STANDARD OPERATING PROCEDURE (UVVIS#1_EnzKin_Gre2p)					
IBG-1 KIT Karlsruher Institut für Technologie		**Institute for Biological Interfaces** IBG-1 | Biomolecular Micro- and Nanostructures			
Short Title	Kinetic parameters of Gre2p	**Page**	1	**of**	9
Title	Measure initial rates of Gre2p and fit with enzyme kinetic models				
Version	1.0		**Created on**	01.03.2021	
Safety instructions	By following the instructions in this SOP you confirm that you have checked the SDS-sheet of the involved chemicals (also reagents of your specific enzyme system) and will protect yourself accordingly when working with these substances. Also, you confirm that you will follow standard lab safety procedure to protect yourself, others, and the environment.		**Related SOP**	UVVIS#2_SpAc_Gre2p	
			Category	PROTOCOL	
			Purpose	describe how to perform experiments to measure the initial rates of an NADPH-dependent enzyme, Gre2p, by following the absorption change of NADPH at 340 nm in a multi-well plate reader to ultimately model enzyme kinetics using e.g. the Michaelis Menten Model	
			Note	It may be necessary to use complex models to fit your data if you observe deviation from Michaelis Menten	
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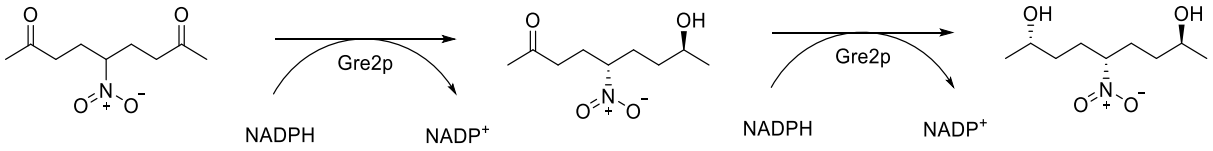
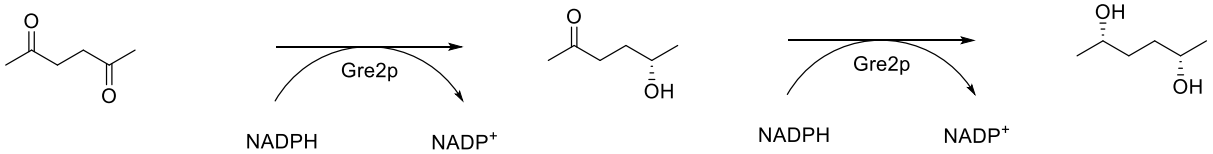


Reaction scheme of the observed reduction of 5-nitrononane-2,8-dione (NDK) to the corresponding hydroxyketone and possible further reaction to the corresponding diol catalyzed by Gre2p. The cofactor NADPH get oxidized to NADP⁺ in the process.



Reaction scheme of the observed reduction of hexane-2,5-dione to the corresponding hydroxyketone and possible further reaction to the corresponding diol catalyzed by Gre2p. The cofactor NADPH get oxidized to NADP⁺ in the process.

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1) Introduction

Enzyme kinetic parameters, K_m and k_{cat}, are determined as follows: 1) measure initial reaction rates with different substrate concentrations (mM/s) 2) plot the rates as a function of the substrate concentration to obtain a "Michaelis-Menten plot" and fit the data with an appropriate enzyme kinetic model. Substrate concentrations have to be chosen so that you get initial rates for five substrate concentrations under K_m and five substrate concentrations above K_m. Also, the reaction should be easily observable but not too fast (at least ten measurement points in a linear slope). To have data to plan with you can research literature or perform a preliminary test with three or four substrate concentrations and two to three different enzyme concentrations.

2) Preparation

The measurement to determine the enzymatic parameters (K_m and k_{cat}) can be performed with a spectrophotometric assay using a UV-Vis plate reader such as the Synergy™ H1 (or MX) Hybrid reader (BioTek, Winooski, VT, USA). Before starting the experiment, plan everything ahead to ensure a smooth procedure.

For the reduction of NDK and Hexane-2,5-dione by Gre2p-His, a substrate range of 50 µM to 50 mM and an enzyme concentration of 25 nM and 10 nM was chosen, respectively. The NADPH concentration has to be chosen so that the absorption is not exceeding a value of 1 (because the detector of the instrument starts to get saturated above that limit). 300 µM NADPH has proven suitable. Controls have to be at least measured in duplicates and the samples in triplicates, better in four replicates. The substrate concentration in the controls has to be high enough, that a possible increase of the NADPH oxidation rate caused by the substrate would be visible, e.g. 10 mM.

