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Abstract

We describe a Multiplex Primer Prediction (MPP) algorithm to build multiplex compatible primer sets for large, diverse, and unalignable sets of target sequences. The MPP algorithm is scalable to larger target sets than other available software, and it does not require a multiple sequence alignment. We applied it to questions in viral detection, and demonstrated that there are no universally conserved priming sequences among viruses and that it could require an unfeasibly large number of primers (~3700 18-mers or ~2000 10-mers) to generate amplicons from all sequenced viruses. We then designed primer sets separately for each viral family, and for several diverse species such as foot-and-mouth disease virus, hemagglutinin and neuraminidase segments of influenza A virus, Norwalk virus, and HIV-1.

Code and Primer Availability

Primer sets are available as supplementary material. The MPP software is available for academic and nonprofit use by contacting the authors.

Introduction

Researchers employ numerous approaches for viral detection and discovery, including metagenomic sequencing (Edwards and Rohwer, 2005; Nakamura, et al., 2009; Victoria, et al., 2009), microarrays (Kistler, et al., 2007; Lin, et al., 2007; Lin, et al., 2006; Palacios, et al., 2007; Quan, et al., 2007; Wang, et al., 2002), or multiplex PCR followed by other methods of characterization such as mass spectrometry (Briese, et al., 2005; Dominguez, et al., 2008; Ecker, et al., 2006; Lamson, et al., 2006; Sampath, et al., 2007), suspension arrays (Fox, 2007; Lenhoff, et al., 2008), or amplicon sequencing (Griffiths, et al., 2002). Multiplex PCR followed by analysis of amplified products fills a niche for viral identification when singleplex PCR has failed or there are a few dozen likely candidates, but the expense of metagenomic sequencing or high density microarrays is unwarranted.(Quan, et al., 2008) However, multiplex primer design for many highly divergent targets is challenging, since no universally conserved primers may exist, and finding sets of primers likely to function well in multiplex (e.g. isothermal T_m's, no primer dimers) adds to the complexity of finding conserved primer candidates. Primer design software that requires a multiple sequence alignment (MSA) as input can be problematic for diverse target sets, as MSAs can be difficult to construct, exhausting memory or available time before an alignment is completed. Even if an alignment does complete for divergent target sets such as all members of a family of RNA viruses or gene homologues across species, alignments may show little nucleotide sequence conservation, and multiple primers are required to amplify all targets. Considering the challenges of primer design for targets showing sequence variation, it is not surprising that many of the PCR-based assays in the literature are predicted to fail to detect desired targets when compared against available sequence data.(Lemmon and Gardner, 2008)

Most currently available multiplex primer prediction tools require multiple sequence alignment (Gadberry, et al., 2005; Jabado, et al., 2006; Jarman, 2004; Linhart and Shamir, 2002; Rose, et al., 2003). None of those tools build multiplex sets in which no primers in the set are predicted to form primer-dimers (although some avoid homodimers). We attempted to run a number of alternative software tools for multiplex or degenerate primer prediction for several species level target sets ranging in size from

41-~6000 sequences: Primaclade (Gadberry, et al., 2005), MuPlex (Rachlin, et al., 2005), FastPCR (Kalendar, 2009), GeneUp (Pesole, et al., 1998), PDA-MS/UniQ software (Huang, et al., 2005), Greene SCPrimer (Jabado, et al., 2006), and HYDEN (Linhart and Shamir, 2002). Only Greene SCPrimer and HYDEN could handle the two smallest, species-level target sets (Norwalk and FMDV), and none of the tools completed for the larger target sets that we attempted to run.

Therefore, we designed MPP software to avoid the requirement of a multiple sequence alignment and to scale better for large and diverse target sets. MPP builds a multiplex-compatible set of primers capable of amplifying all target sequences, attempting to minimize the number primers in the set. We set out to determine a set of highly conserved "universal" primers for viruses, akin to the highly conserved 16S rRNA universal primers for bacteria. Throughout, we use the term "detected" to mean that there should be at least one PCR product. This is a loose definition of "detected" adopted for convenience in the following discussions, and we recognize that a PCR product may prove insufficient for viral characterization. We predicted a set of "universal viral primers" for all available complete genomes of all viruses, and found that the number of universal viral primers would be impractical to implement, even if short, highly conserved priming sequences were used. Then we predicted family-level primer sets for every viral family, as well as for several highly diverse species of RNA viruses, for primers of a traditional length as well as non-traditional, shorter, more highly conserved primers, which are more likely to amplify novel, unsequenced viruses, and which could be an alternative to degenerate primers. While the software uses a greedy algorithm that may settle at a local minimum which is above the true minimal set, it generated primer sets for each viral family with fewer than half the number of primers that would be expected without optimization. Although family-level primer sets for some families are too large to be practical, 64% of the families had primer sets of no more than 20 primer pairs, quite feasible for multiplex PCR.

Finally, we empirically tested a set of 16 multiplexed short (10-mer) primers designed for the Poxviridae family. This demonstrated specific amplification of the expected viral fragment from vaccinia Lister strain. It did not produce a smear of non-specifically amplified products when tested against commercially purified viral template (a mixture of viral and cellular nucleic acids in unknown proportions), as might have been expected from a set of 16 multiplexed, short primers, even though no DNase treatment was used to enrich for encapsidated viral nucleic acids. This suggests that specific amplification with family-level multiplexed sets of short primers might be feasible for viral detection and discovery.

Methods

MPP algorithm for calculating multiplexed primer sets

Here, we outline a greedy algorithm used to calculate conserved sets of multiplexed primers to amplify fragments from each member of a target set of sequences, and provide a more detailed description in the supplemental methods. First, we enumerate all candidate oligos fitting user-specified requirements for length, T_m, and lack of hairpin formation. We rank pairs of these by the number of targets in which that pair occurs within a distance range d_1 bases to d_2 bases of one another, where these might be specified so as to bound a reasonable range for electrophoretic discrimination of bands or

sequencing. The most frequent pair is selected as primers. The process is repeated for the remaining targets that would not have an amplicon from the first pair, with the added consideration that new primers selected not form dimers with other primers already selected, based on nearest neighbor thermodynamic predictions (Markham and Zuker, 2005). There is an option to bin primers into reaction subsets, if desired. If selected, primers are added to a bin until that bin contains b primers, at which point a new bin is begun, following the same process. Binning primers in smaller groups avoids exclusion of the most highly conserved oligos because of primer dimer free energy constraints. The universal set of primers is the set of selected primers to amplify all genomes in the target set. The primers within a bin should be multiplexed into a single PCR reaction, but each bin should be run separately. Other binning strategies could be envisioned, such as starting a new bin with any primer pair that dimerizes with other previously selected primers regardless of the number of primers in the bin, but this could have the possible disadvantage of bin explosion of numerous singleplex or small multiplex reactions.

The script find_amplicons.pl predicts all the amplicons that should be generated by a list of (multiplexed) primers mixed with a set of sequences. The script produces amplicon sequences, their length and position, and the forward+reverse primer combination to yield each product, as well as a summary file of the fragment length distributions enabling a quick assessment of how well each target sequence can be discriminated based purely on amplicon length patterns. We used this script to check whether some viral primer sets are predicted to generate amplicons from the human genome.

For all T_m and free energy predictions, we used the following Unafold settings: [Na+]=0.2 M, [Mg+]=0.0015, annealing temperature=30°C, and strand concentration of each strand=10^{-7} M, making the total strand concentration of both strands=$2x10^{-7}$.

Imposing uniqueness requirements for target-specific priming

The MPP algorithm described here focuses on finding conserved primers, and does not require that the primers be family- or species-specific. For the runs predicting family-specific primers, we designed viral family primers that were unique relative to nontarget viral families by replacing any substring of 17 nt or longer that occurs in any non-target family with a substring of "N"'s, using the suffix array software vmatch (http://www.vmatch.de/). MPP eliminates oligos with N's from consideration as primers. This approach is simple, although it risks being overly strict by eliminating some potentially successful candidate primers, since it disallows those cases where a single non-unique primer pairs with a unique primer, a pair of non-unique primers are too far apart to actually generate an amplicon in a non-target sequence, or candidate oligos partially overlap a non-unique stretch of N's.

Runs predicting universal viral primers

For runs predicting universal viral primers including all viruses at once in the target set, all viral complete genomes and segments downloaded from publicly available sequence databases (Genbank, Baylor, TIGR) as of October 29, 2007 were used. Draft sequences with multiple contigs were merged into a single sequence entry, with contigs separated by 1000 N's, a stretch sufficiently long (greater than d_2) so that primer pairs

would not be designed to fall on different contigs, although there were very few draft sequences in contigs where this was necessary. Because of the large numbers of sequences in two families, only the MP segment sequences from Orthomyxoviridae and the L segment from Bunyaviridae were included, as these are the more conserved segments, reducing the number of targets by 23,017 sequences. The total number of target sequences for these all virus runs were 11,477. We predicted a multiplex set of universal 10-mers without binning the primers into separate reactions, but it was necessary to use a very low x_{dimer} and $x_{homodimer}$ of -11 kcal/mol to be possible to predict multiplexed primers to amplify every target. For the next calculations, we subdivided the primers into sub-reactions with 20 primers per reaction bin, to avoid excluding primers that were predicted to form primer dimers, and raised x_{dimer} and $x_{homodimer}$ to -7 kcal/mol. Primer sets of length 5-18 were predicted (Figure 1). To assess the effect of removing T_m constraints, we generated universal primer sets with length but no T_m requirements, and compared the primer counts to those with T_m constraints (Figure 2).

