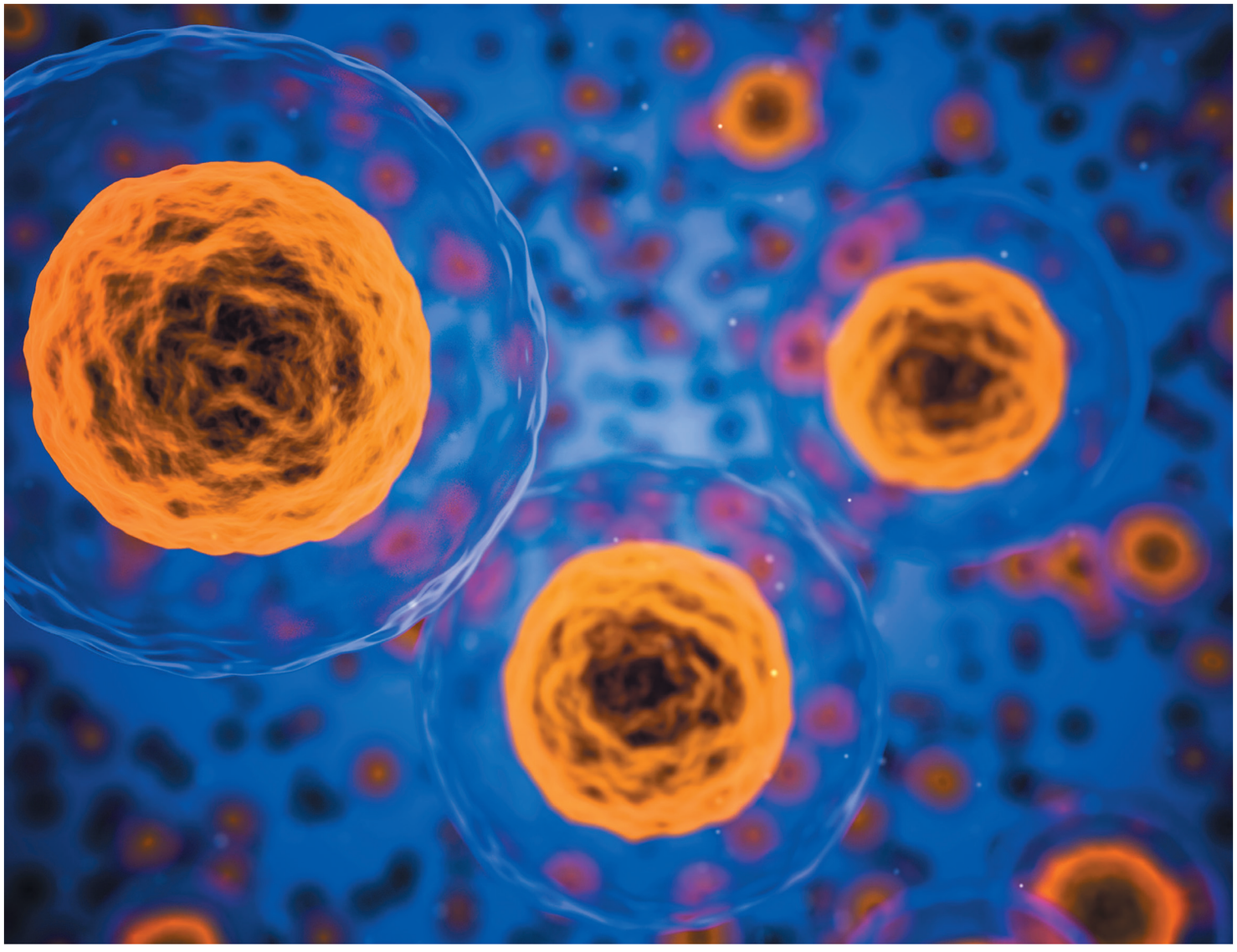

How to Ensure CRISPR Gene Therapies Are Safe and Effective?

