Rapid sample preparation for MicroCal™ ITC and DSC experiments using PD MidiTrap™ G-25 columns

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PD MidiTrap G-25 columns were used for rapid and simple buffer exchange of samples for isothermal calorimetry and differential scanning calorimetry (DSC) studies where a match between the buffers in the sample and reference cells is crucial for reliable results. For both techniques, buffer exchange with either method was essential in order to determine thermodynamic parameters and heat capacity changes. Compared to dialysis, gel filtration of samples on PD MidiTrap G-25 is much faster and requires less buffer while at the same time gives the same high quality analysis data.

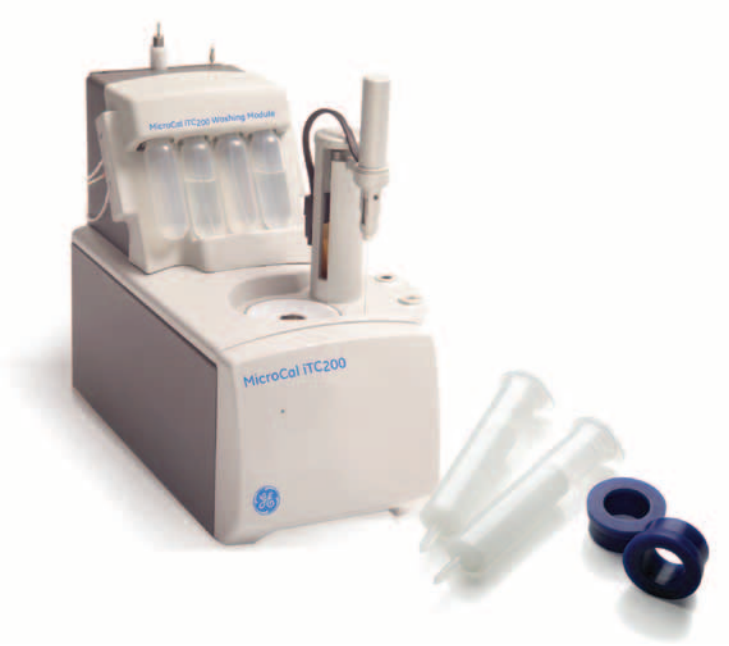

Fig 1. PD MidiTrap G-25 columns were used for buffer exchange before isothermal titration calorimetry of RNAse A using MicroCal iTC$_{200}$ system.

Introduction

To obtain accurate results in isothermal calorimetry (ITC) and differential scanning calorimetry (DSC), buffers must be carefully matched between the sample and the reference cells to minimize heats of dilution. Sample preparation thus usually involves buffer exchange, which can be achieved by dialysis or gel filtration.

Gel filtration with Sephadex™ G-25 medium enables group separation of biomolecules with a molecular weight above 5000 from contaminants such as salts, dyes, and radioactive labels. The medium is designed for desalting and buffer exchange of biological samples such as proteins, and is stable with all commonly used buffers. PD MidiTrap G-25 are prepacked, disposable columns designed for sample preparation of volumes between 0.5 ml and 1 ml (Fig 1). These columns can be operated using two alternative protocols: The gravity protocol provides a simple cleanup of several samples in parallel without any need for a purification system; and the centrifugation protocol allows samples to be run in parallel in a standard centrifuge, giving minimal dilution of the eluted sample.

MicroCal ITC$_{200}$ system (Fig 1) allows direct and label-free measurement of binding affinity and thermodynamics. Heat released or absorbed during biochemical binding events is measured directly, giving information about relative binding affinity (K_b), stoichiometry (n), enthalpy (ΔH), and entropy (ΔS). This information provides valuable insights into the mechanism of binding, including hydrogen bonding, van der Waals interactions, and hydrophobic interactions. Microcal DSC systems measure the instability of biomolecules and can be used both to predict long-term stability and to understand the noncovalent forces responsible for protein or lipid micelle stability.

The sample volume needed for analysis in MicroCal ITC$_{200}$ and MicroCal VP-Capillary DSC is 250 µl and 400 µl, respectively. PD MidiTrap G-25 columns were used for buffer exchange.

Materials and methods

Ribonuclease A (RNase A, Sigma-Aldrich) was prepared in 50 mM potassium acetate buffer, pH 5.5. To compare the results obtained in calorimetry between samples prepared by dialysis and gel filtration, 100 mM NaCl, 50 mM Tris buffer were added to RNase A solution in 50 mM potassium acetate buffer.

