UJEMI PEARLS

CRISPR/Cas13a as a viral nucleic acid detection mechanism for POC testing

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SUMMARY Until the development of the CRISPR-based platforms, SHERLOCK and DETECTR, no molecular diagnostic technique existed that is accurate, fast (i.e. detection within a few hours), inexpensive and portable. Without the need for patient samples to be shipped to laboratories, these nucleic acid detection mechanisms have the potential to revolutionize the world of molecular diagnostics as they bring the ability to provide a rapid and accurate diagnosis to people all over the world, including the most rural and isolated areas. Hence, SHERLOCK and DETECTR can act as a key driver in reducing outbreaks of infectious diseases, and in general disease monitoring. SHERLOCK stands for 'specific high-sensitivity enzymatic reporter unlocking'. It exploits the "collateral effects" of Cas13a's ribonuclease activity in combination with isothermal amplification to detect RNA in a patient's sample. This article details: ***(i)*** the mechanism of SHERLOCK and the progression between its first and second versions, ***(ii)*** its limitations as an all-around multiplexing technique, and ***(iii)*** the bioethical implications that need to be acknowledged alongside its development and eventual public use. The emergence of the novel SHERLOCK platform has the power to impact millions of lives through early viral detection, and it is crucial to understand its mechanism in order to optimize its applications. This will impact the future of point-of-care diagnostic testing, especially for emerging and re-emerging viruses such as dengue virus and Zika virus. However, its limitations and bioethical implications also need to be addressed to ensure that a system is in place that will maintain the integrity of the application's original purpose and the safety of its users.

INTRODUCTION

The possibility of transforming the CRISPR/Cas system as a means for molecular diagnostics has been a hot topic since scientists recognized this potential in 2010. However, earlier efforts to develop this as a diagnostic tool were overshadowed by its ability to edit genomes [1]. In early 2017, an article published in Science highlighted SHERLOCK's ability to distinguish RNA viruses with sheer sensitivity, outperforming that of current diagnostic tools [1].

Clustered regularly interspaced short palindromic repeats (CRISPR) are repeat DNA clusters found in the prokaryotic genome [2]. Within these clusters are palindromic segments of bacterial DNA separated at regular intervals by non-repeating sequences called spacers. These spacer sequences were derived from, and therefore match, segments of viral DNA that previously infected the bacterium [2]. The CRISPR loci contains the genes for certain members of the Cas protein family [3]. The CRISPR/Cas systems are classified into Class 1 and Class 2 based on whether a multi-subunit protein complex is formed (Class 1) or the Cas protein is a single multi-domain effector protein (Class 2) [4]. Depending on the encoded Cas protein's signature genes and protein conservation, they are further divided into six types, Type 1-6 [4]. For instance, Cas9 is a Class 2, Type II protein with target-specific endonuclease activity, and Cas13 is a Class 2, Type VI protein with a non-specific cleavage mechanism [4]. The components of the CRISPR/Cas system are used by prokaryotes as a means to fend off invader [5].

The CRISPR/Cas adaptive immunity system serves to defend microbes against invading viral DNA in three steps, as illustrated in Fig. 1. The first step is *adaptation,*

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which involves the insertion of foreign nucleotide segments (spacers) into the CRISPR array [6,7]. The second step is *transcription and processing* of the CRISPR array into a long pre-CRISPR RNA transcript followed by small mature CRISPR RNA (crRNA) [6,7]. Finally, the third step is *interference*, wherein Cas enzymes are guided by crRNAs to cleave segments of the targeted invading genome [7,8].

In 2012, Jennifer Doudna and Emmanuelle Charpentier were credited for pioneering the "cut and paste" ability which turned the CRISPR/Cas9 system into a gene editing tool [9,10]. A few months later, Feng Zhang was credited as the first to employ this genetic engineering ability on eukaryotes [11]. Following these events, interest was sparked in searching for other novel applications of the CRISPR/Cas system. One of the outcomes, developed by Feng Zhang at the Broad Institute, harnesses the power of the CRISPR/Cas13 system as a molecular diagnostic tool [12]. In its natural state, the protein Cas13a (previously known as C2c2) cleaves RNA through non-specific endonuclease activity, termed "collateral cutting" [1]. By adding reporter molecules that fluoresce when cleaved, the CRISPR/Cas13 system allows for a method to detect the presence of a particular RNA in a sample [1]. This molecular diagnostic tool was termed SHERLOCK, which stands for "Specific High Sensitivity Enzymatic Reporter UnLOCKing [1].

