Published in final edited form as:

Nat Microbiol. 2018 January ; 3(1): 62–72. doi:10.1038/s41564-017-0045-y.

Quantification of diverse virus populations in the environment using the polony method

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Abstract

Viruses are globally abundant and extremely diverse in their genetic make-up and in the hosts they infect. While they influence the abundance, diversity and evolution of their hosts, current methods are inadequate for gaining a quantitative understanding of their impact on these processes. Here we report the adaptation of the solid-phase single-molecule PCR polony method for the quantification of taxonomically relevant groups of diverse viruses. Using T7-like cyanophages as our model, we found the polony method to be far superior to regular quantitative PCR methods and droplet digital PCR when degenerate primers were used to encompass the group's diversity. This method revealed that T7-like cyanophages were highly abundant in the Red Sea in spring 2013, reaching 770,000 phages·ml⁻¹, and displaying a similar depth distribution pattern to cyanobacteria. Furthermore, abundances of two major clades within the T7-like cyanophages differed dramatically throughout the water column: clade B phages that carry the *psbA* photosynthesis gene and infect either *Synechococcus* or *Prochlorococcus* were at least 20-fold more abundant than clade A phages that lack *psbA* and infect *Synechococcus* hosts. Such measurements are of paramount importance for understanding virus population dynamics, their impact on different microbial taxa and for modeling viral influence on ecosystem functioning on a global scale.

The discovery of vast numbers of viruses in oceans and lakes over two decades ago^{1,2} revolutionized the study of environmental viruses. Their abundances have been estimated at 10⁷ viruses·ml⁻¹ seawater with approximately 10³⁰ viruses overall and 10²³ infections occurring every second in the oceans^{3,4}. It is now evident that viruses are abundant in a range of natural ecosystems including terrestrial ecosystems^{5,6} and the human microbiome^{7–9}, and that they likely infect all forms of life. These environmental viruses are extremely diverse, belong to many different families and display a large degree of genetic diversity even within a single family⁴.

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Data availability

Sequences of the picked polonies have been deposited in the GenBank database under Accession Numbers KY788357-KY788381.

Author contributions

N.B. set up the polony method for viruses. N.B., S.G and I.M. designed, performed and analyzed the experiments for method optimization and validation as well as field analyses, and contributed to writing of the manuscript. D.L. conceived the project, participated in experimental design and wrote the manuscript.

The authors declare no competing financial interests.

Viruses impact the abundance, diversity and evolution of their bacterial, archaeal and eukaryotic hosts as well as biogeochemical cycling of matter in the environment^{3,4}. However, a major impediment to gaining a quantitative understanding of their impact on these processes is the lack of suitable tools for identification and quantification of the number of viruses that infect a particular host taxon. Indeed, current methods are either indiscriminant measures of virus-like particles^{1,3,10–12} or use culture-dependent assays^{13–15} that measure only a small portion of the diverse viruses infecting a host taxon^{3,16} (Table 1). This is largely due to their high degree of host specificity^{16,17}, such that no cultured host representative is adequate for the quantification of virus populations that infect diverse members of an entire host taxon. In addition, the use of molecular methods is hampered by the immense genetic diversity within a single virus family. Thus, specific primers and probes are not capable of encompassing the existing diversity within a viral family and can only be used to assess the abundance of a single viral genotype^{18–23}. Furthermore, degenerate primers needed to capture this diversity would yield non-quantitative results due to biases in amplification of diverse templates in quantitative PCR (qPCR) assays, as reported previously for bacteria^{24,25}. Recently, attempts have been made to apply metagenomics to viral quantification, but at this time, these can only provide relative rather than actual abundances of different genotypes^{26,27} (Table 1). Thus, no method currently exists that can quantify diverse viruses contained within a single family that infect a discrete host taxon.

Here we present the development, validation and field testing of the polony method for the identification and quantification of diverse viruses belonging to a distinct virus family and infecting a particular host taxon (termed a genus here following the International Committee on Taxonomy of Viruses). This method, adapted from that developed by Mitra and Church²⁸, is a culture-independent solid-phase PCR amplification method that utilizes highly degenerate primers to encompass the genetic diversity found within a viral genus, yet is sensitive enough to detect a single genome copy present in free viruses. We use the T7-like podoviruses that infect marine cyanobacteria (T7-like cyanophages) as our model system. These viruses contain dsDNA genomes^{29,30} and have a lytic lifestyle^{31–33}, although some members carry an integrase gene^{29,30}. Their cyanobacterial hosts belong to the genera *Synechococcus* and *Prochlorococcus*, which are highly abundant in the oceans and contribute significantly to global primary production³⁴. In this paper we use the term host in the broad sense to signify that members of these cyanobacterial genera are the hosts of these T7-like cyanophages.

Two well-defined phylogenetic clades exist within the T7-like cyanophages^{30,32,35}: clade B cyanophages encode the *psbA* host-like photosynthesis gene (87% of 30 phages investigated) and infect either a *Synechococcus* or *Prochlorococcus* host; while clade A phages lack the *psbA* gene (all 10 phages analyzed) and primarily infect *Synechococcus* hosts. Members of this viral group have a narrow host range^{16,36,37}, and are therefore especially in need of culture-independent quantification. They are well-represented in metagenomic surveys of the oceans^{30,38} yet their numerical abundances (the number of viruses per unit volume) remain unknown.

Results

In our adaptation of the polony method, viruses are dispersed in a thin polyacrylamide gel containing a 5'-acrydite-modified primer poured on a microscope slide (Fig. 1a). PCR reagents are diffused into the gel and a heat step disrupts capsids, making the DNA accessible. Slide PCR is performed and spatially separated amplification spheres result from individual template viruses (Fig. 1a). These amplification spheres, termed polonies for PCR colonies²⁸, are anchored to the gel via the acrydite modified primer. Hybridization with fluorescently labelled probes, complementary to an internal region of the anchored strand of the amplicon, is used for detection with a microarray scanner or epifluorescence binocular. Internal probes prevent enumeration of non-specific amplification spheres including those from primer-dimers. This method, using specific primers and probe for the P-SSP7 phage, worked well with polony formation efficiency of $75\% \pm 1.9\%$ ($n=7$), similar to efficiencies reported for naked DNA²⁸.

Since no single gene is found in all virus types, signature genes that are present in all members of a particular virus genus or family are used to describe viral diversity and phylogeny. The DNA polymerase gene is commonly used for T7-like podoviruses^{39,40} and the cyanophages within them^{30,32,35}. The diversity of this gene among T7-like cyanophages is high, with 45% nucleotide sequence divergence among the most distant clade A and clade B phages. Using available sequence information we designed and tested degenerate primers and probes specific to the T7-like cyanophages yet encompass their known diversity. The best primers had a high degree of degeneracy of over 1000 different combinations, and the best probes had a degeneracy of 16 and 128, with up to 3 inosines (see Supplementary Text).

