Jul 29, 2025

Isothermal titration calorimetry protocol for binding studies of enterovirus EV-A71 2A protease and small inhibitors

DOI
https://dx.doi.org/10.17504/protocols.io.eq2lyqroqvx9/v1

Eda Capkin[1,2,3]

[1]Diamond Light Source; [2]Research Complex at Harwell; [3]ASAP Discovery Consortium

ASAP Discovery

Mary-Ann Xavier
Diamond Light Source, Centre for Medicines Discovery, ASAP D...

DOI: https://dx.doi.org/10.17504/protocols.io.eq2lyqroqvx9/v1

Protocol Citation: Eda Capkin 2025. Isothermal titration calorimetry protocol for binding studies of enterovirus EV-A71 2A protease and small inhibitors. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2lyqroqvx9/v1

Protocol status: Working
We use this protocol and it's working

Created: May 23, 2025

Last Modified: July 29, 2025

Protocol Integer ID: 218847

Keywords: Isothermal titration calorimetry, ITC, EV-71 2A protease, protein-ligand binding, thermodynamics, dissociation constant, drug discovery, binding affinity, calorimetry, protease inhibitors, 2a protease from enterovirus stratin, isothermal titration calorimetry protocol, isothermal titration calorimetry, studies of enterovirus ev, enterovirus stratin, enterovirus ev, binding study, a71 2a protease, ligand concentration, small inhibitors this protocol, small molecule inhibitor, small inhibitor, drug discovery application, binding interaction, protein

Funders Acknowledgements:
National Institutes of Health/National Institute Of Allergy and Infectious Diseases (NIH/NIAID)
Grant ID: U19AI171399

Disclaimer

> The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abstract

This protocol describes an isothermal titration calorimetry (ITC) method for measuring binding interactions between the 2A protease from enterovirus stratin EV-71 and small molecule inhibitors.
Experiments are performed at 25°C using 25 µM protein and 250 µM ligand concentrations with 17 sequential injections. Data analysis determines binding parameters (K_D, stoichiometry) for drug discovery application.

Materials

Materials:
HEPES: HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375
NaCl: Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9888
TCEP: 0.5 M TCEP: Pierce™ Bond-Breaker® TCEP Solution **Thermo Scientific Catalog #**77720
DMSO: Pierce™ Dimethylsulfoxide (DMSO), Sequencing Grade **Thermo Scientific Catalog #**10127403
PCR tubes for sample: BrandTech™ BRAND™ Thin Wall 0.2 mL PCR T **Fisher Scientific Catalog #**13-882-58

Buffer: 50 mM HEPES pH 8.0, 150 mM NaCl, 0.5 mM TCEP

Protocol materials

- HEPES **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H3375
- Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S9888
- 0.5 M TCEP: Pierce™ Bond-Breaker® TCEP Solution **Thermo Scientific** **Catalog #**77720
- Pierce™ Dimethylsulfoxide (DMSO), Sequencing Grade **Thermo Scientific** **Catalog #**10127403
- BrandTech™ BRAND™ Thin Wall 0.2 mL PCR T **Fisher Scientific** **Catalog #**13-882-58
- Human Coxsackievirus A16 strain G10 2A protease **addgene** **Catalog #**228632

Safety warnings

Warning: The buffer solution in protein and compound (detergent%, DMSO%) should be the same to minimize mismatch and dilution.
The concentration of samples should be known and correct.

Protein expression and purification

1 Thre protocol used for expression and purification is the one below using the following plasmid

Human Coxsackievirus A16 strain G10 2A protease **addgene Catalog #**228632

Protocol

NAME

Enterovirus coxsackievirus A16 2A protease small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

Buffer preparation

2 Stock 5X Buffer: 250mM HEPES pH 8.0, 750 mM NaCl, 2.5 mM TCEP

3 Prepare 100 mL of 1X buffer by diluting the 5X stock solution with Milli-Q water.

4 Remove 2 mL from the 1X buffer, then add 2 mL of 100% DMSO to make a 2% DMSO buffer.

Protein preparation

5 Prepare 25 µM protein solution with buffer including 2% DMSO at 400 µL.
The PEAQ-ITC instrument has a cell volume of 300 µL but you can prepare at 400 µL in any case and not to inject air.

Compound preparation

6 100 mM stocks in 100% DMSO

6.1 Take 0.2 µL and add 9.8 µL running buffer without DMSO. This will make a running buffer in 2% DMSO. Then, add 70 uL 2% DMSO + running buffer.
Final concentration: [M] 250 micromolar (µM)

Run settings

7 As a start without any affinity information, 20 -30 µM protein and 200 -300 µM sample concentrations are suggested.

8 Cell (protein) 25 µM at 400 µL

9 Syringe (samples) 250 µM at 80 µL

10 Temperature: 25˚C

11 No of injections: 17

12 Reference power:10 µcal/s

13 Initial delay: 60 seconds

14 1st injection 0.8 µL, rest 2.4 µL

15 Spacing: 150 seconds

16 Stirring Speed (RPM): 750

17 Feedback Mode: High

Instrument preparation and run

18 The syringe is usually left in the rest position and detached from the adaptor. Attach the syringe to the fill port adaptor and place it for the cleaning part. For the cell, put the cleaning part into the cell.

