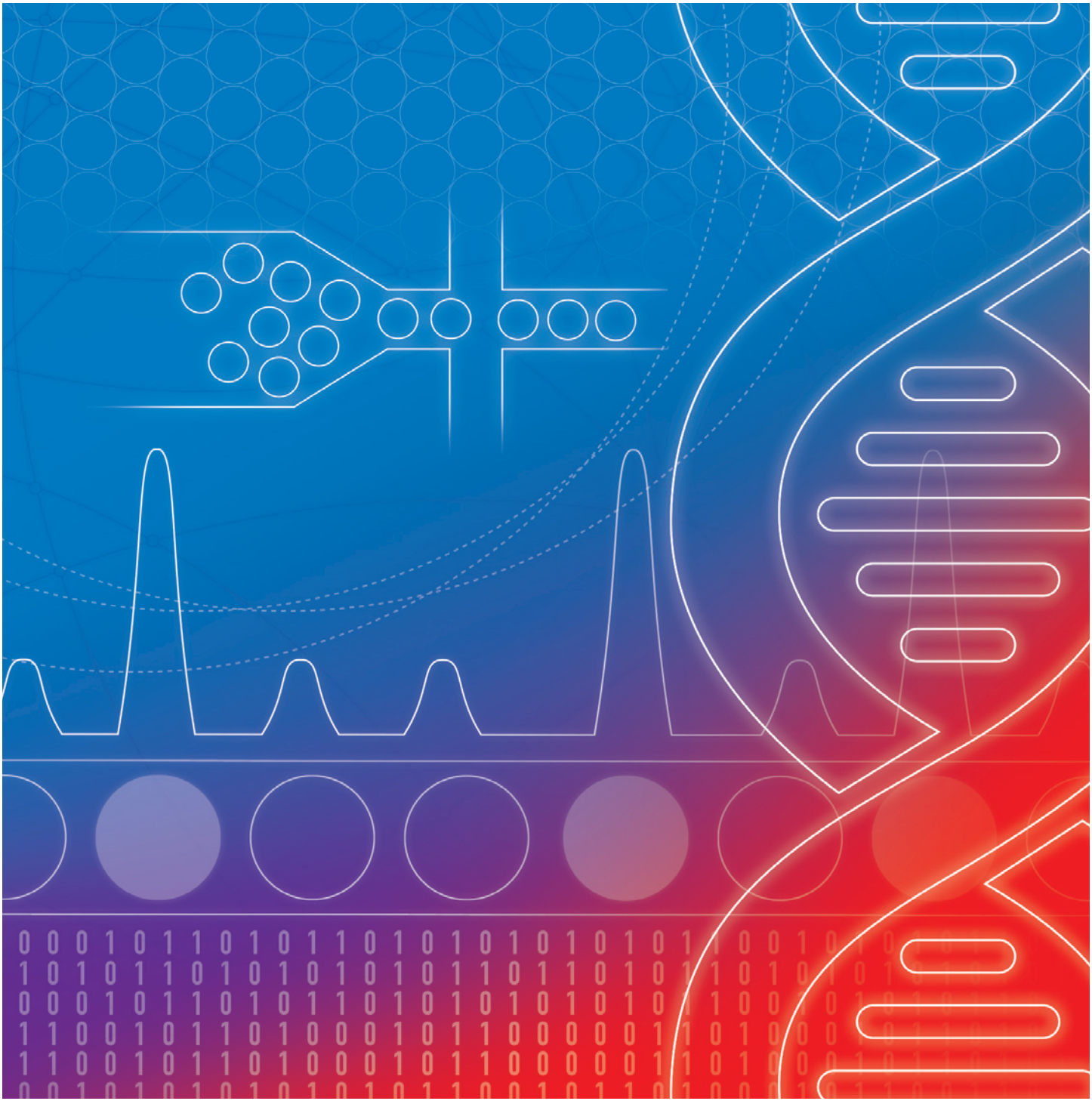
Droplet Digital PCR

RNA: The Next Era in Therapeutic Development

A Droplet Digital™ PCR eBook



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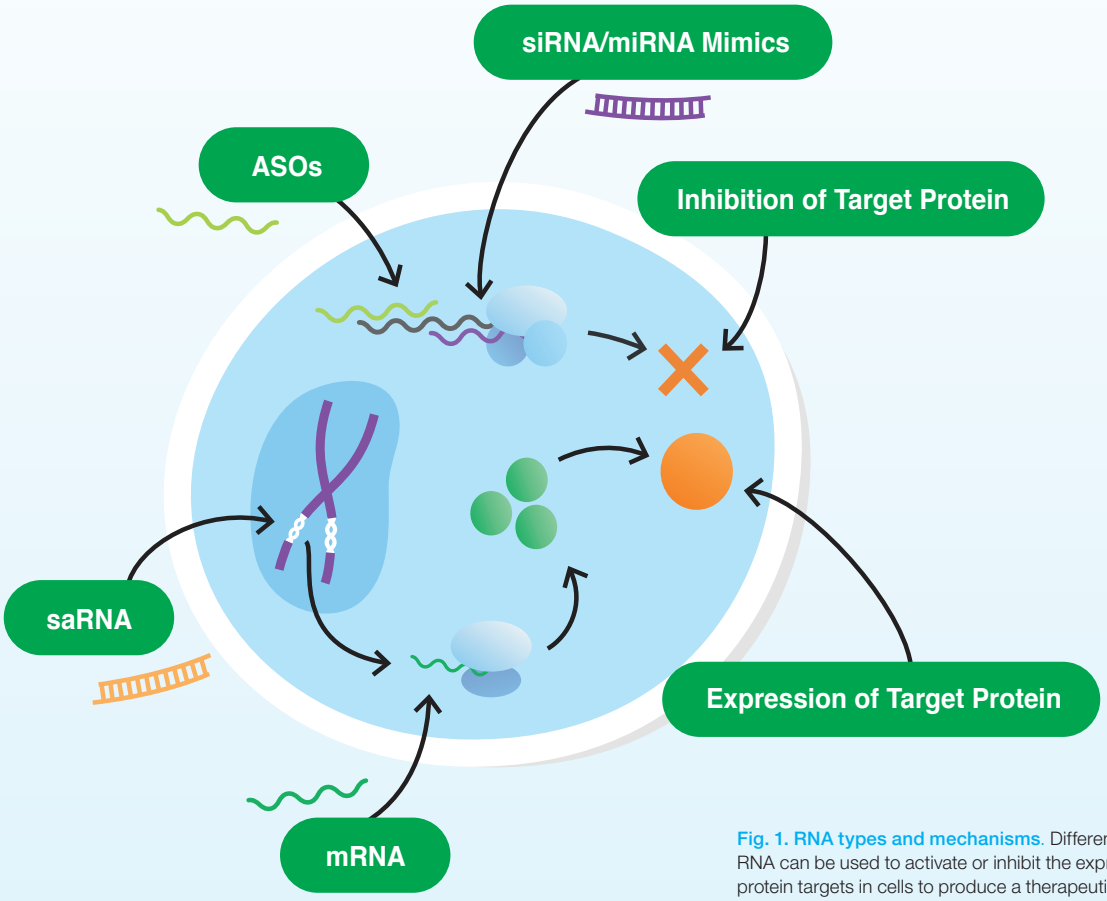

Fig. 1. RNA types and mechanisms. Different types of RNA can be used to activate or inhibit the expression of protein targets in cells to produce a therapeutic effect.

Advances in therapeutic RNAs have exploded in recent years. These novel drugs have the potential to make personalized medicine a reality and, at the same time, make the term "undruggable" obsolete. Collectively, RNA therapeutics consist of multiple types of RNAs that include small activating RNAs (saRNA), antisense oligonucleotides (ASO), microRNAs (miRNA), small interfering RNAs (siRNA), and messenger RNAs (mRNA). These RNAs work by hijacking natural cellular processes that control gene expression and translation (Figure 1). In other words, therapeutic RNAs can be used to provide a sequence-specific modulation of gene expression to cause a desired biological response with a beneficial health outcome.

