Thus, there remains a need for developing assays, such as DSF, that can be used to evaluate the quality of Cas9 molecule/gRNA molecule complexes, e.g., to quantitate productive complex formation, a prerequisite for RNP mediated gene editing.

SUMMARY

In one aspect, the present invention refers to a method of screening for a Cas9 molecule/guide RNA (gRNA) molecule complex, the method comprising: (a) generating a plurality of samples, each sample comprising a Cas9 molecule/gRNA molecule complex generated by combining a Cas9 molecule and one of a plurality of gRNA molecules; (b) detecting a melting temperature (T_m) value of the Cas9 molecule/gRNA molecule complex in each of the plurality of samples; and (c) selecting at least one sample from the plurality of samples based on one or more of (i) a comparison of the T_m values in the plurality of samples to a T_m value of a reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold T_m value, or (ii) a relative ordering of the T_m values of the plurality of samples. Methods are disclosed herein for screening for a Cas9 molecule/gRNA molecule complex for administration to a subject that includes (a) generating a plurality of samples, each sample comprising a Cas9 molecule/gRNA molecule complex generated by combining a Cas9 molecule and one of a plurality of gRNA molecules; (b) detecting a melting temperature (T_m) value of the Cas9 molecule/gRNA molecule complex in each of the plurality of samples; and (c) selecting at least one sample from the plurality of samples based on one or more of (i) a comparison of the T_m values in the plurality of samples to a T_m value of a reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold T_m value, or (ii) a relative ordering of the T_m values of the plurality of samples. In certain examples, the step of detecting the T_m value of the Cas9 molecule/gRNA molecule complex in each sample of the plurality of samples may include assessing each sample in the plurality of samples by differential scanning fluorimetry (DSF). In certain examples, the gRNA may be a chimeric gRNA. In certain examples, the gRNA may be a modular gRNA. In certain examples, the sample may comprise a component comprising an additive, a small molecule, a stabilizing reagent, buffer, pH, salt concentration, glycerol concentration, or other buffer component. In certain examples, a sample comprising a Cas9 molecule/gRNA molecule complex having a T_m of at least 8° C greater than a T_m value of the Cas9 molecule absent the gRNA molecule may be selected.

Disclosed herein in certain examplesare isolated complexes of a Cas9 molecule and a gRNA molecule having a T_m at least 8° C greater than a T_m value of the Cas9 molecule absent the gRNA molecule selected according to the methods provided herein.

Provided herein in certain examplesare compositions including an isolated complex of a Cas9 molecule and a gRNA molecule having a T_m at least 8°C greater than a T_m of the Cas9 molecule absent the gRNA molecule selected according to the methods provided herein. In certain examples, the difference of the T_m of the non-naturally occurring complex of a Cas9 molecule and a gRNA molecule and the T_m of the Cas9 molecule absent the gRNA molecule may be assessed by DSF. In certain examples, the gRNA may be a chimeric or modular gRNA.

Disclosed herein in certain examplesare methods of determining the stability of a Cas9 molecule/gRNA molecule complex including (a) generating a plurality of Cas9 molecule/gRNA molecule complexes, each comprising a Cas9 molecule/gRNA molecule complex generated by combining a Cas9 molecule and one of a plurality of gRNA molecules; (b) detecting a T_m value of each of the Cas9 molecule/gRNA molecule complexes of the plurality of Cas9 molecule/gRNA molecule complexes; and (c) determining one or more of the plurality of Cas9 molecule/gRNA molecule complexes is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is greater than a T_m value of a reference molecule or a T_m reference value.

In certain examplesof the methods herein, the plurality of gRNA molecules may be a library of candidate gRNA molecules. In certain examples, the library of candidate gRNA molecules may comprise a library of tracrRNA molecules or sequences. In certain examples, the library of tracrRNA molecules or sequences may be of differing length.

Disclosed herein in certain examplesare methods of determining a condition that promotes a stable Cas9 molecule/gRNA molecule complex including (a) combining a Cas9 molecule and a gRNA molecule in a sample to form a Cas9 molecule/gRNA molecule complex; (b) detecting a T_m value of the Cas9 molecule/gRNA molecule complex; and (c) determining the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is greater than a T_m value of a reference molecule or a T_m reference value.

