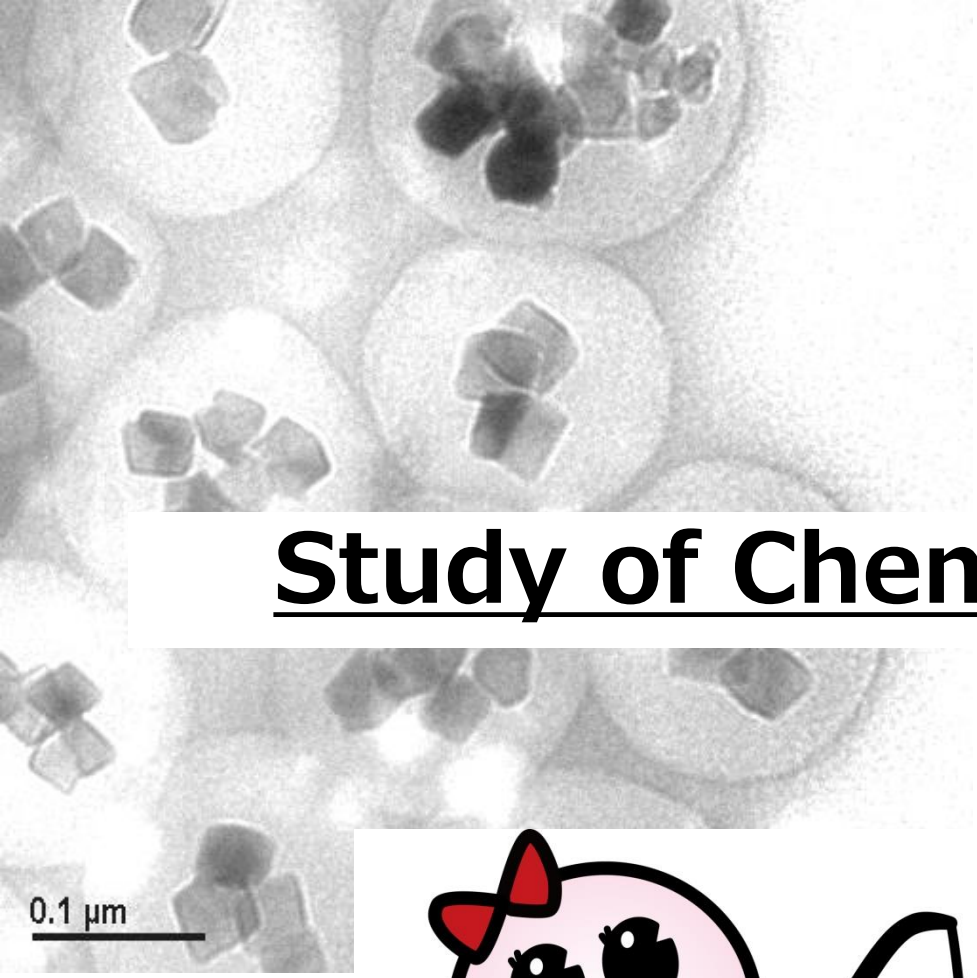
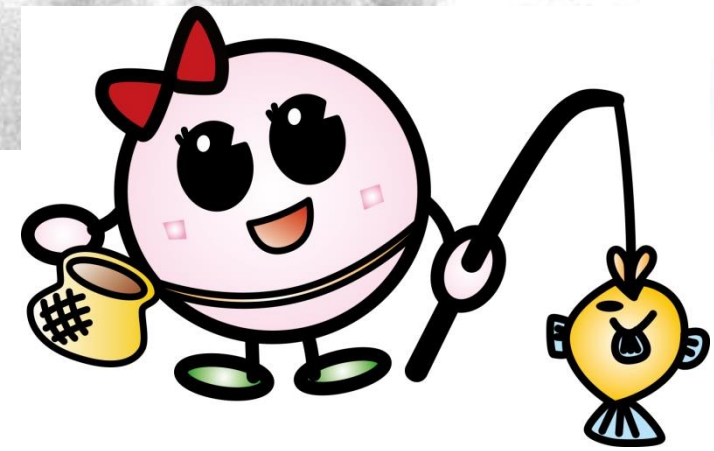
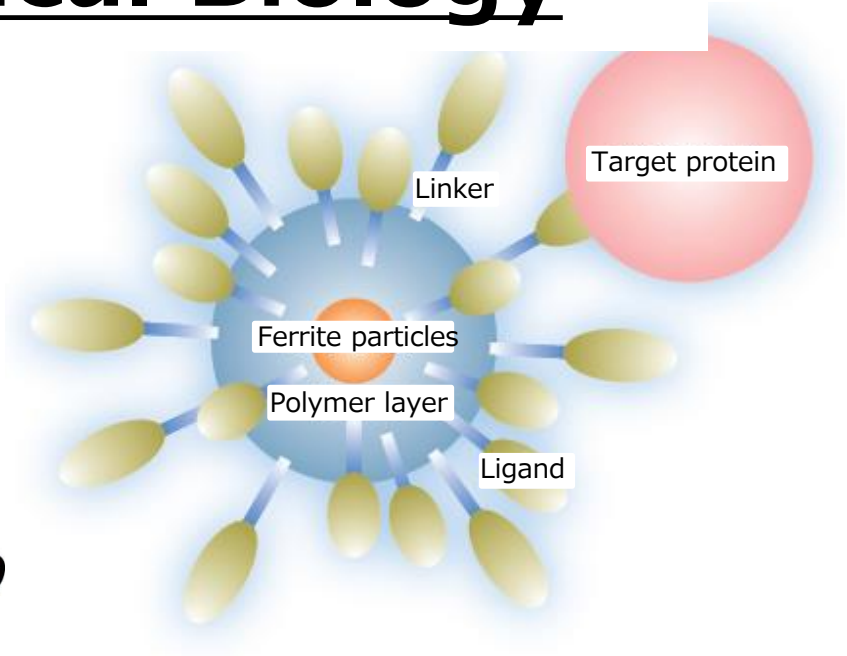


Study of Chemical Biology

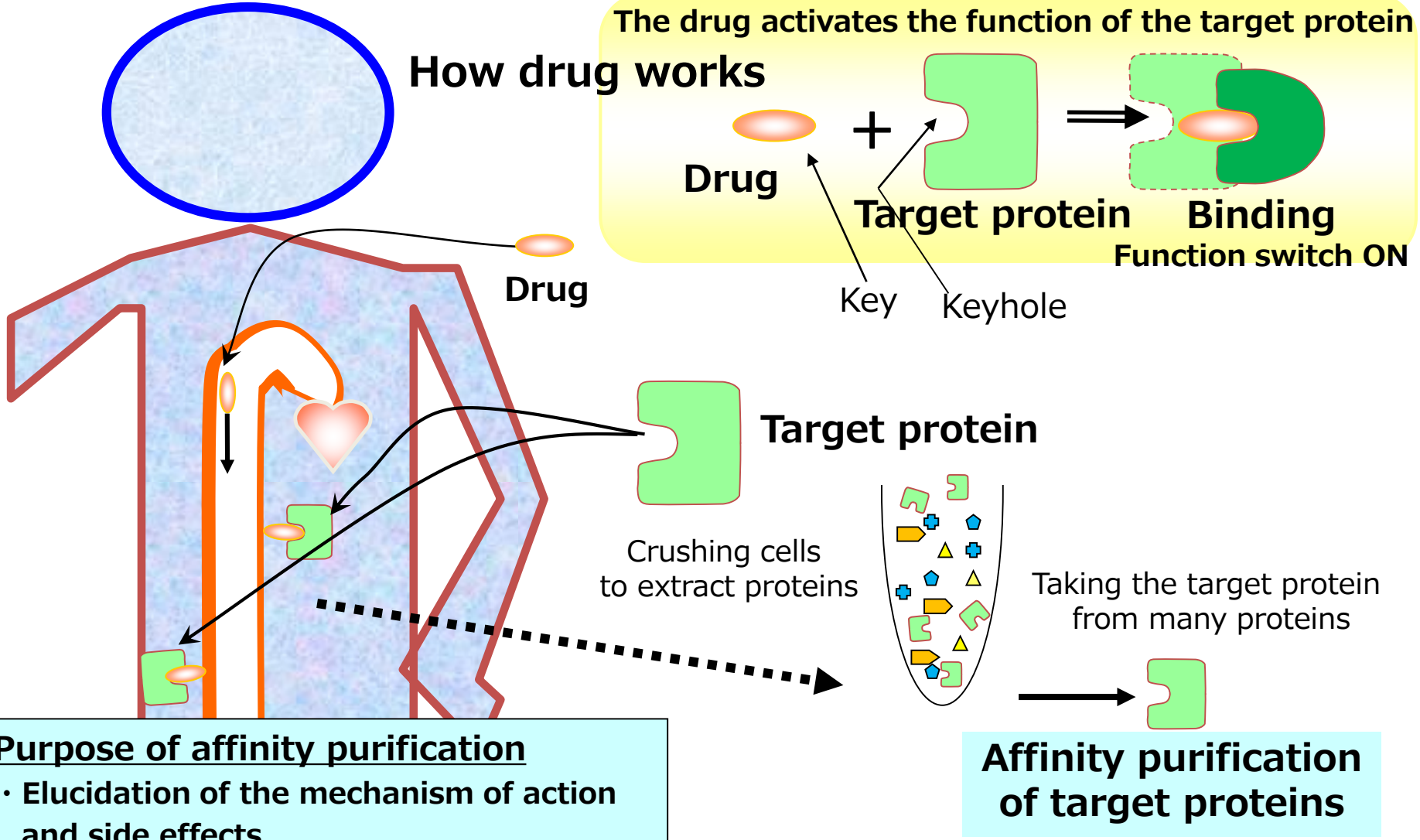




TAMAGAWA SEIKI CO., LTD.

