Benchmarks

Homogenous M13 bacteriophage quantification assay using switchable lanthanide fluorescence probes

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We have developed a rapid and reliable bacteriophage quantification method based on measurement of phage single-stranded DNA (ssDNA) using switchable lanthanide chelate complementation probes. One oligonucleotide probe contains a non-fluorescent lanthanide ion carrier chelate and another probe is labeled with a light absorbing antenna ligand. Hybridization of the non-fluorescent complementation probes in adjacent positions on the released bacteriophage ssDNA leads to high local concentrations of the lanthanide ion carrier chelate and the antenna ligand, inducing formation of a fluorescent lanthanide chelate complex. This method enables monitoring of bacteriophage titers in a 20 min assay with a dynamic range of 10^9 – 10^{12} cfu/mL in a microtiter well format. While designed for titrating filamentous bacteriophage used in phage display, our method also could be implemented in virological research as a tool to analyze ssDNA virus reproduction.

Switchable lanthanide chelate complementation probe technology is a versatile tool for homogenous DNA detection assays due to low background fluorescence levels and high specific signal generation. Complementation probe technology allows detection of low picomolar quantities of target DNA in a homogenous DNA hybridization assay (1) and enables remarkably high signal-to-background ratio (S/B) (maximum of 300) in a closed-tube PCR assay (2). The technology involves the use of two non-fluorescent oligonucleotide probes, one carrying a lanthanide ion and the other, an antenna probe. When hybridized to a DNA target at precisely adjacent positions, the lanthanide ion and antenna probe form a highly fluorescent lanthanide chelate complex.

Phage display (3) is a widely used technique for finding binders by screening large heterologous peptide or protein populations. In phage display, a library of genes is fused to a phage coat protein and expressed on the surface of virus particles. Since the genotype and phenotype are linked by the virion, the displayed protein or peptide with the desired binding characteristics can be easily propagated and isolated. The virus used in phage display is the M13 filamentous bacteriophage of *Escherichia coli*; in fact, the M13 bacteriophage based phage display is the most successful selection technology used in *in vitro* antibody development (4).

Phages selected are then propagated after each selection (panning) round using a bacterial host. Starting from a universal antibody library,

three panning rounds are commonly performed to enrich for the desired target binding antibodies. After each propagation cycle, phage concentration must be determined for better control of the panning process. Phage titers are most often determined by using the plating method, that is the gold standard for the technique. Here, *E. coli* cells are first infected with a dilution series of the produced phage and then either mixed in soft agar and plated, or plated on top of selective agar containing antibiotics. Following overnight incubation at 37°C, either plaques (plaque forming units or pfu) or colonies (colony forming units or cfu) are counted (5). Since phage consists of DNA and proteins that absorb light at the UV region, UV photometry at 265 nm wavelength is also commonly used to measure phage titers (6). Although titrating by absorbance is a rapid technique compared with plating, very pure phage stocks are needed since protein impurities interfere with the measurement. For minimal impurities, phage should be purified using CsCl density-gradient ultracentrifugation, which requires centrifugation times up to 36 h (7).

We have developed a rapid mix-and-measure method for determining phage titer directly in the culture medium, as well as from purified phage preparations. The method is based on measurement of M13 phagemid single stranded DNA (ssDNA) with complementation probe technology and detects ssDNA rather than infectious particles. Therefore, it is important that the standard phage used be the same as, or as similar as possible, to the phage being measured so the amounts of non-infective virus or undetected phage do not influence the results.

Probes were prepared by labeling one 24-mer oligonucleotide (5'-TAT ACGCAAGGCGACAAGGTGCT G(AmC6)-3', Thermo Fisher Scientific, Waltham, MA, USA) at the 3' end with the non-fluorescent heptadentate 7d-DOTA-Eu^{III} carrier chelate (2,2',2''-(10-(3-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclo-dodecane-1,4,7-triyl)tri(acetato)europium^{III}) (8) (Eu^{III} probe) and another 24-mer oligonucleotide (5'-(AmC6)TGCCGCTG-GCGATTTCAGGTTCA-3', Thermo Fisher Scientific) at the 5' end with the tridentate light absorbing Antenna ligand (4-((4-isothiocyanatophenyl)ethynyl)pyridine-2,6-dicarboxylic acid) (1) (Antenna probe). The 7d-DOTA-Eu^{III} and Antenna ligand containing

Figure 1. Principle of the complementation probe-based bacteriophage quantification assay. (A) Bacteriophage are isolated from bacteria by centrifugation and complementation probes are added to the solution. Then phagemid ssDNA is released by lysing the phage at high temperatures and Europium time-resolved fluorescence is measured at +25°C. (B) Molecular structures of the 7d-DOTA-Eu^{III} carrier chelate (1) and the light absorbing Antenna ligand (2). The isothiocyanate group (NCS) is used for coupling the reporter molecules to the oligonucleotides.