We evaluated how the growth of sequence availability could affect the size of the universal set of viral primers, as well as our ability to detect unknown/unsequenced viruses. We predicted a universal viral primer set for all viral genomes and segments available as of January 1, 2004, totaling 9965 sequences, for primers of length 7-15 nt (Figure 2). It was not necessary to exclude any Orthomyxoviridae or Bunyaviridae segments, because these segments were not so deeply sequenced at that time. We then determined how much of the 2007 sequence data would have been detectable using the 2004 primer sets (Figure 3).

To predict how contamination with human DNA might affect the ability to detect specific amplification of viruses, the average number of amplicons for the human genome was also predicted based on these primer sets with 20 primers per bin (Figure 4). The effect of reducing the multiplex size to 10 primers per reaction on human genome amplification was calculated by dividing priming sets in half, with 10 primers per reaction.

Viral family primers

Subdividing viral targets into families, we used an updated set of sequences, downloaded February 5, 2009. For family-level primers, we computed primer sets using 3 alternative parameter settings (Table 1): 1) 17-21-mer primers with T_m=55-60°C, primers not checked for uniqueness, 2) Same length and T_m specifications as in 1) but eliminating from consideration as primers any k-mers ≥17 nt that occur in non-target viral families, as described above, and 3) 10-15-mer primers with T_m=40-45°C.

Species level primers for several divergent species

We also generated primer sets for several species with high sequence diversity: HIV-1, foot-and-mouth-disease virus (FMDV), Norwalk virus, and influenza A segments HA and NA (summary in Tables 2 and 3). MPP parameters were the same as in Table S1, with T_m=35-50°C for 10-mer primers and T_m=55-70°C for 17-18-mer primers. To illustrate the challenges of designing primers from an alignment, we aligned these organisms using MUSCLE (Edgar, 2004) when possible. For the HIV-1 and influenza A segments HA and NA, MUSCLE ran out of memory before completing. An alternative alignment tool, Clustalw (Chenna, et al., 2003), had completed only a small fraction of

the alignment after running for days. So for these large data sets, a random selection of ~35 sequences for each target was aligned with MUSCLE, and this alignment was used to build an HMM (hmmbuild) using HMMer (http://hmmer.wustl.edu/).(Eddy, 1998) The full sequence set was then aligned to the HMM using hmmalign. For Norwalk virus and FMDV, we designed multiplex-degenerate primer sets using Greene SCPrimer (Jabado, et al., 2006) and HYDEN (Linhart and Shamir, 2002), but the other three targets sets were larger than those programs would allow. None of the other primer prediction programs we tried (as indicated in results) would scale to handle even these two smallest target sets.

Emprical testing of Poxviridae multiplex primers

For an empirical demonstration of our algorithm, we tested a multiplex set of 16 short, 10-nt primers designed for the Poxviridae viral family against purified vaccinia virus extracts. The primer sequences are provided in Table 4 along with the predicted single amplicon for vaccinia lister strain. We chose the Poxviridae family multiplex for these first empirical demonstrations because extracted viral nucleic acid was readily available for experiments. All primers were purchased from Integrated DNA Technologies (Coralville, IA) and resuspended to 100 μM stock solutions in TE buffer (pH 8.0, Teknova, Hollister, CA). Working solutions containing equimolar concentrations of each of the 16 primers were used in all experiments. Purified, quantitated vaccinia Lister strain DNA was purchased at a concentration of $1.3x10^4$ copies/μL in nuclease-free water from Advanced Biotechnologies, Inc. (Columbia, MD). All PCR experiments were prepared using the Superscript III RT-PCR kit from Invitrogen (Carlsbad, CA). We selected the RT-PCR kit in order to establish a protocol that could be later be readily applied to additional multiplex viral family reactions with viral DNA and/or RNA. Each 25 uL reaction contained 1× SSIII buffer, 1 U of SSIII RT/Taq enzyme, 4.8 mM $MgSO_4$, 0.1 μM each primer, and a viral template mass of 2.7 pg (~10^4 copies). Tests were performed in triplicate and corresponding negative controls were run under identical conditions except that viral template was replaced with nuclease-free water (Ambion, Austin, TX). All reactions were thermocycled on the Bio-Rad DNA Engine (Hercules, CA) as follows: 1 cycle of 2 min at 94°C; 40 cycles of 15 sec at 94°C, 30 sec at 43.9°C, 1 min at 68°C; 1 cycle of 5 min at 68°C. In line with our goal to create a protocol applicable to both DNA and/or RNA templates, we verified that inclusion of an RT step (1 cycle of 30 min at 45°C) does not alter the outcome of the subsequent PCR when working with DNA template (data not shown). As such, for all Poxviridae 16-plex results reported here, an RT step was not used. We also note that PCRs were first conducted at the standard concentration of $MgSO_4$ (1.6 mM) included in the 1× SSIII buffer, and it was determined that a 4.8 mM $MgSO_4$ concentration was needed for optimal amplification with the short primer multiplex. Lastly, we mention that the selected annealing temperature was determined via annealing temperature gradient experiments. A more detailed discussion on the optimization of reaction conditions for amplification with short primer viral multiplexes is the subject of a separate paper being prepared by the authors (A.L. Hiddessen *et al*.).

This multiplex was compared against the human genome, predicting 233 amplicons between 50 and 1000 bp from the Poxviridae 16-plex. For empirical tests against background human nucleic acids, we followed the same reaction conditions as

above. In these tests, vaccinia DNA was held at a constant concentration of 2.7 pg, and serial dilutions of human genomic DNA (Novagen, Madison, WI), were added to the vaccinia PCR reaction mix, starting at 2.7 pg and titrating down over 4 orders of magnitude to $2.7x10^{-4}$. These experiments were performed using mass ratios of vaccinia:human DNA at 1:1, 10:1, 100:1, 1000:1 and 10000:1.

All PCR experiments were analyzed on 3% agarose TBE gels containing ethidum bromide that were purchased from Bio-Rad (Hercules, CA). Blue juice™ 10× loading dye was purchased from Invitrogen (Carlsbad, CA) and diluted to 2× before use. A 50 bp DNA ladder was purchased from Novagen (Madison, WI). For analysis, 15 µL from each separate 25 µL PCR reaction were combined with 2 µL of of loading dye, and 15 µL of the loading-dye/product mixture was loaded per well and electrophoresed for 1 hr 40 min at 85 V.

For confirmation of amplicon sequence, 9 µL of amplified PCR product was mixed with 4 µL of ExoSap-it (USB, Cleveland, OH) and incubated at 37˚C for 15 min followed by a denaturation step at 80˚C for 15 min. Sanger sequencing was then performed with the BigDye V3.1 Terminator Kit (Applied Biosystems, Inc., Foster City, Ca). Single reactions contained 4 µL of Ready Reaction Mix, 0.2 µM primer, 2 µL 5× Sequencing Buffer, 11.5 µl of nuclease free water (Applied Biosystems, Inc.) and 2 µL of post-Exosap-it PCR product. The sequencing reaction used the following thermocycling profile: 1 cycle of 94˚C for 1 min; 25 cycles of 94˚C for 15 sec, 38˚C for 30 sec, and 60˚C for 4 min. 2 µL of sequencing reaction product was combined with 18 µL of Hi-Di^{Tm} Formamide (Applied Biosystems, Inc.) and run on the ABI 3130 (Applied Biosystems, Inc.). The results were analyzed using Sequencing Analysis v5.2 (Applied Biosystems, Inc.).

Results

Universal viral primers

Predicting a set of universal viral primers that are all mixed in a single large reaction, we predict that 1008 primers of length 10 nt would be required to ensure amplification of at least one fragment of length 80-620 bp from every viral genome. These are predicted to generate a mean and maximum number of amplicons per viral genome of 13.2 and 948, respectively. Binning the primers into smaller sets of 20 primers/bin doubled the number of primers required, as Figure 1 shows the fraction of viral genomes amplified versus the number of primers, for primers ranging from 6-18 nt in length. About 2000 binned 10-mers are required to amplify 100% of sequenced viruses, generating a mean of 1 and maximum of 2.9 amplicons per genome, on average. Using traditional-length 18-mer primers nearly doubles the number to ~3700 primers. The incomplete curve for 7-mers was from inappropriate settings for this oligo length, since some genomes do not contain pairs of 7-mers with the required T_m, as given in the supplemental Tables S1-S2, within the desired amplicon length range. The concave curves showing diminishing returns reflect biased sequence availability rather than any particularly highly conserved primers: the primers that amplify the largest number of sequences are all from influenza, as far more sequences are available for this species than for any other.