Compared to small molecule– or protein-based therapeutics, novel nucleic acid–based therapeutics offer significant advantages, including shortened development timelines, scalability, and flexibility. These benefits became evident with the mRNA-based vaccines and vaccine boosters produced in response to the coronavirus disease 2019 (COVID-19) pandemic (Liu and Wang 2022). An additional benefit of RNA-based approaches includes avoiding the risk of chromosomal integration since the therapeutic RNA does not need to penetrate the host cell nucleus to be effective (Trepotec et al. 2019). Finally, RNA therapeutics offer potential to access clinical targets thought to be out of reach for traditional drug development. Because of this potential to reach new clinically meaningful targets, the RNA therapeutics space has received significant attention and investment, which has led to a rapid expansion of active clinical research and commercialization activities (Figure 2).

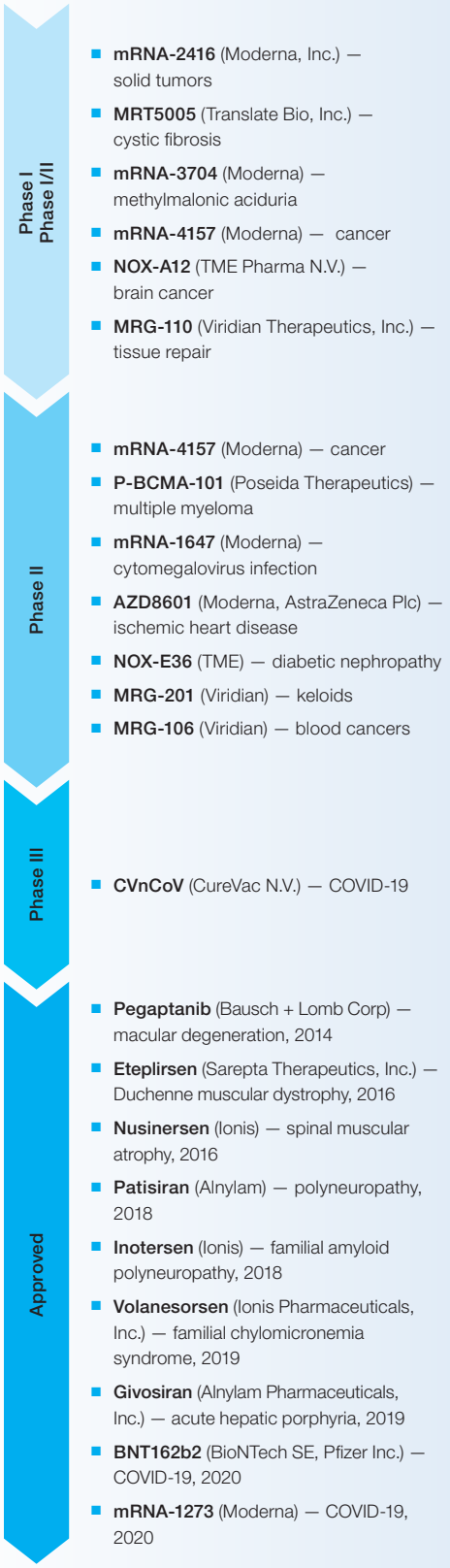

Fig. 2. Progression of therapeutic RNAs through the clinical pipeline. The names of the RNAs are bolded and are followed by their sponsor in parentheses and clinical indications. For the approved products, the year of approval is also shown. Modified from Damase et al. (2021).

RNA Therapeutic Production Basics

A specific sequence and length of RNA, for therapeutic or any other purpose, can be manufactured using a range of nucleic acid synthesis methods (Damase et al. 2021, Whitley et al. 2022). Popular methods include solid-phase chemical synthesis or in vitro transcription from DNA templates using phage RNA polymerase (Karikó et al. 2011). However, there have also been recent advancements in high-yield and large-scale bioproduction methods utilizing either RNA carriers or RNase III–deficient bacteria to produce noncoding RNAs (Ho et al. 2018, Hashiro et al. 2019).

