STANDARD OPERATING PROCEDURE (ITC#3_(r)SIM_Gre2p)					
IBG-1 KIT Karlsruher Institut für Technologie		**Institute for Biological Interfaces** IBG-1 | Biomolecular Micro- and Nanostructures			
Short Title	ITC-(r)SIM Gre2p	**Page**	1	**of**	6
Title	Gre2p (recurrent) single injection kinetics measurements using the ITC				
Version	1.0		**Created on**	26.04.21	
Status	The MicroCal PEAQ-ITC uses methanol and a 10% DECON 90 solution for washing of the cell and syringe. Methanol has the following hazards: H225, H301, H311, H331, H370.  DECON 90 has the following hazards: H290, H315, H319.  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**	SOP_ITC#1_General SOP_ITC#2_MIM_Gre2p	
			Category	PROTOCOL	
			Purpose	To provide instructions on how to measure kinetics of Gre2p's reaction with NDK as substrate and NADPH as cofactor using ITC.	
			Note	If you are not familiar with the ITC in general, read SOP_ITC#1_General first	
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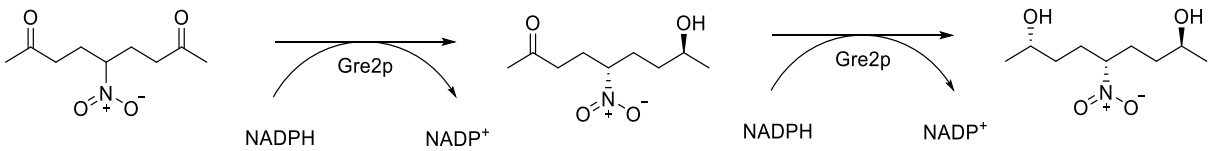

Reaction scheme of the observed reduction of 5-nitrononane-2,8-dione (NDK) to the corresponding hydroxyketone and possible further reaction to the corresponding diol catalyzed by Gre2p. The cofactor NADPH get oxidized to $NADP^+$ in the process.

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1) Introduction

Enzyme kinetics can be measured using ITC (recurrent) single injection experiments (ITC-(r)SIM) and ITC multiple injections experiments (ITC-MIM). K_m can be determined with ITC-SIM experiments, detailed information on the reaction mechanism can be obtained, and the enthalpy of the reaction, $\Delta H_{reaction}$ can be measured to determine k_{cat}. In ITC-SIM experiments, reactant is titrated to a higher concentration of enzyme, than in the ITC-MIM experiment. This approach results in the signal returning to the baseline and the resulting peak is integrated to yield $\Delta H_{reaction}$. In a variation of this experiment, the "recurrent single injection experiment" (ITC-rSIM), insight on product inhibition or activation can be gained. In ITC-rSIM experiments, multiple injections are made – they are NOT the same as ITC-MIM experiments because the signal returns to the baseline. ITC-rSIM experiments allow the distinction between enzyme inactivation and slow-onset inhibition by carefully analyzing the shape of the injection peaks. If the injection depth of the peaks and $\Delta H_{reaction}$ of all recurrent injections are identical, no product inhibition is present. In contrast, enzyme inactivation leads to broader peaks in ITC-rSIM experiments.

2) Preparation of stock solutions

- 100 mM NDK in buffer
  - Store at 4 °C
- 100 mM NADPH in buffer
  - Store frozen and keep dark; freeze again in between measurements
- Gre2p stock (1 – 1.5 mM)
  - Store at – 80 °C; when thawed once store on ice

3) Sample preparation and loading

Before every measurement, the sample solutions have to be prepared freshly by diluting/mixing the stock solutions.

Cell: 10 - 15 µM Gre2p, 10 mM NADPH

- 45 µL buffer + 5 µL Gre2p stock
- Invert multiple times
- 50 µL of the prepared 1:10 dilution + 50 µL NADPH stock + 400 µL buffer
- Invert multiple times and pipette into cell prior to measurement

Syringe: 50 mM NDK

- Mix 40 µL NDK stock with 40 µL buffer in PCR tube
- put tube in loading position

4) Experimental parameters

For the procedure of the actual ITC experiment, check the SOP ITC#1_General. The used parameters are listed in Table 1.

Table 1: Used ITC parameters

Parameter	Value
Temperature (°C)	25
Reference Power (µcal/s)	25
FeedBack	High
Stir Speed (rpm)	500
Initial Delay (s)	60
# of injections	2
Volume (µL)	2
Duration (s)	4
Spacing (s)	600 (dependent on buffer)

5) Troubleshooting

If the listed spacing time of ten minutes is not enough to get the differential power back to the base line (for example when using buffer supplemented with 0.1 % Tween (Figure)), a longer spacing has to be chosen. Figure shows the data from an experiment with sufficient spacing.

