OFFICIAL MEXICAN NORM PROJECT PROY-NOM-000-SAGARPA/SEMARNAT-2017. Good practices for chromosomal insertion of microorganisms of risk 2 by CRISPR-CAS9.

PREFACE

In the elaboration of this Official Mexican Norm intervened:
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- Erwinions

INDEX

0. Introduction

During the latest years, the genomic edition has been cataloged as one of the most important advances in biology. This is a direct effect of the discovery that has allowed experimental development, system CRISPR-Cas9 biotechnology in 2012 and its application.

CRISPR/Cas9 is an immune system of the prokaryotic cell that refers to palindrome short group cycles, regularly molecularly interspaced, which allows a small viral DNA fragment to enter for the infection recognition. When a prokaryote is attacked by a

virus, is injected by its DNA to the host cell. Nevertheless, if the host cell gets to identify the hexogen genome into CRISPR, it acquires the ability to attack the virus, known as Cas9.

Symbolically, CAS 9 is a protein complex of "molecular scissors" in charge of making changes in the genome of some organism, being it cutting, suppressing, modifying, interrupting or incorporating new genetic sequences. However, this complex requires the sequence incorporated in CRISPR to be programmed and cut the target sequence. The most characteristic of this mechanism is its great specificity and accuracy in terms of the location of the cuts and the incorporation of genes at the loci of the desired genome.

This process was standardized by researchers for its use within genetic engineering, pretending the modification or insertion of nucleotides in the genome thanks to the permanent union between the ARN tracr and the ARNcr, genomic sequences within CRISPR that allow the recruitment and programming of Cas9, by means of a fork. In the genetic engineering technique, the cut made by Cas9 in the chain to be modified will not cause degradation. On the other hand, based on DNA damage repair mechanisms, such as repair by homologous and non-homologous ends, it is intended to achieve the modification of the original genome incorporating the desired sequence.

It is thanks to the benefits that this new technique provides and/or is expected to provide (great specificity, control of viral diseases, gene therapy, activation or silencing of genes, etc.) is established within this Official Mexican Standard the generalities experimental development techniques for genome modification using CRISPR-Cas9.

1. 0. Field of application and target

1.1 0.1 This Official Mexican Standard establishes the general method for the chromosomal insertion of microorganisms of risk 2 by CRISPR-Cas9

1.2 0.1 This Official Mexican Standard is of obligatory observance in the National Territory for individuals or corporations that seek to perform this method for either personal or general benefit.

2. Fundament

The method is based on the insertion of exogenous material to a microorganism by means of new technologies. CRISPR-Cas9 is a system used in genetic engineering that allows, unlike conventional methods, to make cuts for correction, elimination, or insertion to genomic DNA.

3. References

This Standard is complemented by the following:
PROJECT OF MEXICAN OFFICIAL STANDARD PROY-NOM-000-SAGARPA / SEMARNAT-2017. Good practices for the genetic transformation of microorganisms of risk 2 by conventional methods.

4. Definitions

For this norm purposes, we understand by:

Host cell: microorganism or cell that will suffer an alteration in its normal genetic material.

DSB: One of the ways of DNA damage that it is given by the double chain break. The abbreviation comes from its english name (double strand break).

CRISPR: an Immune-adaptive sequence of prokaryotic cells.

Chromosomal insertion: Genomic DNA modification of the host cell through the incorporation of the exogen DNA.

Genetic material: macromolecule with animal, vegetal or microbial origin that contains information of one or more genes in the form of nitrogenated base and can be transmitted from generation to generation.
Transformation: the process in which an external DNA is inserted to a host cell, modifying it genetically.

Cas9: Molecular scissors that cause precise double stranded cuts in an objective genome.

ARNcr: Summarised oligonucleotides inside of the cell from the fragment of the viral inserted fragment.

ARNtracr: Oligonucleotides sequence responsible of the recruit of the protein complex Cas9.

sgRNA: ARN oligonucleotide summarised in the laboratory with which it achieves the insertion. It combines the CRISPR sequence with the trans-activator sequence.
Transformation vector: carrier molecule that has inside it a genetic material of interest insert. Through genetic engineering techniques, this molecule is introduced into a cell with the objective of cloning or expressing the vector.