Table of contents

Affinity purification of drug target proteins

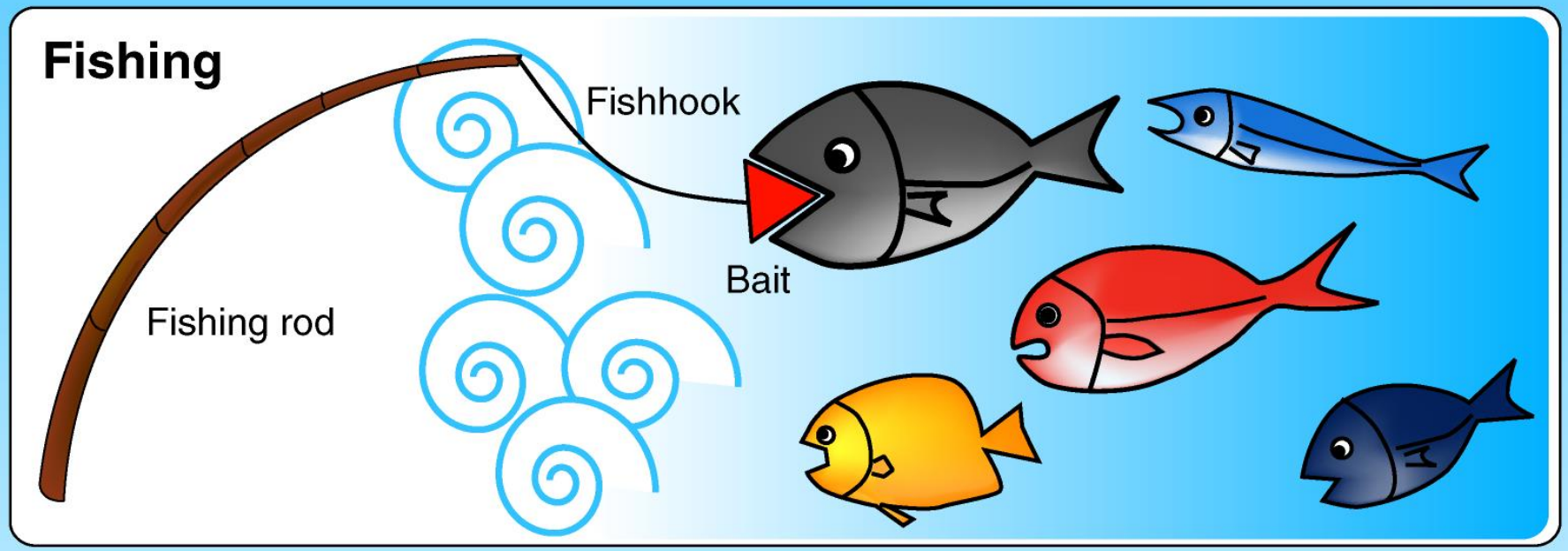


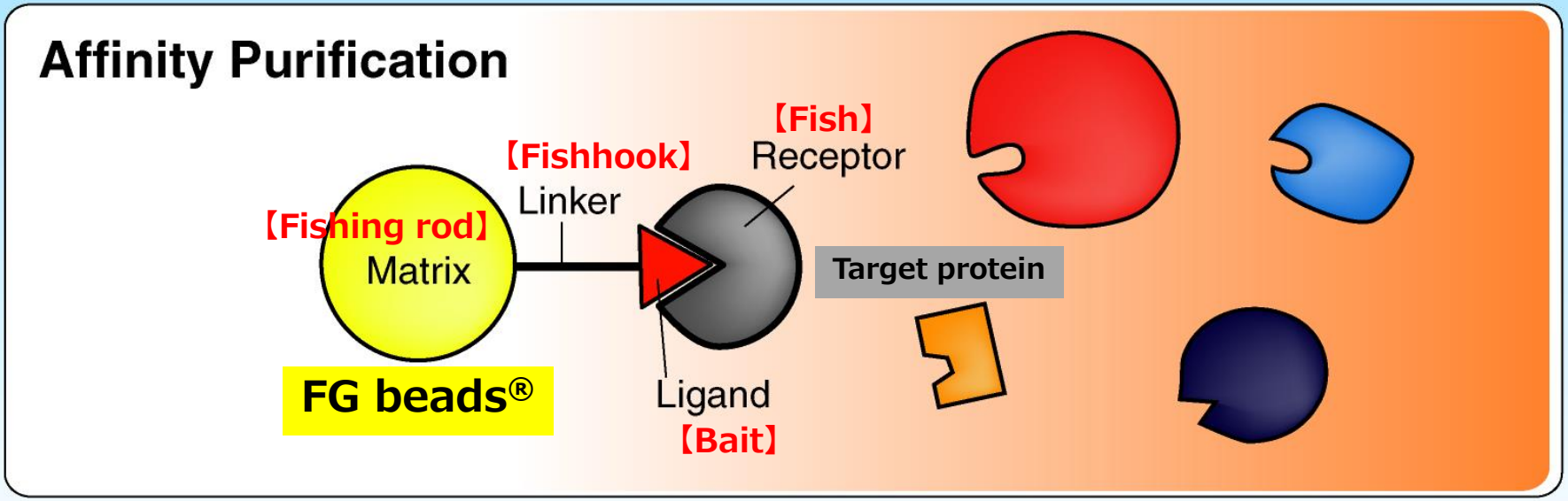
Purpose of affinity purification

- Elucidation of the mechanism of action and side effects
- Analysis of biological reaction network
- Search for new drug discovery targets

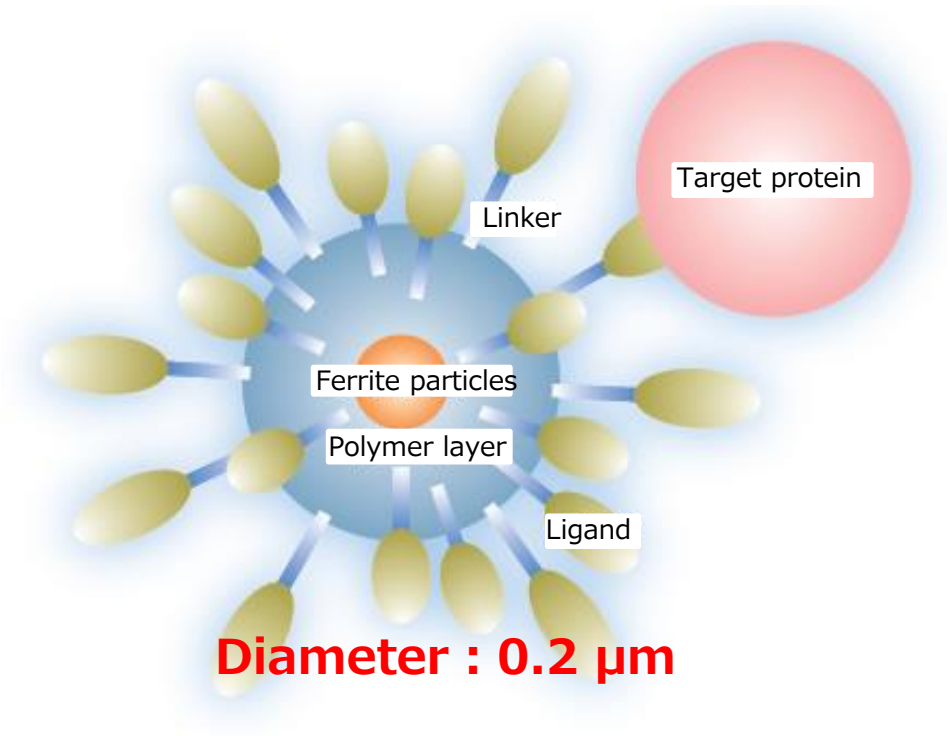
- Many drugs have unknown target proteins.
- Isolation and identification of target proteins are essential for new drug development.

Affinity purification is called Target Fishing and is often compared to fishing.

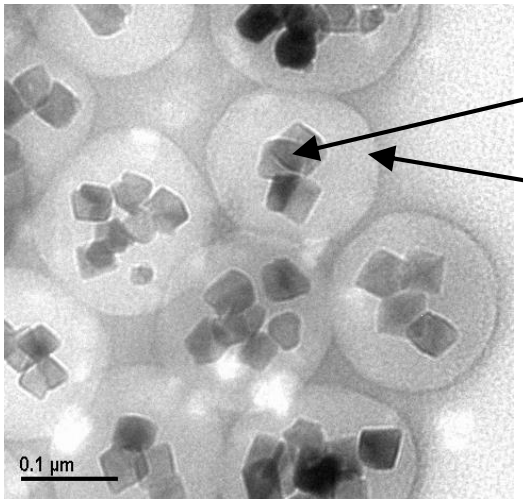




Structure of FG beads®



Diameter : 0.2 μm

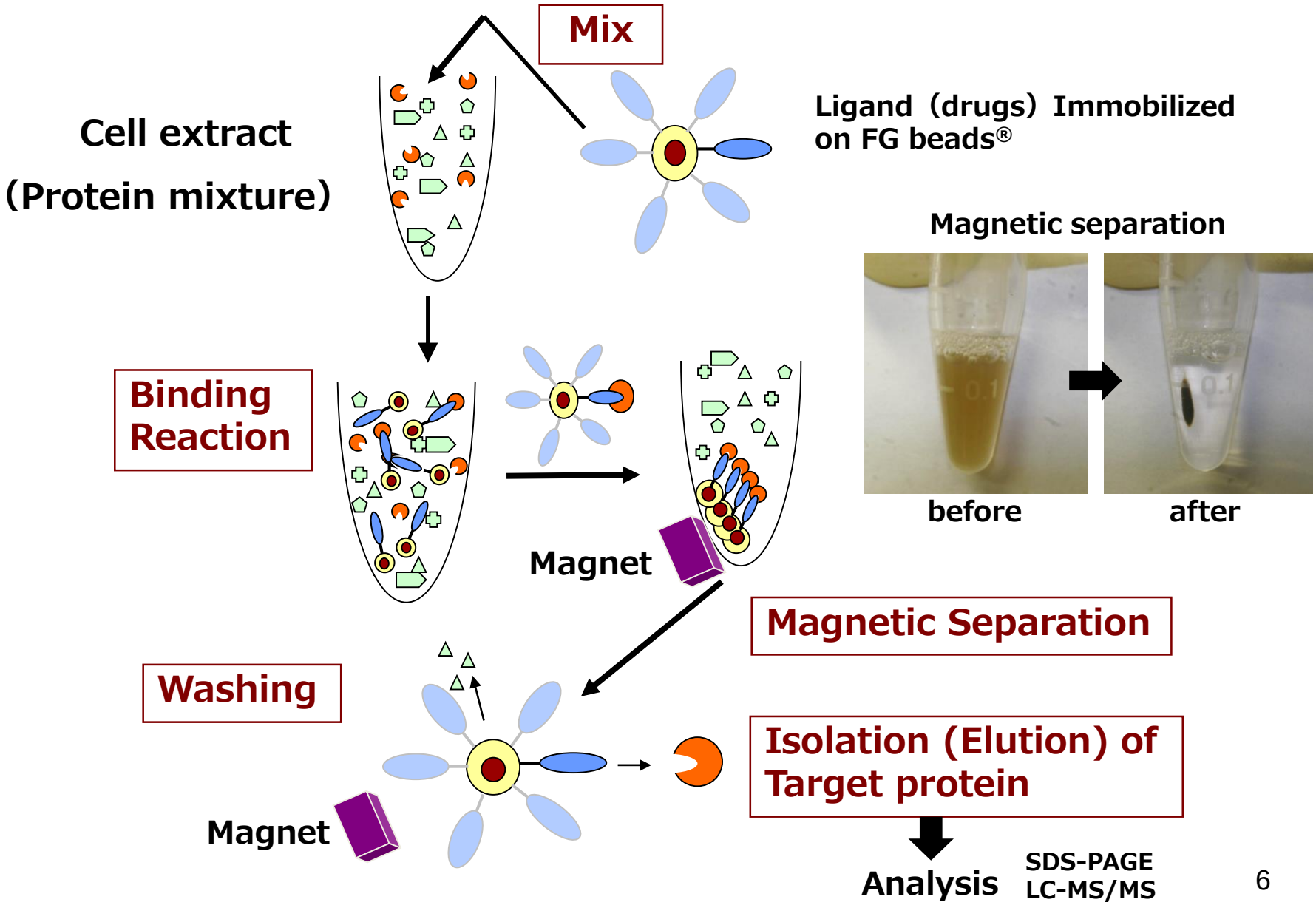


Electric microscope image

Ligand drug is Immobilized via the linker to catch the target protein from many cellular proteins.

