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Assay for quantifying PPM1H phosphatase activity towards LRRK2 phosphorylated Rab proteins and peptides using the Malachite Green method

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ABSTRACT

We describe a sensitive and non-radioactive assay that we deploy for analysing PPM1H protein phosphatase activity using either Rab8A stoichiometrically phosphorylated at Thr72 (the LRRK2 site) or a phospho-peptide encompassing this residue. Our assay relies on malachite green, an inexpensive basic dye, that in the presence of molybdate forms a green complex with orthophosphate hydrolysed from the phospho-substrate during the PPM1H phosphatase reaction. This green malachite orthophosphate complex can be conveniently measured on a spectrophotometer (620 nm). The assay is straightforward and rapid. It involves a single addition step for initiating and terminating the reaction. The assays can be conveniently performed in 96 well plate format and can be adapted to compare the intrinsic activity of different protein phosphatases as well as impact of mutations.

ATTACHMENTS

dgbebgrdf.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

PPM1H phosphatase, LRRK2 phosphorylated Rab protein, Malachite Green method

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49715

MATERIALS TEXT

Reagents:

- Protein Dilution Buffer: [M]**50 Milimolar (mM)** Tris-HCL pH 7.5, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** $MgCl_2$, 5% Glycerol, [M]**7 Milimolar (mM)** beta-mercaptoethanol.
- [M]**40 Milimolar (mM)** HEPES pH 7.5 supplemented with [M]**10 Milimolar (mM)** $MgCl_2$.
- Malachite green assay kit (Sigma MAK307) containing Reagent A, Reagent B, and 1 M stock of orthophosphate standard.
- Recombinant PPM1H phosphatase (Purified as described in protocols.io XXX or purchased https://mrcppureagents.dundee.ac.uk/ (DU62835). Note it is critical that no orthophosphate containing buffers are used in preparing the phosphatase as this could interfere with assay.
- Recombinant PPM1M phosphatase (purchased https://mrcppureagents.dundee.ac.uk/ (DU68141).

  Note: It is critical that no orthophosphate containing buffers are used in preparing the phosphatase as this could interfere with assay.
- Recombinant PPM1J phosphatase (purchased https://mrcppureagents.dundee.ac.uk/ (DU68140).

  Note: It is critical that no orthophosphate containing buffers are used in preparing the phosphatase as this could interfere with assay.
- Wild type and mutant versions of PPM1H as well as other protein phosphatases can be included for this assay
- Recombinant stoichiometrically Thr72 phosphorylated Rab8A (described in protocols.io XXX or purchased https://mrcppureagents.dundee.ac.uk/ (DU68198).

  Note: It is critical that no orthophosphate containing buffers are used in preparing the phosphorylated Rab protein as this could interfere with assay.
- Phospho-peptide encompassing Thr 72 phosphorylated Rab8A (AGQERFRT*ITTAYYR custom synthesis or purchased https://mrcppureagents.dundee.ac.uk/ (EP5830).
- Note that other stoichiometrically phosphorylated protein and peptide substrates can be used for this assay.

Buffers:

- Phosphatase dilution buffer: [M]**50 Milimolar (mM)** Tris-HCL pH 7.5, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** $MgCl_2$, 5% (by vol) Glycerol, [M]**7 Milimolar (mM)** 2-mercaptoethanol.

Equipment:

- 96-well flat-bottom plate
- Spectrophotometric multi-well plate reader (BioTek Epoch, or equivalent)
- Orbital shaker

Prepare a freshly diluted phosphatase mixture (@ 16X final assay concentration):

Immediately prior to undertaking the phosphatase assay prepare a 16X final concentration of PPM1H by diluting concentrated stocks of wild-type or mutant PPM1H or other phosphatase into an ice-cold buffer containing [M]**50 Milimolar (mM)** Tris-HCL pH 7.5, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** $MgCl_2$, 5% (by vol) Glycerol, [M]**7 Milimolar (mM)** 2- mercaptoethanol). **5 µl** of this diluted phosphatase is required per assay. Keep the diluted phosphatase on ice prior to assay. For assays shown below in Figures 2 and 3, the PPM1H, PPM1J and PPM1M phosphatases are used at a final concentration of [M]**25 Nanomolar (nM)** for the phospho-protein assay, and [M]**50 Nanomolar (nM)** for the phospho-peptide assay. This is a 16X stock of these enzymes, which corresponds to [M]**400 Nanomolar (nM)** and [M]**800 Nanomolar (nM)** , respectively.

