Nanotechnology and Computational tool based study of CRISPR/Cas-9 research in Biomedical Engineering

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Abstract*— Invent of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 has taken the medical research worldwide to a newer height, particularly in fighting cancer and more deadly diseases that are currently engulfing the mankind at large. Coupling nanotechnology with CRISPR/Cas-9 system has made a tremendous and significant contribution in further boosting the research in oncobiological research. The authors are making an approach to familiarize the new advances taking worldwide in oncobiological research with the help of Computational tools and nanotechnology. The paper would share the concept of interdisciplinary research coupling biological domain with software-based correction and validation of CRISPR/Cas9 activity in achieving correct sequence edit and cleave as well its delivery to the targeted location. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 is a technology evolved from the type II immune system of archaebacteria. Exploring the functionality of Cas9 protein leads the researcher to a site-specific targeted genetic manipulation. It is a robust technology that is used to make genetic cuts and edits in the DNA but the perfection in editing is yet to be achieved. One of the biggest hurdles in achieving perfection includes the possibility of off-target cleavage that is ruled out by combining computational tools along with nanotechnology-based CRISPR/Cas-9 research. Using nanotechnology and specific designed computational algorithm, the Cas-9 protein is guided to the targeted site that would not only eliminate the probability of guide RNA leading to off-target cleavage but become more accurate in terms of functionality and specificity. Thus, it is an approach to elucidate the possibility of using nanotechnology (biomedical engineering) as a substitute for guide RNA to specify DNA cleavage by Cas-9 and prevent off-target cutting. The paper is an attempt to focus the interdisciplinary area of computer knowledge with biological domain for effective genetic edition and manipulation for oncobiological as well as biomedical engineering particularly targeting tissue engineering.

Keywords—CRISPR, Cas-9, Biomedical instrumentation, Nanotechnology, Guide RNA, and Off-target cleavage

I. INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 has revolutionized genome editing, providing powerful research tools and promising agents for the potential treatment of genetic diseases. Cas9 protein is an enzyme that acts like molecular scissors and is capable of cutting the DNA sequence at specific sites. The natural defense mechanism of bacteria by Cas9 protein is being explored and investigated by worldwide researchers to use it as a scissor for target-specific cleavage. When Bacteriophage would inject its DNA, without any immune system it would get embedded to the genome and that would result in the formation of multiple bacteriophages that would eventually kill the cell but with Cas9 protein defensive mechanism, they are capable of destroying the foreign DNA.

CRISPR cluster contains discrete repetitive sequence *R* and intergenic spacer sequences are arranged at regular intervals. In prokaryotes, exogenous DNA is inserted into the genome, once transcription start, organisms will cut the sequence into pieces which are near to PAM (Protospacer adjacent motif) that is NGG sequence, a short sequence added to CRISPR cluster. Upon insertion of the same exogenous DNA again, Cas9 combines with a short RNA sequence known as guide RNA which is transcribed by CRISPR cluster, the complex will be transported to the nucleus and recognize Protospacer adjacent motif sequence (PAM)[1] to scan the genome-wide. When the complementary DNA against RNA appears, it will be spliced by Cas9, the invasion of exogenous DNA is blocked. The CRISPR/Cas-9 system involves the Cas-9 protein that can bind to the RNA that has been transcribed from the Palindromic host DNA sequence and cleave the foreign DNA combined with RNA spacers, a product obtained from the transcription of the short DNA stretches of the host DNA acquired from extra-chromosomal elements[2]. This system consists of two parts, the trans-activating CRISPR RNA (tracr RNA) which is the transcript of the Palindromic repeats and the CRISPR RNA (crRNA) which is the transcript of the spacer sequences. Tracr RNA and crRNA can be linked together to form a complex known as the guide RNA (gRNA) which is capable of directing the Cas-9 protein to cleave specific sites of the DNA in the PAMs (Protospacer Adjacent Motifs) region.