FG beads®

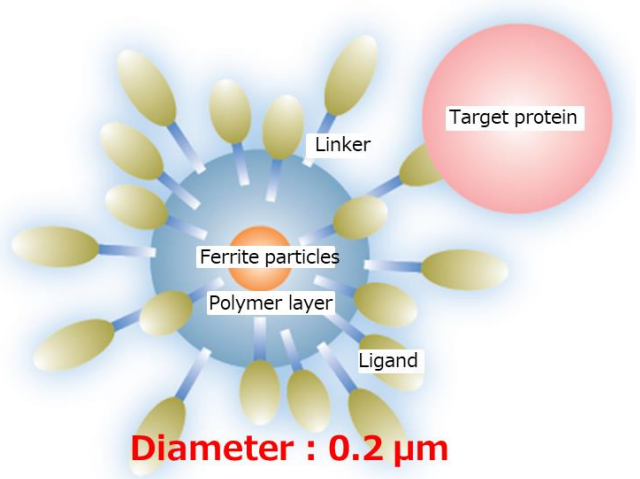
Affinity purification of target proteins by FG beads®



Features of FG beads®

High recovery rate

**FG beads are nano-sized and have a large surface area per weight.
It has high dispersibility and mobility, and the target protein binds efficiently.**

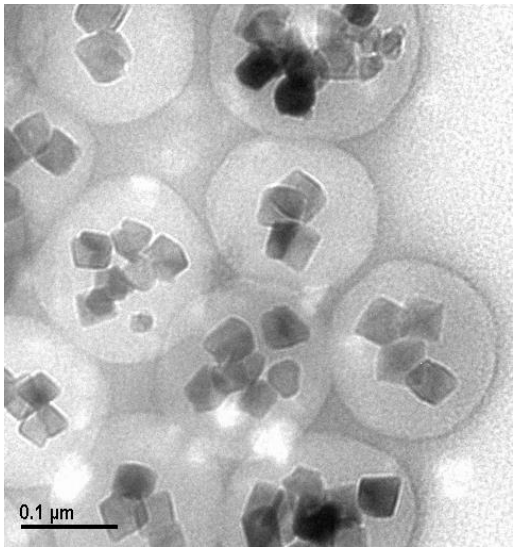


High purity

The surface of the beads is coated with a special polymer, polyGMA (glycidyl methacrylate) and non-specific binding of proteins is extremely low.

Resistance to Organic solvents

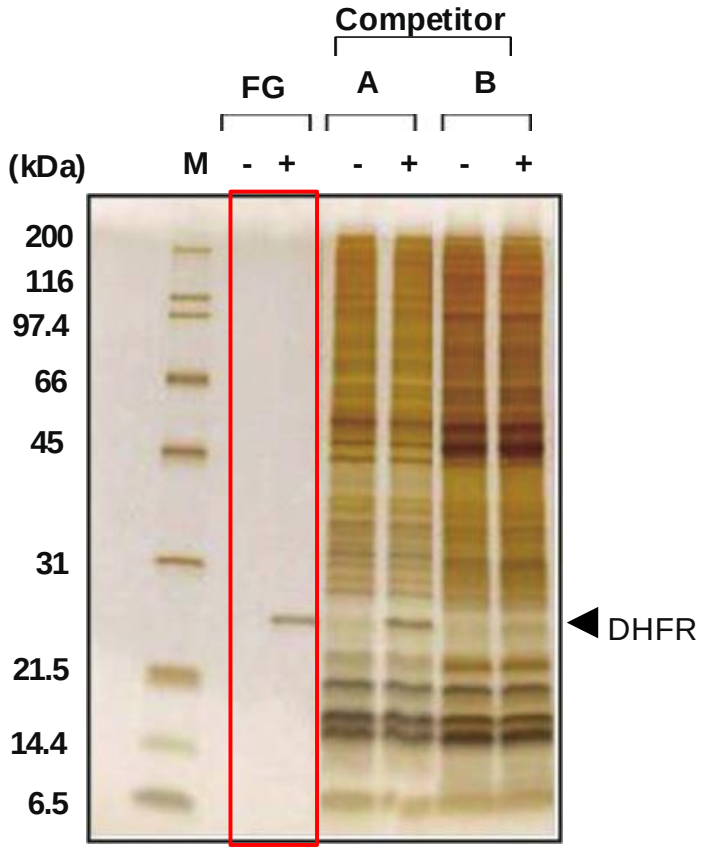
**Ligands can be immobilized in various organic solvents.
Drugs that are insoluble in water can also be immobilized.**

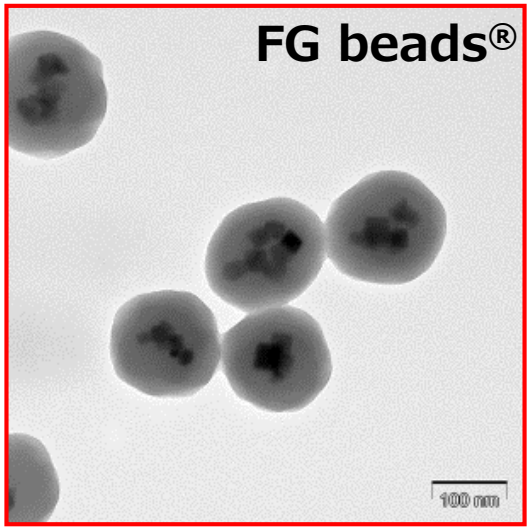


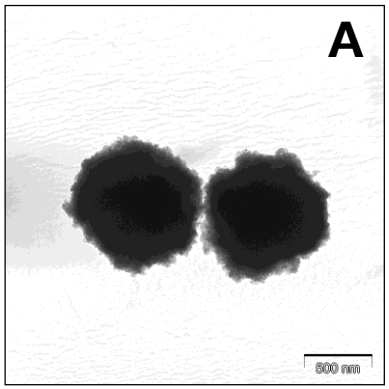
Electric microscope image

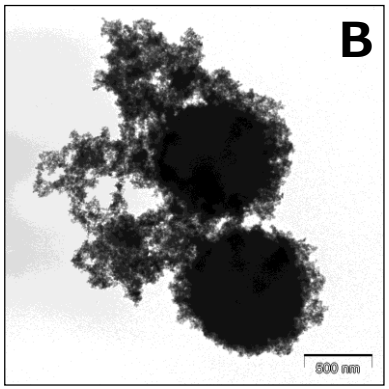
Comparison of FG beads® and other beads

The anticancer drug Methotrexate (MTX) was immobilized on the magnetic beads of each company by the same method, and affinity purification was performed. Each bead was compared by the same weight.



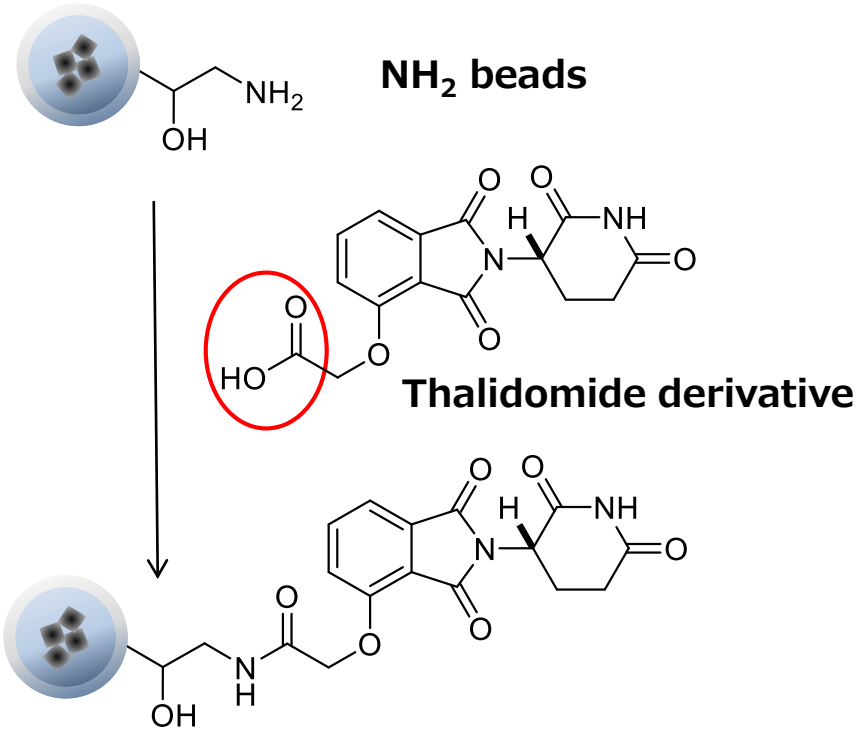


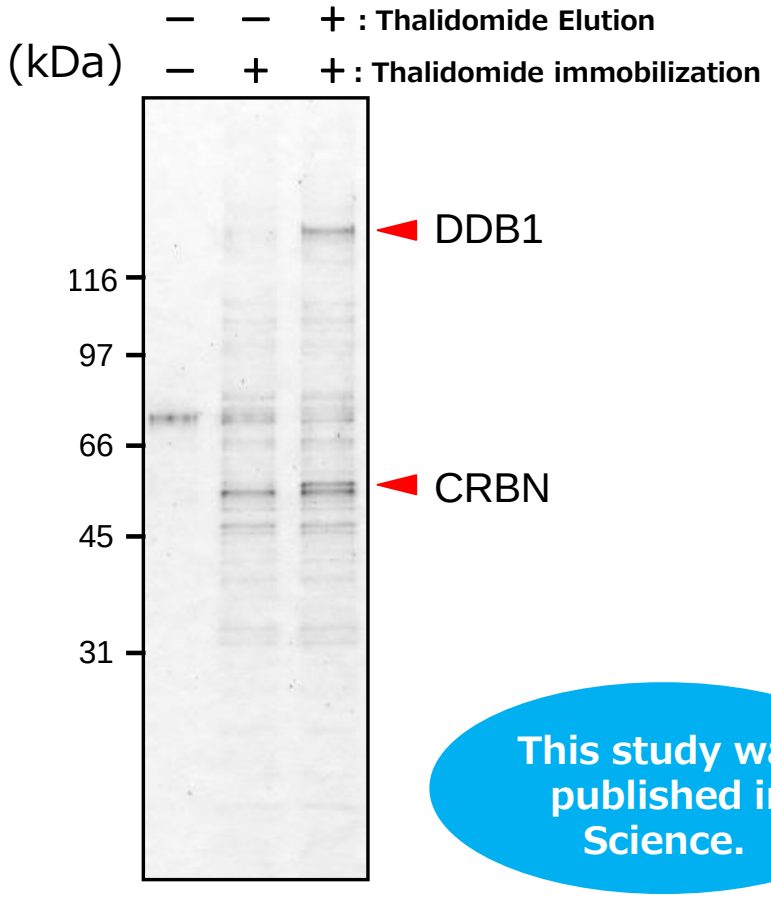




Compared to magnetic beads of other companies, FG beads® have less non-specific binding of proteins, and DHFR, which is the target protein of MTX, can be purified with high purity.

