Biochemistry

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Fire Burn and Cauldron Bubble: What Is in Your Genome Editing Brew?

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Cite This: https://doi.org/10.1021/acs.biochem.2c00431 Read Online

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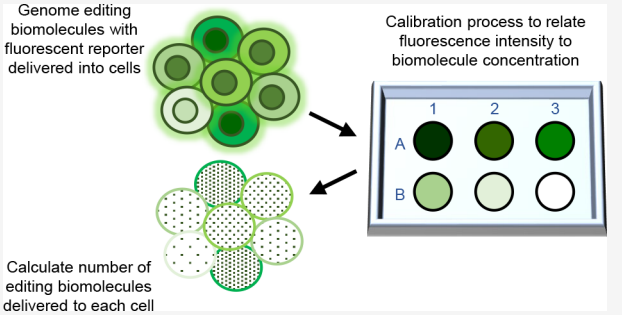
ABSTRACT: Genome editing is a rapidly evolving biotechnology with the potential to transform many sectors of industry such as agriculture, biomanufacturing, and medicine. This technology is enabled by an ever-growing portfolio of biomolecular reagents that span the central dogma, from DNA to RNA to protein. In this paper, we draw from our unique perspective as the National Metrology Institute of the United States to bring attention to the importance of understanding and reporting genome editing formulations accurately and promoting concepts to verify successful delivery into cells. Achieving the correct understanding may be hindered by the way units, quantities, and stoichiometries are reported in the field. We highlight the variability in how editing formulations are reported in the literature and examine how a reference molecule could be used to verify the delivery of a reagent into cells. We provide recommendations on how more accurate reporting of editing formulations and more careful verification of the steps in an editing experiment can help set baseline expectations of reagent performance, toward the aim of enabling genome editing studies to be more reproducible. We conclude with a future outlook on technologies that can further our control and enable our understanding of genome editing outcomes at the single-cell level.



Genome editing is a class of techniques involving nucleic acid damage, repair mechanisms, replication, and/or recombination that introduce site-specific modifications into genomic DNA.[1] The biomolecules used for genome editing are predominantly proteins and nucleic acids that range in their degree of "programmability", the control with which specific nucleotide sequences can be targeted. Current genome editing tools include viral vectors, zinc finger nucleases (ZFNs), meganucleases, transcription activator-like effector nucleases (TALENs), megaTALs, clustered regularly interspaced short palindromic repeat (CRISPR) systems, controlled transposable elements, and peptide nucleic acids (PNAs)[2−5] (Figure 1).

In this Perspective, we focus on the widely used genome editing tool CRISPR/Cas9, originally discovered in bacteria as an adaptive immunity mechanism against viruses. CRISPR/Cas9 was discovered to have the ability to cleave double-stranded DNA in a programmable manner[6] and shortly after was repositioned to have the ability to edit the human genome.[7−9] While our examples focus on the CRISPR/Cas9 system, the concepts we raise around clearly understanding editing biomolecules—stoichiometry, quantities used, reporting, and reagent delivery verification—apply broadly to other genome editing systems as a valuable means of improving the degree of confidence in data that are generated and reported in the genome editing field.

CRISPR/Cas9 is a ribonucleoprotein (RNP) enzyme that consists of a Cas9 nuclease complexed with a guide RNA (gRNA) that facilitates the interaction between the Cas9 and a target nucleotide sequence (Figure 1A). The gRNA was identified in bacteria as a two-part system comprised of a "CRISPR RNA" (crRNA) duplexed with a "trans-activating CRISPR RNA" (tracrRNA) (see the history and biology of CRISPR reviewed in refs 10 and 11). It is possible to chemically synthesize gRNA as a dual-guide system, in which crRNA and tracrRNA are synthesized separately and hybridized together, or as a single-guide system, in which a single polyribonucleotide molecule contains the key components of both the crRNA and tracrRNA sequences.[6−8,12] Commercially synthesized gRNA often includes chemical modifications to the phosphodiester bonds, sugar moieties, or bases, to increase its stability[13−16] and mitigate inflammatory signaling pathways that cause cellular toxicity.[17,18] In the context of genomic DNA in a live cell, Cas9 nuclease activity induces a double-stranded break in DNA,