The endonuclease activity of Cas-9 is guided by a well synthesized single guide RNA molecule to induce breaks at both strands of DNA helix at the specific genomic site. The recognition of a specific genomic site and its cleavage occurs via recognition of 20 nucleotide sequences of sgRNA complementary to the genome site where specific cleavage occurs. Studies demonstrated that multiple mismatches, as well as the bulging site of RNA and DNA, are ignored or tolerated resulting in cleavage of the unwanted genomic site here referred to as off-target sites [3]. Mismatch generation

may be the concern of the off-target cleavage thereby leading to false genomic edition and spicing.
To avoid such generation of mismatches (off-target) two approaches can be adopted:-

a) An optimal sgRNA sequence is designed which has a property to generate the minimal off-target effect. Design is done in such a way that any mismatch is not left unrecognized. Computational biology tools are hereby needed to efficiently detect target off-sites and they are mostly based on integration of oligonucleotides into double-stranded breaks (DSBs) by guide sequence, high-throughput genome-wide translocation sequencing (HTGTS)[4], direct in-situ break labeling (BLA), Integrase-deficient lentiviral vectors (IDLVs), and in-vitro nuclease digested whole genome sequencing (digenome). Computational based methods are designed for sgRNA (single guide RNA) for example CCTop, CRISTA, FORECast and SYNTHEGO are some of the computational tools used by the worldwide researcher for validating the off-target cleavage before the onset of the experiment. The majority of the computational tool considers the distance of mismatch from the PAM (Protospacer adjacent motif) site during validating and evaluating the specificity of candidate sgRNA sequence. To design an optimized CRISPR requires the presence of position-specific mismatch and considers the spatial distribution of mismatches. But some computational tools empirical data neglecting genome context and instead focus on effecting off-target effects for given sgRNA for sequencing.

Out of the discussed computational tools researcher found the CRISTA, a computational tool based on the machine learning approach for predicting the probability of cleavage of a target genome site by given sgRNA. The novelties lie in the fact that it takes into account the possibility of RNA and DNA bulges and accordingly calculates the distances of mismatches from PAM and eventually establish the probability of cleavage off-target sites. While most of the computational tools failed, the CRISTA only takes into consideration the thermodynamic feasibility of sgRNA. Thus, our approach to showcase CRISTA [5] as a higher predictive accurate computational tool as compared to rest for the effective off-target cleavage.

b) Nanoparticles based delivery of Cas9 protein to the target site

Cas9 protein involves off-target mismatches and hence an alternative approach is being established in the form of Nanoparticles as well as a viral particle. However, using viral particles may mimic the immune response that would hinder the delivery system. Hence gold-based CRISPR conjugate is being successfully established for direct injection of the Cas9 ribonucleoprotein to the tumor site.

This paper enlightens the work of biomedical science coupled to computational and nanomaterial synthetic chemistry in achieving high efficacy and better therapeutics in terms of curing the genetic disorder. Working towards the development of novel Nanoparticles as well as user-friendly and robust computational tools would be the futuristic view of the researcher.

II. MECHANISM OF CRISPR

A. CRISPR-CAS Cleavage Mechanism

The CRISPR Cas9 protein acts like a pair of molecular scissors that can cut the DNA at specific sites and thus leading to editing the gene at the sites required. The Cas9 protein is bound with the guide RNA that is artificially created by combining the chemically synthesized trans-activating CRISPR RNA (tracrRNA) and a chemically synthesized CRISPR RNA (crRNA) or a single guide RNA (sgRNA) that consists of both tracrRNA and crRNA as a single construct. The guide RNA also has a special guide sequence that instructs the Cas9 the specific site where it would make the cut in the DNA segment. After the Cas9 is inserted in the genome it scans the whole segment for the target site and the rest is regarded as off-target sites [6]. Once it gets the match on the sequence with the gRNA it unwinds that segment of the DNA and checks whether the sequence is complementary to those on the guide RNA if it isn't complimentary Cas9 continues scanning, but if the strand is complementary to all the bases of the guide RNA then Cas9 cuts the DNA at that specific location. In order to repair the cut damage, the cell often tries to stick them together directly which causes frameshift mutation and that causes a disruption in the gene expression. On the other hand, the cell may often try to repair the damage without causing a mutation and it can be done with the help of a similar matching sequence, scientists can provide a similar sequence with required genomic alterations and the cell tries to incorporate the sequence of the provided segment which causes the required modification in the DNA sequence. This is how the CRISPR Cas9 system can be used to either disrupt gene expression or modify the gene as required.

