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Rapid Titration of Multiple Samples of Filamentous Bacteriophage (M13) on Nitrocellulose Filters

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Phage display is a well-established and powerful tool for the investigation of protein-protein interactions (7,8) and for the selection of human antibodies for in vivo diagnostics and therapy (3,10). Several rounds of selection on immobilized antigen are required to enrich the binding phage. The need to determine phage titers before and after every round represents a major time-consuming factor in this selection procedure.

Usually, phage are titered by infecting *E. coli* plating bacteria with dilution series of phage (6). The infected bacteria are embedded in top-agar. Transient plaques appear after approximately 16 h since the replication of infected bacteria is slowed down by phage synthesis. In case the phage carries an antibiotic resistance gene (as most do), infected bacteria can be plated on agar containing the respective selection substance. This method is preferred over the plaque assay because the colonies are not transient and thus can be identified and counted with better reliability. Alternatively, the number of phage particles can be determined in an ELISA by using an antibody specifically binding to phage proteins (5) and phage of known titer for standardization. This ELISA, however, determines the relative particle number but not colony forming units (cfu) or plaque forming units (pfu), thus not yielding information on infectivity and transmitted resistance, both important parameters in display systems using pIII fusions.

To monitor the panning process, up to now, large numbers of petri dishes had to be used because serial dilutions are required to analyze samples of unknown titer. The number of plates required for each step is further increased if the relation of packaged phagemid to helperphage genomes has to be determined by using different selection markers of phagemid and helperphage.

Despite the fact that this ratio provides valuable information about the performance of the selection system, this control is frequently omitted. In this report, we present a method that can be used to measure cfu on different selection markers. It dramatically reduces the workload and amount of materials required for titration by allowing the assay of multiple phage samples on a single standard agar plate.

For this study, we employed the well-characterized single-chain antibody phage pSEX/M13KO7 for the experiments. pSEX/M13KO7 represents a population of phage that is typically present in panning steps of antibody phage display libraries. It was produced in *E. coli* Top10F[®] bacteria (Invitrogen, Groningen, Netherlands) transfected with the pSEX81 antibody display phagemid (2,9) after infection with the helper phage M13KO7 (Amersham Pharmacia Biotech, Freiburg, Germany), which provides phage proteins necessary for packaging. M13KO7 is a derivative of M13 with a mutation-retarding replication of the helperphage genome (1), thereby resulting in a prevalent packaging of phagemid genomes. The resulting pSEX/M13KO7 phage particle mixture thus mainly contains genomes of the pSEX phagemid, which can express a model scFv antibody fragment to phenoxazone (4) fused to the minor phage coat protein (pIII) and provides ampicillin resistance. A small number of helperphage genomes (providing kanamycin resistance) are also found. Therefore, the helperphage-to-phagemid ratio can be determined by comparing the cfu on ampicillin and kanamycin. Usually, less than 5% of the phage particles contain M13KO7 genomes.

The new method for the titration of phage is also based on cfu determination. First, 16 fields were marked on round nitrocellulose filters (BA 85, 0.45 μ m, $\varnothing$ 82 mm; Schleicher & Schuell, Dassel, Germany) with a pen and placed onto Luria Broth agar plates either containing ampicillin or kanamycin. *E. coli* TG1 bacteria ($A_{600} = 0.6$; Stratagene, Amsterdam, Netherlands) were infected with serial dilutions of pSEX/M13KO7 for 20 min at 37°C. Aliquots of 10 μ L each infection were pipetted into the middle of the fields on the nitrocellulose membranes and incubated overnight at 27°C. In one or a few of the fields out of a serial dilution, single colonies could be identified and conveniently counted (Figure 1). In case the retrieved colonies were too small after overnight incubation, prolonging the growth time at 37°C for 1–2 h could easily increase the colony size. The pSEX/M13KO7 titer was determined to be $2.01 \times 10^9 \pm 2.52 \times 10^8$ cfu/mL packaged phagemids and $1.41 \times 10^6 \pm 1.32 \times 10^5$ cfu/mL helperphage.

For comparison, plaque assays and conventional cfu determinations were carried out for the same sample of pSEX/M13KO7. For the plaque assay, serial dilutions of phage were prepared in Luria Broth medium by serial 1:10 dilutions in ELISA plates. Two milliliters of *E. coli* TG1 bacteria ($A_{600} = 0.6$) were infected with each of the dilutions and incubated as described above. The infected bacteria were mixed with 3.5 mL prewarmed top-agar (Luria Broth medium containing 0.7% agarose), spread over prewarmed Luria Broth agar plates and incubated overnight at 37°C. The number of pfu in the pSEX/M13KO7 suspension was determined to be $2.79 \times 10^9 \pm 3.7 \times 10^8$ pfu/mL. The same phage sample was titered using the antibiotic resistance markers by counting colonies on agar plates after infection. To do this, 2 mL TG1 bacteria ($A_{600} = 0.6$) were infected with phage dilutions prepared as described above. Aliquots of 100 μ L each infection were plated on Luria Broth agar plates containing either ampicillin or kanamycin and incubated overnight at 37°C. The titer of pSEX/M13KO7 particles providing ampicillin resistance was $2.41 \times 10^9 \pm 3.62 \times 10^8$ cfu/mL. Helperphage genomes were present at 1.06×10^6 cfu/mL $\pm 3.09 \times 10^5$ cfu/mL.

