Procedure for Examining Protein Purification Conditions

【Outline of target protein purification experiment】

○ Immobilization of ligand on beads

React ligand solution and beads, masking

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○ Affinity purification of target protein using ligand-immobilized beads

Binding reaction (mixing with protein solution), washing and elution

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○ Analysis

Electrophoresis, silver stain, mass spectrometry

Repeat

Examination of purification conditions

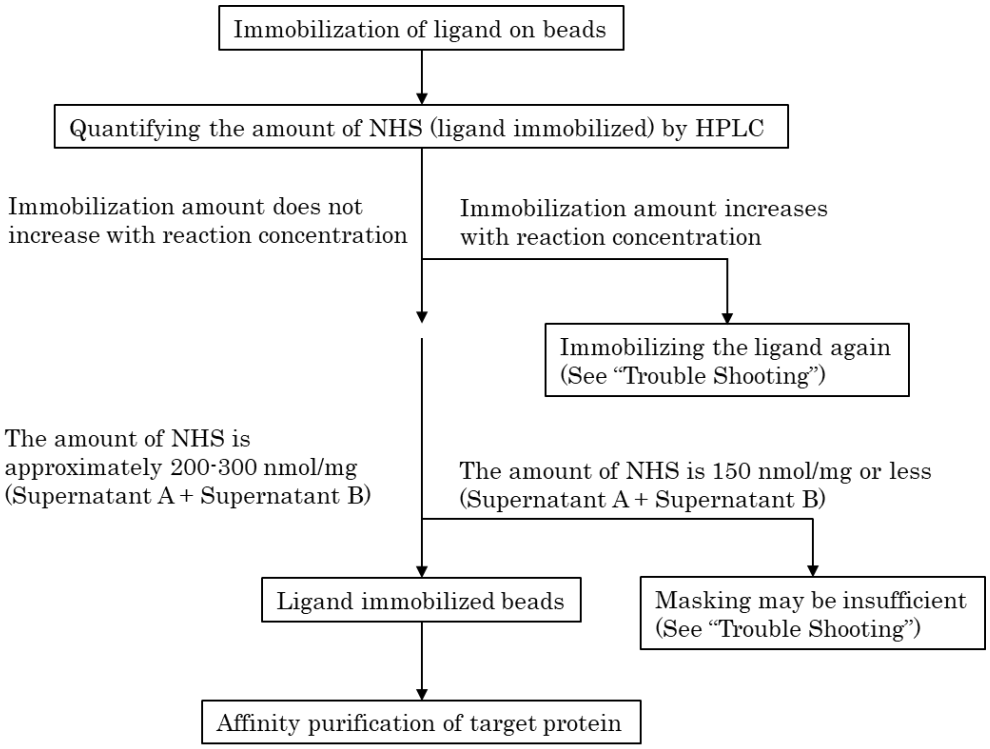
Consideration

- The amount of ligand immobilized
- Buffer composition (salt concentration, etc)
- Protein solution concentration or volume
- Binding reaction time
- Competitive inhibition, drug elution

【Ligand immobilization on beads and quantification of immobilization amount】

○Immobilizing a ligand having NH2 groups on NHS beads

The amount of immobilization can be quantified by HPLC (High Performance Liquid Chromatography). (See protocol 201)



<Quantitative results of amount ligand immobilized>

	Amout of ligand immobilized (nmol/mg)									
Conc. (mM)	MTX	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H	Compound I
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	4.0		10.9	3.6		10.3	7.6	29.2	10.6	13.7
0.2		19.7			21.0					
0.3	9.7		36.0	9.9		36.1	25.2	91.7	34.8	44.7
0.4		43.1								
0.8		92.2								
1	29.2		109.4	34.7	156.7	105.7	82.2	248.7	115.2	150.6

Depending on the structure of the ligand, the amount of immobilization varies greatly even at the same reaction concentration. The optimum amount of immobilization also depends on the structure of the ligand, the buffer composition and the amount of protein solution added at affinity purification.

○Other beads

If using other beads, since the amount of immobilization cannot be quantified by HPLC, the amount of immobilization is qualitatively evaluated by conducting a binding experiment with a protein.

【Results of Protein Purification Experments】

First step

・Immobilization of ligand on beads

・Ligand-binding protein analysis (Affinity purification)

<Expected results>

(1) Non-specific adsorption of protein is observed on non-immobilized beads (0 mM).

(2) No band is observed. (No binding protein band at 0mM beads and also no band on ligand-immobilized beads.)

(3) Many binding protein bands are observed. (Specific bands cannot be determined.)

(4) Some bands of binding proteins that appeared to be ligand-specific is observed.

