Published in final edited form as:

J Clin Virol. 2014 September ; 61(1): 9–19. doi:10.1016/j.jcv.2014.06.013.

Deep Sequencing: Becoming a Critical Tool in Clinical Virology

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Abstract

Population (Sanger) sequencing has been the standard method in basic and clinical DNA sequencing for almost 40 years; however, next-generation (deep) sequencing methodologies are now revolutionizing the field of genomics, and clinical virology is no exception. Deep sequencing is highly efficient, producing an enormous amount of information at low cost in a relatively short period of time. High-throughput sequencing techniques have enabled significant contributions to multiple areas in virology, including virus discovery and metagenomics (viromes), molecular epidemiology, pathogenesis, and studies of how viruses to escape the host immune system and antiviral pressures. In addition, new and more affordable deep sequencing-based assays are now being implemented in clinical laboratories. Here we review the use of the current deep sequencing platforms in virology, focusing on three of the most studied viruses: human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus.

Keywords

Next Generation Sequencing (NGS); Deep Sequencing; Human Immunodeficiency Virus (HIV); Hepatitis C Virus (HCV); Influenza Virus

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Competing interests Miguel E. Quiñones-Mateu developed the novel deep sequencing-based HIV-1 genotyping and coreceptor tropism assay, DEEPGENTMHIV.

Ethical approval Not required

1. Introduction

DNA sequencing has evolved considerably during the last 50 years. The first sequence of a tRNA molecule in 1965 [1] led to the development of more robust DNA sequencing methodologies in 1977 by Walter Gilbert and Allan Maxam (chemical degradation) [2] and Frederick Sanger (chain-termination) [3]. Sanger et al. determined the first complete genome sequence of any organism: the bacteriophage ϕ [3] which was followed by the DNA sequencing of a multitude of organisms, including DNA and RNA viruses, such as Epstein-Barr virus in 1984 [4] and Human Immunodeficiency Virus in 1985 [5]. Thirty years later, more than 2.5 million viral nucleotide sequences have been deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). This information has been instrumental to understand the structure of viral genomes, their biology, evolution, diversity, transmission, pathogenesis, as well evasion from the host immune response, antiviral drugs, and vaccines. Despite the impressive success of “Sanger” or “population” sequencing, and the development of automated DNA sequencing instruments in the mid 1980s, these first-generation sequencing methods are not high-throughput, have limited scalability and are not cost-effective for sequencing a multitude of samples and/or large genomes. A second generation of sequencing technologies has now addressed these limitations.

Following the development of several novel methods for DNA sequencing in the late 1990s, early 2000s [6, 7], different technologies have become commercially available for so-called next generation sequencing (NGS), also known as “second generation”, “massive parallel” or “deep” sequencing. Deep sequencing technologies are able to generate three to four orders of magnitude more information than Sanger sequencing and are significantly less expensive, considering the cost per nucleotide sequenced [8, 9]. Several deep sequencing systems have been developed during the last 10 years, each of them with their own intrinsic performance metrics such as number of reads obtained, read length, accuracy, time to run, cost, etc. [9, 10]. In 2004 454 Life Sciences (Branford, CT) introduced the first instrument based on pyrosequencing [11], prior to being acquired by Roche (Basel, Switzerland). This was followed by the release of the Genome Analyzer (GA, Solexa, Chesterford, UK) in 2005 [12], now Illumina (San Diego, CA). In 2007 the first SOLiD sequencing system was released by Applied Biosystems (Foster City, CA) [13], while the Helicos sequencer [14] and the Ion Torrent Personal Genome Machine [15], were commercialized by Life Technologies (Carlsbad, CA) in 2009 and 2011, respectively. Pacific Biosciences (Menlo Park, CA) introduced the single molecule real-time sequencer in 2011 [16] and more recently Oxford Nanopore Technologies (Oxford, UK) released an ultra-long single molecule sequencer [17]. All these methodologies have continued to evolve, with new chemistries and more potent instruments, resulting in impressive levels of sequencing throughput at constantly lower costs. As a result, the use of deep sequencing continues to expand both in research and clinical settings, and the virology field is no exception [18–20]. In this review we provide an overview of the use of deep sequencing in virology and how these techniques have impacted, and will continue to influence, the study of three of the most important human viral agents that top the list of viral nucleotide sequences in GenBank, i.e., human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus.

2. Current deep sequencing platforms

Many excellent articles have compared and described in detail the different deep sequencing methodologies and instruments, including methods used in template preparation, sequencing, and data analysis [9, 22]. Four platforms dominate the deep sequencing field: 454TM (454 Life Sciences/Roche, Branford, CT) [11], Illumina® (Illumina, Inc. San Diego, CA) [12], Ion TorrentTM (Ion Torrent/Life Technologies, South San Francisco, CA) [15], and PacBio® (Pacific Biosciences, Menlo Park, CA)[16]. In general, all four technologies are able to generate valuable sequence information [26]; however, there are key and significant differences between the amount and quality of the data, and the applications that each system could support (Fig. 1). Selecting a deep sequencing platform depends on the potential application(s) and resources available, including cost of the instrument and reagents, existing infrastructure and personal experience. Although to date most of the published studies in virology have used 454TM or Illumina® systems, perhaps due to the fact that these were the first two methodologies available (Fig. 1), all deep sequencing technologies continue to improve and are being used in a multitude of virological studies [18–20, 24].

3. Applications in general virology

Deep sequencing has been a “shot in the arm” to all genomic studies and virologists have taken particularly advantage of this methodology. First of all, now it is simpler and more affordable than ever to sequence full viral genomes. Likewise, identification and classification of novel and known viruses, unbiased characterization of viral populations without the need for virus culturing (viromes), molecular epidemiology, viral diversity and evolution, transmission and pathogenesis, and in particular medical virology have greatly benefited from the use of deep sequencing. As shown in Figure 2A, the number of publications using deep sequencing in virology has skyrocketed since 2008, particularly those associated with HIV. By allowing the cost-effective study of a greater number of viral variants, including complex viral populations, deep sequencing is the perfect tool for a broad number of applications for studies of HIV, HCV and influenza virus (Fig. 2B).

3.1. Virus discovery

The field of virus discovery has flourished with the advent of deep sequencing methodologies. Coupled with bioinformatics tools, high throughput sequencing has revolutionized the field by allowing the identification and characterization of novel viruses [27] including a novel rhabdovirus associated with acute hemorrhagic fever identified in Central Africa [30] and a new cyclovirus detected in cerebrospinal fluid of patients with central nervous system infections [31]. Together with classical methods of diagnostics such as virus isolation, immunochemistry, and PCR, deep sequencing will continue to play a significant role in the identification of novel viruses, particularly in the face of outbreaks of known and/or new diseases.

3.2. Human, animal, plant, and environmental viromes

Viral metagenomic studies, i.e., the characterization of viral genomes directly from samples, have become extremely popular with the arrival of deep sequencing [28] and numerous viral metagenomic analyses have been published (reviewed in [18, 27–29]). The viral metagenome or “virome” refers to the collection of viruses found in a particular sample from humans, animals, plants or from a specific environmental sample. Virome studies can lead to the discovery of new viruses and/or to their association with known or novel diseases. In the case of the human virome, its composition and impact on human health has been the subject of multiple studies [37, 38]. The healthy human gut virome seems to consist mainly of bacteriophages [39], and can vary among individuals and in response to diet [40]. On the other hand, the human skin virome comprises a high variety of DNA viruses [41]. Interestingly, the human gut, nasopharyngeal, or plasma viromes are highly susceptible to changes associated with diseases such as HIV/AIDS [42, 43], antiviral and immunosuppression therapy [44], or respiratory infections [45, 46]. Similar studies have analyzed the gut virome in different healthy animals, such as pigs [47], wild rodents [48] and pigeons [49], and turkeys [50] and, like in humans; certain diseases seem to affect the animal virome [51, 52]. Numerous viruses have been identified as part of the bat virome, including influenza A viruses [53], hepaciviruses and pegiviruses [54], hepadnaviruses [55], hantaviruses [56] and a series of novel viruses such as cedar virus [57] and a new SARS-like betacoronavirus [58]. These animals have been intensely studied since they are believed to be the reservoir of many potential zoonotic infections [59].

3.3. Molecular epidemiology

Sanger-based DNA sequencing and phylogenetic analyses have been instrumental to characterize the distribution of viruses in a population [63]; however, deep sequencing now allows more complex analysis of huge datasets and even full-length viral genomes. Following multiple zoonotic transmissions, which resulted in distinct lineages in humans, HIV has diversified over time [64, 65]. Accordingly, HIV has been classified into types, groups, subtypes or clades, circulating recombinant forms (CRF) and unique recombinant forms (URF) mainly based on the diversity of the viral *env* and *pol* genes [65–67]. Although to date most of the deep sequencing studies of HIV have focused on drug resistance and tropism, the methodology has been used to characterize novel HIV recombinants [69] and to determine HIV incidence [70]. Interestingly, deep sequencing has been particularly useful in the detection and characterization of HIV superinfection events, which seem to occur at higher rates than previously identified [71].

Seven phylogenetic clades -designated as genotypes- have been identified in HCV, with close to 70 subtypes distributed among the different genotypes [72]. HCV subtypes are epidemiologically distinct, with differences in risk group targeting and geographical distributions that are associated with substantial genetic diversity that reflects their recent epidemic spread [73]. Thus far the reconstruction of the HCV epidemic has been based on Sanger sequences, used to model evolutionary histories of currently circulating variants and to identify historical factors such as widespread use of blood transfusion and other parenterally delivered treatments and vaccination as the facilitators of HCV transmission [74]. However, deep sequencing approaches have not only accelerated the rate at which

HCV sequences are generated, but also represent a substantial advance in sensitivity and molecular resolution to distinguish closely and distantly related HCV genomes. For example, deep sequencing has been used to identify new HCV subtypes and recombinants [75] and to study the history of the HCV epidemic in remote communities [76].

Given the importance of transmission and adaptation of avian influenza viruses, and more recently swine strains for epidemics and pandemics in humans, an important number of studies based on deep sequencing techniques have described avian [77–81] and porcine [82–85] influenza virus evolution. For example, the selection of minority variants with a deletion in the neuraminidase (NA) gene that allows the adaptation of avian influenza viruses from waterfowl to domestic poultry [77], suggests a high frequency of mixed infections and genetic reassortment within these viruses [78, 79, 85]. Other studies described the prevalence and spread patterns of different human influenza viruses in specific geographic areas, based not only on the hemagglutinin (HA) and NA genes but by analyzing all viral segments [86–89].

