Antibodies and Engineered Antibody Fragments against M13 Filamentous Phage to Facilitate Phage-Display-Based Molecular Breeding

Yuki Kiguchi, Hiroyuki Oyama, Izumi Morita, Emiko Katayama, Masatoshi Fujita, Mai Narasaki, Aiko Yokoyama, and Norihiro Kobayashi*

Kobe Pharmaceutical University; 4–19–1 Motoyama-Kitamachi, Higashinada-ku, Kobe 658–8558, Japan.
Received February 27, 2018; accepted April 30, 2018

Antibodies are essential for characterizing various analytes. “Molecular-breeding” approaches enable rapid generation of antibody mutants with desirable antigen-binding abilities. Typically, prototype antibodies are converted to single-chain Fv fragments (scFvs), and random mutations are genetically introduced to construct molecular libraries with a vast diversity. Improved species therein are then isolated via phage display genotype–phenotype-connecting systems to separate them from a large excess of nonspecific scFvs. During these experiments, counting of phage particles is routinely performed. However, current methods depend on the time-consuming overnight cultivation of phage-infected bacteria on agar plates to estimate phage numbers as plaque-forming units (pfu) or colony-forming units, the results of which fluctuate considerably. Immunochemical systems capturing phage particles should be a more convenient and robust alternative. We therefore generated monoclonal antibodies against M13 filamentous phage, which is commonly used for phage display, by employing hybridoma technology. Combinatorial use of two such antibodies (Ab-M13#53 and #71; both specific to the major coat protein pVIII) enabled development of a sandwich enzyme-linked immunosorbent assay (ELISA) that could measure ca. 10^7–10^9 phage pfu/mL. To construct a more convenient system, Ab-M13#71 was converted to the scFv form and further fused with an alkaline phosphatase variant. Using this fusion protein, the sandwich ELISA enabled rapid (within 90 min) and reliable phage counting without reducing the sensitivity, and the results were reasonably consistent with those of infection-based methods. The present anti-phage antibodies and scFvs might also enable visualization of individual phage particles by combining them with sensitive fluorescent staining.

Key words phage display; phage counting; enzyme-linked immunosorbent assay (ELISA); monoclonal antibody; single-chain Fv fragment; fusion protein

Antibodies serve as analytical and diagnostic reagents for monitoring a wide variety of target molecules with excellent sensitivity. Currently, most reagent-grade antibodies with defined primary structures are produced using B-cell hybridoma technology.1–3) This approach supplies native antibodies induced in animals as cloned products with constant binding abilities. However, the limited B-cell clone repertoire in mammals often prevents generation of antibodies with the desired performance.4)

“Molecular-breeding” approaches, i.e., the generation of improved molecules based on genetic engineering, can potentially provide artificial antibodies with higher antigen-binding abilities than native antibodies.4–34) Therein, suitable prototype antibodies are converted to smaller fragments with maintained antigen-binding ability, i.e., single-chain Fv fragments (scFvs)5–11) or Fab fragments, and random or site-directed mutations are genetically introduced to generate libraries of mutants with a vast diversity. Although improved species appear only rarely in the libraries, the genotype–phenotype-connecting systems facilitate their isolation separating them from a large excess of impaired or poorly improved species. Phage display is used more widely than other related systems12–16) (e.g., ribosome-display and cell-surface-display systems),6) because of easier handling and the availability of necessary materials. Here, mutated antibody fragments were expressed on phage particles as fused molecules linked to the exterior coat protein. The Ff class of the filamentous bacteriophages, known as f1, M13, and fd phages (98.5% identical in their DNA sequences)12) that infect F plasmid-containing Gram-negative bacteria, such as Escherichia coli (E. coli) cells, are usually employed for this purpose.13–16) Mostly, the minor coat protein pIII, 3–5 copies of which are incorporated per virion,16) is fused with the antibody fragments4–8,11,14) (Fig. 1A). Phages displaying antibody fragments (Ab-phages; including scFv-phages and Fab-phages) with desirable characteristics are enriched via several rounds of “panning,” i.e., affinity-extraction techniques using immobilized or biotin-labeled antigens.4–8) Using this strategy, we previously succeeded in generating improved scFv mutants specific to cortisol (CS),15) cotinine (CT),16) estradiol-17β (E2),17,18) and Δ9-tetrahydrocannabinol (THC),19) which showed >30-fold, >40-fold, >150-fold, and ca. 10-fold greater equilibrium affinity constants (Kd), respectively, than the corresponding prototype (“wild-type”) scFv.