Disclosed herein in certain examplesare methods of screening for a stable Cas9 molecule/gRNA molecule complex including (a) detecting a T_m value of a Cas9 molecule/gRNA molecule complex via DSF; and (b) determining the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is greater than a T_m value of a reference molecule or a T_m reference value.

Disclosed herein in certain examplesare methods for identifying an optimal gRNA to form a stable Cas9 molecule/gRNA molecule complex including (a) combining a Cas9 molecule and a gRNA molecule in a sample to form the Cas9 molecule/gRNA molecule complex; (b) detecting a T_m value of the Cas9 molecule/gRNA molecule complex; and (c) determining the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA

molecule complex is greater than a T_m value of a reference molecule or a T_m reference value by at least 8°C.

Disclosed herein in certain examplesare methods of determining the stability of a Cas9 molecule/gRNA molecule complex including (a) combining a Cas9 molecule and a gRNA molecule in a sample to form the Cas9 molecule/gRNA molecule complex; (b) detecting a T_m value of the Cas9 molecule/gRNA molecule complex; (c) measuring an activity value of the Cas9 molecule/gRNA molecule complex; and (d) determining the Cas9 molecule/gRNA molecule complex is stable if (i) the T_m value of the Cas9 molecule/gRNA molecule complex is greater than a T_m value of a reference molecule or a T_m reference value and (ii) the activity value of the Cas9 molecule/gRNA molecule complex is greater than an activity value of a reference molecule or an activity reference value.

Disclosed herein in certain examplesare methods of optimizing binding of a gRNA molecule to a Cas9 molecule to form a stable Cas9 molecule/gRNA molecule complex including (a) combining the Cas9 molecule and the gRNA molecule in a sample to form a Cas9 molecule/gRNA molecule complex; (b) detecting a T_m value of the Cas9 molecule/gRNA molecule complex; (c) determining a delta value between the T_m value of the Cas9 molecule/gRNA molecule complex and a T_m value of a reference molecule or a T_m reference value; and (d) determining the Cas9 molecule/gRNA molecule complex is stable if the delta value is at least 8°C and the T_m value of the Cas9 molecule/gRNA molecule complex is greater than the T_m value of the reference molecule or the T_m reference value.

Disclosed herein in certain examplesare methods of detecting a stable Cas9 molecule/gRNA molecule complex including (a) detecting a thermostability value of a reference molecule; (b) combining a Cas9 molecule and a gRNA molecule in a sample to form a Cas9 molecule/gRNA molecule complex; (c) measuring a thermostability value of the Cas9 molecule/gRNA molecule complex; and (d) determining the Cas9 molecule/gRNA molecule complex is stable if the thermostability value of the Cas9 molecule/gRNA molecule complex is greater than the thermostability value of the reference molecule.

In certain examplesof the methods herein, the thermostability value may be a denaturation temperature value and the thermostability reference value may be a denaturation temperature reference value. In certain examplesof the methods herein, the thermostability value may be a T_m value and the thermostability reference value may be a T_m reference value.

In certain examplesof the methods herein, the Cas9 molecule/gRNA molecule complex may be stable if the T_m value of the Cas9 molecule/gRNA molecule complex is at least 1°C, at least 2°C, at least 3°C, at least 4°C, at least 5°C, at least 6°C, at least 7°C, at least 8°C, at least 9°C, at least 10°C, at least 11°C, at least 12°C, at least 13°C, at least 14°C, at least 15°C, at least

16°C, at least 17°C, at least 18°C, at least 19°C, or at least 20°C greater than the T_m value of the reference molecule or T_m reference value. For example, in certain embodiments, the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is at least 8°C greater than the T_m value of the reference molecule or T_m reference value.

In certain examplesof the methods herein, the Cas9 molecule/gRNA molecule complex may be stable if the T_m value of the Cas9 molecule/gRNA molecule complex is about 1°C, about 2°C, about 3°C, about 4°C, about 5°C, about 6°C, about 7°C, about 8°C, about 9°C, about 10°C, about 11°C, about 12°C, about 13°C, about 14°C, about 15°C, about 16°C, about 17°C, about 18°C, about 19°C, or about 20°C greater than the T_m value of the reference molecule or T_m reference value. For example, in certain examples, the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is about 8°C greater than the T_m value of the reference molecule or T_m reference value.