The reaction participants will be added in different volumes (NADPH 10 µL, Gre2p 10 µL, Substrate 80 µL), which has to be taken into consideration when preparing the stock solutions. There are different plate layouts possible. A suggestion is shown in Figure 1.

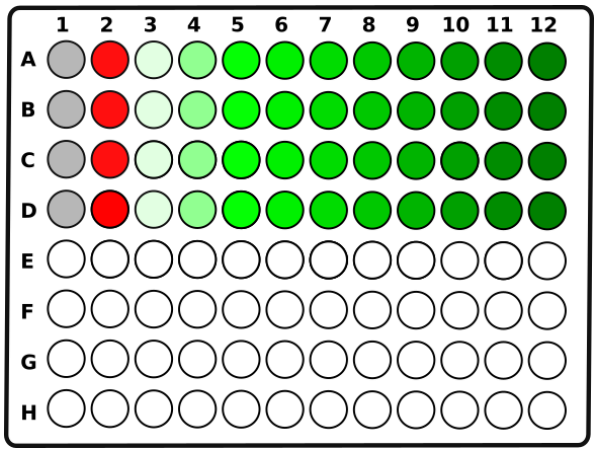

Figure 1: Exemplary plate layout. Grey: only buffer (no enzyme(-E),no substrate (-S),no NADPH (- N)), red: NADPH (300 µM) in buffer(-E,-S,+N) green: 25 nM or 10 nM Gre2p + NADPH (300 µM) + [S] – the color gradient indicates the increasing concentration of substrate from 0.05 to 50 mM (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0)

Table 1: Concentrations of the reaction participants in the final assay and in stock solutions. Because the volumes of the participants are different, the dilution factor is also different.

Reaction participant	Final concentration (in the assay)	Stock concentration
Gre2p	10 nM or 25 nM	100 nM or 250 nM
NADPH	300 microM	3 mM
Substrate	50 microM – 50 mM	62,5 mM

Examples for stock preparation:

NADPH:

- Dissolve 3,9 mg in 1,56 mL buffer

Gre2p:

- Dilute 1.5 mM stock to 50 µM (2 µL in 58 µL buffer)
- Dilute 50 µM stock to 100/250 nM (2/5 µL in 998/995 µL buffer.

NDK/Hexane-2,5-dione

- Dissolve 17.4 µL NDK or 11.7 µL Hexane-2,5-dione in reaction buffer. The mixture has to have a total volume of 1 ml for a stock concentration of 100 mM. Note that NDK can be liquid or solid, depending on the age of the sample, but it has identical properties for our purposes in either state. It is possible to liquidize the NDK by heating it.
- Perform a dilution series. Keep in mind that you need min. 360 µL of each dilution with exception of the 10 mM dilution of which 720 µL are needed because of the controls. It is advised to prepare at least 20 µl extra since excess fluid ensures exact pipetting.

3) Experiment execution

- Prepared the plate (e.g. a 96 well MicroWell™ PolySorp® flat bottom plate by sigma-aldrich) following the pipetting scheme (Figure 1).
- pipet the Gre2p and the NADPH solutions in the wells.
- Gently tap the plate to mix and incubate at RT for five minutes.
- In this time, start up the plate reader, the connected PC and set up the measurement before pipetting the enzyme into the wells.

! The addition of the Substrate is time-sensitive and has to be performed as quickly as possible. To save time and ease the process, this pipetting step is performed with a multichannel pipette. Since you need to pipette 12 different samples, you have to prepare a "master plate" mimicking the final scheme (separated in ingredients, not replicates). Like this you need only four pipetting steps (with the multichannel pipette) instead of 48.

- Check that the plate reader is set up properly and ready to measure
- Pipet from the master plate to the measurement plate
- Quickly place your measurement plate into the plate reader and start the measurement

Table 2: Plate reader parameters (Synergy H1 or MX)

Parameter	**Value**
Temperature	30 °C
Mixing time before every set of measurements	10 s
Measurement total time	2 h (30 min should be also sufficient since only initial rates are of interest)
Interval (shaking + measurements)	Shortest possible (32 s when measuring 48 wells)
Pathlength correction	yes

4) Analysis

A raw data set of such an experiment contains a lot of data (Figure 2).