A Quality Control Case Study with Beta Thalassemia

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Introduction

Each year, about 70,000 new patients are diagnosed with beta thalassemia, making it one of the most common autosomal recessive diseases globally.[1] Beta thalassemia results from a mutation in the hemoglobin beta gene, which causes reduced levels of hemoglobin, decreased red blood cell production, and anemia. Many patients experience dramatic, life-threatening symptoms and require a lifetime of costly medical care. According to a 2008 report from the World Health Organization, roughly 63 percent of beta thalassemia patients depend on regular blood transfusions to live.[2] Those who receive regular transfusions and care can experience a full lifespan, however, these individuals are at ongoing risk of experiencing medical complications from this disease and the blood transfusion.

Researchers have been seeking more effective treatment options to reduce the significant medical burden on this patient population. Thanks to major scientific advances in gene editing in recent years, significantly better options may be available soon. Beta thalassemia is one of many genetic diseases identified as a prime candidate for CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 based gene therapies. While gene editing is still considered a radical approach, directly editing patients' disease-causing genes could represent a one-time, long-term solution to reducing or eliminating disease symptoms.

As new CRISPR-based gene editing strategies are being developed, work is also underway to determine how best to validate them across key DNA, RNA, protein, and cell toxicity parameters. Developers face one major challenge: key genetic hallmarks of a successful therapy are so minute that only ultrasensitive methods for DNA detection such as Droplet Digital PCR (ddPCR) can measure them accurately (**Figure 1**). By choosing an array of quality control methods suited to the challenging task of evaluating these therapies, Developers can ensure that the therapies will be safe and effective – and instill confidence in therapeutic gene editing. By selecting the right quality control regimen, researchers and developers can increase the likelihood that their safe, effective, and genetically precise therapies will gain market approval to help patients with beta thalassemia and other genetic disorders regain a normal life.

Beta Thalassemia: A Disease of Imbalance

Because of its recessive nature, beta thalassemia symptoms vary drastically from patient to patient; patients can present with three levels of disease severity. Individuals carrying one mutated and one normal copy of the beta globin gene are said to have the "minor" form of the disease and may be asymptomatic or suffer mild anemia. If beta globin protein levels are significantly reduced, individuals carrying two mutated copies of the gene may present with the "intermediate" form of beta thalassemia. For the third level of severity, beta globin protein levels are essentially absent so, patients may present with the "major" form of the disease. The intermediate and major patient groups tend to require significant, life-long medical attention. Because the origins of beta thalassemia are genetic, patients requiring significant medical attention are prime candidates for tactics like gene therapy. But to be successful, any therapy must address the causative genetic mutation, as well as the downstream molecular phenomena that heighten symptoms of the disease resulting from improper proportions of proteins in red blood cells that preclude the formation of healthy hemoglobin.