In certain examplesof the methods herein, the Cas9 molecule/gRNA molecule complex may be stable if the T_m value of the Cas9 molecule/gRNA molecule complex is about 1°C to 5°C, about 6°C to 10°C, about 11°C to 15°C, or about 16°C to 20°C greater than the T_m value of the reference molecule or T_m reference value. For example, in certain examples, the Cas9 molecule/gRNA molecule complex is stable if the T_m value of the Cas9 molecule/gRNA molecule complex is about 6°C to 10°C greater than the T_m value of the reference molecule or T_m reference value.

In certain examplesof the methods herein, the T_m value may be detected using a thermal shift assay. In certain examples, the thermal shift assay may be selected from DSF, differential scanning calorimetry (DSC), or isothermal titration calorimetry (ITC).

In certain examplesof the methods herein, the gRNA molecule may comprise a chimeric gRNA molecule. In certain examples, the gRNA molecule may comprise a modular gRNA molecule.

In certain examplesof the methods herein, the Cas9 molecule may be any of the Cas9 molecules disclosed herein. For example, the Cas9 molecule may be a Cas9 molecule selected from Table 1. In certain examples, the Cas9 molecule may be a chimeric Cas9 molecule, or a synthetic or engineered Cas9 molecule. For example, the Cas9 molecule may be a Cas9 molecule with a portion or portions deleted. In certain examples, the Cas9 molecule may comprise a *S. pyogenes* or a *S. aureus* Cas9 molecule.

In certain examplesof the methods herein, the reference molecule may be selected from (a) a reference Cas9 molecule in the absence of a gRNA molecule; (b) a reference Cas9 molecule (e.g., the same Cas9 molecule as the Cas9 molecule in the complex being evaluated) complexed

with a second gRNA molecule (i.e., a gRNA other than the one in the complex being evaluated); and (c) a reference Cas9 molecule/gRNA molecule complex, wherein the reference Cas9 molecule/gRNA molecule was formed under different conditions, e.g., with different proportions of Cas9 molecule and gRNA molecule, than the Cas9 molecule/gRNA molecule complex, or was formed in a different buffer. In certain examples, the reference Cas9 molecule may be the same as the Cas9 molecule of the complex being evaluated. In certain examples, the reference Cas9 molecule may be different from the Cas9 molecule of the complex being evaluated. In certain examples, the reference Cas9 molecule may differ in primary sequence from the Cas9 molecule of the complex being evaluated. In certain examples, the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex may be the same as the gRNA molecule of the complex being evaluated. In certain examples, the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex may be different from the gRNA molecule of the complex being evaluated. In certain examples, the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex may differ in sequence or differs by a modification from the gRNA molecule of the complex being evaluated.

In certain examplesof the methods herein, the T_m reference value may comprise a preselected numerical value for T_m. In certain examples, the T_m reference value may comprise a value correlated with the T_m of any of the reference molecules described herein.

In certain examples, the methods disclosed herein may further include detecting an activity of the Cas9 molecule/gRNA molecule complex; measuring an activity value of the Cas9 molecule/gRNA molecule complex; and determining the Cas9 molecule/gRNA molecule complex is stable if the activity value of the Cas9 molecule/gRNA molecule complex is greater than the activity value of a reference molecule or an activity reference value. In certain examples, the activity may comprise one or more of: an ability to induce indels; an ability to modify a target DNA; a propensity of a preselected repair method; an ability of the gRNA molecule to remain hybridized to the DNA target; and an ability of the gRNA molecule to bind to the Cas9 molecule of the Cas9 molecule/gRNA molecule complex. In certain examples, the activity value may be a binding value and the activity may be the ability of the gRNA molecule to bind to the Cas9 molecule comprising: (a) combining the gRNA molecule and the Cas9 molecule in a sample to form the Cas9 molecule/gRNA molecule complex; (b) measuring a binding value of the Cas9 molecule/gRNA molecule complex; and (c) determining the Cas9 molecule/gRNA molecule complex is stable if the binding value of the Cas9 molecule/gRNA molecule complex is greater than the binding value of a reference molecule or the binding reference value. In certain examples, the

binding value may be measured using a kinetics assay. In certain examples, the kinetics assay may be selected from surface plasmon resonance (SPR) assay, Bio-Layer Interferometry (BLI) assay, or gel band shift assay. In certain examples, the propensity of a preselected repair method may be HDR or NHEJ. In certain examples, the activity of the Cas9 molecule/gRNA molecule complex may be tested using an *in vitro* system; an ex vivo system; an *in vivo* system; a cellular assay; or an animal model.

