<table>
<thead>
<tr>
<th>Short Title</th>
<th>Use of Isothermal Titration Calorimetry (ITC)</th>
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<tbody>
<tr>
<td>Title</td>
<td>Practical instructions for use of the MicroCal PEAQ-ITC in use at IBG-1</td>
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<tr>
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<td>Safety instructions</td>
<td>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.</td>
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<tr>
<td>Related SOP</td>
<td>SOP_ITC#2_MIM_Gre2p SOP_ITC#3_rSIM_Gre2p SOP_ITC#4_BIND_Gre2p</td>
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<tr>
<td>Purpose</td>
<td>To provide guidance in conducting ITC experiments. Isothermal titration calorimetry (ITC) is an extremely sensitive technique to measure the heat released or absorbed during biochemical binding events or enzymatic reactions.</td>
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<td>Note</td>
<td>The practical application of ITC can be cumbersome without the proper preparation of samples and buffers. The ITC at IBG1 was bought end 2019, and used throughout 2020, resulting in this SOP. This SOP covers the steps necessary to obtain reasonable data for binding and kinetic experiments. It assumes that enzyme has been purified previously and that buffer solutions, cofactors or substrates needed for the reaction are prepared. It also assumes the user is informed about general biochemical principles governing binding of small molecules to enzymes and enzyme catalysis.</td>
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<tr>
<td>Author</td>
<td>Gudrun Gygli</td>
</tr>
</tbody>
</table>
## Table of Contents

1) Getting started ............................................................................................................................................. 3  
2) Actions, checks before the experiment ........................................................................................................ 4  
3) Actions, checks during any experiment ......................................................................................................... 6  
4) Binding experiment (ITC-BIND) ....................................................................................................................... 7  
5) Kinetics experiment (single injection, ITC-(r)SIM) ......................................................................................... 8  
6) Kinetics experiment (multiple injections method, ITC-MIM) ......................................................................... 8  
7) Actions, checks after the experiment ............................................................................................................. 9  
8) Shutting down .................................................................................................................................................. 9
1) Getting started

a. If you are a first time user:

- Read this SOP carefully
- READ THE MANUAL (Appendix 1)
- Watch the instruction videos (Appendix 2)
- Contact the author of this document

b. Use:

If the device is off:

- Turn on the computer
- Then the ITC (there are 2 power switches, one at the back of each module)
- Then launch the software

Ignore Windows complaints about a USB drive (B) – that is the ITC, all is well

c. Requirements:

- Do not work with the device unless you have been instructed by the author of this document beforehand!
- You will need MQ water (500 mL), methanol (100 mL), 10% decon 90 (v/v, 100 mL) for the wash module.
- You will need a Hamilton syringe to fill the cell. (! Needs a plastic cover over the needle)
- You will need single PCR-tubes to enable the syringe to aspirate a ligand solution.
- Pipet tips and pipets, 1.5 mL Eppendorfs
- Enzyme, Buffer (approx. 50 mL for 10 experiments, depending on the dilutions required), substrate/ligand solutions (check sample preparation below).
2) Actions, checks **before** the experiment

a. Sample preparation:

Buffer:

- use identical buffer for all your dilutions – meaning you prepare buffer once and freeze it to keep working with the exact same buffer solution throughout the course of your experiments
- keep frozen stocks of the buffer you use for the substrate/cofactor/enzyme dilutions and thaw as needed

Substrate/Cofactor:

- use frozen stock solutions, ideally already at the concentration you will need for your experiment
  
  or
  
  - ensure your stock does not degrade over the course of your series of experiments
  - It is crucial that the sample in the syringe changes as little as possible (especially for binding experiments), so ideally you set up the experiment so that you can use the same (identical) solution (dilution) for the syringe

Enzyme:

- use frozen stock solutions
- do not thaw and re-freeze your enzyme

b. Check before experiment:

- Is the syringe clean?
  - Start a wash cycle if not

- Is the plunger (tip) clean?
  - Contact the author of this document if not – the plunger tip might have to be replaced

- Is the white plastic around the cell and reference cell clean?
  - Flush 3x with water to clean, refill reference cell after this

- Is the valve for loading clean (glas above syringe)?
  - Start a wash cycle if not
Is the Hamilton clean?
  - Flush the Hamilton with water, buffer and/or 10% Decon to clean

Does the plastic cover at the Hamilton syringe tip go a bit over the metal?
  - Use your fingernails to push the plastic cover so that it extends over the metal tip

When has the reference cell been filled last?
  - If it has been more than 1-2 weeks ago, refill it, perform a water-water-titration to check if all is well

c. Fill cell:

  - with Hamilton syringe
  - Volume in cell – for loading: 330-340 – better 400 μL
  - After the automated washing cycle, rinse cell manually with water, then buffer, and then enzyme solution (only recommended if you have enough enzyme or low enzyme concentrations in the cell)

Details (also see the instruction videos by Malvern):

  - do not invert the Hamilton syringe
  - flick to move air bubbles in syringe to the top
  - eject a drop (or more if there is air in the syringe tip)
  - place softly at bottom of cell, lift a few mm
  - begin pipetting slowly until you see liquid at top of cell, then slowly pull out while still injecting slowly to fill the cylinder leading to the cell
  - remove liquid from top of cell funnel (find the “step”, aspirate)

d. Fill the reference cell: same as above, but there is a clearer “step” in the “neck” of the cell (cylindrical).

e. Do before experiment:

  - Perform a water-water titration to check if the system is clean and if you filled the (reference) cell well
3) Actions, checks during any experiment

a. During equilibration:

What is the offset? Should be as close as possible to 41.9 µW/s (or 10 MicroCal/s) ± 2 (or 0.5)

- If you expect a strong exo- or endothermal reaction, you need to adjust the reference power from the default: the value can be set from 1-119 µW/s, adjust to upper third for exothermal, and lower third for endothermal
- If your offset is shifted, your cell is dirty or there is air in the cell
  - Abort, refill cell
  - continue if offset is fine
  - if not:
    - perform water/water injections: the energy per injection should be constant
    - if it is not, troubleshoot with the Malvern Manual

What is your feedback mode?