In conclusion, the cfu titers obtained from the nitrocellulose membrane method were not significantly different from the titers obtained by conventional cfu determination or plaque assay (Table 1). The number of bacteria colonies determined on agar plates with nitrocellulose filters offers sufficient reliability to monitor phage display panning rounds but requires less than one-tenth of the number of agar plates when compared to standard methods. The method allows the investigator to titer multiple dilutions of several different

Table 1. Titration of pSEX/M13KO7 Phage Antibodies: Comparison of Agar Plates versus Nitrocellulose Membranes

Method	Ampicillin				Kanamycin				Plaques			
	Plate	cfu	(mean ± sd)		Plate	cfu	(mean ± sd)		Plate	pfu	(mean ± sd)	
Nitrocellulose	1	2.03 × 10 ⁹			1	1.31 × 10 ⁶						
	2	2.47 × 10 ⁹			2	1.28 × 10 ⁶						
	3	1.89 × 10 ⁹	2.01 × 10 ⁹ ± 2.52 × 10 ⁸		3	1.35 × 10 ⁶	1.41 × 10 ⁶ ± 1.32 × 10 ⁵				n.a.	
	4	1.92 × 10 ⁹			4	1.42 × 10 ⁶						
	5	1.72 × 10 ⁹			5	1.65 × 10 ⁶						
Agar plate	1	2.69 × 10 ⁹			1	1.31 × 10 ⁶			1	2.57 × 10 ⁹		
	2	2.74 × 10 ⁹			2	1.29 × 10 ⁶			2	3.03 × 10 ⁹		
	3	2.16 × 10 ⁹	2.41 × 10 ⁹ ± 3.62 × 10 ⁸		3	8.96 × 10 ⁵	1.06 × 10 ⁶ ± 3.09 × 10 ⁵		3	3.15 × 10 ⁹	2.79 × 10 ⁹ ± 3.7 × 10 ⁸	
	4	1.91 × 10 ⁹			4	5.95 × 10 ⁵			4	2.25 × 10 ⁹		
	5	2.57 × 10 ⁹			5	1.21 × 10 ⁶			5	2.93 × 10 ⁹		

Each determination was done with five independent dilution series of pSEX/M13KO7 phage. The number of infective particles was calculated from the counted colonies and the dilution factor of selected samples. Only fields with a colony number allowing the identification of individual colonies were taken into account (usually up to 50 colonies; see Figure 1 for illustration). In case more than one dilution yielded a field that could be counted, the mean value of the product of colony number and dilution factor is given. Usually, this was possible for two different dilutions per dilution series. n.a., not applicable.

phage samples on a single agar plate. Titration on different antibiotic selection markers can easily be done, too, by preparing two identical NC membranes (Figure 1, B and C). The number of fields on one standard plate can be increased up to 30 by carefully adjusting the incubation temperature or time for the overnight culture, thus optimizing for very small but still clearly visible colonies. It could be argued that a similar assay can be performed without the need of a nitrocellulose filter. We found, however, that the nitrocellulose allows a more reliable recognition and counting of the small colonies. Second, but most important, when pipetting multiple samples directly onto agar plates, the area of the spots covered with bacteria showed large variations in size and shape depending largely on the drying condition of the agar. Further, mixing of samples can occur, in particular closer to the rim of the plate where the agar is not plain due to adhesion to the petridish. These variations were not

Figure 1. Titration on nitrocellulose membranes. (A) Standard petri dish with a titration nitrocellulose membrane with 16 sample fields after overnight culture at 27°C. Serial dilutions of two different pSEX/M13KO7 phage samples were applied, comprising of eight dilution steps each. (B and C) Higher magnification of a typical result with M13KO7 packaged phagemid. The two identically loaded membranes were incubated either on ampicillin (B) or kanamycin (C) plates. The numbers represent successive 1:10 dilution steps.

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observed when samples were pipetted onto plain and dry nitrocellulose filters.

Automated systems using image processing, as established for the generation of gridded gene libraries, might be used to quantify the colonies on the nitrocellulose membranes. In combination with rectangular agar plates, printed nitrocellulose filters, and pipetting robots, automation for high-throughput systems should be achievable.

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