3.4. Viral diversity, transmission, and pathogenesis

All RNA viruses including HIV, HCV, and influenza virus replicate as a multitude of related but nonidentical genetic variants known as quasispecies [90]. These highly diverse viral populations provide numerous advantages to the virus, including escaping the pressure from the host immune system, and resistance to antiviral agents [91]. Prior to the arrival of deep sequencing, viral quasispecies studies relied on the labor-intensive Sanger sequencing of numerous molecular clones [92, 93]. Today, deep sequencing methodologies are capable of generating an extraordinary number of sequences (reads), which make them the ideal tool to study intra- and inter-host viral diversity, virus transmission and adaptation dynamics, and disease progression (Fig. 3).

Recent strategies to study HIV variability have involved deep sequencing of nearly complete viral genomes [94–96] or specific genomic regions [26, 97–107]. Other studies have focused on the analysis of HIV intra-patient evolution, such as the characterization of transmitted HIV and persistence of minority variants [108], estimation of primary infection dates [109], description of intra-host evolution dynamics during the course of infection with or without antiretroviral treatment [95, 98, 103, 107, 110, 111]. Others have analyzed hypermutation patterns [112], viral evolution in different host compartments [113] or in response to the host immune system [114–116], and simultaneous assessment of replication fitness of different drug resistant variants [117]. In the case of HCV, deep sequencing has allowed the study of minority viral variants and intra-host quasispecies diversity [118, 119], the identification of transmitted viral variants [120, 121], and factors associated with HCV pathogenesis. In addition, deep sequencing-based studies allowed the identification of a significant number of non-coding RNAs (ncRNAs) associated with hepatocellular carcinoma caused by HCV infection [123].

Similar to HIV and HCV, deep sequencing provides a better alternative to dissect genetically complex populations of influenza viruses and to detect minority variants with clinical or epidemiological relevance. Deep sequencing-based methods have recently been proposed for the assessment of influenza A viruses diversity and rates of evolution [124–

126], as well as antigenic stability [127] using complete influenza A genomes and exploiting the ability to detect and quantify mutations in heterogeneous viral populations. Others have focused on the evolution of avian influenza strains with potential to become pandemic in humans [128–132], as well as the detection of virulence signatures [133] and reassortment patterns [134]. Many of these studies have aimed at understanding the transmission and adaptation of avian viruses to humans, which could lead to a better preparedness for a potential influenza pandemic.

4. Deep sequencing in the clinical setting

4.1. Drug resistance

HIV, HCV, or influenza virus susceptibility to antiviral drugs has been traditionally assessed using phenotypic assays (i.e., evaluate the ability of the virus to replicate in the presence of the drug) or detecting drug resistance mutations using Sanger sequencing [24, 151]. Despite longer turn-around-time and higher cost, phenotypic assays can assess susceptibility to any antiviral drug without any prior knowledge of the viral sequence. On the other hand, many HIV- mutations associated with reduced susceptibility to antiviral drugs have been identified [152]. Several Sanger-sequencing based HIV genotypic assays have been developed, while a few are commercially available [24]. Unfortunately, Sanger sequencing can only detect minority variants present at frequencies above 15% to 20% of the viral quasispecies [153–157] and thus fail to quantify low-level HIV drug resistant variants. Using deep sequencing, a series of studies have demonstrated the ability to detect minority HIV drug resistant variants at levels as low as 0.1% to 1% of the virus population [26, 106, 158–162]. Most of these studies used the 454TM platform [106, 158, 159, 163, 164], but recently other deep sequencing technologies such as Illumina® [165] and Ion TorrentTM [162, 166] have proved to be useful in the identification of low-level drug resistant HIV variants. Deep sequencing has been used in HIV drug resistance surveillance [167, 168], to study transmission of HIV drug resistant viruses [101, 160, 169–175], and to evaluate the impact of minority variants on treatment efficacy [160, 176–183].

During the last decade treatment of chronic hepatitis C has been based exclusively on the combination of pegylated(peg)-IFN- α and ribavirin (RBV), however, the characterization the HCV lifecycle has led to the identification of a number of potential new targets for direct-acting antiviral (DAA) drugs [184, 185]. Several DAA drugs have been approved for the treatment of HCV infection and many more are in preclinical or clinical development phases [184]. Similar to HIV, reduced susceptibility to DAA drugs has been reported [186–188] and needs to be monitored. Deep sequencing analysis of the HCV NS3 protease in DAA-naïve patients showed that over 80% of the patients carried viruses with at least one DAA resistance mutation [189, 190]. Other studies have shown minority variants that are resistant to inhibitors targeting other HCV genomic regions [75, 190, 191] or have used deep sequencing to characterize the dynamics of drug-resistant variants during and after treatment [192, 193].

Multiple studies have focused on the development of deep sequencing-based assays to determine drug resistance in different influenza virus subtypes, including highly pathogenic avian viruses like H7N9 and H5N1, that could be used in the clinical setting [194–210].

Some have described the role of minority variants resistant to treatment with oseltamivir [211–213]. Transmission of oseltamivir-resistant minority variants prior to the onset of therapy allowed the rapid development of full-blown resistance once treatment started [212], showing the importance of the use of highly sensitive methods to detect drug resistance-associated polymorphisms in the clinical setting. Other studies have focused on surveillance of drug resistance-associated mutations for both NA inhibitors [201, 214–230] and adamantanes [231–234].

4.2. HIV tropism

The discovery that HIV requires a coreceptor to enter target cells, mainly the chemokine receptors CCR5 and CXCR4 [235–237], prompted the development of novel anti-HIV molecules targeting these receptors, as represented by the CCR5-receptor antagonist maraviroc (Selzentry/Celsentri, Pfizer, NY)[238]. Treatment with CCR5 antagonists requires the prior knowledge of the HIV coreceptor tropism in the patient, which led to the development of numerous phenotypic and genotypic approaches to determine HIV coreceptor usage or tropism [24, 239–241]. While a phenotypic assay, a Trofile (Monogram Biosciences)[242, 243] is currently the standard method to determine HIV-1 coreceptor tropism in the U.S., in-house genotypic HIV-1 tropism tests are largely used in Europe [244, 245]. Both approaches have advantages and disadvantages; however, the lack of sensitivity of Sanger sequencing-based assays has led to the use of deep sequencing to increase the ability of genotypic assays to detect minority CXCR4-tropic variants [26, 162, 246–258]. Thus, more sensitive genotypic HIV tropism assays based on deep sequencing have been developed to detect non-R5 variants below 20% of the population in the clinical setting, which have been shown to correlate well with other HIV-1 tropism tests [162, 246, 247, 250]. Finally, using deep sequencing researchers have been able to detect CXCR4-tropic viruses during early HIV infection [257], to study the mutational pathway of the V3 region in HIV during the transition from CCR5 to CXCR4 usage [259], and to identify low-levels of CXCR4-tropic provirus [260].

4.3. Implications of deep sequencing in clinical management

As described above, sequencing-based assays are being increasingly used in the clinical setting and clinical virology laboratories are not the exception. Is deep sequencing going to eventually replace Sanger/population sequencing in clinical laboratories? The short answer may be yes; however, a series of barriers need to be cleared before these assays become fully established. Although historically high costs have been a major hurdle with these methodologies, this can be addressed by (i) batching and multiplexing samples in single sequencing runs and (ii) the continuous decrease in prices of both reagents and instruments. Access to relatively sophisticated technical expertise to perform and analyze deep sequencing data, including bioinformatics solutions, is another key requirement. All this, together with robust implementation and validation processes, is essential to guarantee the success of laboratory-developed tests (LDT) based on deep sequencing in a CLIA-certified environment (to our knowledge, no deep sequencing-based assay has received IVD approval from the U.S. Food and Drug Administration to be used in clinical virology).

Finally, assay sensitivity, error rate, and data reporting and interpretation by clinicians are critical features of any deep sequencing-based test. The intrinsic error (amplification and sequencing) rate of deep sequencing LDTs needs to be calculated, which defines the analytical sensitivity of the assay. In the case of HIV, the two commercially available tests are able to reliably detect minority variants at frequencies as low as 1% of the virus population [162, 250]. Still, what is the clinical significance of these low-abundance variants? HIV genotyping tests based on Sanger sequencing have been used during the last 25 years to make clinical decisions; however, these tests can only detect minority variants present above approximately 20% of the virus population [153, 154]. Thus, all the current knowledge related to antiretroviral treatment monitoring has been based on the detection of variants at this threshold. It is reasonable to assume that under the appropriate selection (i.e., drug pressure), minority variants present at low frequencies (1% to 20%, detected only by deep sequencing) will eventually outcompete other members of the viral population; however, the clinical relevance of these minority members of the viral population is still under debate [176–181]. Additional studies will be needed to elucidate if early detection of these minority variants could influence strategies to control the growth of HIV and other viruses in the clinical setting.

5. Conclusions

There is no doubt that the use of deep sequencing in virology will continue to grow exponentially. We may not have the perfect deep sequencing platform yet, that is, one that combines low error rates, with long reads, and relatively low cost. In addition, the methodology still requires considerable technical expertise, including strong bioinformatics capability. Nevertheless, deep sequencing is poised to revolutionize clinical virology. This technology has already been critical in the discovery of many new viruses, the characterization of virus populations in humans and the potential association of these viromes with the pathogenesis of several diseases. As described here for HIV, HCV and influenza virus, deep sequencing will facilitate the study of virus diversity in many other DNA and RNA viral pathogens. Deep sequencing-based assays are already being used in the clinical setting to monitor HIV-infected individuals [162, 247, 250] and it is only a matter of time before it will be applied routinely in clinical virology laboratories for virtually all viral pathogens.

Acknowledgments

We thank Dr. Paul Olivo for reading and editing the manuscript.

Funding MQM was partially supported by the CWRU/UH Center for AIDS Research (P30 AI036219). MAM was supported by a grant from the Spanish Ministry of Economy and Competitiveness (BFU2010-15194).