Routine counting of phage particles is necessary during phage-display experiments. For example, phage numbers must be properly adjusted for effective panning and evaluation of antigen-binding abilities. To compare antigen-binding activities for different scFv-phages, phage numbers should be normalized with a fixed standard, even though it is difficult to estimate the proportion of phage particles that actually display scFv residues. At present, counting is performed mostly by titrating the infectivity of phages against host bacteria.14,22) Typically, phages to be counted are mixed with bacteria and incubated for infection, and then the infected bacteria are
spread on agar plates to visualize plaques or colonies after overnight cultivation. The number of infectious phages is estimated in terms of plaque-forming units (pfu) or colony-forming units (cfu). These infection-based methods, however, require overnight cultivation before the results can be obtained: this limitation makes us wait until tomorrow for starting next panning round. Furthermore, outcomes tend to fluctuate and show unsatisfactory linearity with dilution series. Considerable amounts of bacteria-contaminated agar waste also compel us careful sterilization and disposal.

A physicochemical counting based on UV absorption of phage proteins and nucleic acids was proposed as a much quicker method that did not require overnight cultivation. However, thorough purification of phages is essential to avoid interference from proteins contained in the culture media. Precipitation with the aid of polyethylene glycol (PEG) is the standard method, but it was pointed out that cesium chloride density-gradient ultracentrifugation (requires >24h) was necessary for reliable determinations. A unique principle was reported several years ago, in which single-stranded phage DNA was quantified based on complementation-probe technology. Although this method enabled a simple mix-and-measure determination of M13 phage, uncommon time-resolved fluorescence measurements were required.

Immunochemical systems capturing phage particles should provide a more convenient, universal, and robust alternative. Because the capsid of filamentous phages is composed mainly of ca. 2700 copies of the major coat protein pVIII (Fig. 1A), it should be relatively easy to develop sensitive sandwich immunoassays. A few reports have demonstrated the production of antibodies recognizing filamentous phages, some of which are marketed. Immunoassay kits are also commercially available for this purpose. However, these assays have not been recognized commonly and thus have not been widespread, because no scientific report describing these methods has been published.

Therefore, it should be worth investigating and reporting publicly the utilities and advantages of the immunochemical approaches to the phage counting. We here established hybridoma clones that secrete antibodies against M13 phage, which is commonly used for displaying antibodies, to obtain genetic sources of anti-phage antibodies necessary for producing various engineered derivatives. To produce a more convenient assay system, one antibody was converted to the scFv form (M13-scFv), and fused with a variant of alkaline phosphatase (ALP) (Fig. 1B). An advanced sandwich enzyme-linked immunosorbent assay (ELISA) system, developed by using this fusion protein (M13-scFv-ALP) as a reporter molecule, enabled rapid and reliable counting of scFv-displaying M13 phage particles. Furthermore, we first demonstrated the data showing that the immunochemical counting easily provided more reliable phage numbers than the conventional infection-based methods and the UV-absorption methods.

![Fig. 1. Schematic Representation of Design and Production of Anti-phage Antibodies](image)

(A) Construction of M13 phage with the primary structure of pVIII shown. (B) Generation of antibodies and engineered antibody fragments against M13 phage.
MATERIALS AND METHODS

Buffers PB: 0.050 M sodium phosphate buffer (pH 7.3); PBS: PB containing 9.0 g/L NaCl; G-PBS: PBS containing 1.0 g/L gelatin; M-PBS: PBS containing 20 g/L skim milk; T-PBS: PBS containing 0.050% (v/v) Tween 20; PVG-PBS: G-PBS containing 1.0 g/L polyvinyl alcohol (PVA) with an average polymerization degree of 500; TBS: 0.025 M Tris–HCl buffer (pH 7.3) containing 9.0 g/L NaCl; G-TBS: TBS containing 1.0 g/L gelatin; T-TBS: TBS containing 0.050% (v/v) Tween 20; and CB: 0.10 M sodium carbonate buffer (pH 8.6).

Phages and Bacteria VCSM13 interference resistant helper phage and E. coli XL1-Blue competent cells were purchased from Agilent Technologies (Hachioji, Tokyo, Japan). The VCSM13 phage was propagated according to the appended direction, directed by PEG-precipitation, and used as an immunogen and for standard and coated antigens in sandwich ELISAs. Phages displaying wild-type scFvs targeting CT, THC, or CS were packaged in our laboratory as described previously.

PCR Primers PCR primers were supplied from Tsukuba Oligo Service (Ushiku, Ibaraki, Japan). The nucleotide sequences of the primers used in this study are shown in Table 1.

Production, Purification, Isotyping, and Biotin-Labeling of Monoclonal Antibodies against M13 Phage The animal experiments described below were performed according to the guidelines established in Kobe Pharmaceutical University. Five female 8-week-old BALB/c mice were immunized 4 times with VCSM13 phage at biweekly intervals: mice were injected subcutaneously with the phage (1×1010 pfu) as suspensions in a mixture of AbISCO-100 adjuvant (Isconova, Uppsala, Sweden) and saline (1: 4, ca. 200 µL). The mouse showing the highest serum titer for anti-phage antibodies received both intraperitoneal and intranasal injections of the phage (1×1010 pfu) suspended in saline. Three days later, spleen cells (ca. 1×106 cells) were prepared and fused with P3/NS1/Ag4-1 (NSI) myeloma cells (ca. 2×105 cells) using the PEG solution described before.

