pNPA Enzyme-Kinetics Protocol for VcCDA

Introduction

Last Updated: *16th Sept 2026*

ADAPTED FROM THE BMG LABTECH PROTOCOL

Assay Principle

The purpose of this SOP is to identify whether the produced VcCDA can catalyse hydrolysis reactions via the use of a pNPA assay. To note this assay measures the esterase activity of the VcCDA enzyme and therefore does not characterise the true kinetics of the deacetylation of the chitobiose. We are relying on the ester groups present, as demonstrated in Fig 1.

Fig 1: Acid/Base Catalytic Mechanism of VcCDA [1]

The assay works by using the enzyme for testing to catalyse the hydrolysis of the acetate ester of pNPA, releasing p-nitrophenol (pNP) and acetic acid. Above its pKa (about 7.2) pNP is observed as a yellow coloured p-nitrophenolate anion, which absorbs near 405 to 410 nm. The rate of absorbance increase is measured as the activity signal. Since the assay run continuously, absorbance needs to be measured in real-time, and the initial linear slope taken for data analysis. To Note: pNPA also hydrolyses on its own, so a no-enzyme blank runs in parallel and is subtracted.

Fig 2: pNPA Reaction Mechanism [2]

[1]https://www.researchgate.net/publication/349018098_An_overall_look_at_insect_chitin_deacetylases_Promising_molecular_targets_for_developing_green_pesticides/figures?lo=1
[2] https://www.bmglabtech.com/en/application-notes/enzyme-kinetic-measurements-performed-on-a-bmg-labtech-microplate-reader/

Materials

- p-nitrophenol (pNP) and p-nitrophenyl (pNPA) acetate
  - p-NPA Vendor: https://www.sigmaaldrich.com/GB/en/product/sigma/n8130
  - pNP Vendor: https://www.sigmaaldrich.com/GB/en/product/sigma/N7660
  - Alternative vendor cause I saw the earliest delivery date might be October for pNP? https://www.thermofisher.com/order/catalog/product/A14376.30
- Clear 96-well microplate
- BMG LABTECH microplate reader
  - (Or any Spectrophotometer)
- MARS Data Analysis Software
  - (or any Data-analysis software that fits Michaelis-Menten and linearised plots)
- 20 mM stock solution of the substrate pNPA in DMSO.
  - Need to prepare in the Fume Hood
  - Store cold and make working dilutions fresh, since pNPA hydrolyses in water.
- 10 mM pNP stock solution in Sodium Phosphate Buffer.

- Used for Standard Curve.

- Sodium Phosphate buffer (50 mM, pH 7.4).
  - Alternatively, we could go for a higher pH buffer like pH 8 to match the optimal pH conditions of VcCDA since only requirement is above pH 7.2. However, Isobel mentioned that the reaction may go too fast at more basic conditions --> Becomes difficult to get a good linear slope.

Procedure

pNP Assay

1. First a 20 mM stock solution of the substrate pNPA in DMSO is prepared.

2. In each well pipette 218 µL phosphate buffer (50 mM, pH 7.4) and 10 µL enzyme preparation is pipetted.

3. 12 µL of pNPA Substrate (Dissolved in DMSO) at different concentrations is added using the onboard injectors (For ClarioStar BMG Labtech Plate Reader). The final volume per well is 240 µL.

   Final pNPA Cocentration is 1mM with final DMSO concentration of 5 %.
   pNPA concentrations range from 0.025 µmol to 0.175 µmol / 240 µL in example BMG Labtech
   The Planas Paper which worked on Vibrio cholerae Deacetylase worked in the range of 0.05–0.8 µM enzyme. [1]

4. The enzymatic reaction starts immediately after adding the substrate. The absorbance is measured at 410 nm for every second for 90 seconds at 37°C.

   Isobel said we may need to lower this too room temperature (25C) if background hydrolysis is too rapid.

5. The blank represents the autohydrolysis of pNPA without enzyme. Add 10 uL buffer instead of enzyme during preparation. 12uL of pNPA DMSO-dissolved substrate and 228uL of phosphate buffer.

   Remember to use the blank to subtract from the measured absorbance to obtain the true absorbance values.

6. Run a pNPA-free negative control that consists only of buffer and enzyme. 230 µL of phosphate buffer (50 mM, pH 7.4) and 10 µL of enzyme preparation is pipetted.