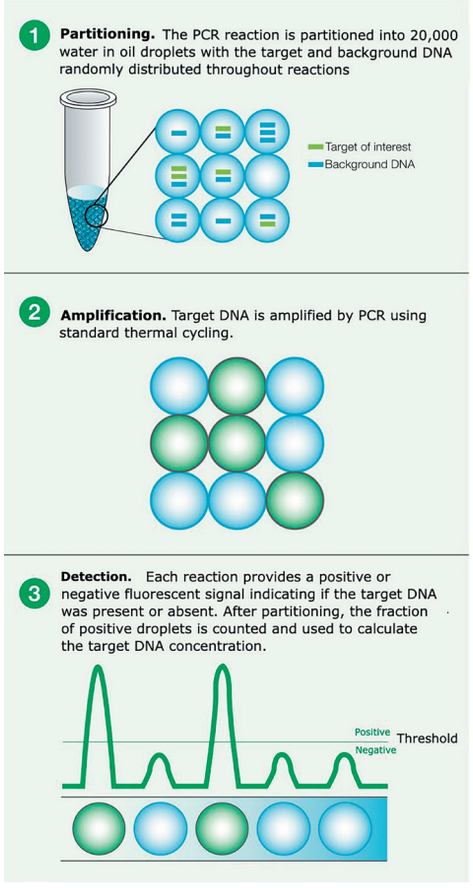

Figure 1: The sensitivity and accuracy of the ddPCR is derived from the initial partitioning step which isolates one or a few nucleic acid strands into individual droplets before running the thermocycling reaction. Some droplets will carry a nucleic acid strand containing the target DNA, which will be amplified during the thermocycling reaction to produce a strong fluorescent signal. In contrast, droplets that do not contain the target sequence will emit background fluorescence only. By counting the strongly fluorescent droplets, it is possible to count the number of target DNA molecules in a cell line, a crucial step when evaluating a new gene editing strategy.

As is well known, the primary role of hemoglobin is to deliver oxygen throughout the body. As a protein tetramer, adult hemoglobin is typically made up of two alpha globin and two beta globin subunits. In the red blood cells of individuals with beta thalassemia, however, hemoglobin contains a reduced number of beta globin subunits or none at all (**Figure 2**). Further, these individuals have smaller red blood cells. Smaller red blood cells combined with fewer beta globin units per cell means that red blood cells in these individuals are unable to carry normal levels of oxygen, resulting in anemia and symptoms including excessive bleeding, increased risk of infection, damage to the spleen, heart, and liver, and deformation and weakening bones.

Unable to bind to low levels of beta globin, free alpha globin subunits begin to precipitate within the red blood cells. These precipitates damage cell membranes, inhibit formation of new blood cells, and cause breakdown of existing red blood cells – all of which render patients' anemia more severe.[3] In fact, clinical studies seeking to understand the determining factors of the severity of beta thalassemia have shown that among patients who had two mutated copies of beta globin gene, the disease was less severe in those whose alpha globin gene was also mutated or deleted.[4] While it is counterintuitive that the deletion of a protein with such a major role in the body could be an asset, patients with balanced alpha and beta globin levels were healthier long-term.

Challenging to Treat with Current Methods

Patients with more severe forms of beta thalassemia currently face treatment for a lifetime of chronic symptoms. Patients must receive care soon after birth at a thalassemia treatment center to get the extensive and highly specialized attention they need. Physicians familiar with the disease can develop a treatment plan and monitor the patient's response. Current treatments can allow patients with intermediate and major disease to manage their anemia. Despite dramatic improvements in treatment options, however, this chronic condition still causes an array of secondary complications that reduce patients' quality of life and put them at an increased risk of early mortality.[5]

The standard strategy to treat these patients hinges on them undergoing blood transfusions approximately every two to four weeks. Transfusions ensure that the blood circulating in the patient's body carries functional hemoglobin that can transport the correct amount of oxygen throughout the circulatory system. This strategy

