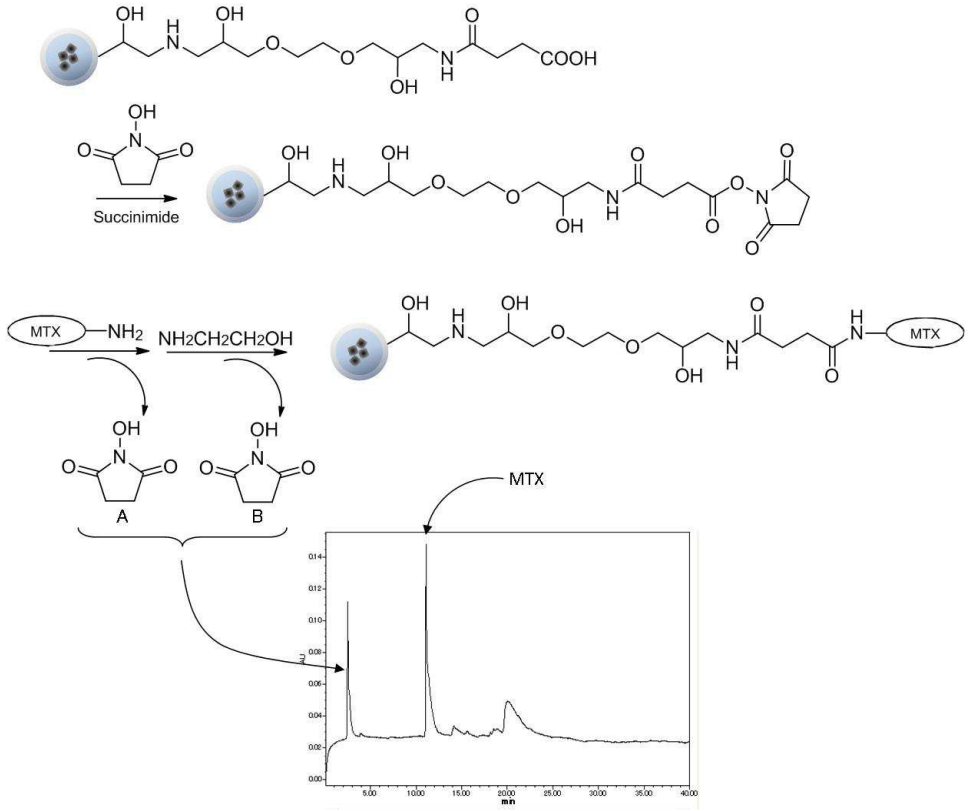
Experiment Protocol 201

Quantifying the amount of ligand immobilization by HPLC (High Performance Liquid Chromatography)

When screening target proteins of ligands, you may need to quantify the amount of ligand immobilization on beads. This experiment protocol describes the method to quantify the amount of immobilization of ligands with NH_2 groups on COOH beads and NHS beads.



1. Materials

1.1 Supernatant A and B to immobilize on NHS beads (Refer to Experiment Protocol 008 and 014)

- Supernatant A(NHS contained solution centrifuged when immobilizing ligands)
- Supernatant B(NHS contained solution centrifuged when masking)

1.2 Reagents

- N-hydroxysuccinimide (NHS) M.W. 115.09
- N,N'-dimethylformamide (DMF)
- Ammonium acetate M.W. 77.08
- Acetic acid M.W. 60.05
- Acetonitrile (For High Performance Liquid Chromatography)

1.3 Apparatus

- Filter unit : Steritop (MILLIPORE SCGPT05RE)
- Micro-tube filter : Ultrafree-MC (MILLIPORE UFC30LG00)
- High Performance Liquid Chromatography (HPLC)(Waters 2695, 2998)
- Symmetry column 5μm C18 4.6×250mm stainless (Waters WAT054275)
- Micro centrifuge

2. Method

2.1 Preparation of reagent solutions

1) Preparation of 0.2 M ammonium acetate solution

1. Add 0.605 g of acetic acid into a 50 ml tube, and dilute this with ultrapure water in a measuring cylinder to 50 ml total. (0.2 M acetic acid)
2. Add 7.708 g of ammonium acetate into a 500 ml beaker, and dilute this with ultrapure water in a measuring cylinder to 400 ml total.
3. Add 0.2 M acetic acid until the pH of the solution becomes 5.70. (Approx. 40 ml)
4. Transfer the solution into a 500 ml measuring cylinder, and dilute this with ultrapure water to 500 ml total.
5. Filter the solution (by using a filter unit), and store it at 4℃.