In certain examples, the reference molecule is selected from any of the reference molecules provided herein. In certain examples, the reference Cas9 molecule/gRNA molecule complex is formed with different proportions of Cas9 molecule and gRNA molecule than the Cas9 molecule/gRNA molecule complex or is formed in a different buffer than the Cas9 molecule/gRNA molecule complex.

Disclosed herein in certain examplesare synthetic Cas9 molecule/gRNA molecule complexes generated using any of the methods described herein.

Disclosed herein in certain examplesare compositions comprising Cas9 molecule/gRNA molecule complexes generated using any of the methods described herein.

Disclosed herein in certain examplesare vector systems comprising a nucleic acid encoding one or more Cas9 molecule/gRNA molecule complexes generated using the any of the methods described herein.

Disclosed herein in certain examplesare methods of delivering a Cas9 molecule/gRNA molecule complex to a target cell comprising delivering any of the Cas9 molecule/gRNA molecule complexes described herein to the target cell. In certain examples, the Cas9 molecule/gRNA molecule complexes may be delivered to the cell by RNP cationic lipid transfection, a viral vector, or RNA transfection. In certain examples, the viral vector may be an AAV vector.

In certain examples, the gRNA molecule in a method, composition, or formulation provided herein may be a unimolecular or chimeric gRNA. In other examples, the gRNA molecule may be a modular gRNA.

In certain examples, the Cas9 molecule in a method, composition, or formulation provided herein may be an *S. pyogenes*, *S. aureus*, or *S. thermophilus* Cas9 molecule, or a Cas9 molecule derived from an *S. pyogenes*, *S. aureus*, or *S. thermophilus* Cas9 molecule. In certain examples, the Cas9 molecule may be labeled, and in certain of these examplesthe label may be a fluorescent dye.

Other features and advantages of the invention will be apparent from the detailed description, drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1I are representations of several exemplary gRNAs.

Fig. 1A depicts a modular gRNA molecule derived in part (or modeled on a sequence in part) from *Streptococcus pyogenes (S. pyogenes)* as a duplexed structure (SEQ ID NOs:39 and 40, respectively, in order of appearance);

Fig. 1B depicts a unimolecular gRNA molecule derived in part from *S. pyogenes* as a duplexed structure (SEQ ID NO:41);

Fig. 1C depicts a unimolecular gRNA molecule derived in part from *S. pyogenes* as a duplexed structure (SEQ ID NO:42);

Fig. 1D depicts a unimolecular gRNA molecule derived in part from *S. pyogenes* as a duplexed structure (SEQ ID NO:43);

Fig. 1E depicts a unimolecular gRNA molecule derived in part from *S. pyogenes* as a duplexed structure (SEQ ID NO:44);

Fig. 1F depicts a modular gRNA molecule derived in part from *Streptococcus thermophilus (S. thermophilus)* as a duplexed structure (SEQ ID NOs:45 and 46, respectively, in order of appearance);

Fig. 1G depicts an alignment of modular gRNA molecules of *S. pyogenes* and *S. thermophilus* (SEQ ID NOs:39, 45, 47, and 46, respectively, in order of appearance).

Figs. 1H-1I depicts additional exemplary structures of unimolecular gRNA molecules.

Fig. 1H shows an exemplary structure of a unimolecular gRNA molecule derived in part from *S. pyogenes* as a duplexed structure (SEQ ID NO:42).