(4) → **Examination of specificity**

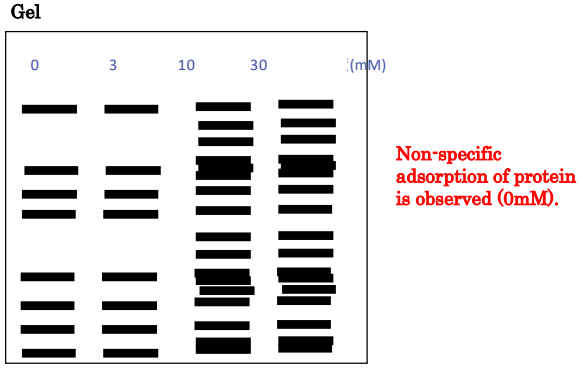
・Competitive inhibition

・Drug elution

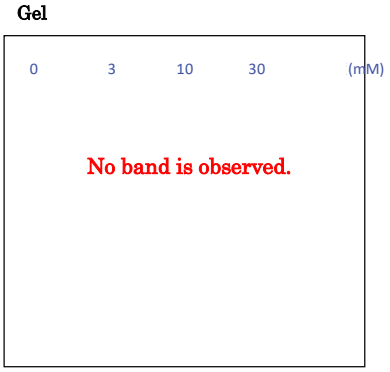
→ **Mass spectrometry**

(1), (2), (3) → **Trouble shooting**

(1)

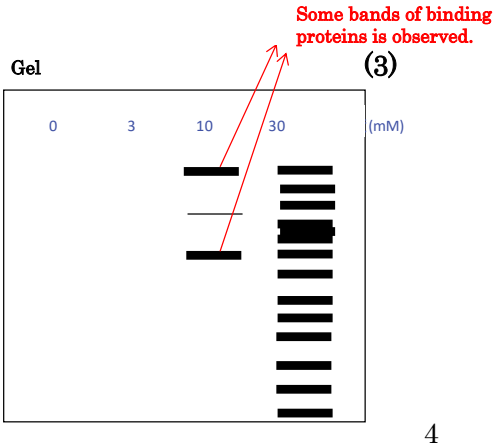


(2)



(2)

(4)



【Trouble Shooting】

(1) Non-specific adsorption of protein is observed on non-immobilized beads (0 mM)

Cause	Countermeasures
Insufficient masking	Use dehydrated DMF
	Repeat the masking process. (When using COOH beads, repeat the masking process from the NHS activation process.)
	Use an ultrasonic devices to sufficiently disperse the beads in the masking process and check it carefully.
Insufficient washing	Repeat washing the beads more or increase buffer volume in the affinity purification process.
Insoluble fruction is contained in cell extract.	The cell extract centrifuge (15,000 rpm, 4℃, 30 minutes or longer) before mixing into beads.

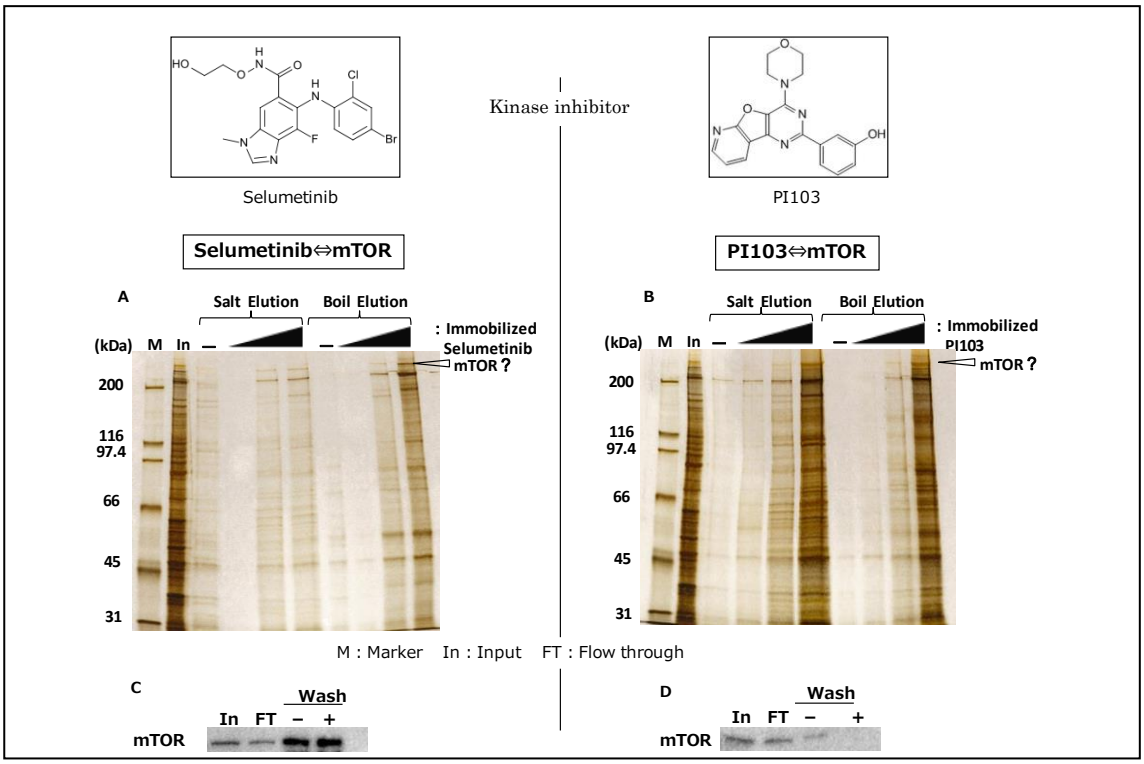
(2) No band is observed. (No binding protein band at 0mM beads and also no band on ligand-immobilized beads.)