5. Symbols and abbreviations:

When this NOM refers to the following abbreviations and symbols they will stand for:

mM millimolar
µL microliters
mL milliliter
mm millimeter
g grams
pg picograms
pH Hydrogen potential
% percentage
UFC colony creator units
h hours
min minutes
OD_{600} optic density to 600 nanometers
LB Luria Bertani
°C degree Celsius
rpm revolutions per minute
bp bases
G guanine
C cytosine

A adenine
T thymine
N any nitrogenous base

6. Reagents and materials

6.1 Reagents

Ultrapure Water

Culture Way

Lennox Broth

FORMULA

INGREDIENTS AND QUANTITY

Water 1000 ml
Tryptone 10 g
Yeast extract 5 g
calcium chloride 5 g

PREPARATION

In case of using the components, put these in one liter of water and boil until they dissolve completely.

Distribute in sterilizable glass recipients in a way that it doesn't exceed ¾ of its volumetric capacity.

Sterilize in autoclaving at 121 ± 1,0 ºC during 15 minutes. The final pH of it must be 7,0 ± 0,2 at 25ºC.

If the culture medium is immediately used, cool at 45ºC ± 1,0 ºC in water and maintain it at that temperature until its using. The medium must not merge more than one time.

In case of dehydrated mediums follow the fabricant's instructions.

Culture Medium

Agar Lennox Broth

FORMULA

INGREDIENTS QUANTITY

Water 1000 ml
Tryptone 10 g
Yeast extract 5 g
Calcium Chloride 5 g
Agar 20 g

PREPARATION

In case of using the components, put these in one liter of water and boil until they dissolve completely.

Distribute in sterilizable glass recipients in a way that it doesn't exceed ¾ of its volumetric capacity.

Sterilize in autoclaving at 121 ± 1,0 ºC during 15 minutes. The final pH of it must be 7,0 ± 0,2 at 25ºC.

If the culture medium is used immediately, cool to 45ºC ± 1,0 ºC in water.

Distribute the volume, approximately 20.25 mL in Petri boxes.

In case of dehydrated mediums follow the fabricant's instructions.

6.2 Materials

Crushed ice
Sterile microtubes 1.5 ml
Electroporation cell 2 mm

7. Equipment and instruments

Electroporator that allows discharges till 3.0 kV and the pulse selection in a 1.0 to 4.0 degree with a precision of ± .1 ms.

Bell of biological security with ultraviolet light for its sterilization.

Micropipet with a rank of sample-take between 20 - 200 µL.

Micropipet with a rank of sample-take between 0.2 - 20 µL.

Computer with minimum 1GB of RAM, 250 MB available in the hard disk drive and resolution of 1024*768.

8. sgRNA design

8.1 Select the place to digest through Cas9 inside the genomic sequence of interest.

8.2 Identify all the combinations of sequences of 23 pb, in 5' 3' sense, that contains the PAM protospacer en the 3' extreme. Procure that it is in a rank of +/- 50 pb starting from the site where it decides to do the cut.

8.3 From what was obtained in 8.2, calculate the % of GC in the detected sequences.

8.4 Choose those sequences that have a GC content between 40% and 60%. In case of not finding, increase the rank to +/-100 pb.

8.5 Select one of the sequences obtained from the analysis and realize a local alignment search of sequences against a base of biological data to corroborate that inside the genome that is going to be modified it only exists one equal fragment. Repeat this step until obtaining only one positive result. In the last instance, select the one with minor coincidences.

8.6 Incorporate the chosen fragment in 8.4, inside a genetic circuit for its expression. The recommended order is U6 promotor, gRNA, the template of guide RNA, terminator.

8.7 Synthesize the genomic circuit.

9. DNA template design for insertion through homologous recombination

The DNA template design for homologous recombination varies depending on the objective of the experimental development. However, it is important to have in consideration the following aspects:

9.1 Establish the size of the insert.

9.2 Select an ascending and descending homologous sequence beginning from the site that's going to be digested by Cas9.

9.2.1 In case of being a little insert of approximately 100-200 bp, use one single-stranded oligonucleotide with 50 - 80 bp on each homologous arm.