Figure 2. Performance of the complementation probe-based bacteriophage quantification assay. Complementation probes hybridize to the released bacteriophage ssDNA and form a fluorescent lanthanide chelate complex. (A) Phage assay standard curve prepared using purified scFv-phage. (B) Concentration of the scFv-phage in the culture media was monitored during 20 h culture.

isothiocyanate-groups were coupled to the free amino groups of the carbon linkers attached to the oligonucleotides, forming a covalent thiourea bond as described in Karhunen et al. (1). The probes were designed to hybridize to adjacent positions on the anti-sense strand of the chloramphenicol acetyl-transferase gene, a selectable marker on

the phagemid pEB91 (9), packed into the M13 phage with VCSM13 helper phage (Stratagene, La Jolla, CA, USA). Hybridization of the probes leads to a high local concentration of the reporter molecules (7d-DOTA-Eu^{III} and Antenna ligand), inducing formation of a fluorescent mixed chelate complex by self-assembly of the reporter molecules. Principles of

the assay and reporter molecule structures are shown in Figure 1.

A stock of M13 bacteriophage displaying an anti-lysozyme single-chain fragment variable (scFv) antibody (amL3/4-42-pEB91-clone, Kd: 0.8 nM) isolated from the single-framework library, published by Brockmann et al. (9), was prepared according to the

protocol described by Korpimäki et al. (10) using VCSM13 and *E. coli* XL-1 blue from Stratagene. Infective titer (cfu/mL) of the prepared scFv-phage stock was determined by the plating method based on antibiotic selection and colony counting. A standard curve was created by measuring a dilution series of the scFv-phage that covered $0-2.7 \times 10^{12}$ cfu/mL using the complementation probe assay. The reaction mixture consisted of 10 nM Eu^{III} probe, 5 nM Antenna probe and scFv-phage in assay buffer (25 mM TRIS-HCl, 900 mM NaCl, 0.1% Tween 40, 0.05% NaN₃, 30 μ M DTPA at pH 7.5). The phage were lysed at 95°C for 1 min and the reaction mixture was cooled 1–3°C/min followed by 5 min incubation at +25°C before measurement. Europium time-resolved fluorescence (Eu TRF) was measured using a 340 nm excitation filter, 615 nm emission filter, 0.4 ms delay, and 0.4 ms measurement time with a 1420 Victor Multilabel Counter (Perkin Elmer Life and Analytical Life Science, Turku, Finland).

The detection limit of the standard, defined as the lowest measurement point giving higher signal than the background + 3 $\times$ SD of the background signal, was 1.14×10^9 cfu/mL. The average coefficient of variation of all standard curve measurement points (three individual assays with three replicates) was 6.4%, indicating reliable measurement performance. When the scFv-phage titer on the standard curve exceeded the concentration of the probes, the signal started to decrease because the probes were hybridized to different scFv-phage ssDNA molecules. (Figure 2A)

The titer of the emerging scFv-phage was also monitored directly in the culture medium. A sample was withdrawn from the phage culture and prepared as described in Figure 1A every two hours during 20 h phage culture. Prepared samples were diluted 1:7 in assay buffer containing 10 nM and 5 nM Eu^{III}, and Antenna probes, respectively. Eu TRF of the reactions was measured (three parallel reactions at each measurement point) and converted to cfu/mL according to the phage standard curve. At 4 h, the phage titer had already exceeded the detection limit; the maximum titer was reached by 8–10 h (Figure 2B). Only 20 min was needed for measuring the titer at each measurement point.

Phage quantification with complementation probes is a robust method

for titer determination, even when measurements are performed directly in culture supernatant. It drastically decreases the total assay and hands-on time compared to the plating method and gives more accurate counts of the phagemid-containing virions than absorbance measurements since contaminating proteins and helper phage do not interfere with the assay. In addition, variation in absorbance measurements can occur since the length of the phage filament is dependent on the size of the packed phagemid; this difficulty is avoided when using the complementation assay. Since the complementation probe based phage assay measures ssDNA directly, the assay should be applicable to quantification of any ssDNA phage species.

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Competing interests

Authors Dr. Lamminmäki and Prof. Soukka are inventors on patent application WO2010/109065 relating to assay methods based on the switchable lanthanide luminescence.

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