Reliable titration of filamentous bacteriophages independent of pIII fusion moiety and genome size by using trypsin to restore wild-type pIII phenotype

Geir Åge Løset[1], Solveig G. Kristinsson[1,2], and Inger Sandlie[1]

[1]Department of Molecular Biosciences, University of Oslo and [2]Affitech AS, Oslo Research Park, Oslo, Norway

BioTechniques 44:551-554 (April 2008)
doi 10.2144/000112724

Phage display holds a key position in the use of combinatorial library approaches for the purpose of protein engineering and discovery. However, modifying the pIII protein of the phage can severely and negatively influence the infectiousness of the phage particle. This concern is particularly relevant when large pIII fusions in combination with multivalent display systems are in use. We here describe the use of trypsin to restore wild-type pIII phenotype as a small modification to the standard titration protocol. The results show that the trypsin treatment has a very large but heterogeneous effect on the phage infection efficiency, depending on the pIII fusion domain and the valence of display.

The filamentous phage is widely used in basic and applied science (1), and collections of such phages (libraries) display different proteins or peptides as fusions to a phage coat protein, most often to pIII. It is invariably necessary to measure the phage particle titers, when library diversity and complexity is determined, during selection procedures and when comparing the performance of single clones that display dfferent proteins. Usually, phage titers are scored by infecting *Escherichia coli* with serial dilutions of phage and given as plaque-forming or colony-forming units (pfu and cfu, respectively). In either case, the numbers obtained depend on the infection efficiency of the phage particles. Notably, pIII is the critical phage component mediating the early events of host entry (2). Hence, infection efficiency depends both on the number of pIII fusion proteins displayed as well as the nature of the pIII fusion partner. Even fusion partners with high homology may affect infection efficiency differently, thus obscuring the results obtained. Alternatively, the number of phage particles can be determined by an infection-independent procedure, namely enzyme-linked immunosorbent assay (ELISA), which uses antibodies that bind specifically to the phage coat. The signal obtained needs to be correlated to that of samples with known titer, and if the number of phage particles is low, ELISA lacks the necessary sensitivity. Moreover, phage particle size varies with genome size (3), making comparison of the signal from different vector systems difficult.

Here we report a very simple modification to the standard infection-based titration protocol that allows for a reliable and accurate phage particle titer determination. This involves pre-incubating the phage samples with trypsin to remove the pIII fusion moiety. In our hands, this small modification has a major impact on the resulting titers, simply by ensuring that wild-type (wt) pIII phenotype is restored with a minimum of handling and expense. Proteolytic removal of the fusion moiety is rapid, and the digestion step described is done as a short 5 min extension of the standard protocol.

The phagemids used have been described in a previous study (4). All are ampicillin-resistant (amp^R), encode T-cell receptor (TCR) or antibody domains fused to pIII, and range in size from 4.9 kb to 6.6 kb. Phagemid rescue and apolyethyleneglycol precipitation were done with M13K07 (GE Healthcare, Uppsala, Sweden),

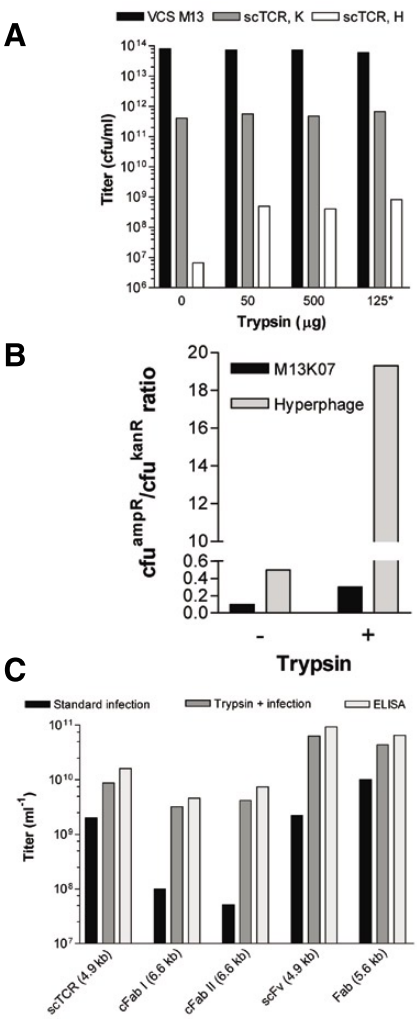

Figure 1. Comparison of different titration methods. (A) A fixed amount of VCSM13- or a scTCR-encoding phagemid (pFKPDN-scTCR Vαβ7A10B2, described in Reference 4), rescued by M13K07 (K) or Hyperphage (H), was digested with 0, 50, or 500 µg acetylated trypsin, or by a trypsin/EDTA premix corresponding to 125 µg trypsin. Digestion was done for 1 h at room temperature before the samples were titrated. One of two independent experiments is shown. (B) The phagemid to helper phage ratios of the M13K07 or Hyperphage-rescued phagemid in (A) were determined with or without trypsin digestion. One of two independent experiments is shown. (C) Comparison of the titers of Hyperphage-rescued phagemids displaying different pIII fusions. The titers were determined by the standard or the modified infection protocol, or by ELISA. The various phagemid sizes are given within brackets and the standard curve in the ELISA was determined from a separate pSEX81-scFv anti-phOx sample titrated several times by standard spot titration. One of two independent experiments is shown. Key to graph: scTCR, pSEX-scTCR Vαβ4B2A1; cFab I, pFABDFN-HαLβ4B2A1; cFab II, pFABDFN-HαLβ7A10B2; scFv, pSEX81-scFv anti-phOx; Fab, pFAB-Display anti-phOx; all described in Reference 4.

