this article presents a complete story that could be easily followed and repeated by other scientists. With a few minor revisions (outlined below), the protocol will be ready for video preparation for JOVE.

Minor Concerns:
1. Lines 61-63: Some words are missing from this sentence.
Thank you for noticing the missing words. Now we fix the sentence to ” TmPPase shows both high similarity in addition to the complete conservation of the position as well as identity of all catalytic residues to the protist mPPases[3,9] and to the solved structure of *Vigna radiata*[10] mPPase.”

2. Lines 69-71: The addition of a chemical scheme showing the chemical reaction producing the blue-colored phosphomolybdenum species would help support the assay design. This could be added into the components of Figure 1 or placed separately in the article.
We do not add the chemical scheme as we already add a reference (https://www.sciencedirect.com/science/article/pii/S000326701500882X?via%3Dihub) in the sentence for the interested reader.

3. Lines 90-91: Some description of the state of the protein that was added to the reaction needs to be inserted into the description. Was it frozen? Stored at what temperature? In what solution?
We add a note to describe the state of the protein that was used for the reactivation.

4. Lines 117-118: A brief description of the necessary precautions should be added.
A brief description of the necessary precautions has been added.

5. Lines 123-134: Do these other solutions need to be made fresh each time? Shelf-life and storage conditions should be added for solution A, B, and PPi standard.
Not necessary, they can be stored on ice for a week before use. We have been added this description to the text in line 207-208.

6. Line 154-155: I do not understand this note. Seems like all strips would be incubated at one time for 5 min. I do not understand the 20 s interval. The blue arrows in Figure 1 do not help with this interpretation. More description of this information would be helpful, as this interval seems to be key to the overall design of the plate.
The strips would be placed on the heating block one at a time with 20 s interval between strips. We added a description to the note in line 271 and Figure 1 for clarity.

7. Lines 167-171: Very nice note. Interesting addition.
Thank you for the compliment.

8. Lines 216-218: Some extra information about the assay should be added, including over what range of concentrations is the absorbance linear? What is the sensitivity of the assay? This will help with the adaptation of the assay to other systems.
We added all the necessary information to the text (line 362-367).

9. Line 236: Is Figure 8 present? Does this mean Figure 3?
No, it should be Figure 4. Sorry for the mistake.

10. Lines 275-276: The authors need to provide experimental support for this claim, especially as the sensitivity of the assay was not ever confirmed in the current article.
We added the information about the sensitivity of the assay in line 366 and provide a reference from our previously published paper.

Yours sincerely,

Adrian Goldman, Ph. D.
Leadership Chair in Membrane Biology.