7. For data analysis, take the initial linear slope over the 10 to 70 s window, skipping the injection or mixing peak.

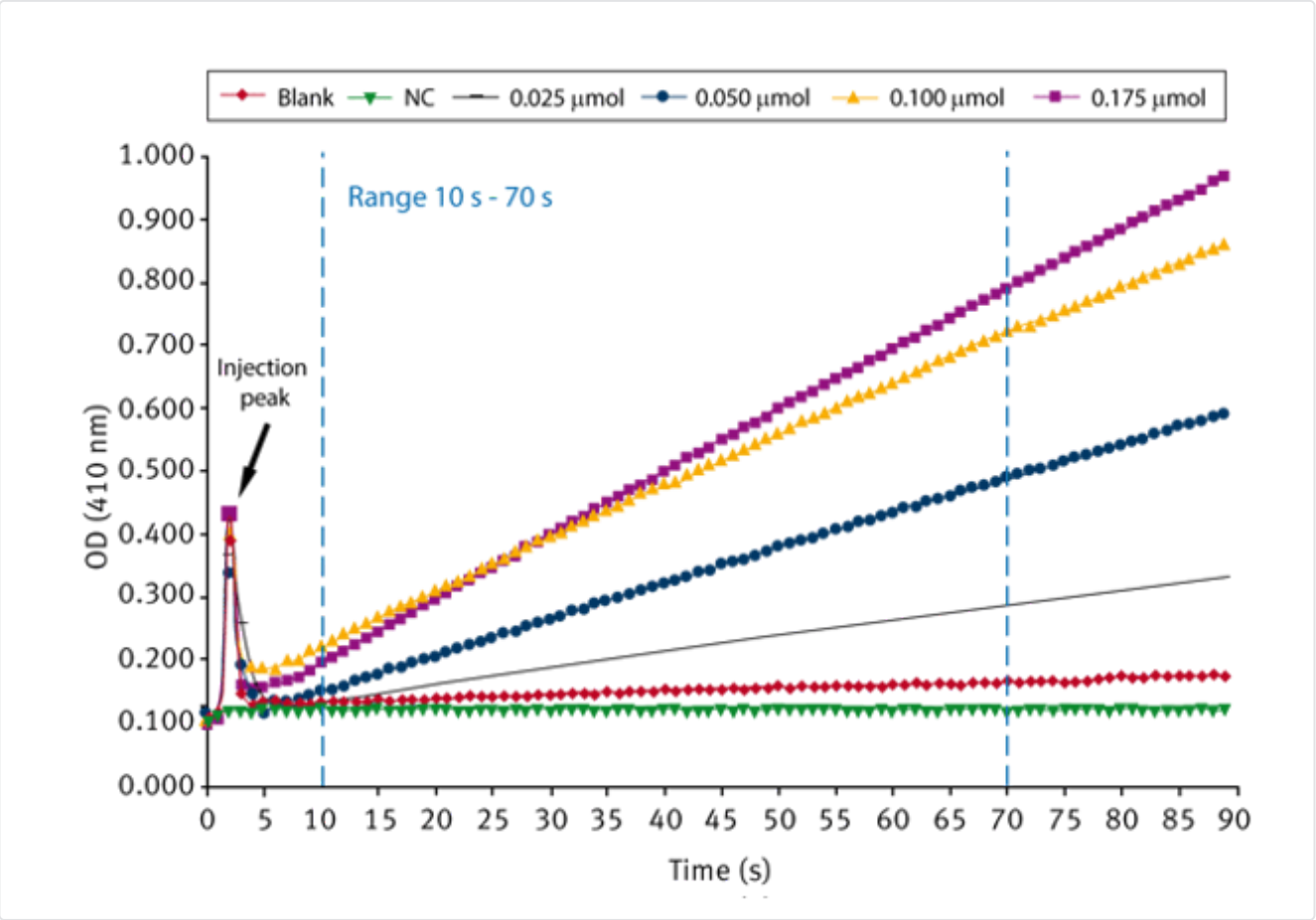


Fig 3: Example. Signal curves of esterase-catalysed reactions using different concentrations of pNPA

[3] https://pubs.acs.org/ancham/article/90/18/10654/820176/Screening-Assay-for-Directed-Evolution-of-Chitin

Instrumentation & Injection Settings (For Plate Reader)

Plate Reader Settings

	A	B
1	**Instrument Setting**	**Value**
2	Measurement type	Absorbance, well mode
3	Number of intervals	90
4	Kinetic interval time	1 s (90 s total read)
5	Flashes per well	20
6	Positioning delay	0.5 s
7	Target temperature	37 C
8	Filter	410-10 nm

Injection Settings

	A	B
1	**Injection Setting**	**Value**
2	Volume of pump 1 and 2:	Individual
3	Pump speed:	310 µl/s
4	Injection start time:	2.0 s

pNP Standard Curve

For the calculation of the initial reaction rates, it is necessary to convert the OD values per minute into µmol of product per minute. Therefore, a standard curve of the product using the same reaction conditions for the kinetic measurements has to be performed.

