Biotechnology and Bioengineering
DOI 10.1002/bit. 21208

The Si-tag for Immobilizing Proteins on a Silica Surface

Koji Taniguchi,[1] Kazutaka Nomura,[1] Yumehiro Hata,[1] Tomoki Nishimura,[1] Yasuo Asami,[1] and Akio Kuroda[1,2]*

[1]*Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan*

[2]*SORST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan*

*To whom correspondence should be addressed: Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan.

Tel: (+81)82-424-7758; Fax: (+81)82-424-7047

E-mail: akuroda@hiroshima-u.ac.jp

Running title: *Silica tag for protein immobilization*

Received June 22, 2006; Accepted September 5, 2006

Abstract: Targeting functional proteins to specific sites on a silicon device is essential for the development of new biosensors and supramolecular assemblies. Using intracellular lysates of several bacterial strains, we found that ribosomal protein L2 binds tightly to silicon particles, which have surfaces that are oxidized to silica. A fusion of *E. coli* L2 and green fluorescence protein adsorbed to the silica particles with a K_d of 0.7 nM at pH 7.5 and also adsorbed to glass slides. This fusion protein was retained on the glass slide even after washing for 24 h with a buffer containing 1 M NaCl. We mapped the silica-binding domains of *E. coli* L2 to amino acids 1-60 and 203-273. These two regions seemed to cooperatively mediate the strong silica-binding characteristics of L2. A fusion of L2 and firefly luciferase also adsorbed on the glass slide. This L2 silica-binding tag, which we call the "Si-tag", can be used for one-step targeting of functional proteins on silica surfaces.

Keywords: silicon device; protein targeting; silica; ribosomal protein L2; protein array; protein orientation; green fluorescent protein; luciferase

INTRODUCTION

The ability to target proteins to specific sites and anisotropically immobilize them on a silicon biodevice while preserving their function is necessary for the development of new biosensors such as nanowire sensor arrays (Patolsky et al., 2004; Zheng et al., 2005) and silicon-based optical sensors (Lin et al., 1997). One of the most exciting tools in proteomics is protein microarray technology, wherein a large number of proteins or peptides can be immobilized on a slide glass or a solid substance for high-throughput analysis of biochemical properties and biological activities (Hanash, 2003). Proteins have mainly been immobilized on silica surfaces by nonspecific adsorption (Bussow et al., 1998; Ge, 2000; Lueking et al., 1999; Martin et al., 1998; Mendoza et al., 1999) or covalent bond formation between readily available functional groups on protein molecules (e.g., -NH_2) and complimentary coupling groups on the solid surfaces (e.g., aldehyde or epoxide) (MacBeath and Schreiber, 2000; Zhu et al., 2000). The nonspecific nature of these approaches inevitably requires the use of purified proteins. In addition, proteins may be denatured when the interaction with the surface is too strong.

Several efforts have been made to immobilize proteins with a controlled orientation. Immobilized protein A or G has been used to target immunoglobulins to the silica surface of a protein chip (Kanno et al., 2000; Turkova, 1999). Also, a poly-His tag has been used to immobilize proteins on silica surfaces via

surface-chelated metal ions (Cha et al., 2005). These methods, however, can require the modification of the silica surface. Addition of a sequence of nine arginine residues (poly-Arg) to a protein has been used for directed adsorption of a fusion protein onto a glass slide or silica resin without loss of enzymatic activity (Fuchs and Raines, 2005). This method seems to be effective for targeting proteins to silica surfaces without chemical modification of the surface; however, poly-Arg tagged protein is slowly released from the silica surface, and a tag that binds more strongly than poly-Arg is needed for targeting of proteins to silica surfaces.

In this report, we tested the intracellular proteins from several bacterial strains as a silica-binding protein. We found that ribosomal protein L2 binds the most strongly to silica surfaces. We further demonstrated that an L2-protein fusion binds to silica surfaces 20- to 100-fold more strongly than poly-Arg-tagged proteins. The L2-protein fusion can be used for one-step targeting of proteins to a silica surface, which can be applied to protein array and biosensor technologies.

MATERIALS AND METHODS

Materials

Silicon particles were obtained from Junsei Chemical (Tokyo, Japan). Silica particles (diameter 0.8 μm) were obtained from Soekawa Chemicals (Tokyo, Japan). Plasmid pGFPuv was from Clontech (Palo Alto, CA, USA) and pET21-b was from Novagen

(Madison, WI, USA). B-PER lysing solution was purchased from PIERCE (Rockford, IL, USA). All other reagents were from Wako Chemical (Osaka, Japan) or Sigma Chemical (St. Louis, MO, USA) and of highest available quality.

Identification of Silica-binding Proteins

E. coli, P. aeruginosa, and *P. putida* were grown on 2xYT medium at 37°C for 18 h. Cells were collected by centrifugation and disrupted in the presence of 0.25 mg/ml of lysozyme by ultrasonication (Branson, CT, USA). The lysate was subjected to centrifugation at 100,000 x *g* for 15 min. The protein concentration of the cleared supernatant was adjusted to 1 mg/ml with a buffer containing 25 mM Tris-HCl (pH 7.5), 0.5% Tween 20, and 1 M NaCl. Silicon particles (10 mg) were mixed with 1 ml of the diluted supernatant and incubated with rotary mixing for 30 min at 4°C. The silicon particles were precipitated by centrifugation at 4400 x *g*, washed three times with 1 ml of the buffer, and suspended in a SDS-sample buffer. After boiling for 5 min, proteins were separated by 12.5% SDS-PAGE. To perform the mass spectrometric analysis, the protein bands were excised from Coomassie blue-stained gels. In-gel digest of the proteins was performed using sequencing-grade trypsin (Promega, Madison, WI, USA). For matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) analysis, the peptide extracts were directly eluted from C18 ZipTip microcolumns (Millipore, Bedford, MA, USA)

with 1.5 μl of saturated cyano-4-hydroxycinnamic acid directly onto the MALDI target and analyzed with a MALDI-TOF apparatus (Bruker, Bremen, Germany). The peptide fingerprints obtained by MALDI-TOF were used for protein searches using Mascot (Matrix science Ltd, London, UK).