Identification of Thalidomide target proteins





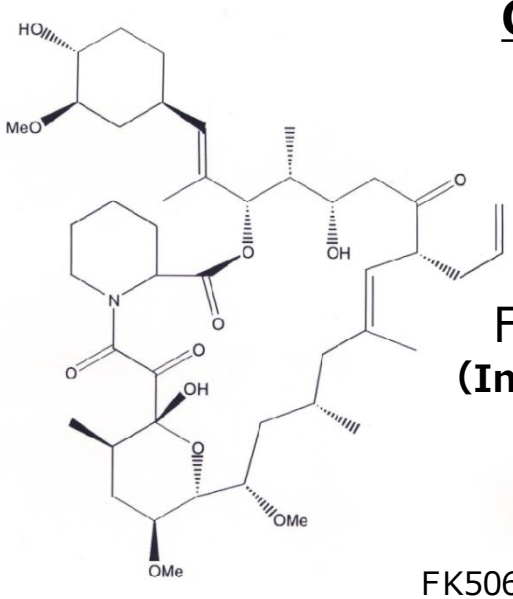
This study was published in Science.

<Elucidation of the mechanism that causes malformations>

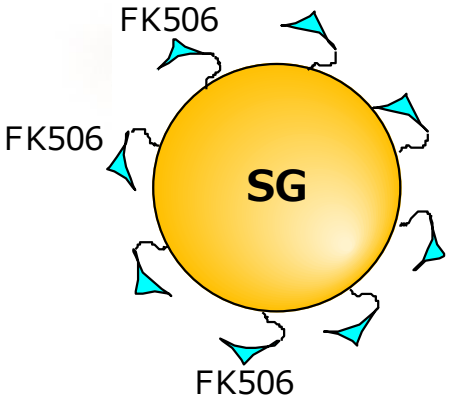
Cereblon (CRBN) and DDB1 were identified when thalidomide derivatives were immobilized on FG beads® and affinity purification was performed from human cell extracts. Cereblon is a component of an enzyme involved in proteolysis, and thalidomide has been found to cause malformations by inhibiting the action of this enzyme.

T. Ito *et al*., Science 327 (2010) 1345

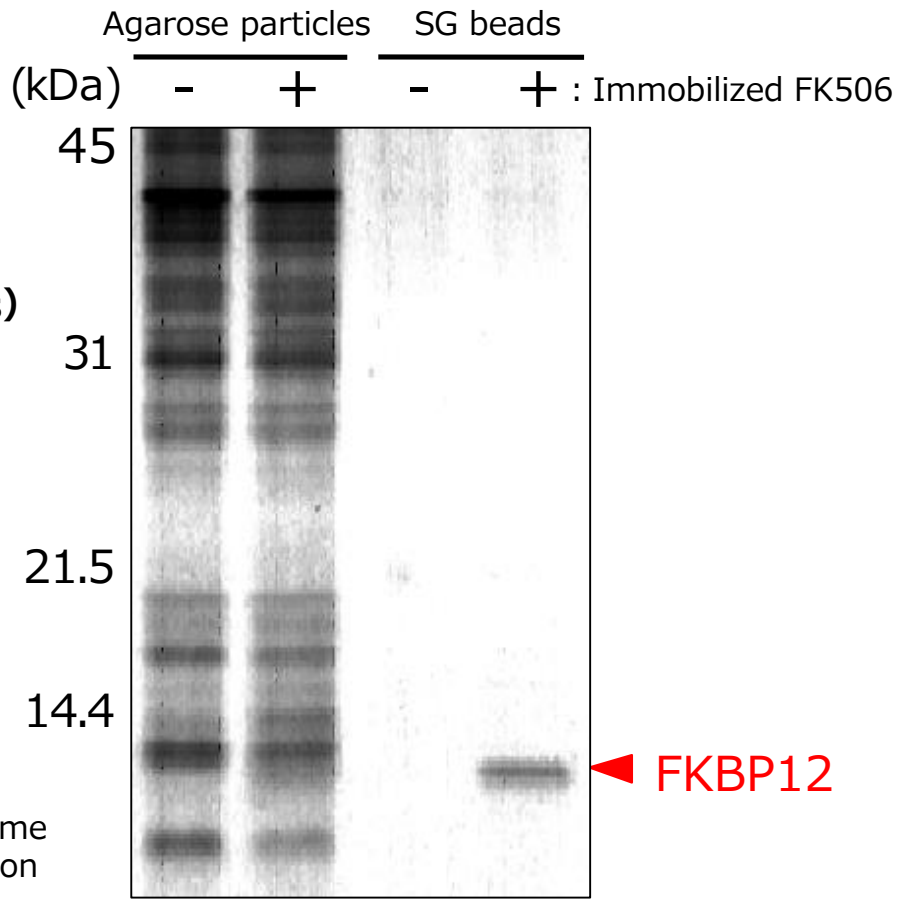
Purification of FK506 binding proteins



FK506
(Immunosuppressants)



Comparison of agarose particles and SG beads

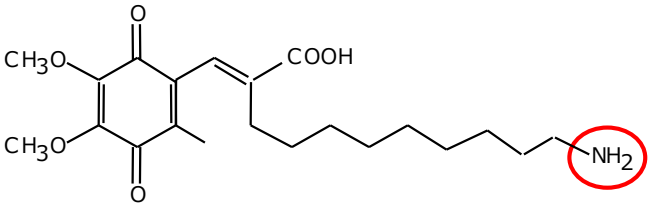


SG beads :
SG beads do not have ferrite in the bead core. It has the same characteristics as FG beads, but is recovered by centrifugation instead of magnetic separation.

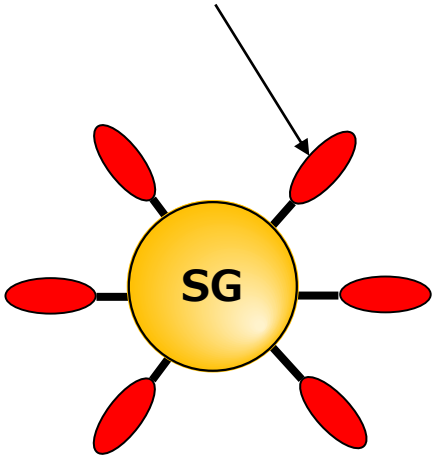
The FK506 derivative was immobilized on SG beads, affinity purification was performed, and FKBP12 was purified. Compared to agarose particles, which have been often used for protein affinity purification, there is no non-specific binding of proteins, and it was possible to purify with high purity.

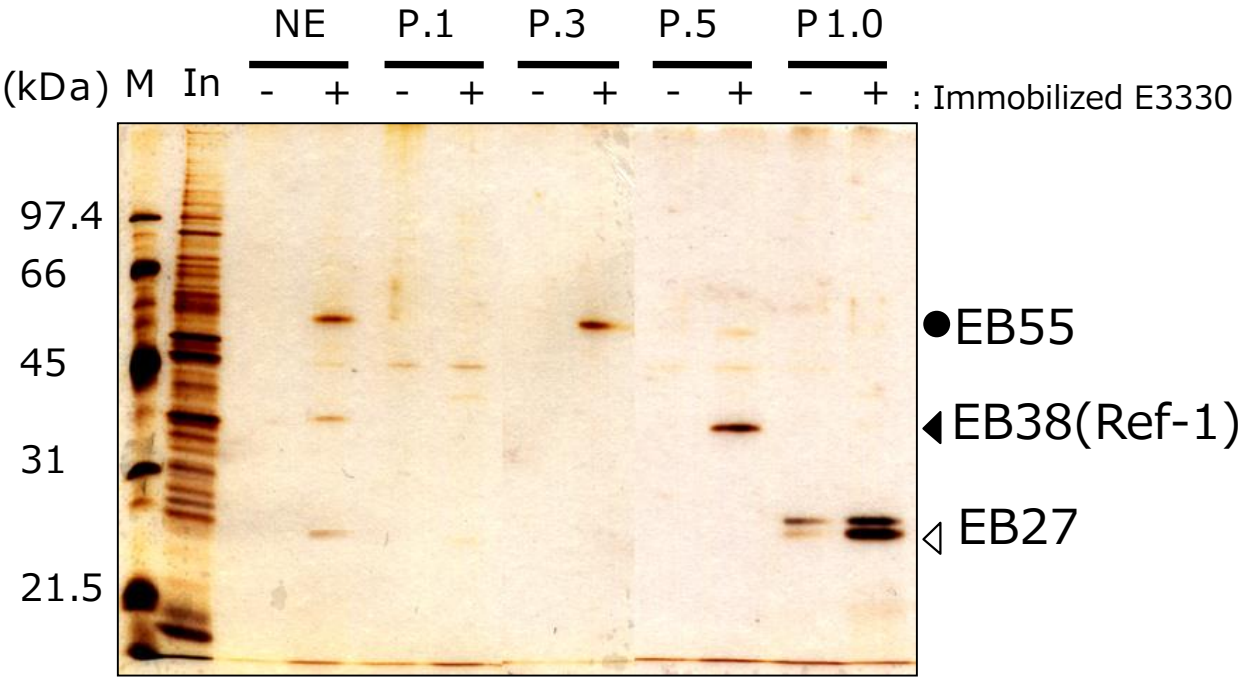
N. Shimizu *et al*., Nature. Biotech. 18 (2000) 877

Purification of E3330 binding proteins



E3330 amino derivative
(Anti-inflammatory agent)





※Fractionation by gradually increasing the salt (KCl) concentration during nuclear extract fractionation

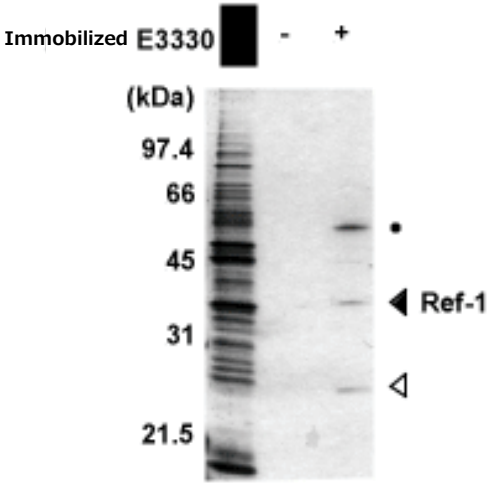
NE-: No fractionation with KCl
P.1-: Fractionation with 0.1 M KCl
P.3-: Fractionation with 0.3 M KCl
P.5-: Fractionation with 0.5 M KCl
P1.0-: Fractionation with 1.0 M KCl

The E3330 derivative was immobilized on SG beads, an anti-inflammatory drug, and affinity purification was performed from the nuclear extract of Jurlat cells to purify three types of binding proteins. As a result of analysis, Ref-1 was found to be the target protein.