8. From a 10 mM pNP stock solution, dissolved in phosphate buffer (50 mM, pH 7.4), different dilutions of pNP ranging from 0.0025 µmol to 0.1 µmol need to be prepared for a volume of 228 µL.

9. Add 12 µL of DMSO to simulate the DMSO Concentration in the kinetic samples.

10. The optical density is measured using an absorbance 410-10 nm filter. Plot absorbance against micromoles pNP.

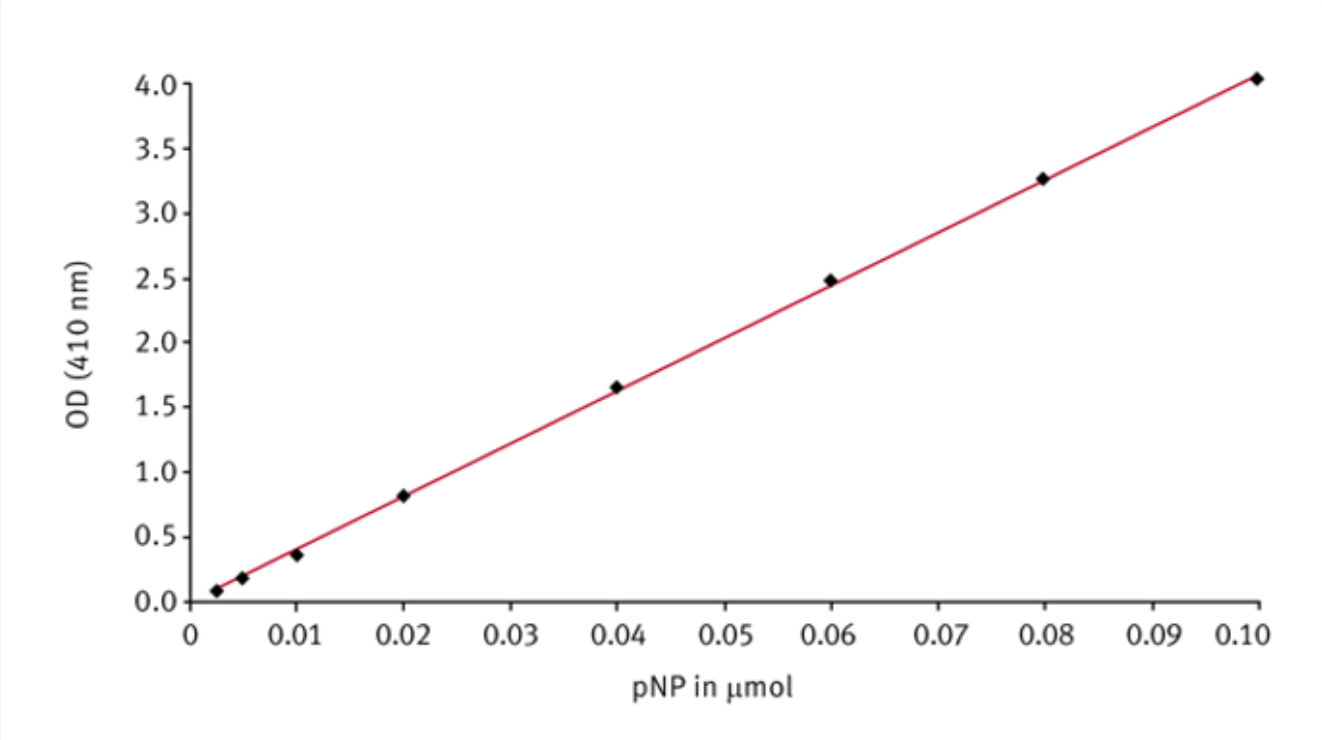


Fig. 4: Example. pNP standard curve using 240 µL volume with 16.6 % DMSO.
The slope of the standard curve represents the extinction coefficient for pNP under the conditions mentioned. The coefficient is used to calculate the initial reaction velocities.

Data Analysis

11. For each well, take the initial linear slope (OD per minute) within the 10 to 70 s Linear window.

12. Subtract the mean blank slope.

13. Convert the corrected slope to micromoles pNP per minute using the standard-curve slope. This is the initial velocity.

14. Plot velocity against substrate to fit Michaelis-Menten and read Km and Vmax.

15. Report Km as a concentration (mM). If using micromoles per well; divide by the 0.240 mL well volume to get concentration.

    *The MARS Data Analysis Software (or any Data-analysis software that fits Michaelis-Menten and linearised plots) should be able to handle this part. Below is just a more manual version.

References

Aragunde, H., Biarnés, X. and Planas, A. (2018). Substrate recognition and specificity of chitin deacetylases and related family 4 carbohydrate esterases. *International Journal of Molecular Sciences*, [online] 19(2), p.412. doi:10.3390/ijms19020412.

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