Increasing availability of sequence data on universal primer sets

The increase in sequence data requires approximately 700 more 10-mer primers to amplify all sequenced viruses in 2007 compared to 2004 (Figure 2). While the increase in the number of sequences used between the two dates was only ~15%, the number of primers required increased by 48%, illustrating the substantial increase in diversity represented by the additional sequence data. Figure 2 also shows that removing all T_m constraints allows fewer primers to be used since no conserved primers are eliminated due to T_m, as some AT rich subsequences tend to be fairly conserved. Figure 3 shows that a universal primer set predicted using the 2004 sequence data would amplify only 35% of the 2007 sequences using primers of at least 10 nt in length. Shorter primers increase this fraction to over 60%, due to the higher likelihood of occurrence and conservation of shorter oligos, but even so, a multiplex of 7-mers is not guaranteed to amplify a fragment from every virus.

Predicting effects of contamination from human DNA

If all human DNA cannot be removed from the sample, simulations indicate that on average, multiplexes of 10-mer primers are expected to produce hundreds of amplicons from the human genome, which would appear as a smear on a gel (Figure 4). With primers of length 11-14, there is a large variation among bins. While most short sequences do occur in the human genome (Herold, 2008), an amplicon requires that two occur in proximity, and primers ≥11 bases make this a sporadic event for bins with only 10 primers. Nonetheless, imperfect sample purification to eliminate eukaryotic nucleic acids could be problematic for universal viral priming using primers shorter than 15 bases, particularly for multiplexes of 10 or more primers.

Family identification by sequencing products from universal 10-mer primer set versus randomly amplified fragments

If universal viral primers amplified fragments from a newly emerged virus, would the product sequences show similarity to others in the same family? We predicted amplicon sequences from 10 newly emerged viruses (Australian bat lyssavirus, Crimean Congo hemorrhagic fever, Nipah, Hendra, Venezuelan equine encephalitis, West Nile, Ebola, Hanta, hepatitis C, and Marburg viruses) using the universal 10-mer primer set and BLASTed (blastn) (Altschul, et al., 1997) those amplicon sequences against either other species in the same target family or viruses in other families. On average 87% of the amplicons had BLAST hits in the correct family, with an average of 135 hits per amplicon. In contrast, only 11% of amplicons had hits in other, non-target families, with an average of 2.5 hits per amplicon. For comparison, 72% of randomly selected fragments of 200 bp (or 65% and 78% for 100 bp and 600 bp fragments, respectively) had hits in the correct family with an average of 59 hits per amplicon, and 2.8% had hits in other families with an average of 2.4 hits per amplicon. Thus, universal primers do amplify more conserved regions than randomly selected fragments. These rough calculations depend heavily on available sequence data, and are likely to significantly overestimate our ability to characterize a truly novel virus, extrapolating from results of viral metagenomic sequencing in which over 60% of the sequences cannot be identified based on BLAST comparisons to existing sequence databases.(Edwards and Rohwer, 2005)

Viral family primers

The number of family-level primers for each family, and the number of genomes available for generating those primer sets, is given in Table 1, for 3 alternative settings: short primers of 10-15 nt with T_m 40-45°C, standard length primers of 17-21 nt and T_m 55-60°C, and the same but requiring that each primer subsequence ≥17 nt be unique to the target viral family relative to other viral families. At least one amplicon of 200-800 bp was required from every genome. Hypothetically, the worst case scenario to amplify a target set of N sequences would require 2N primers. MPP requires on average only 37% or 45% of this number, for primers of length 10-15 nt or 17-21 nt without the requirement for family specific primers respectively (averaged across families, omitting those families for which the computations were not run to completion). The most diverse families, in particular Bunyaviridae, Geminiviridae, Polydnaviridae, Reoviridae, Retroviridae, Siphoviridae, and Orthomyxoviridae require so many primers that actually applying family-level amplification is probably infeasible. For these, more restricted target sets may be necessary, such as limiting to a single segment for the segmented families or to subclades, and possibly the incorporation of primers with degenerate or inosine bases. Some families with many genomes can be amplifed with relatively few primers, such as Coronaviridae, Hepadnaviridae, Poxviridae, Togaviridae, Microviridae, and Polyomaviridae. Using primers of length 10-15-mers or 17-21-mers, 66% or 63%, respectively, of the viral families have primer sets of 40 or fewer primers (20 primer pairs), which is feasible for typical multiplexes. The sizes of the family-level primer sets show a trend for an increase with the number of available sequences in the family (Figure 5, p=0.14). There is no clear relationship between the number of primers in the set and whether the genomes are single or double stranded RNA or DNA. All family primer sequences are available as supplementary information.

Species-level primers for divergent RNA viruses

Primer design with the MPP software indicates that relatively few primers are required to amplify all sequenced genomes of HIV-1, FMDV, and Norwalk virus (Table 2). Influenza A HA and NA segments demand large numbers of 10-mer or 17-18-mer primers, so one could break these into subgroups, possibly by serotype, as shown for several HA serotypes in Table 3. The percentage of genomes amplified versus the number of primers used, for primers of either 10-mers or 17-18-mers, is shown in Figure 6. This plot shows that a large fraction of targets are amplified with only 2 primers, and the addition of subsequent primers shows diminishing returns in amplifying fewer, more divergent targets not detected by the initial, more conserved, primer pair, although the true diminishing returns depend on the extent to which available sequence data is an unbiased representation of diversity.

The more traditional method of attempting to find primers from a multiple sequence alignment would be problematic, probably requiring manually designed primer multiplexes or highly degenerate primers. For HIV-1, for example, there is not a single position with 100% conservation across all sequenced isolates. Dropping the required conservation down to 95% (58 of the 1175 genomes could disagree with a consensus base at any position), there are 3 conserved regions of at least 18 bases, with positions relative to the consensus: ACAGGAGCAGATGATACAGTA starting at position 3665;

TATGGAAAACAGATGGCAGG starting at 7347; and CTATGGCAGGAAGAAGCG starting at 9071. These regions are too far apart to be used as primers for most polymerases used in diagnostic PCR protocols, where amplicons must typically be less than 300 bases long for efficient amplification. A recently published study (Casabianca, et al., 2007) selected primers from the 5' LTR U5 end to the Gag-Pol start (5′-TAGCAGTGGCGCCCGA -3′ and 5'- TCTCTCTCCTTCTAGCCTCCGC -3'), but a comparison against available genomic data indicates that 487 of the 1175 genomes (41%) do not contain a sequence match for this primer pair, so may fail to be amplified.

For influenza A segment HA, the size of the longest conserved region from the 95% consensus is only 5 bases, and for segment NA, only 6 bases, insufficient for even a single primer. For FMDV and Norwalk virus, the longest 100% conserved regions are 9 and 6 bases, respectively. The MPP software makes it straightforward for a non-expert to predict a multiplex-compatible set of primers to amplify all targets, even for enormous and heterogeneous target sets that cannot be aligned.

Comparison with other software

For comparison, we considered other software options for designing primers for these hetergeneous viruses. We tried Primaclade (Gadberry, et al., 2005), but the webserver timed out for Norwalk and FMDV alignments. The link to the MuPlex (Rachlin, et al., 2005) server was not functional, and in any case the file size limit of 500 Kb would have been exceeded by all but the Norwalk data set. FastPCR (Kalendar, 2009) and GeneUp (Pesole, et al., 1998) were the only programs that did not require an MSA as input. The GeneUp program is no longer being maintained, and the web server is not functional. The FastPCR software did not complete "group-specific PCR" for the smallest data set, Norwalk virus, after running for 18 hours, and for "multiplex PCR" gave the error message "No compatible combination of pair primers for multiplex PCR found." The PDA-MS/UniQ software (Huang, et al., 2005) was not available for download or on a public web server. CODEHOP (Rose, et al., 2003) requires protein alignment as input so is not appropriate for whole-genome (nucleotide) alignments.

SCPrimer (Jabado, et al., 2006) did generate a number of degenerate primer candidates from the multiple sequence alignments for Norwalk and FMDV, requiring the user to manually select a combination of forward and reverse groups from a set of options. We ran SCPrimer using length, T_m, etc. settings mirroring or more lenient than those we used for Table 3 (T_m=55-65°C, GC%=20-80%, length 17-25 bp, 100% coverage, product size 80-620 bp, allowed T_m difference 10°C, others left as defaults). HYDEN (Linhart and Shamir, 2002) also generated degenerate candidates, although it does not check T_m and the length is limited to a single value rather than a range (we set it at 18 rather than 17 because of the lack of T_m control, and allowed 0 mismatches). The GreeneSCPrimer option requiring the fewest total primers for the Norwalk set required 18 primers, 4 of which had either 2-fold or 4-fold degeneracy so the actual number of priming sequences would be 26, compared to a total of 20 non-degenerate primers predicted by MPP (Supplementary information). HYDEN generated 4 degenerate primers covering only 34 of 41 sequences, each with 3- or 4-fold degeneracy for Norwalk, which translates to 15 priming sequences in the reaction. One would need to find primers to amplify the remaining seven sequences. Small degenerate priming sets (e.g. 4 primers in this case) are less expensive to purchase, but because of dilution effects from the many

sequence combinations actually present (15 priming sequences in the PCR), sensitivity may be reduced compared to nondegenerate priming. However, using a smaller set of degenerate signatures such as those from HYDEN may be preferable, and is a capability that could improve MPP in a future version.