2) Preparation of 10 mM ammonium acetate solution: Mobile phase

Dilute 50ml of 0.2 M ammonium acetate solution in a measuring cylinder to 1l total.

3) Preparation of standard curve for succinimide (NHS)

100 mM : 1M item used for immobilizing HOSu: 100 µl + DMF 900 µl
10 mM : 100mM 100 µl + DMF 900 µl
1 mM : 10mM 100 µl + DMF 900 µl
100 µM : 1mM 100 µl + DMF 900 µl
10 µM : 100µM 100 µl + DMF 900 µl
0 mM : DMF only

- Use 10 mM or lower only for standard curve.

4) Preparation of buffer to dilute a sample

Prepare the solution with the buffer composition when starting the gradient.

Buffer :10 mM ammonium acetate (47 ml) (94% of the total)
+ acetonitrile (3 ml) (6% of the total)

2.2 Preparation of a sample

1) Mix 20 µl of a sample (standard curve, supernatant A and B) with180 µl of buffer to dilute the sample.
2) Perform filtering to prevent beads being mixed into the HPLC. (For each 200µl: Use a micro tube filter, and centrifuge at 5000×g for 1 min)
3) Add 150 µl of the filtrate into each vial, cover the vial, and install it to an auto-sampler of the HPLC.

2.3 Preparation of HPLC and programming

1) Switch on the HPLC (HPLC main body, column oven, and PC), and clean the passages.
2) Install the column. (by pouring the solvent. Be careful about the direction of the arrow. Confirm that liquid flows out.)
3) Perform the buffer displacement in the column under the condition of 94% of 10mM ammonium acetate and 6% of acetonitrile. (Wait until pressure stabilizes: approx. 1350psi)
4) Perform the programming.
   ↓ Start up HPLC control software (Empower)
   ↓ Set up the wizard (Capacity of a sample to be analyzed: 50 µl, the number of samples, etc.)
   ↓ Set up the method (Gradient conditions: Fig. 1, Column: 40℃)

Method：Gradient 40minutes (Including washing processes)

Time (minute)	Flow velocity(ml/min)	10 mM ammonium acetate (%)	Acetonitrile (%)	Curve
-	1	94	6	-
10	1	60	40	6
12	1	20	80	6
20	1	20	80	6
22	1	94	6	6
40	1	94	6	6

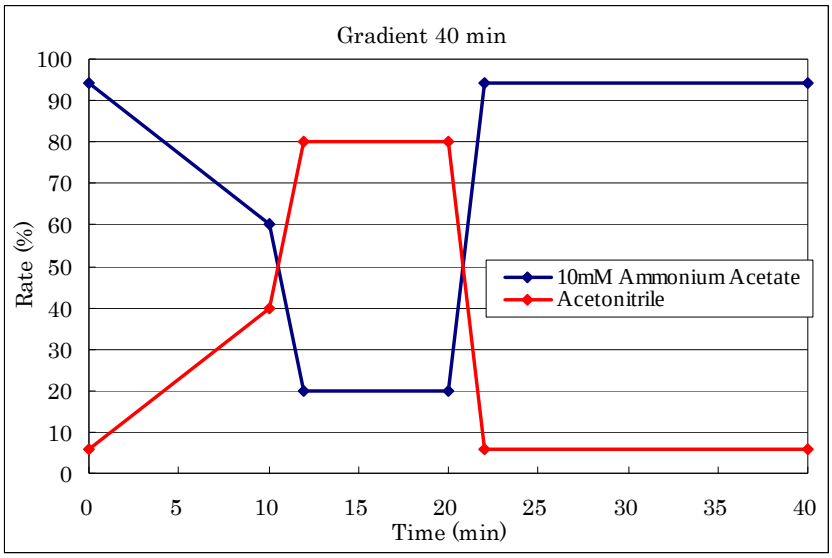


Fig. 1 Change of ammonium acetate solution mixing ratio of gradient 40 minutes

2.4 Analysis

1) Select the wizard and the method you set up, and start the measurement.
2) After the measurement, analyze at 260nm, and convert the peak area into a quantitative value.
3) Wash the column with 100% acetonitrile (1ml/min) for one hour.

2.5 Data analysis

1) From the peak area of a sample of supernatant A, calculate the amount of succinimide separated when immobilizing the ligands.
2) Obtain the actual amount (μM) of ligand immobilization by subtracting, from the above 1), the amount of succinimide when the amount of ligands to be added is 0 mM (ligands are not immobilized) as the background. (Convert into mol/mg.)
3) From the peak area of a sample of supernatant B, calculate the amount of succinimide separated when masking.