Construction of GFP, L2-GFP, and Poly Arg-tagged GFP

A DNA fragment encoding GFP was amplified with primers G1 and G2 and with pGFPuv as a template and then inserted into the *Hin*dIII and *Not*I sites of pET21-b. The resulting plasmid was designated pETGFP. A DNA fragment encoding ribosomal protein L2 was amplified with primers R1 and R2 and with *E. coli* chromosomal DNA as a template and then inserted into the *Eco*RI and *Sac*I sites of pETGFP. The resulting plasmid was designated pETL2GFP. For the addition of poly-Arg to the N-terminus of GFP, synthetic oligonucleotides G3 and G4 were annealed and inserted into *Nhe*I and *Eco*RI sites of pETGFP, and the resulting plasmid designated pETR9GFP. DNA sequences of the primers are shown in Table I.

Construction of Deletion Mutants of L2-GFP

For the construction of L2(1-60)-GFP, L2(61-202)-GFP, and L2(203-273)-GFP, DNA fragments encoding deletion mutants of ribosomal protein L2 were amplified with

primer sets R1/R3, R4/R5, and R2/R6, respectively, and then inserted into the *Eco*RI and *Sac*I sites of pETGFP. The resulting plasmids were designated pETL2(1-60)GFP, pETL2(61-202)GFP, and pETL2(203-273)GFP, respectively. For the construction of L2 (1-60, 203-273)-GFP, an inversed PCR was performed with primers R7 and R8 and with pETL2GFP as a template, and the amplified DNA was ligated to obtain pETL2(1-60, 203-273)GFP.

Construction of L2-Luc Plasmids

A DNA fragment encoding luciferase was amplified with primers L1 and L2 and with luciferase T7 DNA (Promega) as a template. The amplified fragment was replaced with the *Sal*I/*Not*I fragment of pETL2GFP, and the resulting plasmid designated pETL2Luc.

Expression and Purification of the Recombinant Proteins

The plasmids described above were introduced into *E. coli* Rosetta™ (DE3) pLysS (Novagen). GFP, L2-GFP, and R9-GFP proteins with a C-terminal His tag were expressed in 2xYT medium for 6 h in the presence of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and disrupted with lysozyme and ultrasonication. Polyhistidine-tagged proteins were purified by chromatography on a HiTrap Chelating column (Amersham

Biosciences, Piscataway, NJ, USA). The fractions containing the recombinant proteins were obtained by elution with a buffer consisting of 20 mM phosphate buffer (pH 7.4), 0.5 M imidazole, and 15% glycerol. The fractions containing fusion proteins were then applied to an HS cation exchange column (Perspective Biosystems, Cambridge, MA, USA) equilibrated with 20 mM HEPES-NaOH (pH 7.5) containing 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol. The fusion proteins were eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. L2-Luc was also purified using a HiTrap Chelating column chromatography and elution with 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl, 0.5 M imidazole, and 15% glycerol. The purity of the recombinant proteins was estimated by SDS-PAGE to be greater than 95%.

Kinetic Analysis of L2-GFP and R9-GFP to a Silica Particle

Proteins (GFP, L2-GFP, or R9-GFP) were diluted to the indicated concentrations in 1 ml of a buffer containing 0.5% Tween-20, 0.5 M NaCl, and 25 mM of phosphate (pH 6.0), Tris-HCl (pH 7.0, 7.5, or 8.0), or glycine-NaOH (pH 9.0). The diluted proteins were then mixed with 0.1 mg of silica particles. After a 15-min incubation, the silica particles were precipitated by centrifugation and then washed with the same buffer. The amount of GFP that bound to the silica particle was determined by measuring the reduction in GFP fluorescence in solution. The dissociation constant (K_d) and

maximum binding of L2-GFP and R9-GFP to silica particles were determined by Scatchard analysis.

Absorption of Protein on a Glass Slide

An aliquot of protein solution was spotted onto a slide glass (Matsunami, Tokyo, Japan). The slide glass was shaken in a solution of 25 mM Tris-HCl (pH 8.0), 0.5% Tween 20, and 1 M NaCl at room temperature. At the indicated times, the slide was removed from the buffer, and GFP fluorescence remaining on the slide was visualized with an image analyzer (Typhoon; Amersham Biosciences). Luciferase and L2-Luc were also adsorbed on the slide glass and washed as described above. To detect luciferase activity, a substrate solution (3 μl) containing 2 mM ATP, 2 mM luciferine, 120 mM Tris-HCl (pH 7.4), and 16 mM $MgCl_2$ was added, and the luminescence was monitored for 0.5 s with high-sensitivity CCD camera (Spectral Instruments, Tucson, AZ).

One-step Targeting of Protein on the Glass Slide

E. coli recombinant culture (0.2 ml) expressing L2-GFP (Si-tagged GFP) was mixed with equal volume of B-PER lysing solution. After 10 min, 13 μl of 1 M Tris-HCl (pH 7.5) and 14 μl of 5 M NaCl was added to the mixture. An aliquot of the mixture was spotted onto a glass slide using a Stampman (Nippon Laser and Electronics Lab,

Nagoya, Japan). After the glass slide was washed with wash buffer (25 mM Tris-HCl [pH 8.0], 0.5 % Tween 20, and 0.5 M NaCl), the fluorescence was analyzed with the Typhoon image analyzer (Amersham Bioscience). Proteins retained on the glass slide were removed by the SDS-sample buffer and analyzed by SDS-PAGE.

RESULT AND DISCUSSION

Silica-binding Proteins in *Escherichia coli, Pseudomonas aeruginosa*, and *Pseudomonas putida*

Cleared supernatants from *E. coli, P. aeruginosa*, and *P. putida* intracellular lysates were mixed with silicon particles and incubated with rotary mixing for 30 min at 4°C. The silicon particles were precipitated by centrifugation and wahsed three times with a buffer containing 0.5% Tween 20 and 1 M NaCl. Proteins retained on the silicon particles were analyzed by SDS-PAGE (Fig. 1). Of the *E. coli* intracellular proteins, ribosomal protein L2 appeared to bind strongly to the silicon particle. Also, OprF and L2 from *P. aeruginosa* and S9 and L2 from *P. putida* proteins appeared to bind to the silicon particle. These proteins also bound to silica particles (data not shown). Because the silicon surface is oxidized to silica under the assay conditions, we concluded that these proteins bind to silica.