Using these degenerate primers and probes, well-formed polonies resulted from viral templates while no-template controls were free of polonies (Fig. 1b). Furthermore, the empirical testing of different probes for clade-specific polony detection enabled us to find probes that differentiate between clade A and clade B T7-like cyanophages (Fig. 1c). However, efficiency of polony formation was lower than when using specific primers and probes ($51\% \pm 6.1\%$ rather than $75\% \pm 1.9\%$) for the P-SSP7 phage.

Next we assessed polony formation efficiency using 5 different T7-like cyanophages from across the phylogenetic tree. The reproducibility of the virus-to-polony conversion efficiency for a single virus was very high and linear for 2-3 orders of magnitude (Fig. 1d). However, different virus-to-polony conversion efficiencies were observed for diverse viruses (between 18-62%) with a difference of 3.4-fold (Fig. 1d). These differences in efficiency occurred during polony formation (slide PCR), likely due to differences in primer annealing to different template sequences, and not polony detection with hybridization probes since specific and degenerate probes detected the same polonies (Fig. 1c).

Quantification of T7-like cyanophages in seawater samples using the polony method requires taking differences in virus-to-polony conversion efficiencies into consideration (Fig. 1d). The average number of T7-like cyanophages is calculated from the average of the 5 conversion efficiencies ($y=0.404x$), or from the average of 10,000 bootstrap resamplings of

these slopes (see Methods). Confidence intervals are determined from the 95% quantiles of the bootstrap analysis, which result in maximal differences of up to 2.2-fold.

The number of polonies that can be accurately quantified on a single slide is restricted by overlapping polonies and is dependent on polony size. We empirically set this at 1200 polonies per slide for the T7-cyanophage system. A reliable lower estimate is achieved with 10 polonies per slide. These bounds correspond to the detection of between ~10,000 to over a million T7-like cyanophages·ml⁻¹ seawater. The lower limit can be significantly improved by concentrating viruses at least 40- to 100-fold prior to the polony procedure using a slightly modified iron flocculation method⁴¹, which achieved concentration efficiencies of 93%±13% (n=5). This provides an overall dynamic quantification range of over 4 orders of magnitude, ranging from 10² to over 10⁶ T7-like cyanophages·ml⁻¹ seawater. Note that samples can be diluted to increase the upper limit.

Next we wished to compare the polony method to other methods that could theoretically be used for the quantification of environmental viruses. Quantitative qPCR is a simple, accurate method for quantifying single genotypes from the environment^{18,42} (defined here as having greater than 95-97% nucleotide identity in the signature gene). However, qPCR is highly problematic for accurate quantification of diverse templates with degenerate primers, due to differences in primer annealing and amplification efficiencies of various templates within a population, as found previously for bacteria^{24,25}. Methods that employ physical separation of template molecules, such as the polony method and droplet digital PCR (ddPCR)²⁰, are expected to largely circumvent these problems as a single molecule is amplified at one site, preventing template competition. Furthermore, enumeration of amplicons is carried out on a presence/absence basis. As such, differences in amplification are not important as long as enough amplicon is produced per template molecule to pass the threshold for detection.

We compared the polony method with ddPCR, SYBR Green and Taqman qPCR methods using both specific and degenerate primers and probes at two DNA template concentrations, 10⁴ and 10⁶ copies·ml⁻¹, for both the Syn5 and P-SSP7 phages. All methods performed well with specific primers and probes (Fig. 2a, Supplementary Fig. 1). However, only the polony method detected viral DNA at these concentrations using degenerate primers and probes (Fig. 2a, Supplementary Fig. 1). Furthermore, a simulation based on 25% differences in amplification efficiencies found for two phages (Supplementary Text), showed that large disparities of 625-fold in apparent abundances would be reported after 40 cycles for the same amount of initial template (Fig. 2b). Surprisingly, no concentration of template was detectable using Taqman qPCR or Taqman-based ddPCR with degenerate primers and probes. This was due to both low efficiency amplification with degenerate primers and lack of amplicon detection with degenerate probes (Supplementary Fig. 2). See Supplementary Text for a discussion of detection limitations of the qPCR methods.

These comparisons show that the polony method is far superior to other methods when degenerate primers and probes are required to gain a population level view of a diverse virus group. This is both in terms of the ability to detect viruses at environmentally relevant concentrations and in the accuracy of quantification.

We now wished to apply this method to field samples. Prior to doing so, we first tested common preservation and storage conditions. We found that storage at -80 °C without fixatives is best and that samples can undergo multiple freeze-thaw cycles without detriment to quantification (Supplementary Text, Supplementary Fig. 3).

Next we assessed the abundances of clade A and clade B T7-like cyanophages in the viral fraction (<0.2 µm filtrate) of surface coastal waters in the Gulf of Aqaba, Red Sea. Since the level of viral patchiness at the phage genus level is unknown, multiple samples were collected from the same site at 2 minute intervals. Total T7-like cyanophages were highly abundant with average abundances of 662,100±112,300 and 181,400±13,900 per milliliter seawater in September 2012 (n=5) and May 2014 (n=3) respectively (Fig. 3a), and were significantly different on the two sampling dates (t-test, p=0.0005). Little sample-to-sample variability existed across the samples collected on each date (Fig. 3b). These data demonstrate high reproducibility and low short-term temporal variation, indicating that a single sample is representative of viral abundance in that body of water at that time.

A comparison of the abundances of T7-like cyanophages from clade A and clade B revealed that clade B cyanophages were considerably more abundant than clade A cyanophages (t-test, p<0.001 for both dates) by as much as 40- and 180-fold (Fig. 3). Quantification of phages at abundances of ~10³ viruses·ml⁻¹, as found for clade A phages in May 2014, was quite variable due to low number of colonies per slide (Fig. 3a). Concentrating samples 50-fold improved reproducibility and reliability of quantification at these low abundances (Supplementary Fig. 4).

Importantly, differences found in viral abundance between sampling dates (p=0.004) and between clade A and clade B phages (p<0.0001 for both dates) are significant even when we take the uncertainty of the composition of phage populations on each date into consideration. This was determined from 95% confidence intervals of the quantiles from 10,000 bootstrap resamplings.