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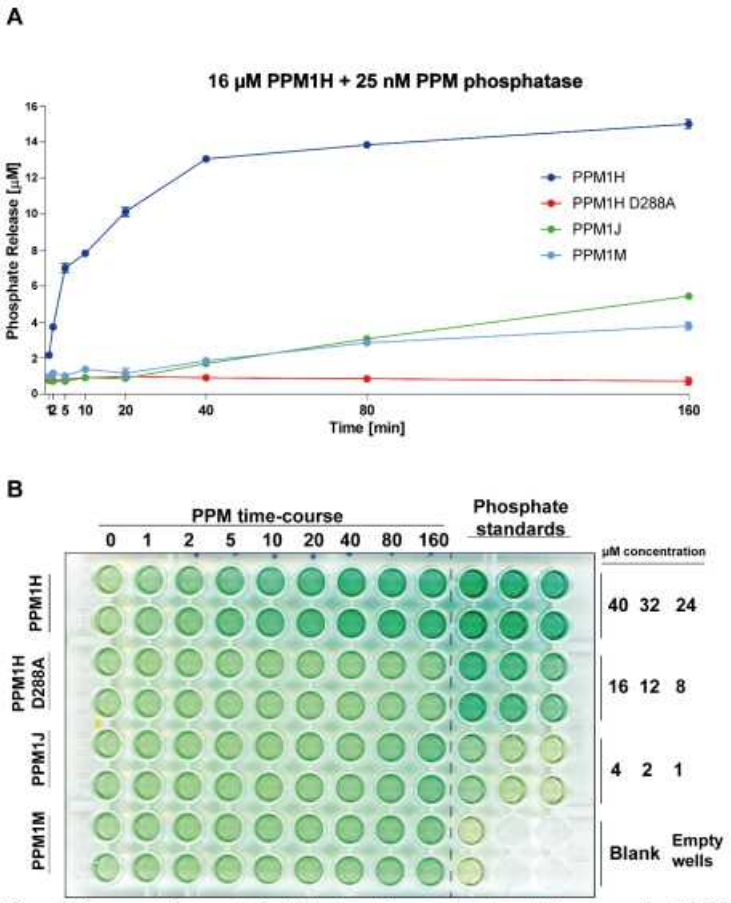

Figure 2: Representative result for PPM phosphatase dephosphorylation assay using 25 nM PPM phosphatases and 16 µM recombinant phosphorylated Rab8A. **(A)** Plotted concentrations of released phosphate (µM) from the phosphatase assay vs time (min). **(B)** 96-well plate after the completed malachite green assay. 25 nM PPM1H WT, PPM1H D288A, PPM1J WT, or PPM1M WT were incubated with 16 µM recombinant phospho-Rab8A protein for the indicated timepoints.

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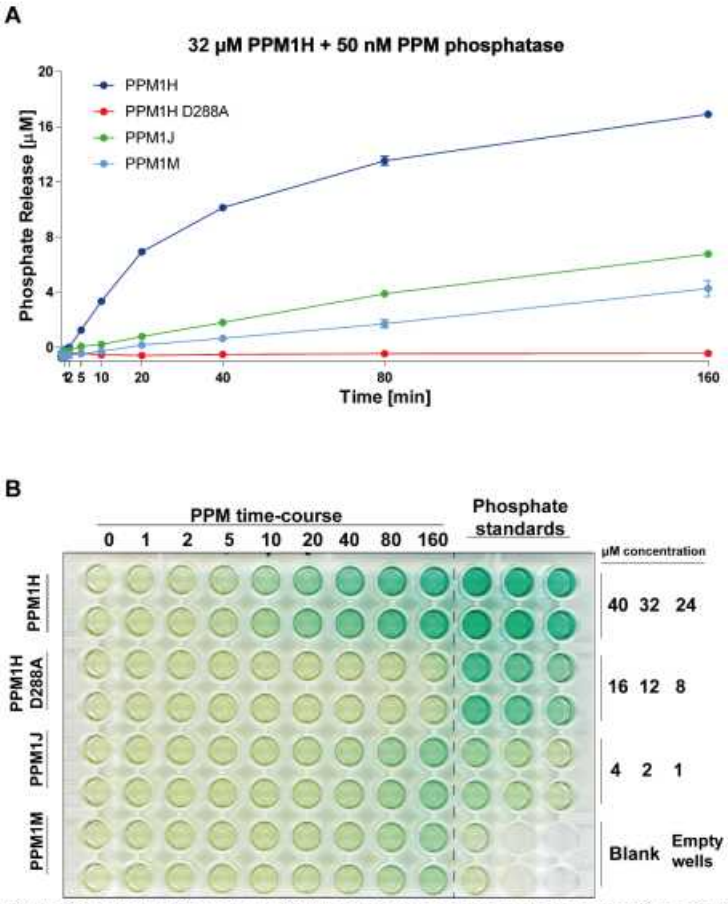