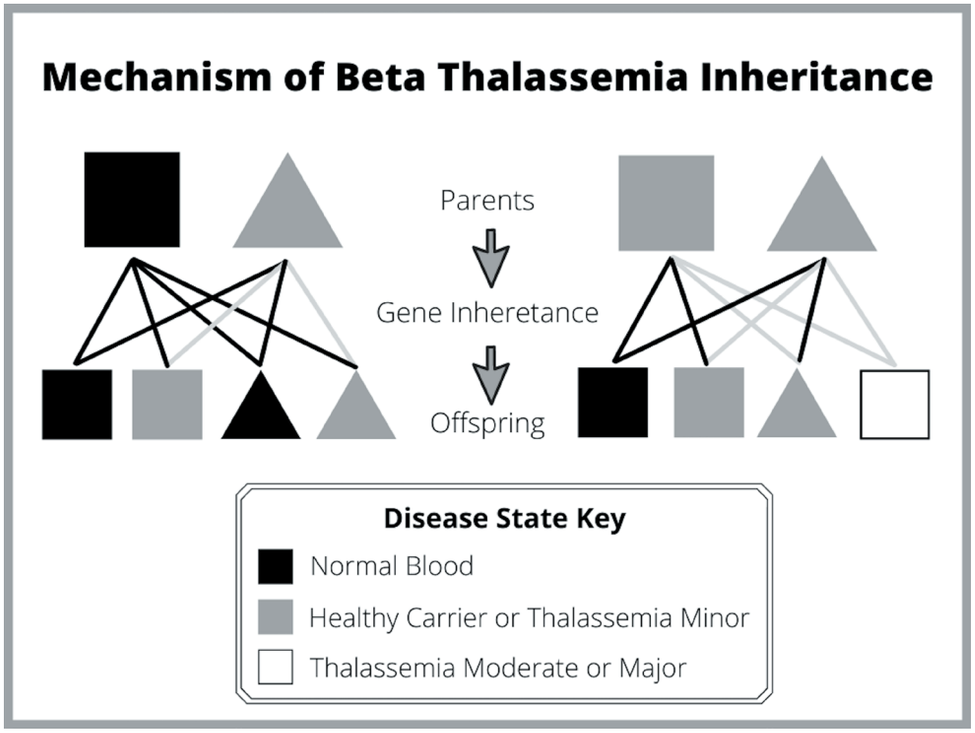

Figure 2: Beta-thalassemia is an autosomal recessive disease originating at the beta-globin gene locus. In the case of couples where one parent is a carrier of one mutant beta-globin gene and one wild type gene and the other parent carries two wild type genes, 50 percent of the offspring will have normal blood and 50 percent will be carriers of the mutant gene and may present with beta-thalassemia minor symptoms. If both parents are carriers of a mutant beta-globin gene, then 25 percent of offspring will have normal blood, 50 percent will be carriers of the mutant gene, and 25 percent will have two mutant copies of the beta-globin gene and will present with the moderate or major form of the disease.

has a major drawback: iron buildup in the body, also known as iron overload.[6] Excess iron causes tissue damage leading to a broad spectrum of severe and life-threatening complications like heart failure, liver cirrhosis, hypothyroidism, diabetes mellitus, growth retardation, and failure or delay of sexual maturation. To combat the dangerous side effects of iron overload, transfusion-dependent patients must take iron chelating medications, as well as additional disease management drugs to treat their disease symptoms as needed. In the face of these risks, patients are completely dependent on these procedures to alleviate their disease and face the potential of pain or organ damage throughout their lives.

Considering that a lifetime of blood transfusions comes with substantial challenges for beta thalassemia patients, scientists and clinicians are exploring alternative treatment possibilities for these individuals. One strategy relies on fetal globin, a hemoglobin subunit that, like beta globin, pairs with alpha globin but is typically only produced at high levels during fetal development and infancy.[7] As individuals age, a switch occurs, and the body stops producing high levels of fetal globin and starts producing high levels of alpha globin. Typically, in patients with beta thalassemia, symptoms begin at the time of the switch, prompting clinicians to turn to drugs that stimulate fetal hemoglobin production, such as hydroxyurea, 5-azacytidine, and short chain fatty acids. These therapies help compensate for the lack of beta globin and can reduce or eliminate the need for blood transfusions in intermediate-to-severe patients. While these treatments can also alleviate some of the systemic complications in vital organs, only a portion of patients responds to them.

> *"In fact, clinical studies seeking to understand the determining factors of the severity of beta thalassemia have shown that among patients who had two mutated copies of beta globin gene, the disease was less severe in those whose alpha globin gene was also mutated or deleted."*

Hematopoietic stem cell transfusion is another strategy that can offer dramatic benefits. In this approach, chemotherapy or radiation are used to eliminate the patient's bone marrow, and then replace it with a donor's bone marrow. However, this high-risk procedure is generally only performed on individuals with the major form of the disease who aren't responding to any other form of treatment. Even then, it can be difficult to find a matched donor who can aid in a drastic procedure that must be repeated throughout the patient's lifetime.