or Hyperphage (Progen, Heidelberg, Germany) helper phage (both kanamycin-resistant, kan^R) as described previously (5,6). Notably, Hyperphage is a modified M13K07 without endogenous pIII expression, hence all pIII molecules displayed by Hyperphage-rescued particles are fusions. Regular M13K07 encodes wt pIII and therefore directs display of varying, but lower, numbers of pIII fusion proteins. Titration based on infection efficiency was done by spot titration (7), and ELISA was done as described in Reference 4. Acetylated trypsin was from Sigma-Aldrich (St. Louis, MO, USA) and a trypsin/EDTA premix was obtained from BioWhittaker (Lonza Group Ltd., Visp, Switzerland). SDS PAGE and Western blot analysis was performed as described previously (4).

We first did a titration comparison of particles derived from a phagemid rescued with either M13K07 or Hyperphage helper phages. The procedure was carried out in the presence or absence of trypsin treatment, and VCSM13 (Stratagene, La Jolla, CA, USA) was included as a control, because the filamentous phage particle is virtually protease-resistant. Whereas the infectious titers (cfu^{ampR}) of the M13K07-rescued sample and VCSM13 (cfu^{kanR}) were unaffected by trypsin treatment, the Hyperphage-derived sample showed an approximately 120-fold increase (Figure 1A). There was no significant difference between the trypsin digestion conditions, hence the trypsin/EDTA premix, a standard reagent in most laboratories, offers the most convenient alternative.

Knowledge of packaged phagemid to helper phage ratios provides valuable information when working with phagemid display, and this ratio is determined by cfu using different selection markers on the phagemid and helper phage DNA. When mapping the phagemid to helper phage ratios by means of the cfu^{ampR}/cfu^{kanR} counts, trypsin digestion dramatically increased the ratio by 40-fold for the Hyperphage-rescued sample whereas there were only minor effects for the M13K07-rescued sample (Figure 1B). This may be explained by the fact that

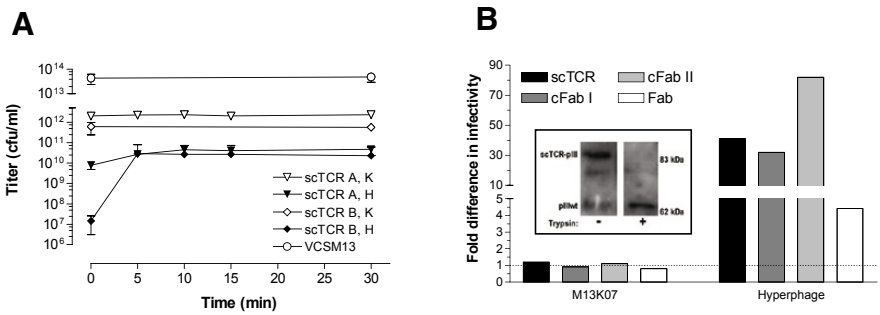

Figure 2. Adjustment of the trypsin digestion conditions and assessment of the effect on titers. (A) Two phagemids harboring different scTCRs fused to pIII rescued by M13K07 (K) or Hyperphage (H) were titrated after trypsin digestion (using the trypsin/EDTA premix) for various times ranging from 5 to 30 min. The experiment was performed in triplicate and the mean ± SD is shown. Key to graph: scTCR A, pFKP-DN-scTCR Vαβ4B2A1; scTCR B, the same clone as shown in Figure 1A; both are described in Reference 4. (B) Phagemids that display different pIII fusions (scTCR, cFab, or Fab) were rescued by M13K07 or Hyperphage followed by titration with or without 5 min trypsin digestion (using the trypsin/EDTA premix). The difference in infectious titer is given as the output with trypsin digestion divided by the output without trypsin digestion; one of two independent experiments is shown. *Inset*: 2×10^7 Hyperphage-rescued cfu^{ampR}, either untreated or treated by trypsin for 10 min, were separated by SDS PAGE and blotted onto a polyvinylidine fluoride membrane, followed by anti-pIII detection. One out of two independent experiments is shown. Key to graph: scTCR, pSEX-scTCR Vαβ7A10B2; cFab I, pFABDFN-HαLβ4B2A1; cFab II, pFABDFN-HαLβ7A10B2; Fab, pFAB-Display anti-phOx; all are described in Reference 4.

M13K07-rescued particles primarily derive their infectivity from wt pIII, which they display at various levels. Such complementation is not feasible in most phage genome-based display systems, or when using Hyperphage for phagemid rescue.