19 In the ITC Control software, select Wash for both cell and syringe, and start the procedure

20 There are two Hamilton syringes, one is used for water and buffer wash/injections, and the other is used only for protein not to dilute protein with buffer solution

21 After the cleaning, manually wash 4-5 times with water, then perform 4 – 5 times manual injections with buffer to equilibrate the cell.

22 After the wash, manually inject protein solution into the cell till you can see from the top (300 µL).

23 In the software, check the syringe position. If it is not ready to “sample load”, click the ‘plunger down’.

24 Place the sample containing the eppendorf in the holder and insert the fill port adaptor attached syringe.

25 Then, select the ‘sample load’ to fill the syringe with sample.

26 Detach the fill adaptor and place the syringe into the cell.

27 Enter the sample and protein concentration for the syringe and cell sections in the software, respectively.

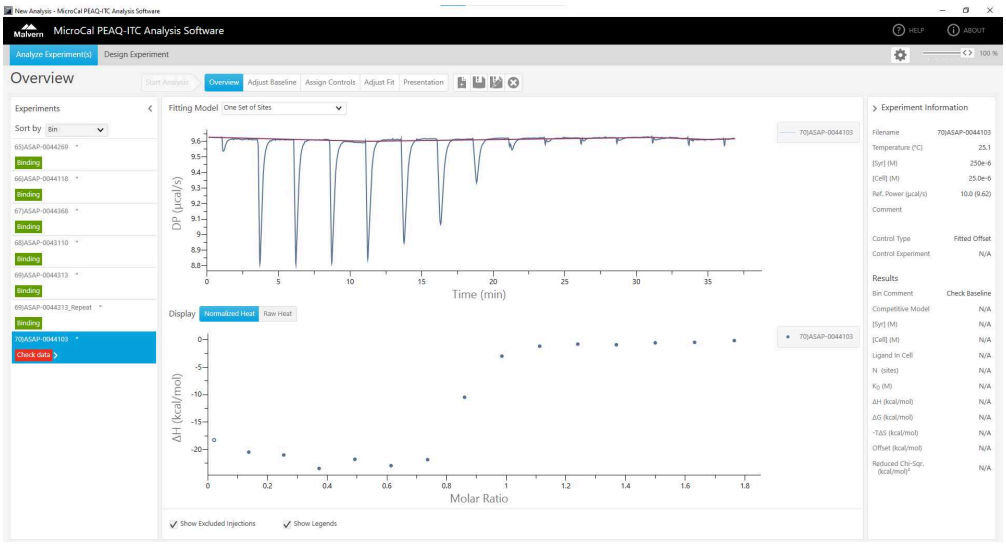
28 Start the run

Results expected and data analysis

29 Data Analysis

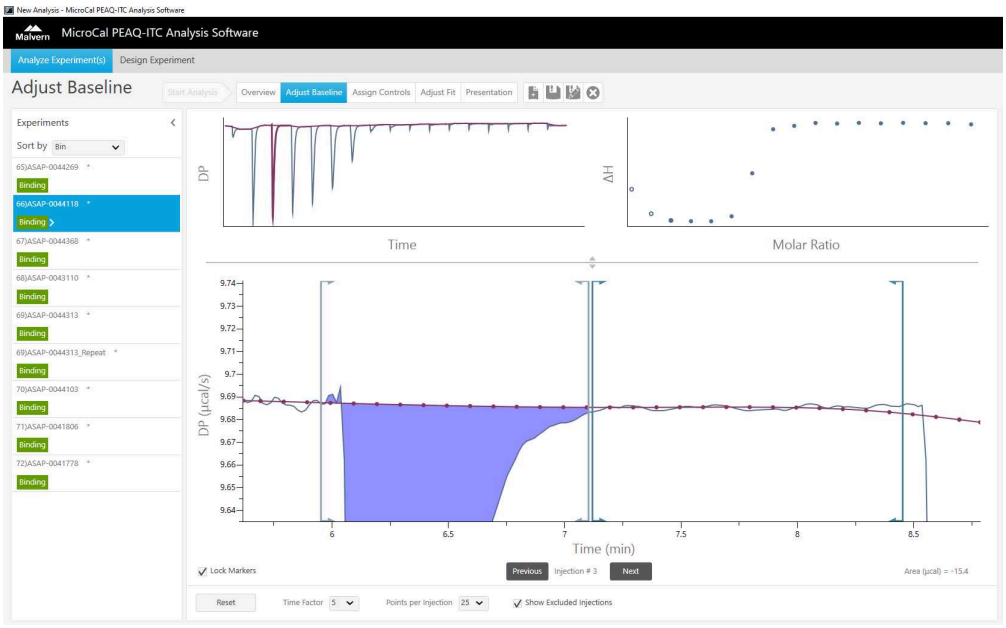
30 Open the ITC Analysis Software and upload the result.