References

1. Holley RW, Apgar J, Everett GA, Madison JT, Marquisee M, Merrill SH, et al. Structure of a Ribonucleic Acid. *Science*. 1965; 147:1462–1465. [PubMed: 14263761]
2. Maxam AM, Gilbert W. A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the United States of America*. 1977; 74:560–564. [PubMed: 265521]

3. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*. 1977; 74:5463–5467. [PubMed: 271968]
4. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, et al. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature*. 1984; 310:207–211. [PubMed: 6087149]
5. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature*. 1985; 313:277–284. [PubMed: 2578615]
6. Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P. Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry*. 1996; 242:84–89. [PubMed: 8923969]
7. Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, et al. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nature biotechnology*. 2000; 18:630–634.
8. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, et al. Performance comparison of benchtop high-throughput sequencing platforms. *Nature biotechnology*. 2012
9. Liu L, Li Y, Li S, Hu N, He Y, Pong R, et al. Comparison of next-generation sequencing systems. *Journal of biomedicine & biotechnology*. 2012; 2012:251364. [PubMed: 22829749]
10. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, et al. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC genomics*. 2012; 13:341. [PubMed: 22827831]
11. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005; 437:376–380. [PubMed: 16056220]
12. Bennett S. Solexa Ltd. *Pharmacogenomics*. 2004; 5:433–438. [PubMed: 15165179]
13. Valouev A, Ichikawa J, Tonthat T, Stuart J, Ranade S, Peckham H, et al. A high-resolution, nucleosome position map of *C. elegans* reveals a lack of universal sequence-dictated positioning. *Genome research*. 2008; 18:1051–1063. [PubMed: 18477713]
14. Bowers J, Mitchell J, Beer E, Buzby PR, Causey M, Efcavitch JW, et al. Virtual terminator nucleotides for next-generation DNA sequencing. *Nature methods*. 2009; 6:593–595. [PubMed: 19620973]
15. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, et al. An integrated semiconductor device enabling non-optical genome sequencing. *Nature*. 2011; 475:348–352. [PubMed: 21776081]
16. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, et al. Real-time DNA sequencing from single polymerase molecules. *Science*. 2009; 323:133–138. [PubMed: 19023044]
17. Howorka S, Cheley S, Bayley H. Sequence-specific detection of individual DNA strands using engineered nanopores. *Nature biotechnology*. 2001; 19:636–639.
18. Radford AD, Chapman D, Dixon L, Chantrey J, Darby AC, Hall N. Application of next-generation sequencing technologies in virology. *The Journal of general virology*. 2012; 93:1853–1868. [PubMed: 22647373]
19. Barzon L, Lavezzo E, Costanzi G, Franchin E, Toppo S, Palu G. Next-generation sequencing technologies in diagnostic virology. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*. 2013; 58:346–350. [PubMed: 23523339]
20. Barba M, Czosnek H, Hadidi A. Historical perspective, development and applications of next-generation sequencing in plant virology. *Viruses*. 2014; 6:106–136. [PubMed: 24399207]
21. Gullapalli RR, Lyons-Weiler M, Petrosko , Dhir R, Becich MJ, LaFramboise WA. Clinical integration of next-generation sequencing technology. *Clinics in laboratory medicine*. 2012; 32:585–599. [PubMed: 23078661]
22. Metzker ML. Sequencing technologies - the next generation. *Nature reviews. Genetics*. 2010; 11:31–46.
23. Park JY, Kricka LJ, Fortina P. Next-generation sequencing in the clinic. *Nature biotechnology*. 2013; 31:990–992.

24. Gibson RM, Schmotzer CL, Quinones-Mateu ME. Next-Generation Sequencing to Help Monitor Patients Infected with HIV: Ready for Clinical Use? *Current infectious disease reports*. 2014; 16:401. [PubMed: 24585216]
25. Quinones-Mateu ME, Endris V. The Sequencing Continuum for Clinical Research: From Sanger to Next Gen. *Science*. 2014; 343:1159.
26. Archer J, Weber J, Henry K, Winner D, Gibson R, Lee L, et al. Use of Four Next-Generation Sequencing Platforms to Determine HIV-1 Coreceptor Tropism. *PloS one*. 2012; 7:e49602. [PubMed: 23166726]
27. Lipkin WI, Firth C. Viral surveillance and discovery. *Current opinion in virology*. 2013; 3:199–204. [PubMed: 23602435]
28. Mokili JL, Rohwer F, Dutilh BE. Metagenomics and future perspectives in virus discovery. *Current opinion in virology*. 2012; 2:63–77. [PubMed: 22440968]
29. Julian TR, Schwab KJ. Challenges in environmental detection of human viral pathogens. *Current opinion in virology*. 2012; 2:78–83. [PubMed: 22440969]
30. Grard G, Fair JN, Lee D, Slikas E, Steffen I, Muyembe JJ, et al. A novel rhabdovirus associated with acute hemorrhagic fever in central Africa. *PLoS pathogens*. 2012; 8:e1002924. [PubMed: 23028323]
31. Tan le V, van Doorn HR, Nghia HD, Chau TT, Tu le TP, de Vries M, et al. Identification of a new cyclovirus in cerebrospinal fluid of patients with acute central nervous system infections. *mBio*. 2013; 4:e00231–00213. [PubMed: 23781068]
32. Phelps NB, Mor SK, Armien AG, Batts W, Goodwin AE, Hopper L, et al. Isolation and molecular characterization of a novel picornavirus from baitfish in the USA. *PloS one*. 2014; 9:e87593. [PubMed: 24586283]
33. Stenglein MD, Sanders C, Kistler AL, Ruby JG, Franco JY, Reavill DR, et al. Identification, characterization, and in vitro culture of highly divergent arenaviruses from boa constrictors and annulated tree boas: candidate etiological agents for snake inclusion body disease. *mBio*. 2012; 3:e00180–00112. [PubMed: 22893382]
34. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, Henriquez JA, et al. Characterization of a canine homolog of hepatitis C virus. *Proc Natl Acad Sci U S A*. 2011; 108:11608–11613. [PubMed: 21610165]
35. Burbelo PD, Dubovi EJ, Simmonds P, Medina JL, Henriquez JA, Mishra N, et al. Serology-enabled discovery of genetically diverse hepaciviruses in a new host. *J Virol*. 2012; 86:6171–6178. [PubMed: 22491452]
36. Breitbart M, Salamon P, Andresen B, Mahaffy JM, Segall AM, Mead D, et al. Genomic analysis of uncultured marine viral communities. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:14250–14255. [PubMed: 12384570]
37. Lecuit M, Eloit M. The human virome: new tools and concepts. *Trends in Microbiology*. 2013; 21:510–515. [PubMed: 23906500]
38. Delwart E. A roadmap to the human virome. *PLoS pathogens*. 2013; 9:e1003146. [PubMed: 23457428]
39. Minot S, Bryson A, Chehoud C, Wu GD, Lewis JD, Bushman FD. Rapid evolution of the human gut virome. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:12450–12455. [PubMed: 23836644]
40. Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, et al. The human gut virome: inter-individual variation and dynamic response to diet. *Genome research*. 2011; 21:1616–1625. [PubMed: 21880779]
41. Foulongne V, Sauvage V, Hebert C, Dereure O, Cheval J, Gouilh MA, et al. Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. *PloS one*. 2012; 7:e38499. [PubMed: 22723863]
42. Li L, Deng X, Linsuwanon P, Bangsberg D, Bwana MB, Hunt P, et al. AIDS alters the commensal plasma virome. *Journal of Virology*. 2013; 87:10912–10915. [PubMed: 23903845]
43. Handley SA, Thackray LB, Zhao G, Presti R, Miller AD, Droit L, et al. Pathogenic simian immunodeficiency virus infection is associated with expansion of the enteric virome. *Cell*. 2012; 151:253–266. [PubMed: 23063120]

44. De Vlaminc I, Khush KK, Strehl C, Kohli B, Luikart H, Neff NF, et al. Temporal response of the human virome to immunosuppression and antiviral therapy. *Cell*. 2013; 155:1178–1187. [PubMed: 24267896]
45. Wylie KM, Mihindukulasuriya KA, Sodergren E, Weinstock GM, Storch GA. Sequence analysis of the human virome in febrile and afebrile children. *PloS one*. 2012; 7:e27735. [PubMed: 22719819]
46. Alquezar-Planas DE, Mourier T, Bruhn CA, Hansen AJ, Vitcetz SN, Mork S, et al. Discovery of a divergent HPIV4 from respiratory secretions using second and third generation metagenomic sequencing. *Scientific reports*. 2013; 3:2468. [PubMed: 24002378]
47. Shan T, Li L, Simmonds P, Wang C, Moeser A, Delwart E. The fecal virome of pigs on a high-density farm. *Journal of Virology*. 2011; 85:11697–11708.. [PubMed: 21900163]
48. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, et al. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*. 2008; 456:53–59.. [PubMed: 18987734]
49. Phan TG, Vo NP, Boros A, Pankovics P, Reuter G, Li OT, et al. The viruses of wild pigeon droppings. *PloS one*. 2013; 8:e72787. [PubMed: 24023772]
50. Day JM, Ballard LL, Duke MV, Scheffler BE, Zsak L. Metagenomic analysis of the turkey gut RNA virus community. *Virology journal*. 2010; 7:313. [PubMed: 21073719]
51. Belak S, Karlsson OE, Blomstrom AL, Berg M, Granberg F. New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary microbiology*. 2013; 165:95–101. [PubMed: 23428379]
52. Li L, Pesavento PA, Shan T, Leutenegger CM, Wang C, Delwart E. Viruses in diarrhoeic dogs include novel kobuviruses and sapoviruses. *The Journal of general virology*. 2011; 92:2534–2541. [PubMed: 21775584]
53. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, et al. New world bats harbor diverse influenza A viruses. *PLoS pathogens*. 2013; 9:e1003657. [PubMed: 24130481]
54. Quan PL, Firth C, Conte JM, Williams SH, Zambrana-Torrel CM, Anthony SJ, et al. Bats are a major natural reservoir for hepaciviruses and pegiviruses. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:8194–8199. [PubMed: 23610427]
55. Drexler JF, Geipel A, Konig A, Corman VM, van Riel D, Leijten LM, et al. Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:16151–16156. [PubMed: 24043818]
56. Guo WP, Lin XD, Wang W, Tian JH, Cong ML, Zhang HL, et al. Phylogeny and origins of hantaviruses harbored by bats, insectivores, and rodents. *PLoS pathogens*. 2013; 9:e1003159. [PubMed: 23408889]
57. Marsh GA, de Jong C, Barr JA, Tachedjian M, Smith C, Middleton D, et al. Cedar virus: a novel Henipavirus isolated from Australian bats. *PLoS pathogens*. 2012; 8:e1002836. [PubMed: 22879820]
58. Yang L, Wu Z, Ren X, Yang F, He G, Zhang J, et al. Novel SARS-like betacoronaviruses in bats, China, 2011. *Emerging Infectious Diseases*. 2013; 19:989–991. [PubMed: 23739658]
59. Smith I, Wang LF. Bats and their virome: an important source of emerging viruses capable of infecting humans. *Current opinion in virology*. 2013; 3:84–91.. [PubMed: 23265969]
60. Bolduc B, Shaughnessy DP, Wolf YI, Koonin EV, Roberto FF, Young M. Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated Yellowstone hot springs. *Journal of Virology*. 2012; 86:5562–5573.. [PubMed: 22379100]
61. Hurwitz BL, Sullivan MB. The Pacific Ocean virome (POV): a marine viral metagenomic dataset and associated protein clusters for quantitative viral ecology. *PloS one*. 2013; 8:e57355. [PubMed: 23468974]
62. Al Rwahnih M, Daubert S, Urbez-Torres JR, Cordero F, Rowhani A. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. *Archives of Virology*. 2011; 156:397–403. [PubMed: 21140178]
63. Leitner, T. *The molecular epidemiology of human viruses*. 1st ed. Springer; New York: 2002.