Safer, longer-lasting options that have the potential to cure this disease are needed. Furthermore, because the disease is a result not just of too little beta globin, but also of toxic precipitates of alpha globin, precision approaches that both restore beta globin production and the balance between the alpha and beta globin protein levels would offer exceptional advantages.

Gene Editing to Treat Beta Thalassemia

Gene editing represents a direct method to repair the genetic mutations involved in beta thalassemia. Among gene editing strategies available, CRISPR-Cas9 can modify the genome in a highly targeted fashion, making it a particularly attractive option for beta thalassemia treatment. Through this strategy, a patient's own hematopoietic stem cells are collected and modified by a CRISPR-Cas9 system, and subsequently grafted back to the patient.* Molecularly speaking, three potential gene modifications can be made to restore functional hemoglobin, meaning that several precision medicine approaches may be used to treat beta thalassemia:

- Re-starting the production of fetal globin to restore the fetal hemoglobin tetramer
- Adding a functional copy of beta globin to restore the adult hemoglobin tetramer
- Deleting a copy of alpha globin to reduce alpha globin precipitates

There are currently three active clinical trials listed on clinicaltrials.gov evaluating the safety and efficacy of two ex vivo gene therapies that use CRISPR/Cas9 to increase fetal globin production.[8] Like the drugs that stimulate fetal globin production listed above, these promising gene therapies are designed to re-start the transcription of the fetal globin gene, raising its protein levels in patients so that the subunits can form tetramers with alpha globin counterparts, thereby reducing the alpha globin precipitates that worsen beta thalassemia patients' anemia. Based on individuals with beta thalassemia who naturally continue to produce fetal globin into adulthood, successful gene editing would also be expected to leave patients with a milder, more manageable clinical status.[9]

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Numerous additional studies are evaluating the merit of using CRISPR to manipulate either the alpha or beta globin loci to treat beta thalassemia. These approaches seek to either restore functional adult hemoglobin levels or reduce the alpha globin precipitates. Making either modification alone comes with significant challenges and, unfortunately, may not fully eliminate symptoms of the disease.

The rationale for editing the alpha globin locus is based on the fact that beta thalassemia patients lacking functional copies of the alpha globin gene tend to have less severe symptoms.[10] Thus, gene editing tactics to reduce alpha globin levels are being evaluated to determine if the modification reduces the harmful alpha globin precipitates and improves patients' anemia. This approach only works in patients expressing partial levels of the beta globin gene.[4] Some patients may still require ongoing care to manage their disease, particularly if the alpha-beta balance is not fully restored, or if hemoglobin levels are not high enough to deliver sufficient oxygen throughout the body.

On the other hand, repairing the mutated beta globin gene would help to improve the alpha-beta globin balance and reduce precipitates. This modification is difficult to execute because the CRISPR/Cas9 strategy must be tailored specifically to each patients' mutation. When the modification is carried out correctly, however, it has been shown to raise beta globin expression in patient-derived cells.[11] On the other hand, this approach does not generally raise beta globin levels up to wild type levels, and it has been suggested that future strategies would do well paired with tactics to either raise fetal globin levels or lower alpha globin levels to achieve maximum therapeutic benefit.[11]

A Two-Pronged Approach

While individual gene editing of the alpha or beta loci alone can partially address the underlying mechanisms that cause beta thalassemia, modifying both could potentially return patients' alpha and beta globulin levels to a healthy, balanced state. This would be particularly beneficial for patients with severe disease who produce no natural beta globin protein. However, there is no denying that this approach is complicated because it requires the simultaneous deletion of an alpha globin gene and the correction of beta globin gene. The treatment will not fully work without both edits. And if the edits occur in the wrong place, as observed in other studies, the therapy can damage healthy genes.[12]