In addition to needing to be a specific sequence, the RNA produced for a therapeutic purpose must also have sufficient stability in vivo. Because of the inherent instability of RNA, multiple strategies have been developed for RNA protection, transport, and delivery to the intended target (Whitley et al. 2022). Liposomes, created using cationic lipids, can encapsulate RNAs in particles that protect and stabilize the RNA while also aiding in delivery to the cell since the particles closely resemble the cell membrane composition (Damase et al. 2021). Other protection and delivery strategies include the use of polymer nanomaterials, silica, carbon, or gold nanoparticles to stabilize and help deliver RNA into cells (Damase et al. 2021).

Challenges in Therapeutic RNA Development and Production

Overall, methods for RNA synthesis, protection, and delivery have progressed substantially in recent years to provide a foundation for the evolving development and commercial production of RNA therapeutics. However, significant challenges still exist in target identification, validation, real-world production, and quality control.

It is well known that RNAs can drive pathogenesis in disease by modulating gene expression. For example, in individuals with spinal muscular atrophy, somatic mutations in the *SMN1* gene will transcribe mRNAs that, when translated, will produce nonfunctional SMN1 protein variants (Hill and Meisler 2021). Likewise, short noncoding miRNAs, which control protein expression through RNA degradation mechanisms, are often deregulated in cancer (Fabris et al. 2016). Understanding and attributing the role of a specific RNA to a disease state is a complex process that involves basic studies of mechanism of action using cell culture and complex animal models. This level of target identification and attribution, or validation, is key to developing an effective and successful RNA therapeutic. Although target identification and validation are a constant challenge, each disease and potential RNA therapeutic study is somewhat unique.

In the development phase, challenges beyond target identification and validation must also be considered and addressed. The degree to which the RNA was chemically modified during development must be studied and optimized. It has been shown that these modifications can be a make or break for the molecule in terms of RNA stability, pharmacokinetics, and ultimately translation potential and therapeutic benefit (Yu et al. 2020). For example, chemical modifications to the RNA nucleosides and phosphate groups that make up the backbone can drastically increase the translation efficiency. Specifically, it has been shown that replacement of uridine with synthetically modified substitutes can aid translation efficiency (Liu and Wang 2022). Similarly, it is known that the addition of long chains of adenine nucleotides, or poly(A) tails, to mRNA molecules produced in cells increases their stability. This fact has been leveraged by adding such poly(A) tails onto synthetically

produced RNA to improve stability and processing of the RNA inside target cells. The overall architecture and length of the poly(A) tail is a feature that must be optimized and validated to deliver consistent overall translational efficacy and half-life of the RNA therapeutic molecule (Trepotec et al. 2019).

Once a specific RNA has been identified and its chemical composition optimized to elicit the desired therapeutic response, the production of that RNA presents additional challenges. Specifically, its production must be scaled to supply enough of the therapeutic to support clinical testing and eventually commercial production. Scale-up challenges must be addressed to produce drug substance that is equivalent to the small-scale material. It is not uncommon for processes, especially those used for downstream purification, to change dramatically during the transition from small- to large-scale production (Karalė et al. 2021).

Purity of the RNA product is another challenge that must be managed to deliver a product that is free of potential contaminants that could compromise therapeutic safety and efficacy. Multiple types of contaminants need to be considered, controlled, and quantitatively monitored. These range from process-derived byproducts such as unincorporated nucleoside triphosphates, small abortive transcripts, and template plasmid DNA, to potential microbial contamination such as *Mycoplasma* (Karikó et al. 2011). Each of these contaminants need to be managed because they can elicit a strong host immune response to the therapeutic and consequently reduce the overall RNA safety and efficacy.

> …incorporating a set of robust analytical methods from the start can help deliver successful development and production…

Analytics Is the Key for RNA Success

As noted, the road to successful development of an RNA-based therapeutic is littered with potential obstacles. However, incorporating a set of a robust analytical methods from the start can help deliver successful development and production with an absence of problematic contaminants (Yu et al. 2020).

Analytical assays can be divided into either proteomic or genomic methods. As the name implies, proteomic methods measure and quantify proteins. They can be used to functionally assess a therapeutic RNA by measuring the translated protein product and subsequent biological consequence. Though these types of assays are useful for assessing RNA function, not much information can be gained about the quality or biochemical characteristics of the RNA itself. These types of proteomic assays most commonly consist of antibody-based approaches for detection of the specific end protein product, either through ELISA, flow cytometry, or immunohistochemistry.