RESEARCH QUESTIONS

Once the SHERLOCK platform is commercialized for public use, there is expected to be a significant decline in disease epidemics. For instance, the 2015/16 Zika virus (ZIKV) pandemic would have been minimized if there were fast and accessible methods to diagnose infected individuals in ZIKV-positive areas [12]. Viruses like ZIKV are difficult to detect since viral titers are low and symptoms are similar to other arboviruses [13]. With high sensitivity and a low cost of $0.61 per test, SHERLOCK can quickly and accurately diagnose individuals even in the most rural areas, surpassing the ability of previously used diagnostic techniques [14]. In addition, due to its widespread accessibility, the non-invasive samples required, and its user-friendly nature, this detection platform has the potential to be integrated with a centralized database for real-time disease monitoring that is updated through consumer self-testing and smartphones use [15]. This article examines the molecular diagnostic platform, SHERLOCK, created by the Zhang Lab at the Broad Institute. First, it explores the mechanism of SHERLOCK, along with the improvements that have already been made between the first and second versions, released in 2017 and 2018, respectively. Next, it addresses its limitations as an all-around multiplex diagnostic technique. Lastly, this article discusses the bioethical implications to be considered for its

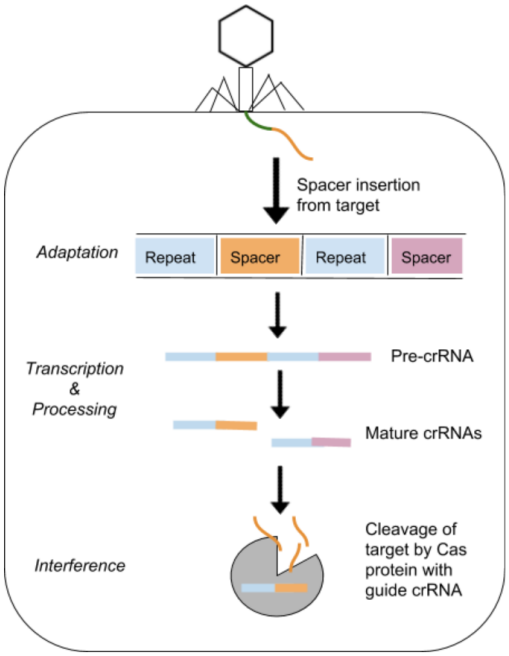


FIG. 1 *(i) Adaptation* involves inserting fragments of the invading viral genome into the CRISPR loci in the form of a spacer. *(ii) Transcription and processing* involves transcribing the CRISPR locus into a long pre-cRNA molecule, followed by processing into smaller mature crRNA molecules. *(iii) Interference* involves detection and degradation of the target sequences by the crRNA-guided Cas proteins. Adapted from [8]

development and public use. Future directions and improvements will also be suggested. The main goal of this paper is to address the ability of the CRISPR/Cas13 system to be used as a viral molecular diagnostic technique.

PROJECT NARRATIVE

How does Sherlock work? What is the mechanism? With attomolar sensitivity, SHERLOCK is an *in vitro* nucleic acid detection platform that allows real-time detection of a viral target sequence through isothermal nucleic acid amplification followed by Cas13a-mediated collateral cleavage of reporter RNA molecules [1]. First, the patient sample (urine, serum, or saliva), is taken and amplified via recombinase polymerase amplification (RPA), which is detected by Cas13a (**Fig 2**) [12]. Next, Cas13a, an RNA-guided RNase, is paired with a single CRISPR RNA (crRNA) guide to allow for specific RNA sensing [1]. Once the target sequence is recognized, Cas13a endonuclease activity is activated, resulting in the collateral cleavage of the target sequence, as well as neighbouring non-target RNAs such as reporter molecules [1]. This is the basic mechanism of SHERLOCK.