Special Issue: Gene Editing

Received: July 19, 2022
Revised: September 28, 2022

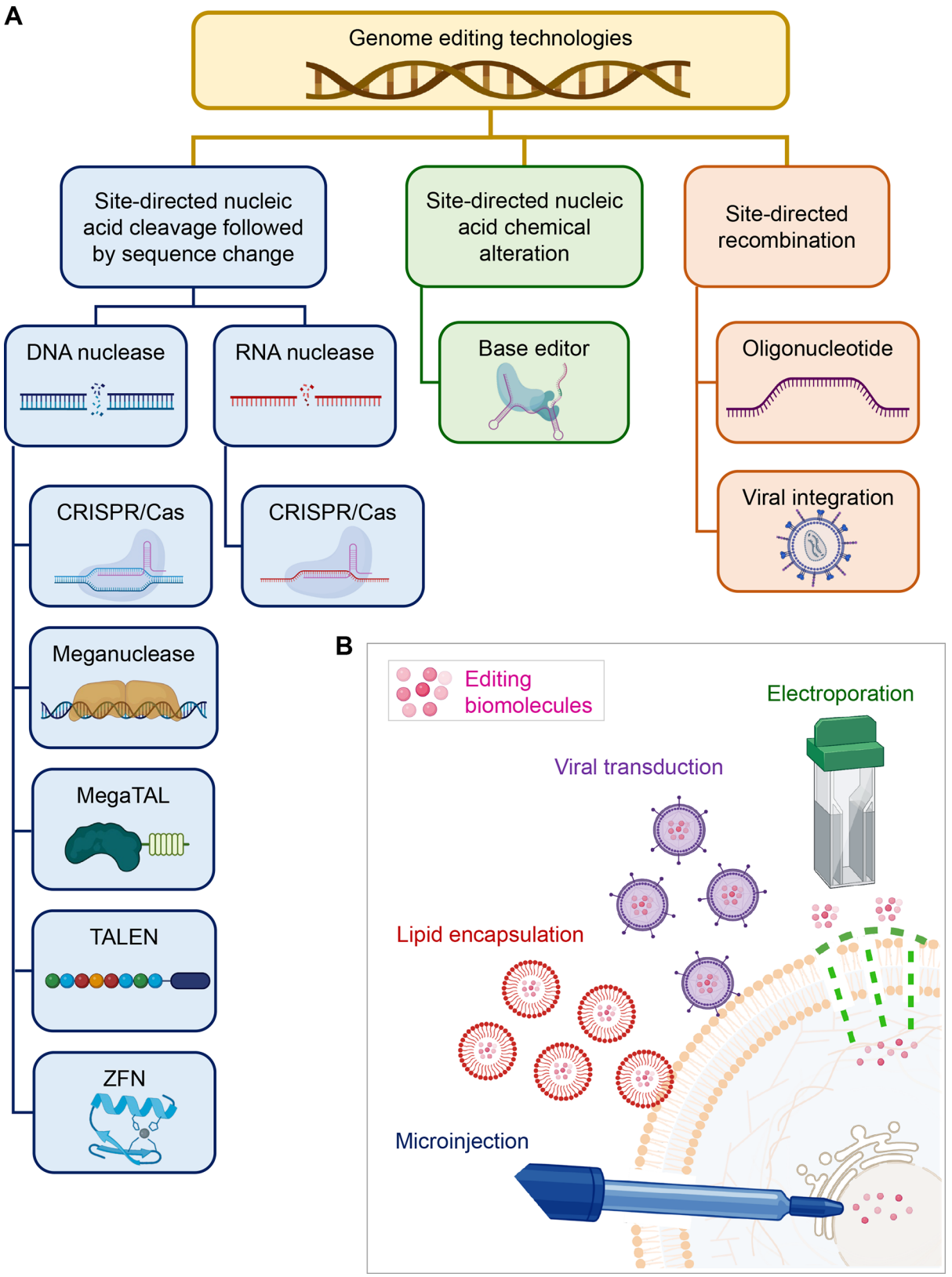


Figure 1. Genome editing tools and delivery methods. (A) Classes of genome editing tools currently used in laboratory studies. Schematic adapted from ref 1. (B) Delivery methods for introducing CRISPR/Cas9 and other genome editing biomolecules into cells.

which may then be repaired by the DNA damage response mechanisms of a cell. Through experimental design, a researcher can attempt to bias DNA repair through the cellular processes of non-homologous end joining (NHEJ) or homology-directed repair (HDR) to create an intended sequence change (see ref 19 for a review of repair pathways).

CRISPR/Cas9 can be delivered to cells through physical and chemical methods, such as microinjection, electroporation, and

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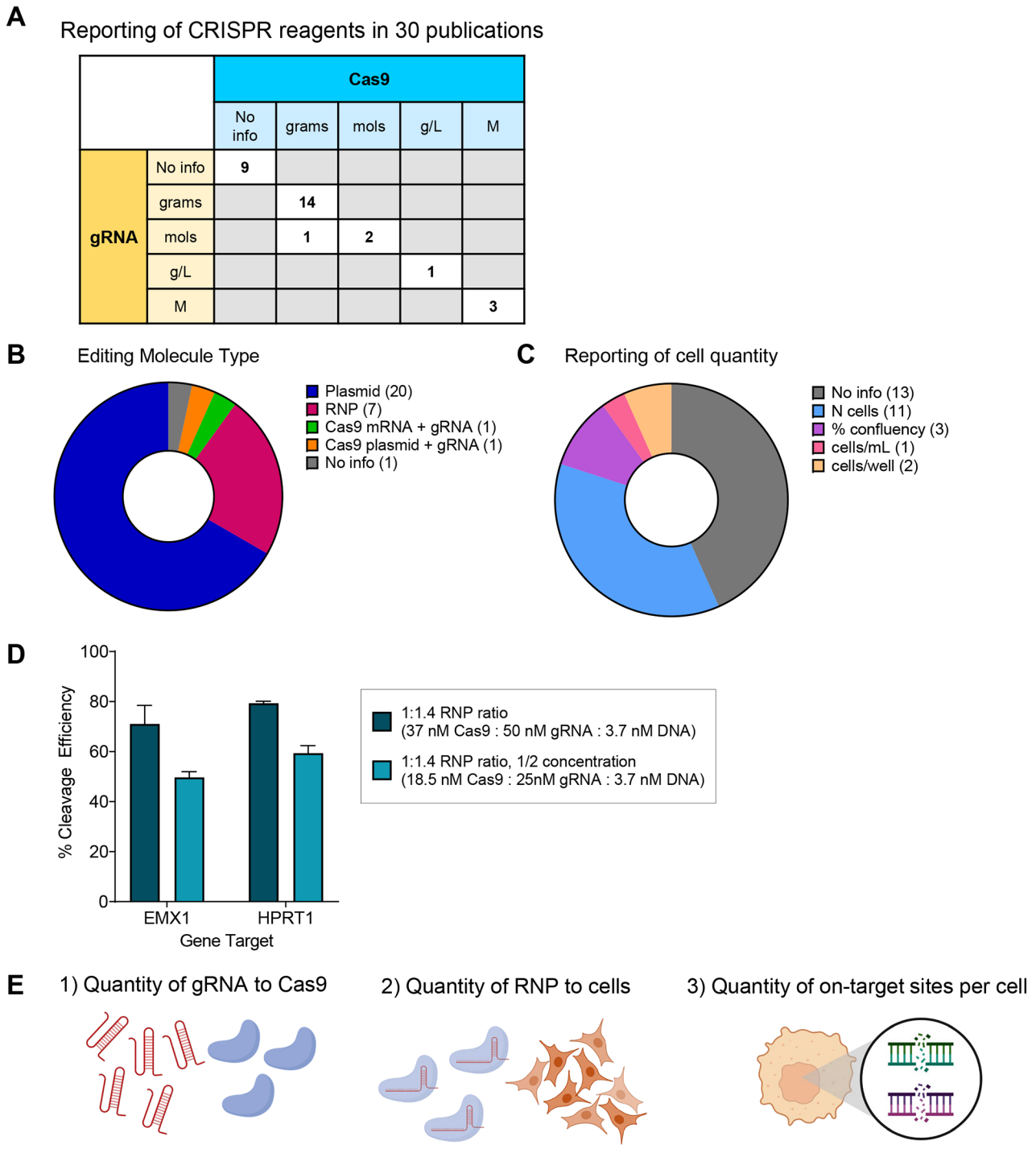

