iJRASET
International Journal For Research in Applied Science and Engineering Technology



INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 13 **Issue:** V **Month of publication:** May 2025

DOI: https://doi.org/10.22214/ijraset.2025.71666

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 7.538
Volume 13 Issue V May 2025- Available at www.ijraset.com

Advances in Gene Editing Techniques

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Abstract: ***This review highlights advancements in gene editing techniques, including CRISPR-Cas9, base editing, prime editing, PASTE, and modified CRISPR. CRISPR enables gene insertion and substitution by creating double-stranded breaks in the genome using guide RNA. Base editing alters single base pairs, while prime editing corrects transition mutations by adding up to 50 nucleotides with a nickase.***
PASTE identifies specific genome sites for insertion, adding up to 50 base pairs. These techniques rely on guide RNA and reverse transcriptase to modify the genome. Despite their potential, challenges remain, such as off-target effects, editing efficiency, immunogenicity, and the need for safe delivery systems. Applications include animal breeding, disease treatments (e.g., metabolic disorders, cancer, cardiovascular diseases), immunity enhancement, plant breeding for better traits, and gene therapies. In therapeutics, these techniques also contribute to drug development from biological sources, showing great promise for future medical and agricultural advancements.

I. INTRODUCTION

Genome editing techniques enable scientists to alter DNA sequence which leads to change in physical traits. This became possible on development of enzymes act as molecular scissors to cut DNA sequence and make a double stranded break in DNA followed by addition, removal and alteration in sequence that can be helpful in case to treat genes related disorders. The development in gene editing techniques occur firstly in late 1900s, advances in them occur till 2009; the time when more advanced technique is developed i.e. CRISPR which is more effective, cost sensitive and efficient is used majorly till date by most of scientist to make any alteration in genome sequence. (1) The development of gene editing technologies in the past and present are techniques involving Zinc Finger Nuclease (ZFN), Transcription Activator Like-Effector Nuclease (TALLEN), Mega nucleases (MNs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR with their modifications is also there. These developments are parallel to application like advancement in technologies with immense speeds, scope in resolving problems related to biomedical sciences, and development of better-quality organisms and plants, in formulation of biomedicines to treat disorders and in therapeutic applications.

Techniques development also creates awareness for their use with safety, efficacy and high-quality outcomes and efficient delivery of alternative genes. (2) CRISPR is the most advanced technique which became a key tool for many scientists for modification in gene sequence involved in many fields such as plant, human disease, agricultural and synthetic biology because of its ability to modify gene and protein characters either in vivo or invitro.

Valuation of this technique increased due to its easy implementation, bioavailability in fields, availability of reagents, high specificity and increase rate of gene editing. CRISPR can make a double stranded break (DSBs) in DNA sequences afterward alter of insert sequences complementarity to guide RNA (gRNA). Cas9 gRNA consists of two non-coding RNA sequence; one is a uniform trans-activating CRISPR RNA (tacr RNA) which recognize by Cas9 and CRISPR RNA (crRNA) which have locus specific sequence, these form a duplex called as single-guide RNA (sg RNA) which in turn provide guidance for insertion, deletion or alteration in DSBs site in genome for gene manipulation.(3) There are many uses of CRISPR in including plant, animal, bacteria and human.

Its implementation in wide variety of uses including industrial use, horticulture, breeding and cancer, gene therapies. Because of these vast uses of CRISPR it receives exceptional attention among all developed technologies. Regarding severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, it demonstrated significant promises in early and simple detection techniques, potential therapy and vaccine development. Future prospect, usage ethics and rules, governance implication and related issues is also somewhat clarified by review.(4)

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ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 7.538
Volume 13 Issue V May 2025- Available at www.ijraset.com