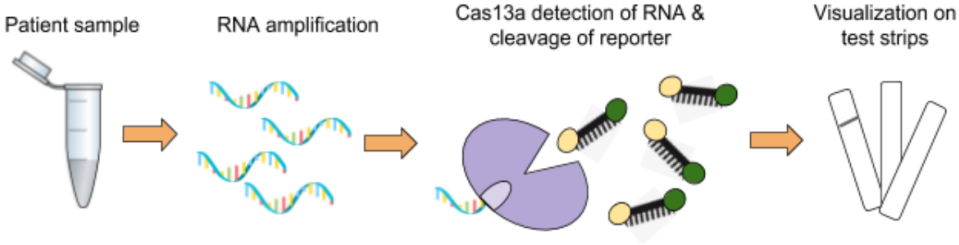
When SHERLOCKv2 was released in 2018, one major improvement was the addition of the lateral flow read-out for easy visualization of results (**Fig 3)** [16]. As each end of the reporter carries a different a label, when the reporter molecule is cleaved, a unique signal is created, and the cleaved reporter molecules collect at a specific location on the paper strip [12]. These cleaved reporter molecules form a visible band, indicating that the sample tests positive. On the other hand, if target RNA is not found in the sample, reporter molecules remain intact and collect at a different detection line on the paper strip, indicating a negative sample [12].

Besides the addition of the lateral flow read-out, SHERLOCKv2 boasts other improvements that allow for a more robust detection system. Regarding quantitative measurement, SHERLOCKv2 boasts enhanced sensitivity through the use of more dilute primer concentrations in the preamplification steps [16]. In the original version, exponential preamplification steps quickly saturated reporters and compromised sensitivity. However, once diluted primer concentrations were used in the preamplification, SHERLOCK was able to detect input down to 2 aM [16].

Another advance in SHERLOCKv2 is the combination of Cas13a with Csm6, an auxiliary CRISPR-associated nuclease, which resulted in a 3.5-fold increase in detection signal sensitivity in terms of signal-to-noise ratio. Because Csm6 is activated by the products of Cas13a collateral activity, Csm6 amplified the detection signal by assisting in the cleavage of reporter molecules [12].

A further improvement in the second version is the elimination of a nucleic acid extraction and isolation step, which required a lab and professionally trained personnel [12]. Through a new process called HUDSON, which stands for Heating Unextracted Diagnostic Samples to Obliterate Nucleases, a chemical (containing TCEP and EDTA) and heat treatment inactivates ribonucleases found in human bodily fluids that would otherwise

FIG. 2 SHERLOCK Procedure. First, patient samples are collected. Then, the samples undergo isothermal RNA amplification via recombinase polymerase amplification. Next, the Cas13a enzyme with a programmed guide RNA, and reporter molecules are added to the sample. When the target viral RNA is found, Cas13a activates its cleavage mechanism and randomly slices nearby RNA, including reporter molecules. Because each end of the reporter molecules carries a different label, Cas13a separates these signatures, creating a unique signal within the sample. The sample is then applied to a flow detection system for easy visualization of results.



degrade the viral RNA [12]. Patient samples would first undergo HUDSON before the RNA amplification step of SHERLOCK. Pairing HUDSON with SHERLOCK allowed for the distinction between similar ssRNA viruses such as ZIKV and DENV, and the detection of a single point mutation linked to microcephaly in the ZIKV genome [12]. The full timeline of HUDSON-to-SHERLOCK takes under two hours to complete, including the visual read-out [12]. Tested with either blood, serum, plasma or saliva, it takes around 20 minutes for inactivation, roughly 20 minutes for the RPA reaction, and around 1 hour for T7 transcription and Cas13 detection on the paper strips [12]. In summary, noticeable advancements between the first and second versions of SHERLOCK such as the addition of the lateral flow read-out, usage of dilute primer concentrations, combination of Cas13a with Csm6, and the elimination of the nucleic acid extraction and isolation step have resulted in a more robust detection system.

What are the limitations of SHERLOCK? What suggested improvements can be made? One current limitation that prevents SHERLOCK from differentiating between all classes of viruses is that Cas13a is specifically an RNA-guided enzyme that only cleaves ssRNA [6]. *In vitro* experimental results involving the chosen Cas13a enzyme, LshCas13 (previously known as LshC2c2), showed that dsRNA substrates were not efficiently cleaved, and dsDNA substrates were not cleaved at all [6]. Hence, relying on Cas13a limits the diagnostic ability to only ssRNA viruses such as dengue virus, Zika virus, West Nile virus, and yellow fever virus [6,12]. SHERLOCKv2 explored a detection system based on Cas12, another protein with collateral activity, which exhibits ssDNA cleavage [17]. After preamplification and RPA steps, Cas12a was able to produce a detectable signal at 2aM using input concentrations of about 100nM. With a combination of Cas13a, Cas13b and Cas12a, three targets were detected (a synthetic ssDNA, ZIKV ssRNA, and DENV ssRNA) [16]. This combination has not yet been tested in the presence of a natural ssDNA virus. This is a good step forward, however, the ability of this combination of orthogonal enzymes to distinguish more than three viruses at once has yet to be demonstrated.