Figure 2. Genome editing tools and delivery methods. Inputs in the editing process. (A) Analysis results of 30 publications using CRISPR/Cas9 to edit HEK293 or HEK293T cells. Units reported in these publications are presented for gRNA (rows) and Cas9 (columns), with the number of publications that reported each combination shown in the matrix. (B) Pie chart of the number of publications in the analysis that used a given type of editing biomolecule format. (C) Pie chart of the number of publications that reported a given type of units for the cells used in the editing reaction, with N defined as the number or quantity of the cell population. (D) IVC assay using published commercial protocols[52] showing the percent cleavage efficiency of two human gene targets *EMX1* and *HPRT1* with the indicated concentrations of the Cas9, gRNA, and template DNA. Error bars are means ± the standard deviation of two replicate reactions. (E) Key input quantities necessary for accurately reporting on and being able to reproduce an RNP editing reaction.

lipid encapsulation (Figure 1B). For more information about delivery mechanisms, including viral methods, which will not be discussed in this Perspective, we refer readers to a recent review.[20] CRISPR/Cas9 can be delivered in a variety of formats. The three most common are (1) a DNA plasmid(s) encoding Cas9 and gRNA, (2) Cas9 mRNA co-delivered with gRNA, and (3) RNP comprised of Cas9 protein complexed with gRNA prior to cellular delivery. In the following sections, we call attention to two biochemical aspects of CRISPR/Cas9: the quantities of inputs in the editing reaction and the fluorescent

https://doi.org/10.1021/acs.biochem.2c00431
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Table 1. Calculation of Plasmid Copy Numbers in 1 μg across Various Published Cas9 Plasmids

	1	2	3	4	5	6	7	8
plasmid name	eSpCas9(1.1)	xCas9(3.7)	pSpCas9(BB)-2A-GFP (PX458)	SpCas9-HF1	Sniper-Cas9	lentiCRISPR v2	LentiCRISPR v2 GFP	lentiCRISPR v2 hygro
source	Addgene #71814	Addgene #108379	Addgene #48138	Addgene #138556	Addgene #138559	Addgene #52961	Addgene #82416	Addgene #98291
nucleotide length of plasmid map	8505	9553	9229	12838	12838	14873	13131	15261
MW[a] of plasmid (g/mol)	5.26×10^6	5.90×10^6	5.70×10^6	7.93×10^6	7.93×10^6	9.19×10^6	8.11×10^6	9.43 × 106
moles of plasmid[b] in 1 μg	1.90×10^{-13}	1.69×10^{-13}	1.75×10^{-13}	1.26×10^{-13}	1.26×10^{-13}	1.09×10^{-13}	1.23×10^{-13}	1.06×10^{-13}
plasmid copy number[c] in 1 μg	1.15×10^{11}	1.02×10^{11}	1.06×10^{11}	7.59×10^{10}	7.59×10^{10}	6.55×10^{10}	7.42×10^{10}	6.39×10^{10}
x-fold change from spCas9 plasmid 1	1.00	0.89	0.92	0.66	0.66	0.57	0.65	0.56

[a]MW based on a 618 g/mol average molar mass of double-stranded DNA for fully protonated polymeric bases.[53] [b]Moles of plasmid calculated by 1 μg divided by plasmid MW. [c]Plasmid copy number, or molecules of plasmid, calculated with Avogadro's number (6.022×10^{23}).

biomolecules that could be used as a reference to confirm successful delivery into cells. We look at pitfalls in failing to document the editing formulation completely and how the choice of a reporter molecule can yield different interpretations about how successfully the editing reagent was delivered into cells. Our aim with this Perspective is to leave readers with insight and methods to facilitate more informed decisions on reporting editing formulations and verifying reagent delivery. Doing so will result in a more accurate understanding of how an experiment was designed, greater confidence in reported outcomes, and improved reproducibility of genome editing protocols.

KNOWLEDGE AND REPORTING OF INPUTS IN THE EDITING PROCESS

Genome editing with CRISPR/Cas9 is dependent on several factors, such as the interaction affinities between the Cas9 and gRNA biomolecules that assemble to form the RNP complex, the interactions between the RNP and native cellular proteins, and the interactions between the RNP and chromatin. In the cell nucleus, CRISPR/Cas9 searches for and binds chromatin for dwell times that are influenced by both the degree of homology between the gRNA and DNA nucleotide sequences[21] and chromatin accessibility. While the importance of biomolecule quantities and concentrations for genome editing may be understood, they are not often clearly reported in the methods published in the literature. When this information is reported, the units may be listed in different ways. A brief survey of leading vendors that list commercially available Cas9 and gRNA reagents shows that there is variation in how the units are reported. Different vendors report different units for these products ranging from grams to moles to molar concentrations.

We investigated how CRIPSR/Cas9 formulations are reported in the literature with an analysis of 30 publications (Figure 2A) that used CRIPSR/Cas9 for genome editing (Cas9 and variants such as Cas9 nickase and base editors) and genome engineering (dCas9, a "dead" catalytically inactive Cas9 variant that binds but does not cleave DNA).[22−51] We limited our search to recent papers published from 2019 to 2021 for which the full text was available through PubMed Central and that involved the widely used experimental human cell lines HEK293 and/or HEK293T in some aspect of the study. These publications were found through PubMed searches for "(HEK293) AND (Cas9)" and "(HEK293) AND (Cas9) AND (RNP)".