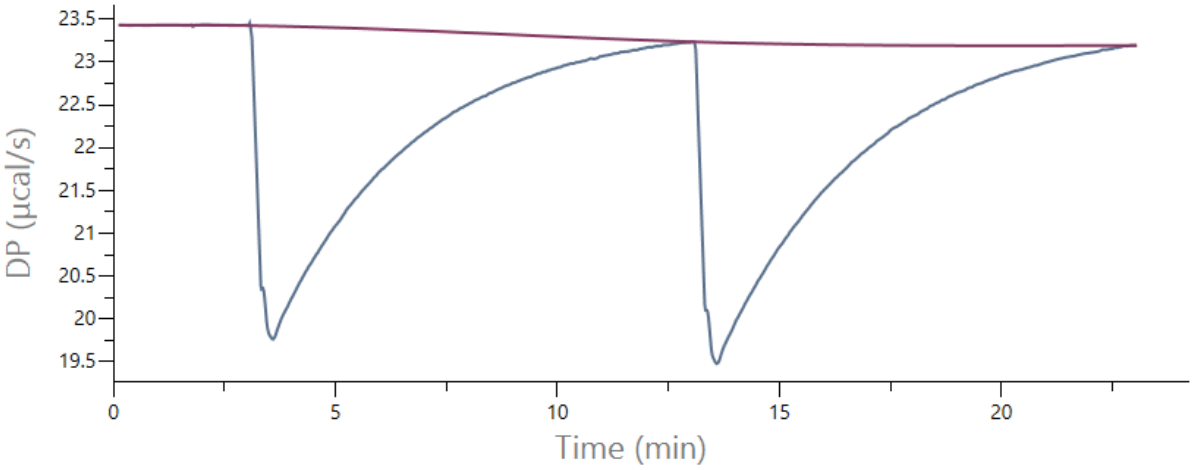

Figure 1: Differential power (DP) to time plot with insufficient spacing. The DP has not reached the baseline yet.

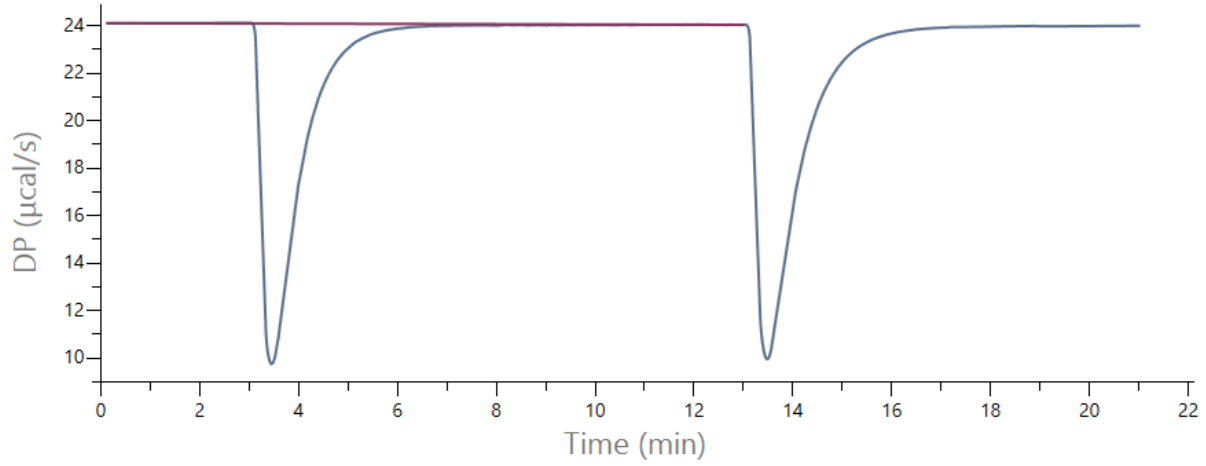

Figure 2: Differential power (DP) to time plot with sufficient spacing. The DP has reached the baseline before the second injection.

If the fit of the MicroCal PEAQ-ITC Analysis Software (Version 1.30) does not work, you can change the data to “ok” and also force the software to use K_m determined by the multiple injection experiments. It may be possible to “force” the fit in this manner and still reach a converging result (fit parameters are stable and do not change if “fit” is clicked multiple times).

6) Enzyme information for STRENDA compliance

Table 2: 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	Depending on the sample in 100 mM KPi, 1xPBS or 100 mM HEPES buffer
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 % NDK: NDK was synthesized as previously described,[7,8] for determination of purity NMR was used (see spectra in SI)
Measured Reaction	Gre2p+NADPH + NDK -> Gre2p + NADP+ + NDK-alcohol, see page 1 of this SOP
Assay pH	7.5, measured at 25 °C for the respective Buffer
Buffer	Depending on the sample in 100 mM KPi, 1xPBS or 100 mM HEPES buffer
Substrate concentration range	10 mM NADPH, NDK: 0.5 mM
Enzyme concentration	Depending on the individual sample in buffer between 10 - 15 µM initially in the cell
Activity and Methodology	
Directly measured using ITC	