9.2.2 In case of being a big insert, use a double-stranded plasmid with homology on each arm of approximately 800 bp.

9.3 Contain the desired DNA sequence that must be transformed into the host cell at the same time that the gRNA and the Cas9 protein system.

9.4 Avoid that the template contains the PAM sequence.

10. Electroporation

It must be followed the electroporation protocol established inside the NOM "PROYECTO DE NORMA OFICIAL MEXICANA PROY-NOM-000-SAGARPA/SEMARNAT-2017. Buenas Prácticas para la transformación genética de microorganismos de riesgo 2 por métodos convencionales".

11. Results report

The results report must be issued in base of what is established inside the PROYECTO DE NORMA OFICIAL MEXICANA PROY-NOM-164-SEMARNAT/SAGARPA-2012, QUE ESTABLECE LAS CARACTERÍSTICAS Y CONTENIDO DEL REPORTE DE RESULTADOS DE LA O LAS LIBERACIONES REALIZADAS DE ORGANISMOS GENÉTICAMENTE MODIFICADOS, EN RELACIÓN CON LOS POSIBLES RIESGOS PARA EL MEDIO AMBIENTE Y LA DIVERSIDAD BIOLÓGICA Y, ADICIONALMENTE, A LA SANIDAD ANIMAL, VEGETAL Y ACUÍCOLA.

12. Risks evaluation

12.1 Activity outside the target.

The principal challenge is the activity outside the target. Because of the CRISPR-Cas9 DSB activity is modulated only with one RNA guide (gRNA) of 20 nucleoids and is tolerable with some mismatches of pair bases between gRNA and the objective DNA. There is a possibility that it could cut in genomic locations just partially complementaries to the gRNA. These off-target activities vary between different DNA sites due to variations in their nucleotide compositions and genomic context. Any activity outside the target could cause undesirable consequences and is not acceptable. To address this problem, a number of methods have been proposed, from the selection of gRNA through various bioinformatic tools, as dictated in section 8 of this same trade, to the use of shorter gRNA, and inducible CRISPR-Cas9, as well as the use of the CRISPR-Cas9 protein directly

12.2 Efficiency variations

The effectiveness of DSB activities directed by CRISPR-Cas9 varies widely depending on the nucleotide compositions and the genomic context of the target protospacer DNA sites as well as the secondary structure of the gRNA. Some characteristics of gRNA sequences have been identified that significantly affect efficiency based on the analyses of more than 200 gRNA Similar results were also obtained in other independent studies, showing significant variations in the efficiency between gRNA directed to adjacent proto-spacer sites in the same plasmid. Unfortunately, until now, there is no reliable bioinformatic method to widely predict

the efficiencies of CRISPR-Cas9 and gRNA in a target DNA site. At present, the effectiveness of a gRNA can be determined realistically only by experimental methods. Several technologies have been developed for the screening of the efficacy and specificity of the gRNA, including the integral technologies (NGS and GUIDE-Seq). In addition, the editing efficiency of the genome is also affected by the binding of non-homologous ends (higher) and HDR (lower) DNA repair mechanisms. HDR efficiencies can be increased by varying the insert/donor size, modifying DNA donors by inhibiting NHEJ activity. Recently, it was shown that NHEJ can also be used to facilitate the large number of DNA fragments (IRES-GFP) knock-in with higher efficiencies than HDR.