Genomic methods measure and quantify nucleic acids and thus are a great asset for assessing the RNA itself as well as its functionality in a cell from a transcriptional perspective. Common genomic assays utilized in RNA therapeutics development and production include in situ hybridization, which currently stands as the gold standard for single molecule RNA visualization (Yu et al. 2020). However, this method is highly dependent on the probe and detection marker, which can exhibit high background and limit reliability (Yu et al. 2020).

RNAs can also be detected by absorbance of ultraviolet (UV) light or by tandem mass spectrometry (MS/MS), often after separation by liquid chromatography (LC). LC-UV exhibits high specificity. LC-MS/MS also offers high specificity over a wide dynamic range but can have limited sensitivity for specific RNAs. However, both require substantial sample preparation to achieve desirable results (Yu et al. 2020).

Reverse transcription PCR (RT-PCR) is a genomic method that offers excellent selectivity and sensitivity for the detection and quantification of target RNA sequences. However, the quantitative capability of RT-PCR is highly dependent on the type of PCR utilized. Real time, or quantitative, PCR (qPCR) measurements are dependent on the generation of a reference standard curve, and accuracy can be affected by low copy numbers of the template RNA sequence. Droplet Digital PCR (ddPCR™), on the other hand, is a unique type of PCR that can provide absolute quantification of RNAs, making it ideal for low template copy number applications (Kojabad et al. 2021).

Due to these favorable characteristics, ddPCR technology has proven to be an invaluable analytical asset and is extensively used at various stages of the RNA development and production process, from basic RNA therapeutics discovery, where it can be used in gene expression studies to identify and validate attribution of RNAs to specific pathologies, to downstream production quality control (QC), where it can be used to characterize and validate poly(A) tail content of RNAs. In one study, a two-step reverse transcription–based ddPCR assay targeting the poly(A) tail content was utilized to determine that mRNA fragments present in the Pfizer-BioNTech COVID-19 vaccine (BNT162b2) were primarily derived from premature transcriptional termination that occurred during manufacturing (Patel et al. 2023). These truncated transcripts did not exhibit viable expression of the SARS-CoV-2 spike protein antigen. Therefore, this study suggested that mitigation of this truncated species could enhance vaccine potency. Studies such as this demonstrate the power of Droplet Digital PCR in RNA therapeutics.

As is the case in almost any discovery and production process, having the right set of analytical assays to help understand, characterize, and monitor the process is critical to timely progress and reliable production.

Summary and Conclusions

RNAs are a relatively new class of therapeutic molecules that have made huge advancements in recent decades and offer hope to reach what were previously considered undruggable targets. And, offering rapid and cost-effective development, this space is poised to continue growing in the coming years. However, RNAs have a unique set of challenges that must be considered and ultimately solved if their full potential is to be realized. The minimization of host immune reactions, maximization of RNA delivery and subsequent uptake into target cells, and optimization of RNA pharmacokinetics all must be considered to improve the chance of clinical success.

As with other therapeutic classes of compounds and biological agents, successful development of RNA therapeutics is heavily dependent on a robust analytical portfolio. These analytics can be employed to answer some of the difficult questions around optimal therapeutic RNA composition, such as the appropriate number of backbone modifications, poly(A) tail length and design, and absence of host immune system activators. Deploying several genomic and proteomic assays can increase confidence during discovery and clinical translation of the therapeutic.

RT-PCR, especially ddPCR technology, has emerged as an invaluable analytical asset for therapeutic RNA development and production. Droplet Digital PCR, with its inherent benefit of absolute quantification and high sensitivity and specificity, even when template copy numbers are low, has proved valuable from the early discovery stage through quality control–based assays of the final manufactured product (Figure 3). Application of well-chosen assays, including ddPCR technology–based assays, will support the development and production of safe and effective RNA-based therapeutics.

This new class of therapeutics stands poised to redefine the term druggable and provide cost-effective, rapidly adaptable solutions to improve the quality of life for millions.