II. ADVANCED TOOLS AND TECHNIQUE FOR GENE EDITING

A. CRISPR Cas-9

CRISPR Cas system is an adaptive system found primarily in prokaryotes, to provide them with immunity against invading viruses and plasmid by cleaving their nucleic acids. CRISPR is spotlighted in 2012 as its role in gene editing when George church, Jennifer Doudna, Emmanuelle Charpentier, and Feng Zhang described it as tool for modifying genome at specific regions. CRISPR is firstly identified accidentally in E. coli in 1987 by a Japanese scientist, Yoshizumi Ishino during examining of alkaline phosphate converting gene found repeated sequence with spacer sequence in genome. In 1993 researchers led by j. d. van Embden discover various spacer sequence in different M. tuberculosis species strain and classify them on that basis, technique known as 'spacer oligonucleotide typing' of spoligotyping. Francisco Mojica and Ruud Jansen firstly refer them as CRISPRs.(5) CRISPR technique work by firstly creating a double stranded break in DNA at specific site on analyzing the entire genome sequence, afterwards two components of CRISPR one of which is Cas9 nuclease which is able to make a cut or break in double strand while the other is single guide RNA (sgRNA) which guide for complementarity for insertion, alteration of genetic sequence. After insertion or alteration, cells have inbuilt repair mechanisms which repair sequence by two ways; either by high-fidelity homology-directed repair (HDR) or by error prone non-homologous end joining (NHEJ). Breaking by nuclease initiate at 3 base pair upstream of 'NGG' protospacer adjacent motif (PAM). Output of desired result of gene modification can occur by modifying the sgRNA sequence so it results complementarity sequence also alter, and desired results can be achieved. The CRISPR system has been classified in many subunits of type 1,3 and 4. Researchers have been searching for betterment of CRISPR by PAM advancement so different breaking points be found for increase in chances of gene editing and by developing variants of nuclease used i.e. Cas. Many variants of Cas are developed including Cas12a (Cpf1), staphylococcus aureus Cas (SaCas9), Neisseria meningitidis Cas9 (NmCas9) and these allowed new target loci points for breaks. Alteration in Cas PAM preference generates increased spectrum of gene editing. There are also some modifications present for sgRNA that include, truncated guide RNAs (truRNA), ribozyme-gRNA-ribozyme (RGR), polycistronic tRNA-gRNA (PTG/Cas9) which have desired guiding sequences for desired outcomes resulting for gene editing. Genome repair mechanism works by NHEJ, through processes independent of homology between the two ends, this mechanism serves to unite the two ends of DNA. At or close to the tip junction location, this pathway produces Indel-type alterations (insertion or deletion of one or a few nucleotides), which are inherently error-prone. The final product of the target gene will thus have compromised functionality because of these alterations. In other words, gene knockout is frequently the outcome of Cas9-NHEJ's combined action. With numerous uses, gene knockout is highly interesting in the fields of genetics, cell biology, biomedicine, and many more. And the other is HDR, a repair route based on homologous recombination, can be used when the ends produced by the cleavage are homologous to one another or to a third molecule. Occasionally, the Cas9 enzyme's cleavage of the target DNA does not produce ends that are somewhat similar. Nevertheless, the HDR repair procedure may still be initiated in these circumstances. This requires the introduction of a donor DNA molecule, which needs to be homologous with both ends that resulted after the incision. CRISPR is a widely used technique applied in many fields for gene modification in maybe plants, microbes, organisms, animals and for welfare in humans. CRISPR has very vast applications in plants, for hybrid models creations, desired trait species, improved quality, improve resistance, and wide environmental conditions surviving. In microbes or organisms, it is used in improving positive used traits, for testing, and for research related studies. In animals specifically in humans it applicable in treatment of vast variety of diseases including cancer, HIV-AIDS, metabolic disorders, cardiovascular diseases, neurogenerative disease, and many more. Despite of advance technique than ZFN, TALLEN, CRISPR has also some limitations and challenges that have to be overcome in future research that are reduction in off-targets effect while breaking or adding of sequence, relatively high rate of HDR should be implemented for DNA repair than NHEJ, improvement of allele specific editing by editing alleles without interruption on other alleles hence normal functioning can take place, new delivery methods and advancement in old methods should be accomplished for better editing of gene sequence for desired editing. (3,6–13)

B. Base Editing: Single Nucleotide Substitution

This technique is advancement of CRISPR as in this technique in place of Cas9 another variant is used which is catalytically dead version of streptococcus pyogenes Cas9 (dCas9) and firstly is developed at David Liu's lab at broad institute. The base editing technique has overcome the limitations of CRISPR involves breaking of double strand of genome which repair by two ways: NHEJ (non-homologous end-joining) or HDR (homology-directed repair), out of them HDR is occurring prevalently for repairment at DSBs according to desired DNA template for alteration, in case of eukaryotes HDR is not more efficient way for alteration and repairment of DNA sequences.