Hybridoma cells were selected in hypoxanthine-aminopterin-thymidine medium in 96-well cluster dishes, and anti-phage antibodies in the culture supernatants were screened by ELISA as described below. Selected hybridomas were expanded and cloned by limiting dilution and inoculated into pristane-treated male 8-week-old BALB/c mice. After ca. 2 weeks, ascites fluids were collected and the immunoglobulin G (IgG) fractions were prepared using HiTrap protein G HP columns (GE Healthcare Japan, Hino, Tokyo, Japan). A portion of the purified antibody was biotinylated using the EZ-Link NHS-LC-Biotin reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.): the biotinylation reagent and the antibody (molar ratio of 10:1) were mixed in CB at room temperature for 120 min, and then unreacted reagent was removed by dialysis against PBS. The antibody isotypes were determined using the Immunopure Monoclonal Antibody Isotyping Kit II (Thermo Fisher Scientific).

ELISAs to Screen for Hybridomas That Secrete Anti-phage Antibodies First, 96-well microplates (#3590; Corning, Corning, NY, U.S.A) were coated with VCSM13 phage (ca. 1×1010 pfu/well) and blocked with M-PBS at 37°C for 60 min. Supernatants of hybridoma cultures, diluted adequately with G-PBS, were added (100 µL/well) and incubated at 37°C for 60 min. After washing the wells with T-PBS, goat anti-mouse IgG antibody labeled with peroxidase (POD) (Jackson ImmunoResearch, West Grove, PA, U.S.A.; 16 ng/well) was added and incubated similarly. The wells were washed and the POD activities were measured using o-phenylenediamine (o-PD) as the hydrogen donor.

Cloning of Ab-M13#71 Variable Region Genes The Vh gene of Ab-M13#71 was cloned by rapid amplification of cDNA 5′-ends (5′RACE). Total RNA was extracted from the hybridoma cells (ca. 1×105 cells), and reverse-transcribed using Superscript II reverse transcriptase (Thermo Fisher Scientific) with an oligo-dT primer. The cDNAs obtained were used in 5′RACE with subclass-specific forward primers (my2b-GSP1, 2, and 3). The Vl gene fragment was amplified using the universal reverse primer Vl-1/III (38) and the κ-chain specific forward primer mκ-GSP. The product was then amplified using the Vl-amp-Rev and mκ-GSP primers to add a restriction site. The Vh and Vl gene fragments obtained were subcloned into the XmaI and SalI sites of pBluescript II (Toyobo, Kita-ku, Osaka, Japan) and the nucleotide sequences of the resulting subclones were determined by a standard method.

Assembly and Expression of the M13-scFv Gene The Vh and Vl genes of Ab-M13#71 were amplified separately to add restriction sites, a linker sequence to combine the Vh and Vl domains, and a FLAG tag for detection and purification purposes. These reactions were performed in a 100-µL mixture containing the recombinant plasmid prepared above (50 ng), reverse and forward primers (see below; Table 1. Nucleotide Sequences of PCR Primers Used in This Study

| Primer          | Sequence (5′−3′; Restriction site)* |
|-----------------|-----------------------------------|
| my2b-GSP1       | GCTGGCCCGGTTGCGGCAAC               |
| my2b-GSP2       | ACACTGTCGGAGCAGGGAT                |
| my2b-GSP3       | GGAATCCCGGGAGTACCCCTGACCAGGC      (Xmal) |
| mκ-GSP         | GGATCCCGGTTGAGGTGGAAGATG          (Xmal) |
| Vl-1/III        | GACATTGTTGACGYCARTCT               |
| Vl-amp-Rev      | ACTAGTCGACAGCATTTGATGCYACARTCT (SalI) |
| M13#71Vh-Rev    | ATGTGTTATTCTCAGGGCCCCAAACCCGGGCTGAGGGAGGTGGTCAGTTGCGACAGTCG    (NcoI) |
| M13#71Vh-For    | CGGGCGCATACCCCTCGGCAACCCGGCCATGGGAGGTGGTGGAGGGGCTGAGCTGGTC |
| M13#71Vl-Rev    | CAGGCGGAGGTTGATACCGGCGGTTGCGGACATTGTTGATGACTCAGTCTCA |
| M13#71Vl-For    | GATTTGGGTCCTACATTTGTTGACCTATTCATCAGTCTTTATATTATATCATCATCATCATTTATATACT (SalI) |
| M13#71Vl-For-2  | GATTTGGGTCCTACATTTGTTGACCTATTCATCAGTCTTTATATTATATCATCATCATCATTTATATACT (SalI) |