4) Obtain the amount (μM) of carboxyl groups on which ligands are not immobilized by subtracting, from the above 3), the amount of succinimide of a sample of supernatant B without masking as the background. (Convert into mol/mg.)

5) Obtain the amount of surface carboxyl groups of carboxylated beads by totaling the amount of ligand immobilization (A) obtained in the above 2) and the amount of carboxyl groups on which ligands are not immobilized (B) obtained in the above 4). (Convert into mol/mg.)

3. Supplements (Actual measurement data: Example MTX)

3.1 HPLC peak

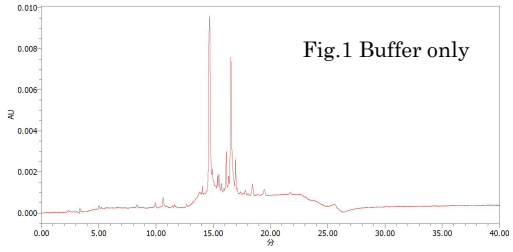


Fig.1 Buffer only

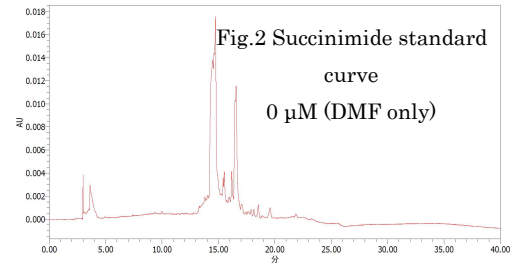


Fig.2 Succinimide standard curve
0 μM (DMF only)

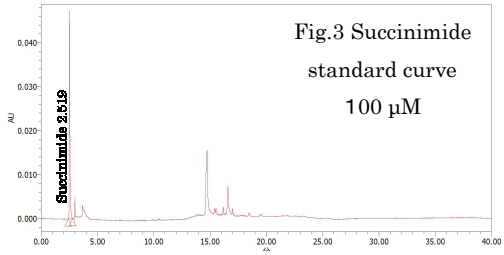


Fig.3 Succinimide standard curve
100 μM

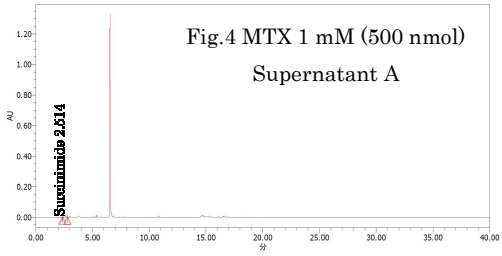


Fig.4 MTX 1 mM (500 nmol)
Supernatant A

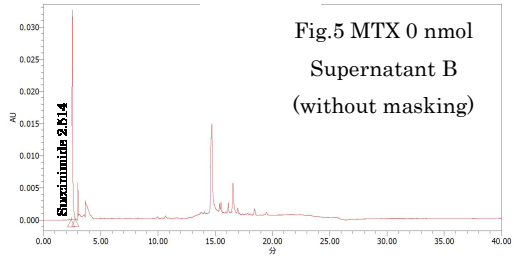


Fig.5 MTX 0 nmol
Supernatant B
(without masking)

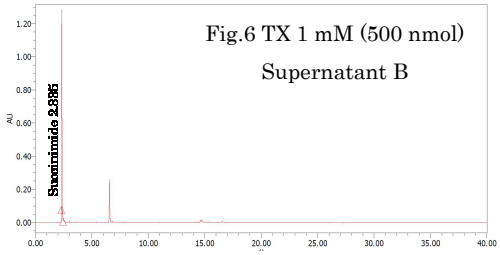


Fig.6 TX 1 mM (500 nmol)
Supernatant B

[HPLC Analysis: Sample 20 μl + Buffer 180 μl]

Fig.1. Sample = Buffer
Analyzed only the buffer with a ratio of 10mM acetic acid ammonium : acetonitrile = 94:6

Fig.2. Sample = Succinimide standard curve 0 μM
Create the standard curve in DMF because succinimide to be separated when immobilizing MTX and when masking is dissolved in DMF.
Succinimide 0 μM is only in DMF. Analyzed DMF only. No peak appeared 3 minutes before.

Fig. 3. Sample = Succinimide standard curve 100 μM
Analyzed succinimide 100 μM. A peak appeared at 2.519 minutes.