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Base editing techniques are available for both DNA and RNA for alternating the sequence. Base editing techniques include mutation at single nucleotides resolution, all four transition mutations C→T, G→A, A →G, T →A can be worked in genome using CRISPS base editors. The cytosine base editor (CBE) can install C-G to T-A mutation, where adenine base editor able to edit A-T to G-C sequence. In RNA nucleotide alteration can be done by RNA base editor. In base editing the both base editors have priority for firstly searching for desired sequence in genome have protospacer adjacent motif (PAM), once the spacer sequence is located the dCas9 will bind to that sequence at specific location and start analyzing in sequence for alteration site on single strand of DNA on locating that site it form a loop by stretching the strand at location and with help of base editors it alters the bases, on the other strand of DNA naturally occurring deaminase enzyme alter the sequence complimentary to its respective site on editing strand. Both CBE and ABE have their respective enzyme for working as analyzers and modulators for alternation of base in genome sequence. This advancement of CRISPR to base editing led to treatment of human disease generally due to SNPs (single nucleotide polymorphisms). Base editing techniques can be applied to many fields for research, in plant and crop development, disease treatment in humans and plants, and in many ways base editing can be useful tool for gene editing. CBE and ABE currently have 4 mutation possibilities which allow the cure of many diseases caused by mutations. Therapeutically advantageous of base editors in decreasing off target activity, enhancing product purity of CBEs, broadening the targeting scope and minimizing bystanders editing. Due to the availability of low possibilities, the need of advancement lead to development of prime editing.(13–17)

C. Prime Editing

The conventional CRISPR Cas9 technique use two components; one is Cas9 protein that make break in double strand of DNA while the other is sgRNA(single guide RNA) which have desired sequence that can be used as template strand by reverse transcriptase to generate sequence on DNA strand at break point, which self-repair by two mechanisms either by HDR (alter sequence desirably) or NHEJ (randomly insertion and deletion can occur) to edit genome. Another mutated version of Cas9 generally use is dCas9 (dead Cas9) which is able to form complex that can interfere activity of CRISPR by inhibiting or activating, this dCas9 is not able to make double strand break in place of which it make a single stranded break to repair or alter DNA gene sequence this technique is developed by Liu known as Base Editing which include deaminase protein for fusion with dCas9. But the technique BE can correct only four mutations and two transversion and can cause bystander editing which led to undesirable product. To fill all these gaps a new technique has evolved known as Prime Editing. PE is described by Anzalone et al. in Nature. That overcomes the major limitations i.e. providing customized sequence at desired site. It also involves single stand break in DNA and by using pegRNA (prime editing guide RNA) that drives Cas nuclease, pegRNA have both spacer complimentary to DNA other strand PBS (primer binding site) and sequence to introduce in gene modification process. Three generations of prime editors are successfully trials in human. PE1 Moloney murine leukemia virus reverse transcriptase (M-MLV RT), which bind with pegRNA that in case guide modifications, Penta mutant RT linked to the nickase [Cas9(H840A)-MMLV RT (D200N/L603W/T330P/T306K/ W313F)] was described as a second-generation PE (PE2) which have 1.6 – 5.2-fold more efficiency in mutation insertion than PE1. Two major problems that limit gene editing are which DNA strand will act as template during DNA repair and other is which strand get attached with DNA flaps that can be edited or unedited. Base editing in plants and animals shows that made of nick with unedited strand is more effective. This method applied to PE using nickase, which was directed by sgRNA and had spacer that exclusively matched the modified sequence, this system is known as PE3, which increases efficiency to three folds. PE was also performed for insertion up to 44 bp and deletion up to 80 bp. Further, optimization can be achieved by exploiting more active Cas9 or RT variants by rational modification, in vitro evolution approaches and fusion with additional or combinatorial functional protein domains. In plants this technique is applicable in creation of herbicide resistant germplasm, creation of effective alleles, insertion of protein tags, regulation of protein expressions, creation of disease-resistant germplasm. In animals applicable to treat muscular dystrophy, Leber congenital amaurosis, tyrosinemia, antitrypsin deficiency, phenyl ketone urea. PE is applied for hybrid seed production, quality improvement, regulatory concerns, genetic gain, speed breeding.(13,18,18–22)