a) Underlined in the nucleotide sequences.
50 pmol each), and Ex Taq DNA polymerase (TaKaRa Bio, Kusatsu, Shiga, Japan) (2.5 U). The \( V_h \) fragment was amplified with the M13\#71V\( _{h-}\)Rev and M13\#71V\( _{h-}\)For primers, and the \( V_l \) fragment was amplified with the M13\#71V\( _{l-}\)Rev and M13\#71V\( _{l-}\)For primers. The cycling profile was as follows: 35 cycles of 95°C (1 min), 64°C (1 min), and 72°C (2 min) followed by a single incubation at 72°C (10 min). The amplified products were then spliced by overlap extension PCR based on previous procedures. The resulting product was then amplified with the M13\#71V\( _{h-}\)Rev and M13\#71V\( _{l-}\)For-2 primers to add tandem TAA stop codons, and subcloned into the pEXmide 5 expression vector. The resulting plasmid, containing the entire fragment of this recombinant plasmid, which includes the scFv (Sigma-Aldrich, St. Louis, MO, U.S.A.), was transformed into XL1-Blue cells and a cloned transformant was grown for protein expression. The soluble M13-scFv gene (5\( _{uni2032} \)I\( _{uni2032} \)Spe\( _{uni2032} \)I DNA fragment for that was digested from the recombinant plasmid prepared above. The resulting plasmid, containing the M13-scFv-ALP\( \) gene (5\( _{uni2032} \)I\( _{uni2032} \)Spe\( _{uni2032} \)I\( _{uni2032} \)Sfi\( _{uni2032} \)I), was transformed into E. coli XL1-Blue cells. A cloned transformant was grown and protein expression was induced. The fusion protein in the periplasmic extracts was used without purification for ELISAs, whereas the fusion protein was affinity-purified for analysis by gel electrophoresis.

**Sandwich ELISA for Counting Phage Particles**

(a) **Assay using IgG-form Ab-M13\#71 as the reporter** (Fig. 2A). Initially, 96-well microplates (N3590, Corning) were coated overnight at 4°C with 5.0-μg/mL solution of affinity-purified Ab-M13\#53 in PBS (100 μL/well) and blocked with Block Ace (DS Pharma Biomedical, Suita, Osaka, Japan) at 37°C for 60 min. Wells were washed three times with T-PBS, and then suspensions containing various amounts of VCSM13 phage (as standards) or scFv-phages in PVG-PBS with 20% (v/v) ethanol were added (100 μL/well) and the plates were incubated at 37°C for 30 min. After washing the wells, a 60-ng/mL solution of biotin-labeled Ab-M13\#71 in PVG-PBS was added (100 μL/well) and incubated at 37°C for 30 min. The wells were washed and incubated with 1.0-μg/mL solution of POD-labeled streptavidin (Jackson ImmunoResearch) in PVG-PBS (100 μL/well) at 37°C for 30 min. The wells were washed and POD activity in the wells was determined with a colorimetric assay using o-PD. 17–21) (b) **Assay using M13-scFv as the reporter** (Fig. 2B). Ab-M13\#53-coated plates were incubated with phages as described above. After washing the wells three times with T-PBS, scFv-M13 diluted in PVG-PBS was added (100 μL/well) and incubated at 37°C for 30 min. The wells were washed similarly and incubated with a 0.20-μg/mL solution of POD-labeled streptavidin (Jackson ImmunoResearch) in PVG-PBS (100 μL/well) at 37°C for 30 min. The wells were washed and POD activity in the wells was determined with a colorimetric assay using o-PD. 17–21) (c) **Assay using M13-scFv-ALP’ as the reporter** (Fig. 2C). Ab-M13\#53-coated plates were incubated with phages as described above. After washing the wells three times with T-TBS, scFv-ALP’ diluted in G-TBS was added (100 μL/well) and the plates were incubated at 37°C for 30 min. The wells were washed, and ALP’ activity in the wells was determined by a colorimetric assay using p-nitrophenyl phosphate (p-NPP) as the chromogen.

In these assays, the concentrations of the reporter anti-
bodies were adjusted to produce bound enzyme activities around the upper asymptote of an absorbance of 2−4 after a 30-min enzyme reaction.