Characterization of a L2-Green Fluorescent Protein (GFP) Fusion Protein

Because GFP does not bind to silica particles in the presence of 0.5% Tween 20 and

0.5 M NaCl (data not shown), we generated a fusion of *E. coli* L2 and GFP (L2-GFP) to perform kinetic analyses of the interaction between L2 and a silica surface. We found that L2-GFP bound to the silica particles at pH values above 7.0 (Fig. 2). The dissociation constants (K_d's) of L2-GFP to the silica particle at pH 7.5, 8.0, and 9.0 were 0.7, 0.55, and 0.46 nM, respectively (Fig. 3). The maximum amounts of L2-GFP bound to the silica particle at pH 7.5, 8.0, and 9.0 were 25, 32, and 31 μg L2-GFP protein/mg silica particle, respectively (Fig. 3).

Addition of a poly-Arg tag has been used for direct adsorption of protein on glass slides or silica particles without loss of enzymatic activity (Fuchs and Raines, 2005). Poly-Arg tagged GFP (R9-GFP) bound to silicon particles with K_d's at pH 7.5, 8.0, and 9.0 of 123, 18, and 25 nM, respectively (data not shown). The maximum amounts of R9-GFP bound to silica particles at pH 7.5, 8.0, and 9.0 were 5.9, 14, and 16 μg GFP protein/mg silica particles, respectively. These results indicated that L2-GFP binds to the silica particles 30- to 200-fold more strongly than R9-GFP. Furthermore, we found that L2-GFP but not GFP or R9-GFP is retained on the slide glass after washing with a buffer containing 1 M NaCl for 24 h (Fig. 4).

Silica-binding Domains of L2

We next identified the silica-binding sites of L2 by constructing deletion mutants of L2 fused to GFP. We found that L2 (1-60)-GFP and L2 (203-273)-GFP bound to the

silica particles but that L2 (61-202)-GFP did not, suggesting that the silica-binding sites are localized on L2 between residues 1–60 and 203–273 (Fig. 5). The K_d values for L2(1-60)-GFP and L2(203-273)-GFP were 19- and 26-fold higher than that of L2-GFP, respectively, and that of L2(1-60, 203-273)-GFP was 2-fold higher than that of L2-GFP. These results suggested that these two silica-binding domains cooperatively mediate the strong silica-binding characteristics of the intact L2 protein. The maximum amount of bound protein was almost the same for L2(1-60, 203-273)-GFP as for L2-GFP.

Ribosomal protein L2 is highly conserved (Watanabe and Kimura, 1985). Tryptic digestion of the *Bacillus stearothermophilus* L2 and 23S rRNA complex produces a protected fragment (60–206) that contains the RNA-binding domain (Watanabe and Kimura, 1985). Here, we found that the N- and C-terminal regions but not the RNA-binding region are involved in silica binding. We showed amino acids residues that are responsible for the adhesion to silica (Fig. 5). The two silica-binding domains contain no homologous sequence, but many positively charged amino acids residues. The acid-base ionization constants (pKa's) for *E. coli*, *P. aeruginosa,* and *P. putida* L2 proteins were 10.9, 11.2, and 11.1, respectively. This is not surprising because the N- and C-terminal regions of L2 contain many positively charged amino acids residues. The RNA-binding domain without the N- and C-terminal regions has been crystallized, and its structure has been solved, but it

was not possible to crystallize intact L2, suggesting that the N- and C-terminal regions do not form an organized structure (Watanabe and Kimura, 1985). We believe that the positively charged and non-organized structure may be important for the strong silica binding characteristics of L2. *P. putida* S2 protein also shows a high pKa constant of 11.2; however, the pKa for OprF of *P. putida* is 5.0. The reason for the strong silica-binding characteristic of OprF was unclear.

Characterization of L2-Luciferase

We next constructed a fusion of L2 and luciferase (L2-luciferase). Aliquots of purified luciferase (luciferase and L2-luciferase) were spotted. The luminescence intensity generated from the proteins was almost same, but after the slide glass was washed in buffer, only L2-luciferase was retained on the slide glass (Fig. 6). This indicated that the L2 fusion, which we tentatively designate the "Si-tag", can be applied to the targeting of functional enzymes on a silica surface.

Purified enzyme activities of intact luciferase and L2-luciferase were assayed in solution. We found that L2 reduced luciferase activity by 60-70% (Fig. 7). We also compared fluorescence activity of purified GFP and L2-GFP. In this case, L2 reduced fluorescence activity of GFP by 30% (data not shown). Therefore, fusion by L2 (Si-tag) affects enzyme activity, but still has evident superiority to the poly-Arg tag for the immobilization on a silica surface.

One-step Targeting of L2-GFP Fusion Protein on a Slide Glass

"Protein chips" have been created using glass slides, precision robots, and other off-the-shelf equipment. This technology enables the rapid screening of thousands of small-molecule drug candidates to determine their potential effects on specific proteins. We predicted that the Si-tag would be a powerful method to produce protein chips. We cultivated *E. coli* producing L2-GFP (Si-tagged GFP), lysed them with a B-PER lysing solution and Tris-buffered saline, and directly spotted the lysate on the glass slide (Fig. 8A). After the slide was washed, we examined the bound fluorescence (Fig. 8B). Si-tagged GFP appeared to represent the majority of the protein bound to the glass slide (Fig. 8C). These results show that a Si-tagged protein produced in *E. coli* can be targeted onto a silica surface without purification.

The Si-tag, here we tentatively designated, can be applied to immobilization of proteins on silica surfaces, which is important for the development of protein microarray as well as silicon-based biosensors (Lin et al., 1997; Patolsky et al., 2004; Zheng et al., 2005).

References

Bussow K, Cahill D, Nietfeld W, Bancroft D, Scherzinger E, Lehrach H, Walter G. 1998. A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. Nucleic Acids Res 26:5007-5008.

Cha T, Guo A, Zhu XY. 2005. Enzymatic activity on a chip: the critical role of protein orientation. Proteomics 5:416-419.

Fuchs SM, Raines RT. 2005. Polyarginine as a multifunctional fusion tag. Protein Sci 14:1538-1544.

Ge H. 2000. UPA, a universal protein array system for quantitative detection of protein-protein, protein-DNA, protein-RNA and protein-ligand interactions. Nucleic Acids Res 28:e3.

Hanash, S. (Ed.). 2003. Proteomics 3: issue11 & all pape.

Kanno S, Yanagida Y, Haruyama T, Kobatake E, Aizawa M. 2000. Assembling of engineered IgG-binding protein on gold surface for highly oriented antibody immobilization. J Biotechnol 76:207-214.