For FMDV, MPP predicted 6 non-degenerate multiplex compatible primers to amplify all targets. GreeneSCPrimer generated a number of candidates, and manual inspection identified that the best of those primer combinations would require 6 primers, one of which had 2-fold and another had 3-fold degeneracy, totaling 9 actual priming sequences in a reaction (Supplementary information). This compares with 2 primers each with 4-fold degeneracy using HYDEN (8 priming sequences in a reaction), to amplify 98% (183 of 187) targets. Again, the small number of signatures predicted by HYDEN is desirable for some applications, although the degeneracy is high and these must be supplemented to pick up the few outlying sequences.

In summary, no software except MPP could run on the larger target sets we examined. Only HYDEN and GreeneSCPrimer completed for only the two smallest target sets. These softwares selected smaller sets of degenerate primers than the non-degenerate MPP primers. If target sets are sufficiently conserved so that a reasonable MSA can be built, and degenerate primers are acceptable, these tools may be preferable over MPP. But for larger and more diverse target sets, only MPP completed.

Poxviridae experimental results

Using the experimental conditions described in the Methods section, we tested the Poxviridae 16-plex (Table 5) against vaccinia virus, Lister strain DNA. As assessed by agarose gel analysis, we achieved specific amplification of the predicted 617 bp amplicon for the target vaccinia genome (lane 3, Figure 7). The band does not appear in the no template control shown in lane 4 (Figure 7).

To confirm that the amplicon observed in gel analysis was a specifically amplified product from vaccinia virus, Lister strain, we sequenced the product according to the procedures described above. An analysis of the high-quality sequence read data taken from the electropherogram yielded a 95% identity (maximum) to vaccinia virus, Lister strain (AY678276.1) with a query coverage of 99% and an e-score of 0 (no data shown). These values indicate that the sequenced product was vaccinia DNA and not amplification from an exogenous nucleic acid source, e.g. host cell.

Notably, our multiplex reaction did not produce a smear (lane 3, Figure 7) that would be indicative of non-specific priming of either the target or exogenous host cellular nucleic acids. While the exact manufacturer's extraction protocol is proprietary, it is generally known that a standard sucrose gradient ultracentrifugation step is used to enrich for the viral capsids prior to viral nucleic acid extraction, However, to the best of our knowledge, no nuclease digestions are performed prior to viral capsid lysis. Furthermore, while the viral 'extract' contains a mixture of both viral DNA and cellular nucleic acids, the exact proportions of host cell and viral nucleic acids cannot be determined, Thus, our results indicate that a multiplex of 16 primers of 10 nt each amplifies only the specific predicted band from vaccinia.

In some applications, such as clinical or biodetection applications, the exogenous nucleic acids from other eukarytotic sources, notably human sources, may be present in varying and unknown concentrations. To test the effects of background human genomic

DNA on the performance (amplification specificity) of the Poxviridae 16-plex primer set, we conducted PCR reactions across a series of mass ratios of vaccinia Lister DNA:human genomic DNA at 1:1, 10:1, 100:1, 1000:1 and 10000:1. Our algorithm predicted 233 amplicons between 50 and 1000 bp from the human genome. In experiments, this would appear as a 'smear' on the agarose gel, which indeed was observed for the mass ratios of 1:1 and 10:1 (Figure 7, lanes 6 and 7, respectively). However, even at a ratio of 10:1 vaccinia:human DNA (lane 7), the 617 bp vaccinia amplicon is clearly visible on the gel, despite the numerous non-specific amplicons. At ratios of 100:1, 1000:1 and 10000:1 (lanes 8, 9 and 10, respectively, Figure 7), the smear is drastically reduced to non-existent (at the resolution of the agarose gel), and the vaccinia amplicon is readily visible. For comparison, we tested the 16-plex primers against the same mass of human DNA in the absence of vaccinia DNA at 2.7 pg (the 1:1 ratio mass), and 0.027 pg (100:1 mass) and $2.7x10^{-4}$ pg (10000:1 mass) (lanes 12, 13, and 14, respectively, Figure 7). The data show a similar smear at 2.7 pg as was observed when vaccinia DNA was present (lane 6). However, the low intensity 617 bp amplicon from vaccinia is visible in lane 6 (with vaccinia) while a similar amplicon is clearly not present in lane 12 (without vaccina). As a further point of comparison, we tested a single-plex pair of vaccinia-specific standard length 20-nt primers previously used in a TaqMan reaction for vaccinia detection (Beer, et al., 2007)and also observed smears at 1:1 and 10:1 vaccinia to human DNA (data not shown). Thus, specific amplification with a short-primer multiplex may be feasible for viral detection if combined with a probe-based detection method such as TaqMan® or Luminex bead based suspension arrays (http://www.luminexcorp.com/).

Discussion and Conclusions

Primer design for amplification and detection of divergent target sequences can be challenging, and this problem will only grow as sequencing technologies improve. Some methods are limited in scalability, particularly those requiring a multiple sequence alignment as input. Developing a PCR multiplex is often a tedious mix-and-match process from among primers originally designed to work in singleplex. We describe the MPP algorithm based on hashing of conserved k-mer subsequences that requires no multiple sequence alignment and where multiplex-compatible primer sets are built de novo to avoid primer dimer and hairpin formation. The algorithm seeks to minimize the number of primers to amplify all targets, although because the algorithm is heuristic and not an exhaustive search of all combinations of primers, the smallest primer sets may not always be selected. Such an exhaustive search would be NP complete, and not practical for large target sets. The software handles a large number of input sequences, although for very diverse targets the predicted primer multiplex may be too large to be empirically feasible. For many target sets, such as those including all the genomes of a species, predicting a universal primer set requires only minutes up to a few hours, although for inputs with thousands of sequences a run may take days. We used MPP to design a universal primer set for a target set of all virus genomes, and showed that even if short primers are used, thousands of priming sequences would be required to amplify all sequenced viruses. We then applied MPP to design multiplex family level primers for every viral family, as well as for some diverse species target sets too large for other available primer prediction software.

In addition to finding primers for viral detection, another application of MPP is to design multiplex primers for homologues in a gene family. The user can specify an appropriate product length range to ensure amplification of an adequate span across the gene. MPP could also be used to design multiplex primer sets for unrelated target sequences, for example, multiple bacterial and viral species or gene families, in a single reaction. Since no sequence alignment is required, there is no need for any sequence conservation among targets. Recent work showed that a large scale multiplex of 800 primer pairs specifically designed to detect a diverse set of genes from 9 pathogens improved sensitivity on a microarray by up to 1000 fold.(Palka-Santini, et al., 2009) The MPP software can be used to design multiplex primer sets for similar work. We demonstrated the application of the algorithm experimentally using vaccinia, amplifying the specific expected band with a Poxviridae short primer multiplex PCR, and confirmed the band's expected sequence. Although one or a small number of unexpected products were also observed at concentrations too low to allow for sequencing, we were not able to determine their source, possibly from mismatched hybridization with target, primer dimer chains, differences between our isolate and the sequence in Genbank, or contamination. We used traditional purification methods, and went to no special effort to purify encapsidated viral nucleic acids from those of the host cells. This demonstrates that viral family short primer multiplex PCR can specifically amplify intended products in a single-step PCR reaction, and could be useful if combined with a downstream method of product analysis such as sequencing or hybridization to a specific probe.

While short primer PCR multiplexes may enable amplification of diverse target sets, they will not match the specificity of longer primers. However, a highly conserved short primer multiplex could amplify all members of a family, followed by product hybridization to a planar or suspension array to provide species and strain discrimination. Our results indicate that short primer multiplex analysis should be combined with information about amplicon sequence (e.g. probe hybridization, amplicon mass or base content, or sequencing) since electrophoretic banding patterns may contain one or several unexpected bands.