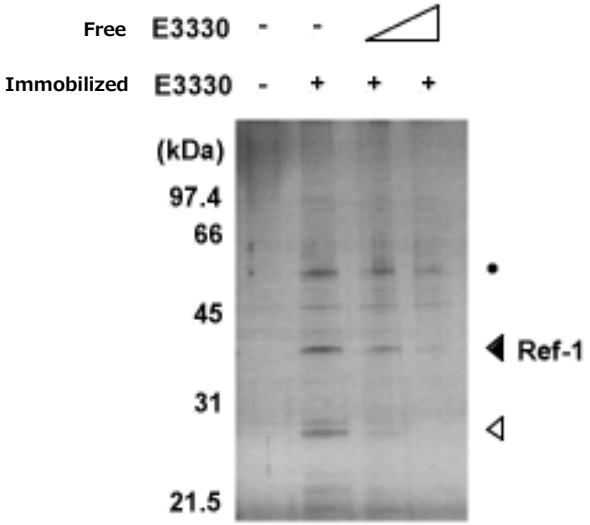
N. Shimizu *et al*., Nature. Biotech. 18 (2000) 877

Purification of E3330 binding proteins

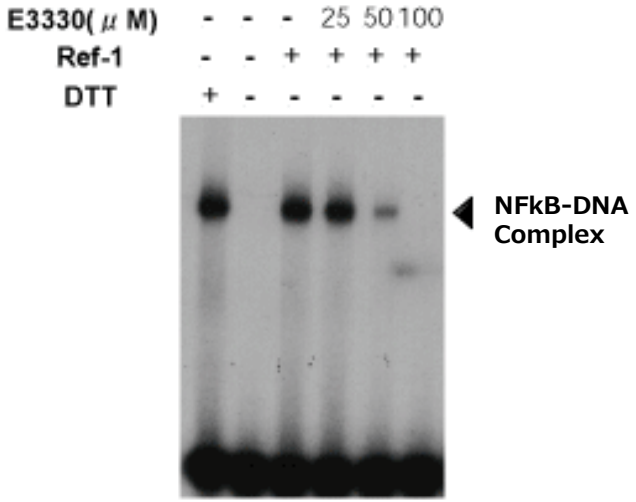
A **Affinity purification**



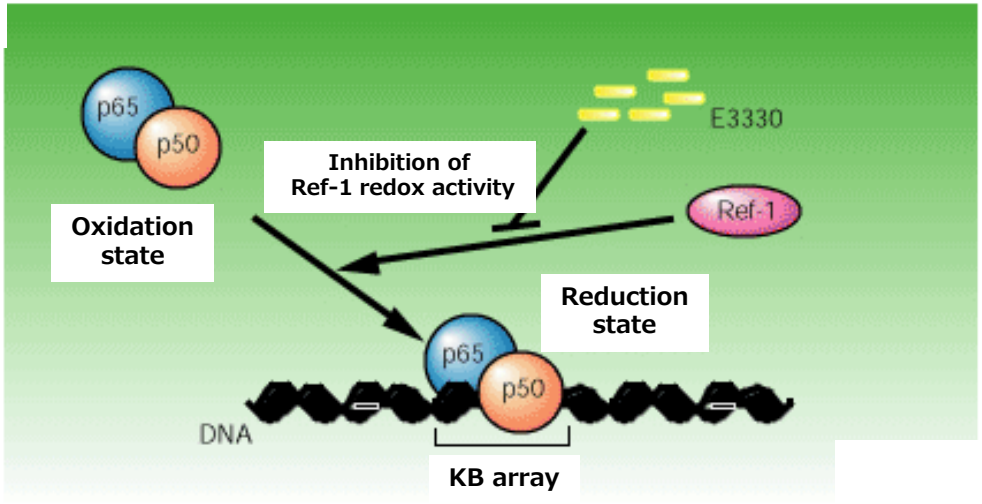
B **Competitive inhibition by E3330**



C **Electrophoresis Mobility Shift Assay**



D



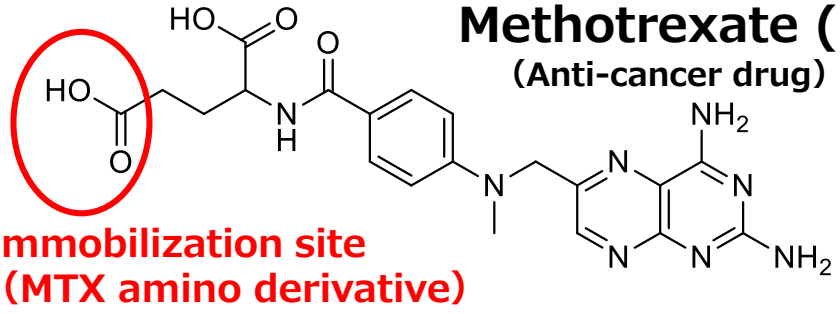
Ref-1 reduces NF-kB in the nucleus by redox activity and enhances its DNA binding ability. E3330 inhibits this redox activity by binding to Ref-1.

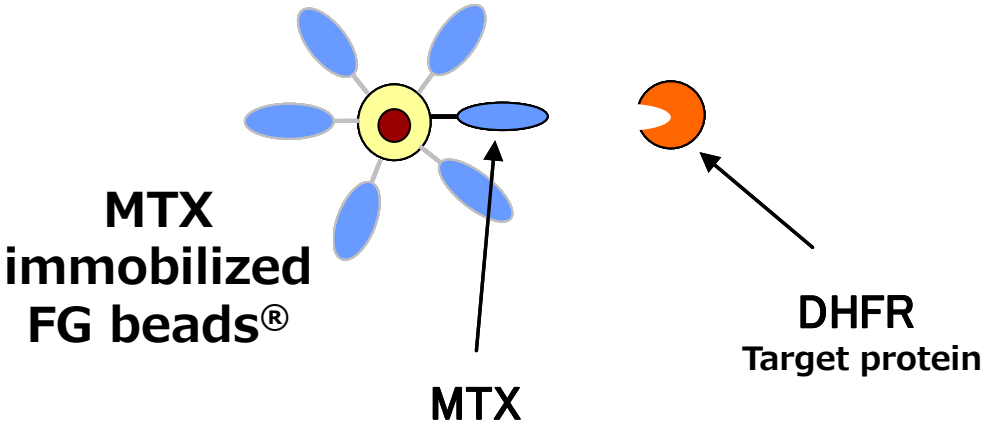
⬇

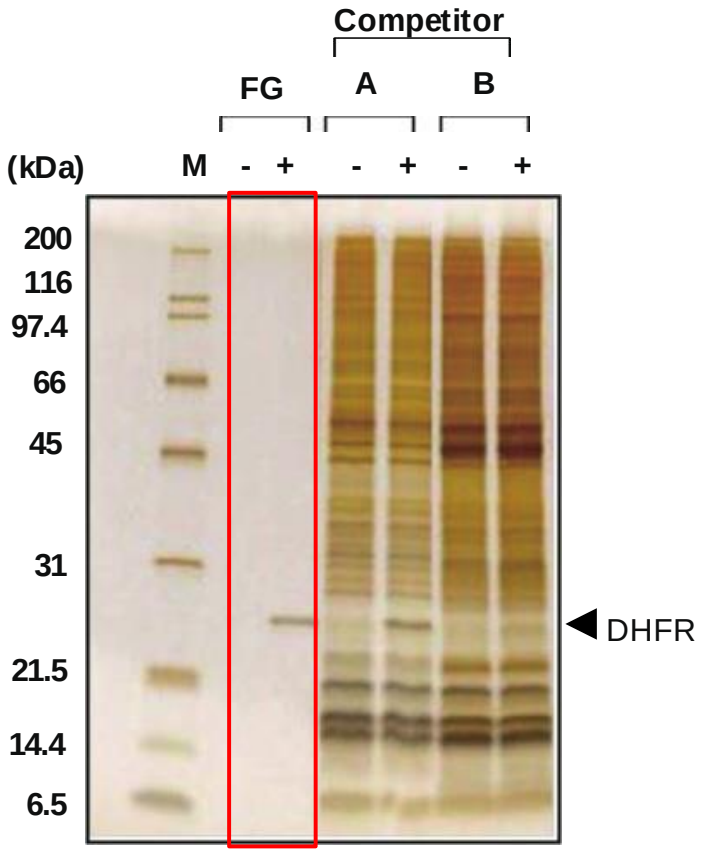
The research was revealed that E3330 inhibits the transcription promoting activity of NF-kB, suppresses the gene expression of inflammatory cytokines, and exerts an anti-inflammatory effect.

N. Shimizu *et al*., Nature. Biotech. 18 (2000) 877

Purification of MTX binding proteins







The anticancer drug methotrexate (MTX) was immobilized on the magnetic beads of each company by the same method, and affinity purification was performed.
Compared to magnetic beads of other companies, there is less non-specific adsorption of proteins, and DHFR, which is the target protein of MTX, can be purified with high purity.