Figure 3: Representative result for PPM phosphatase dephosphorylation assay using 50 nM PPM phosphatase and 32 µM phospho-Rab8A peptide. **(A)** Plotted concentrations of released phosphate (µM) from the phosphatase assay vs time (min). **(B)** 96-well plate after the completed malachite green assay. 50 nM PPM1H WT, PPM1H D288A, PPM1J WT, or PPM1M WT were incubated with 32 µM phospho-Rab8A peptide for the indicated timepoints.

If undertaking the phosphatase assay with a phospho-protein substrate - prepare the Thr72 phosphorylated Rab8A Substrate (@ 1.067X final assay concentration)

Prior to undertaking the phosphatase assay prepare a 1.067X final concentration of recombinant stoichiometrically Thr72 phosphorylated Rab8A GTPgS or GDP by diluting the protein in a buffer **[M]40 Milimolar (mM)** HEPES and **[M]10 Milimolar (mM)** MgCl2). **75 µl** of this substrate solution is required per assay. For the assay shown in Figure 2 we employed **[M]16 Micromolar (µM)** Thr72 phosphorylated Rab8A GTPgS, so a stock of **[M]17.1 Micromolar (µM)** substrate was prepared for this study. Keep substrate on ice until transfer to the 96 well plate.

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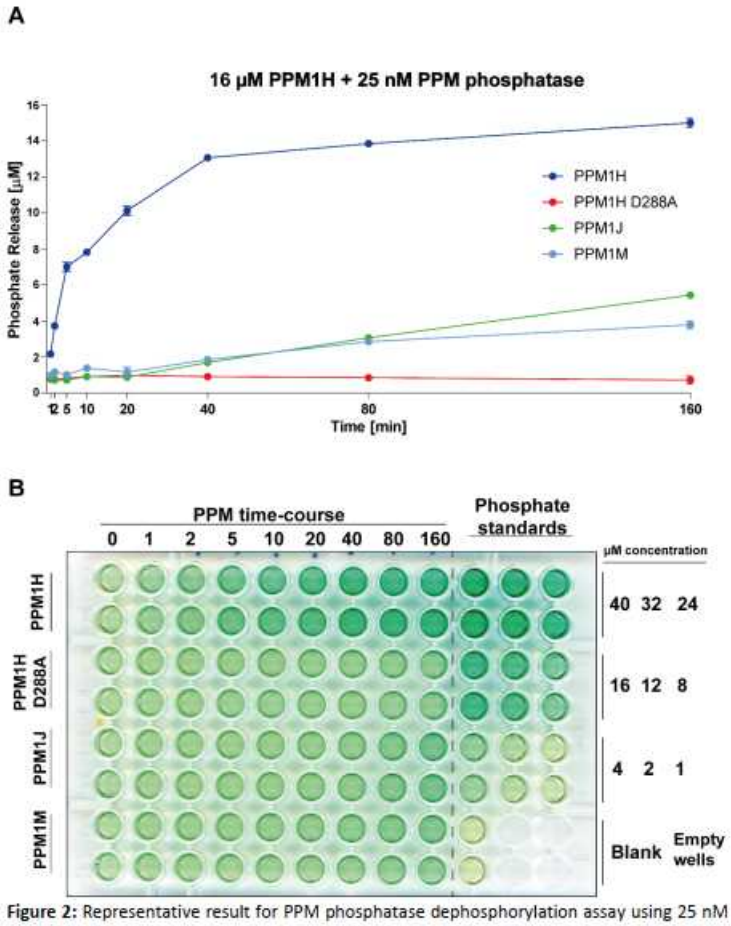