Sodium chloride and Tris were removed from RNase A by buffer exchange. For buffer exchange by dialysis, a Spectra/Por™ (Spectrum™ Laboratories Inc.) natural cellulose dialysis membrane tubing was soaked for 30 min in 50 mM of potassium acetate buffer to remove the sodium azide preservative, after which 1 ml of RNase A sample was carefully injected into the tubing. The dialysis buffer (50 mM potassium acetate buffer, pH 5.5) was replaced three times over the course of a day (300 ml each), and left overnight at 4˚C on a stir plate.

For buffer exchange by gel filtration, PD MidiTrap G-25 columns (GE Healthcare Life Sciences) were used according to the manufacturer's recommended gravity protocol, using 50 mM potassium acetate buffer, pH 5.5 as buffer. A volume of 1 ml of RNase A was used per column. Sample preparation was complete within 30 min and less than 20 ml of buffer was used altogether.

Protein concentrations before and after buffer exchange were determined with a spectrophotometer using an extinction coefficient of 9800/cm/M at 280 nm. Cytidine 2'-monophosphate (2'-CMP, Sigma-Aldrich) at a concentration of 795 µM was prepared in 50 mM potassium acetate buffer, pH 5.5. Calorimetric analysis was performed in either MicroCal ITC$_{200}$ or MicroCal VP-Capillary DSC.

ITC

Three different experiments were performed with MicroCal ITC$_{200}$ system. The first used RNase solution in potassium acetate buffer with added Tris and NaCl before buffer exchange. The second used RNase in potassium acetate buffer after buffer exchange with dialysis. The third experiment used RNase in potassium acetate buffer after gel filtration. The sample cell was filled with 250 µl of 60 µM RNase A in indicated buffer. The titration syringe was filled with 40 µl of 795 µM 2'-CMP in 50 mM potassium acetate buffer. The reference cell was filled with deionized water. For each experiment, 40 µl of titrant was injected into the sample cell in aliquots of 2 µl at a rate of 0.5 µl/s. The following settings were used: Stir speed 1000 rpm, reference power 5 µcal/s, feedback mode/gain set to high, temperature 25˚C, initial delay 60 s. Data were analyzed with Origin software, v7 (OriginLab™ Corporation) for ITC.

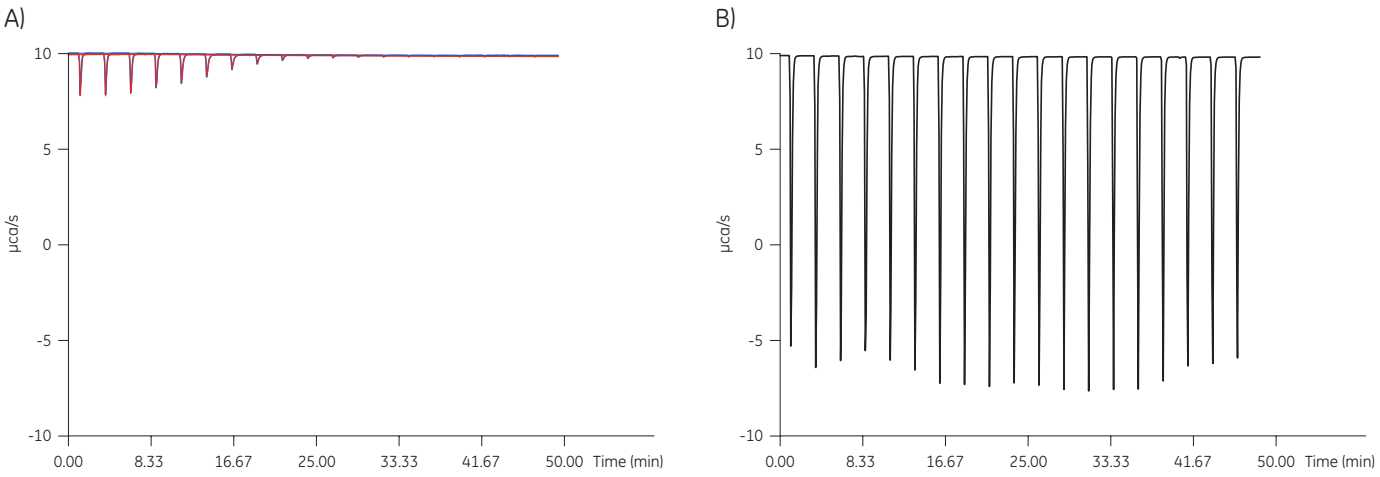

Fig 2. A) Overlapping profiles of raw ITC data of the interaction between 60 µM RNase A and 795 µM 2′-CMP obtained after dialysis (red line) and after gel filtration (blue line) using the gravity protocol. **B)** Raw ITC data of the interaction without buffer exchange by dialysis or gel filtration.