What is claimed is:

1. A method of screening for a Cas9 molecule/guide RNA (gRNA) molecule complex, the method comprising:

(a) generating a plurality of samples, each sample comprising a Cas9 molecule/gRNA molecule complex generated by combining a Cas9 molecule and one of a plurality of gRNA molecules;

(b) detecting a melting temperature (Tm) value of the Cas9 molecule/gRNA molecule complex in each of the plurality of samples; and

(c) selecting at least one sample from the plurality of samples based on one or more of (i) a comparison of the Tm values in the plurality of samples to a Tm value of a reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value, or (ii) a relative ordering of the Tm values of the plurality of samples.

2. The method according to claim 1, wherein the step of detecting the Tm value of the Cas9 molecule/gRNA molecule complex in each sample of the plurality of samples includes assessing each sample in the plurality of samples by differential scanning fluorimetry (DSF).

3. The method according to claim 1, wherein the gRNA is a chimeric gRNA.

4. The method according to claim 1, wherein the gRNA is a modular gRNA.

5. The method of claim 1, wherein the Cas9 molecule/gRNA molecule complex is selected if the Tm value of the Cas9 molecule/gRNA molecule complex is at least 1°C, at least 2°C, at least 3°C, at least 4°C, at least 5°C, at least 6°C, at least 7°C, at least 8°C, at least 9°C, at least 10°C, at least 11°C, at least 12°C, at least 13°C, at least 14°C, at least 15°C, at least 16°C, at least 17°C, at least 18°C, at least 19°C, or at least 20°C greater than the Tm value of the reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value.

6. The method of claim 5, wherein the Cas9 molecule/gRNA molecule complex is selected if the Tm of the Cas9 molecule/gRNA molecule complex is at least 8°C greater than the Tm value of the reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value.

7. The method of claim 1, wherein the Cas9 molecule/gRNA molecule complex is selected if the Tm value of the Cas9 molecule/gRNA molecule complex is about 1°C, about 2°C, about 3°C, about 4°C, about 5°C, about 6°C, about 7°C, about 8°C, about 9°C, about 10°C, about 11°C, about 12°C, about 13°C, about 14°C, about 15°C, about 16°C, about 17°C, about 18°C, about 19°C, or about 20°C greater than the Tm value of the reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value.

8. The method of claim 1, wherein the Cas9 molecule/gRNA molecule complex is selected if the Tm value of the Cas9 molecule/gRNA molecule complex is 1°C to 5°C, 6°C to 10°C, 11°C to 15°C, or 16°C to 20°C greater than the Tm value of the reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value.

9. The method of claim 8, wherein the Cas9 molecule/gRNA molecule complex is selected if the Tm value of the Cas9 molecule/gRNA molecule complex is 6°C to 10°C greater than the Tm value of the reference Cas9 molecule/gRNA molecule complex or a pre-determined threshold Tm value.

10. The method of any of claims 1-9, wherein the Tm value is detected using a thermal shift assay.

11. The method of claim 10, wherein the thermal shift assay is selected from differential scanning fluorimetry (DSF), differential scanning calorimetry (DSC), or isothermal titration calorimetry (ITC).

12. The method of any of claims 1-11, wherein the Cas9 molecule is a Cas9 molecule selected from Table 1.

13. The method of any of claims 1-11, wherein the Cas9 molecule is a chimeric Cas9 molecule, or a synthetic or engineered Cas9 molecule, e.g., a Cas9 molecule with a portion or portions deleted.

14. The method of any of claims 1-13, wherein the Cas9 molecule comprises a *S. pyogenes* or a *S. aureus* Cas9 molecule. 15. The method of claim 1, wherein the reference Cas9 molecule is the same as the Cas9 molecule of the complex being evaluated.

16. The method of claim 1, wherein the reference Cas9 molecule is different from the Cas9 molecule of the complex being evaluated.

17. The method of claim 1, wherein the reference Cas9 molecule differs in primary sequence from the Cas9 molecule of the complex being evaluated.

18. The method of any of claims 1 and 15-17, wherein the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex is the same as the gRNA molecule of the complex being evaluated.

19. The method of any of claims 1 and 15-17, wherein the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex is different from the gRNA molecule of the complex being evaluated.

20. The method of claim 19, wherein the gRNA molecule of the reference Cas9 molecule/gRNA molecule complex differs in sequence or differs by a modification from the gRNA molecule of the complex being evaluated.