D. PASTE: Drag and Drop Edit for Long Insertion

This technique PASTE (Programmable Addition via Site-specific Targeting Elements) is a new tool which can insert long base pairs sequence in genome without creating double stranded break in DNA at target specific site. It was discovered by Jonathan Gootenberg and Omar Abudayyeh, they showed its ability to add or insert long sequence up to 46bp in genome sequence. PASTE uses serine integrase protein by bacteriophage for insertion of sequence, it acts by analyzing for proper landing site (AttB) which is not common in genome, but they must analyze initially for bounding by the integrase's attachment site (AttP).

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For this PASTE use prime editor's reverse transcriptase to make multiple copies for these landing site in genome near target specific sequence, prime editor splices it into 50bp pairs and attachment site for AttB ca able to recognize 46bp. Action of it occur by insertion of sequence without creating any double stranded break in genome. PASTE can introduce large DNA fragments range from 5.6~36 kb into human immortalized cells. Development of PASTE can also promise in therapeutic application by knocking in large sequence without DSBs, such as in treatment of several harmful big gene's mutation. Like CRISPR it can also be useful in many ways for like gene therapies, hybrid models, disease treatment, and gene modified organisms, plants or animals for betterment.(13,18,23–27)

E. Future challenges for gene editing and their clinical safety, efficacy and delivery

Although many techniques are evolved over time which make easily manipulation of genome sequence that help in creation of desired trait, still they have some challenges to overcome in upcoming research that are providing upgraded PAM spacer sequence for alteration of desired sequence in genome, secondly in increasing the efficiency of gene editing by developing tools and discovering more able tools for make precise and good edit, availability of techniques outcome in public awareness hence more and more development and application can occur in present scenario of treating various diseases.

Despite of exciting discoveries there are many opportunities are developed for editing but their limitations has also to be considered which should be altered or cope up for providing better edits, safety in providing gene edits to market availability has also been in consideration hence does not lead to development of new disease or create more complex conditions for livelihood, efficacy and deliveries of edit technique to organism is of about great importance for creating ease in treatment and availability for them. Afterall there should be some ethics in developing gene edits and their application in treating disease has to be maintained so that it does not lead to devastating effects.(28–31)

III. APPLICATIONS

A. Clinical Trials

- Blood Disorder: sickle cell disease (SCD) and beta thalassemia are two genetic illnesses caused by variations in a gene that encodes for a portion the hemoglobin molecules. (32)
- Cancer: through the process of the targeting of oncogenes, mutant tumor suppressor genes, and immune cell engineering firm cancer immunotherapy, CRISPER-Cas9 is being researched as a treatment for a variety of malignancies'. (33)
- HIV/AIDS: CRISPR-based treatments have been researched in certain clinical trials focusing on and eliminating HIV DNA from infected cells, which could result in a functional cure.(34)
- Genetic Disease: Because of capable breakthroughs in gene manipulation, monogenic diseases resulting from mutation of a singular gene such as cystic fibrosis, Duchenne muscular dystrophy, and primary immunodeficiency disorders stand a chance of prevention and treatment.(35)
- Viral infection: By directly targeting virulence or drug-resistant genes, the CRISPR-Cas system can eradicate bacteria while limiting the spread and transfer of harmful genes among microorganisms. This opens new avenues for research on preventing and treating bacterial multidrug resistance at the gene level.(36)