Since free DNA is found in seawater samples, we wished to assess whether colony formation resulted from encapsidated viral DNA. A DNase digestion protocol that digests free but not encapsidated DNA was used for the purpose (Supplementary Text, Supplementary Fig. 5a). No differences in colony abundances were found with and without DNase treatment, indicating that colonies in field samples originated from encapsidated DNA (Supplementary Text, Supplementary Fig. 5b).

To further verify that colonies resulting from field samples arose from amplification of T7-like cyanophages, we sampled (picked 28) and sequenced 25 colonies from samples collected in September 2012 (Methods). This confirmed that the colonies resulted from the amplification of the DNA polymerase gene from T7-like podoviruses: 24 clustered phylogenetically with known T7-like cyanophages throughout the phylogenetic tree and one clustered outside the known diversity for cyanophages from this genus (Fig. 4). A high degree of sequence diversity was found among the sequenced colonies with nucleotide identities as low as 54% between clade A and clade B colonies and 62-66% identity for colonies within each of the two clades. Thus, these combined findings demonstrate that the

polony method can reliably detect and quantify diverse T7-like cyanophages from complex marine communities.

Once the polony method had been set-up and validated, we determined, for the first time, numerical abundances of T7-like cyanophages along a depth profile in the marine environment. This was done during the spring bloom in April 2013 at Station A, a well-studied site in the centre of the Gulf of Aqaba, Red Sea^{43,44} (Fig. 5). Water column stratification had begun after winter mixing and there was a relatively shallow deep chlorophyll maximum at 55-65 m depth. Cyanobacterial abundances were greatest at 40-60 m with approximately 75,000-85,000 *Synechococcus* and *Prochlorococcus* cells·ml⁻¹ (Fig. 5).

Throughout the top 140 m of the water column T7-like cyanophages numbered more than 300,000 phages·ml⁻¹. A subsurface maximum of ~770,000 T7-like cyanophages·ml⁻¹ was found at 60 m depth (Fig. 5d, e) and coincided with the deep chlorophyll maximum and the peak in cyanobacterial abundances. Indeed, their general distribution pattern followed that of the cyanobacteria. At all depths clade B phages were far more abundant than clade A phages, from 20-fold more at the surface to 110-fold more at 140 m (Fig. 5f).

Discussion

The development of the polony method for viral ecology has provided the first quantitative assessment of diverse environmental viruses at the population level for a particular viral genus. We found that T7-like cyanophages were extremely abundant both in coastal surface samples as well as throughout the photic zone in the middle of the Gulf of Aqaba, ranging from ~180,000 to 770,000 phages·ml⁻¹. These abundances, from just this single family of cyanophages, are up to 10-fold higher than those of their cyanobacterial hosts.

Comparing these cyanophage abundances to those reported previously is difficult as the various methods intrinsically measure different things (Table 1). Polony quantification encompasses diverse genotypes within a viral type (genus in this case) and enumerates intact phages that are both infective and non-infective. In comparison, qPCR^{18,42}, digital PCR¹⁹, phageFISH²³, and like methods^{20–22}, also enumerate infective and non-infective phages but target a single genotype within a viral family (Table 1). Metagenomics also assesses infective and non-infective phages, providing relative abundances of different phage types at a variety of taxonomic levels within the community^{27,30,38}. In contrast, commonly used culture dependent measures provide estimates of infective phages across families as well as across genotypes within each phage family, but are limited to those capable of infecting the host(s) used for the assay. This is particularly problematic for the quantification of host-specific viruses such as the T7-like cyanophages. Nonetheless, a comparison to the latter method provides an important perspective. In coastal samples collected in September 2012, the polony method detected over ~660,000 T7-like cyanophages·ml⁻¹ (Fig. 3), whereas culture dependent measures using two cyanobacterial hosts estimated ~24,000 phages·ml⁻¹ of all cyanophage types (see Fig. 5 of ref¹⁶). These findings highlight the large degree to which culture-dependent methods underestimate cyanophage abundances. This is particularly apparent since only the T7-like cyanophages have been quantified so far and that

other cyanophage genera, such as T4-like and TIM5-like cyanophages^{16,36,37,45} have yet to be considered in the combined quantification of cyanophages by the polony method.

Quantification of cyanophage abundances, as obtained using the polony method, is important for documenting population sizes and distribution patterns over temporal and spatial scales. This information is crucial for gaining insight into how phage populations fluctuate along environmental gradients and with changes in host population sizes. Furthermore, central to understanding the impact of viruses on their hosts is assessing the probability of members of virus populations encountering members of their host population. This is an essential first step prior to the initiation of an infection cycle and governs whether downstream consequences to the host populations can ensue, including host mortality, selection for cells resistant to infection and delivery of genetic material. Encounter rates are directly related to the actual densities of both host and virus, and for any given virus to host ratio, the higher their densities the greater the chance of contact. It should be noted that relative viral quantification, determined in relation to other viruses, does not provide us with the means to adequately address these fundamental questions in viral ecology. An example is seen from the comparison of relative abundances of clade A and clade B phages to their actual abundances (compare Fig. 5d,e to Fig. 5f). While measurements of population sizes showed that both phage clades had a subsurface peak and that this peak coincided with peak abundances of cyanobacteria, the ratio of clade B to clade A phages increased continuously from the surface down to more than 100 m, and missed the spatial structure of these populations with depth.

The random sequencing of just 25 polonies amplified from a single site in September 2012 returned sequences that spanned the phylogenetic tree of T7-like cyanophages. These findings emphasize that a high degree of diversity of this cyanophage genus is present in water samples. Therefore, targeting a single genotype within the T7-like cyanophages using other methods would greatly underestimate the abundance of this group as a whole. Furthermore, the polony method can be used to link both abundance and diversity in a single sample.

Previous studies using non-quantitative or relative quantification approaches to investigate surface samples in different oceanic regimes reported that T7-like cyanophages from clade B are found in higher proportions than those from clade A^{38,46,47}, including in the Red Sea³⁵. These trends are the same as those we found in surface samples using our quantitative approach. Here we further show that the dominance of clade B phages extends throughout the photic zone. This finding challenges previous hypotheses that the *psbA* gene would primarily be advantageous for phages in high irradiance upper surface layers^{48,49}. Thus, it seems feasible that the dominance of clade B phages at depth is related to other factors beyond encoding photosynthesis genes or that the *psbA* gene is beneficial throughout the photic zone since photosynthetic energy production in cyanobacteria is not limited to the upper surface layers.