Fig.4. Sample = MTX 1mM supernatant A
Supernatant when the amount of MTX-NH_2 to be added is 1mM. A peak of succinimide appeared at 2.514 minutes. A peak of MTX appeared at around 6.5 minutes. Calculate the amount of MTX-NH_2 immobilization from the peak area of succinimide.

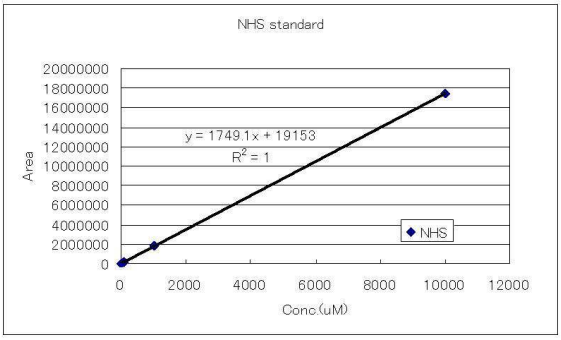
Fig. 5. Sample = MTX 0mM supernatant B (without masking)
Supernatant B when the amount of MTX-NH_2 to be added is 0mM and when masking is not performed (DMF instead of aminoethanol)
A peak of background succinimide appeared at 2.514 minutes.

Fig. 6. Sample = MTX 1mM supernatant B
Supernatant B when the amount of MTX-NH_2 to be added is 1mM and when masking is performed.
A peak of succinimide appeared at 2.335 minutes. The peak position appeared several minutes ahead

Experiment Protocol 201

3.2 HPLC Quantitative Value

	Succinimide solution	Area	Peak (min)
Standard curve solution	0 μM	0	-
	10 μM	19368	2.523
	100 μM	180527	2.519
	1000 μM	1823563	2.515
	10000 μM	17504457	2.513



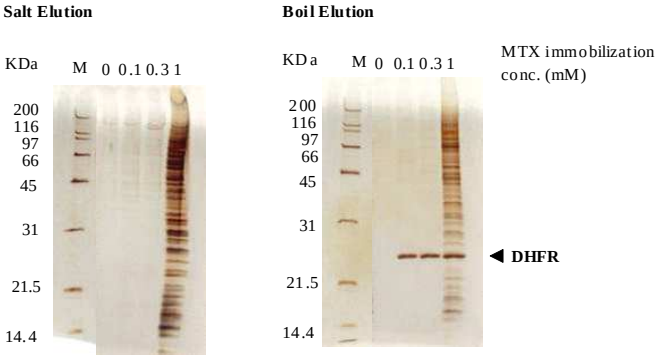
[Reference]
Standard curve :y = 1800x (位

(Note)
Succinimide peak position
Standard curvr, SupernatantA : 2.510~2.540 min
SupernatantB : 2.330~2.340 min
(It shifts.)

	Conc. of chemical compound at immobilization (mM)	MTX-NH2 (nmol/500ul)	NHS sup quantitative conc. with HPLC (uM)	Succinimide value of 200ul (nmol)	Background removal (nmol)	Immobilized MTX-NH2 at 1mg of beads (nmol/mg)	COOH or NHS value (nmol/mg)	Average (nmol/mg)
259NHS Sup A	0	0	79	15.9	0.0	0.0	201.5	211.7
	0.1	50	83	16.6	0.8	0.8	216.8	
	0.3	150	99	19.9	4.0	4.0	212.7	
	1	500	136	27.2	11.3	11.3	215.9	
259NHS Sup B	0		1008	201.5				
	0.1		1080	216.0				
	0.3		1044	208.7				
	1		1023	204.6				

200ul = 1mg of beads

3.3 Affinity purification by MTX immobilized beads



Condition
Beads : COOH beads(S259PSSNENCOOH)
Amount of beads : 0.5mg
Binding, Washing buffer : 150 mM KCl(200μl)
Elution buffer : 1M KCl(30μl)
Protein extract : HeLa cytoplasmic compartment
Protein Conc. : 3mg/ml(200 μl)
Reaction time : 2h

4. Notes

- Because micro-tube filters are not resistant to organic solvent, use them after diluting the organic solvent with buffer.
- The value of supernatant A plus B shows the whole amount of COOH (NHS) of COOH (or NHS beads) groups. When this value is significantly lower than 200 nmol/mg, it is considered that the masking was not performed correctly or NHS was separated by water contamination. In such a case, perform the immobilization again using new NHS beads.