Lin VS, Motesharei K, Dancil KP, Sailor MJ, Ghadiri MR. 1997. A porous silicon-based optical interferometric biosensor. Science 278:840-843.

Lueking A, Horn M, Eickhoff H, Bussow K, Lehrach H, Walter G. 1999. Protein microarrays for gene expression and antibody screening. Anal Biochem 270:103-111.

MacBeath G, Schreiber SL. 2000. Printing proteins as microarrays for high-throughput function determination. Science 289:1760-1763.

Martin BD, Gaber BP, Patterson CH, Turner DC. 1998. Direct Protein Microarray Fabrication Using a Hydrogel "Stamper". Langmuir 14: 3971-3975.

Mendoza LG, McQuary P, Mongan A, Gangadharan R, Brignac S, Eggers M. 1999. High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA). Biotechniques 27:778-80, 782-6, 788.

Patolsky F, Zheng G, Hayden O, Lakadamyali M, Zhuang X, Lieber CM. 2004. Electrical detection of single viruses. Proc Natl Acad Sci U S A 101:14017-14022.

Turkova J. 1999. Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. J Chromatogr B Biomed Sci Appl 722:11-31.

Watanabe K, Kimura M. 1985. Location of the binding region for 23S ribosomal RNA on ribosomal protein L2 from *Bacillus sterothermophilus*. Eur J Biochem 153:299-304.

Zheng G, Patolsky F, Cui Y, Wang WU, Lieber CM. 2005. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. Nat Biotechnol 23:1294-1301.

Zhu H, Klemic JF, Chang S, Bertone P, Casamayor A, Klemic KG, Smith D, Gerstein M, Reed MA, Snyder M. 2000. Analysis of yeast protein kinases using protein chips.

Nat Genet 26:283-289.

Figure legends

Fig. 1. Silica-binding proteins in (A) *E. coli*, (B) *P. aeruginosa*, and (C) *P. putida*. Lane 1, cleared supernatant prepared from cell lysates. Lane 2, proteins that bound to silicon particles in the presence of 0.5% Tween and 1 M NaCl.

Fig. 2. Effects of pH on the binding of L2-GFP to silica particles. 1 ml of 3 μg/ml L2-GFP was mixed with a 0.1 mg of silica particles (diameter 0.8 μm) at various pH's. After incubation for 15 min, the silica particles were precipitated by centrifugation and washed. The amount of L2-GFP bound to the silica particle was plotted as a function of the pH.

Fig. 3. Scatchard analysis of L2-GFP binding to silica particles. The binding assay was performed as described in Fig. 2 at pH 7.5, 8.0, and 9.0.

Fig. 4. Immobilization of GFP, L2-GFP, and R9-GFP on a slide glass. Protein solution (GFP, L2-GFP, or R9-GFP) at a concentration of 10 nM was used to draw the letters "GFP" on a glass slide. The slide was dipped in a solution of 25 mM Tris-HCl (pH 8.0), 0.5% Tween 20, and 1 M NaCl solution and shaken at room temperature. After 24 h, the slide was removed from the buffer, and GFP fluorescence remaining on the slide glass was visualized with an image analyzer.

Fig. 5. Silica-binding domains of L2 and their dissociation constants. The K_d values of GFP-L2 deletion mutant fusions for silica particles were measured. The amino acid residues of the silica-binding domains (1-60, 203-273) were listed.

Fig. 6. Immobilization of L2-luciferase on a glass slide.

(A) An aliquot (3 μl) of luciferase solution was spotted on a glass slide and mixed with substrate solution (3 μl) containing 2 mM ATP, 2 mM luciferine, 120 mM Tris-HCl (pH 7.4), and 16 mM $MgCl_2$. Luminescence was obtained for 0.5 s with a high-sensitivity CCD camera. **(B)** The slide was washed with 30 mM Tris-HCl (pH 8.0) containing 1 M NaCl and then overlaid with substrate solution. The luminescence was obtained for 0.5 s with a high-sensitivity CCD camera as described in (A).

Fig. 7. Bioluminescence activities of luciferase and L2-luciferase. Purified luciferase (100 pmol) and L2-luciferase (100 pmol) were mixed with indicated amounts of ATP in 100 μl of 25 mM Tris-HCl [pH 7.4], 5mM $MgCl_2$, and 0.5 mM luciferin. The bioluminescence was measured by using a multiplate luminometer (Wallac, Massachusetts, USA).

Fig. 8. One-step targeting of L2-GFP on silica surfaces. (A) Schematic representation of one-step targeting. A culture of *E. coli* harboring pETL2GFP (0.2 ml) was mixed with the same volume of B-PER lysing solution. After 10 min, 13 μl of 1 M Tris-HCl (pH 7.5) and 14 μl of 5 M NaCl were added to the mixture. An aliquot (0.2 μl) of the mixture was spotted on the glass slide. The slide glass was washed with 30 mM Tris-HCl (pH 7.5) containing 1 M NaCl and 0.5% Tween 20 and then examined with a fluorescence image analyzer. **(B)** Fluorescence image of the bound protein. GFP fluorescence remaining on the slide glass was visualized by with the image analyzer. **(C)** Purity of the targeted protein. The targeted proteins were removed with SDS-sample buffer, and the purity of the targeted protein was analyzed by SDS-PAGE.

Acknowledgement

This work was supported in part by integrated research on quantum information and life science in Hiroshima University and by the Special Coordination Funds from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

TABLE I *Sequences of primers*

Primer	DNA sequence
G1	AGAAAAGCTTAGTAAAGGAGAAGAACTTTTCACT
G2	TCATGCGGCCGCAAGCTCATCCATGCCATGTGTA
G3	CTAGCCGTCGCCGTCGTCGCCGTCGTCGTCGCAAG
G4	AATTCTTGCGACGACGACGGCGACGACGGCGACGG
R1	CATCGAATTCT ATGGCAGTTGTTAAATGTAAA
R2	AGTT GAGCTC GT TTTGCTACGGCGACGTACGA
R3	AGTTGAGCTCGTCTGCTTGTGGCCACCACCGA
R4	CATCGAATTCTGCTTACCGTATTGTTGACTTC
R5	AGTTGAGCTCGTCAGCATATGCTCAGCATTGC
R6	CATCGAATTCTCGCGTTCTGGGTAAAGCAGG
R7	GTTCTGGGTAAAGCAGGTGC
R8	CTGCTTGTGGCCACCACCGC
L1	CCGGGTCGACATGGAAGACGCCAAAAAC
L2	GTTGCGGCCGCCAATTTGGACTTTCCGCC