Another limitation is regarding the lateral flow readout. Currently, the team has shown the presence or absence of a single viral target. To enable multiplexed detection, the researchers plan to incorporate a colorimetric readout containing a single test strip with multiple test lines for different genetic targets [16]. The authors have not yet explored the mechanism that will allow the non-specific cleave mechanism to assign the presence of a particular RNA signature to a certain colour. The authors used the cleavage a FAM-biotin reporter which aggregates in different locations depending on whether it was cleaved [16]. The long-term goal of this technology is to combine microfluidics and smartphone technology to allow individuals to self-test at any time and place (refer to **Fig 4**). The ultimate application of this multiplexed detection is to reach a new level of point-of-care

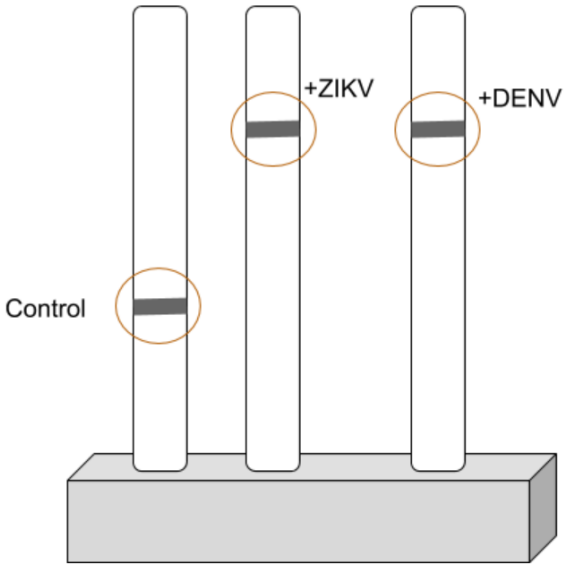


FIG. 3 SHERLOCKv2 Lateral Flow Test Strips The lateral flow test strips introduced in SHERLOCKv2 provide an easy visualization to indicate whether a sample tests positive or negative for the target virus. A band that appears on the lower detection line indicates that the target virus was not present in the patient sample, while a band on the upper detection line indicates that the patient samples contain the virus. Adapted from [12].

self- and patient testing where results could be automatically uploaded to a centralized database [18]. This would provide information for disease monitoring to help prevent outbreaks, and inform citizens and travellers of the circulating viruses in their local area [18].

Bioethical implications. Prior to the release of SHERLOCK for public use, it is crucial for its developers to ensure that donor human samples collected by researchers to develop and validate the instruments are used ethically. It is also pertinent to address that at this early stage in development, SHERLOCK may produce false-positive signals, which could result in considerable ramifications to the user [16].

Before donor samples can be added to a biobank, donors need to be well informed of the purpose of the research, along with expected benefits and risks, before providing the researcher with consent to use their samples [20]. In addition, the privacy and identifiability of the donor is a top concern. Permanent anonymization is guaranteed to protect personal information as it erases any link to other data, however this eliminates the ability to re-contact the donor [19]. For this reason, it has become an acceptable standard practice to employ various forms of coding to achieve a sufficient level of privacy [19]. This method of coding donor data would be ideal for the development of SHERLOCK as developers will be able to notify donors as required, such as for cases of false-positives.

False-positive cases through the SHERLOCK platform may occur due to an overwhelming volume of RNAses in the patient sample [12]. To reduce the rate of false-positive readouts and aid in the robustness of the diagnostic tool, the creators of SHERLOCK have combined Csm6 with Cas13 detection on lateral flow [16]. Another cause of false-positive, as well as false-negative, signals occurs during SHERLOCK applications in low-resource settings where mechanical or chemical disruption of RNA is used for detection instead of the extraction of pure nucleic acids [20]. False-positive signals may be caused by nuclease carryover which may degrade the reporter, and false-negative signals may occur due to nucleases that degrade the target viral RNA [20]. Hence, the addition of RNAse Inhibitors or use of HUDSON pretreatments are crucial to maintain the sensitivity and specificity of the SHERLOCK nucleic acid detection [20].