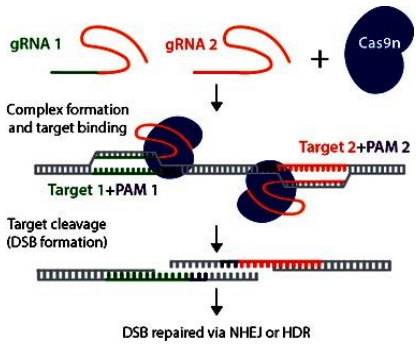


Figure-1: Working Mechanism of CRISPR/Cas-9 system to induce double-stranded breaks in DNA strands [7]

B. PAM Recognition

The Protospacer adjacent motif (or PAM for short) is a short DNA sequence of 2 to 6 base pairs in length that must be present in the DNA region needed to be targeted for cleavage by the CRISPR/Cas9 endonuclease system. The location of PAM is found 3 to 4 nucleotides downstream

from the cut site. Targeted genomic location for edition and alteration are specified by the presence of nuclease specific PAM sequence [11].

CRISPR mediated edition of a specific genomic location is restricted by the presence of nuclease specific PAM sequences. It is evident from Streptococcus Pyrogenes that Cas-9 recognizes 5'-nGG-3' sequence without the presence of GG sequence. The presence of consensus G sequence leads to disruption of the endonuclease activity of Cas-9 protein. Research evidence indicates that Cas-9 endonuclease does not recognize a specific PAM rather recognizes different PAM sequences originating from different bacterial species, eventually making the researcher opting a wide array of nuclease for CRISPR activity and thereby generating multi-targets and editing facility.

C. Single guide RNA (SgRNA) and its need

The Cas-9 protein being a pair of molecular scissors need an indication as to which segment of the DNA sequence to cleave and this is where the use of single guide RNA (SgRNA) comes in. When the Cas9 cuts the viral DNA and stores it for CRISPR repeats it excludes the PAM sequence so as to ensure the bacterial genome is not treated as a target [12]. This is very essential for scientists to know while designing guide RNA. The guide RNA is a chimeric complex composed of the scaffold sequence of trans-activating CRISPR RNA and the custom-designed CRISPR RNA sequence [13]. However, the SgRNA may also be said to be a necessary evil as research has found out that it directly impacts the cleavage efficacy and also responsible for off-target cleavage so the need to engineer the right gRNA is a crucial step for any Cas-9 mediated cleavage.

D. Effective and correct binding of SgRNA with Cas9 endonuclease system in delivering precise target specific cuts.

The Cas-9 protein first needs to bind with the guide RNA so during the making of SgRNA which is a scaffold sequence that the Cas9 recognizes and binds to a 20 bp spacer sequence (user-defined) is inserted in the SgRNA[9-10]. In DNA the Cas9 binds to the PAM sequence where different Cas proteins are used for different PAM sequences. Once expressed the Cas9 and SgRNA form a ribonucleotide complex based on interactions between the scaffold sequence and positive grooves on Cas9 resulting in its conformational change. Furthermore interaction of Cas-9 with gRNA shifts the molecule from its inactive state (non-DNA binding confirmation) to a more active (DNA binding conformation) state. Cas9 only cuts a given segment if it shares sufficient homology with the given target DNA.

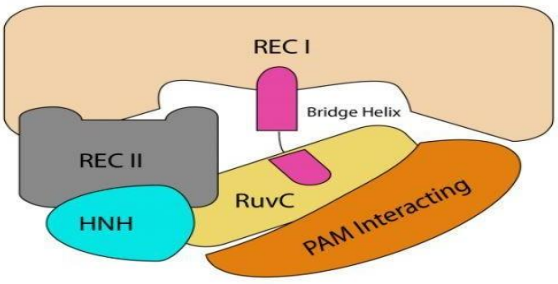


Figure-2: Structural Domains of Cas-9[12]

Once the Cas9-gRNA complex binds a putative DNA target, the seed sequence (8-10 bases at the 3′ end of the gRNA targeting sequence) will begin to anneal to the target DNA. If the seed and target DNA sequences match, the gRNA will continue to anneal to the target DNA in a 3′ to 5′ direction. The end result of Cas9-mediated DNA cleavage is a double-strand break (DSB) within the target DNA (3-4 nucleotides upstream of the PAM sequence).[8]