RESULTS AND DISCUSSION

Establishment of Hybridoma Clones Generating Antibodies against M13 Phage

Initially, we planned immunization with a recombinant pVIII, prepared in our laboratory, after conjugation with keyhole limpet hemocyanin. However, a commercially available anti-M13 antibody did not recognize the recombinant pVIII (data not shown), suggesting that epitopes on monomeric pVIII differ from those on pVIII assembled in the M13 phage capsid. Therefore, we immunized BALB/c mice, the most common spleen donor used for cell fusions, with whole VCSM13 phage particles (Fig. 1) mixed with AbISCO-100, a saponin-based adjuvant.33,34) VCSM13 is a derivative of M13 phage that has the same pVIII sequence as wild-type M13 phage41,42) (Fig. 1A), and is commonly used as a helper phage in phage-display systems12–16) using the phagemid vectors.43,44) Serum titers for the anti-phage antibodies were elevated in all mice, and a final immunization was given to the mouse that showed the highest titer. Splenocytes from this mouse were used for a cell fusion experiment. Among several hybridoma clones established by limiting dilution, clones #53 and #71 were found to produce anti-phage antibodies suitable for developing sandwich ELISA systems. It is noteworthy that immunization with the AbISCO-100 adjuvant provided more hybridomas secreting anti-phage antibodies than immunization using the more common Freund’s adjuvant (data not shown).

Characterization of Antibodies and scFvs against M13 Phage

The isotypes of monoclonal antibodies Ab-M13/#53 and Ab-M13/#71, secreted from hybridoma clones #53 and #71, were determined to be γ3, κ and γ2b, κ, respectively. Both antibodies were specific to the pVIII protein as shown by immunoblot analysis using gel-separated coat proteins of VCSM13 phage (Fig. 3A), although the fine epitope structures recognized were not determined.

To establish more sensitive ELISAs, smaller antibody fragments that can bind multiply on a phage virion, due to less susceptibility to steric hindrance, should be advantageous. Therefore, Ab-M13/#71, having the more common heavy chain subclass, was converted to M13-scFv. Specifically, the V_H and V_L genes were cloned and combined via linker1 encoding the common (GGGGS)_3 sequence9,10) in the orientation of 5^V_H-linker1-V_L-3 (Fig. 1). The resulting gene construct was expressed in E. coli cells with a FLAG tag (DYKDDDDK)45) added at the C-terminus. The nucleotide sequences of the V_H and V_L genes revealed the primary structures of these domains. The V_H and V_L sequences belonged to subgroup IIC and I, respectively, based on the Kabat definition.46) The complementarity-determining regions (CDRs) of the V_H and V_L domains, defined according to Kabat’s rule,46) were as follows: V_H-CDR1, NTYVH; V_H-CDR2, RIDPANGKTYDPFNQD; V_H-CDR3, DPYYIGSTYVSFFDV; V_L-CDR1, KASQNVRTDVA; V_L-CDR2, LASKRHT; and V_L-CDR3, LQHRSYPLT. The binding parameters, $k_a$, $k_d$, and $K_a$ of M13-scFv against VCSM13 phage at 25°C were 1.65×10^4 M^−1 s^−1, 1.41×10^2 s^−1, and 1.17×10^6 M^−1, respectively. To establish more convenient assay systems omitting the second binding reaction for monitoring the FLAG tag of scFv molecule, M13-scFv was then fused with ALP', a variant of E. coli ALP with a D101S substitution.

Fig. 3. Electrophoretic Characterization of Antibodies and Engineered Fragments  
(A) Immunoblot analysis to determine the specificities of monoclonal anti-phage antibodies. VCSM13 phage particles (ca. 1×10^10 or 1×10^9 pfu in lanes 1 and 2, respectively) were heated to 70°C for 10 min and separated by SDS-PAGE. Proteins separated on the gel were transferred to a polyvinylidene difluoride membrane, which was then blocked for 120 min at room temperature with Block Ace. Ab-M13/#53 or #71, diluted 1:1000 with G-PBS, was incubated with the membrane at room temperature for 60 min. After washing with T-PBS, the bound antibodies were detected by incubation at room temperature for 60 min with a POD-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch). Captured POD activity was visualized using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine. (B) SDS-PAGE of the affinity-purified antibody fragments. Lane M, M_r marker; lane 1, M13-scFv; and lane 2, M13-scFv-ALP'.
that increases its enzyme activity,\textsuperscript{31} via the peptidase-resistant linker2 (GSTSGSGKSEGKG).\textsuperscript{47} The resulting fusion protein (M13-scFv-ALP'\textsuperscript{3}) retained immunoreactivity against VCSM13 phage and sufficient enzyme activity for practical use, and was stable at $-20^\circ$C more than a year (data not shown). Several rounds of freezing and thawing did not decrease its utility. Both the M13-scFv and M13-scFv-ALP'\textsuperscript{3} proteins migrated as single bands on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with nearly the expected relative molecular masses ($M_r$: 27878.8 and 77005.6, respectively) (Fig. 3B).

Construction of Sandwich ELISAs for Counting M13 Phage

The sensitivities of three types of sandwich assays (Figs. 2A–C) were compared. In assay A, we used a biotin-labeled version of Ab-M13#71, which is a whole IgG molecule. In assays B and C, we used the engineered fragments M13-scFv and M13-scFv-ALP', respectively, as the reporter. To capture phages, affinity-purified Ab-M13#53 was directly coated on the microplates. Dose–response curves were constructed for VCSM13 phage titrated as the number of pfu, using the infection-based method. Therefore, the values for the present assays A–C reflect the immunochemical equivalence with VCSM13 phage normalized to pfu values. To prevent self-aggregation and adsorption to the solid phase by the phage particles, standard phages were diluted with buffer containing PVA and ethanol.