Previous studies have shown the utility of short primer singleplex PCR, using 9-mers or 10-mers, followed by gel electrophoresis for genetic fingerprinting of eukaryotes and bacteria (Caetano-Anolles, et al., 1991; Williams, et al., 1990). For viruses, with much smaller and more diverse genomes, the large numbers of 9-mer or 10-mer primers required to generate at least one band from every virus as predicted by our analyses implies that primer size would need to be as short as 5-mers to rely on a gel banding pattern using only one priming sequence for fingerprinting viruses (unpublished analyses). However, the analyses here predict that imperfect sample purification to eliminate eukaryotic nucleic acids could be problematic for universal viral priming using primers shorter than 15 bases, particularly for multiplexes of 10 or more primers. Nanda et al. (Nanda, 2007; Nanda, et al., 2008) was able to achieve sufficient viral isolation from cell culture samples to allow viral identification using viral PCR with priming sequences as short as pentamers, so the problem of contaminating host nucleic acids for specific, short primer PCR of viruses is not insurmountable. They found specific pentamer PCR to be several logs more sensitive than non-specific amplification, provided that they purified encapsidated viral nucleic acids prior to PCR. Another method that has been used for virus discovery is VIDISCA (*Vi*rus *di*scovery cDNA-AFLP) using

restriction enzyme digestion, adaptor ligation, and PCR by priming with the adaptor sequence.(de Vries, et al., 2008; Pyrc, et al., 2008) This method, like pentamer priming, requires prior separation of encapsidated viral nucleic acids, as it generates fragments from any DNA present, viral, host or otherwise. Multiplex PCR with primers 10-15 nt in length may be yet another alternative strategy lying between these non-specific methods and PCR with ≥18-mers, as we have shown that it can more specifically target a candidate viral family without requiring initial nuclease digestions steps to remove non-viral nucleic acids.

In summary, we applied the MPP software to generate multiplex-compatible primer sets for every viral family and several viral species and experimentally demonstrated specific, short primer multiplex amplification.

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Table 1: Primer counts for viral family primer sets, as described in the methods. Grey shaded entries indicate where calculations were not run to completion. In other cases (not shaded) where fewer than 100% of targets were predicted to be amplified, the algorithm failed to find primer pairs that met all the required specifications for the remaining targets, that is, primers in the right length, T_m,, and amplicon length range, with hairpin and dimer avoidance with other primers already selected to be in the set, could not be found. For 10-15-mers, T_m=40-45°C, and for 17-21-mers, T_m=55-60°C. For both, the amplicon length range was 200-800 bp.

Family	Number of Target Sequences	Number 10-15-mer primers	% of Targets Amplified	Number of 17-21-mer primers	% of Targets Amplified	Family Specific Number of 17-21-mer primers	% of Targets Amplified
Adenoviridae	78	24	100.00%	36	100.00%	42	100.00%
Alloherpesviridae	6	4	100.00%	8	100.00%	8	100.00%
Ampullaviridae	1	2	100.00%	2	100.00%	2	100.00%
Arenaviridae	154	90	100.00%	142	100.00%	146	99.35%
Arteriviridae	129	10	100.00%	12	100.00%	12	100.00%
Ascoviridae	4	4	100.00%	6	100.00%	6	100.00%
Asfarviridae	10	2	100.00%	2	100.00%	2	100.00%
Astroviridae	26	12	100.00%	16	100.00%	16	100.00%
Baculoviridae	52	20	100.00%	44	100.00%	48	100.00%
Barnaviridae	1	2	100.00%	2	100.00%	2	100.00%
Bicaudaviridae	2	2	100.00%	4	100.00%	4	100.00%
Birnaviridae	219	22	100.00%	26	100.00%	30	100.00%
Bornaviridae	13	4	100.00%	4	100.00%	4	100.00%
Bromoviridae	321	124	100.00%	165	100.00%	139	93.46%
Bunyaviridae	1265	345	93.04%	183	75.81%	129	62.13%
Caliciviridae	210	48	100.00%	80	100.00%	82	100.00%
Caulimoviridae	66	56	100.00%	58	100.00%	60	100.00%
Chrysoviridae	16	18	100.00%	24	100.00%	24	100.00%
Circoviridae	594	20	100.00%	34	100.00%	34	100.00%
Closteroviridae	61	53	100.00%	64	100.00%	64	100.00%
Comoviridae	80	68	100.00%	78	100.00%	90	100.00%
Coronaviridae	279	24	100.00%	30	100.00%	34	100.00%
Corticoviridae	1	2	100.00%	2	100.00%	2	100.00%
Cystoviridae	21	19	100.00%	24	100.00%	24	100.00%
Dicistroviridae	25	22	100.00%	24	100.00%	26	100.00%
Endornaviridae	6	8	100.00%	8	100.00%	8	100.00%
Filoviridae	39	8	100.00%	7	100.00%	12	100.00%
Flaviviridae	2866	102	100.00%	142	99.97%	158	99.97%

Flexiviridae	195	121	100.00%	170	100.00%	164	92.31%
Fuselloviridae	6	2	100.00%	4	100.00%	4	100.00%
Geminiviridae	1429	162	100.00%	172	94.47%	204	91.11%
Globuloviridae	2	2	100.00%	4	100.00%	4	100.00%
Hepadnaviridae	2141	16	100.00%	17	100.00%	22	99.91%
Hepeviridae	134	8	100.00%	18	100.00%	21	100.00%
Herpesviridae	98	27	100.00%	48	100.00%	54	100.00%
Hypoviridae	7	4	100.00%	8	100.00%	8	100.00%
Inoviridae	33	30	100.00%	32	100.00%	34	100.00%
Iridoviridae	13	8	100.00%	14	100.00%	14	100.00%
Leviviridae	12	12	100.00%	14	100.00%	14	100.00%
Lipothrixviridae	8	8	100.00%	8	100.00%	8	100.00%
Luteoviridae	99	22	100.00%	26	100.00%	28	100.00%
Malacoherpesvirid	1	2	100.00%	2	100.00%	2	100.00%
Marnaviridae	1	2	100.00%	2	100.00%	2	100.00%
Metaviridae	4	4	100.00%	4	100.00%	4	75.00%
Microviridae	105	10	100.00%	14	100.00%	16	100.00%
Mimiviridae	1	2	100.00%	2	100.00%	2	100.00%
Myoviridae	95	90	100.00%	112	100.00%	116	100.00%
Nanoviridae	206	70	100.00%	88	99.51%	78	91.26%
Narnaviridae	9	14	100.00%	18	100.00%	18	100.00%
Nimaviridae	3	2	100.00%	2	100.00%	2	100.00%
Nodaviridae	41	22	100.00%	26	100.00%	26	100.00%
Ophioviridae	15	22	100.00%	26	100.00%	28	100.00%
Orthomyxoviridae	32988	318	99.76%	35	74.41%	78	82.15%
Papillomaviridae	272	115	100.00%	167	94.85%	216	99.63%
Paramyxoviridae	252	56	99.21%	80	99.21%	83	99.21%
Partitiviridae	74	72	89.19%	88	98.65%	90	97.30%
Parvoviridae	136	48	100.00%	62	100.00%	62	100.00%
Phycodnaviridae	10	6	100.00%	8	100.00%	12	100.00%
Picobirnaviridae	2	4	100.00%	4	100.00%	4	100.00%
Picornaviridae	842	103	100.00%	119	100.00%	126	100.00%
Plasmaviridae	1	2	100.00%	2	100.00%	2	100.00%
Podoviridae	90	75	100.00%	92	100.00%	96	96.67%
Polydnaviridae	232	331	99.57%	307	80.17%	266	68.10%
Polyomaviridae	669	28	100.00%	34	100.00%	38	100.00%
Potyviridae	452	110	100.00%	178	100.00%	196	100.00%
Poxviridae	148	10	100.00%	20	100.00%	22	100.00%
Reoviridae	3127	169	52.13%	195	58.68%	155	46.56%
Retroviridae	1652	118	99.94%	193	100.00%	227	100.00%
Rhabdoviridae	104	58	100.00%	68	100.00%	76	100.00%
Roniviridae	2	2	100.00%	2	100.00%	2	100.00%
Rudiviridae	5	6	100.00%	6	100.00%	6	100.00%

Sequiviridae	9	6	100.00%	10	100.00%	10	100.00%
Siphoviridae	236	156	100.00%	214	99.58%	220	0.00%
Tectiviridae	10	4	100.00%	4	100.00%	6	100.00%
Tetraviridae	6	8	100.00%	12	100.00%	12	100.00%
Togaviridae	140	23	100.00%	38	100.00%	46	99.29%
Tombusviridae	81	55	100.00%	68	100.00%	70	100.00%
Totiviridae	38	48	100.00%	54	97.37%	58	100.00%
Tymoviridae	24	13	100.00%	29	100.00%	34	100.00%

Table 2

Virus Species	Number of sequences	Number of 10-mers	Number of 17-18 -mer primers in set	Longest conserved region from MSA (nt)
NA segment of Influenza A*	6375	52	120	5
HA segment of Influenza A*	5440	73	153	1
HIV-1	1175	6	16	0
FMDV	187	4	6	9
Norwalk	41	7	20	6

* For Influenza A HA and NA segments, all complete sequences, including lab strains, from all hosts, countries, and serotypes were downloaded from the NCBI Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/multiple.cgi) on January 18, 2008.