The polony method enables quantitative assessment of thousands of viruses in a single sample, providing great statistical power in a cost-effective, high-throughput and relatively simple manner. This is the method of choice for numerical quantification of viral taxa (or

that of any organism type) that exhibit a significant degree of diversity and require degenerate primers to adequately identify and sample that diversity. Quantification is restricted, however, to the viral genus or family under investigation and requires prior knowledge of their diversity for appropriate primer and probe design so that all known members of a group are included and others are excluded.

It is important to note that qPCR and ddPCR are the preferred methods for quantification of single genotypes with specific primers as they are more simple and have greater precision than the polony method. Additionally, metagenomics is better suited for relative quantification at the community level, at least within a single sample, as it captures and catalogues the relative composition and diversity of the entire community all at once.

Our use of the polony method is aimed at assessing cyanophages and their impact on their cyanobacterial hosts and to gain insights into viral-mediated cycling of matter from autotrophs to heterotrophs. The method can also be adapted to determine the abundances of entire virus families irrespective of host taxa infected (for example all T7-like phages) or other viral genera within a family (T7-like phages that infect pelagibacter, roseobacter or other host taxa) and other virus families (T4-like phages that infect various host taxa). Indeed, it can be adapted to investigate any DNA or RNA virus type infecting any prokaryotic or eukaryotic taxon from ecosystems ranging from aquatic and terrestrial environments to the human microbiome. Thus, its application will provide the first glimpses into population sizes of a suite of virus types at the virus family and host taxon level in diverse ecosystems worldwide. This method will enable the determination, not only of taxon-specific viral distribution patterns and abundances at any point in time, but also of their rate of production and decay. As such data generated with this method will facilitate modelling of contact rates between viruses and their host taxa and the formulation of quantitative global models of the impact of viruses on host populations and ecosystem functioning.

Methods

Preparation of viral lysates

Development of the polony method for quantification of T7-like cyanophages was carried out mainly with the Syn5 phage which infects *Synechococcus* sp. strain WH8109 and the P-SSP7 phage which infects *Prochlorococcus* sp. strain MED4. Additional T7-like cyanophages were used for some assays and include the S-TIP37 phage that infects *Synechococcus* sp. strain WH8109 and the P-TIP2, P-TIP38 and P-TIP42 phages, all of which infect *Prochlorococcus* sp. strain MED4. Viral lysates were prepared by propagating phages on their hosts. Cell debris were removed by filtration and the <0.2µm filtrates were collected and stored at 4 °C.

Seawater sample collection and handling

Red Sea samples were collected in the Gulf of Aqaba on 19 September 2012 and 13 May 2014 from the pier of the Interuniversity Institute for Marine Sciences in Eilat, Israel. Samples for the depth profile were collected using the Research Vessel, Sam Rothberg, on 4

April 2013 at ~1:00 PM local time from Station A (29°27.97' N, 34°55.68' E) in the middle of the Gulf of Aqaba. Water column salinity, temperature and in-situ chlorophyll *a* fluorescence levels were determined from the Sea-Bird Scientific CTD (SBE 19plus V2 SeaCAT). Mediterranean Sea samples were collected on 29 December 2012 and 4 February 2013 from the shore or 1 mile off shore opposite the Ruppin School of Marine Sciences at the Michmoret campus on the coast of Israel.

Seawater samples were poured over 20 µm mesh plankton netting into amber collection bottles over. Samples for polony analysis were also filtered through 0.2 µm acrodisc syringe filters (Pall Life Sciences) and the filtrate, containing the viral fraction, was collected in sterile polypropylene 50 ml tubes (Greiner Bio-One). The samples were kept at room temperature for 12-24 hours during transportation back to the lab and analyzed fresh, or frozen in liquid nitrogen and stored at -80 °C.

Samples for determining *Synechococcus* and *Prochlorococcus* abundances by flow cytometry were fixed in 0.125% glutaraldehyde for 20 minutes prior to flash freezing in liquid nitrogen and subsequent storage at -80 °C. The LSR II flow cytometer (Becton Dickinson) was used to enumerate populations based on their autofluorescence and side scatter. It should be noted that surface populations of *Prochlorococcus* are underestimated due to low levels of pigments per cell.

Sample concentration

Phage lysates and seawater samples were concentrated 50-fold using a slight modification of the iron chloride flocculation method⁴¹. FeCl₃ was added to the samples at a final concentration of 0.18 mM and incubated at room temperature for at least 1 h. Flocculated phages were centrifuged for 10 min at 17,000 Xg at 10 °C. The flocculants were resuspended in a buffer containing 0.125 Tris, 0.1 M Na₂EDTA and 0.125 M oxalic acid, pH 6. Magnesium chloride was omitted from this buffer (in comparison to the published method) due to the formation of precipitant in the buffer within a short period of time.

The efficiency of concentration was initially tested using P-SSP7 phage lysates at a concentration of 10⁵ phage·ml⁻¹. We simulated total virus-like particle concentrations in the oceans by adding 10⁷ phage·ml⁻¹ of the Syn9 phage, which belongs to the *Myoviridae* family of tailed phages.

Primers and probes for polonies

Degenerate primers and probes for polony analyses were designed based on the known diversity of T7-like cyanophages^{35,40,46} and empirically tested (see Supplementary Text). The primers used in this study after optimization were 341Fd-15-NNN and 534Rd (see Supplementary Table 1) and yield amplicons for different T7-like cyanophages that are 578-584 bp in length. We used metagenomics datasets and clone libraries to bioinformatically verify that these primers match environmental sequences that phylogenetically cluster with the T7-like cyanophages but not other T7-like phages. The forward primer (341Fd-15-NNN), without considering the 3 random nucleotides at the 5' end, and reverse primer (534Rd) have degeneracies of 1024 and 2048, respectively. The reverse primer is acrydite modified at the 5' end.

Degenerate probes for polony analyses are complementary to the 5'-acrydite anchored strand and are modified with either Cy5 or Cy3 at the 5' end. They were designed to be internal to the amplicon to ensure specific detection of T7-like cyanophages and prevent the detection of non-specific polonies and primer-dimers. These probes were also designed to differentiate between clade A and clade B T7-like cyanophages and were positioned in the region of amino acid 405 of DNA polymerase (see Supplementary Table 1). The probes were designed with a maximum of 3 inosines which enabled us to reduce the degree of degeneracy of the probes to 16 for the clade A probe and 128 for the clade B probe. The probes are modified with Cy3 or Cy5 fluorophores at their 5' end.

Primers and probes are HPLC purified.

Polony procedures

The polony method was initially developed by Mitra and Church²⁸. The methods and reagents described here for slide preparation, polony amplification and hybridization, are slight modifications of those found at http://arep.med.harvard.edu/polony/polony_protocols. All pre-PCR procedures were carried out in a sterile environment. All samples were analysed in duplicate polony assays.

