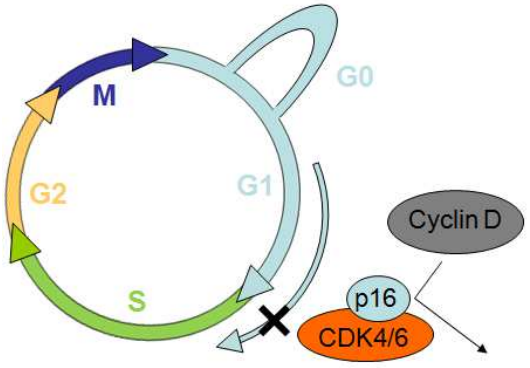
Nacalai USA
Innovations for Life Sciences.

Immunoprecipitation

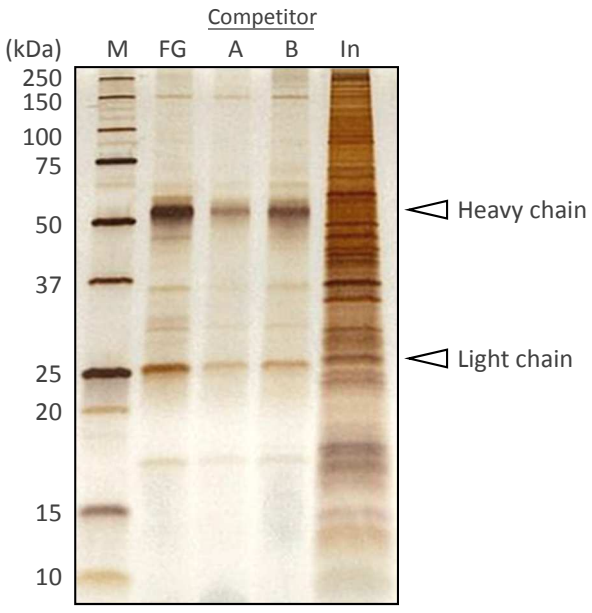
Summary
Immunoprecipitation is an effective approach for the detection of a slight amount of endogenous protein in cells. This time, we performed immunoprecipitation of p16 of HeLa cell, by using Protein G beads.
p16 is known as one of inhibitors of cyclin-dependent kinase (CDK), and is also referred to as p16 INK4a because it belongs to INK4 family. The cell cycle is composed as shown in the right figure. CDK4/6 is activated by forming a complex with CyclinD, phosphorylates target protein, and induces progress in the cell cycle from G1 phase to S phase. p16 works to stop the progress of the cell cycle by competitively inhibiting the complex formation of CDK4/6 and Cyclin D.



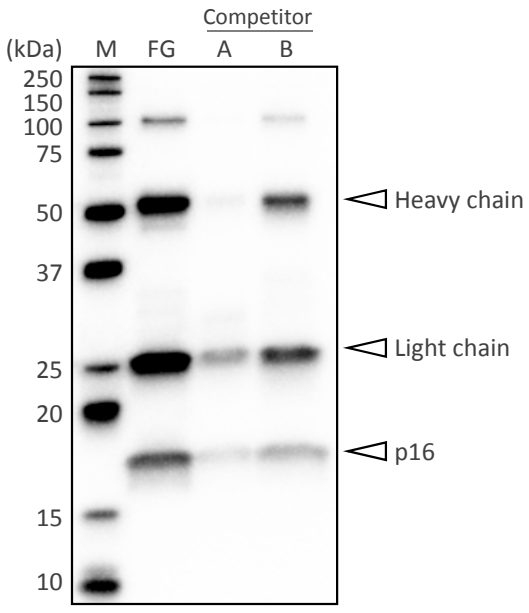
Inhibition pattern of cell cycle progression by p16

It is known that p16 gene functions as a tumor suppressor gene, and is mutated or deleted in a human cancer cell in most cases. When a cell receives oncogenic stress or signals, an expression of p16 gene increases, the function of CDK4/6 is inhibited, and the cell cycle progression is stopped. This is regarded as a self-defense mechanism to protect normal cell from canceration.

Result
Using 0.1mg of Protein G beads from each company and 5ug of Anti-p16 antibody, we immunoprecipitated p16 from HeLa cell extracts and performed silver staining and Western blotting after SDS-PAGE. As the result of the Western blotting, we confirmed that the p16 was purified and FG beads achieved the largest recovery amount. The result of the silver staining showed that the proteins interacting with the p16 were co-immunoprecipitated because plural clear bands were detected.



Silver staining



Western blotting

Materials and Method

- **Materials**

1. Protein G beads
2. HeLa cell extracts (cytosolic fraction) … 3mg/ml
3. PBS(-) (137mM NaCl, 2.7mM KCl, 8.1mM Na2HPO4・12H2O, 1.5mM KH2PO4)
4. Wash buffer A (10mM HEPES-NaOH (pH7.9), 50mM KCl, 0.2mM EDTA, 10%(v/v) glycerol)
5. Wash buffer B (20mM HEPES-NaOH (pH7.9), 150mM KCl, 1mM MgCl2, 0.2mM CaCl2, 0.2mM EDTA, 10%(v/v) glycerol, 0.1% NP-40, 0.2mM PMSF)
6. SDS sample buffer (62.5mM Tris-HCl (pH6.8), 0.005% BPB, 2% SDS, 10% glycerol, 5% 2-mercapto ethanol)
7. Anti-p16 antibody (Abcam)
8. Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (GE)
9. Transfer buffer (25mM Tris, 192mM Glycine, 20%(v/v) Methanol)
10. Blocking buffer (Thermo)
11. TBS-T buffer (20mM Tris-HCl (pH7.5), 500mM NaCl, 0.1% Tween-20)

- **Methods 1 (Binding antibody)**

1. Wash
   Transfer 0.1mg of beads to a tube.
   Wash beads with 200ul PBS(-) 2 times at 4^{o}C
2. Binding Antibody
   Add antibody (5ug) diluted in 200ul PBS(-) to beads.
3. Reaction
   Mix for 30min at room temperature
4. Wash
   Wash antibody binding beads with Wash buffer A 2 times at 4^{o}C

- **Methods 2 (Immunoprecipitation)**

1. Add sample solution
   Add 200ul HeLa cell extracts
2. Reaction
   Resuspend beads and incubate with rotation for 120min at 4^{o}C.
3. Wash
   Separate magnetically and remove supernatant. Wash beads with Wash buffer B 3times at 4^{o}C. Separate magnetically and remove supernatant.
4. Elution
   Add 40ul SDS sample buffer and resuspend beads. Boil for 5min and remove the beads.
5. Analyze the samples by SDS-PAGE and silver staining

- **Methods 3 (Western blotting)**

1. Perform SDS-PAGE and place the gel in Transfer buffer for about 10min.
2. Transfer the protein from the gel to a PVDF membrane.
3. Block the membrane with the Blocking buffer for 15min at room temperature.
4. Dilute the primary antibody with the Blocking buffer to 1/150.
5. Incubate for 60min at room temperature.
6. Wash the membrane with TBS-T buffer 3 times.
7. Dilute the secondary antibody with the TBS-T buffer to 1/2000.
8. Incubate for 60min at room temperature.
9. Wash the membrane with TBS-T buffer 5 times.
10. Detect with a chemiluminescence substrate.

- **FG beads information**

Product name	Protein G beads
Product number	TAS8848N1173
Storage temperature	2-8^{o}C
Storage buffer	10mM HEPES (pH7.9), 50mM KCl, 1mM EDTA, 10% glycerol
Size of beads	190nm ± 20nm
IgG binding capacity	> 100ug mouse IgG/mg of beads