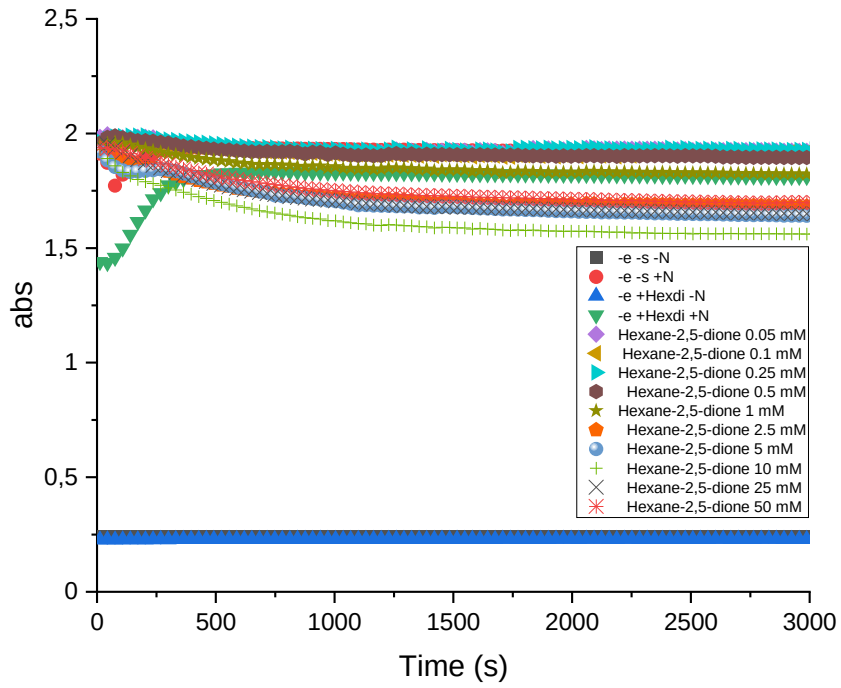

Figure 2: Exemplary dataset of one replicate. After a initial equilibration period of about 400 s, every sample shows decrease in absobtion at λ = 340 nm. The controls without NADPH form a baseline. Note, that the abs values are caulculated with a beseline correction to 10 mm. The mesured values do not exceed a value of 0.8 and the detector of the instrument is not saturated.

The decrease in NADPH absorption at 340 nm corelates to its oxidation to $NADP^+$ and therefore the activity of Gre2p.

The analysis procedure in bullet points:

- Find the earliest and steepest linear decrease in absorption (to get initial rates)
    - At the begin the absorption is unstable which is also observable in the controls. The first interval of 10+ measurement points with linear decrease has to be chosen. This is preferably the same interval for all samples, but can also be slightly altered.

- Perform a linear fit through this interval of minimal ten points (yields initial rate). This can be done with most data analysis software but origin is advised since the more complex fits later on are not possible with non-scientific software like excel (or with your custom python script).
    - The quality of this fit differs depending on the data (Figure 3). R^2 = 0,92 can be used as a threshold under which the data should not be used, but it is important to control every fit and make sure the datapoints do not "curve around the linear fit", as they do in Figure 3c.

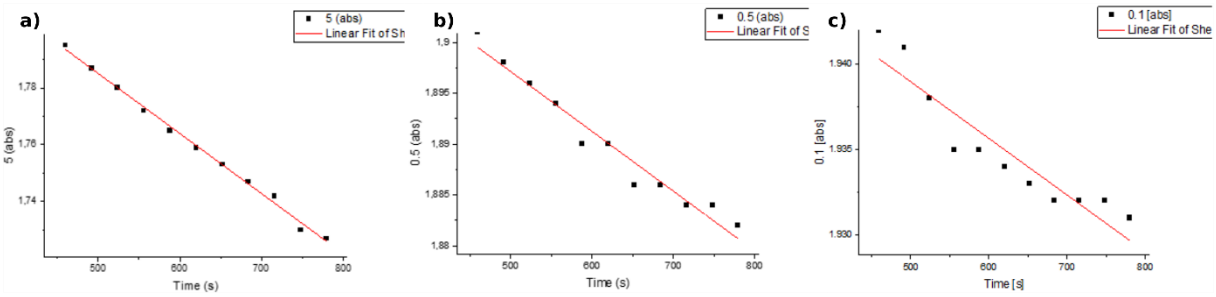

Figure 3: Examples of different linear fits of rate measurements. a) good fit (R^2 = 0,99516), b) an acceptable fit (R^2 = 0.95782), c) a poor fit which should not be used (R^2 = 0.86264), also, the datapoints "curve around the linear fit", with some systematically above and some below the curve.

- The slope of the fit (initial rate, in mM/s) can be used to calculate the rate in which NADPH is oxidized (using its extinction coefficient of 6.22 $(mmol*cm*L)^{-1}$).
    - Subtract the rate of the control without Gre2p from these rates to only use the decrease caused by the enzyme in further calculations
- Calculate the rates per enzyme ($mol_{Substrat}/(s*mol_{enzyme})$ = s^{-1}) using the final enzyme concentration in the assay (e.g. 10 nM) and the calculated NADPH decrease (e.g. X mM/s).