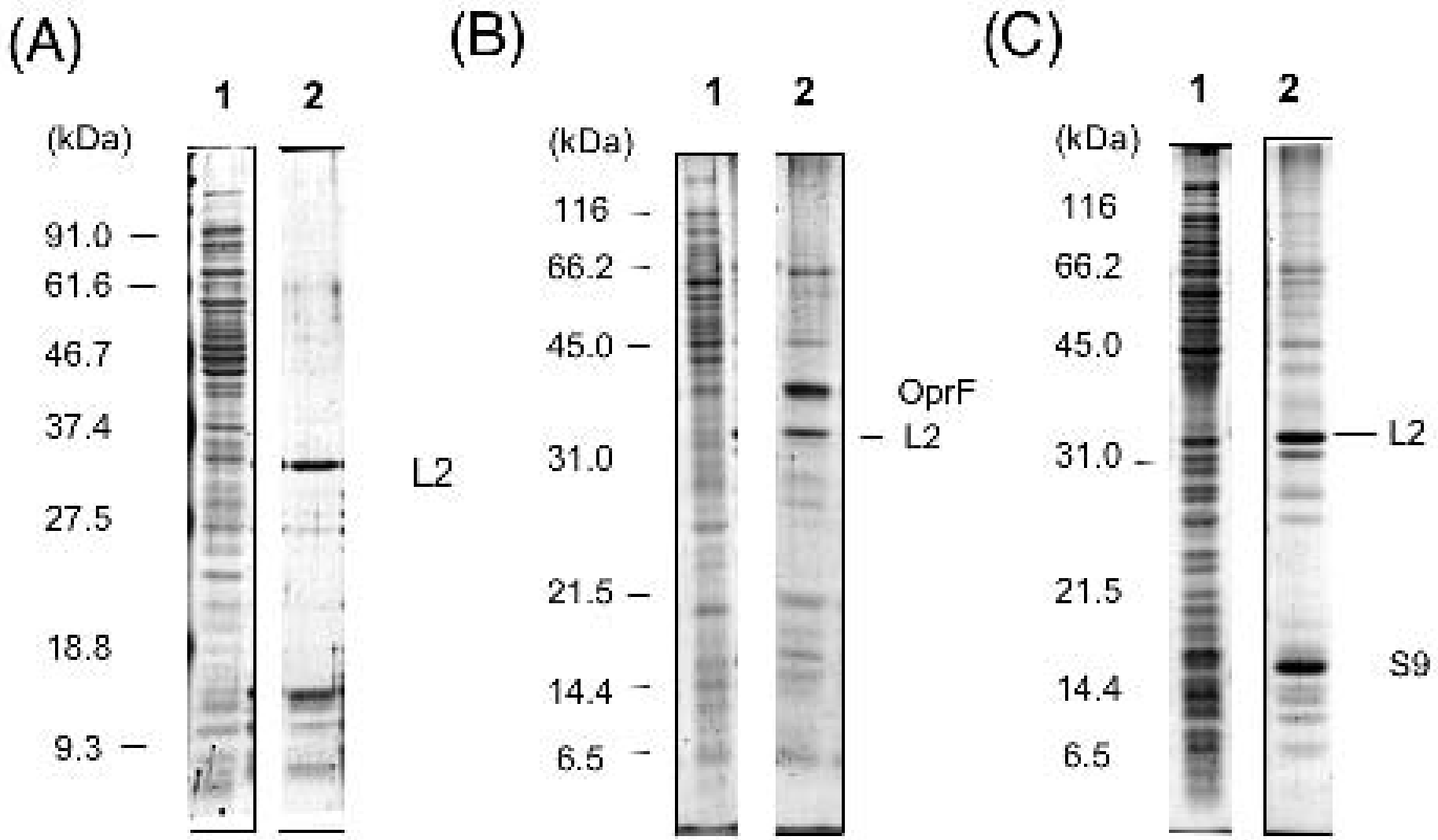


Figure 1 (Kuroda)

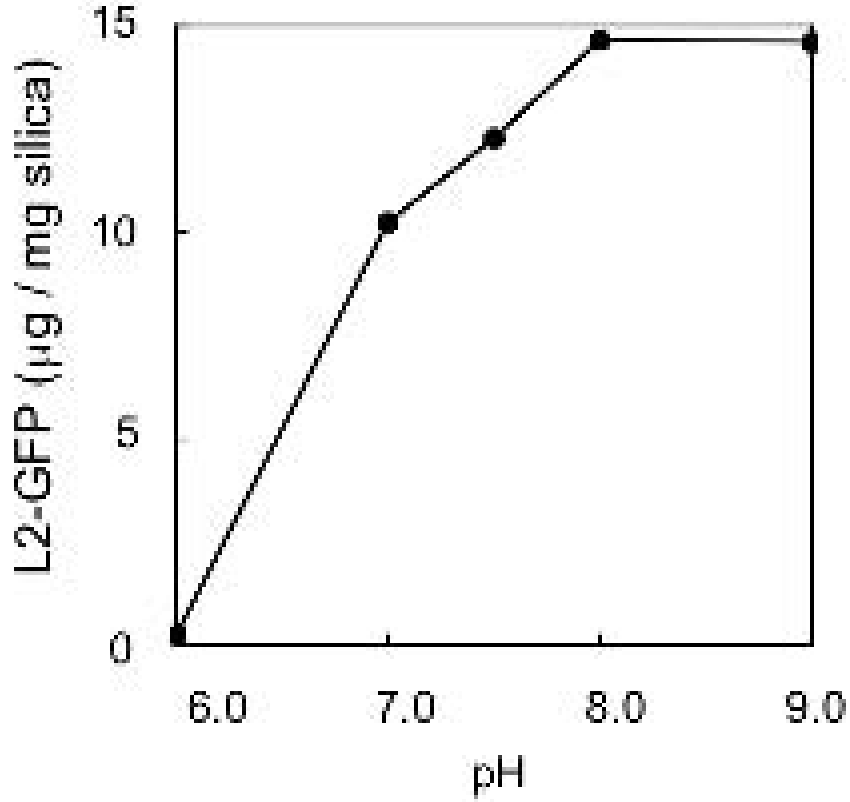


Figure 2 (Kuroda)