64. Hemelaar J. Implications of HIV diversity for the HIV-1 pandemic. *J Infect.* 2013; 66:391–400. [PubMed: 23103289]
65. Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global trends in molecular epidemiology of HIV-1 during 2000–2007. *Aids.* 2011; 25:679–689. [PubMed: 21297424]
66. Lal RB, Chakrabarti S, Yang C. Impact of genetic diversity of HIV-1 on diagnosis, antiretroviral therapy & vaccine development. *Indian J Med Res.* 2005; 121:287–314. [PubMed: 15817945]
67. Quinones-Mateu ME, Arts EJ. Recombination in HIV-1: Update and implications. *AIDS Reviews.* 1999; 1:89–100.
68. Kijak GH, McCutchan FE. HIV diversity, molecular epidemiology, and the role of recombination. *Curr Infect Dis Rep.* 2005; 7:480–488. [PubMed: 16225787]
69. Bruselles A, Rozera G, Bartolini B, Prosperi M, Del Nonno F, Narciso P, et al. Use of massive parallel pyrosequencing for near full-length characterization of a unique HIV Type 1 BF recombinant associated with a fatal primary infection. *AIDS Res Hum Retroviruses.* 2009; 25:937–942. [PubMed: 19751146]
70. Park SY, Goeken N, Lee HJ, Bolan R, Dube MP, Lee HY. Developing high-throughput HIV incidence assay with pyrosequencing platform. *Journal of Virology.* 2014; 88:2977–2990. [PubMed: 24371062]
71. Redd AD, Quinn TC, Tobian AA. Frequency and implications of HIV superinfection. *The Lancet infectious diseases.* 2013; 13:622–628. [PubMed: 23726798]
72. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: Updated criteria and genotype assignment web resource. *Hepatology.* 2014; 59:318–327. [PubMed: 24115039]
73. Simmonds P. Genetic diversity and evolution of hepatitis C virus--15 years on. *J.Gen.Virol.* 2004; 85:3173–3188. [PubMed: 15483230]
74. Simmonds P. The origin of hepatitis C virus. *Curr Top Microbiol Immunol.* 2013; 369:1–15. [PubMed: 23463195]
75. Newman RM, Kuntzen T, Weiner B, Berical A, Charlebois P, Kuiken C, et al. Whole genome pyrosequencing of rare hepatitis C virus genotypes enhances subtype classification and identification of naturally occurring drug resistance variants. *J Infect Dis.* 2013; 208:17–31. [PubMed: 23136221]
76. Forbi JC, Purdy MA, Campo DS, Vaughan G, Dimitrova ZE, Ganova-Raeva LM, et al. Epidemic history of hepatitis C virus infection in two remote communities in Nigeria, West Africa. *J Gen Virol.* 2012; 93:1410–1421. [PubMed: 22456613]
77. Croville G, Soubies SM, Barbieri J, Klopp C, Mariette J, Bouchez O, et al. Field monitoring of avian influenza viruses: whole-genome sequencing and tracking of neuraminidase evolution using 454 pyrosequencing. *J Clin Microbiol.* 2012; 50:2881–2887. [PubMed: 22718944]
78. Dugan VG, Dunham EJ, Jin G, Sheng ZM, Kaser E, Nolting JM, et al. Phylogenetic analysis of low pathogenicity H5N1 and H7N3 influenza A virus isolates recovered from sentinel, free flying, wild mallards at one study site during 2006. *Virology.* 2011; 417:98–105. [PubMed: 21658737]
79. Marchenko VY, Alekseev AY, Sharshov KA, Petrov VN, Silko NY, Susloparov IM, et al. Ecology of influenza virus in wild bird populations in Central Asia. *Avian Dis.* 2012; 56:234–237. [PubMed: 22545553]
80. Van Borm S, Rosseel T, Vangeluwe D, Vandenbussche F, van den Berg T, Lambrecht B. Phylogeographic analysis of avian influenza viruses isolated from Charadriiformes in Belgium confirms intercontinental reassortment in gulls. *Arch Virol.* 2012; 157:1509–1522. [PubMed: 22580556]
81. Yu X, Jin T, Cui Y, Pu X, Li J, Xu J, et al. Coexistence of influenza H7N9 and H9N2 in poultry linked to human H7N9 infection and their genome characteristics. *J Virol.* 2014
82. Clavijo A, Nikooinenejad A, Esfahani MS, Metz RP, Schwartz S, Atashpaz-Gargari E, et al. Identification and analysis of the first 2009 pandemic H1N1 influenza virus from U.S. feral swine. *Zoonoses Public Health.* 2013; 60:327–335. [PubMed: 22978260]
83. Lange J, Groth M, Schlegel M, Krumbholz A, Wiczorek K, Ulrich R, et al. Reassortants of the pandemic (H1N1) 2009 virus and establishment of a novel porcine H1N2 influenza virus, lineage in Germany. *Vet Microbiol.* 2013; 167:345–356. [PubMed: 24139631]

84. Lorusso A, Vincent AL, Harland ML, Alt D, Bayles DO, Swenson SL, et al. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. *J Gen Virol.* 2011; 92:919–930. [PubMed: 21177926]
85. Lycett SJ, Baillie G, Coulter E, Bhatt S, Kellam P, McCauley JW, et al. Estimating reassortment rates in co-circulating Eurasian swine influenza viruses. *J Gen Virol.* 2012; 93:2326–2336. [PubMed: 22971819]
86. Barrero PR, Viegas M, Valinotto LE, Mistchenko AS. Genetic and phylogenetic analyses of influenza A H1N1pdm virus in Buenos Aires, Argentina. *J Virol.* 2011; 85:1058–1066. [PubMed: 21047959]
87. de la Rosa-Zamboni D, Vazquez-Perez JA, Avila-Rios S, Carranco-Arenas AP, Ormsby CE, Cummings CA, et al. Molecular characterization of the predominant influenza A(H1N1)pdm09 virus in Mexico, December 2011–February 2012. *PLoS One.* 2012; 7:e50116. [PubMed: 23209653]
88. Fordyce SL, Bragstad K, Pedersen SS, Jensen TG, Gahrn-Hansen B, Daniels R, et al. Genetic diversity among pandemic 2009 influenza viruses isolated from a transmission chain. *Virol J.* 2013; 10:116. [PubMed: 23587185]
89. Lin J, Kang M, Zhong H, Zhang X, Yang F, Ni H, et al. Influenza seasonality and predominant subtypes of influenza virus in Guangdong, China, 2004–2012. *J Thorac Dis.* 2013; 5:S109–117. [PubMed: 23977430]
90. Eigen, M.; Biebricher, CK. Sequence space and quasispecies distribution. In: Domingo, E.; Holland, JJ.; Ahlquist, P., editors. *RNA genetics*. CRC Press; Boca Raton: 1988. p. 211–245.
91. Domingo E, Menendez-Arias L, Quinones-Mateu ME, Holguin A, Gutierrez-Rivas M, Martinez MA, et al. Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. *Prog. Drug Res.* 1997; 48:99–128. [PubMed: 9204684]
92. Najera I, Holguin A, Quinones-Mateu ME, Munoz-Fernandez MA, Najera R, Lopez-Galindez C, et al. Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. *J. Virol.* 1995; 69:23–31. [PubMed: 7983713]
93. Quinones-Mateu ME, Holguin A, Dopazo J, Najera I, Domingo E. Point mutant frequencies in the pol gene of human immunodeficiency virus type 1 are two- to threefold lower than those of env. *AIDS Res. Hum. Retroviruses.* 1996; 12:1117–1128. [PubMed: 8844016]
94. Gall A, Ferns B, Morris C, Watson S, Cotten M, Robinson M, et al. Universal amplification, next-generation sequencing, and assembly of HIV-1 genomes. *J Clin Microbiol.* 2012; 50:3838–3844. [PubMed: 22993180]
95. Henn MR, Boutwell CL, Charlebois P, Lennon NJ, Power KA, Macalalad AR, et al. Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS Pathog.* 2012; 8:e1002529. [PubMed: 22412369]
96. Bimber BN, Dudley DM, Lauck M, Becker EA, Chin EN, Lank SM, et al. Whole-genome characterization of human and simian immunodeficiency virus intrahost diversity by ultra-deep pyrosequencing. *J Virol.* 2010; 84:12087–12092. [PubMed: 20844037]
97. Fisher R, van Zyl GU, Travers SA, Kosakovsky Pond SL, Engelbrech S, Murrell B, et al. Deep sequencing reveals minor protease resistance mutations in patients failing a protease inhibitor regimen. *J Virol.* 2012; 86:6231–6237. [PubMed: 22457522]
98. Hedskog C, Mild M, Jernberg J, Sherwood E, Bratt G, Leitner T, et al. Dynamics of HIV-1 quasispecies during antiviral treatment dissected using ultra-deep pyrosequencing. *PLoS One.* 2010; 5:e11345. [PubMed: 20628644]
99. Lataillade M, Chiarella J, Yang R, DeGrosky M, Uy J, Seekins D, et al. Virologic failures on initial boosted-PI regimen infrequently possess low-level variants with major PI resistance mutations by ultra-deep sequencing. *PloS one.* 2012; 7:e30118. [PubMed: 22355307]
100. Mild M, Hedskog C, Jernberg J, Albert J. Performance of ultra-deep pyrosequencing in analysis of HIV-1 pol gene variation. *PloS one.* 2011; 6:e22741. [PubMed: 21799940]
101. Mitsuya Y, Varghese V, Wang C, Liu TF, Holmes SP, Jayakumar P, et al. Minority human immunodeficiency virus type 1 variants in antiretroviral-naïve persons with reverse transcriptase codon 215 revertant mutations. *J Virol.* 2008; 82:10747–10755. [PubMed: 18715933]