  Equation: $$rate\ per\ enzyme = \frac{NADPH\ decrease}{[Gre2p]}$$
- Use the calculated rates per enzyme for a kinetic model (e.g.: MichaelisMenten) and test which model is the best for your data.
    - Exclude the data resulting from poor linear fits for this step
    - The fits used can be found in origin using the path Analysis → Fitting → Nonlinear Curve Fit → Open Dialog → Category: Enzyme kinetics → Function: *choose desired model here*

- Exemplary Models and the underlying equations:

  Michaelis Menten: $y = \frac{V_{max}\, x}{K_M + x}$

  Substrate Inhibition: $y = \frac{V_{max}\, x}{K_M + x(1+\frac{x}{K_i})}$

- To compare the models, you can use the "Adj. R-Square" value provided by origin. The higher this value is, the better the fit.
- Exemplary data for a fit with the Michaelis Menten model (Figures 4 and 5)

- Calculate the means and standard deviation from the replicates of the enzyme parameters v_{max} (k_{cat}), K_m and others like K_i depending on the model you used to fit your data.

Summary

	Vmax		Km			Statistics	
	Value	Standard Error	Value	Standard Error	Va	Reduced Chi-Sqr	Adj. R-Square
kt-(cat)	4,07539	0,29225	0,60097	0,23897	0,0	0,2549	0,88592
	3,88042	0,27943	1,48841	0,51899	0,1	0,14752	0,93187
	4,79034	0,28139	2,05551	0,48429	0,	0,13091	0,9554
	3,65985	0,34035	1,40903	0,56238	0,1	0,23908	0,86432

Figure 4: Exemplary summary of a Michaelis Menten model by origin. The Adj. R-Square which indicates the quality of the fit is highlighted by the red box.

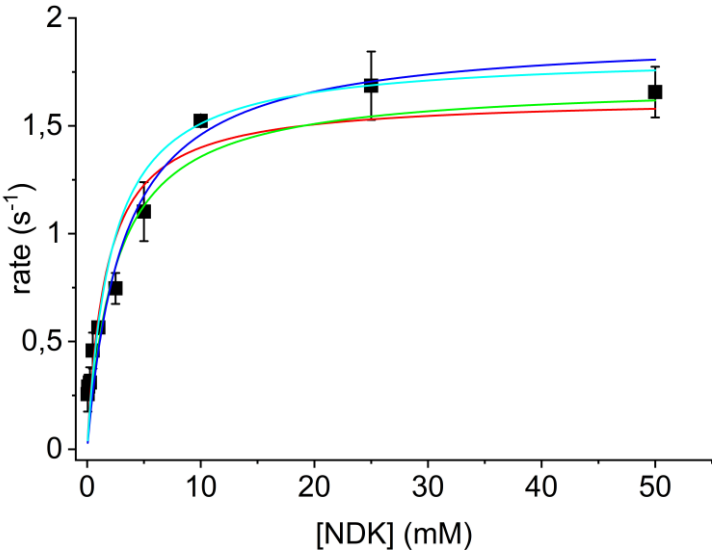

Figure 5: Example of a data-set fitted with the classical Michaelis Menten model

5) Enzyme information for STRENDA compliance

Table 3: Enzyme and assay information summarized following the STRENDA Guidelines as much as possible.

Data	Value
Name of reaction catalyst	**G**enes de **r**espuesta a **e**stres (stress-response gene) (Gre2p)
EC number	1.1.1.283
NCBI Taxonomy ID of organism of origin	1294304
GenBank Sequence ID	AJT71311.1
Artificial modification	C-terminal hexahistidine-tag
Storage conditions	
Enzyme purity	Apparently homogeneous by SDS-PAGE
Storage temperature	-80 °C, flash frozen
pH	7.5, measured at 25 °C
Buffer	100 mM KPi
Enzyme concentration (of frozen stock)	Depending on the sample in buffer 1.1 to 1.5 mM
Samples are thawed	On ice or at room temperature
Assay conditions	
Substrate purity	NADPH: IWT Reagents with a purity of 99.6 % Hexane-2,5-dione: Fluka analytical with a purity of 99.0 % NDK was synthesized as described here: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-DPTOJTZVUD-UHFFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ
Measured Reaction	Gre2p+NADPH + NDK -> Gre2p + NADP+ + NDK-alcohol, see page 1 of this SOP Gre2p+NADPH + Hexane-2,5-dione -> Gre2p + NADP+ + Hexane-2,5-diol, see page 1 of this SOP
Assay pH	7.5, measured at 25 °C for the respective Buffer
Buffer	100 mM KPi
Substrate concentration range	300 µM NADPH, NDK/Hexane-2,5-dione: 50 µM – 50 mM
Enzyme concentration	10 nM for experiment with Hexane-2,5-dione, 25 nM for experiment with NDK
Activity and Methodology	
See details described above.	