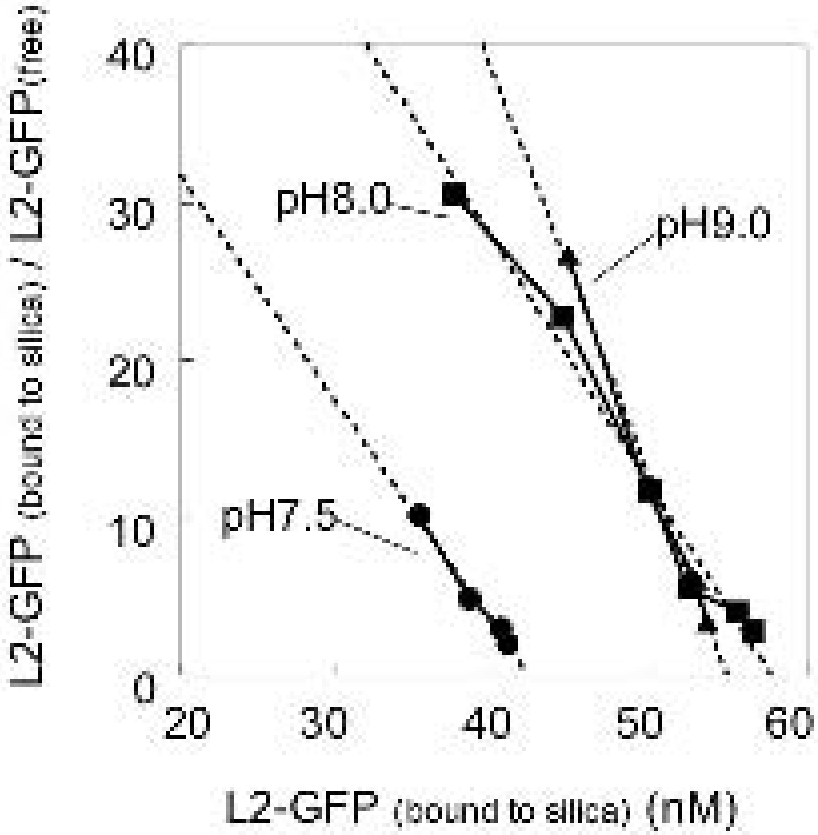


Figure 3 (Kuroda)

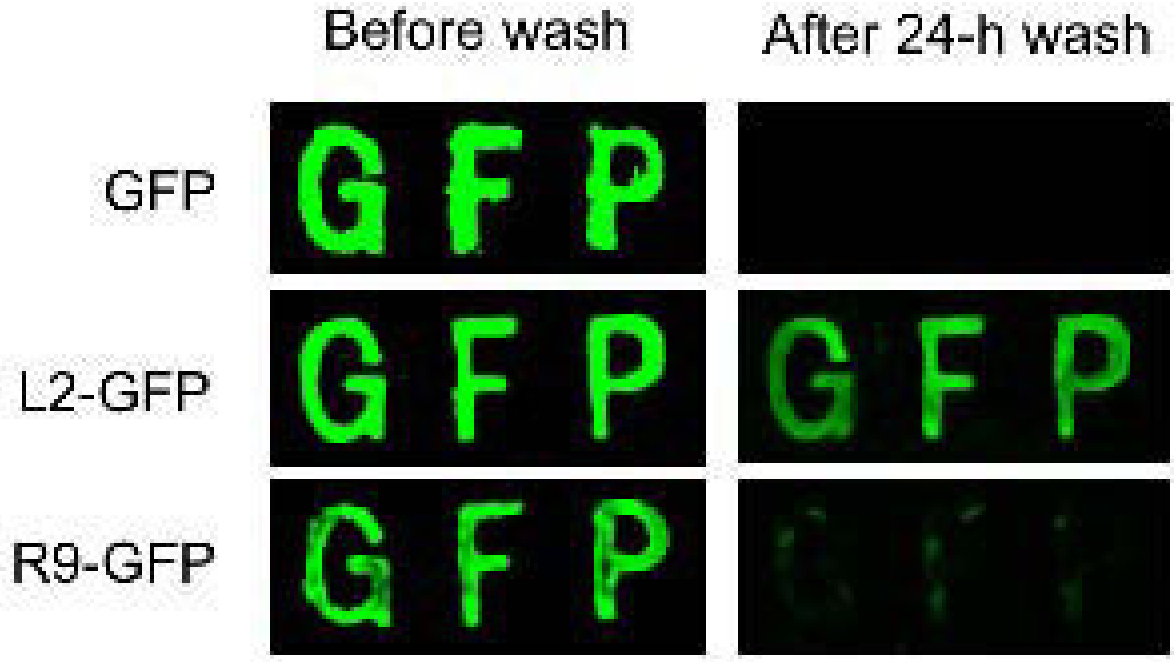
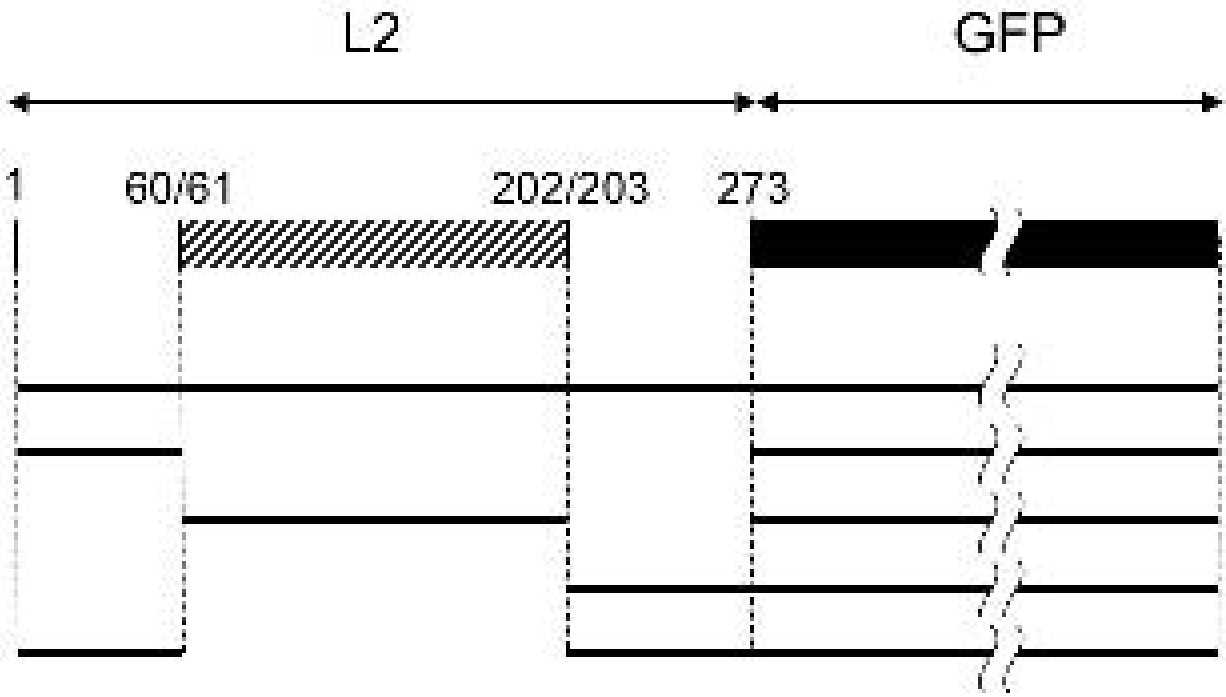


