CENTER FOR
MACROMOLECULAR
INTERACTIONS

ITC200: CMI Getting Started Guide to Isothermal Titration Calorimetry

Introduction

Isothermal Titration Calorimetry (ITC) is a label-free method for measuring binding of any two molecules that release or absorb heat upon binding. ITC can be used to measure the thermodynamic parameters of biomolecular interactions, including affinity (K_A), enthalpy (ΔH), entropy (ΔS), and stoichiometry (**n**). Energetically favorable binding reactions have negative free energy values, $\Delta G = RTlnK_D$. ΔG, has two energetic components, enthalpy (ΔH) and entropy (ΔS) and their contributions are expressed as: $\Delta G = \Delta H - T\Delta S$. In an ITC experiment, ΔH of binding is measured directly. The microcalorimeter has two cells: one contains water and acts as a reference cell, the other contains the sample, into which a binding partner is titrated using an injection syringe. Heat sensing devices detect temperature differences between the cells when binding occurs in the sample cell and give feedback to the heaters, which compensate for this difference and return the cells to equal temperature.

Heats measured in an ITC are a combination of heats of binding and heats of dilution, so a control experiment to measure heats of binding must be performed. Therefore, one experiment includes two titrations:

- Component A (in the syringe) injected into component B (in the cell).
- Component A (in the syringe) injected into buffer (in the cell).

Instrument Overview

The CMI has a **Microcal ITC200** from Malvern Pananalytical.

Applications

- Equilibrium binding: K_A, K_D
- Stoichiometry of binding: n
- Macromolecular and small molecule binding

Key Features

- Truly modification-free (no labels or immobilization)
- No size limits
- Can gain insights into the mechanism of binding
- No consumables to purchase

Required Supplies

- Pipettes (recommended)
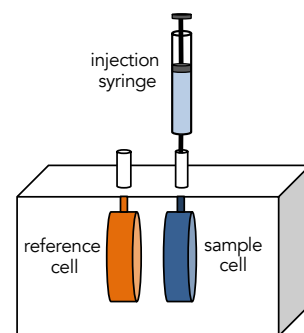
Sample Preparation

Assay Buffers
- The two binding partners must be in **identical buffers** to minimize heats of dilution which can mask heats of binding.
- **Additional buffer (matched to samples) is required** for baselines, dilutions and rinsing the cell.
- DMSO has high heats of dilution and should be matched extremely well between the cell and the syringe.
- Small differences in pH will cause high heats of dilution.
- Reducing agents can cause erratic baseline drift and artifacts. TCEP is recommended over βMe and DTT. Avoid or keep ≤ 1 mM, especially if ΔH is small.
- Using degassed buffers will reduce the introduction of air bubbles and improve results.

Samples
- Required volumes for one experiment:
  - ≥ 300 μL protein for sample cell (202 μL + ~ 80-90 μL for filling).
  - ≥ 100-120 μL ligand for syringe (40 μl syringe + 20 μl for filling, for each injection)
- *typical starting concentrations:*
  - 5-50 μM in the cell (at least 10x K_d)
  - 50-500 μM in the syringe (≥10x concentration in cell for 1:1 stoichiometry)
    - Aim for a **c value** between 10-100 for optimal fit (see below)
- Molar concentration should be accurately measured.
  - Errors in Cell concentration affect stoichiometry.
  - Errors in the Syringe concentration will directly translate to errors in the K_D, and affect ΔH and n.
- Protein aggregates will interfere with ITC.
  - Centrifuge or filter samples before use.
  - Assess protein heterogeneity via light scattering.
  - Purify protein samples with soluble aggregates by size-exclusion chromatography.

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c value
$$c = n \bullet [M]_{cell}/K_D$$
n = stoichiometry
$[M]_{cell}$=concentration in the cell
K_D = dissociation constant

c value should be between 10-100
<10   can't determine n, but can still fit K_D
>1000 can determine n but not K_D

Getting Started

Resources
Additional resources are available at the instrument, including Microcal ITC200 Getting Started Guide and User Manual.