One recent study reported a promising strategy to make these dual modifications.[13] The scientists used a modified CRISPR/Cas9 construct, called CRISPR/Cas9 D10A nickase, to cut out one copy of the alpha globin A2 gene and replace it with a new copy of the beta globin gene.[13] Since the edits had to be precise, a guide RNA was used to direct the Cas9 variant to cut the gene sequence flanking the alpha globin A2 locus, remove the gene, and then facilitate the integration of an exogenous copy of the beta globin gene in the exact same location. Errors that removed or introduced even just one base pair could have rendered the new gene out of frame, leading to a nonfunctional protein.

By restoring the balance between alpha and beta globin protein, this strategy enables the formation of more adult hemoglobin tetramers. As such, it has the potential to deliver a unique therapeutic benefit to patients with beta thalassemia. However, this complex edit does require rigorous quality control. For example, the researchers had to establish that their CRISPR construct was able to achieve a seamless gene edit to produce on-target integration. They also needed to determine on average how many copies of the beta globin gene individual cells received. To achieve this level of precision, quality control assessments required the extreme sensitivity and accuracy of ddPCR.

The Role of ddPCR in Gene Editing Quality Control

Droplet digital PCR technology offers broad advantages when assessing CRISPR-based gene editing systems. As a highly sensitive and precise tool, ddPCR is ideal for detecting and quantifying rare variants in the genome, including those resulting from CRISPR modifications.[13] It can also detect gene inversions and excisions in sequences of a wide range of lengths.[14] Furthermore, ddPCR has been shown to detect CRISPR edits made by both nonhomologous end joining and homology directed repair, making it a suitable tool for the evaluation of a variety of possible gene editing systems.

When compared to other tools for DNA assessment, ddPCR offers distinct advantages in some cases, and plays a vital role when paired with