Purification of MTX binding proteins

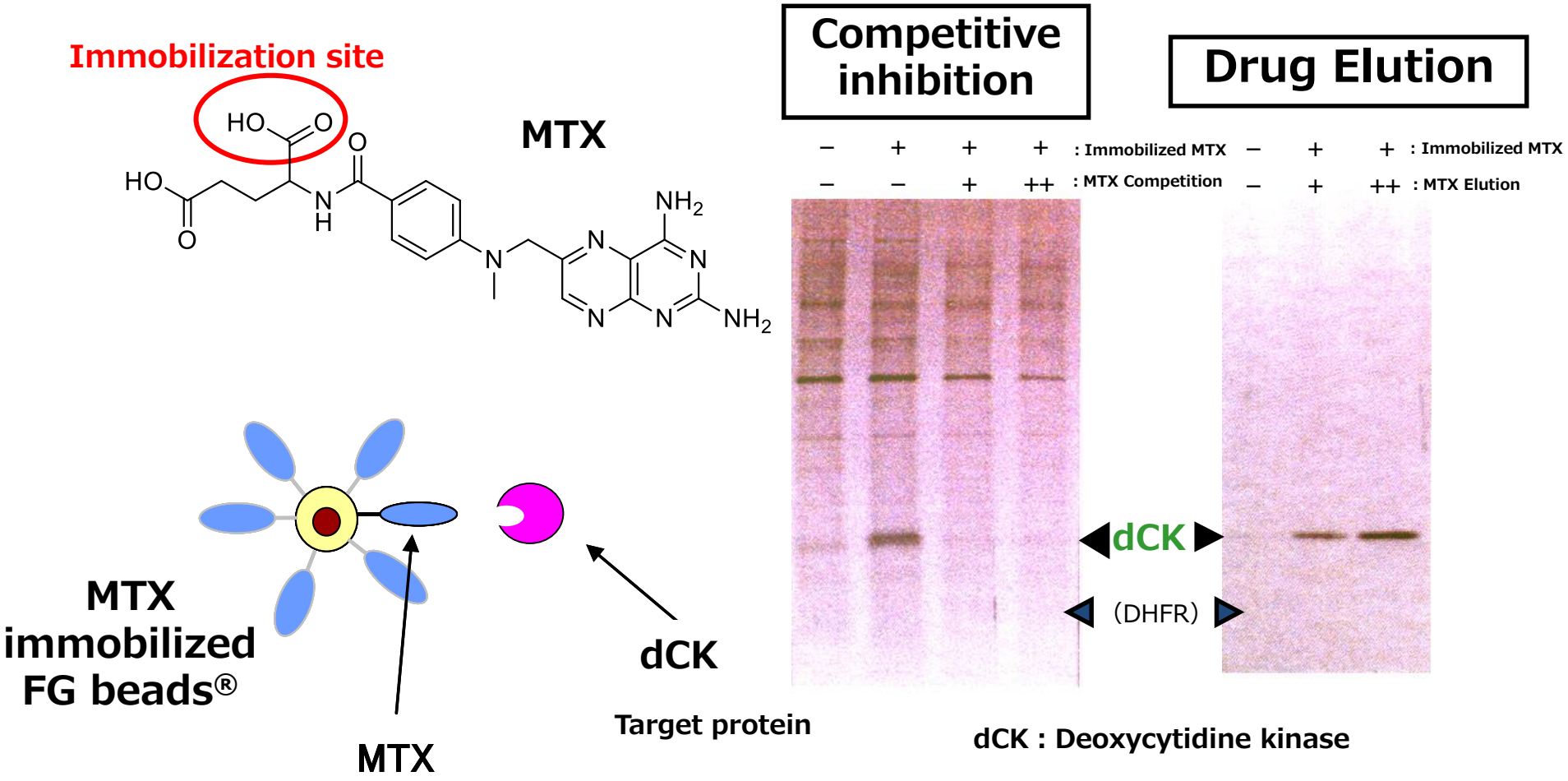
<Applied therapy with MTX>

- Administration of MTX in combination with other anticancer agents synergistically enhances the therapeutic effect for intractable leukemia and lymphoma.
- MTX is used for chronic rheumatism (immunosuppressive effect)

The DHFR-mediated action of MTX cannot explain these effects.

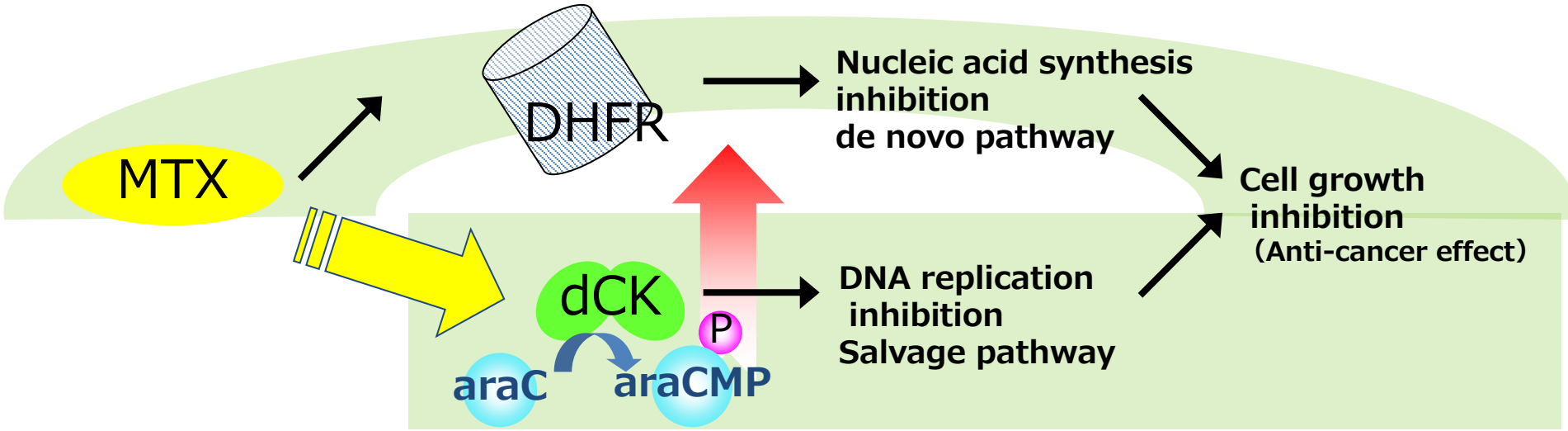
Search for new target proteins for MTX

Purification of MTX binding proteins



Affinity purification was performed by changing the immobilizing site between MTX and beads, and a new target protein, dCK, was identified.

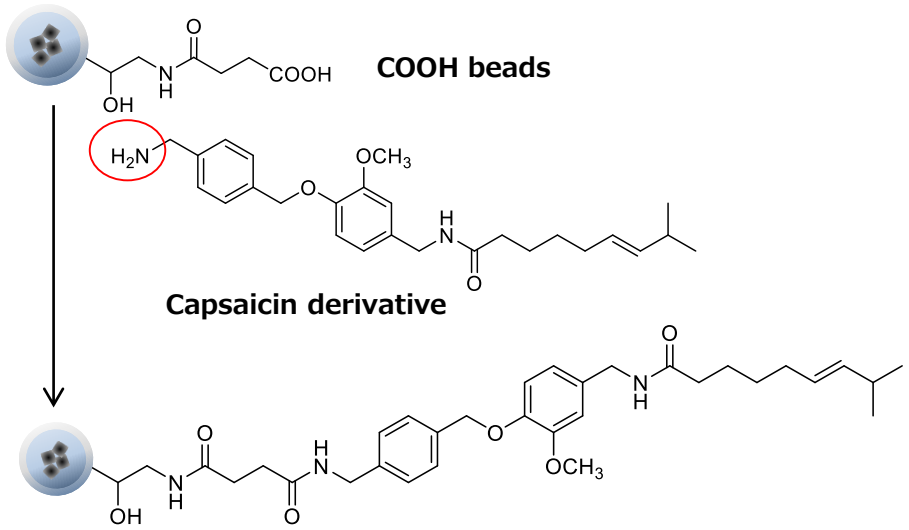
Purification of MTX binding proteins

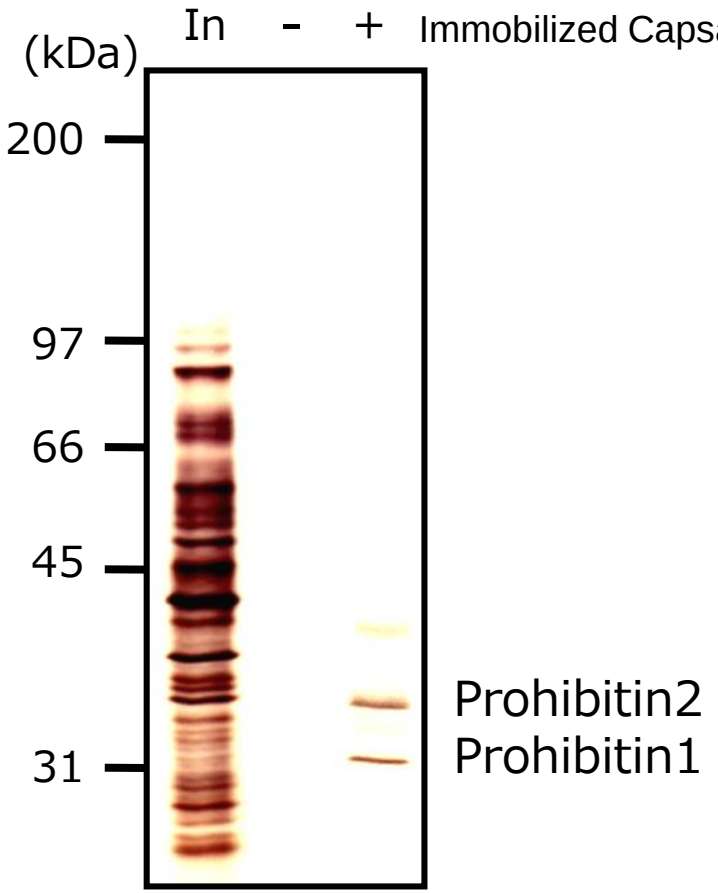


The reseach was elucidated the molecular mechanism of high-concentration combination therapy for malignant lymphoma of MTX and the anticancer drug araC.
MTX not only acts on DHFR to inhibit cell proliferation, but also promotes phosphorylation of araC and inhibits DNA replication, thereby inhibiting cell proliferation and exerting an anticancer effect.

H. Uga *et al*., Mol. Pharmacol. 70 (2006) 1832

Purification of Capsaicin binding proteins



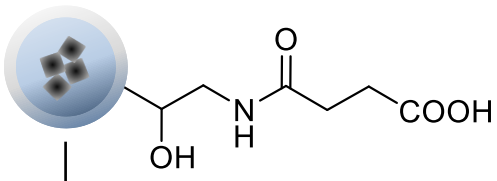


<Leukemia cell growth inhibitory action mechanism of capsaicin>

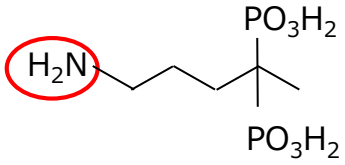
Prohibitin1 and Prohibitin2 were identified by immobilizing a capsaicin derivative on FG beads® and performing affinity purification from human leukemia cells NB4. This elucidated the mechanism by which capsaicin induces apoptosis in leukemic cells.