General Care and Maintenance
- The instrument should generally be left ON at all times.
- Wash the instrument thoroughly before and after every run.
- Treat the syringe with care, since it is extremely fragile and expensive.
- Perform water-in-water titrations at the end of the day to check the system

Startup
1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (eCommons ID and password).
3. The ITC200 instrument should generally be left ON. If not, turn it on using the power switch on the back.
4. Start the MicroCal ITC200 software.
5. After system initialization, check that all systems are working and that the red light on the front of the instrument is on.
6. Make sure that wash station bottles and methanol are at least ½ full.
   a. Methanol bottle should contain 100% Methanol.
   b. Water and Buffer bottles should **both** contain filtered and degassed ultrapure water.
7. Empty the waste bottle if it is more than ½ full.
8. Fill the reference cell with water if it has not been filled in the past week.
9. In the ITC200 software, select Instrument Controls Tab.
10. Run **Cell Water Rinse (long).**
11. Run **Syringe Wash (long).**
    a. Follow the on-screen directions for attaching the fill port adapter.
    b. <u>**Do not over-tighten fill port adapter**</u>.

Data Collection

1. Setup
   a. In the ITC200 software, select **Setup** Tab.
   b. Enter Data File path. eg. C:\ITC200\Data\PInameLab\YourName.
   c. Enter Setup File Path (optional).
   d. Current User should be Default User (do not create user profiles).
2. Design
   a. Select **Advanced Experimental Design** Tab.

    b. **Enter Experimental Parameters:**   recommended starting parameters

i)	Total # Injections	19
ii)	Cell Temperature (°C)	20
iii)	Reference Power (μcal/s)	6-7 (if ΔH is unknown)
iv)	Initial Delay (sec)	60
v)	Syringe concentration (mM)	
vi)	Cell concentration (mM)	
vii)	Stirring Speed (RPM)	750 (using NEW twisted paddle syringe)
viii)	Data File Name	
ix)	Feedback Mode/Gain	high

    c. **Enter Injection Parameters:**

		1st injection	remaining injections
i)	Volume (μL)	0.4	2
ii)	Duration (sec)	0.8	4 (2X injection volume)
iii)	Spacing (sec)	150 - 180	150 - 180
iv)	Filter Period (sec)	5	5

3. Fill Syringe:
    a. Select **Instrument Controls** Tab.
    b. Run **Syringe Fill** Method.
        i)   Place a tube with ≥60 μl syringe sample in the tube holder at load position.
        ii) Follow on screen directions for filling syringe.
        iii) Do not forget to remove the wash module attachment after filling.
    c. Manually wash the cell with buffer that is matched identically to the sample buffer, at least three times using the Hamilton syringe.
        i)   Take care as to not bend the Hamilton syringe when placing inside the cell (it is fragile and expensive).
    d. When syringe fill is complete, **disconnect the fill port adapter**:
        i)   Move pipette to rest position.
        ii) Loosen retaining nut.
        iii) Remove fill port adapter.
        iv) Gently tighten retaining nut.
4. Manually Fill Cell:
    a. Be very careful not to introduce air bubbles.
    b. Fill the Hamilton syringe with ~ 300 μL sample for cell, remove air bubbles.
    c. Carefully insert the syringe into the sample cell until it touches the bottom.
    d. Lift the syringe up ~ 1-2 mm.
    e. Slowly fill the cell with sample (until sample emerges from top).
    f. Gently aspirate and dispense about 50 μL sample to dislodge any air bubbles.
    g. Remove the syringe slowly while ejecting.
    h. Place the tip of the syringe on the ledge at top of metal cell stem and remove excess solution.
5. Place pipette into cell port.
6. Click start to begin assay.
    a. In 5-10 min, the differential power (DP) should stabilize near the set reference power (±1 μcal/sec).

    b. If DP does not stabilize near the reference power, then check that the sample cell and the reference cell are filled or **clean the cell again**.

7. Select Real Time Plot to view raw data.
8. After every run, **clean the cell and syringe again before the next titration**.
9. Analyze data in Origin (see Data Analysis Section below, after shutdown).
10. At the end of the day, follow the Shutdown Procedure.

