

2017:101-118; and Sergeeva et al., CRISPR Gene Editing. Springer; 2019:213-232) have several shortcomings. The wide range in editing efficiency of different gRNA sequences necessitates a lengthy and expensive process of synthesizing, cloning, and screening various gRNA plasmids. Targeted NGS, which is the gold standard for quantifying CRISPR edits, is resource-intensive and expensive while other screening approaches such as western blot analysis, T7 endonuclease I assays, and size-based PCR amplicon analysis (VanLeuven et al., Biotechniques. 2018;64(6):275-278) lack speed, sensitivity, and ability to accurately differentiate weak gRNAs from more efficient ones. (Sentmanat et al., Scientific Reports. 2018;8(1):1-8). Furthermore, efficient stable integration of the transfected Cas9 DNA (Lino et al., Drug Deliv. 2018;25(1):1234-1257) can have undesirable outcomes for engineered CHO cell lines for manufacturing therapeutic proteins. Thus, there remains a need in the art for an efficient strategy to achieve multiplex knockouts.

3. SUMMARY

In certain examples, the present disclosure is directed to methods of producing a cell comprising edits at two or more target loci, wherein the method comprises combining two or more guide RNAs (gRNAs) capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form a ribonucleoprotein complex (RNP); serially transfecting a population of cells with the RNP until at least about 10% indel formation is achieved at each target locus; and isolating a cell comprising edits at two or more target loci by single cell cloning of the cell from the population of serially transfected cells. In one aspect, the present disclosure provides a method of producing a cell comprising edits at two or more target loci: (a) combining two or more guide RNAs (gRNAs) capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form a ribonucleoprotein complex (RNP); (b) serially transfecting a population of cells with the RNP until at least about 50% indel formation is achieved at each target locus; and (c) isolating a cell comprising edits at two or more target loci by single cell cloning of the cell from the population of serially transfected cells. In certain examples, the gRNA is an sgRNA. In certain examples, the gRNA comprises a crRNA and a tracrRNA. In certain examples, the crRNA is an XT-gRNA.

In certain examples of the methods of producing a cell comprising edits at two or more target loci described herein, the population of cells is serially transfected with the RNP until at least about 20% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 30% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 40% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 50% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 60% indel formation is achieved at each target locus.

In certain examples of the methods of producing a cell comprising edits at two or more target loci described herein, the ratio of moles of RNP to number of transfected cells is between about 0.1 pmol per 10^6 cells to about 5 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.15 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.17 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.2 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 1 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 2 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 3 pmol per 10^6 cells.

In certain examples of the methods of producing a cell comprising edits at two or more target loci described herein, three or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, four or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. . In certain examples, five or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, six or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, seven or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, eight or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, nine or more

gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus. In certain examples, ten or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.

In certain examples, the cell is a T cell, an NK cell, a B cell, a dendritic cell, a CHO cell, a COS-7 cell; an HEK 293 cell, a BHK cells, a TM4 cell, a CV1 cell; a VERO-76 cell; a HELA cells; or an MDCK cell.

In certain examples of the methods producing a cell comprising edits at two or more target loci described herein, the two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are identified via a efficiency screen comprising: (a) transfecting a population of cells with a population of RNPs, where each RNP comprises a gRNA capable of directing CRISPR/Cas9-mediated indel formation at a target locus; and (b) sequencing the target loci to identify gRNAs based on their efficiency in directing CRISPR/Cas9-mediated indel formation. In certain examples, the sequencing is performed using Sanger sequencing.

In certain examples, the present disclosure is directed to a cell composition, wherein the cell comprises edits at two or more target loci, wherein the edits are the result of: combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP; serially transfecting a population of cells with the RNP until at least about 10% indel formation is achieved at each target locus; and isolating the cell comprising edits at two or more target loci by single cell cloning of the cell from the population of serially transfected cells

In certain examples, the present disclosure is directed to a host cell composition, wherein the host cell comprises: a nucleic acid encoding a non-endogenous polypeptide of interest; and edits at two more target loci, wherein the edits are the result of: combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP; serially transfecting a population of cells with the RNP until at least about 10% indel formation is achieved at each target locus; and isolating the host cell comprising edits at two or more target loci by single cell cloning of the host cell from the population of serially transfected cells.