B. Disease Treatment

- Cardiovascular Disease: Within the field of cardiovascular disease research, genome-editing tools are just starting to be widely applied. It is not hard to foresee what is soon to come: the
- generation of many novel cellular and animal models, functional interrogation of molecular pathways involved in cardiovascular development and disease pathogenesis and attempts to prevent and treat.(34)
- Metabolic disease: (1) Correction of genetic aberrations leading to metabolic diseases. Insulin receptor gene defects could potentially be treated using CRISPR/Cas9, one possible use of this technology. (2) Adding new genes that enhance the body's ability to metabolize food more efficiently. For instance, using CRISPR/Cas9, one may insert genes that encode for enzymes to improve the digestibility of fat or carbohydrates.(37)

C. Plant Development

Enhanced Crop Traits

- Disease resistance: The production of crops resistant to bacterial, fungal, and viral illnesses has been made easier by gene editing. CRISPR has been used to create resistance to bacterial blight, tobacco mosaic virus, and rice blast fungus.(37)

- Increased Yield and Quality: The capacity to precisely modify plant genomes makes it possible to improve desired characteristics including stress tolerance, yield, disease resistance, and nutritional value.(38)
- Stress Tolerance: Crops made via genome editing could be more resistant to environmental challenges including drought, heat, cold, and salinity.(39)
- Nutritional Enhancement: CRISPR-Cas9 and other innovative gene editing technologies are essential for enhancing crops. They boost nutritional content, decrease postharvest losses, increase resilience against biotic and abiotic challenges, and permit precise alterations to plant endogenous genes.(40)

D. Examples of Application

- Rice: Rice yield, stress resistance, and nutritional value have been effectively improved using CRISPR/Cas9 technology. (41)
- Wheat: Wheat yield and disease resistance have been raised by means of gene editing.(40)
- Other Crop: Based on CRISPER, one effective method to boost the nutritional value of crops and make them safe for those with allergies or sensitivity is genome editing using maize, tomatoes, and oilseed rape.(37)

E. Therapeutic Application

Correcting Genetic Defects:

- Gene Therapy: With the help of gene editing, researchers may precisely target and alter the genes causing hereditary illnesses, perhaps leading to cures or markedly better patient outcomes.(42)

F. Developing New Cancer Treatment

- Targeting Cancer Cells: While sparing healthy tissues, gene editing allows one to specifically target and eradicate cancer cells.(43)
- Immunotherapy: Increases the immune system's capacity to identify and eliminate cancer cells by genetically modifying T cells to target cancer cells.(44)

G. Enhancing Drug Delivery and Targeting

- Nanoparticles: Treatment efficacy can be increased by designing nanoparticles to transport therapeutic chemicals to body parts, including cancers.(45)
- Alzheimer's Disease: Alzheimer's disease is being studied for management and treatment using nanomedicine, including nanoparticles.(45)

H. Animal Breeding

- Disease resistance: Livestock resistant to some diseases, like ALV in chickens, PRRSV in pigs, and Mycobacterium infections in cows, can be produced using gene editing. Researchers can create animals with improved immunity by focusing on genes linked to illness risk.(46,47)

I. Enhanced Production Traits

- Meat Quality: Gene editing improves the quality and production of meat products by modifying genetic variables that affect muscle growth, fat distribution, and softness.(48)
- Milk Production: By using genetic modifications, scientists can increase milk production and improve its composition to meet dietary requirements by, for example, raising the amount of protein or fat.(49)
- Feed Efficiency: By improving an animal's capacity to transform feed into lean tissue, precision gene editing can promote quicker growth rates and more economical use of resources.(50)

J. Other Application

- Sex Control: Selective breeding of chosen genders is made possible using gene editing to determine the sex of progeny.(39)
- Research Animal Models: Gene editing can be used to produce animal models for the study of human illnesses, which will aid in the creation of new drugs.(51)

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IV. CONCLUSION

Gene editing is an emerging technology with far-reaching consequences across a wide range of fields. Using technologies and tools like CRISPR-Cas9, base editing, prime editing, and PASTE, scientists are opening unprecedented possibilities for accuracy in genetic manipulation. Though there are some limitations, such as ethical issues, technical challenges, and regulatory complexities, the applications of gene editing are significant. These involve improving breeding in animals, promoting disease cure, enhancing plant growth, supporting innovative clinical tests, and advancing therapeutic interventions. As technology keeps developing, it has the potential to revolutionize science, agriculture, medicine, and biotechnology, ensuring a healthier and more sustainable future.