As a reliable diagnostic tool needs to be both sensitive and specific, it is imminent to conduct SHERLOCK clinical trials in which results are compared with currently available tests. The results of these clinical trials must be approved by the U.S. Food and Drug Administration (FDA) as a direct-to-consumer test [21]. FDA oversight for direct-to-consumer tests for moderate to high medical purposes, which may have a higher impact on medical care, include the assessment of analytical validity, clinical validity, as well as a comparison of the company's claims with how well it works [21]. Because SHERLOCK is

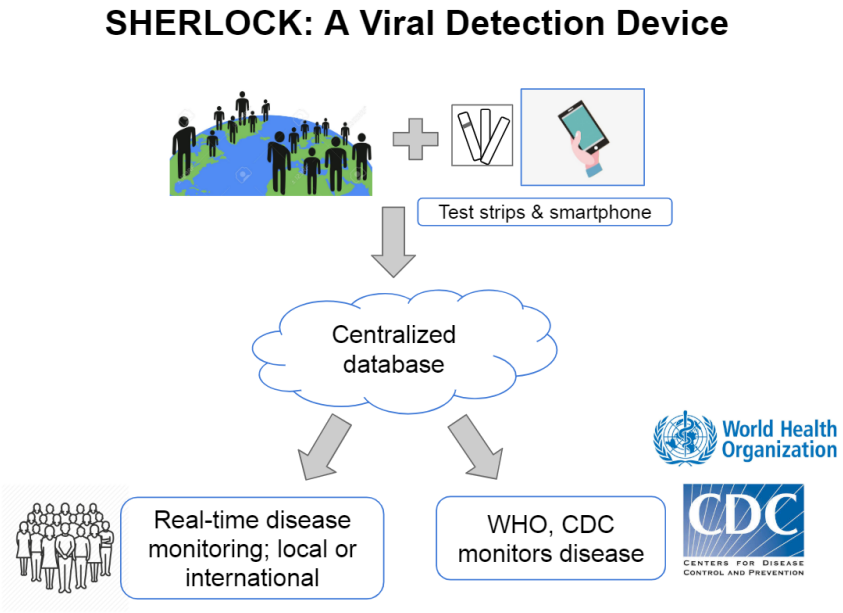


FIG. 4 Future Potential for SHERLOCK Once SHERLOCK's multiplex capability is further developed, microfluidics can be paired with smartphone technology to allow individuals to self-test at their own convenience. The data can be sent to a centralized database which will inform the user of their result, and the coded information can be processed to be used for real-time disease monitoring.

looking to diagnose viral infections, a false-positive result may greatly impact the user through health and safety risks such as inappropriate or unnecessary medication provided, unnecessary stress, anxiety or depression, and unwarranted financial burden [22].

Therefore, ethical implications such as the privacy of donor information, as well as the potential for false-positives must be considered if SHERLOCK aims to become a point-of-care self-testing diagnostic technique.

CONCLUSIONS

Overall, the SHERLOCK platform opens new frontiers in molecular virology as it is the first molecular diagnostic technique that is cheap ($0.61 per test), easily accessible, accurate and user-friendly. Due to SHERLOCKv2 improvements, this platform eliminates the need for laboratory-based instrumentation, which was previously identified as the barrier which prevented access to diagnostic testing to those in rural areas. Hence, this highly sensitive and rapid form of testing will allow timely responses to prevent or significantly reduce the impact of future disease outbreaks. Further, once the limitations of SHERLOCK with respect to multiplexing are overcome, it will become a more accessible point-of-care diagnostic tool through the integration of microfluidics and smart-phone technology. This will bring about a new form of self-test that individuals can complete independently at their own convenience, and test results can be determined through a smartphone application, which transmits information to a centralized database. This database can then be monitored by a single organization, such as the World Health Organization, to more precisely predict and track disease outbreaks worldwide. General information from this database could also be processed into an up-to-date graphic, accessible on a smartphone, for the public to learn which diseases are circulating in their local area or specific locations internationally. Bioethical implications that come with widespread access to SHERLOCK should be considered before and after its availability for public use to prevent dire consequences for its users that may offset any epidemiological advantages. Therefore, the significant number of potential applications for the SHERLOCK viral diagnostic platform highlights the potential of this new technology in the molecular virology field.