DSC

Three different experiments were performed with MicroCal VP-Capillary DSC system. The first used RNase solution in potassium acetate buffer with added Tris and NaCl before buffer exchange. The second used RNase in potassium acetate buffer after buffer exchange with dialysis. The third experiment used RNase in potassium acetate buffer after gel filtration The sample solution of 60 µM RNase A in indicated buffer and reference solution (50 mM potassium acetate buffer) were placed in a 96-well plate (400 µl each). The samples were scanned from 20˚C to 110˚C at a scan rate of 250˚C/h, with 15 min equilibration before each scan. Data were analyzed with Origin v7 for DSC, following buffer subtraction and concentration correction of the protein scans.

Results

ITC

To compare the effectiveness of dialysis and buffer exchange, the same batch of RNase A sample was prepared by either dialysis or gel filtration using PD MidiTrap G-25 columns. The raw ITC data generated from both methods were compared (Fig 2A). The profile from dialysis (red line) and gel filtration (blue line) were overlayed and very small changes were seen. For comparison purposes, Fig 2B shows the profile obtained from RNase A without buffer exchange by dialysis or gel filtration. The excessive heats of dilution observed here (black line) made it difficult to determine thermodynamic parameters from the the RNase A and 2′-CMP interaction. Overall, both dialysis and buffer exchange methods were successful in removing the low-molecular contaminants (i.e., NaCl, Tris) and gave the same high quality data. The thermodynamic parameters were also found to be in excellent agreement.

DSC

DSC scans generated from the same RNase A sample prepared by either dialysis (red line) or gel filtration (blue line) were compared (Fig 3). Both profiles clearly overlapped, with the heat capacity of the baseline close to zero and a more linear pre- and post-transitional baseline. For comparison purposes, a scan of RNase A sample without buffer exchange by dialysis or gel filtration is shown (black line). The observed nonlinear pre- and post-transitional baseline is indicative of poor solvent matching, and thus it was not possible to determine the enthalpy and heat capacity change accurately from this profile.

As a result of more accurately matched buffers, it was possible to determine not only the T_m, but also the enthalpy and heat capacity change (from the red and blue profiles), which were found to be in excellent agreement between the two methods.

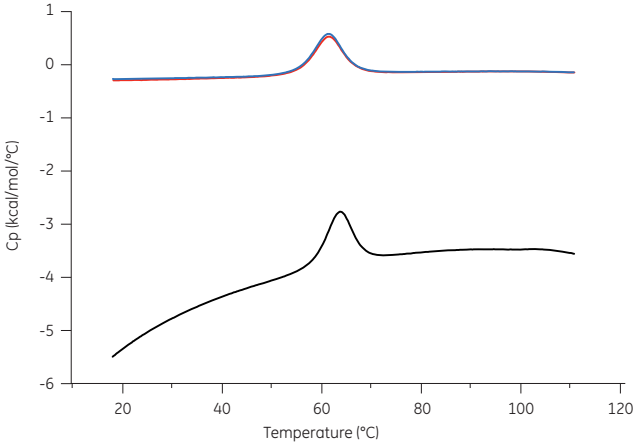

Fig 3. DSC scans obtained from 60 µM RNase A without buffer exchange by dialysis or gel filtration (black line), after dialysis (red line) and after gel filtration (blue line) using the gravity protocol.

Conclusions

In order to obtain high-quality calorimetric data, sample preparation is crucial. Buffer matching as well as removal of low-molecular weight contaminants contribute to minimize high heats of dilution in both ITC and DSC experiments.

The simplicity and efficiency of PD MidiTrap G-25 columns is well suited to buffer exchange by gel filtration and provides the same high quality data as dialysis. Moreover, gel filtration gives faster buffer exchange, is easier to use, and requires significantly lower buffer volumes than dialysis.

Ordering information

Product	Code number
PD MidiTrap G-25	28-9180-08
MicroCal iTC$_{200}$ System	28-4289-55
MicroCal VP-Capillary DSC System	28-4289-48

For more information on our wide range of products for sample preparation, visit www.gelifesciences.com/sampleprep. To discover more about our offering of MicroCal instruments for label-free measurement of binding affinity and thermodynamics, visit www.gelifesciences.com/microcal