Next, a panel of phagemids of different sizes, all rescued with Hyperphage and displaying various pIII fusion moieties, were titrated by the standard or modified spot titration protocol, or by ELISA (Figure 1C). In all cases, trypsin treatment changed the titers obtained to nearly equal that given by ELISA. Protease removal of the fusion moiety was rapid, and the digestion step could be reduced to 5 min (Figure 2A). A final side-by-side comparison of the standard versus the modified spot titration protocol, with 5 min trypsin digestion using phagemids harboring different pIII fusions and rescued by either M13K07 or Hyperphage, clearly showed the effect on the resulting titers (Figure 2B). The increase in titers of Hyperphage-rescued particles after trypsin treatment varied from about 5-fold to 90-fold. This reflects the influence of the fusion moiety on the infection efficiency of pIII. Notably, the increase in titer was 120-fold for the particles displaying the scTCR fusion shown in Figure 1A, which underscores the importance of the improved titration procedure. A Western blot analysis of the trypsin-treated scTCR variant indeed confirmed the removal of the fusion moiety and a corresponding increase in the intensity of the band corresponding to wt pIII (Figure 2B).

Many library generation and selection regimens benefit from combining phagemid and phage genome display systems (8). It is at present feasible to use phagemid-based libraries in combination with pIII-modified helper phages such as Hyperphage, Ex-phage, and Phaberge (9) (leading to multivalent display) during the initial selection rounds and then to switch to the standard helper phages leading to low-valence display (mixture of wt pIII and pIII fusions). A reliable infection-based titration protocol for phagemids is therefore needed. The phagemids used herein are all derived from the pSEX series (10), which have a trypsin site engineered into the linker connecting the displayed moiety to pIII. This was introduced for the purpose of ensuring efficient elution of phage from immobilized antigen. Here we demonstrate the importance of the site for accurate titration. The procedure may be equally useful in other full-length pIII phage

display systems, however. The phage particle is highly resistant to even very high temperatures, and a short, high-temperature pre-incubation to denature the pIII fusion moiety before protease digestion (11) should be sufficient.

ACKNOWLEDGMENTS

The authors would like to thank Affitech AS for providing the phagemids and Bjarne Bogen for providing the T-cell clones harboring the receptors included in this work. The work was supported in part by the Norwegian Research Council (grant no. 174796).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Sidhu, S.S. and S. Koide.** 2007. Phage display for engineering and analyzing protein interaction interfaces. Curr. Opin. Struct. Biol. *17*:481-487.
2. **Karlsson, F., C.A.K. Borrebaeck, N. Nilsson, and A.-C. Malmborg-Hager.** 2003. The mechanism of bacterial infection by filamentous phages involves molecular interactions between TolA and phage protein 3 domains. J. Bacteriol. *185*:2628-2634.
3. **Marzec, C.J. and L.A. Day.** 1983. DNA and protein lattice-lattice interactions in the filamentous bacteriophages. Biophys. J. *42*:171-180.
4. **Løset, G.A., E. Lunde, B. Bogen, O.H. Brekke, and I. Sandlie.** 2007. Functional phage display of two murine α/β T-cell receptors is strongly dependent on fusion format, mode and periplasmic folding assistance. Protein Eng. Des. Sel. *20*:461-472.
5. **Welschof, M., P. Terness, S.M. Kipriyanov, D. Stanescu, F. Breitling, H. Dorsam, S. Dubel, M. Little, and G. Opelz.** 1997. The antigen-binding domain of a human IgG-anti-F(ab′)2 autoantibody. Proc. Natl. Acad. Sci. USA *94*:1902-1907.
6. **Rondot, S., J. Koch, F. Breitling, and S. Dubel.** 2001. A helper phage to improve single-chain antibody presentation in phage display. Nat. Biotechnol. *19*:75-78.
7. **Koch, J., F. Breitling, and S. Dübel.** 2000. Rapid titration of multiple samples of filamentous bacteriophage (M13) on nitrocellulose filters. BioTechniques *29*:1196-1198, 2002.
8. **Bradbury, A.R. and J.D. Marks.** 2004. Antibodies from phage antibody libraries. J. Immunol. Methods *290*:29-49.
9. **Soltes, G., M. Hust, K.K.Y. Ng, A. Bansal, J. Field, D.I.H. Stewart, S. Dubel, S. Cha, and E.J. Wiersma.** 2007. On the influence of vector design on antibody phage display. J. Biotechnol. *127*:626-637.
10. **Breitling, F., S. Dubel, T. Seehaus, I. Klewinghaus, and M. Little.** 1991. A surface expression vector for antibody screening. Gene *104*:147-153.
11. **Jespers, L., O. Schon, K. Famm, and G. Winter.** 2004. Aggregation-resistant domain antibodies selected on phage by heat denaturation. Nat. Biotechnol. *22*:1161-1165.

Received 1 October 2007; accepted 2 December 2007.

Address correspondence to Geir Åge Løset, Programme for Cell Biology, Department of Molecular Biosciences, P.O. Box 1041 Blindern, University of Oslo, Norway. e-mail: g.a.loset@imbv.uio.no