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REFERENCES

1. Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA, Myhrvold C, Bhattacharyya RP, Livny J, Regev A, Koonin EV, Hung DT, Sabeti PC, Collins JJ, Zhang F. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science. 2017 Apr 28;356(6336):438–42.
2. Ishino Y, Krupovic M, Forterre P. History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology. Margolin W, editor. J Bacteriol. 2018 Apr 1;200(7):e00580-17.
3. Haft DH, Selengut J, Mongodin EF, Nelson KE. A Guild of 45 CRISPR-Associated (Cas) Protein Families and Multiple CRISPR/Cas Subtypes Exist in Prokaryotic Genomes. PLoS Comput Biol [Internet]. 2005 Nov [cited 2019 Feb 26];1(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1282333/
4. Yao R, Liu D, Jia X, Zheng Y, Liu W, Xiao Y. CRISPR-Cas9/Cas12a biotechnology and application in bacteria. Synthetic and Systems Biotechnology. 2018 Sep 1;3(3):135–49.
5. Barrangou R, Marraffini LA. CRISPR-Cas Systems: Prokaryotes Upgrade to Adaptive Immunity. Molecular Cell. 2014;54(2):234–44.
6. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV, Zhang F. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science. 2016 Aug 5;353(6299):aaf5573.
7. Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin EV. Diversity and evolution of class 2 CRISPR–Cas systems. Nat Rev Microbiol. 2017 Mar;15(3):169–82.

8. Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie. 2015 Oct 1;117:119–28.
9. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science. 2012 Aug 17;337(6096):816.
10. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature. 2011 Mar 1;471(7340):602–7.
11. Lander ES. The Heroes of CRISPR. Cell. 2016;164(1):18–28.
12. Myhrvold C, Freije CA, Gootenberg JS, Abudayyeh OO, Metsky HC, Durbin AF, Kellner MJ, Tan AL, Paul LM, Parham LA, Garcia KF, Barnes KG, Chak B, Mondini A, Nogueira ML, Isern S, Michael SF, Lorenzana I, Yozwiak NL, MacInnis BL, Bosch I, Gehrke L, Zhang F, Sabeti PC. Field-deployable viral diagnostics using CRISPR-Cas13. Science. 2018 Apr 27;360(6387):444–8.
13. Zika Virus [Internet]. CDC. 2014 [cited 2019 Mar 9]. Available from: https://www.cdc.gov/zika/hc-providers/preparing-for-zika/clinicalevaluationdisease.html
14. CohenApr. 13 J, 2017, Pm 2:00. New CRISPR tool can detect tiny amounts of viruses [Internet]. Science | AAAS. 2017 [cited 2019 Mar 11]. Available from: https://www.sciencemag.org/news/2017/04/new-crispr-tool-can-detect-tiny-amounts-viruses
15. CRISPR Could Be the Future of Disease Diagnosis - OneZero [Internet]. [cited 2019 Oct 9]. Available from: https://onezero.medium.com/crispr-could-be-the-future-of-disease-diagnosis-b91c4aa88b41
16. Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science. 2018 Apr 27;360(6387):439–44.
17. Chen JS, Ma E, Harrington LB, Costa MD, Tian X, Palefsky JM, Doudna JA. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science. 2018 Apr 27;360(6387):436–9.
18. Li M, Diamandis EP. Technology-driven diagnostics: From smart doctor to smartphone. Critical Reviews in Clinical Laboratory Sciences. 2016 Jul 3;53(4):268–76.
19. Budimir D, Polašek O, Marušić A, Kolčić I, Zemunik T, Boraska V, Jerončić A, Boban M, Campbell H, Rudan I. Ethical aspects of human biobanks: a systematic review. Croat Med J. 2011 Jun;52(3):262–79.
20. Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO, Zhang F. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nature Protocols. 2019 Oct 1;14(10):2986–3012.
21. Research C for DE and. Direct-to-Consumer Tests. FDA [Internet]. 2018 Nov 3 [cited 2019 Oct 6]; Available from: http://www.fda.gov/medical-devices/vitro-diagnostics/direct-consumer-tests
22. Sharkey CM. Direct-to-Consumer Genetic Testing: The FDA's Dual Role as Safety and Health Information Regulator. DEPAUL LAW REVIEW. 68:43.