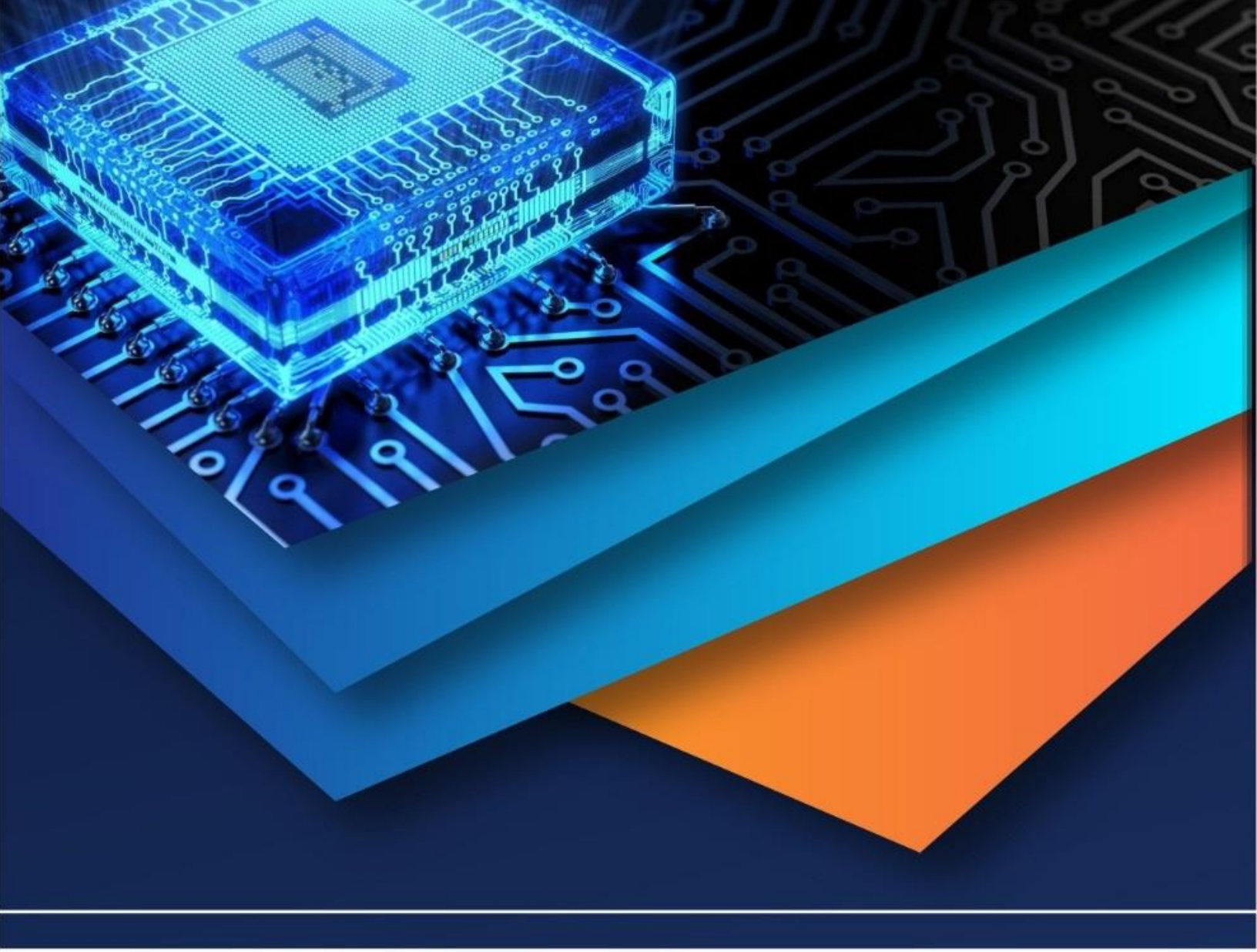
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