Slide preparation and casting the gel—Polony gels were cast into a 25-40 µm deep oval well (24 x 14 mm at the longest axes) in the Teflon coating of custom-made glass microscope slides (Thermo Fisher Scientific). The slides were pretreated with Bind-Silane (GE Healthcare Life Sciences) to facilitate attachment of the polyacrylamide gel to the glass surface of the slide. Slides were placed in a mixture containing 0.4% Bind Silane and 0.022% acetic acid for 1 hour with gentle shaking and were then rinsed three times in MilliQ water and once in 100% ethanol. Slides were air-dried and stored for up to 3 months in a vacuum desiccator. Prior to gel casting the slides were irradiated with UV light for 15 min.

The 10% acrylamide gel mix contained 9 parts 40% acrylamide solution (IEF acrylamide from GE Healthcare Life Sciences or BioRad cat #161-0140) and 1 part acrylamide/bisacrylamide (38:2) (Sigma-Aldrich). The gel mix was filtered through a 0.22 µm PVDF syringe filter unit (Millex-GV, Millipore) and combined with 0.2% bovine serum albumin (BSA), 5'-acrydite-modified reverse primer (Eurofins MWG Operon) (see Supplementary Table 1 for primer sequences) and up to 2.5 µl of phage template in seawater. The 5'-acrydite-modified primers were used at a concentration of 1 µM for specific primers and 20 µM for degenerate primers. The acrydite modification becomes covalently attached to the polyacrylamide matrix and serves to anchor the primer and resulting amplicon to the gel.

Gels (11.6 µl) were cast immediately after the addition of 0.1% ammonium persulfate and 0.1% tetramethylethylenediamine (TEMED). Gels were covered with a cover slip (18x30 mm) and allowed to polymerize for 30 min in a chamber filled with argon. After removal of the cover slip, the gels underwent 3 sequential washes of 10 min with gentle shaking: two in MilliQ water and one in 0.025% Tween-20 (Sigma-Aldrich). The slides were air-dried for 20-30 min.

PCR reagents and conditions—Twenty μ l of the following mix of PCR reagents were diffused into the gel: 1x *Taq* buffer with magnesium chloride, 0.25 mM deoxyribonucleotide triphosphate (dNTP) mix, 0.2% BSA, 0.1% Tween-20, unmodified forward primer (see Supplementary Table 1) and Jumpstart *Taq* polymerase (Sigma-Aldrich). When using specific primers, 0.5 μ M forward primer and 3.4–6.8 U of *Taq* polymerase were used, whereas 15 μ M forward primer and 13.4 U of *Taq* polymerase were used with degenerate primers. The mix was applied to the center of the gel and covered immediately with a cover slip. A Secure-Seal hybridization chamber (Grace Biolabs, SA500) was applied to the top of the slide and was filled with approximately 600 μ l mineral oil (Sigma-Aldrich).

PCR cycling was initiated with a 5 min heat step at 94 °C that served both to disrupt phage capsids and make the DNA accessible for PCR and as a hot-start for the Jumpstart *Taq* polymerase. PCR cycling then consisted of 50 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and elongation at 72 °C for 2 min when using degenerate primers. Polony formation with specific primers was carried out for 35 cycles with the annealing step at 60 °C for 30 s. The PCR procedure was ended with a 6 min elongation step at 72 °C. Thermocycling was carried out in a twin-tower slide thermocycler (DNA Engine with dual block slide chamber, BioRad).

After PCR cycling, the secure-seal chambers were removed and the slides were washed in hexane for 5 min. After removing the cover slips the slides were washed twice for 4 min with gentle shaking in wash buffer E (10 mM Tris (pH7.5), 50 mM KCl, 2 mM EDTA (pH8.0), 0.01% Triton X-100).

Hybridization of probes to PCR amplicons—PCR amplicons were denatured in a solution of 70% formamide in 1X SSC buffer (150 mM NaCl, 15 mM sodium acetate) for 15 min at 70 °C with gentle shaking. The unanchored strand of DNA was washed out of the gel with three successive washes at room temperature with gentle shaking: once in MilliQ water for 3 min and twice in wash buffer E for 4 min.

The gels were dried and a Frame-Seal slide chamber (BioRad, SLF1201) was applied to the slide. Hybridization mix (140 μ l) containing the fluorescently labelled probe (see Supplementary Table 1 for probe sequences) was applied to the center of the gel, and covered with the chamber seal. The hybridization mix consisted of a 0.6 μ M 5'-Cy5-modified probe (Sigma) in 6x SSPE (900 mM NaCl, 90 mM NaH_2PO_4 , 6 mM EDTA) and 0.1% Triton X-100. The gel was heated to 94 °C for 6 min in the thermocycler and then hybridized at 55 °C for 15 min for specific probes and 42 °C for 30 min for degenerate probes. After removal of the Frame-Seal chamber the slide was subjected to 3–4 successive washes in wash buffer E at room temperature with gentle shaking for approximately 25–30 min each wash. In cases where a high level of fluorescence background was still found, an additional wash was carried out overnight.

Stripping of probes is carried out using the same procedure as described for amplicon denaturation, and annealing of additional probes is carried out as described above. Slides can be stored in buffer E at 4 °C for at least 2 years.

Microarray scanning and image analysis—Prior to scanning the slides excess buffer E was removed with a short centrifugation step of 1 min at 45 Xg at room temperature. Gels were scanned on a GenePix 4000B microarray scanner (Axon Instruments) using GenePix Pro software (version 5.0). Good signal-to-noise ratios were achieved for Cy5 detection using the 635 nm laser and Cy3 with the 532 nm laser with the following settings: PMT Gain of 550, 100% power, 10 μ m pixel resolution, and 20 μ m focus position.

Images were acquired in TIFF format and analyzed using ImageJ software⁵⁰. A 0.1 cm² grid was overlaid on the images and manual tagging tools were used to count colonies in each grid. Grids in which the gel was damaged were not included in the analyses. Gels were not used when colony distribution was not fairly even across the gel or in which colonies were overlapping due to high concentrations. The average number of colonies per grid area was used to determine the total number of colonies in the gel.

Colony picking and sequencing

A microarray image of the gel was used to map colonies onto a piece of paper at a 1:1 scale that was then placed under the slide to facilitate colony picking. The probe was removed in a 70% formamide solution at 70 °C using the same procedure as described for amplicon denaturation. An additional wash in MilliQ water was carried out for 3-5 min. Since the PCR amplicon is covalently attached to the gel, it was necessary to physically excise a piece of the gel with the colony. Approximately 2 mm of the gel was excised using two sterile toothpicks when the gel was still moist. Picking is best carried out when colonies are at least 3 mm apart. With the protocol used here with degenerate primers for T7-like cyanophages, this generally occurred when there were fewer than 50 colonies per slide.