102. Mortier V, Dauwe K, Vancoillie L, Staelens D, Van Wanzeele F, Vogelaers D, et al. Frequency and predictors of HIV-1 co-receptor switch in treatment naive patients. *PLoS One*. 2013; 8:e80259. [PubMed: 24244665]
103. Recordon-Pinson P, Raymond S, Bellocave P, Marcelin AG, Soulie C, Descamps D, et al. HIV-1 dynamics and coreceptor usage in Maraviroc-treated patients with ongoing replication. *Antimicrob Agents Chemother*. 2013; 57:930–935. [PubMed: 23208718]
104. Rozera G, Abbate I, Bruxelles A, Vlasi C, D'Offizi G, Narciso P, et al. Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations. *Retrovirology*. 2009; 6:15. [PubMed: 19216757]
105. Varghese V, Shahriar R, Rhee SY, Liu T, Simen BB, Egholm M, et al. Minority variants associated with transmitted and acquired HIV-1 nonnucleoside reverse transcriptase inhibitor resistance: implications for the use of second-generation nonnucleoside reverse transcriptase inhibitors. *J Acquir Immune Defic Syndr*. 2009; 52:309–315. [PubMed: 19734799]
106. Shao W, Boltz VF, Spindler JE, Kearney MF, Maldarelli F, Mellors JW, et al. Analysis of 454 sequencing error rate, error sources, and artifact recombination for detection of Low-frequency drug resistance mutations in HIV-1 DNA. *Retrovirology*. 2013; 10:18. [PubMed: 23402264]
107. Rozera G, Abbate I, Bruxelles A, Vlasi C, D'Offizi G, Narciso P, et al. Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations. *Retrovirology*. 2009; 6:15. [PubMed: 19216757]
108. Yin L, Liu L, Sun Y, Hou W, Lowe AC, Gardner BP, et al. High-resolution deep sequencing reveals biodiversity, population structure, and persistence of HIV-1 quasispecies within host ecosystems. *Retrovirology*. 2012; 9:108. [PubMed: 23244298]
109. Poon AF, McGovern RA, Mo T, Knapp DJ, Brenner B, Routy JP, et al. Dates of HIV infection can be estimated for seroprevalent patients by coalescent analysis of serial next-generation sequencing data. *Aids*. 2011; 25:2019–2026. [PubMed: 21832936]
110. Armenia D, Vandenbroucke I, Fabeni L, Van Marck H, Cento V, D'Arrigo R, et al. Study of genotypic and phenotypic HIV-1 dynamics of integrase mutations during raltegravir treatment: a refined analysis by ultra-deep 454 pyrosequencing. *J Infect Dis*. 2012; 205:557–567. [PubMed: 22238474]
111. Tsibris AM, Korber B, Arnaout R, Russ C, Lo CC, Leitner T, et al. Quantitative deep sequencing reveals dynamic HIV-1 escape and large population shifts during CCR5 antagonist therapy in vivo. *PLoS One*. 2009; 4:e5683. [PubMed: 19479085]
112. Knoepfel SA, Di Giallonardo F, Daumer M, Thielen A, Metzner KJ. In-depth analysis of G-to-A hypermutation rate in HIV-1 env DNA induced by endogenous APOBEC3 proteins using massively parallel sequencing. *J Virol Methods*. 2011; 171:329–338. [PubMed: 21111003]
113. Lee GQ, Swenson LC, Poon AF, Martin JN, Hatano H, Deeks SG, et al. Prolonged and substantial discordance in prevalence of raltegravir-resistant HIV-1 in plasma versus PBMC samples revealed by 454 “deep” sequencing. *PloS one*. 2012; 7:e46181. [PubMed: 23049972]
114. Wu X, Zhou T, Zhu J, Zhang B, Georgiev I, Wang C, et al. Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. *Science*. 2011; 333:1593–1602. [PubMed: 21835983]
115. Xiao M, Prabakaran P, Chen W, Kessing B, Dimitrov DS. Deep sequencing and Circos analyses of antibody libraries reveal antigen-driven selection of Ig VH genes during HIV-1 infection. *Exp Mol Pathol*. 2013; 95:357–363. [PubMed: 24158018]
116. Zhang Y, Yuan T, Li J, Xu J, Shao Y, Chen Z, et al. The potential of the human immune system to develop broadly neutralizing HIV-1 antibodies: implications for vaccine development. *Aids*. 2013; 27:2529–2539. [PubMed: 24100711]
117. Brumme CJ, Huber KD, Dong W, Poon AF, Harrigan PR, Sluis-Cremer N. Replication fitness of multiple nonnucleoside reverse transcriptase-resistant HIV-1 variants in the presence of etravirine measured by 454 deep sequencing. *J Virol*. 2013; 87:8805–8807. [PubMed: 23720723]
118. Ninomiya M, Ueno Y, Funayama R, Nagashima T, Nishida Y, Kondo Y, et al. Use of illumina deep sequencing technology to differentiate hepatitis C virus variants. *J Clin Microbiol*. 2012; 50:857–866. [PubMed: 22205816]

119. Gregori J, Salicru M, Domingo E, Sanchez A, Rodriguez-Frias F, Quer J. Inference with viral quasispecies diversity indices: Clonal and NGS approaches. *Bioinformatics*. 2014
120. Escobar-Gutierrez A, Vazquez-Pichardo M, Cruz-Rivera M, Rivera-Osorio P, Carpio-Pedroza JC, Ruiz-Pacheco JA, et al. Identification of hepatitis C virus transmission using a next-generation sequencing approach. *J Clin Microbiol*. 2012; 50:1461–1463. [PubMed: 22301026]
121. Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A, et al. Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. *PLoS Pathog*. 2011; 7:e1002243. [PubMed: 21912520]
122. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet*. 2013; 45:164–171. [PubMed: 23291588]
123. Hou W, Bonkovsky HL. Non-coding RNAs in hepatitis C-induced hepatocellular carcinoma: Dysregulation and implications for early detection, diagnosis and therapy. *World J Gastroenterol*. 2013; 19:7836–7845. [PubMed: 24307777]
124. Bidzhieva B, Zagorodnyaya T, Karagiannis K, Simonyan V, Laassri M, Chumakov K. Deep sequencing approach for genetic stability evaluation of influenza A viruses. *J Virol Methods*. 2014
125. Bhatt S, Holmes EC, Pybus OG. The genomic rate of molecular adaptation of the human influenza A virus. *Mol Biol Evol*. 2011; 28:2443–2451. [PubMed: 21415025]
126. Tsai KN, Chen GW. Influenza genome diversity and evolution. *Microbes Infect*. 2011; 13:479–488. [PubMed: 21276870]
127. Warren S, Wan XF, Conant G, Korkin D. Extreme evolutionary conservation of functionally important regions in H1N1 influenza proteome. *PLoS One*. 2013; 8:e81027. [PubMed: 24282564]
128. Bourret V, Croville G, Mariette J, Klopp C, Bouchez O, Tiley L, et al. Whole-genome, deep pyrosequencing analysis of a duck influenza A virus evolution in swine cells. *Infect Genet Evol*. 2013; 18:31–41. [PubMed: 23660486]
129. Crusat M, Liu J, Palma AS, Childs RA, Liu Y, Wharton SA, et al. Changes in the hemagglutinin of H5N1 viruses during human infection--influence on receptor binding. *Virology*. 2013; 447:326–337. [PubMed: 24050651]
130. Hoper D, Kalthoff D, Hoffmann B, Beer M. Highly pathogenic avian influenza virus subtype H5N1 escaping neutralization: more than HA variation. *J Virol*. 2012; 86:1394–1404. [PubMed: 22090121]
131. Mertens E, Dugan VG, Stockwell TB, Lindsay LL, Plancarte M, Boyce WM. Evaluation of phenotypic markers in full genome sequences of avian influenza isolates from California. *Comp Immunol Microbiol Infect Dis*. 2013; 36:521–536. [PubMed: 23891310]
132. Wilker PR, Dinis JM, Starrett G, Imai M, Hatta M, Nelson CW, et al. Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses. *Nat Commun*. 2013; 4:2636. [PubMed: 24149915]
133. Waybright N, Petrangelo E, Lowary P, Bogan J, Mulholland N. Detection of human virulence signatures in H5N1. *J Virol Methods*. 2008; 154:200–205. [PubMed: 18929600]
134. Deng YM, Caldwell N, Barr IG. Rapid detection and subtyping of human influenza A viruses and reassortants by pyrosequencing. *PLoS One*. 2011; 6:e23400. [PubMed: 21886790]
135. Xiao YL, Kash JC, Beres SB, Sheng ZM, Musser JM, Taubenberger JK. High-throughput RNA sequencing of a formalin-fixed, paraffin-embedded autopsy lung tissue sample from the 1918 influenza pandemic. *J Pathol*. 2013; 229:535–545. [PubMed: 23180419]
136. Taubenberger JK. The virulence of the 1918 pandemic influenza virus: unraveling the enigma. *Arch Virol Suppl*. 2005:101–115. [PubMed: 16355870]
137. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. *Nature*. 2005; 437:889–893. [PubMed: 16208372]
138. Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science*. 2005; 310:77–80. [PubMed: 16210530]