Typical dose–response curves showed that assays A–C afforded similar measurable ranges covering ca. $10^7$–$10^{10}$ pfu/mL, which are suitable for practical use. The midpoint of the dose–response curves, defined as the dose of standard VCSM13 phage showing intermediate enzyme activity between those of the upper and lower plateau phases (estimated based on the asymptotes), was $3.1 \times 10^8$, $2.1 \times 10^8$, and $5.1 \times 10^8$ pfu/mL for assays A, B, and C, respectively (Fig. 4). Assay B showed higher binding than assay A for the middle- and high-dose area ($>1 \times 10^8$ pfu/mL), but showed lower binding for low-dose area ($<1 \times 10^7$ pfu/mL) (Fig. 4A). The former observation could reflect easier accessibility of the scFvs due to their smaller molecular mass (ca., 1/6 of IgGs), enabling more reporter binding per captured phage virion, whereas the latter observation could be attributed to stronger binding of bivalent IgGs due to the avidity effect.

Among these systems, assay C allowed for the most rapid measurement, which finished within 90 min, because of the lower number of incubation steps involved. The limit of detection, defined as the lowest measurement point with 3-fold higher enzyme activity than the standard deviation (S.D.) of zero determination ($n=10$), was $1.0 \times 10^7$ pfu/mL, which was ca. 100-fold lower than that of the DNA-targeting method.\textsuperscript{25}

Practical Application of Sandwich ELISA Using M13-scFv-ALP'

Four kinds of scFv-phages, each displaying anti-Ct,\textsuperscript{18} anti-$E_\gamma$,\textsuperscript{19,20} anti-THC,\textsuperscript{21} or anti-CS\textsuperscript{22} scFv, packaged with VCSM13 as a helper phage, were conventionally titrated in terms of the cfu by the infection-based method on selective agar. Concurrently, phage concentrations were determined by assay C and UV spectrometry. Assay C yielded phage counts with much less bias away from the cfu values. Thus, while the UV method resulted in >15-fold larger values (very likely overestimated due to a trace amount of proteins contaminated), those of assay C were 3.7-fold smaller (on average), and both biases were observed systematically for all scFv-phages tested (Table 2). The reason for the latter bias is reasonably consistent with the fact that the first progeny phage particles appear 10 min\textsuperscript{6} or even faster\textsuperscript{48} after infection at 37°C (thus, before being plated on agar in the usual infection-based counting), and considering this, assay C should provide rather reliable phage numbers. The coefficient of variation (CV) of assay C (4.6–12%) was obviously lower than that of the infection-based method (13–21%). The linearity of the assay values for serially diluted phages was satisfactory, not only for PEG-precipitated scFv-phages (Fig. 5A), but also for scFv-phages in culture media (Fig. 5B): the latter of which indicated that assay C enables determination of phage concentrations in cultures without purification. Therefore, scFv-phage
propagation was monitored during the common phage rescue procedure. As shown in Fig. 5C, a reasonable time course was obtained, where ca. 50% of the maximum propagation was observed after 10 h. The present ELISA does not distinguish between the scFv-phages and helper phages. In usual protocols for phagemid-based phage preparation in which expression of the scFv-III is controlled with a lac promoter,46 however, excess helper phages are removed by changing medium (for removing glucose and adding kanamycin, the latter of which is necessary for selecting helper-phage-infected bacteria) after phagemid-transformed bacteria are infected with helper phages (see caption of Fig. 5C). Therefore, possible helper phage contamination does not cause significant overestimation: in fact, the ELISA readouts for 0.50 h and 1.0 h after the infection (as differential absorption) were −0.009 and −0.010.

CONCLUSIONS

We developed a sandwich ELISA for counting M13 phage displaying antibody fragments using a newly established monoclonal anti-phage antibody and an ALP fused antibody fragment. This assay showed practical sensitivity with convenient colorimetric endpoint detection, and enabled much more rapid, precise, and potentially more reliable phage counting than the conventional infection-based methods. The present assay will facilitate phage-display-based molecular breeding to generate not only antibody reagents but also other functional proteins. In the breeding approach for antibodies, efficient selection and isolation of rare improved Ab-phages from libraries with vast diversity is crucially important. We expect that the present anti-phage antibodies and their fragments might enable observation of individual phage particles by common optical microscopy. By staining phage capsids and captured antigens in complexes of Ab-phages and relevant antigens with different fluorescent dyes, improved Ab-phages might be visually identified.