This literature analysis included editing applications that were either in vitro, where editing biomolecules were applied to cells or tissue in culture, or in vivo, where it was applied to the zygotes of mouse and zebrafish model organisms. The studies used various editing formulations [plasmid, RNA, plasmid and RNA, and RNP (Figure 2B)] and various delivery methods (lipid encapsulation, viral transduction, electroporation, and microinjection). In cases in which a DNA plasmid encoded Cas9 and gRNA on the same vector, we reported the units reported for the plasmid as the units for both Cas9 and gRNA (i.e., "5 μg of plasmid" is noted as 5 μg of Cas9 and 5 μg of gRNA). We arranged the results in a matrix in which the intersection of each row and column lists the total number of publications that reported a given unit of gRNA and a given unit of Cas9 (Figure 2A).

Our analysis shows that the units of Cas9 and gRNA editing inputs are reported in various ways: mass (grams), moles, mass concentration (grams per liter), and concentration (molar). The gaps were concerning, as we found nine of the 30 publications provided no units for either Cas9 or gRNA quantities used. We also examined whether the publications reported any information about the units of cells (cells in culture or animal embryos) used for the editing reaction. This included cells that were used directly for genome editing or cells that were used indirectly as harvest cells for generating viral vectors from plasmids that encapsulated the genome editing reagents. We found that 11 of the 30 publications reported a discrete cell quantity used in the editing process (Figure 2C), and three of the publications reported on cell quantity as a concentration (cells per milliliter or cells per well). Three publications reported on cells as a percent confluency. Again, concerningly, 13 of the 30 publications did not report any information about the units of cells used in the genome editing process. Approximately half of the publications, 16 of 30, provided units for all three inputs of Cas9, gRNA, and cells. Eight publications provided no information at all about any of the three inputs.

This analysis presents a major concern for CRISPR/Cas9 editing studies and the genome editing field overall: How is one to have a complete understanding of the editing reaction, or attempt to reproduce a formulation reported in the literature, if

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the quantities of editing biomolecules and cells involved are not explicitly reported?

Incomplete reporting of the input quantities and concentrations used in an editing reaction is likely not intentional. We want readers to reflect on how factors external to the actual experiment, such as a measurement device, may influence the way the units of a reagent are reported in a protocol. Take for example a DNA spectrophotometer, which reports on DNA concentration in units of grams per liter (often nanograms per microliter or micrograms per microliter). Given that concentration by mass is how the DNA plasmid stocks would be recorded and annotated in the lab, it is seemingly natural (and sensible) to report on plasmid units for an experiment in terms of mass, i.e., that "1 μg of plasmid" was used in an editing reaction. The main issue here is that plasmids come in different "sizes", or more specifically, different nucleotide lengths. This means that a given mass of plasmid solution will contain different numbers of plasmid molecules (and hence different numbers of Cas9 and/or gRNA gene copies) depending on its nucleotide length. We examine this issue with examples of Cas9 plasmids that were used in the publications from our literature analysis (Table 1). We encourage researchers to report sufficient information about their editing biomolecule reagents so that subsequent readers of the published methods can find the necessary details and make the relevant calculations for their use case.

A second example of incomplete reporting of editing reagent quantities is the frequent use of ratios as a way to report on the Cas9 and gRNA amounts used in an editing reaction. In our analysis, we found RNP formulation listed a stoichiometry value for the Cas9:gRNA ratio, such as "1:2". This importantly informs on the relative amount of the molecules used to form RNP complexes, but stoichiometry alone without further concentration information is not sufficient to understand the amount of Cas9 and gRNA used. Numerical values and units of Cas9 and gRNA (such as molar quantities) and information about the amount of cells used for editing are needed to understand the following: At what scale is the RNP being presented to the cells? How many editing molecules are being presented to what quantity of cells, and to what quantity of available copies of target DNA?

An in vitro cleavage (IVC) assay[54] can illustrate the pitfalls of a heavy reliance of Cas9:gRNA ratios to report an editing formulation. In an IVC assay, a solution of assembled RNP is incubated with a purified DNA template containing the target sequence to be recognized and cleaved by the RNP. The resulting fragments can be used to assess cleavage efficiency or the fraction of DNA total template that was cleaved by the RNP by the end of a defined incubation time period. Following a commercial IVC protocol,[52] it is clear how the same RNP Cas9:gRNA ratio of 1:1.4 has different results in cleavage efficiency when the RNP concentration relative to the DNA template is halved (Figure 2D). While IVC results are not a prediction of RNP cleavage efficiency in a cellular environment, this assay can provide a baseline expectation of RNP "quality" via cleavage functionality and if cleavage functionality is altered under various conditions or with variation to the RNP. Explicit reporting of the quantities of Cas9, gRNA, and cells used in a genome editing experiment is critical for having a complete understanding of the editing reaction and facilitating the reproduction of the protocol and formulation (Figure 2E).

FLUORESCENT BIOMOLECULES TO VERIFY DELIVERY

Genome editing in mammalian cells requires functional editing biomolecules to be present in the cell nucleus. If we consider the process for genome editing with CRISPR/Cas9 more carefully, then we understand that successful intended editing has achieved the following. (1) The editing biomolecules of sufficient quality were introduced into the cell. (2) Irrespective of the format in which they were delivered, the final form of Cas9 protein and gRNA is localized in the cell nucleus. (3) The editing biomolecules were functional in the cellular environment, such that (4) double-stranded breaks were induced at the intended DNA target site that was subsequently repaired by the cell and (5) at a level detectable by the researcher. If editing was unsuccessful, these concepts separating biomolecule delivery and localization from the outcome of detectable editing can now serve as quality control steps toward understanding what led to the absence of detectable editing. We encourage our readers to consider the concept "Were the editing biomolecules delivered?" separately from "Are the biomolecules functional at their intended location?" and "Was there detectable editing?" This allows a researcher to examine whether the editing biomolecules actually entered the cell (and cell nucleus) and/or to try to determine whether the editing molecules were functional in the cellular environment.