Figure 4 (Kuroda)



Position (aa)	K_d (nM)
1-273	0.7
1-60	13.5
61-202	-
203-273	18.4
1-60, 203-273	1.6

1-60: MAVVKCKPTSPGRRHVVKVVNPELHKGKPFAPLLEKNSKSGGRNNNGRITT RHIGGGHKQ

203-273: VLGKAGAARWRGVRPTVRGTAMNPVDHPHGGGEGRNFGKHPVTPWGVQ TKGKKTRSNKRTDKFIVRRRSK

Figure 5 (Kuroda)

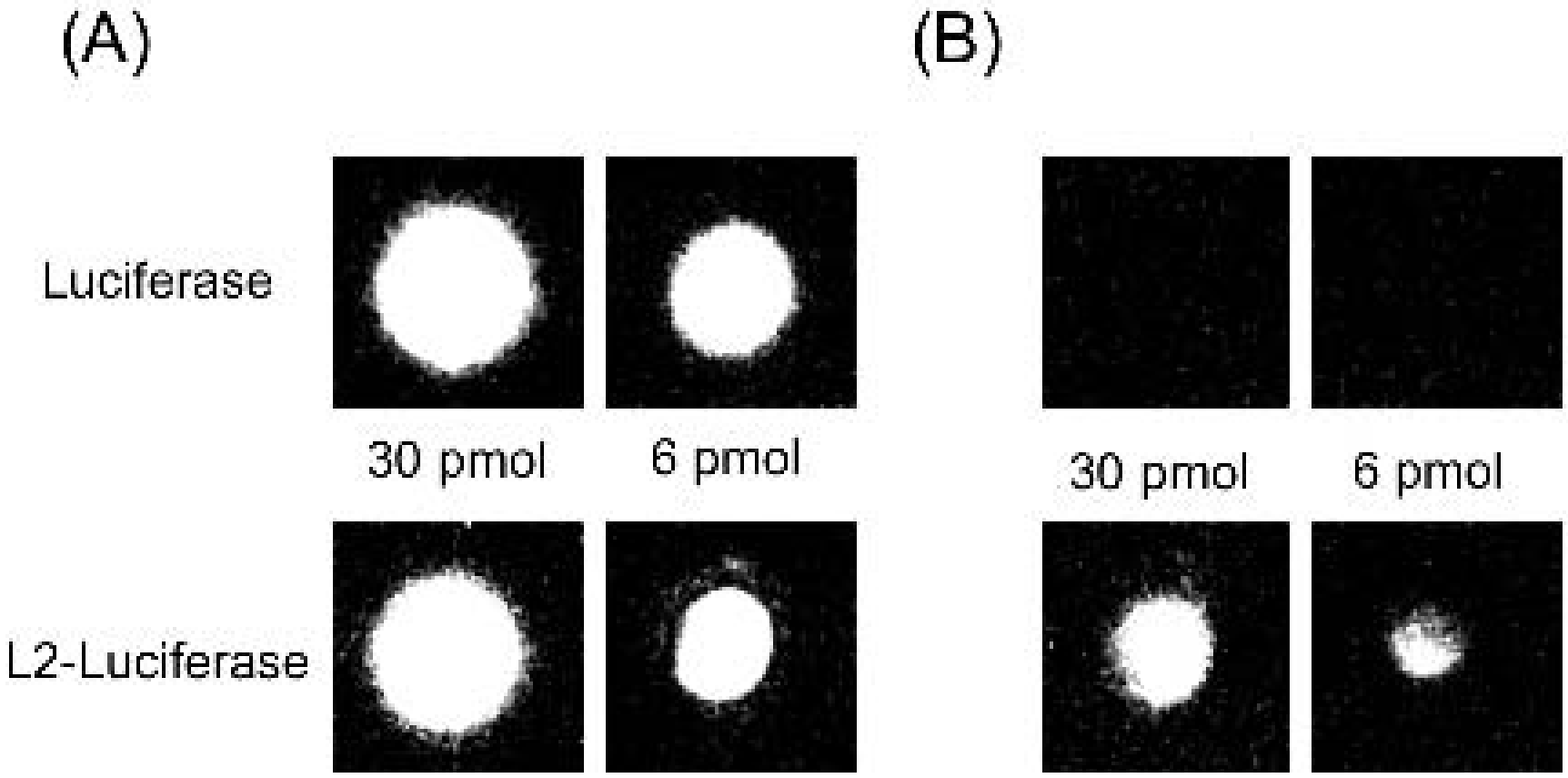


Figure 6 (Kuroda)