Acknowledgments This work was supported in part by Grants from the Japan Society for the Promotion of Science (JSPS) (JSPS KAKENHI Grant Number 16K08954). We would like to thank Dr. Eskil Söderlind (Avena Partners AB, Sweden) and Professor Carl A. K. Borrebaeck (Lund University, Sweden) for providing the pEXmide 5 vector.

Conflict of Interest The authors declare no conflict of interest.
REFERENCES

1) The Immunoassay Handbook: Theory and applications of ligand binding, ELISA and related techniques, 4th Edition. (Wild D ed.) Elsevier, the Netherlands (2013).
2) Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity, Nature, 256, 495–497 (1975).
3) Therapeutic Monoclonal Antibodies: From bench to clinic. (Zhi-qiang A ed.) Wiley, Hoboken, NJ (2009).
4) Kobayashi N, Oyama H. Antibody engineering toward high-sensitivity high-throughput immunosensing of small molecules. Analyst, 136, 642–651 (2011).
5) Antibody Engineering: Methods and protocols. (Lo BKC ed.) Humana Press, Totowa, NJ (2004).
6) Handbook of therapeutic antibodies. (Dübel S ed.) Wiley-Blackwell, Hoboken, NJ (2010).
7) Bradbury ARM, Sidhu S, Dübel S, McCafferty J. Beyond natural antibodies: the power of in vitro display technologies. Nat. Biotechnol., 29, 245–254 (2011).
8) Chiu ML, Gilliland GL. Engineering antibody therapeutics. Curr. Opin. Struct. Biol., 38, 163–173 (2016).
9) Skerra A, Plückthun A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. Science, 240, 1038–1041 (1988).
10) Bird RE, Hardman KD, Jacobson JW, Johnson S, Kauffman BM, Lee SM, Lee T, Pope SH, Riordan GS, Whitlow M. Single-chain antigen-binding proteins. Science, 242, 423–426 (1988).
11) Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. ScFv antibody. Principles and clinical application. Clin. Dev. Immunol., 11 (2004).
12) Smith GP, Petrenko VA. Phage display. Chem. Rev., 97, 391–410 (1997).
13) Phage display a laboratory manual. (Barbas III CF, Burton DR, Scott JK, Silverman GJ eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (2001).
14) Phage display. (Clackson T, Lowman HB eds.) Oxford University Press, Oxford, NY (2004).
15) Rakonjac J, Bennett NJ, Spagnuolo J, Gagie D, Russel M. Filamentous bacteriophage: biology, phage display and nanotechnology applications. Curr. Issues Mol. Biol., 13, 51–76 (2011).
16) Hamzeh-Mivehroud M, Alizadeh AA, Morris MB, Church WB, Dastmalchi S. Phage display as a technology delivering on the promise of peptide drug discovery. Drug Discov. Today, 18, 1144–1157 (2013).
17) Oyama H, Morita I, Kiguchi Y, Morishita T, Fukushima S, Nishi-mori Y, Niwa T, Kobayashi N. A single-step “breeding” generated a diagnostic anti-cortisol antibody fragment with over 30-fold enhanced affinity. Biol. Pharm. Bull., 40, 2191–2198 (2017).
18) Oyama H, Morita I, Kiguchi Y, Banzono E, Ishii K, Kubo S, Watanabe Y, Hirai A, Kaucer C, Ohta M, Kobayashi N. One-shot in vitro evolution generated an antibody fragment for testing urinary cotinine with more than 40-fold enhanced affinity. Anal. Chem., 89, 988–995 (2017).
19) Kobayashi N, Oyama H, Kato Y, Goto J, Söderlin E, Borreback CÅ, K. Two-step in vitro antibody affinity maturation enables extracellular prokaryotic assays with more than 10-fold higher sensitivity. Anal. Chem., 82, 1027–1038 (2010).
20) Oyama H, Yamaguchi S, Nakata S, Niwa T, Kobayashi N. “Breeding” diagnostic antibodies for higher assay performance: A 250-fold affinity-matured antibody mutant targeting a small biomarker. Anal. Chem., 85, 4930–4937 (2013).
21) Morita I, Oyama H, Yasuo M, Matsuda K, Katagi K, Ito A, Tatsuda H, Tanaka H, Morimoto S, Kobayashi N. Antibody fragments for on-site testing of cannabinoids generated via in vitro affinity maturation. Biol. Pharm. Bull., 40, 174–181 (2017).
22) Molecular cloning a laboratory manual. 2nd edition. (Sambrook J, Fritsch EF, Maniatis T eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
23) Day LA, Wiseman RL. A comparison of DNA packaging in the virions of fd, Xf, and PFI, The Single-Stranded DNA Phages. (Denhardt DT, Dressler D, Ray DS eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY pp. 605–625 (1978).
24) Smith GP, Scott JK. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol., 217, 228–257 (1993).