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If undertaking the phosphatase assay with phospho-peptide substrate - prepare the phosphopeptide encompassing phosphorylated Thr72 of Rab8A (AGQERFRT*ITTAYYR) (@ 1.067X final assay concentration)

Prior to undertaking the phosphatase assay prepare a 1.067X final concentration of phosphopeptide encompassing phosphorylated Thr72 of Rab8A (AGQERFRT*ITTAYYR) by diluting the stock peptide into a buffer [M]**40 Milimolar (mM)** HEPES and [M]**10 Milimolar (mM)** $MgCl_2$). **75 µl** of this substrate solution is required per assay. For the assay shown in Figure 3 we employed [M]**32 Micromolar (µM)** phospho-peptide, so a stock of [M]**34.13 Micromolar (µM)** substrate was prepared for this study. Keep substrate on ice until transfer to the 96 well plate.

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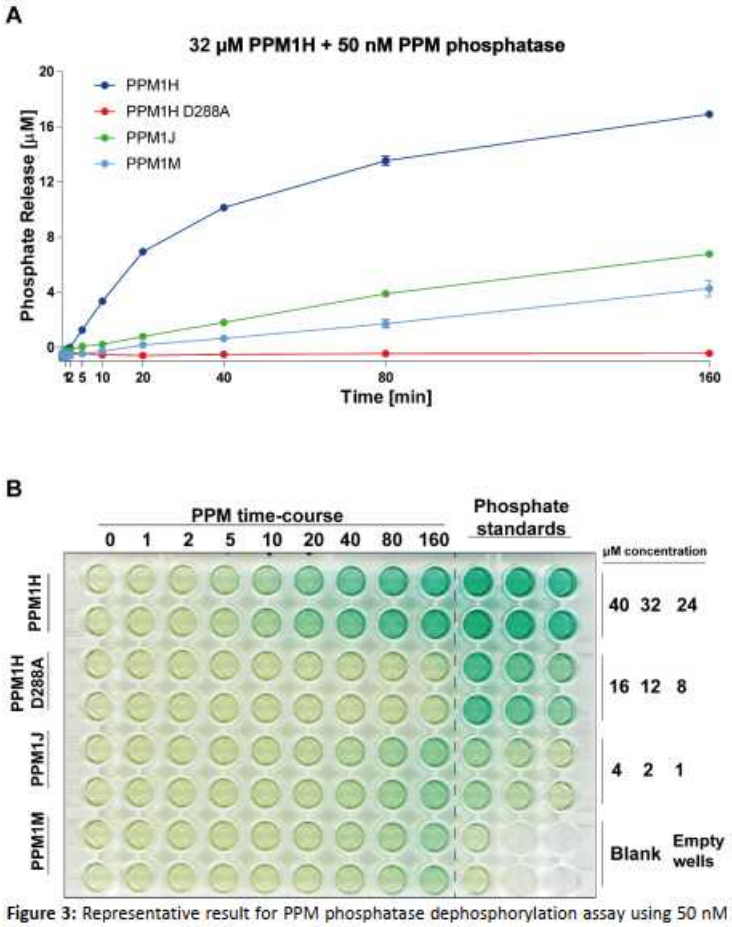

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Malachite green reagent:

Prior to phosphatase assay prepare a stock of malachite green reagents by mixing 1 volume of Reagent B with 100 volumes of Reagent A at **Room temperature** . We deploy these reagents from a malachite green assay kit (Sigma MAK307). Vortex the resulting mixture and keep the resulting mixture called malachite green reagent at **Room temperature** . This malachite green reagent is stable for several hours. **20 µl** of this final mix is needed per phosphatase assay.