We note here that depending on the delivery method used, certain cellular processes may confound proper localization and functionality of the biomolecules. For instance, lipid and viral encapsulation methods require intracellular uptake through the endosomal pathway. This requires the biomolecules to successfully undergo "endosomal escape": the editing biomolecules must leave the endosomal compartment before biochemical alterations (including a decrease in pH) cause the vesicles to undergo a transition into lysosomes, risking an impact to the functionality of the biomolecules, and their eventual degradation. We direct readers to a recent review[55] that examines the challenges of endosomal escape for delivering RNA therapeutics with lipid encapsulation. Another example of incorrect localization is if a researcher mistakenly tries to deliver Cas9 into eukaryotic cells that does not contain a nuclear localization signal (NLS). On its own, the bacterially derived Cas9 protein is not capable of traversing a nuclear membrane and therefore needs to be fused to an NLS peptide so it can interact with the necessary nuclear chaperone proteins. For a given editing application, it is helpful to conceptualize and separately evaluate the steps of delivery, localization, and functionality of the editing biomolecules. Employing a stepwise verification strategy with this framework can help identify key sources of variability and elucidate where in the process challenges are occurring toward observing detectable editing.

One way to evaluate the successful delivery of editing biomolecules into cells is to use a reporter biomolecule coupled with a visual detection system such as a microscope, which allows the researcher to visually inspect whether the editing biomolecules entered cells. We describe here different types of reporters that may be used in gene editing experiments and possible methods that could be used early in the experiment design process to test biomolecule delivery and functionality.

In some cases, a reporter for delivery can be introduced into the genome editing formulation as a separate molecule from the biomolecules used for genome editing. Some examples are the small molecules lucifer yellow (LY) (Figure 3A) and fluorescein

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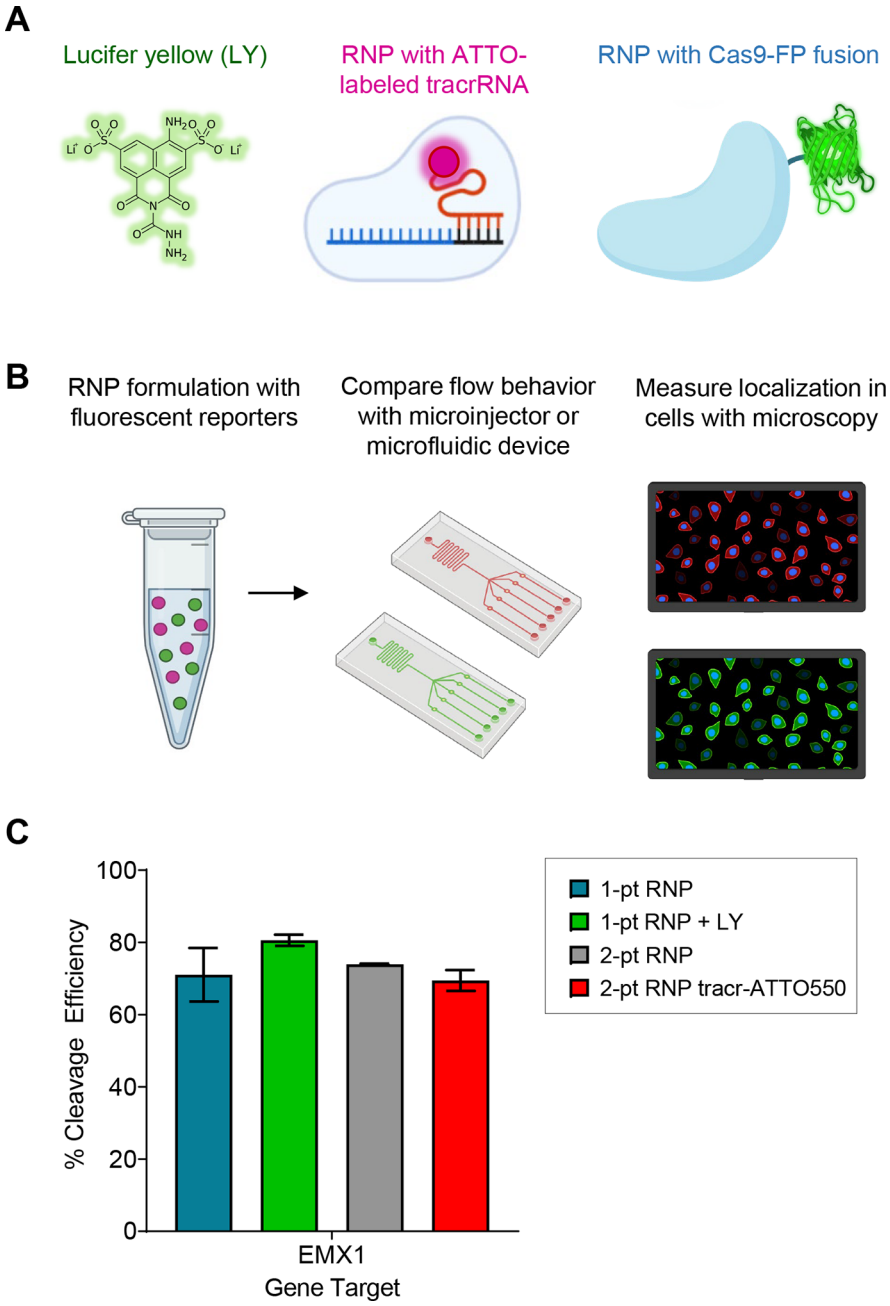