Cause	Countermeasures
Ligand is not immobilized.	Check the immobilization protocol again.
	Use an ultrasonic devices to sufficiently disperse the beads in ligand immobilized process and check it carefully.
Low amount of ligand immobilized	Increase the ligand concentration in ligand immobilized process.
No or low target protein in protein solution.	Increase the concentration or amount of the protein solution.
	Use another protein solution.
Weak affinity between ligand and target protein	Reduce the salt concentration in the binding /washing buffer.
	Reduce the surfactant concentration in the binding / washing buffer to 0.1% or less.

(3) Many binding protein bands are observed. (Specific bands cannot be determined.)

Cause	Countermeasures
Large amount of ligand immobilized	Reduce the ligand concentration in ligand immobilized process.
Ligand is hydrophobic and easy to bind proteins	Increase the salt concentration in the binding /washing buffer.
	Try competitive inhibition or drug elution.

◎If the affinity between the ligand and the target protein is weak, the bound protein once recovered may be came off by repeating wash (and increasing the salt concentration of the buffer, etc.).



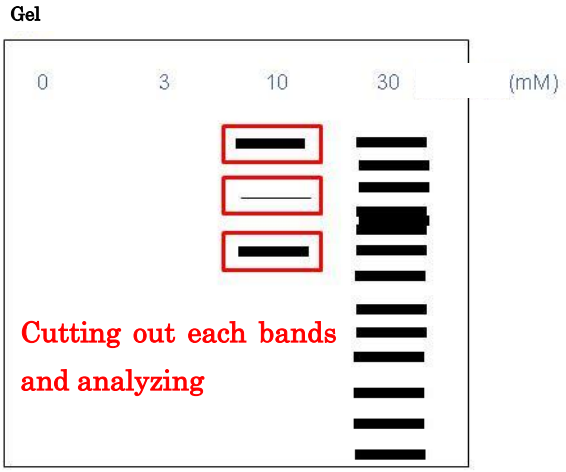
In the case of PI103, the mTOR that was once recovered came off by washing the beads after the binding reaction and could not be purified. In the case of Selumetinib, mTOR does not come off even after washing, and it can be purified. It is considered that the affinity between PI103 and mTOR is weak.

◎If many binding protein bands are observed, a specific binding protein can be identified by a method called Shotgun Proteomics in Mass Spectrometry.

【Two Methods for Mass Spectrometry】

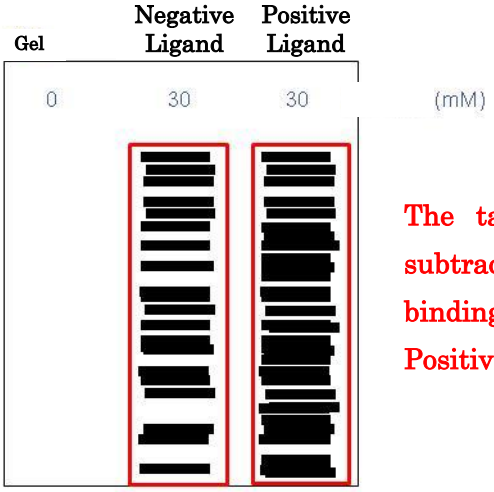
Method 1 Gel-separated protein bands are excised each, and the fragment peptide obtained by protease treatment is mass-analyzed to identify the bound protein.

➡ Only a few bands of protein that bind to the ligand are analyzed.



Method 2 All binding proteins eluted from the beads without gel separation are treated with proteases and the resulting fragment peptides are comprehensively analyzed. (Shotgun proteomics)

➡ Analysis is possible even if the number of proteins is large.



The target binding protein can be identified by subtracting the Negative Ligand (inactive compound) binding protein from the analysis result of the Positive Ligand (active compound) binding protein.

〈Examples of mass spectrometry sample〉

- Purified samples of positive ligand-immobilized beads and negative ligand-immobilized beads.
- Purified samples of compound-immobilized beads and control beads (0 mM).

・Elution samples of drug elution: No compound and with compound.

・Elution samples of competitive inhibition: No competitive inhibition and competitive inhibition.