Phosphate standards:

Prior to phosphatase assay, it is important to prepare a serial dilution of orthophosphate from [M]**0 Micromolar (µM)** - [M]**40 Micromolar (µM)** that can be used as a standard curve to calculate concentration of phosphate released by protein phosphatase activity. It is important to make up the phosphate in the same buffers as used for the phosphatase assay i.e. buffer [M]**40 Milimolar (mM)** HEPES pH 7.5 and [M]**10 Milimolar (mM)** $MgCl_2$. We normally prepare a 9 point standard curve with concentrations of phosphate of [M]**0 Micromolar (µM)** , [M]**1 Micromolar (µM)** , [M]**2 Micromolar (µM)** , [M]**4 Micromolar (µM)** , [M]**8 Micromolar (µM)** , [M]**12 Micromolar (µM)** , [M]**16 Micromolar (µM)** , [M]**24 Micromolar (µM)** , [M]**32 Micromolar (µM)** and [M]**40 Micromolar (µM)** by diluting the 1 M stock orthophosphate standard provided in the malachite green assay kit (Sigma MAK307) in

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[M]**40 Milimolar (mM)** HEPES pH 7.5 and [M]**10 Milimolar (mM)** MgCl2. These standards are prepared in

80 µl aliquots in duplicate for each concentration. These can be kept at **Room temperature** .

Assay for quantifying PPM1H phosphatase activity towards LRRK2 phosphorylated Rab proteins and peptides using the Malachite Green method

30m

1 Setup the assays in a 96-well flat-bottomed plate (see Figure 1, Figure 2B and Figure 3B):

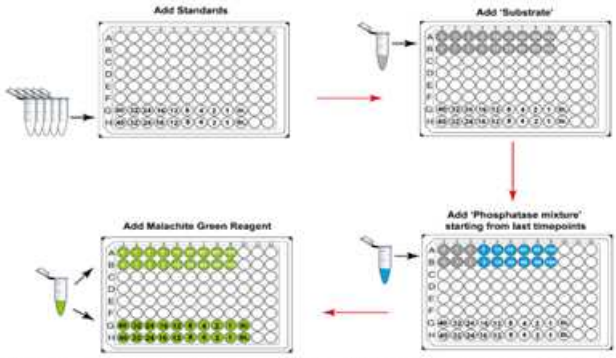

Figure 1. Example of phosphatase assay workflow. Phosphate standards are marked in white, substrate mixture marked in grey, phosphatase mixture marked in blue, Malachite Green reagent marked in green.

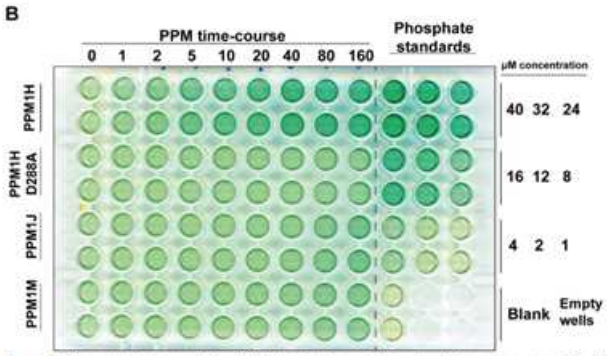

Figure 2: Representative result for PPM phosphatase dephosphorylation assay using 25 nM PPM phosphatases and 16 µM recombinant phosphorylated Rab8A. **(A)** Plotted concentrations of released phosphate (µM) from the phosphatase assay vs time (min). **(B)** 96-well plate after the completed malachite green assay. 25 nM PPM1H WT, PPM1H D288A, PPM1J WT, or PPM1M WT were incubated with 16 µM recombinant phospho-Rab8A protein for the indicated timepoints.

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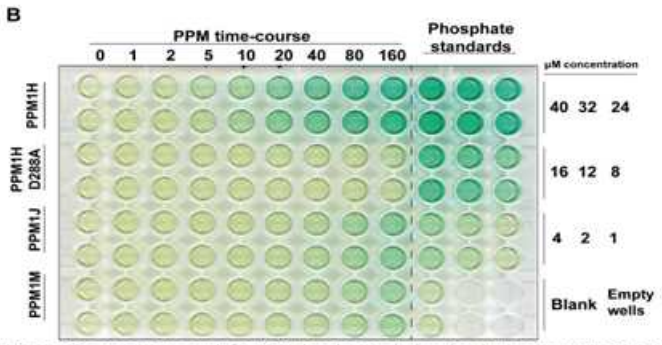

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1.1 Label the plate with the appropriate time points above each well to ensure no mix-up.