Figure 3. Verifying reagent delivery. (A) Example of three types of fluorescent reporters used to confirm delivery of the solution into cells: lucifer yellow (LY), tracrRNA conjugated to an ATTO fluorophore, and Cas9 fused to a fluorescent protein (FP) such as GFP or mCherry. (B) Possible evaluation methods by which fluorescent reporters for RNP delivery could be measured. (C) IVC assay with RNP formulated in a 1:1.4 Cas9:gRNA ratio, 366 nM Cas9, 500 nM two-part gRNA with tracrRNA-ATTO550, and 4.52 mM lucifer yellow (LY), testing the impact of fluorescent tracers on the cleavage efficiency of the DNA template EMX1.[78] Error bars are means ± the standard deviation of two replicate reactions.

isothiocyanate (FITC) injected into mammalian adherent cells[56−58] and phenol red used in zebrafish embryo injections.[59] A second type of delivery verification can be a "positive control" of a similarly sized reporter molecule applied to a parallel plate (i.e., GFP plasmid, GFP mRNA, or GFP protein). A third type of reporting is to co-express a fluorescent reporter from the same vector as the Cas9 and/or gRNA sequence.[12,60] A fourth type of reporting is to have a fluorescent reporter conjugated to the RNP itself. One could label the Cas9, as in Cas9-GFP or Cas9-mCherry,[61] delivered in the format of either purified protein, mRNA, or DNA plasmid.[7,12] One could also label the gRNA of the RNP complex, by having the tracrRNA of a dual-guide gRNA system conjugated to a fluorophore such as ATTO550 or ATTO647 (Figure 3A), each of which is a commercially available reagent.

Before choosing a reporter as a verification step for the successful delivery of a reagent into cells, we invite the reader to ask some key questions. First, is the reporter molecule of a representative size with respect to the editing biomolecules being used? Diffusion and flow rates of biomolecules are partly dependent on their size, as well as charge. If the reporter and editing biomolecules have differences in these aspects, one could be misled in terms of the amount of editing reagent that was actually presented to and delivered into the cell. Some possible

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ways to determine whether various reporters show differences in flow or intracellular localization behavior would be to have a single solution with the candidate reporters as in Figure 3B, where an external reporter such as LY is in the same solution as a fusion reporter, such as tracrRNA-ATTO550 that is part of the RNP complex. One could test whether these two reporter molecules exhibit differences in each other with respect to their flow behavior or degree of localization in cells. One could use a method such as microinjection into a free solution or a transfection system with cells.

Any measured differences between the candidate reporters with respect to flow or intracellular localization behavior can help with making a more informed decision about which reporter may be the best choice for the intended use case for verifying the successful delivery of the editing formulation. Not all reporters are alike: different choices can lead to different interpretations of the quantity of editing biomolecule that was presented to and ultimately delivered into the cell. We note here a recent publication in which differences in delivery efficiency were revealed with tracrRNA-ATTO550, showing that RNP was unable to enter the cell nucleus effectively depending on the delivery instrument used.[62]

A second key question to ask about a reporter molecule is whether it is complexed with the RNP (as with Cas9-GFP fusions or ATTO-labeled tracrRNA) or separate from it (as with LY or GFP plasmid, mRNA or protein). If the reporter is separate from the RNP, is it co-delivered with the RNP into the same target cells or delivered into a parallel "positive control" sample of cells?

While labeling the RNP itself with a reporter is perhaps the most direct way of measuring RNP delivery, there could be a possible impact of the reporter interfering with the biological function of the RNP, such as the ability of the RNP components to form a complex effectively, or the ability of the RNP to interact with a DNA target sequence and cleave it.

We found an instance in which Cas9-GFP was reported not to have a difference compared to unlabeled RNP[63] and an in vitro study examining the interactions of various fluorescent reporters with lipid bilayers assembled from egg phosphatidylcholines.[64] This latter study concluded that a fluorophore could give the mistaken interpretation that it was labeling a membrane-bound protein, when it was in fact the fluorophore itself and not the protein that was interacting with the lipid bilayer. We were unable to find a publication that built on this type of evaluation to study the impact of reporter molecules on RNP functionality in cells. However, the IVC assay can serve as a useful in vitro preverification step. Using the example RNP formulation from Figure 2D, one could test whether a candidate tracer affects the cleavage efficiency of the DNA target in an in vitro context. Comparing RNP with both single- and dual-guide systems, either unlabeled, containing LY, or tracrRNA-ATTO550, one could test whether there are effects of either reporter on the cleavage efficiency of a DNA template in vitro that would give information for understanding if a reporter is impacting RNP activity (Figure 3C) and deciding on a delivery control strategy appropriate for the given application.

To conclude, a reporter molecule is useful to confirm successful delivery of editing cargo, but there is no one size fits all solution in terms of the best reporter a researcher should choose.

Fluorescent biomolecules are presented as one effective approach that utilizes detection instrumentation commonly available in research laboratories. Each form of reporter has its advantages and possible disadvantages that need to be considered and tested to determine if there are impacts on interpretation of delivery, RNP cleavage functionality, and editing efficiency. Additional concepts, strategies, and technologies may be needed to have confidence in editing biomolecule delivery for in vivo editing of a multicellular organism, where the intention is for molecules to reach the physical location of and be delivered into the intended cell(s) and/or tissue(s).

FUTURE DIRECTIONS: SINGLE-CELL MANIPULATION

Genome editing technologies have already allowed for great strides in biological research. Bulk level editing manipulations and measurements—i.e., genome editing formulations applied to a cell population, followed by population level genomic analyses—are state-of-the-art editing approaches for obtaining fundamental insight into the biosciences. Bulk editing methods provide a path for developing human therapeutics and other products requiring large-scale production of edited cells. Given the value of the already existing bulk methods, and the value in understanding population level behavior, one may ask, "What is the value of scaling genome editing efforts to the single cell?" We argue that while single-cell manipulations and measurements are not relevant for every use case, the knowledge to be gained from working at single-cell resolution is of scientific value. Single-cell technological capabilities are worth pursuing as additional tools for the field of genome editing.