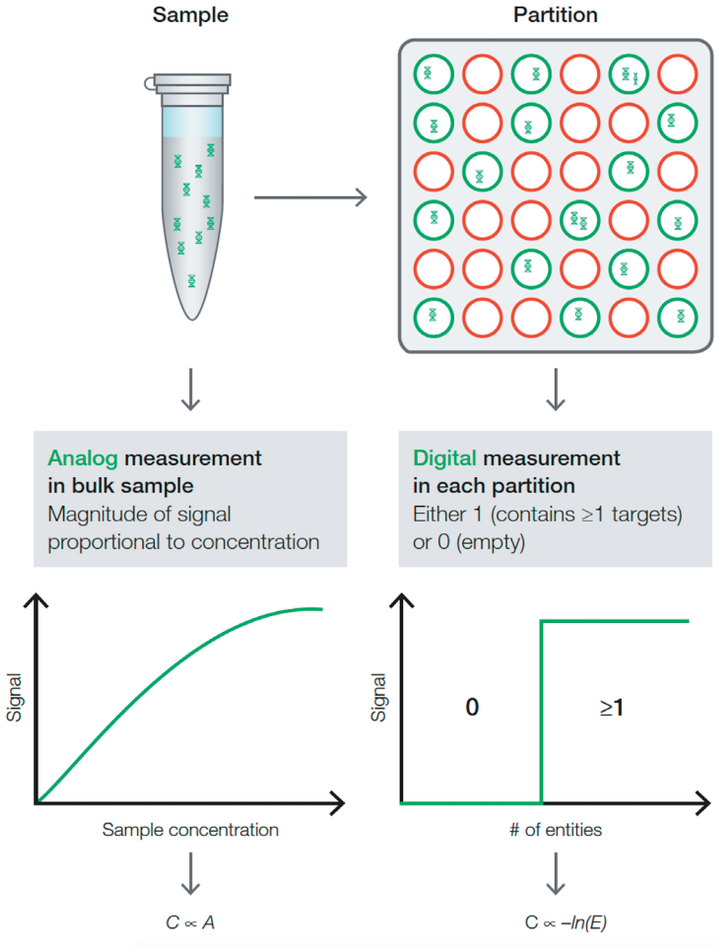

Figure 3: qPCR's sensitivity is limited because the technique is based on the analysis of bulk samples and measures signals produced by multiple target DNA sequences simultaneously. ddPCR offers higher sensitivity because the technique partitions samples into droplets that contain one or a few nucleic acid strands each. Subsequently, a quantitative, "digital" measurement can be made based the number of droplets that contained a target sequence and produced a positive signal.

others. For example, compared to qPCR, ddPCR is more sensitive and accurate due to the assay design (**Figure 3**). To interpret a qPCR assay, researchers must run their bulk sample alongside a standard curve to interpret their results, thereby introducing potential error into the assay and reducing the degree of assay sensitivity. In contrast, ddPCR is extremely sensitive and accurate because it partitions samples into droplets that contain one or a few nucleic acid strands each before amplification, and then counts the number of droplets that contained the target sequence after amplification. In this way, the assay can provide an absolute count of the number of copies of gene targets in a sample or quantifying the number of copies of target sequence per cell. As noted above, the inherent variability of qPCR prevents detection of target sequences present at lower than two to three copies per cell. The different capabilities of these two assays were highlighted in the study of the dual CRISPR edit mentioned above.[13,14] It was essential that the researchers used ddPCR to evaluate the integration of their construct, because it occurred at a frequency of 0.8 copies per cell – lower than the limit of detection of qPCR.

On the other hand, NGS and ddPCR can be combined to get a complete picture of how a CRISPR-based gene editing system is functioning. NGS can scan the genome and identify the off-target locations where a new gene is inserted, whereas ddPCR can measure the frequency of on-target insertion and gene copy number.

In Summary: CRISPR for Therapeutic Gene Editing

- ❖ Therapeutic gene editing represents a promising option for genetic diseases like beta thalassemia that can't be easily treated.
- ❖ New, potentially curative therapies for beta thalassemia use CRISPR-based gene editing to alter the expression of one of more human globin genes to raise hemoglobin levels and/or to restore the balance between alpha and beta globin protein levels in patients. This is just one of many genetic diseases that CRISPR gene therapy may address.
- ❖ QC testing using precise, quantitative methods such as ddPCR will be crucial to evaluating these therapies moving forward to maximize benefit to patients and clinical success.

Figure 4: Key highlights from the article

However, in instances where either technology may be used, ddPCR is faster, less labor-intensive, and less expensive.[15] It is also capable of detecting rare events without being impacted by read depth.

The Future of CRISPR in Gene Editing

With roughly 30 clinical trials in the works that aim to use CRISPR to correct a genetic disease, there is a distinct possibility that the first CRISPR-based gene therapy could be approved in the next decade.[16] Between now and then, biopharmaceutical developers must continue to hone their protocols to develop safer and more efficient gene editing systems and consequent precision therapies for diseases.

Quality control measures that are sensitive and precise will be key to achieving gene-editing therapies because they can test crucial parameters both dramatic and subtle. Also key to success will be the raising of testing methods that are matched for specific tasks to gold standard status, e.g., building on the sensitivity of ddPCR to evaluate the quantity and quality of therapeutic gene modifications.