In certain examples of the compositions disclosed herein, the gRNA is an

sgRNA. In certain examples of the compositions disclosed herein, the gRNA comprises a crRNA and a tracrRNA. In certain examples of the compositions disclosed herein, the crRNA is an XT-gRNA. In certain examples of the compositions disclosed herein, the population of cells is serially transfected with the RNP until at least about 20% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 30% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 40% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 50% indel formation is achieved at each target locus. In certain examples, the population of cells is serially transfected with the RNP until at least about 60% indel formation is achieved at each target locus.

In certain examples of the compositions disclosed herein, the ratio of moles of RNP to number of transfected cells is between about 0.1 pmol per 10^6 cells to about 5 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.15 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.17 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 0.2 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 1 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 2 pmol per 10^6 cells. In certain examples, the ratio of moles of RNP to number of transfected cells is about 3 pmol per 10^6 cells.

In certain examples of the compositions disclosed herein, the cell is a T cell, an NK cell, a B cell, a dendritic cell, a CHO cell, a COS-7 cell; an HEK 293 cell, a BHK cells, a TM4 cell, a CV1 cell; a VERO-76 cell; a HELA cells; or an MDCK cell.

In certain examples of the compositions disclosed herein, the two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are identified via an efficiency screen comprising: transfecting a population of cells with a population of RNPs, where each RNP comprises a gRNA capable of directing CRISPR/Cas9-mediated indel formation at a target locus; and sequencing the target loci to identify gRNAs based on their efficiency in directing CRISPR/Cas9-mediated indel formation. In certain examples, the sequencing is performed using Sanger sequencing.

In certain examples, the methods for producing a polypeptide of interest described herein comprise: culturing a host cell composition comprising: a nucleic acid

encoding a non-endogenous polypeptide of interest; and edits at two or more target loci, wherein the edits are the result of: (1) combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP; (2) serially transfecting a population of cells with the RNP until about 10% indel
 5 formation is achieved at each target locus; and (3) isolating the host cell comprising edits at two or more target loci by single cell cloning of the host cell from the population of serially transfected cells; and isolating the polypeptide of interest expressed by the cultured host cell.

In certain of the above described examples, the methods provided in the present
 10 disclosure further comprise purifying the product of interest, harvesting the product of interest, and/or formulating the product of interest.

In certain of the above described examples, the cell is a mammalian cell. In certain of the above described examples, the mammalian cell is a CHO cell.

In certain of the above described examples, the cell expresses a product of
 15 interest. In certain of the above described examples, the product of interest expressed by the mammalian cells is encoded by a nucleic acid sequence. In certain of the above described examples, the nucleic acid sequence is integrated in the cellular genome of the mammalian cells at a targeted location. In certain of the above described examples, the product of interest expressed by the cells is further encoded by a nucleic acid sequence that
 20 is randomly integrated in the cellular genome of the mammalian cells.

In certain of the above described examples, the product of interest comprises a protein. In certain of the above described examples, the product of interest comprises a recombinant protein. In certain of the above described examples, the product of interest comprises an antibody or an antigen-binding fragment thereof. In certain of the above
 25 described examples, the antibody is a multispecific antibody or an antigen-binding fragment thereof. In certain of the above described examples, the antibody consists of a single heavy chain sequence and a single light chain sequence or antigen-binding fragments thereof. In certain of the above described examples, the antibody is a chimeric antibody, a human antibody or a humanized antibody. In certain of the above described examples, the antibody
 30 is a monoclonal antibody.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E. gRNA screening process and indel analysis for detecting knockout efficiencies. Figure 1A shows a workflow to screen for potent gRNAs for each
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