Understanding the information from single-cell measurements is undoubtedly challenging; there is prevalent literature to date showing the processes of genome organization and gene expression are dynamic and stochastic (recently reviewed in refs 65 and 66). There is heterogeneity across single cells of a population and within a single cell over time. These factors make single-cell studies of genome editing outcomes more difficult to understand than the population averaged outcomes. However, when focused on the population average, one may fail to see the population distribution, in which deviations from the mean are due to subpopulations of cells exhibiting different biology. Importantly with single-cell genomic measurements, one can detect allelic frequencies and the co-occurrence of variants within a single cell. For example, detecting 50% editing at a single genomic locus in a bulk measurement does not indicate how many cells were actually edited. At the single-cell level, a bulk measurement of 50% edited alleles could be distributed in a number of ways. At one end, 100% of the cells could be heterozygous, containing one edited and one unedited allele. At the other end, 50% of the cells could be homozygous for the edited alleles, meaning that 50% of the cells are homozygous unedited.

The actual allelic distribution that results from a genome editing experiment likely lies somewhere between these two extremes. If the goal is to determine which sequences co-occur within a single cell, then one cannot use bulk genomic measurements alone; single-cell genomic measurements must be used to report which of the series of alleles detected in bulk are co-occurring in the same cell. A single-cell level resolution of the genome may substantially alter the interpretation of (1) how many total cells were edited and (2) whether the edited cells have sequences that make them fit for purpose. Depending on the application, relatively few edited cells may be able to produce sufficient product or phenotype to be useful for a given application. Conversely, relatively few edited cells with specific

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features may be undesired or make the cells unsuitable for an application. The heterogeneity of cell populations is an active area of study in cancer, where mutational subgroups may shift and gain dominance during a therapeutic course of treatment. We argue that the heterogeneity in a cell population also needs to be examined with CRISPR/Cas9 genome editing.

As we mentioned above, cells will be subjected to CRISPR/Cas9-induced DNA damage in different locations (off-target activity) and will repair that damage in different ways, and with different sequence changes. To better understand how cellular systems respond to genome editing biomolecules, to evaluate the on-target potency of editing nucleases, and to develop more fit for purpose approaches to genome editing, one needs precise control over the dose of the reagents delivered into single cells. We are not advising that the genome editing field as a whole should move from bulk to solely single-cell manipulation and measurements, nor are we advising that every research question requires single-cell manipulation. For instances in which editing needs to be high throughput for the number of cells and/or various editing conditions or formulations, currently available technology for single-cell manipulation would not be sufficient. However, for those research applications in which it could be advantageous or informative to have control over dose delivery or high-resolution data on the distribution of genomic and phenotypic outcomes with relatively few cells, we ask those readers to consider the following ideas for future needs and technological advancements that would allow precision manipulation to be coupled with precision measurement:

1. Deliver "treatment" and "control" conditions side by side to cells in the same environment (Figure 4A).
   - Technology requirement: The ability to identify, index, and sort cells, so that each condition delivered to cells is distinguishable and can be observed or assayed separately.
   - Possible innovations on existing technologies: CRISPR/Cas9 optogenetic variants (recently reviewed in refs 67 and 68) coupled with photostimulation instrumentation that delivers a controlled illumination pattern to part of a field of view;[69,70] direct microinjection.[56,71]
2. Reproducible controlled delivery of a solution to a single cell, and the capability to shift the dose delivered in controlled increments (Figure 4B).
   - Technology requirement: Ability to control and produce parameters reproducibly (i.e., for electroporation, N pulses, voltage, pulse duration; for injection, dimensions of microinjection needle, aperture area, pressure, and duration).
   - Possible innovations in existing technologies: Microinjection with pressure and duration control to deliver editing reagents, electroporation coupled with single-cell electroporation in microchannels,[72] nanostraws,[73−75] electrowetting,[76] injections using microfluidically formed droplets,[77] and injections with a cantilever connected to a microfluidic reservoir.[56,71]
3. Measure the volume of the editing solution and the number of editing molecules delivered into a single cell (Figure 4C).
   - Technology requirement: The ability to quantify the volume of a solution and the quantity of biomolecules delivered to a cell. If using a visual readout such as fluorescent reporters, this may require a calibration standard to convert a measured fluorescence intensity into known quantities of volumes and editing biomolecules delivered to the cell.
   - Possible innovations of existing technologies: Microfluidic flow measurement analysis, calibration slide with photostable fluorescent material,[78] microspheres with assigned equivalent reference fluorophore units (ERFs),[79,80] and adoption of ASTM F3294-18 that provides a guide of standards for using microscopy-based methods to make quantitative fluorescence intensity measurements in cell-based assays.[81]