25) Lehmsuuvari A, Manninen J, Huovinen T, Soukka T, Lamminäki U. Homogenous M13 bacteriophage quantification assay using switchable lanthanide fluorescence probes. Biotechniques, 53, 301–303 (2012).
26) Bhardwaj D, Singh SS, Abrol S, Chaudhary VK. Monoclonal antibodies against a minor and the major coat proteins of filamentous phage M13: their application in phage display. J. Immunol. Methods, 179, 165–175 (1995).
27) Kneissel S, Queitsch I, Petersen G, Behraing O, Michael B, Dübel S. Epitope structures recognised by antibodies against the major coat protein (gP8) of filamentous bacteriophage fd (Inoviridae). J. Mol. Biol., 288, 21–28 (1999).
28) “Anti-M13 pHi monoclonal antibody.”: https://www.ncbi.nlm.nih.gov/products/e0833-anti-m13-piimonoclonal-antibody#Product%20Information
29) “Anti-M13 gP8 monoclonal antibody.”: https://www.abdesignlabs.com/store/index.php?dispatch=products.view&product_id=29040.
30) “Phage titration ELISA.”: https://www.progen.com/media/downloads/DESCRIPTIONS/PRPHAGE_en%20_V9.pdf#search=%23phage%20titration%20ELISA%20%20gP8%20%27
31) Suzuki C, Ueda H, Tsumoto K, Mahoney WC, Kamagai I, Nagamune T. Open sandwich ELISA with V6/ V-alkaline phosphatase fusion proteins. J. Immunol. Methods, 224, 171–184 (1999).
32) Oyama H, Morita I, Kiguchi Y, Miyake S, Moriuchi A, Akisada T, Niwa T, Kobayashi N. Gaussia luciferase as a genetic fusion partner with antibody fragments for sensitive immunoassay monitoring of clinical biomarkers. Anal. Chem., 87, 12387–12395 (2015).
33) “Abisco-100 vaccine adjuvant.”: http://www.violinet.org/vaxjo/vaxjo_detail.php?c=vaxjo_id=89.
34) Sjölander A, Cox JC, Barr IG. ISCOMs: an adjuvant with multiple functions. J. Leukoc. Biol., 64, 713–723 (1998).
35) Köhler G, Howe SC, Milstein C. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. Eur. J. Immunol., 6, 292–295 (1976).
36) Kobayashi N, Banzono E, Shimoda Y, Oyama H, Kumihito T, Morita I, Ohta M. A monoclonal antibody-based enzyme-linked immunosorbent assay for human urinary cotinine to monitor tobacco smoke exposure. Anal. Methods, 3, 1995–2002 (2011).
37) Frohman MA, Dash MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. U.S.A., 85, 8998–9002 (1988).
38) Nicholls PJ, Johnson VG, Blanford MD, Andrew SM. An improved method for generating single-chain antibodies from hybridomas. J. Immunol. Methods, 165, 81–90 (1993).
39) Jirholt P, Ohlin M, Borreback CÅ, Söderlin E. Exploiting sequence space: shuffling in vivo formed complementarity determining regions into a master framework. Gene, 215, 471–476 (1998).
40) Oyama H, Tanaka E, Kawanaka T, Morita I, Niwa T, Kobayashi N. Anti-idiotypic scFv-enzyme fusion proteins: a clonable analyte-mimicking probe for standardized immunosassays targeting small biomarkers. Anal. Chem., 85, 11553–11559 (2013).
41) “Capsid protein GSP-P69541 (CAPSD_BPM1).”: http://www.rcsb.org/pdb/protein/P69541.
42) “VCMS13 interference-resistant helper phage, complete genome.”: https://www.ncbi.nlm.nih.gov/nuccore/AY598820?report=genbank.
43) O’Connell D, Becerill B, Roy-Burman A, Daws M, Marks JD. Phage versus phagemid libraries for generation of human monoclonal antibodies. J. Mol. Biol., 321, 49–56 (2002).
44) Qi H, Lu H, Qiu H-J, Petrenko V, Liu A. Phagemid vectors for phage display: properties, characteristics and construction. *J. Mol. Biol.*, 417, 129–143 (2012).

45) Knappik A, Plückthun A. An improved affinity tag based on the flag® peptide for the detection and purification of recombinant antibody fragments. *Biotechniques*, 17, 754–761 (1994).

46) Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. *Sequences of Proteins of Immunological Interest*. U. S. Department of Health and Human Services, National Institutes of Health, U. S. Government Printing Office, Washington, DC (1991).

47) Whitlow M, Bell BA, Feng S-L, Filipula D, Hardman KD, Hubert SL, Rollence ML, Wood JF, Schott ME, Milenic DE, Yokota T, Schlom J. An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability. *Protein Eng. Des. Sel.*, 6, 989–995 (1993).

48) Ploss M, Kuhn A. Kinetics of filamentous phage assembly. *Phys. Biol.*, 7, 045002 (2010).