Table 3

Influenza A HA Serotype	Number of sequences	Number of 18-mer primers in set
H1	1080	24
H2	108	8
H3	1972	15
H5	1325	16
H7	256	8

Table 4. Sequence and size (nt) information for the 16 primers in the predicted family-level multiplex for Poxviridae viruses used in these experiments. The forward and reverse primers (FP, RP) in bold are predicted to amplify vaccinia Lister with the indicated product length (617 bp amplifon size).

Primer sequence	**Primer size (nt)**
CGGAGACCAA (FP 617)	10
TCGTCGTCCA (RP 617)	10
TGTTGGTGTG	10
TCGTCTACGA	10
TGTGGTCCTT	10
CCATGTTCGC	10
TCGAGGAGAA	10
CCGGCTCCAG	10
TGAACCTGGT	10
GCGCACGTAC	10
TCACGCATCT	10
GGGAAACAGC	10
ACCGTTGTCA	10
TACCATCGTC	10

AACCTGTGCA	10
GGCGGAGGTA	10

Figure 1: Hundreds to thousands of primer sequences are required to amplify all viruses. Percent of viral genomes detected vs number of primers required. Calculated for primers of different sizes, and based on sequence data as of April 25, 2007. Parameters used are given in supplemental Tables S1-S2.

Figure 2: Effect of T_m constraints and sequence database size on size of universal primer set. The number of primers in the universal set for all viruses is shown, as a function of primer size. Calculations were based either on sequence data available April 2007 and imposing T_m constraints in primer selection, or without T_m or GC% constraints, or based on January 2004 sequence data with T_m constraints. For primer size of 5 with T_m constraints, a constraint of $T_m > 0$ was used, delivering primers with GC% of 80-100%.

Figure 3: Primers from old sequence data miss many newly sequenced viruses. Fraction of viral genomes available on April 25, 2007 that would have been detected using primer sets developed based on the sequence data available on January 1, 2004.

Figure 4: Average number of bands predicted from PCR against the human genome using the universal viral primers for the runs as described in Figure 2, average +/- standard deviation of the top 10 bins for each primer length. For bin size of 10, we divided the universal primer bins of 20 primers/bin into two bins each.

Figure 5: Number of primers required to amplify fragments from each complete genome or segment in the family versus the number of available sequences in that family. Primer parameters were length 17-21-mers, T_m between 55-60°C.

Figure 6: The percentage of genomes amplified versus the number of primers used, for primers of either 10-mers or 17-18-mers, for several highly diverse virus species, A) HIV-1, FMDV, and Norwalk, and B) Influenza A HA and NA segments. The 2 most highly conserved primers amplify a large fraction of genomes, and additional primers show diminishing in detecting the remaining, more divergent sequences.

Figure 7. PCR amplification of vaccinia DNA, Lister strain with the 16-plex Poxviridae primer set, with (lanes 6-10) or without (lanes 3,4) human DNA present in the reaction, for a reaction containing 4.8 mM $MgSO_4$, 0.1 μM each primer, and 2.7 pg (~10^4 copies) of vaccinia lister DNA, and using an annealing temperature of 43.9˚C. Lane 3 shows specific amplification of vaccinia lister DNA followed by the no template control in lane 4. Lanes 6-10 represent the experiments with mass ratios of vaccinia:human DNA of 1:1, 10:1, 100:1, 1000:1 and 10000:1, respectively. Lanes 12-14 represent amplification of human DNA only (no vaccinia present in reaction) for the same masses used at the 1:1, 100:1 and 10000:1 ratios (or 2.7 pg, 0,027 pg and 2.7×10^{-4} pg human DNA, respectively). Lanes 5, 11 and 15 did not contain any sample. The arrow on the left points to the expected band (617 bp). The arrows on the right correspond to the 50 bp DNA ladder (lanes 1 and 16 contain the same ladder)

Figure 1

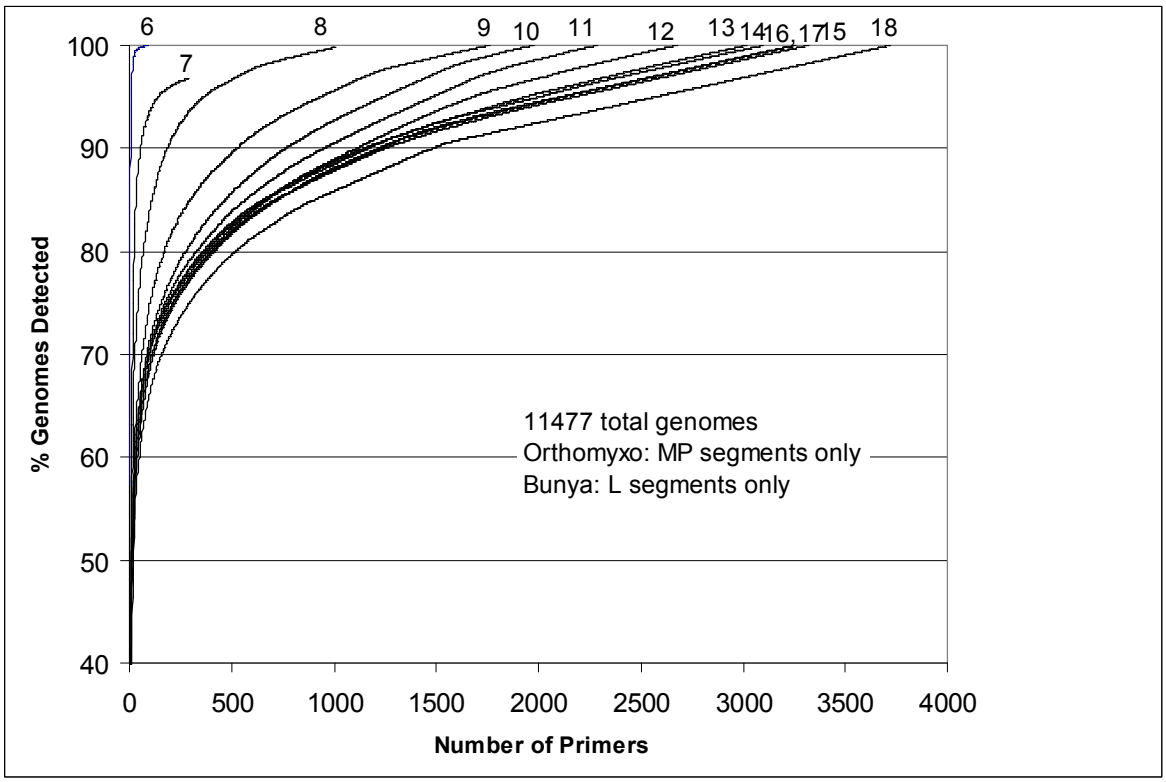

6
8
9
10
11
12
13
14
16,17
15
18
7
100
90
80
70
60
50
40
% Genomes Detected
11477 total genomes
Orthomyxo: MP segments only
Bunya: L segments only
0
500
1000
1500
2000
2500
3000
3500
4000
Number of Primers