The piece of gel is placed in a 24 μ l PCR reaction volume with 1X Opti-Buffer, 3 mM MgCl₂, 0.2 mM dNTPs and 2 U BIO-X-ACT Short DNA polymerase (Bioline). PCR of the colony is carried out using 2 μ M of the same forward and reverse primers as for colony formation but without the acrydite modification. After an initial 5 min denaturation step at 95 °C, 40 PCR cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s and elongation at 70 °C for 1 min were carried out followed by a final elongation step at 70 °C for 5 min. The resulting amplicons were extracted from agarose gels using the QIAquick Gel Extraction kit (Qiagen), cloned into the PCRII Topo TA cloning kit (Invitrogen) and transformed by electroporation into competent DH5 α *E. coli* cells. Plasmid DNA was sequenced by Sanger sequencing.

Quantitative PCR assays

Comparisons of quantitative PCR assays were carried out with specific and degenerate primer sets designed at the same positions on the DNA polymerase gene. Genomic DNA of the Syn5 and P-SSP7 phages were used for most analyses. S-TIP37 phage DNA was used for SYBR Green qPCR assays instead of P-SSP7 due to the lack of suitability of P-SSP7 for this assay (see Supplementary Text). Standard curves of dilution series were carried out for each PCR assay type and used for quantification.

SYBR Green qPCR—SYBR Green qPCR reactions were carried out in duplicate 20 µl reaction volumes containing 1X LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics), 0.5 µM both forward and reverse specific primers or 20µM forward and 10 µM reverse degenerate primers (Sigma). The phage gDNA templates were added in 5 µl volumes per reaction.

Reactions were carried out in a LightCycler® 480 system (Roche Diagnostics). Thermocycling consisted of an initial denaturation step of 10-15 min at 95 °C, and 45 cycles of denaturation at 95 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 45 s. Fluorescence plate read measurements for quantification were performed at the end of each cycle at 84 °C for the Syn5 and S-TIP37 phages (see Supplementary Text) when using both specific and degenerate primers sets. Amplification efficiencies were calculated from the slope of the threshold cycle values (Cp) plotted against the logarithm of starting gDNA concentrations by using the following equation: $E = 10^{(-1/\text{slope})} - 1$.

TaqMan qPCR—Duplicate qPCR assays were carried out in 20 µl reactions containing 1X LightCycler® 480 Probes Master Mix (Roche Diagnostics), 5 µl phage genomic DNA template in 10 mM Tris-HCl (pH 8.0), 0.5 µM phage-specific forward and reverse primers or 5 µM degenerate primers. Probes were added at a concentration of 0.1 µM for both specific and degenerate probes (Roche, Eurofins MWG Operon). Both primer and probe concentrations were experimentally optimized. The real-time qPCR probes are dual-labeled with a reporter fluorophore (FAM) and a dark quencher dye. Sequences of primers and probes are listed in Supplementary Table 1.

All qPCR experiments were performed using the LightCycler® 480 system (Roche Diagnostics) using the same thermocycling conditions as for the SYBR Green qPCR assays but without fluorescence plate reads. qPCR data analysis was done using the LightCycler® 480 software version 1.5 (Roche Diagnostics).

Droplet digital PCR—Digital droplet PCR assays were carried out using a QX100 Droplet Digital PCR System (BioRad) in duplicate TaqMan-type PCRs as assays based on nucleic acid dyes could not be used with degenerate primers (see Supplementary Text). The 20 µl ddPCR reaction mixture consisted of 1X ddPCR master mix (Bio-Rad), 5 µl phage genomic DNA template, 0.5 µM phage-specific forward and reverse primers or 5µM degenerate primers, and 0.1 µM of either specific or degenerate probe (Roche, Eurofins MWG Operon). Primer and probe concentrations used were those found to be best by the TaqMan procedure.

In order to generate droplets, the reaction volume was mixed with 70 µl of droplet generation oil (Bio-Rad) using the QX100 droplet generator (Bio-Rad). The droplets generated from each sample were transferred to a 96-well plate for PCR amplification, which was carried out on a Sensoquest labcycler (Sensoquest GBH). The thermocycle conditions for ddPCR were the same as those used for SYBR Green and TaqMan qPCR reactions except that fluorescence plate reads were not performed.

Following amplification, the droplets were read on the QX100 droplet reader. Data analysis was done using the QuantaSoft software version 1.6.6. (Bio-Rad). Samples for which one of the technical replicates gave a high random signal while the other replicate was zero were not included in the analyses. The assays were carried out at the Microarray Unit of the Faculty of Life Sciences at Bar Ilan University.

Testing storage and preservation conditions

In order to test storage and preservation conditions for field samples to be analyzed with the polony method, four independent samples were collected from the Mediterranean Sea on 4 February 2013 and analyzed within 24 h of collection. Aliquots of the samples were stored at -80 °C either without fixative or fixed in 0.1% glutaraldehyde or 0.8% formaldehyde in Eppendorf tubes and analyzed after 5 months and 18 months of storage. Three to four independent samples were analyzed for cyanophages belonging to clade B.

In order to assess whether it is possible to freeze and thaw stored samples multiple times, we carried out 1, 2 and 5 freeze-thaw cycles 5 months after storage of the above unfixed samples. Three independent field samples were analyzed for clade B cyanophages. It should be noted that freeze-thaw cycles made phage DNA susceptible to DNase treatment, determined for both the Syn5 phage and field samples, indicating that while the polony quantification was not detrimentally affected by the freeze-thaw cycles, this likely disrupted phage capsids and made the DNA accessible to degradation by DNase.

DNase treatment

Experiments to assess the most appropriate concentration of DNase I to use in seawater samples were carried out with Syn5 phage lysates (2×10^7 phage·ml⁻¹) and plasmid DNA (TOPO TA, Invitrogen) containing an insert of the Syn5 portal protein gene (50 ng·ml⁻¹) in 0.02 µm-filtered and autoclaved Mediterranean Sea seawater. The Syn5 phages or plasmid DNA were incubated with DNase I (bovine pancreas, Sigma) in separate reactions at concentrations ranging from 0-40 Kunitz units·ml⁻¹ at 37°C for 1 h. The enzyme was inactivated by the addition of 50 mM EDTA (pH 8.0).