Data Analysis

1. Open **Microcal Analysis Launcher** and click on ITC200 to launch Origin 7.0 (or open Origin 7 directly).
2. Import Data:
   a. Click on **Read Data.**
   b. Select data files to be added (include experimental titration and control titration).
   c. Click on Add files, then OK.
3. Review Raw Data:
   a. The software will calculate baselines and the area of each peak.
   b. Select Window → RawITC.
   c. Examine the baseline and peaks.
   d. (Optional) Click Adjust Integrations to modify the baselines or peak boundaries.
4. Review Data Table:
   a. Select Window→ "Filename".
   b. Examine the Data Table.
      i) DH   DeltaH of each injection.
      ii) INJV   Injection volume.
      iii) X   Concentration of syringe contents in the cell after each injection.
      iv) Mt   Concentration of the cell contents in the cell after each injection.
      v) XMt   Molar Ratio (X/Mt).
      vi) NDH   DeltaH kcal/mole of injectant.
5. Fit Data:
   a. Select Window → DeltaH.
   b. **Remove First Injection.**
      i) Click Remove Bad Data and place the cross-hair over the first data point.
      ii) Double-click or click Enter to remove the data.
   c. **Subtract Reference Data** using point-for-point or average heats of dilution.
      i) Subtract each heat of dilution (point-for-point subtraction).
         (1) Select Subtract Reference Data.
         (2) Identify the Data and Reference files.
         (3) Click Ok.
      ii) Subtract average heats of dilution.
         (1) Calculate the average of the Reference Data (from Data Table perform column statistics).
         (2) Select Math → Simple Math.
         (3) Enter a minus (-) sign as the operator.

(4) Enter the average heats of dilution as Y2.
(5) Click OK.

    d. **Fit Binding Curve.**
      i) Select One Set of Sites.
      ii) Fitting Dialog will open.
      iii) Perform 200 iterations (or more) until the data converge.
      iv) Click Done.
      v) Choose alternate models, but always use the simplest model that explains your system.
    e. Create Publication Quality Figure.
      i) Select Analysis → Final Figure.
      ii) Edit as needed by Right-clicking and selecting.
        (1) Layer Contents (to add or remove curves, eg. if fit is missing).
        (2) Plot Details (to adjust formatting).

6. **Save as Project** to keep fitting parameters.
7. **Export Data:**
    i) Each window can be exported as either a text file or as an image file.
    ii) Data Table Window (named with the filename) has integrated raw data and the fits.

Shutdown

1. Clean the instrument.
    a. Clean the cell using the **Cell Water Rinse (long).**
    b. Clean the syringe using the **Syringe Wash (Long)** protocol.
    c. If you suspect precipitation in the cell, wash the cell with detergent:
      i) Follow the **Detergent Soak and Rinse** protocol using 10-20% Contrad 70.
      ii) Remove the detergent manually from the cell (using the Hamilton syringe).
      iii) Wash the cell using the wash module.
2. **Run a water-in-water titration**:
    a. Fill the syringe with filtered, degassed water.
    b. Fill the cell with filtered, degassed water.
    c. Detach the wash module from the syringe.
    d. In the ITC200 software, go to Setup Tab.
      i) Enter Data File path: C:\ITC200\Data\1_CMI_WaterTitrations.
      ii) Enter Setup File Path: C:\ITC200\Setup\1_CMI_WaterTitrations.
    e. In the ITC200 software, select Advanced Experimental Design Tab.
    f. Load the file *Water_15cycle_RP6.inj* or set up the experiment with the following parameters:
      i) 15 injections, 20 °C, Reference Power 6 μcal/sec, Initial Delay 60 sec.
      ii) Data File Name: YYYYMMDDwaterINITIALS (eg. 20150519waterKLA).
      iii) 2 μL injections, 120 sec spacings.
    g. Start the water-in-water titration (runtime ~ 30 min).
3. During the run, check that:
    a. The differential power (DP) is within 1 μcal/sec of the set reference power.
    b. Check that the heats of dilution are minimal and uniform (<0.02 μcal/sec).

   c. **If these fails, repeat from Step 1 <u>until the instrument is clean</u>**.
4. Run the **Syringe Wash (Long or Short)** protocol.
5. Leave water in the cell.
6. Leave the instrument power on (unless instructed otherwise).
7. Sign in the paper logbook.
8. Logoff from PPMS!

<u>Data Management</u>

Technology	Isothermal Titration Calorimetry (ITC)
Instrument	Microcal ITC200
Recommended Repository	[Generalist Repository](#)

Data Collection Software

Current Version	MicroCal iTC200, Version 1.30.0		
Data Files (Type, ~size)	experiment file (readable text file)	.itc	250 KB/measurement

Data Analysis Software

Current Version	Origin 7 (MicroCal Analysis Module), Version 7.0552		
Data Files (Type, ~size)	analysis file	.opj	350 KB/file
Readable Exports	graph image	.jpg	200 KB

Book time and Report Problems through the PPMS system: https://ppms.us/hms-cmi
- rates are based on *booked and real-time usage.*

Contact cmi@hms.harvard.edu with questions.

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