III. DEMERITS OF CRISPR

Some of the commonly known demerits of CRISPR include Cas-9 off-target cleavage, in-del mutations caused during DNA repair after Cas-9 cleavage, not being able to recognize sequences of length more than 20 base pairs and the system is not efficient enough to be implemented in humans as of yet. However, in this paper, we have concentrated more on the off-target cleavage which is a chief concern to the researchers and at the same time, we have touched upon the possibility of predicting mutations caused during the DNA repair. Once Cas-9 has finished the cleavage mechanism the DNA tries to repair itself by either Homology Direct Repair (HDR) or Non-Homologous End Joining (NHEJ) but in mammalian cells NHEJ is seen as a major effective means of DNA repair [5] which is more prone to in-del mutations and thus arises the need of computational tools which not only makes the DNA editing accurate but predicts the possibility of most probable mutations that can take place post the DNA cleavage.

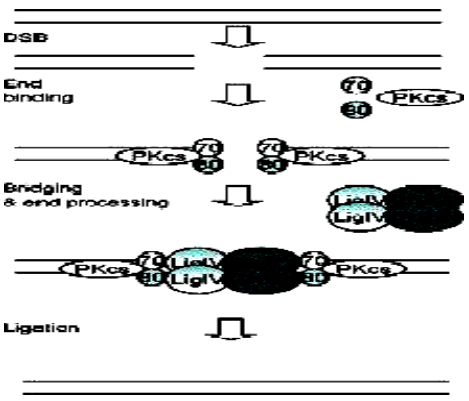


Figure-3:- NHEJ Working Mechanism

IV. NEED FOR COMPUTATIONAL TOOL

The demerits are a prime reason accounting for the inefficiency of CRISPR in its domain. However, with modern computational tools, the mechanism of action of CRISPR was not only improved but its probable outcomes were also predicted more accurately. A machine learning algorithm relies on a range of data inputs, and incorporation of a set of features that can be used to predict cleavage efficiencies. The primary demerit of Cas-9 is that it cleaves off-target DNA and so in order to counter that, researchers started implementing machine learning algorithms via computational tools to build up a more accurate cleavage result and eliminating the off-target demerits. We'll analyze some of the well-known and most accurate machine learning tools for CRISPR that are available for usage and determine their validity by comparing their outputs for our desired results. The main reason behind the demerit of off-target cleavage is thought to be in the designing of the single guide RNA for the Cas-9 protein. Among the well known computational tools, CRISTA is being seen as the most novel approach because of its ability to take into consideration DNA bulges, which is often neglected by most tools. This has created a significant impact on improving accuracy because DNA bulges are very common phenomena that tend to hamper the desired result of our DNA manipulation. In this review, we'll be comparing CRISTA, CCTop, Synthego, and FORECast, all of which rely on machine learning algorithms to provide us with predictive outputs for DNA editing.

CCTop is a tool used to determine suitable CRISPR/Cas9 target sites in a given sequence(s) and predict its potential off-target sites. CCTop identifies and ranks all candidate sgRNA target sites according to their off-target quality and displays full documentation. CCTop[13] is an experimentally validated system for the rapid selection of high-quality target sites for gene inactivation, non-homologous end-joining as well as homology-directed repair and from 2017 to 2019, the number of citations for CCTop has increased from 0 to 5 each year and researchers are even using CCTop in real-world cases for Pathology [14]. However, the demerits of CCTop are that it does not take into account the bulges in DNA proximal to the PAM region and this neglect often results in the rise of false-positive rates.

This was, however, corrected in the most modern approach i.e CRISTA. The only known novel tool that takes into account the bulges and proximal and distal to the PAM regions although the distal bulges can be overruled. CRISTA is the combination of datasets obtained from using several genome-wide unbiased methods for CRISPR-Cas9 cleavage sites profiling: GUIDE-Seq, HTGTS, and BLESS. CRISTA is based on learning a regression model using the Random Forest algorithm, and further allows the examination of the importance of features that determine the variation of cleavage efficiency. The ability of the machine learning framework is to differentiate between cleaved and uncleaved sites can also be examined with CRISTA's classical learning algorithms. The comparison chart of CRISTA over CCTop, and other alternate tools are given below[2].