139. Bartolini B, Chillemi G, Abbate I, Bruselles A, Rozera G, Castrignano T, et al. Assembly and characterization of pandemic influenza A H1N1 genome in nasopharyngeal swabs using high-throughput pyrosequencing. *New Microbiol.* 2011; 34:391–397. [PubMed: 22143812]
140. Deyde VM, Gubareva LV. Influenza genome analysis using pyrosequencing method: current applications for a moving target. *Expert Rev Mol Diagn.* 2009; 9:493–509. [PubMed: 19580433]
141. Hoper D, Hoffmann B, Beer M. A comprehensive deep sequencing strategy for full-length genomes of influenza A. *PLoS One.* 2011; 6:e19075. [PubMed: 21559493]
142. Kampmann ML, Fordyce SL, Avila-Arcos MC, Rasmussen M, Willerslev E, Nielsen LP, et al. A simple method for the parallel deep sequencing of full influenza A genomes. *J Virol Methods.* 2011; 178:243–248. [PubMed: 21946287]
143. Rutvisuttinunt W, Chinnawirotpisan P, Simasathien S, Shrestha SK, Yoon IK, Klungthong C, et al. Simultaneous and complete genome sequencing of influenza A and B with high coverage by Illumina MiSeq Platform. *J Virol Methods.* 2013; 193:394–404. [PubMed: 23856301]
144. Hoper D, Hoffmann B, Beer M. Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. *J Clin Microbiol.* 2009; 47:674–679. [PubMed: 19109473]
145. Ren X, Yang F, Hu Y, Zhang T, Liu L, Dong J, et al. Full genome of influenza A (H7N9) virus derived by direct sequencing without culture. *Emerg Infect Dis.* 2013; 19:1881–1884. [PubMed: 24206919]
146. Archer J, Baillie G, Watson SJ, Kellam P, Rambaut A, Robertson DL. Analysis of high-depth sequence data for studying viral diversity: a comparison of next generation sequencing platforms using Segminator II. *BMC Bioinformatics.* 2012; 13:47. [PubMed: 22443413]
147. Flaherty P, Natsoulis G, Muralidharan O, Winters M, Buenrostro J, Bell J, et al. Ultrasensitive detection of rare mutations using next-generation targeted resequencing. *Nucleic Acids Res.* 2012; 40:e2. [PubMed: 22013163]
148. Kuroda M, Katano H, Nakajima N, Tobiume M, Aina A, Sekizuka T, et al. Characterization of quasispecies of pandemic 2009 influenza A virus (A/H1N1/2009) by de novo sequencing using a next-generation DNA sequencer. *PLoS One.* 2010; 5:e10256. [PubMed: 20428231]
149. Levine M, Sheu TG, Gubareva LV, Mishin VP. Detection of hemagglutinin variants of the pandemic influenza A (H1N1) 2009 virus by pyrosequencing. *J Clin Microbiol.* 2011; 49:1307–1312. [PubMed: 21307217]
150. Watson SJ, Welkers MR, Depledge DP, Coulter E, Breuer JM, de Jong MD, et al. Viral population analysis and minority-variant detection using short read next-generation sequencing. *Philos Trans R Soc Lond B Biol Sci.* 2013; 368:20120205. [PubMed: 23382427]
151. Kwong AD, Najera I, Bechtel J, Bowden S, Fitzgibbon J, Harrington P, et al. Sequence and phenotypic analysis for resistance monitoring in hepatitis C virus drug development: recommendations from the HCV DRAG. *Gastroenterology.* 2011; 140:755–760. [PubMed: 21255574]
152. Shafer RW. Rationale and uses of a public HIV drug-resistance database. *The Journal of infectious diseases.* 2006; 194(Suppl 1):S51–58. [PubMed: 16921473]
153. Larder BA, Kohli A, Kellam P, Kemp SD, Kronick M, Henfrey RD. Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing. *Nature.* 1993; 365:671–673. [PubMed: 8413632]
154. Church JD, Jones D, Flys T, Hoover D, Marlowe N, Chen S, et al. Sensitivity of the ViroSeq HIV-1 genotyping system for detection of the K103N resistance mutation in HIV-1 subtypes A, C, and D. *The Journal of molecular diagnostics : JMD.* 2006; 8:430–432. quiz 527. [PubMed: 16931582]
155. Halvas EK, Aldrovandi GM, Balfe P, Beck IA, Boltz VF, Coffin JM, et al. Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. *Journal of clinical microbiology.* 2006; 44:2612–2614. [PubMed: 16825395]
156. Leitner T, Halapi E, Scarlatti G, Rossi P, Albert J, Fenyo EM, et al. Analysis of heterogeneous viral populations by direct DNA sequencing. *Biotechniques.* 1993; 15:120–127. [PubMed: 8363827]

157. Grant RM, Kuritzkes DR, Johnson VA, Mellors JW, Sullivan JL, Swanstrom R, et al. Accuracy of the TRUGENE HIV-1 genotyping kit. *Journal of clinical microbiology*. 2003; 41:1586–1593. [PubMed: 12682149]
158. Dudley DM, Chin EN, Bimber BN, Sanabani SS, Tarosso LF, Costa PR, et al. Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. *PloS one*. 2012; 7:e36494. [PubMed: 22574170]
159. Fisher R, van Zyl GU, Travers SA, Kosakovsky Pond SL, Engelbrech S, Murrell B, et al. Deep sequencing reveals minor protease resistance mutations in patients failing a protease inhibitor regimen. *Journal of Virology*. 2012; 86:6231–6237. [PubMed: 22457522]
160. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, Baxter JD, et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. *The Journal of infectious diseases*. 2009; 199:693–701. [PubMed: 19210162]
161. Wang C, Mitsuya Y, Gharizadeh B, Ronaghi M, Shafer RW. Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. *Genome research*. 2007; 17:1195–1201. [PubMed: 17600086]
162. Gibson RM, Meyer AM, Winner D, Archer J, Feyertag F, Ruiz-Mateos E, et al. Sensitive Deep Sequencing-based HIV-1 Genotyping Assay to Simultaneously Determine Susceptibility to Protease, Reverse Transcriptase, Integrase, and Maturation Inhibitors, as well as HIV-1 Coreceptor Tropism. *Antimicrobial Agents and Chemotherapy*. 2014; 58:2167–2185. [PubMed: 24468782]
163. Avidor B, Girshengorn S, Matus N, Talio H, Achsanov S, Zeldis I, et al. Evaluation of a benchtop HIV ultradeep pyrosequencing drug resistance assay in the clinical laboratory. *Journal of clinical microbiology*. 2013; 51:880–886. [PubMed: 23284027]
164. Stelzl E, Proll J, Bizon B, Niklas N, Danzer M, Hackl C, et al. Human immunodeficiency virus type 1 drug resistance testing: Evaluation of a new ultra-deep sequencing-based protocol and comparison with the TRUGENE HIV-1 Genotyping Kit. *Journal of Virological Methods*. 2011; 178:94–97. [PubMed: 21907239]
165. Willerth SM, Pedro HA, Pachter L, Humeau LM, Arkin AP, Schaffer DV. Development of a low bias method for characterizing viral populations using next generation sequencing technology. *PloS one*. 2010; 5:e13564. [PubMed: 21042592]
166. Chang MW, Oliveira G, Yuan J, Okulicz JF, Levy S, Torbett BE. Rapid deep sequencing of patient-derived HIV with ion semiconductor technology. *Journal of Virological Methods*. 2013; 189:232–234. [PubMed: 23384677]
167. Ji H, Li Y, Graham M, Liang BB, Pilon R, Tyson S, et al. Next-generation sequencing of dried blood spot specimens: a novel approach to HIV drug-resistance surveillance. *Antivir Ther*. 2011; 16:871–878. [PubMed: 21900719]
168. Ji H, Li Y, Liang B, Pilon R, MacPherson P, Bergeron M, et al. Pyrosequencing dried blood spots reveals differences in HIV drug resistance between treatment naive and experienced patients. *PLoS One*. 2013; 8:e56170. [PubMed: 23409150]
169. Bellecave P, Recordon-Pinson P, Papuchon J, Vandenhende MA, Reigadas S, Tauzin B, et al. Detection of Low-Frequency HIV Type 1 Reverse Transcriptase Drug Resistance Mutations by Ultradeep Sequencing in Naive HIV Type 1-Infected individuals. *AIDS Res Hum Retroviruses*. 2013
170. Gonzalez S, Tully DC, Gondwe C, Wood C. Low-abundance resistant mutations in HIV-1 subtype C antiretroviral therapy-naive individuals as revealed by pyrosequencing. *Curr HIV Res*. 2013; 11:43–49. [PubMed: 23305169]
171. Ji H, Liang B, Li Y, Van Domselaar G, Graham M, Tyler S, et al. Low abundance drug resistance variants in transmitted HIV drug resistance surveillance specimens identified using tagged pooled pyrosequencing. *J Virol Methods*. 2013; 187:314–320. [PubMed: 23159670]
172. Lataillade M, Chiarella J, Yang R, Schnittman S, Wirtz V, Uy J, et al. Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naive subjects in the CASTLE study. *PLoS One*. 2010; 5:e10952. [PubMed: 20532178]

173. Messiaen P, Verhofstede C, Vandenbroucke I, Dinakis S, Van Eygen V, Thys K, et al. Ultra-deep sequencing of HIV-1 reverse transcriptase before start of an NNRTI-based regimen in treatment-naïve patients. *Virology*. 2012; 426:7–11. [PubMed: 22305619]
174. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, Baxter JD, et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naïve patients significantly impact treatment outcomes. *J Infect Dis*. 2009; 199:693–701. [PubMed: 19210162]
175. Geretti AM, Conibear T, Hill A, Johnson JA, Tambuyzer L, Thys K, et al. Sensitive testing of plasma HIV-1 RNA and Sanger sequencing of cellular HIV-1 DNA for the detection of drug resistance prior to starting first-line antiretroviral therapy with efavirenz or efavirenz. *J Antimicrob Chemother*. 2013
176. Li JZ, Paredes R, Ribaudo HJ, Svarovskaia ES, Kozal MJ, Hullsiek KH, et al. Relationship between minority nonnucleoside reverse transcriptase inhibitor resistance mutations, adherence, and the risk of virologic failure. *AIDS*. 2012; 26:185–192. [PubMed: 22179227]
177. Li JZ, Kuritzkes DR. Clinical implications of HIV-1 minority variants. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2013; 56:1667–1674. [PubMed: 23446628]
178. Li JZ, Paredes R, Ribaudo HJ, Svarovskaia ES, Metzner KJ, Kozal MJ, et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA : the journal of the American Medical Association*. 2011; 305:1327–1335. [PubMed: 21467286]
179. Stekler JD, Ellis GM, Carlsson J, Eilers B, Holte S, Maenza J, et al. Prevalence and impact of minority variant drug resistance mutations in primary HIV-1 infection. *PloS one*. 2011; 6:e28952. [PubMed: 22194957]
180. Li JZ, Paredes R, Ribaudo HJ, Kozal MJ, Svarovskaia ES, Johnson JA, et al. Impact of minority nonnucleoside reverse transcriptase inhibitor resistance mutations on resistance genotype after virologic failure. *The Journal of infectious diseases*. 2013; 207:893–897. [PubMed: 23264671]
181. Codoner FM, Pou C, Thielen A, Garcia F, Delgado R, Dalmau D, et al. Added value of deep sequencing relative to population sequencing in heavily pre-treated HIV-1-infected subjects. *PloS one*. 2011; 6:e19461. [PubMed: 21602929]
182. Gonzalez S, Tully DC, Gondwe C, Wood C. Low-abundance resistant mutations in HIV-1 subtype C antiretroviral therapy-naïve individuals as revealed by pyrosequencing. *Current HIV research*. 2013; 11:43–49. [PubMed: 23305169]
183. Beerenwinkel N, Gunthard HF, Roth V, Metzner KJ. Challenges and opportunities in estimating viral genetic diversity from next-generation sequencing data. *Frontiers in microbiology*. 2012; 3:329. [PubMed: 22973268]
184. Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. *J Hepatol*. 2012; 56(Suppl 1):S88–100. [PubMed: 22300469]
185. Scheel TK, Rice CM. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat Med*. 2013; 19:837–849. [PubMed: 23836234]
186. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology*. 2011; 53:1742–1751. [PubMed: 21374691]
187. Franco S, Bellido R, Aparicio E, Canete N, Garcia-Retortillo M, Sola R, et al. Natural prevalence of HCV minority variants that are highly resistant to NS3/4A protease inhibitors. *J Viral Hepat*. 2011; 18:e578–582. [PubMed: 21914079]
188. Aparicio E, Franco S, Parera M, Andres C, Tural C, Clotet B, et al. Complexity and catalytic efficiency of hepatitis C virus (HCV) NS3 and NS4A protease quasispecies influence responsiveness to treatment with pegylated interferon plus ribavirin in HCV/HIV-coinfected patients. *J Virol*. 2011; 85:5961–5969. [PubMed: 21471227]
189. Bartolini B, Giombini E, Zaccaro P, Selleri M, Rozera G, Abbate I, et al. Extent of HCV NS3 protease variability and resistance-associated mutations assessed by next generation sequencing in HCV monoinfected and HIV/HCV coinfected patients. *Virus Res*. 2013; 177:205–208. [PubMed: 23954579]

190. Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, Osaki Y, et al. Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. *PLoS One*. 2011; 6:e24907. [PubMed: 21966381]
191. Franco S, Casadella M, Noguera-Julian M, Clotet B, Tural C, Paredes R, et al. No detection of the NS5B S282T mutation in treatment-naïve genotype 1 HCV/HIV-1 coinfecting patients using deep sequencing. *J Clin Virol*. 2013; 58:726–729. [PubMed: 24140031]
192. Chevaliez, S.; Rodriguez, C.; Soulier, A.; Ahmed-Belkacem, A.; Hezode, C.; Pawlostky, JM. 18th International Symposium on HCV and related viruses. Seattle, USA: 2011. Molecular characterization of hepatitis C virus resistance to telaprevir by means of ultra-deep pyrosequencing.
193. Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. *J Clin Microbiol*. 2012; 50:3267–3274. [PubMed: 22837328]
194. Bao JR, Huard TK, Piscitelli AE, Tummala PR, Aleemi VE, Coon SL, et al. Reverse-transcription polymerase chain reaction/pyrosequencing to characterize neuraminidase H275 residue of influenza A 2009 H1N1 virus for rapid and specific detection of the viral oseltamivir resistance marker in a clinical laboratory. *Diagn Microbiol Infect Dis*. 2011; 71:396–402. [PubMed: 22000086]
195. Chantratita W, Sukasem C, Sirinavin S, Sankuntaw N, Srichantaratsamee C, Pasomsub E, et al. Simultaneous detection and subtyping of H274Y-positive influenza A (H1N1) using pyrosequencing. *J Infect Dev Ctries*. 2011; 5:348–352. [PubMed: 21628810]
196. Deng YM, Caldwell N, Hurt A, Shaw T, Kelso A, Chidlow G, et al. A comparison of pyrosequencing and neuraminidase inhibition assays for the detection of oseltamivir-resistant pandemic influenza A(H1N1) 2009 viruses. *Antiviral Res*. 2011; 90:87–91. [PubMed: 21376084]
197. Deyde VM, Nguyen T, Bright RA, Balish A, Shu B, Lindstrom S, et al. Detection of molecular markers of antiviral resistance in influenza A (H5N1) viruses using a pyrosequencing method. *Antimicrob Agents Chemother*. 2009; 53:1039–1047. [PubMed: 19124660]
198. Deyde VM, Okomo-Adhiambo M, Sheu TG, Wallis TR, Fry A, Dharan N, et al. Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses. *Antiviral Res*. 2009; 81:16–24. [PubMed: 18835410]
199. Duan S, Boltz DA, Li J, Oshansky CM, Marjuki H, Barman S, et al. Novel genotyping and quantitative analysis of neuraminidase inhibitor resistance-associated mutations in influenza A viruses by single-nucleotide polymorphism analysis. *Antimicrob Agents Chemother*. 2011; 55:4718–4727. [PubMed: 21730113]
200. Duwe S, Schweiger B. A new and rapid genotypic assay for the detection of neuraminidase inhibitor resistant influenza A viruses of subtype H1N1, H3N2, and H5N1. *J Virol Methods*. 2008; 153:134–141. [PubMed: 18725246]
201. Gubareva LV, Trujillo AA, Okomo-Adhiambo M, Mishin VP, Deyde VM, Sleeman K, et al. Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. *Antivir Ther*. 2010; 15:1151–1159. [PubMed: 21149922]
202. Lackenby A, Democratis J, Siqueira MM, Zambon MC. Rapid quantitation of neuraminidase inhibitor drug resistance in influenza virus quasispecies. *Antivir Ther*. 2008; 13:809–820. [PubMed: 18839782]
203. Mok CK, Chang SC, Chen GW, Lo YL, Chen SJ, Wu HS, et al. Pyrosequencing reveals an oseltamivir-resistant marker in the quasispecies of avian influenza A (H7N9) virus. *J Microbiol Immunol Infect*. 2013
204. Okomo-Adhiambo M, Sheu TG, Gubareva LV. Assays for monitoring susceptibility of influenza viruses to neuraminidase inhibitors. *Influenza Other Respir Viruses*. 2013; 7(Suppl 1):44–49. [PubMed: 23279896]
205. Operario DJ, Moser MJ, St George K. Highly sensitive and quantitative detection of the H274Y oseltamivir resistance mutation in seasonal A/H1N1 influenza virus. *J Clin Microbiol*. 2010; 48:3517–3524. [PubMed: 20668122]

206. Qi Y, Fan H, Qi X, Zhu Z, Guo X, Chen Y, et al. A novel pyrosequencing assay for the detection of neuraminidase inhibitor resistance-conferring mutations among clinical isolates of avian H7N9 influenza virus. *Virus Res.* 2013
207. Tamura D, Nguyen HT, Sleeman K, Levine M, Mishin VP, Yang H, et al. Cell culture-selected substitutions in influenza A(H3N2) neuraminidase affect drug susceptibility assessment. *Antimicrob Agents Chemother.* 2013; 57:6141–6146. [PubMed: 24080660]
208. Whitehead TA, Chevalier A, Song Y, Dreyfus C, Fleishman SJ, De Mattos C, et al. Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. *Nat Biotechnol.* 2012; 30:543–548. [PubMed: 22634563]
209. Wu NC, Young AP, Dandekar S, Wijersuriya H, Al-Mawsawi LQ, Wu TT, et al. Systematic identification of H274Y compensatory mutations in influenza A virus neuraminidase by high-throughput screening. *J Virol.* 2013; 87:1193–1199. [PubMed: 23152521]
210. Okomo-Adhiambo M, Nguyen HT, Sleeman K, Sheu TG, Deyde VM, Garten RJ, et al. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. *Antiviral Res.* 2010; 85:381–388. [PubMed: 19917319]
211. Dharan NJ, Fry AM, Kieke BA, Coleman L, Meece J, Vandermause M, et al. Clinical and virologic outcomes in patients with oseltamivir-resistant seasonal influenza A (H1N1) infections: results from a clinical trial. *Influenza Other Respir Viruses.* 2012; 6:153–158. [PubMed: 22118629]
212. Ghedin E, Holmes EC, DePasse JV, Pinilla LT, Fitch A, Hamelin ME, et al. Presence of oseltamivir-resistant pandemic A/H1N1 minor variants before drug therapy with subsequent selection and transmission. *J Infect Dis.* 2012; 206:1504–1511. [PubMed: 22966122]
213. Inoue M, Barkham T, Leo YS, Chan KP, Chow A, Wong CW, et al. Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. *Emerg Infect Dis.* 2010; 16:1633–1636. [PubMed: 20875299]
214. Tellez-Sosa J, Rodriguez MH, Gomez-Barreto RE, Valdovinos-Torres H, Hidalgo AC, Cruz-Hervet P, et al. Using high-throughput sequencing to leverage surveillance of genetic diversity and oseltamivir resistance: a pilot study during the 2009 influenza A(H1N1) pandemic. *PLoS One.* 2013; 8:e67010. [PubMed: 23843978]
215. Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients - Seattle, Washington, 2009. *MMWR Morb Mortal Wkly Rep.* 2009; 58:893–896. [PubMed: 19696719]
216. Oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis--North Carolina, 2009. *MMWR Morb Mortal Wkly Rep.* 2009; 58:969–972. [PubMed: 19745803]
217. Arvia R, Corcioli F, Simi L, Orlando C, De Santis R, Facchini M, et al. Monitoring the susceptibility to oseltamivir of Influenza A(H1N1) 2009 virus by nested-PCR and pyrosequencing during the pandemic and in the season 2010–2011. *J Virol Methods.* 2012; 184:113–116. [PubMed: 22659066]
218. Chen LF, Dailey NJ, Rao AK, Fleischauer AT, Greenwald I, Deyde VM, et al. Cluster of oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infections on a hospital ward among immunocompromised patients--North Carolina, 2009. *J Infect Dis.* 2011; 203:838–846. [PubMed: 21343149]
219. Correia V, de Andrade HR, Santos LA, Lackenby A, Zambon M. Antiviral drug profile of seasonal influenza viruses circulating in Portugal from 2004/2005 to 2008/2009 winter seasons. *Antiviral Res.* 2010; 86:128–136. [PubMed: 20083142]
220. Dharan NJ, Gubareva LV, Meyer JJ, Okomo-Adhiambo M, McClinton RC, Marshall SA, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *Jama.* 2009; 301:1034–1041. [PubMed: 19255110]
221. Duwe SC, Wedde M, Birkner P, Schweiger B. Genotypic and phenotypic resistance of pandemic A/H1N1 influenza viruses circulating in Germany. *Antiviral Res.* 2011; 89:115–118. [PubMed: 21070812]