Bio-Rad offers comprehensive products and services, including ddPCR technology solutions, to support the development and production of RNA therapeutics.

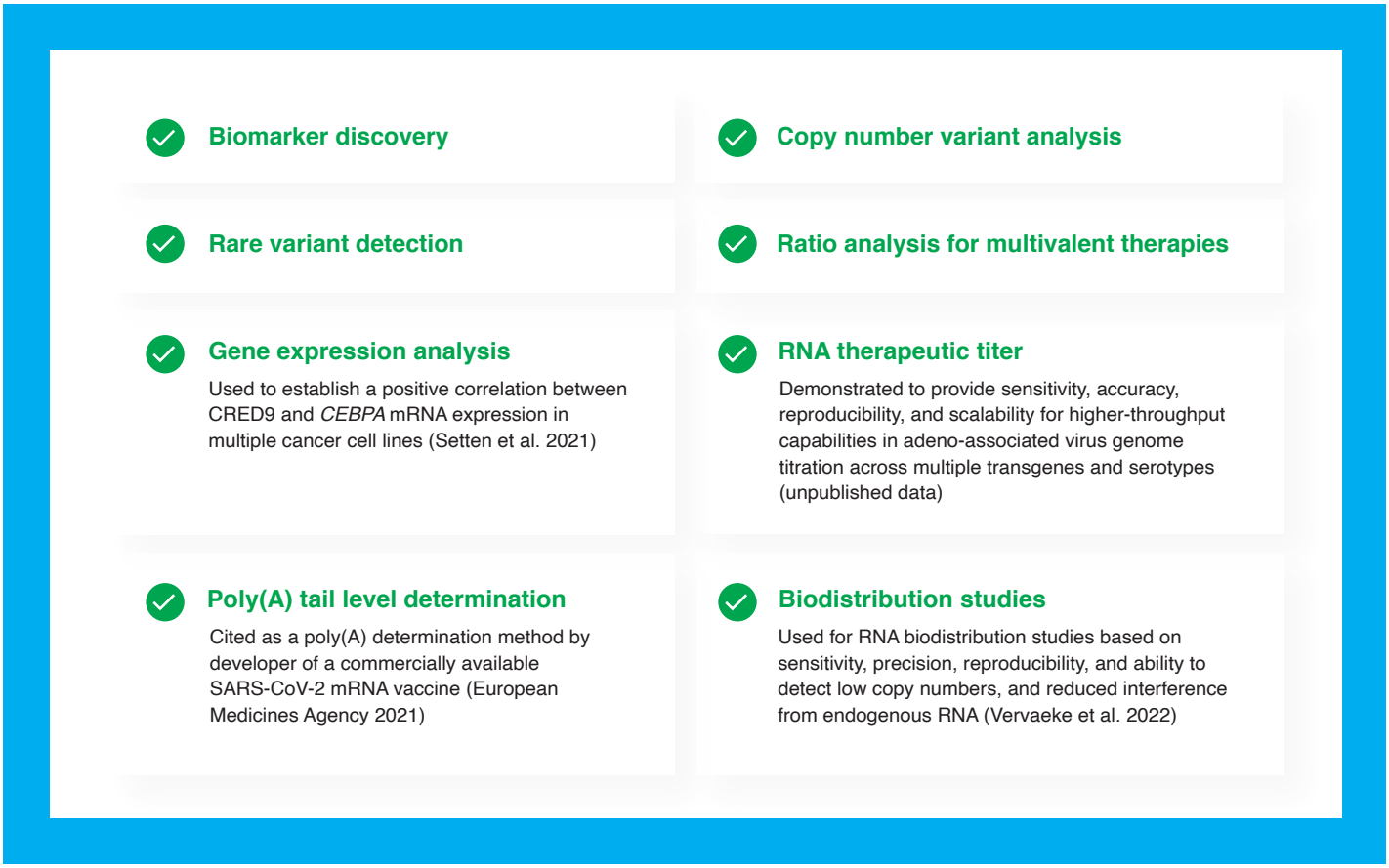

Fig. 3. Droplet Digital PCR supports the development, testing, and production of RNA therapeutics in manifold ways.

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