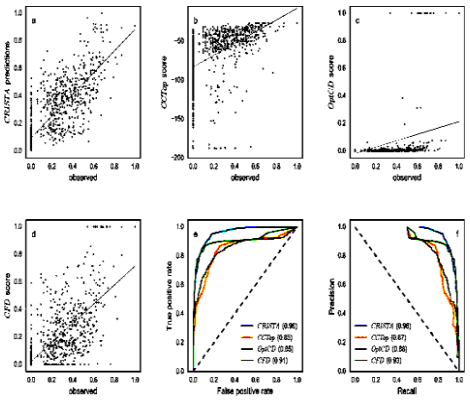
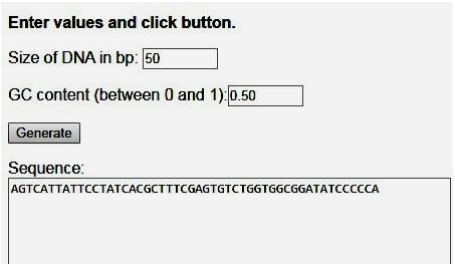
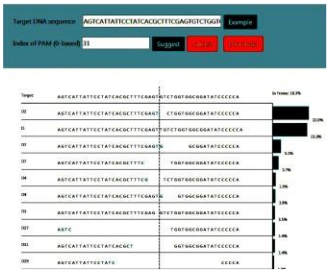


Figure-4: Graphical Comparison of CRISTA vs other available tools in terms of the predictive algorithm [7]

This gives us a clear picture of how CRISTA is more beneficial because of its ability to interpret the propensity while taking into account the bulges and modified PAM sites 17, 34 and 22. Apart from CRISTA and CCTop we also have two online alternatives namely FORECast and Synthego.



The FORECast is the result of the combination of a dataset of over 40,000 genome libraries worldwide and combining them in different situations to obtain a strong database that would be capable of predicting the most probable mutations that can occur for the host sequence with a certain PAM region. We've randomly generated a DNA sequence 50 base pair long with GC content between 0.5 and used that sequence in FORECast to obtain the most probable result of manipulation. A key point to note is FORECast includes a feature of suggesting the PAM site of the given DNA sequence which proves to be highly beneficial during the time of CRISPR engineering and designing of single guide RNA. We have used this sequence and run it through FORECast with its own PAM suggestion to obtain the output.



The output gives us a clear picture of the comparison between the most likely DNA in-del mutations to the least likely DNA in-del mutations. Lastly comes Synthego, an online tool devised by MIT to obtain (i) Knockout guide design (ii) Verify sgRNA design and (iii) ICE Analysis. Synthego is an efficient tool used to design guide RNA as it suggests us the best gRNA sequence depending on the genome host and the gene that we are trying to manipulate. It also gives us visual interface on each gRNA sequence's on-target vs off-target score and ranks them from the highest efficiency to lowest for that particular gene. One can also order the gRNA sequences online from Synthego to be delivered to their lab. We've taken *Homo sapiens* as genomic host and an arbitrary gene TMOD2 and used them to find a gRNA sequence that will be the most efficient when we are trying to manipulate the TMOD2 gene in humans. Apart from the top 4 gRNAs, there is also an option to compare the top 4 gRNAs to the rest of the guide RNA sequences.

V. NEED FOR NANOTECHNOLOGY IN CRISPR

With the demerit of lack of specificity in CRISPR/ Cas-9 technology and the alerting inefficacy of the off-target cleavage the need to correct that has lead multiple researchers to come up with various approaches. One such viable approach might be the coupling of nanotechnology with CRISPR wherein instead of relying upon the guide RNA to lead the Cas-9 protein to the required site of cleavage we'll be using Nanotechnology to directly injects the Cas-9 protein into the required specific sequence to avoid any kind of off-target cleaving. Researchers have also confirmed that the main reason behind this demerit is guiding RNA's inefficiency. Thus with this approach, we'll be able to correct this error.

The gene editing with CRISPR associated protein has tremendous potential to treat diseases. It is observed that gene editing therapies are achieved by 2 forms:

I. Gene editing by CRISPR/ Cas-9 system based on NHEJ that permanently silences disease-causing genes by mimicking in-del mutations.

II. Other is HDR that corrects mutation on the gene resulting in a specific caused disease as that of their normal sequence.