222. Eshaghi A, Bolotin S, Burton L, Low DE, Mazzulli T, Drews SJ. Genetic microheterogeneity of emerging H275Y influenza virus A (H1N1) in Toronto, Ontario, Canada from the 2007–2008 respiratory season. *J Clin Virol.* 2009; 45:142–145. [PubMed: 19451021]
223. Hoang Vu MP, Nguyen CT, Nguyen le KH, Nguyen TK, Le QM. Oseltamivir resistance among influenza viruses: surveillance in northern Viet Nam, 2009–2012. *Western Pac Surveill Response J.* 2013; 4:25–29. [PubMed: 24015368]
224. Hurt AC, Ernest J, Deng YM, Iannello P, Besselaar TG, Birch C, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res.* 2009; 83:90–93. [PubMed: 19501261]
225. Longtin J, Patel S, Eshaghi A, Lombos E, Higgins R, Alexander D, et al. Neuraminidase-inhibitor resistance testing for pandemic influenza A (H1N1) 2009 in Ontario, Canada. *J Clin Virol.* 2011; 50:257–261. [PubMed: 21251873]
226. Miller HB, Gose RB, Nagata MT, Sciulli RH, Whelen AC. Pacific region influenza surveillance for oseltamivir resistance. *J Clin Virol.* 2012; 54:73–75. [PubMed: 22296793]
227. Nguyen HT, Trujillo AA, Sheu TG, Levine M, Mishin VP, Shaw M, et al. Analysis of influenza viruses from patients clinically suspected of infection with an oseltamivir resistant virus during the 2009 pandemic in the United States. *Antiviral Res.* 2012; 93:381–386. [PubMed: 22330888]
228. Okomo-Adhiambo M, Demmler-Harrison GJ, Deyde VM, Sheu TG, Xu X, Klimov AI, et al. Detection of E119V and E119I mutations in influenza A (H3N2) viruses isolated from an immunocompromised patient: challenges in diagnosis of oseltamivir resistance. *Antimicrob Agents Chemother.* 2010; 54:1834–1841. [PubMed: 20194700]
229. Pontoriero A, Baumeister E, Campos AM, Savy VL. Virological surveillance and antiviral resistance of human influenza virus in Argentina, 2005–2008. *Rev Panam Salud Publica.* 2011; 30:634–640. [PubMed: 22358415]
230. Esposito S, Molteni CG, Daleno C, Valzano A, Fossali E, Da Dalt L, et al. Clinical importance and impact on the households of oseltamivir-resistant seasonal A/H1N1 influenza virus in healthy children in Italy. *Virol J.* 2010; 7:202. [PubMed: 20738882]
231. Bright RA, Medina MJ, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, et al. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet.* 2005; 366:1175–1181. [PubMed: 16198766]
232. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. *Jama.* 2006; 295:891–894. [PubMed: 16456087]
233. Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, et al. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J Infect Dis.* 2007; 196:249–257. [PubMed: 17570112]
234. Higgins RR, Eshaghi A, Burton L, Mazzulli T, Drews SJ. Differential patterns of amantadine-resistance in influenza A (H3N2) and (H1N1) isolates in Toronto, Canada. *J Clin Virol.* 2009; 44:91–93. [PubMed: 19019729]
235. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature.* 1996; 381:667–673. [PubMed: 8649512]
236. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science.* 1996; 272:872–877. [PubMed: 8629022]
237. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature.* 1996; 381:661–666. [PubMed: 8649511]
238. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, et al. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* 2005; 49:4721–4732. [PubMed: 16251317]
239. Rose JD, Rhea AM, Weber J, Quinones-Mateu ME. Current tests to evaluate HIV-1 coreceptor tropism. *Curr. Opin. HIV AIDS.* 2009; 4:136–142. [PubMed: 19339953]

240. Poveda E, Briz V, Quinones-Mateu M, Soriano V. HIV tropism: diagnostic tools and implications for disease progression and treatment with entry inhibitors. *AIDS*. 2006; 20:1359–1367. [PubMed: 16791010]
241. Weber J, Piontkivska H, Quinones-Mateu ME. HIV type 1 tropism and inhibitors of viral entry: clinical implications. *AIDS Rev*. 2006; 8:60–77. [PubMed: 16848274]
242. Whitcomb JM, Huang W, Fransen S, Limoli K, Toma J, Wrin T, et al. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. *Antimicrob. Agents Chemother*. 2007; 51:566–575. [PubMed: 17116663]
243. Reeves JD, Coakley E, Petropoulos CJ, Whitcomb JM. An Enhanced-Sensitivity Trofile™ HIV Coreceptor Tropism Assay for Selecting Patients for Therapy with Entry Inhibitors Targeting CCR5: A Review of Analytical and Clinical Studies. *Journal of Viral Entry*. 2009; 3:94–102.
244. Obermeier M, Symons J, Wensing AM. HIV population genotypic tropism testing and its clinical significance. *Current Opinion in HIV and AIDS*. 2012; 7:470–477. [PubMed: 22832711]
245. Poveda E, Alcamí J, Paredes R, Córdoba J, Gutiérrez F, Llibre JM, et al. Genotypic determination of HIV tropism - clinical and methodological recommendations to guide the therapeutic use of CCR5 antagonists. *AIDS Reviews*. 2010; 12:135–148. [PubMed: 20842202]
246. Swenson LC, Mo T, Dong WW, Zhong X, Woods CK, Thielen A, et al. Deep V3 sequencing for HIV type 1 tropism in treatment-naïve patients: a reanalysis of the MERIT trial of maraviroc. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2011; 53:732–742. [PubMed: 21890778]
247. Swenson LC, Mo T, Dong WW, Zhong X, Woods CK, Jensen MA, et al. Deep sequencing to infer HIV-1 co-receptor usage: application to three clinical trials of maraviroc in treatment-experienced patients. *The Journal of infectious diseases*. 2011; 203:237–245. [PubMed: 21288824]
248. Saliou A, Delobel P, Dubois M, Nicot F, Raymond S, Calvez V, et al. Concordance between two phenotypic assays and ultradeep pyrosequencing for determining HIV-1 tropism. *Antimicrobial Agents and Chemotherapy*. 2011; 55:2831–2836. [PubMed: 21464245]
249. Kagan RM, Johnson EP, Siaw MF, Van Baelen B, Ogden R, Platt JL, et al. Comparison of Genotypic and Phenotypic HIV Type 1 Tropism Assay: Results from the Screening Samples of Cenicriviroc Study 202, a Randomized Phase II Trial in Treatment-Naïve Subjects. *AIDS Research and Human Retroviruses*. 2013; 30:151–159. [PubMed: 23875707]
250. Kagan RM, Johnson EP, Siaw M, Biswas P, Chapman DS, Su Z, et al. A genotypic test for HIV-1 tropism combining Sanger sequencing with ultradeep sequencing predicts virologic response in treatment-experienced patients. *PloS one*. 2012; 7:e46334. [PubMed: 23029482]
251. Swenson LC, Moores A, Low AJ, Thielen A, Dong W, Woods C, et al. Improved detection of CXCR4-using HIV by V3 genotyping: application of population-based and “deep” sequencing to plasma RNA and proviral DNA. *Journal of Acquired Immune Deficiency Syndromes*. 2010; 54:506–510. [PubMed: 20512044]
252. Swenson LC, Daumer M, Paredes R. Next-generation sequencing to assess HIV tropism. *Current Opinion in HIV and AIDS*. 2012; 7:478–485. [PubMed: 22832710]
253. Archer J, Braverman MS, Taillon BE, Desany B, James I, Harrigan PR, et al. Detection of low-frequency pretherapy chemokine (CXC motif) receptor 4 (CXCR4)-using HIV-1 with ultra-deep pyrosequencing. *AIDS*. 2009; 23:1209–1218. [PubMed: 19424056]
254. Vandenbroucke I, Van Marck H, Mostmans W, Van Eygen V, Rondelez E, Thys K, et al. HIV-1 V3 envelope deep sequencing for clinical plasma specimens failing in phenotypic tropism assays. *AIDS research and therapy*. 2010; 7:4. [PubMed: 20804564]
255. Archer J, Rambaut A, Taillon BE, Harrigan PR, Lewis M, Robertson DL. The evolutionary analysis of emerging low frequency HIV-1 CXCR4 using variants through time--an ultra-deep approach. *PLoS computational biology*. 2010; 6:e1001022. [PubMed: 21187908]
256. Raymond S, Saliou A, Nicot F, Delobel P, Dubois M, Cazabat M, et al. Frequency of CXCR4-using viruses in primary HIV-1 infections using ultra-deep pyrosequencing. *AIDS*. 2011; 25:1668–1670. [PubMed: 21673557]

257. Abbate I, Vlassi C, Rozera G, Bruselles A, Bartolini B, Giombini E, et al. Detection of quasispecies variants predicted to use CXCR4 by ultra-deep pyrosequencing during early HIV infection. *AIDS*. 2011; 25:611–617. [PubMed: 21160417]
258. Lee GQ, Harrigan PR, Dong W, Poon AF, Heera J, Demarest J, et al. Comparison of population and 454 "deep" sequence analysis for HIV type 1 tropism versus the original trofile assay in non-B subtypes. *AIDS Research and Human Retroviruses*. 2013; 29:979–984. [PubMed: 23350534]
259. Bunnik EM, Swenson LC, Edo-Matas D, Huang W, Dong W, Frantzell A, et al. Detection of inferred CCR5- and CXCR4-using HIV-1 variants and evolutionary intermediates using ultra-deep pyrosequencing. *PLoS pathogens*. 2011; 7:e1002106. [PubMed: 21731496]
260. Rozera G, Abbate I, Bruselles A, Vlassi C, D'Offizi G, Narciso P, et al. Archived HIV-1 minority variants detected by ultra-deep pyrosequencing in provirus may be fully replication competent. *AIDS*. 2009; 23:2541–2543. [PubMed: 19907212]
261. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*. 2011; 28:2731–2739. [PubMed: 21546353]

Highlights

- Deep sequencing is revolutionizing the clinical virology field.
- More affordable deep sequencing methodologies allow their use in clinical laboratories.
- Most deep sequencing-based studies are related to HIV, HCV, and influenza virus.
- Deep sequencing assays permit early identification of minority drug resistant variants
- Clinical significance of minority viral variants is still under debate.

Figure 1.

Principal characteristics of the four most used deep sequencing platforms to date: 454™ (GS Junior and GS FLX+ systems), Illumina® (MiSeq v2 and HiSeq 2500 systems), Ion Torrent™ (Ion Personal Genome Machine®, 318 v2 chip and Ion Proton™, I chip systems), and Pacific Biosciences® (PacBio RS II SMRT®). Information about virus-related publications was obtained from the respective companies (personal communications and/or websites) and PubMed (<http://www.ncbi28nlm.nih.gov/pubmed/>) search as of March 27th, 2014.

Figure 2. Scientific publications based on deep sequencing. (A) Cumulative number of publications associated with HIV, HCV, or influenza virus and deep sequencing during the last 13 years. The size of the circles corresponds to the number of publications in each given year, e.g., 57 HIV studies using deep sequencing were published in 2013. The total number of publications per virus is indicated. (B) Percentage of scientific publications per research area. Search in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) as of March 27th, 2014 using “HIV”, “HCV”, “influenza”, “deep sequencing”, “pyrosequencing”, “epidemiology”, “quasispecies”, “pathogenesis”, “transmission”, “drug resistance”, “tropism”, and/or “vaccine” as keywords.

Figure 3. Comparison of phylogenetic analyses based on Sanger or deep sequencing. Neighbor-joining phylogenetic trees were constructed using (A) Sanger sequencing of 105-bp fragments corresponding to the HIV-1 V3 region of gp120 (*env* gene) from 12 HIV-infected individuals or (B) deep sequencing reads with a frequency >1 corresponding to the same 105-bp fragments (Gibson and Quiñones-Mateu, unpublished results). Each color-coded dot represents a unique variant, frequency is not depicted. Bootstrap resampling (1,000 data sets) of the multiple alignments tested the statistical robustness of the trees, with percentage values above 75% indicated by an asterisk. s/nt, substitutions per nucleotide. Phylogenetic trees were constructed using MEGA 5.05 [261].