By holding therapies to the highest standard possible throughout research, development and manufacturing, scientists can effectively and efficiently screen out cell lines with off-target edits and other undesirable properties. By reliably delivering the highest quality gene therapies to patients, developers increase their therapy's chances of attaining clinical success. With each new successful therapy, the field will gain traction, ushering in a new generation of therapies to correct otherwise intractable genetic diseases like beta thalassemia (**Figure 4**). JoPM

References

1. Weatherall DJ. The challenge of haemoglobinopathies in resource-poor countries. Br J Haematol. 2011;154(6):736-744. doi:10.1111/j.1365-2141.2011.08742.x
2. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86(6):480-487. doi:10.2471/blt.06.036673
3. Bank A. Understanding globin regulation in beta-thalassemia: it's as simple as alpha, beta, gamma, delta. J Clin Invest. 2005;115(6):1470-1473. doi:10.1172/JCI25398
4. Mettananda S, Gibbons RJ, Higgs DR. α-Globin as a molecular target in the treatment of β-thalassemia. Blood. 2015;125(24):3694-3701. doi:10.1182/blood-2015-03-633594
5. Betts M, Flight PA, Paramore LC, Tian L, Milenkovic D, Sheth S. Systematic Literature Review of the Burden of Disease and Treatment for Transfusion-dependent β-Thalassemia. Clin Ther. 2020;42(2):322-337.e2. doi:10.1016/j.clinthera.2019.12.003
6. Beta Thalassemia. National Organization for Rare Disorders. 2018. Visited December 2021. https://rarediseases.org/rare-diseases/thalassemia-major/
7. Musallam KM, Taher AT, Cappellini MD, Sankaran VG. Clinical experience with fetal hemoglobin induction therapy in patients with β-thalassemia. Blood. 2013;121(12):2199-2372. doi:10.1182/blood-2012-10-408021
8. National Institutes of Health. clinicaltrials.gov Search. U.S. National Library of Medicine. Visited December 2021. https://clinicaltrials.gov/ct2/results?cond=beta+thalassemia&term=crispr&cntry=&state=&city=&dist=&Search=Search&recrs=a&recrs=d&recrs=f
9. Traxler EA, Yao Y, Wang YD, et al. A genome-editing strategy to treat β-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. Nat Med. 2016;22(9):987-990. doi:10.1038/nm.4170
10. Badens C, Joly P, Agouti I, et al. Variants in genetic modifiers of β-thalassemia can help to predict the major or intermedia type of the disease. Haematologica. 2011;96(11):1712-1714.
11. Cosenza LC, Gasparello J, Romanini N, et al. Efficient CRISPR-Cas9-based genome editing of β-globin gene on erythroid cells from homozygous β039-thalassemia patients. Mol Ther Methods Clin Dev. 2021;21:507-523. Published 2021 Apr 3. doi:10.1016/j.omtm.2021.03.025
12. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. Mol Ther Nucleic Acids. 2015;4(11):e264. Published 2015 Nov 17. doi:10.1038/mtna.2015.37
13. Pavani G, Fabiano A, Laurent M, et al. Correction of β-thalassemia by CRISPR/Cas9 editing of the α-globin locus in human hematopoietic stem cells. Blood Adv. 2021;5(5):1137-1153. doi:10.1182/bloodadvances.2020001996
14. Watry HL, Feliciano, CM, Gjoni K et al. Rapid, precise quantification of large DNA excisions and inversions by ddPCR. Sci Rep 10, 14896 (2020). doi.org/10.1038/s41598-020-71742-z
15. Peng C, Zheng M, Ding L, et al. Accurate Detection and Evaluation of the Gene-Editing Frequency in Plants Using Droplet Digital PCR. Front Plant Sci. 2020;11:610790. Published 2020 Dec 14. doi:10.3389/fpls.2020.610790
16. National Institutes of Health. clinicaltrials.gov Search. U.S. National Library of Medicine. Visited November 2021. https://clinicaltrials.gov/ct2/results?term=CRISPR&Search=Apply&recrs=b&recrs=a&recrs=f&recrs=d&age_v=&gndr=&type=&rslt=

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Footnote

* The targeting of CRISPR-Cas9 systems relies on specially designed guide RNA to direct the Cas9 endonuclease to cut DNA at the precise location where the edit is to be made. When paired with nonhomologous end-joining or homology directed repair, the system can insert a new DNA sequence into the location of interest.