It has been observed that the latter approach is more effective that former and hence most therapeutics are based on HDR with a word of caution regarding its delivery mechanism. The earlier adeno-associated virus was proposed for delivering of Cas-9 protein to tumor site but due to its pre-existing immunity, that approach has got its limitation. Furthermore, such viral approaches to delivering Cas-9 may cause reverse clinical implications and reduce the efficacy of HDR therapeutics. Thus, non-viral delivery through the nanotechnology approach became the buzz. Delivery strategies by the tip of acetamide and polyethyleneimine both synthesized by the Nanoparticles approach has been the most successful Cas-9 ribonucleic protein into tumors. However, it is also observed that introducing a macromolecule is challenging. Thus, the need for a vehicle with pure synthetic Nanoparticles like that of gold would play a pivotal role in delivering Cas-9 ribonucleic protein and induce HDR therapeutics. This gold-CRISPR conjugate nanoparticle as termed CRISPR-gold is composed of gold nanotechnology conjugated with DNA further complexed with donor DNA, Cas-9 ribonucleic proteins and endosomal disruptive polymer also known as poly(N-(N-(N-2)aminoethyl)aspartate)[15].

CRISPR-gold is designed via endocytosis since the polymer is cationic that complexes with CRISPR-gold. The component of CRISPR-gold that, post endocytosis, the polymer triggers endosomal disruption that causes the release of CRISPR-gold complex in the cytoplasm. Once the CRISPR-Gold complex enters the cytoplasm, the glutathione concentration within cytoplasm increases releasing DNA from the core of the gold CRISPR composite that further causes rapid release of Cas-9 ribonucleic protein and donor DNA to target site. The CRISPR-gold nanoparticle delivery mechanism is shown in **Figure 5**.

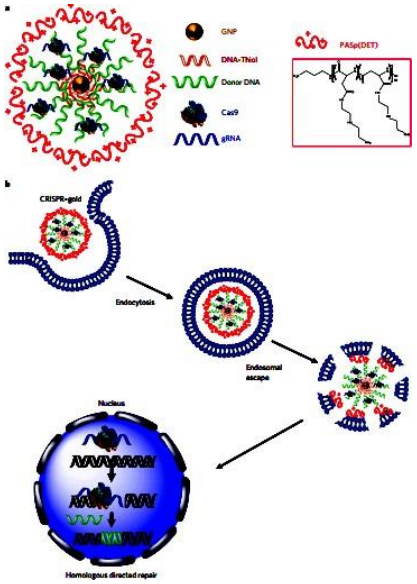


Figure 5: The Figure shows the mechanism of CRISPR-Gold nanoparticle delivery.

VI. SALIENT FEATURES OF MACHINE LEARNING TOOL

After comparing all the results from CCTop, CRISTA, FORECast, and Synthego, we are certain that machine learning is the best approach for making CRISPR more efficient. The most important features of the machine learning algorithm in the field of CRISPR technology includes:

i. The prediction of off-target cleavage
ii. Best output to design guide RNA sequence
iii. Predicting in-del mutations for DNA repairs
iv. Verifying guide RNA designs
v. A large and ever-growing database
vi. Chance to reduce the error rate

VII. CONCLUSION

The computational tool and nanotechnology based approach for the safe and efficient delivery of the Cas9 RNP to the target location for better therapeutics approach in mitigating the clinical manifestation of the genetic disorder is the buzz among the researcher working in the interdisciplinary subject's paper. After careful assessment of the drawbacks of the CRISPR/Cas-9 genetic editing and manipulation system in achieving full efficacy we believe that a better understanding of the user friendly and robust computational tool need to be established before CRISPR/Cas-9 implementation or injection in the *invivo* system, and hence the need of the suitable computational algorithm based approach has taken the researcher in the biomedical domain to use the computational tool beforehand. Similar to its validation check it is also important to deliver the efficient CRISPR/Cas-9 system *invivo* and hence Nanoparticles based approach is also considered with such materials that would generate or mimic least or no immune response against the foreign synthetic nanoparticles.

Our paper shows a clear state of where our current modern computational biology is and how far we've revolutionized genetic manipulation with the applications of CRISPR combined with the machine learning algorithms. Not only is this approach viable and experimentally validated but it's being updated constantly every day in order to attain more datasets for further in-depth processing reviews and higher specificity, eliminating the off-target demerits and reducing the chance of mutative errors. Thus, considering all of the conditions and examining the present state of our standing we can robustly claim that it is found beyond doubt computational biology would be the prime factor that would improve the efficacy of CRISPR. This could be the door to next-generation genetic control where we would have CRISPR babies and higher grade genetically modified organisms.

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