Figure 2

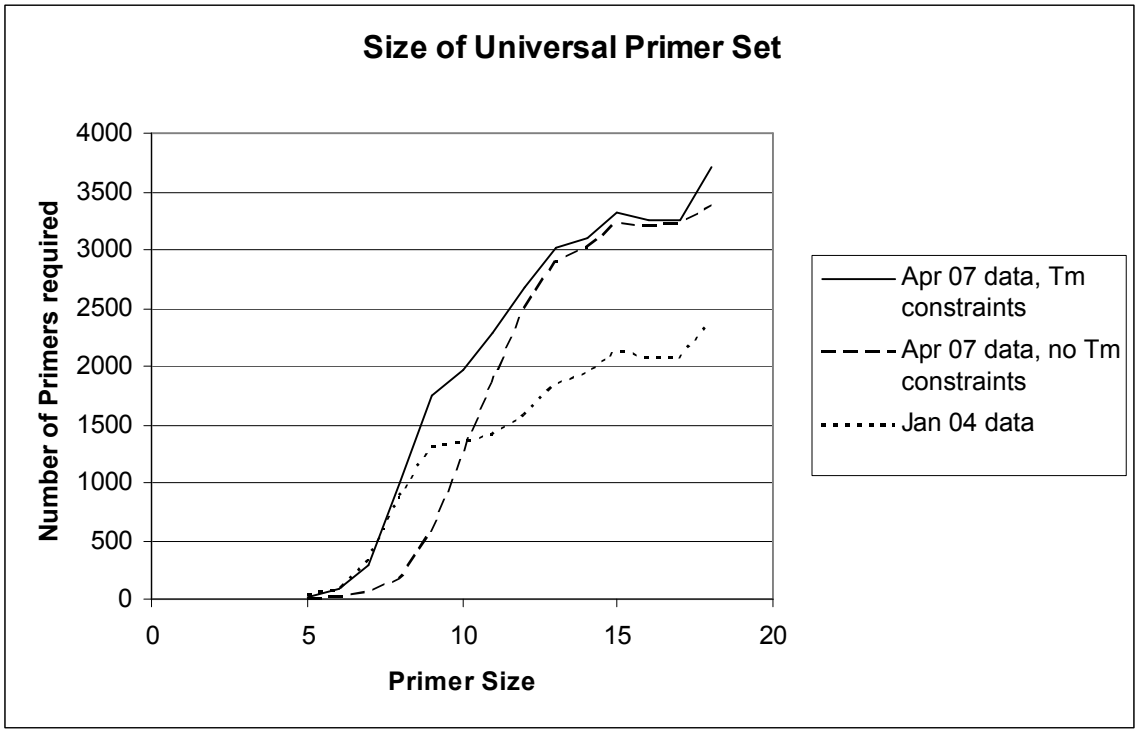


Figure 3

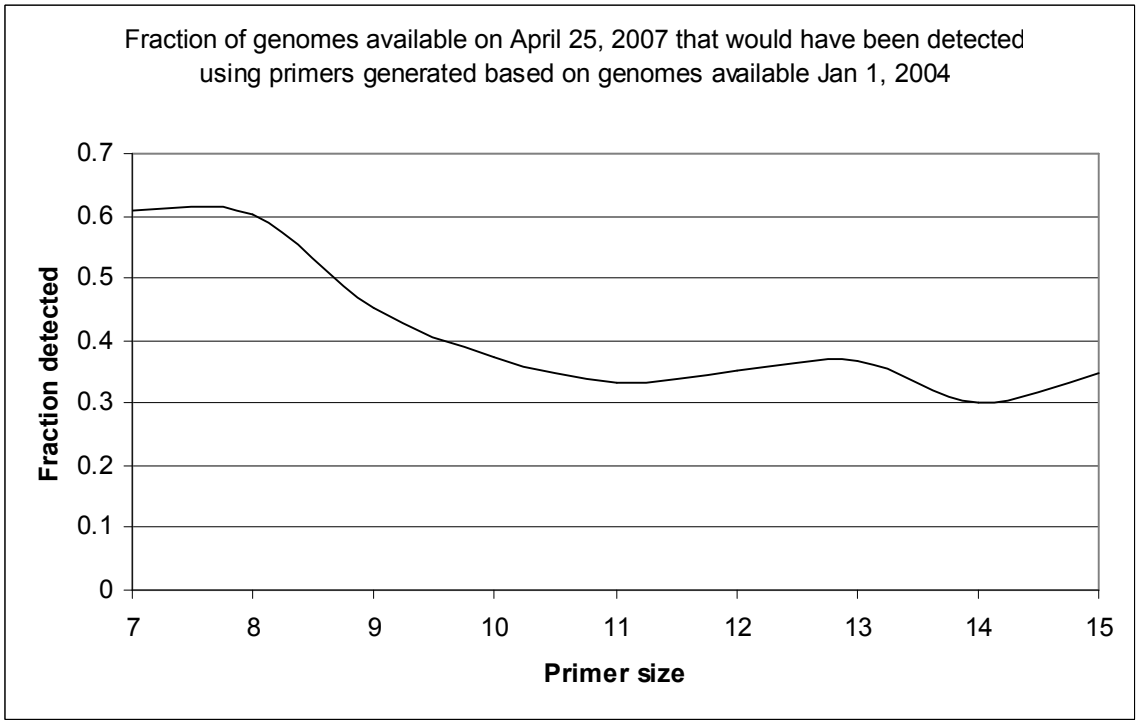


Figure 4

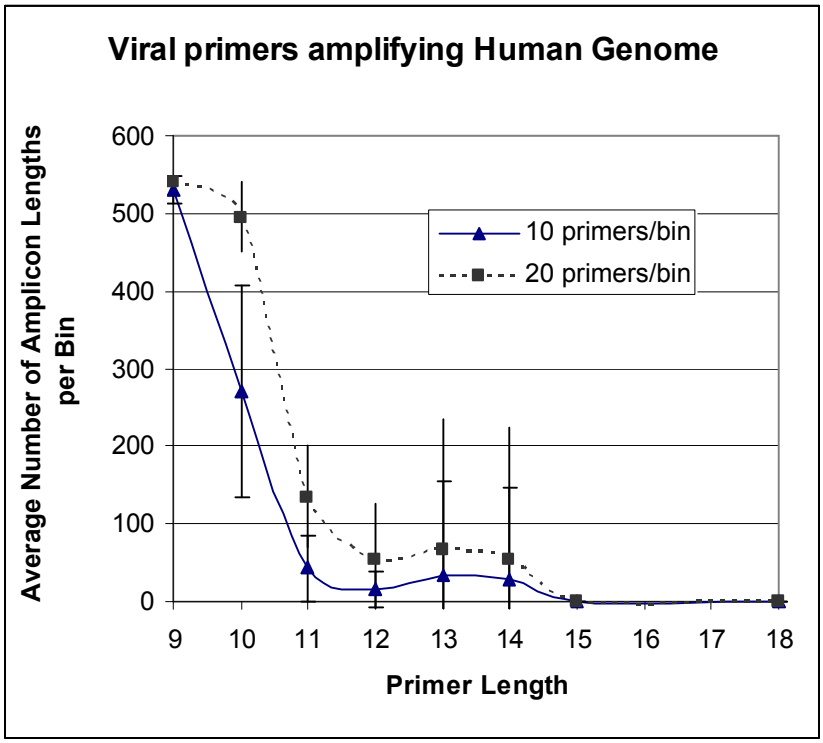
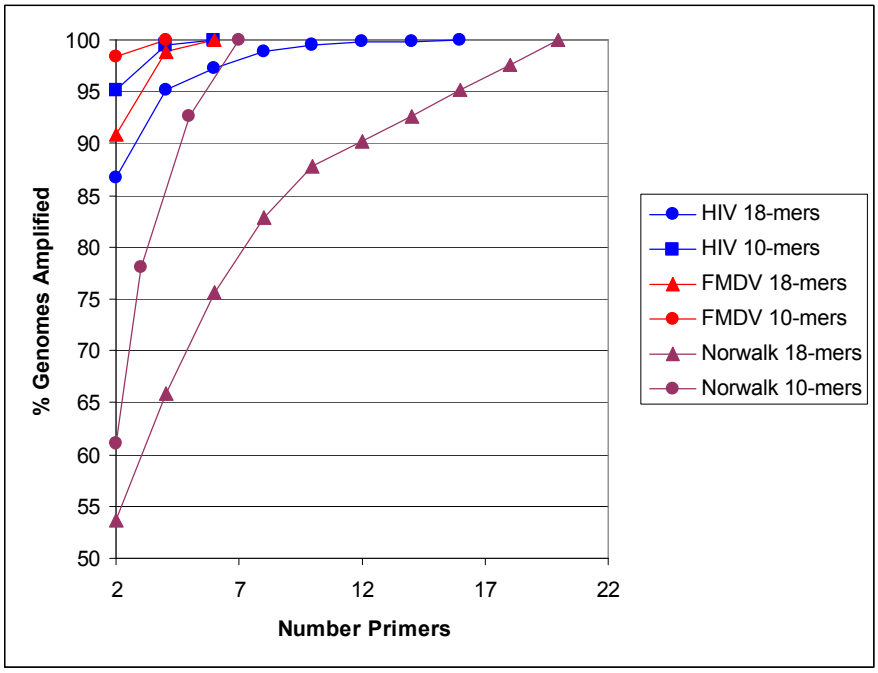

Viral primers amplifying Human Genome
Average Number of Amplicon Lengths per Bin
600
500
400
300
200
100
0
9
10
11
12
13
14
15
16
17
18
Primer Length
10 primers/bin
20 primers/bin

Figure 5


Number of 17-21-mer Primers
100
10
1
1
10
100
1000
10000
Number of Target Sequences
ssDNA
dsDNA
ssRNA
dsRNA

Figure 6

A


100
95
90
85
80
75
70
65
60
55
50
% Genomes Amplified
2
7
12
17
22
Number Primers
HIV 18-mers
HIV 10-mers
FMDV 18-mers
FMDV 10-mers
Norwalk 18-mers
Norwalk 10-mers