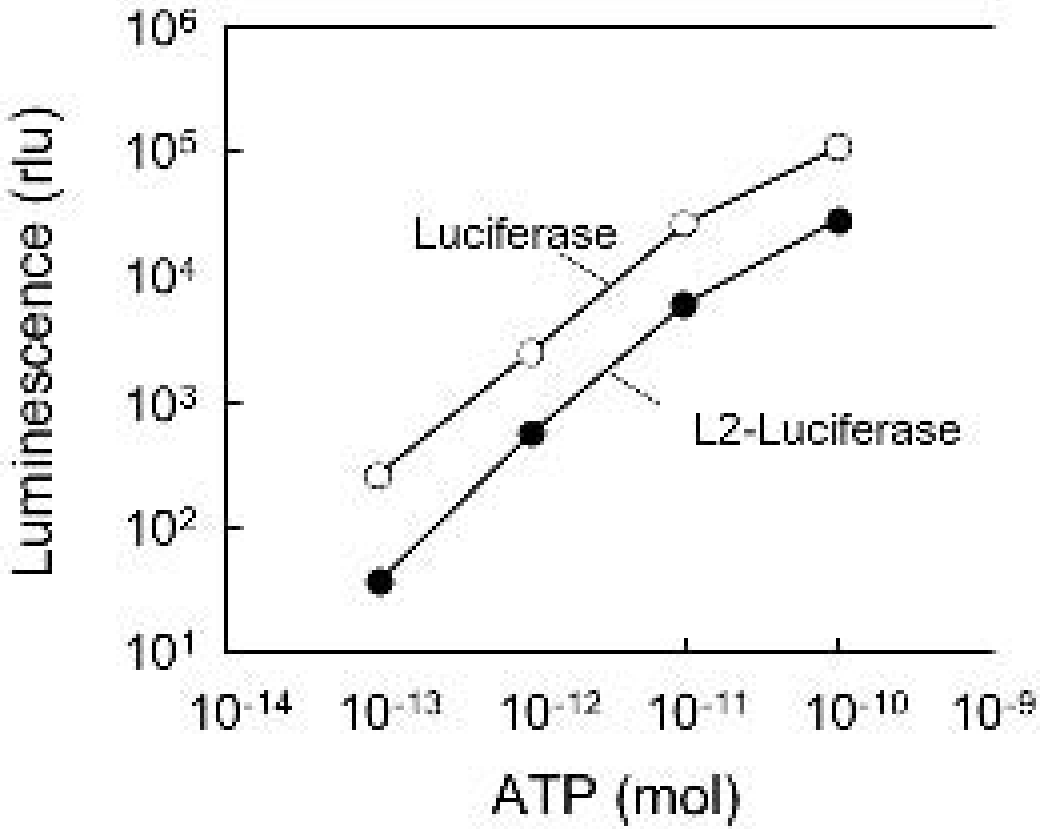


Figure 7 (Kuroda)

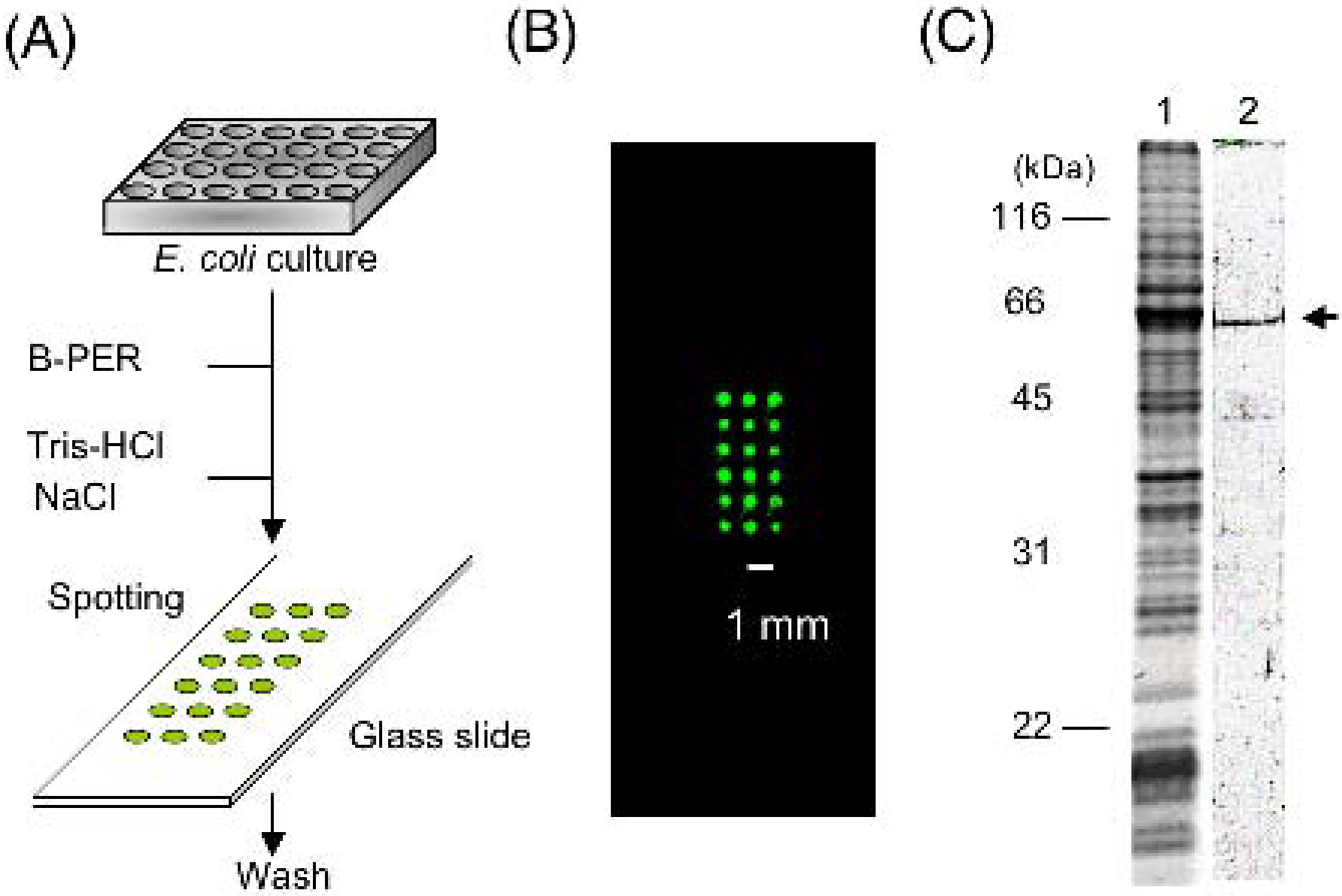


Figure 8 (Kuroda)