C. Kuramori *et al*., Biochem. Biophys. Res. Commu. 379 (2009) 519

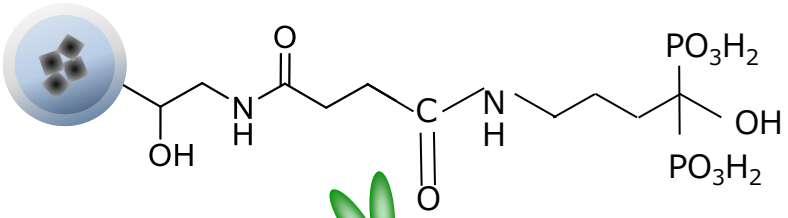
Purification of Alendronate binding proteins

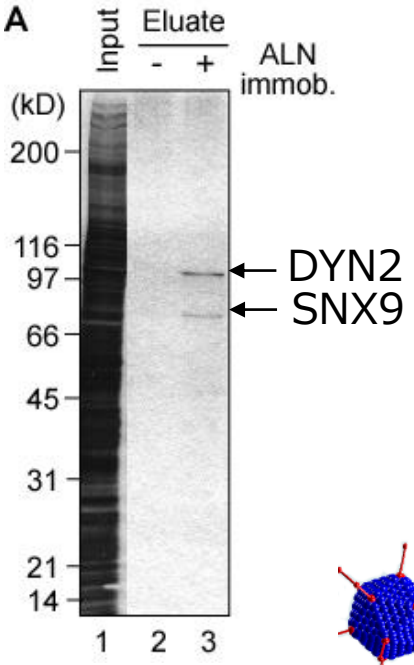


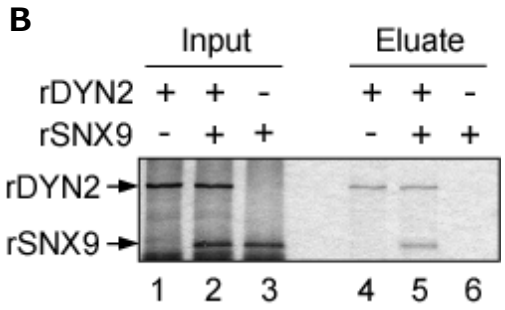
COOH beads

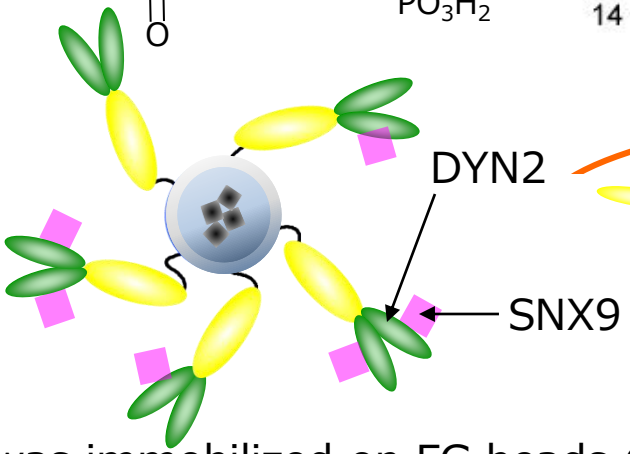


Alendronate (Osteoporosis drug)

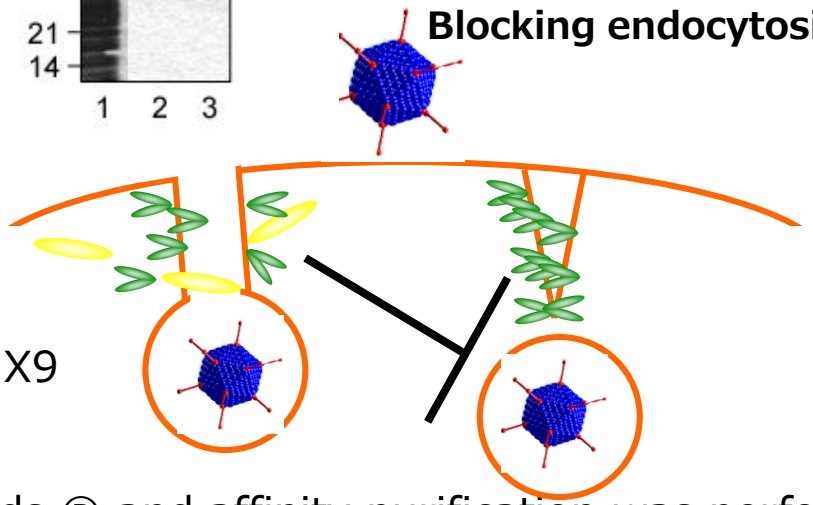








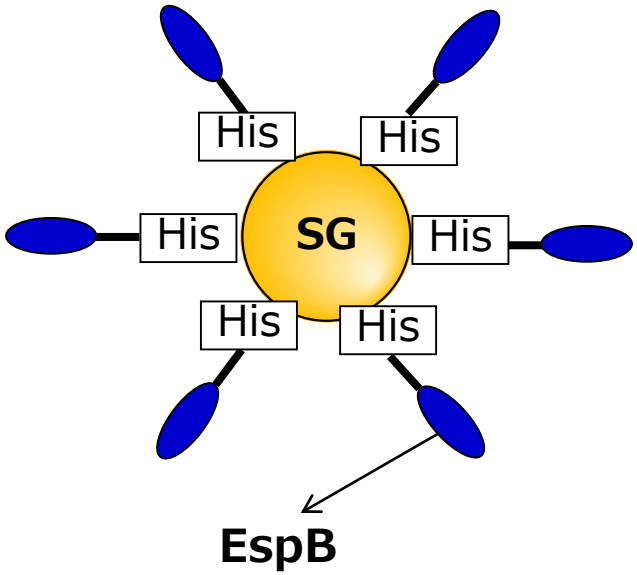
Blocking endocytosis by ALN



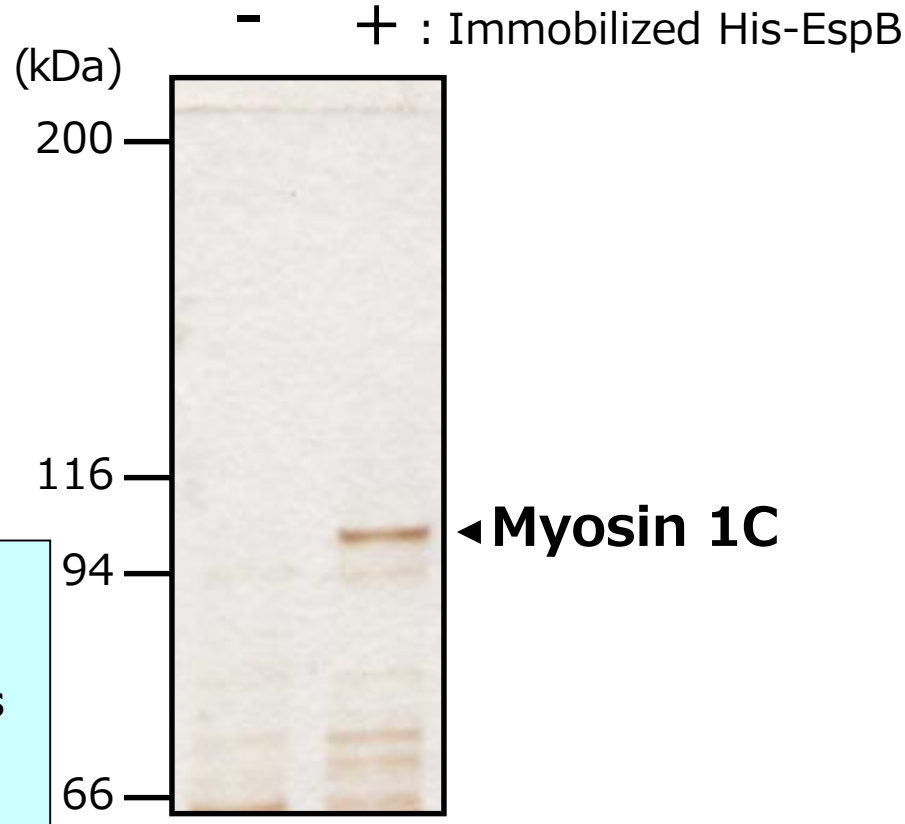
Alendronate was immobilized on FG beads ® and affinity purification was performed to identify dynamin (DYN) 2 and SNX9. Dynamin was not a protein involved in osteoporosis, but analysis of a novel binding protein led to the discovery of its potential use as an antiviral drug.

Y. Masaike *et al*., Mol. Pharmacol. 77 (2009) 262

Purification of EspB binding proteins



(Pathogenic protein of enterohemorrhagic Escherichia coli “O157”)



Mechanism of action for EspB :
Suppression of microvilli destruction of intestinal epithelial cells and phagocytosis of macrophages by inhibition of myosin function

Recombinant protein expressed with His-tag: EspB was immobilized on SG beads (Ts beads), and affinity purification was performed from HeLa cell extract, and Myosin1C was identified. This led to the elucidation of the infectious molecular mechanism of pathogenic Escherichia coli.

Y. Iizumi *et al*., Cell Host & Microbe, 2 (2007) 383

Elucidation of mechanism by searching for targets

※The studies of the red letters are introduced in this document.

Achievements in the Handa Laboratory

Pharmaceuticals

- Anti-cancer drug (MTX, Thalidomide, Taxol)
- Osteoporosis (KF21232, Bisphosphonate)
- Anti-inflammatory drug (E3330, Aspirin)
- Immunosuppressant (FK506)
- Anti-rheumatic agent (Bucillamine)
- Anti-analgesic (Salicylic acid)
- Cardiac stimulant (Vesnarinone)
- Anti-influenza drug (Tamiflu)
- Anti-Alzheimer's drug (Hellisenon D)

Biological substances

- Vitamins (Vitamin K2)
- Amino acids (Leucine, Arginine, Methionine)
- Heme-related substances
- Double-stranded DNA, Single-stranded DNA
- Bioactive peptide

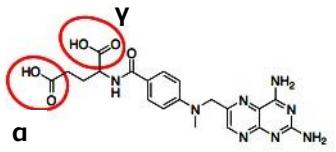
Virulence factors / Toxic substances

- Pathogenic Escherichia coli toxin (EspB)
- Environmental hormones (Phthalic acid, Atrazine, Bisphenol A)
- Viral factor (Integrated DNA sequence of AAV)

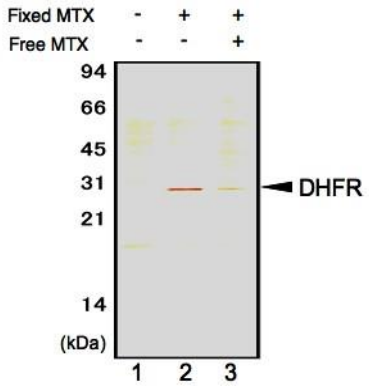
New target protein for anticancer drug MTX