B

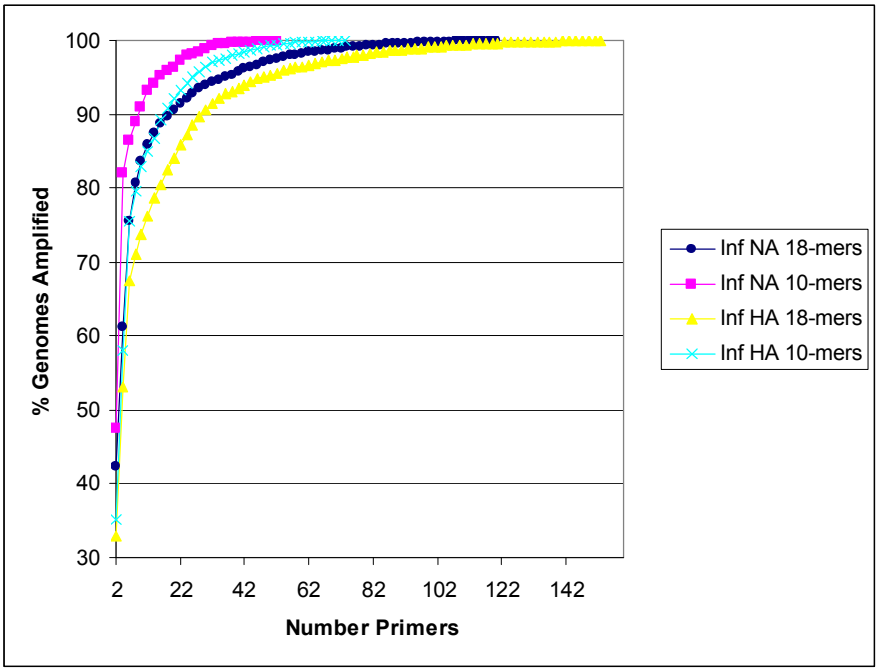
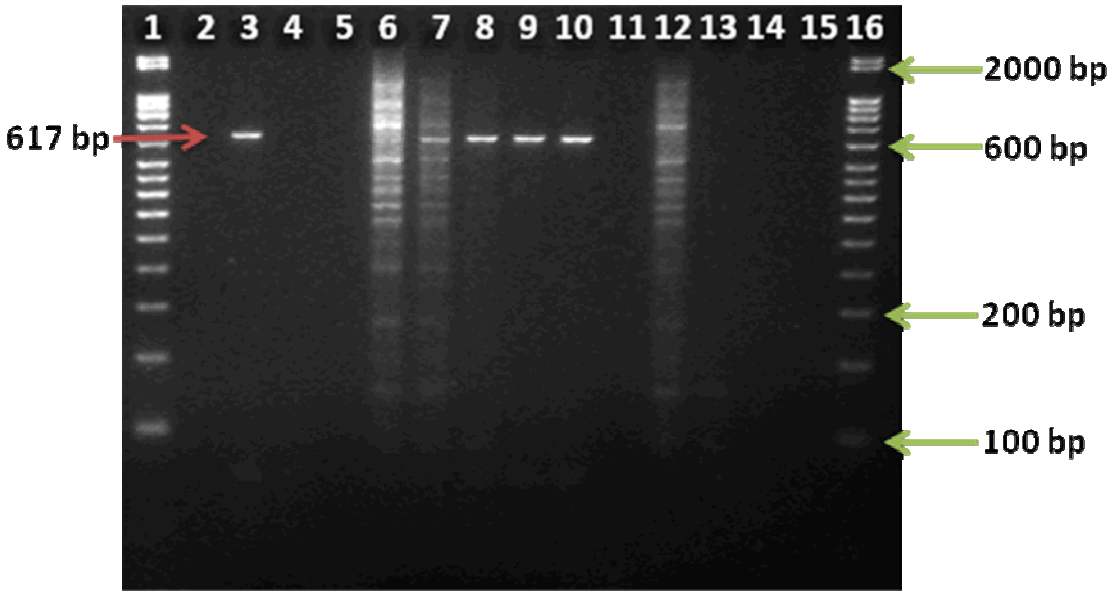

100
90
80
70
60
50
40
30
% Genomes Amplified
2
22
42
62
82
102
122
142
Number Primers
Inf NA 18-mers
Inf NA 10-mers
Inf HA 18-mers
Inf HA 10-mers

Figure 7



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Supplemental Methods: MPP Algorithm for calculating highly conserved, multiplexed primer sets

To select highly conserved universal primers for a set of targets, we have coded the following algorithm in PERL. The source code is available for non-profit use by contacting the authors. The universal set of primers should produce at least one amplicon in each of the target sequences. The process is diagrammed in Figure S1 and described as follows:

1) We first enumerate all candidate oligos that occur in the target set of sequences that fall in the user-specified parameter range for T_m, hairpin and homodimer avoidance (free energy values above the thresholds x_{hair} and $x_{homodimer}$, respectively), do not contain homopolymer strings longer than 4 bases, do not contain non-ATCG bases, and are not composed entirely of dinucleotide repeats. T_m's and free energies for hairpin and homodimer avoidance (see below) are calculated using Unafold. (Markham and Zuker, 2005) These candidate oligos are stored in a hash h_1 with the oligo sequences as keys and values containing bit vectors composed of the following: the number of genomes containing that oligo, the identity of the last target sequence added to the count (to speed the initial hash construction), and the identities of each target sequence containing that oligo. Target sequences are referenced by number in a lookup table. This representation stores the oligo frequencies efficiently for subsequent calculations, but does not track the location of each oligo in each sequence, to reduce memory requirements. Currently, oligo sequences in the keys are represented as character strings (8 bits/character), although a more memory efficient implementation should store A,C,T, and G using only 2 bits each, providing a four-fold memory savings.

2) The oligos are sorted by the frequency of target sequences in which they occur. A set s_1 of the 100 most frequent oligos whose homodimer and hairpin free energies exceed $x_{homodimer}$ and x_{hair}, respectively, is constructed. Since free energy calculation is relatively time consuming, these are only calculated as needed until the set s_1 has been populated with 100 oligos.

3) Next, two more hashes are constructed like the first for each oligo i in s_1, using only the subsequences from the original targets that lie between d_1-d_2 nucleotides of the oligo s_{1i}. One is a hash h_{2u} for the upstream oligos, and the other is a hash h_{2d} for the downstream oligos. After sorting both h_{2u} and h_{2d} by frequency in the target sequences, the oligo j is chosen for each oligo i that occurs most frequently either upstream or downstream, giving the most frequent oligo pairs (i,j). Again, only oligos j with homodimer and hairpin free energies exceeding $x_{homodimer}$ and x_{hair} and (i,j) dimer free energy exceeding x_{dimer} are allowed, so if the most frequent j is not energetically acceptable, the next most frequent j is selected until an acceptable oligo can be found. The most frequent pair (i*,j*) is selected from among all (i,j) pairs. If j* is from h_{2u}, then it becomes primer p_1 and the reverse complement of i* becomes primer p_2, while if j* is from h_{2d} then i* and the reverse complement of j* are used as primers. These primers are added to the reaction bin.

4) The list of target sequences yet to be detected is updated, eliminating any with valid amplicons in the specified size range d_1-d_2 generated by any combination of primers already selected. The values of hash h_1 are also updated by subtracting from the oligo frequency counts those targets detected and removing their target identities.

5) The process from steps 2-4 is repeated for the targets not yet detected until all targets have a valid amplicon, adding the constraint at the end of step 3 that primers p_1 and p_2 must have dimer free energies exceeding x_{dimer} with any primer already selected, in order to be added to the bin. If either primer in the pair is predicted to dimerize with any primer already selected in that bin, then the next most frequent pair (i*,j*) is checked for acceptability as primers, until a suitable pair is found. In step 4, since primers in a bin are intended to be mixed in multiplex, targets can be detected by any combination of primers in any orientation relative to one another, not just those that were selected as a pair. We call these unintended detections "serendipitous hits," and update the list of yet-to-be-detected targets and h_l accordingly.
6) If the option to bin primers in subsets of b primers is selected, primers are added to a bin until that bin contains a maximum of b primers, at which point a new bin is begun. Occasionally, the same primer may be selected more than once in the same bin, paired with a different oligo, resulting in an odd number of primers. Binning primers in small groups avoids exclusion of the most highly conserved oligos because of primer dimer free energy constraints. The code also runs faster because there are fewer primer dimer free energy calculations that must be performed every time a new primer is added to a bin, and because fewer primers are discarded due to free energy constraints so that acceptable primers are found sooner.

For all analyses in this paper, T_m and ΔG were calculated using Unafold with $[Na^+]$=0.2 M, $[Mg^{+2}]$=0.0015 M, T_{anneal}=30°C, strand concentration of each strand of 1e-07 M, and with the "DNA" option.

Table S1: Parameter values used in the predictions of universal viral primers. $x_{homodimer}$, x_{dimer}, and x_{hair} are the minimum allowed value of nearest neighbor predicted free energies of homodimer, primer dimer, and hairpin formation, respectively, for primers in a bin. d_1 and d_2 are the minimum and maximum size of required amplicons. Amplicons outside of this size range are allowed, but do not count toward detection.

Parameter	Value
$x_{homodimer}$	-7 kcal/mol
x_{dimer}	-7 kcal/mol
x_{hair}	-5 kcal/mol
d_1	80
d_2	620

Table S2: T_m (°C) settings used in predicting universal primer set for all viruses for the runs considering all 11,477 virus sequences as a single target set.

Length	T_m min	T_m max
6	10	35
7	25	40
8	30	45
9	35	50
10	35	50
11	35	50
12	35	50
13	40	55
14	45	60
15	50	65
16	50	70
17	50	70
18	55	70

Figure S1: Diagram of alignment-free algorithm used for predicting universal primer sets.