The amount of phage and plasmid DNA was determined by Taqman qPCR after diluting the samples 100-fold in 10 mM Tris buffer (pH 8.0) to eliminate problems of amplification efficiency in seawater samples. Reactions (25 µl) contained 1X LightCycler® 480 Probes Master Mix (Roche Diagnostics), 0.5 µM Syn5 portal protein forward and reverse primers and 0.1 µM probe (Roche Diagnostics) (see Supplementary Table 1 for primer sequences), 10 µl diluted Syn5 phage or plasmid DNA template. Thermocycling consisted of 15 min at 95 °C, and 45 cycles of 95 °C for 10 s and 60 °C for 30 s. Fluorescence measurements were conducted at the end of each cycle at 60 °C. The experiment was carried out twice with duplicate analyses for each sample.

Triplicate seawater samples for DNase treatment were collected and filtered freshly over 0.2 µm syringe filters. Samples (20 µl) were treated freshly (without freezing and thawing) with DNase I at 5 Kunitz units/ml and subjected to the polony procedure. The number of polonies were compared to those in the same freshly collected samples that had not been treated with DNase.

Phylogenetic analyses

Multiple nucleotide alignments were carried out with MUSCLE in the MEGA7 program⁵¹ and verified visually. Nucleotide sequences (263 positions) were used to generate optimal Neighbor-Joining and Maximum Likelihood trees in the MEGA7 program. Evolutionary distances were computed using the Maximum Composite Likelihood method and the units are the number of nucleotide substitutions per site. Gaps and missing data were excluded from the analyses. Bootstrap analyses (1000 repetitions) for both Neighbor-Joining and Maximum Likelihood trees were carried out and values greater than 50% are presented.

Statistics

Two-tailed, paired t-tests were performed to assess significance of differences in storage and preservation conditions, freeze-thaw cycles and DNase treatment. The data were normally distributed as determined from the Shapiro-Wilk test. The alpha was corrected for multiple comparisons using the Bonferroni correction but did not change the significance of the results.

When normally distributed, as determined from the Shapiro-Wilk test, one-way analysis of variance (ANOVA) was carried out to determine whether there were differences among samples collected 2 min apart on the same day. When differences among samples were found, Bonferroni Post-Hoc tests were performed to determine which of the samples were different. For a non-normally distributed sample, May 2014 sample 2 clade A phages, the Mann-Whitney-Wilcoxon Test was used to compare it to sample 3.

Regression curve analyses and its use to assess population abundances

Regression curve analyses of phage-to-polony conversions were carried out for 5 cultured phages that are spread across the phylogenetic tree (Fig. 1b). The number of input phages was determined from epifluorescence microscopy counts of SYBR Green stained particles¹⁰. Polony formation was carried out after removing free DNA from phage lysates using the DNase I protocol described above. Degenerate primers and probes for the DNA polymerase gene were used for polony amplification. The number of samples used for regression curve analyses for each phage were as follows: n=37 for Syn5, n=8 for P-SSP7, n=8 for P-TIP38, n=7 for P-TIP2 and n=24 for P-TIP42.

Major axis regressions were carried out for each of the 5 phages using the 'Lmodel2' package in R⁵². Intercepts were set to zero as no-phage controls did not yield colonies. The individual curves, slope and R^2 values are presented in Fig. 1d. The average of the 5 slopes ($y=0.404x$) is used to calculate the average number of phages from the number of colonies measured in seawater samples. Alternatively, the average of 10,000 bootstrap resamplings of the 5 slopes is used to calculate the abundance of phage in seawater samples (see below). These two methods give the same results, with less than 1% variation between the methods. The average of at least two technical replicate slides is used per sample. The 95% confidence intervals of the method for single samples (as for the depth profile) are the 95% quantiles of the bootstrap analyses.

Two-tailed t-tests were used to assess whether the numbers of phages on different dates were significantly different and whether the number of clade A and clade B phages were significantly different in the same seawater sample. We also wanted to assess whether significant differences were maintained when we take into account the uncertainty of the composition of the phage population with respect to differences in phage-to-polony conversion efficiencies. To do this we carried out 10,000 bootstrap resamplings using the ‘stats’ package in R52. One bootstrap sampling was carried out as follows: Measured polony abundances were randomly sampled n number of times with replacement (n =the number of independent samples) and the average of the n samplings was calculated; the average of 5 random samplings of the polony-to-phage conversion efficiency slopes (for 5 slopes), with replacement, was also calculated; the product of these two averages was then determined. Bootstrap confidence intervals of the quantiles were determined using the ‘stats’ package in R52.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Kun Zhang and George Church for help with initial set up of the polony method in our lab, Brady Cunningham for advice on iron chloride flocculation, Lindell lab members for many helpful discussions and ideas during the development of the method, Ido Izhaki, Gitai Yahel and Maksim Bocharenko for advice on statistical analyses, Roy Kishony for use of lab facilities, and the Interuniversity Institute for Marine Sciences of Eilat and the Ruppin School of Marine Sciences for access to sampling facilities and Bar Mosevitzky for sample collection in September 2012. We also thank Sarit Avrani, Oded Beja, Mya Breitbart, Michael Carlson, Yael Mandel-Gutfreund, Gazalah Sabehi, Daniel Schwartz, Dror Shitrit and Joshua Weitz for comments on this or an earlier version of the manuscript. This research was funded by the European Council FP6 Marie Curie Reintegration grant no. 046549, the Israel Science Foundation Individual grant no. 749/11, the European Research Council Consolidator Grant 646868 and the Mallat Family Fund and Cullen Fund from the Technion awarded to D.L.

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Figure 1. Development of the polony method for viral ecology.

(a) Diagram of the polony method adapted for the quantification of diverse viruses from seawater samples. (b) Polony formation for the P-SSP7 phage and a no-phage control using degenerate primers for the DNA polymerase gene. Slides are representative of 34 replicate experiments. (c) Polony formation with degenerate primers of a clade A (Syn5) and a clade B (P-SSP7) phage hybridized with genotype-specific and degenerate clade-specific probes. Polonies on the same slide were sequentially hybridized to the three different probes. Slides are representative of 6 replicate experiments. (d) Virus-to-polony conversion efficiencies and standard curves of polony formation using degenerate primers and probes determined for 5 different phages spread across the phylogenetic tree (see Fig. 4). The number of input phages was determined by epifluorescence microscopy counts of lysates. The lysates underwent DNase treatment to remove free DNA prior to the assays.

Figure 2. Comparison of the polony method to other quantitative PCR methods.