Anti-cancer drug

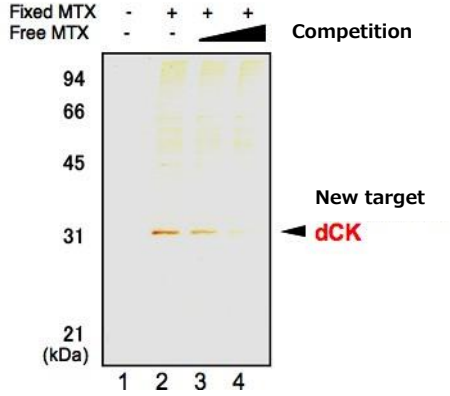
Methotrexate (MTX)



MTX-α immobilized beads

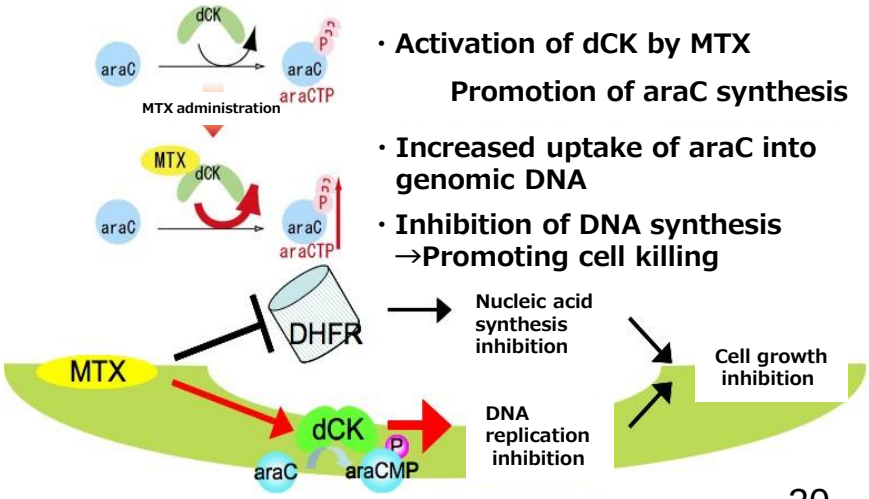


MTX-γ immobilized beads



Mechanism of MTX and AraC combination therapy

Activation of dCK by MTX

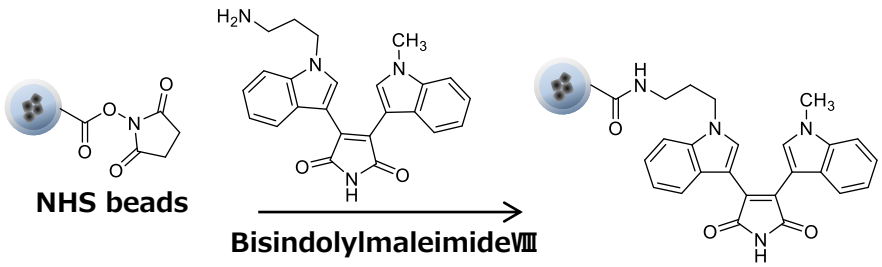


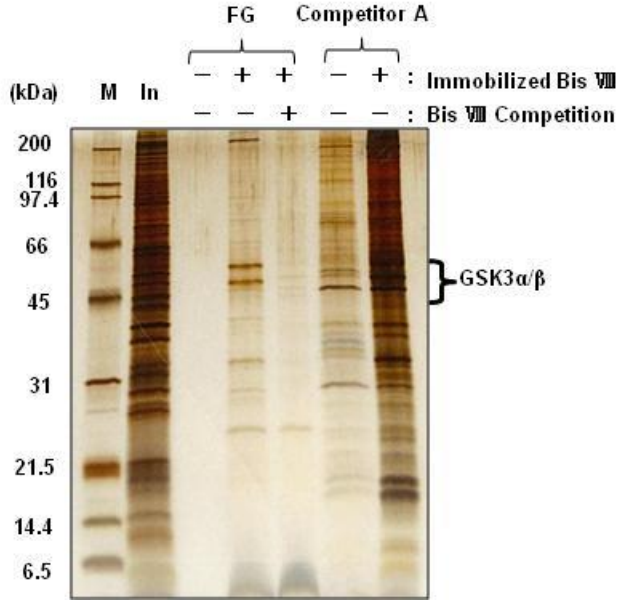
- **Activation of dCK by MTX**
  Promotion of araC synthesis
- **Increased uptake of araC into genomic DNA**
- **Inhibition of DNA synthesis →Promoting cell killing**

H. Uga *et al*., Mol. Pharmacol. 70 (2006) 1832

Purification of inhibitor drug binding proteins

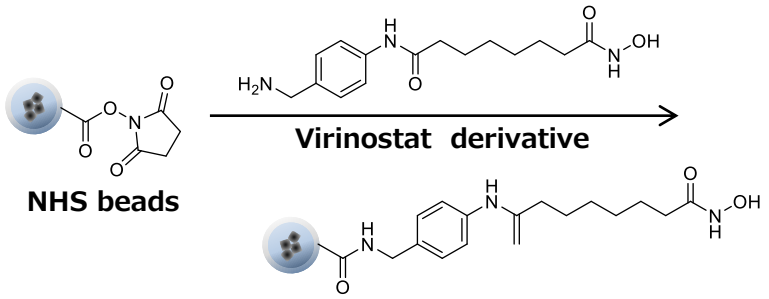
①Kinase inhibitor BisindolylmaleimideⅧ

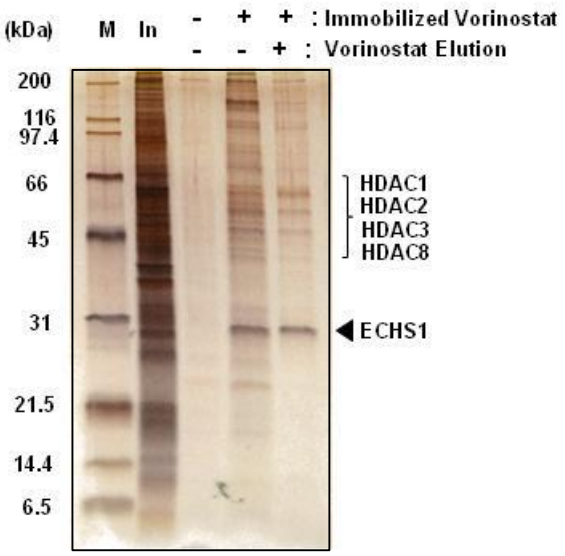




Bisindolylmaleimide Ⅷ (BisⅧ) was immobilized on FG beads®, and magnetic beads A of another company, and the bound proteins was purified from the HeLa cell extract.
As a result, several binding proteins were purified, and GSK3α/β was identified as the main binding protein of BisⅧ by Western blotting and MS analysis.

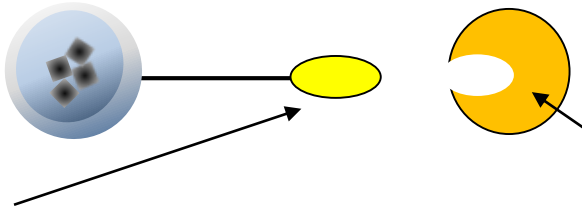
②HDAC inhibitor Vorinostat





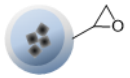
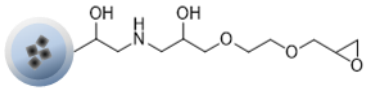
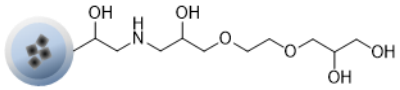
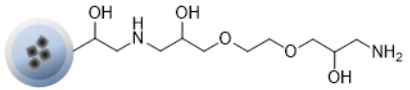
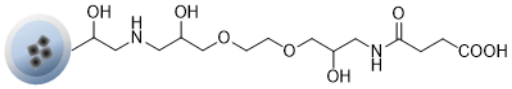
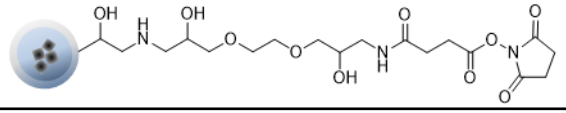
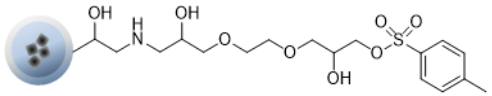
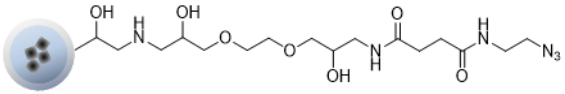
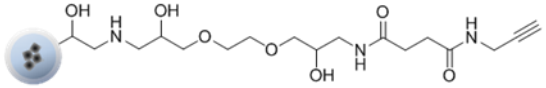
A derivative of the HDAC inhibitor Vorinostat (AHA) was immobilized on FG beads® and the bound proteins was affinity purified from the HeLa cell extract.
As a result, Four types of HDACs were recovered by Western blotting and ECHS1 was identified by MS analysis.

Combination of Ligand and Target molecule

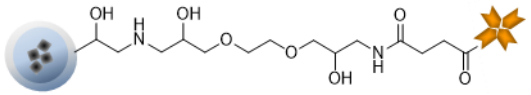
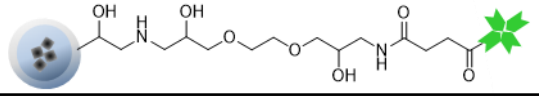
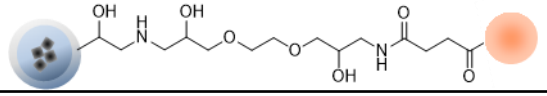
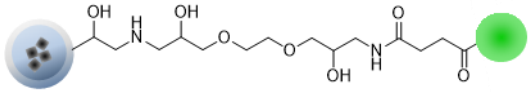


Ligand		Target molecule
Chemical Compound (drug)	⟷	Protein DNA
Protein	⟷	Chemical substance Protein Peptide DNA RNA
Peptide	⟷	Protein
DNA	⟷	DNA RNA DNA-binding protein Chemical substance
Antibody	⟷	Antigen

FG beads® lineup

Product number	Product name	Structure	Ligand
TAS8848N1010	Plain beads		DNA
TAS8848N1110	Linker beads		R-NH2 Amino group R-OH Phenolic hydroxyl group
TAS8848N1120	OH beads		R-COOH Carboxyl group
TAS8848N1130	NH_2 beads		R-COOH Carboxyl group
TAS8848N1140	COOH beads		R-NH2/R-NHR Amino group
TAS8848N1141	NHS beads		R-NH2/R-NHR Amino group
TAS8848N1150	Ts beads		His-Tag protein
TAS8848N1160	Azide beads		Alkyne compounds
TAS8848N1161	Alkyne beads		Azide compounds

FG beads® lineup

Product number	Product name	Structure	Ligand
TAS8848N1170	Streptavidin beads		Biotinylated compounds Biotinylated substances
TAS8848N1171	NeutrAvidin beads		Biotinylated compounds Biotynylated substances
TAS8848N1172	Protein A beads		IgG
TAS8848N1173	Protein G beads		IgG