- Off: your signal will take longer to reach the baseline again, and you will have “rounded” peaks - the spacing between your injections has to be larger, you will have lower “electronic noise” in the measurement – typically, this setting is not needed
- High: your signal will reach the baseline faster because the ITC will “help”, you can use smaller spacing between your injections, you will get “harder” peaks, the “electronic noise” will be slightly larger – is a MUST for multiple reactions, interactions to be able to reach the resolution needed to separate them

b. Check during experiment:

- Does your injection cause the signal to cross 0? -> abort, restart with lower concentrations
- Is your peak due to binding/the reaction or simply a “solvation peak” due to the ligand BUFFER mismatch? -> perform controls!
4) Binding experiment (ITC-BIND)

a. If you have no knowledge of the $K_D$ of your system:
   o Start with approx. 100 $\mu$M of enzyme in the cell and 10 mM of ligand in the syringe. Use the “design experiment” tool of the Malvern MicroCal PEAQ-ITC Analysis Software to adjust the concentrations and obtain good data.
   o Else: use 100 $\mu$M enzyme in the cell and a concentration of ligand 5x $K_D$ in the syringe, see what happens.

b. Check during experiment (binding):
   o Do the injection peaks overlap (does the signal NOT reach the baseline again)? If yes, your spacing is too short

c. Perform controls!
   o Ligand into buffer titration
     • Is there a huge solvation peak in the ligand-titration? This could be due to DMSO/Glycerol/Acetonitrile/Ethanol added to make ligand soluble – resolubilize without these
   o Buffer into enzyme titration
     • Should only give titration peaks similar to the buffer into buffer titration
   o Buffer into buffer titration
     • Should look like the water-water titrations.

d. Basic Troubleshooting:
   o Check the section Troubleshooting in the Manual.
   o Also check the “Getting Started” section of this SOP.
   o If your $K_D$ or N (sites) values keep changing with changing ligand concentration, something is wrong.
   o If the released energy is too small or too large, adjust the enzyme concentration.
5) Kinetics experiment (single injection, ITC-(r)SIM)
Same as multiple injections method (ITC-MIM), but with approx. 10x higher [E] and larger injection volume of titrant (2 μL or more). If you repeat a single injection experiment, you are doing a "recurrent SIM", which can give you information on enzyme inactivation or slow onset inhibition.

6) Kinetics experiment (multiple injections method, ITC-MIM)

a. If you have no knowledge of the $K_m$ of your system:
   - Start with approx. 0.1 μM of enzyme in the cell and 10 mM of ligand in the syringe. Use the “design experiment” tool of the Malvern MicroCal PEAQ-ITC Analysis Software to adjust the concentrations and obtain good data.
   - Else: use 0.1 μM enzyme in the cell and a concentration of ligand 5x $K_m$ in the syringe, see what happens.

b. Check during experiment (kinetics, MIM):
   - Is the plateau between injections stable?
   - If not, decrease enzyme concentration

c. Perform controls!
   - Ligand into buffer titration
     - Is there a huge solvation peak in the ligand-titration? This could be due to DMSO/Glycerol/Acetonitrile/Ethanol added to make ligand soluble – resolubilize without these
     - Is there a stable baseline, or does it shift, imitating the experiment with enzyme?
       (i) This is highly problematic and will lead to misinterpretation of the kinetics!

d. Basic Troubleshooting:
   - Check the section Troubleshooting in the Manual.
   - Also check the “Getting Started” section of this SOP.
   - If your $K_m$ values keep changing with changing substrate concentrations, something is wrong.
7) Actions, checks after the experiment
   a. Check after each experiment:
      o Is the syringe clean? Is the plunger clean?
      o Is the white plastic around the cell and reference cell clean?
      o Is the valve for loading clean (glass above syringe)?
      o Is the Hamilton clean? If there is (protein) precipitation, your measurement quality is doubtful

   b. Washing after/between experiment:
      o If you have approx. 100 µM enzyme in the cell: wash the cell manually with buffer containing 0.1 % tween
      o Wash the cell manually with buffer
      o Wash the cell manually with water
      o Start one of the automated cleaning protocols:
          ▪ Rinse: Water
          ▪ Wash: Water + detergent
          ▪ Soak: Water+detergent, heats up to 60°C (only for cell)
      o If you are planning further experiments in the next days or weeks:
          ▪ Fill cell with MQ water, close lid
      o If you are not planning further experiments for the next 3 weeks:
          ▪ fill cell with 10% (m)ethanol/water, close lid

8) Shutting down
   a. Washing
      o If you are planning further experiments in the next days or weeks: Fill cell with MQ water, close lid
      o If you are not planning further experiments for the next 3 weeks: fill cell with 10% (m)ethanol/water, close lid

   b. Sample cell in 10% ethanol or methanol if not in use for longer period of time
      o Fill cell, close with white lid if not in use and “resting”

   c. Only turn the device off if you know it will not be used in the next month
      o Close software
      o Shutdown PC
      o Turn off ITC (2 switches at the back of each module)