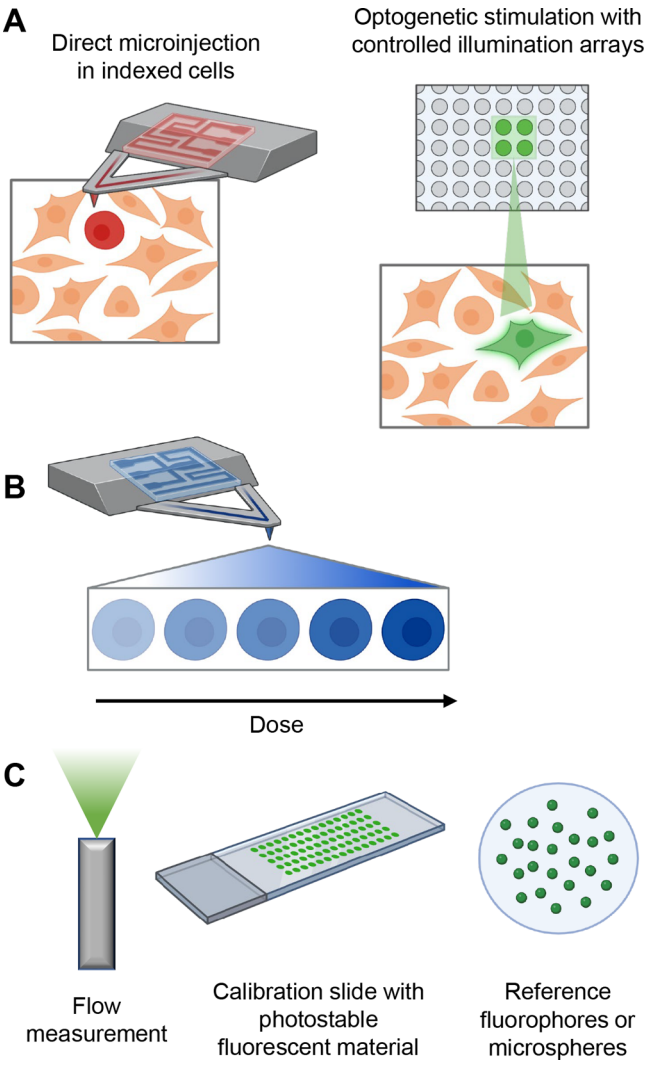

Figure 4. Single-cell manipulation and measurement. (A) A conceptual model of possible future advancement in genome editing experiments is to target specific cells in a population for delivery of the treatment condition and culture them alongside cells with the control condition, in the same environment. This could be done with direct microinjection or photostimulation with controlled arrays to direct the excitation wavelength to a specific area in a field of view. (B) Controlled delivery of a given quantity of reagent, and the ability to increment it intentionally, will allow for highly controlled dose response experiments. (C) Possible tools for relating the fluorescence intensity of a reporter to the volume of solution or number of editing molecules delivered to a cell.

https://doi.org/10.1021/acs.biochem.2c00431
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Table 2. Recommendations for improved reporting of editing formulations

concept	recommendation
editing formulation	• report the formulation type (plasmid, RNA, RNP, etc.)
	• report any co-introduced reagents (HDR donor or fluorescent tracer)
reagent source	• report the source for each reagent used
	• from a donating lab: include citation to previous work where available
	• commercially purchased: vendor and item number
	• formulated in lab: details on how reagent was generated
numerical values and units	• reporting molar amounts is recommended; this could be reported as the starting molarity and the volume used, or the final molarity in the editing formulation
	• if reporting relative ratios (stoichiometry) of editing biomolecules
	• report the molecule identity to which the ratio corresponds, for example, 1:1.4 Cas9:gRNA
	• report numerical value and units of mass, concentration, and/or molarity for at least one component in the formulation so that the other component values can be calculated (see Figure 2D for an example)
	• if a plasmid is used, sufficient information should be provided to calculate the number of plasmid molecules in the formulation. This could be reported as follows
	• molar amount of plasmid, for example, 100 nM plasmid
	• mass amount of plasmid with nucleotide length, for example, 1 μg of plasmid, 8505 bp
	• mass amount of plasmid with molecular weight (MW), for example, 1 μg of plasmid, 5.26×10^6 g/mol
	• mass amount of plasmid with sequence or reference by which a reader can calculate the nucleotide length or MW of the plasmid construct, for example, 1 μg of plasmid, Addgene #71814
cell quantity	• reporting the number of cells that were treated with editing formulation is recommended
	• if reporting cell quantity in other units, sufficient information should be provided to obtain the cell number. This could be reported as follows
	• cell concentration and volume, for example, 200 μL of 1×10^6 cells/mL
	• cells per well and plate dimensions, for example, 1×10^5 cells/well in a six-well format (9.6 cm^2)
	• percent confluency and plate dimensions, for example, 70% confluency in a six-well format (9.6 cm^2)
delivery method	• report the delivery system used (lipid encapsulation, microinjection, electroporation, etc.)
	• report the source/vendor, instrument information, and the delivery parameters (include values and units)
assessments	• separate the concepts of delivery, localization, and editing when designing, executing, and interpreting experimental results
	• report the values and calculation used when describing a measure of performance (percent editing, delivery efficiency, etc.)
	• report on the time point(s) at which measurements were made

There is broader significance to the biochemistry measurement concepts discussed in this paper. Reproducibility and confidence in measurements within the genome editing field need to be accelerated in three major avenues: (1) developing relevant control materials, (2) standardizing processes to document and share experimental protocols and accompanying data, and (3) converging upon unified language for genome editing terminology and definitions. Toward this end, the NIST Genome Editing Program (which contains the public−private partnership NIST Genome Editing Consortium) is currently leading international efforts with academia, industry, and government stakeholders to identify and address existing gaps in the standards and measurement needs of the genome editing field (https://www.nist.gov/programs-projects/nist-genome-editing-consortium).[82−84] A companion effort being led by the U.S. National Institutes of Health is the NIH Common Fund Somatic Cell Genome Editing (SCGE) Program (https://commonfund.nih.gov/editing). The goal of SCGE is to reduce the burden of disease caused by genetic changes by accelerating technology development to improve the safety and efficacy of genome editing approaches. The recently launched SCGE toolkit (https://scge.mcw.edu/toolkit/) serves as a platform to house the data and metadata generated by the program's participating laboratories and serves as a model for integrating the data collection practices recommended in this Perspective.

The U.S. Food and Drug Administration has also recently issued a draft guidance on incorporating genome editing into human therapy products, with a section devoted to genome editing chemistry, manufacturing, and control recommendations.[85] At its core, genome editing is fundamentally a biochemical process. Designing experiments that are better fit for purpose with clear reporting of editing biomolecule formulations will lead to greater confidence in the resulting data, more accurate interpretation of the results, and improved reproducibility of experimental protocols. We have summarized our major recommendations discussed in this Perspective in Table 2. We ask readers to reflect on the concepts discussed in this Perspective and consider these recommendations early in the research process, as well as during the preparation of results for dissemination.

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Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acs.biochem.2c00431
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■ ACKNOWLEDGMENTS

The authors thank Natalia Kolmakova for guidance with the IVC protocols and analysis. The authors also thank Patricia Kiesler and Christopher Sims of the NIST Material Measurement Laboratory for critical reading of the manuscript. Figures in this paper were created with Biorender.com. Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by NIST, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

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https://doi.org/10.1021/acs.biochem.2c00431
Biochemistry XXXX, XXX, XXX−XXX