31 Check the overall heat change plot over the 17 injections.



Data evaluation and adjustment for an ITC data

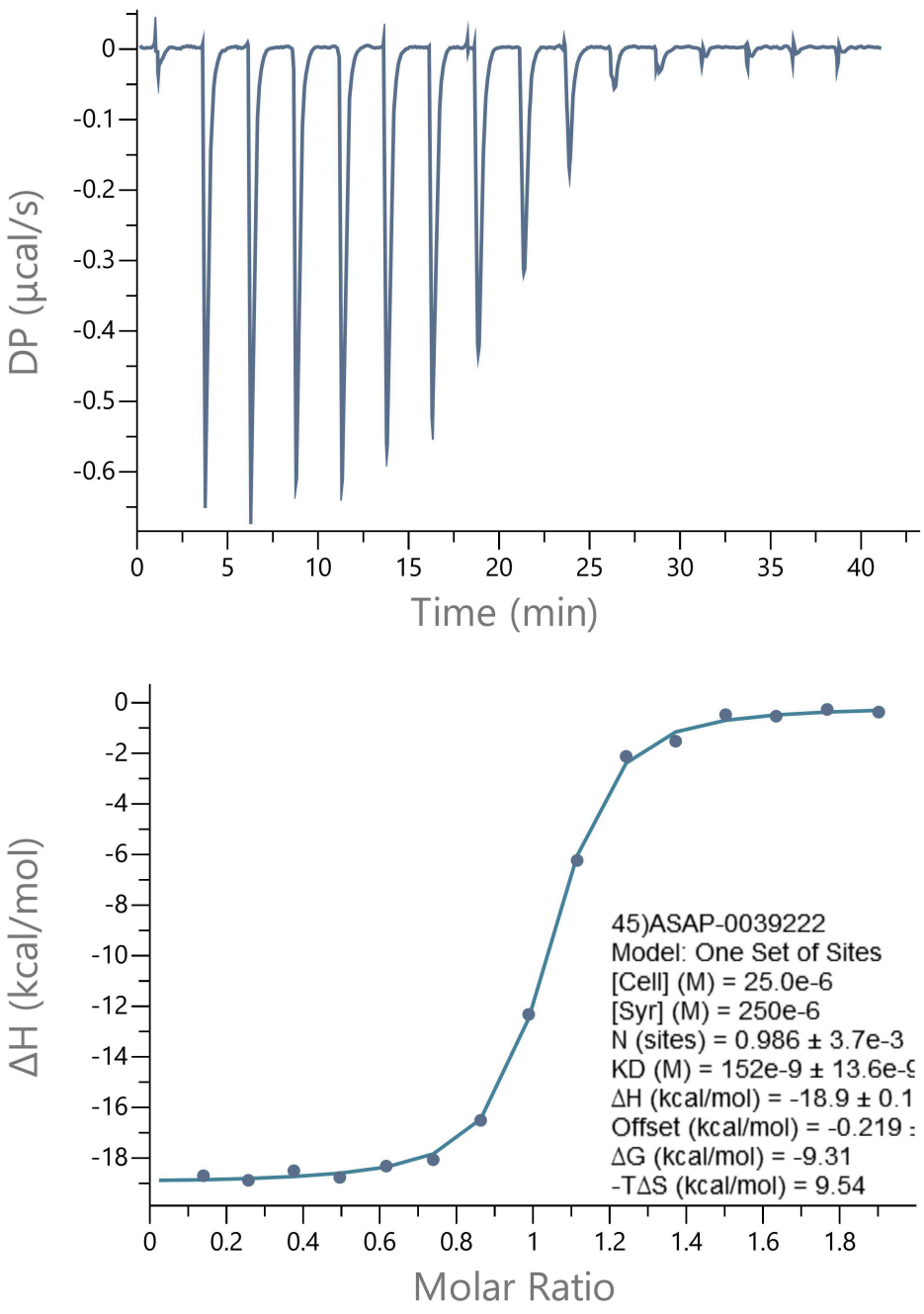
32 Check the baseline and adjust it if it is noisy.



Baseline adjustment in the raw titration plot

33 Assign control as blank or fitted offset to subtract the blank response.

34 Then, check the K_D, N values, and other parameters.



Adjusted titration plot and binding isotherm with thermodynamic parameters

35 After the completion of the run, start the cleaning process as stated in steps 28 to prepare for next run.

Protocol references

1. ITC200: CMI Getting Started Guide to Isothermal Titration Calorimetry (https://cmi.hms.harvard.edu/sites/g/files/omnuum4016/files/2025-03/CMI%20ITC200%20Getting%20Started%20Guide.pdf)
2. Manual: MicroCal PEAQ-ITC user manual (https://www.malvernpanalytical.com/en/learn/knowledge-center/user-manuals/man0573en)