1.2

Add **75 µl** of required phospho-substrate to a sufficient number of wells required to perform the planned number of assays.

> We recommend undertaking each assay condition in at least duplicate, preferably in triplicate.

1.3 Keep plate and aliquoted samples at **Room temperature** .

1.4

Add **80 µl** of the serial dilution of orthophosphate standards to another set of wells in duplicate.

1.5 Place the 96-well plate at room temperature on an orbital shaker with slight agitation.

2

Initiate the phosphatase assay reaction by briefly pausing the orbital shaker and adding **5 µl** of phosphatase mixture to the desired wells of the 96-well plate for each timepoint.

3

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Mix well by pipetting up and down before restarting the orbital shaker.

If a time course study is being undertaken, we recommend initiating the assay with the longest timepoint first and working backwards so that all assays are terminated at a similar time.

4

When the desired time point to terminate the reaction is reached, stop reactions by adding **20 µl** of malachite green reagent to each phosphatase reaction and mixing by pipetting up and down.

The malachite green reagent is acidic and will denature the phosphatase and therefore stop the reaction.

5

For the 'zero' timepoint add **20 µl** of the malachite green reagent to **75 µl** of the phospho-substrate mixture followed by **5 µl** of the phosphatase mixture.

6

30m

Incubate the 96-well plate at **Room temperature** for **00:30:00** to allow the green colour to develop.

7 Measure the absorbance at 620 nm using spectrophotometric multi-well plate reader.

8 Plot absorbance (620nm) versus phosphate standard concentrations to create a standard curve.

9 Determine concentration of phosphate released for each assay using the standard curve.

10 Subtract the zero timepoint from concentration of phosphate released from timecourse.

11 Plot the amount of phosphate released (µM) vs time (min) (See Figure 2A and Figure 3A that shows a time course of PPM1H dephosphorylation of **16 Micromolar (µM)** stoichiometrically Thr72 phosphorylated Rab8A.

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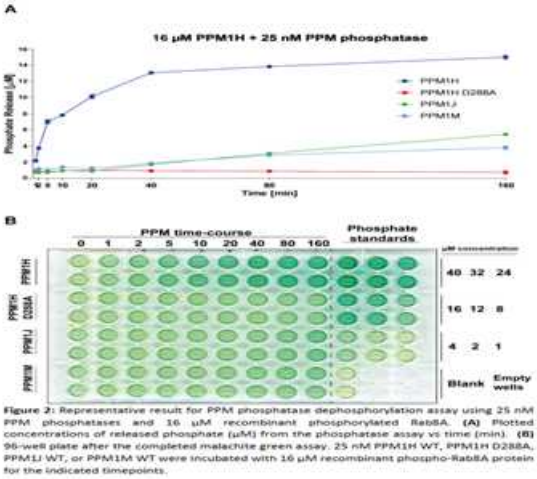

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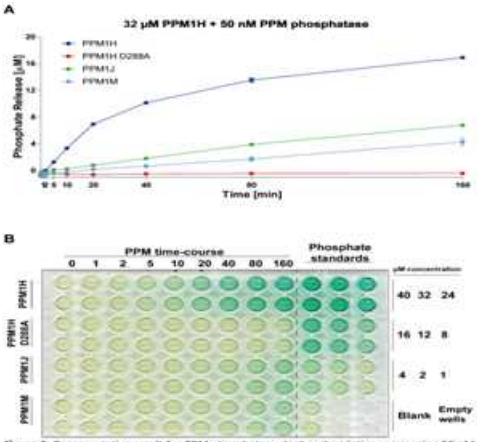

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These assays also demonstrate that catalytically inactive PPM1H[D288A] mutant fails to dephosphorylate the phospho-Rab8A protein or phopsho-Rab8A peptide. The assays also demonstrate that PPM1M and PPM1J phosphatases that are closely related to PPM1H poorly dephosphorylate the phosphoRab8A protein and peptide substrate).

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