(a) Comparison of quantification using the polony, droplet digital PCR (ddPCR) and the SYBR Green and Taqman qPCR methods with specific and degenerate primer and probe sets. Two concentrations of genomic DNA from the Syn5 phage were used: S1=10⁴ copies·ml⁻¹ and S2=10⁶ copies·ml⁻¹ (50 and 5,000 copies per reaction, respectively). Results shown are representative of 2 independent experiments for the polony and SYBR qPCR methods and 3 independent experiments for the Taqman qPCR and ddPCR methods. Two technical replicates were carried out for each method in each experiment. The same results were also obtained for the P-SSP7 phage (see Supplementary Fig. 1). (b) Simulation of differences in viral quantification using degenerate primers in qPCR assays for two phage genotypes that amplified with different efficiencies. The simulation is based on the 25% difference in amplification efficiencies in SYBR Green qPCR assays of 46.6 ± 3.3 and 72.2 ± 2.5% for Syn5 and S-TIP37, respectively (n=5 independent experiments). Differences of 625 fold are found after 40 cycles.

Figure 3. Analysis of T7-like cyanophages in coastal waters from the Red Sea using the polony method.

Samples were collected off the pier of the Interuniversity Institute for Marine Sciences on 19 September 2012 and 13 May 2014. (a) Averages and standard deviations of independent samples collected at 2 minute intervals in September 2012 (n=5) and in May 2014 (n=3).

Significant differences were found in abundances for total T7-like cyanophages on the two dates ($p=0.0005$, two-tailed t-test) and between clade A and clade B phages on each sampling date ($p=0.0002$ for Sept and $p=7.9 \times 10^{-5}$ for May, two-tailed t-test). (***) $p<0.001$.

(b) Sample-to-sample variability for samples collected at 2 minute intervals. Each bar consists of results for a different sample and sample numbers (x-axis) represent the order of collection. Clade A and clade B phage abundances are shown in red and blue respectively. Averages and standard deviations of the technical replicates (polony slides per sample) are shown for each sample. For the September 2012 samples n=6, 6, 8, 4 and 5 technical replicates for samples 1, 2, 3, 4 and 5, respectively, for analysis of both clade A and clade B phages. For the May 2014 samples n=2, 3 and 4 technical replicates for samples 1, 2 and 3, respectively, for clade A phages; and n=3, 4 and 4 technical replicates for samples 1, 2 and 3, respectively, for clade B phages.

Figure 4. Neighbor-Joining tree of the DNA polymerase gene from T7-like cyanophages and sequenced polonies.

The neighbor-joining (NJ) tree was inferred from 263 nucleotide positions for cyanophage isolates (appearing in ref30,35) and polonies from this study (designated as P# #). Bootstrap values >50% for NJ and maximum likelihood (ML) trees are shown (NJ/ML). The T7 DNA polymerase gene was used as an outgroup. Blue diamonds denote polonies identified with the clade B probe, red diamonds denote polonies identified with the clade A probe and green circles denote cyanophages used in virus-to-polony conversion efficiency analyses. Twenty-five polonies were picked and sequenced from September 2012 Red Sea samples. Clade A

and clade B phage clusters are marked on the right. The scale bar shows nucleotide substitutions per site.

Figure 5. Depth profiles of T7-like cyanophages and cyanobacteria collected from the Gulf of Aqaba, Red Sea during the spring bloom on 4 April 2013.

(a) Density of the water column determined from in-situ measurements of salinity and temperature, (b) chlorophyll *a* fluorescence determined from in-situ measurements, (c) cyanobacterial abundances determined from flow cytometry, (d) abundances of clade A T7-like cyanophages determined by the polony method after 50-fold concentration, (e) abundances of clade B T7-like cyanophages determined by the polony method, and (f) the ratio of clade B to clade A T7-like cyanophages. Results are from a single sample collected at each depth with 3 technical replicates for cyanobacterial counts and 2 technical replicates for cyanophage abundances.

Table 1
Comparison of current virus quantification methods with the polony method

Method type	Method#	Basis of method*	Level of discrimination	Comments
Particle-based	Transmission electron microscopy (TEM)1,12	Negative staining, virion morphology imaging	<ul style="list-style-type: none">Discriminates between viruses with different virion morphologiesIndiscriminant with respect to virus family and host type	<ul style="list-style-type: none">Requires high degree of expertise with electron microscopy
	Virus-like particles (VLP)10,11	Nucleic acid stained particles in viral fraction, epifluorescence microscopy or flow cytometry detection	<ul style="list-style-type: none">All dsDNA virusesIndiscriminant with respect to virus family and host type	
Culture-dependent	Plaque assay13,14	Infectivity, clearings in culture on semi-solid plates	<ul style="list-style-type: none">Quantifies viruses that infect host strain used for assay	<ul style="list-style-type: none">Limited to hosts that have been cultured
	Most probable number (MPN)13	Infectivity, lysis of liquid culture	<ul style="list-style-type: none">No differentiation between virus families (except viral tagging that provides relative quantif. of types)	<ul style="list-style-type: none">Not representative of viruses infecting diverse hosts within a host taxon of interest
	Viral tagging15	Adsorption to host, sequencing		
Sequence-based	Quantitative PCR (qPCR)18,42	PCR with specific primers, SYBR Green or Taqman PCR	<ul style="list-style-type: none">Quantify single virus genotype	<ul style="list-style-type: none">Require knowledge of virus sequence for primer and probe design
	Digital (dPCR)19 and droplet digital PCR (ddPCR)20	PCR with specific primers of spatially partitioned viruses		
	Loop-mediated isothermal amplific. (LAMP)21,22	Strand displacement amplification at constant temperature with multiple specific primers		
	PhageFISH23	Hybridization with multiple specific long probes		
	Metagenomics26,27	Sequencing of viral DNA after amplification	<ul style="list-style-type: none">Discriminates between all virus typesHost discrimination is possible for viruses with known hosts	<ul style="list-style-type: none">Relative quantification only.Time consuming. ExpensiveHost discrimination inferred from sequence information for viruses with known hosts
	Polony method	In-gel PCR of spatially separated viruses with specific and degenerate primers and probes for signature genes &	<ul style="list-style-type: none">Discriminates between viruses belonging to different families or generaDiscriminates between host taxa the viruses infect	<ul style="list-style-type: none">Requires knowledge of signature gene sequence diversity of virus taxa for primer and probe designHigh-throughputHost discrimination inferred from sequences of signature genes of viruses with known hosts

#The numbers refer to literature references for the methods
* All methods quantify infective and non-infective viruses except for the plaque and MPN assays which quantify infective viruses

Signature gene – gene common to all viruses within the taxonomic group of interest