12.3 PAM restrictions

The accuracy of the CRISPR-Cas9 DSB activity requires the presence of a PAM, which serves as an essential site for the landing of the CRISPR-Cas9-sgRNA complex in the proto-spacer DNA and initiates the interrogation of the target DNA. It has been discovered that CRISPR-Cas9s from different bacterial species have different PAMs with various lengths and nucleotide compositions as well as each one determines the different cut-off frequencies of CRISPR-Cas9 for a given genome.
None of the MAPs identified so far, not even the combination of all the known MAPs could cover sequences of a complete genome, which in some cases would restrict the use of CRISPR-Cas9 technology. To alleviate such limitations of MAP, progress has been made in modifying the CRISPR-Cas9 PAMs through a directed evolution based on bacterial selection or by a rational design based on the structure. Variants of SpCas9 (Streptococcus pyogenes Cas9) have been created to recognize different PAMs other than "NGG", including a variant that recognizes a PAM of 2 nucleotides "YG". The PAM of 2 nucleotides would significantly increase the genome editing coverage. However, many PAMs nested in a target DNA are inhibitors of CRISPR-Cas9 in vivo activities. The ideal strategy would be to customize a PAM sequence adapted only to the desired target DNA site through the alteration of the amino acids that interact with PAM, which has been made possible by identifying the amino acids responsible for PAM recognition in both SpCas9 as in SaCas9 Cas9 of Staphylococcus aureus.

13. Concordance with international standards

This standard was made in accordance with the Cartagena Protocol on Biosafety of the Convention on Biological Diversity, issued by the Secretariat of the Convention on Biological Diversity of the United Nations Organization, specifically with articles 1, 2 , 4, 15, 16, 19 and Annex III of the same instrument, by virtue of the following:

I.- The general objective of the protocol is to help ensure an adequate level of safe practices of living modified organisms resulting from modern biotechnology that may have adverse effects on biological diversity, taking into account the risks to human health. This NOM was made in accordance with these principles, by listing within its objectives that this method is done in a biosecure and ethical manner.

II.- The risk assessment prepared in this NOM is in accordance with the guidelines established in Article 15 and Annex III of the Protocol, since it establishes that risk assessments will be carried out in accordance with it's a scientific procedure, something that the NOM covers up in the risk assessment section and is validated because the evaluation was made with sources that guarantee that the methods have been previously used, and have been successful.

III.- According to the fifth paragraph of Article 16 of the Protocol states that the Parties must "specific living modified organisms or specific traits of living modified organisms that may have adverse effects for the conservation and sustainable use of biological diversity, taking also take into account the risks to human health", which is established both in the introductory paragraphs of this NOM, and in the section on Risk Assessment in its three parts talking about microorganisms at risk 2 by CRISPR-Cas9. They must also "adopt appropriate measures for the treatment of those living modified organisms or specific traits", which is also compiled according to what is established by the Risk Assessment section, example in the idealization of CRISPR-Cas9, establishing that for a better embodiment, customized variants are used to adapt specifically to the target DNA site, as well as establishing several points to cover.

IV.- This NOM establishes that for compliance with article 19 of the Cartagena Protocol, the administrative authorities in charge of functions and responsibilities in

genetic matters, are those established in the section on "Observance of the norm", which is fulfilled in the following NOM, the the obligation to comply by the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food and the Ministry of Environment and Natural Resources. Each Party will designate a national focal point that will be responsible for the liaison with the Secretariat on its behalf.

V.- In order to comply with the provisions of Annex III of the Cartagena Protocol, several points applicable to this NOM have been included in the section on "Risk assessment", the first being the objective of the risk assessment precisely, "to determine and evaluate the possible adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely receiving environment, also taking into account risks to human health". In this NOM, the above is covered in its full risk assessment, since it foresees what is necessary to carry out in order to avoid adverse effects when performing the "Method for homologous recombination in microorganisms at risk 2 by CRISPR-Cas9" as it would be to use a series of methods, from the selection of ARNsg through various bioinformatic tools, as dictated in section 8 of this same trade, to the use of shorter gRNA, and inducible CRISPR-Cas9, as well as the use of the CRISPR-Cas9 protein directly.

It also covers the first general principle of the Protocol, which establishes that the risk assessment must be carried out in a transparent and scientifically competent manner, as well as taking into account the advice of experts and the guidelines established by international organizations, since these extremely complicated methods have been investigated in depth and by means of reliable sources that have endorsed the method of homologous recombination in microorganisms of risk 2 by CRISPR-Cas9, which demonstrates the scientific support necessary for the realization of this method.It also complies with the sixth general principle of the Protocol, given that the risk assessment of this NOM specifically talks about the precautions that must be taken into account when carrying out the method,
as for example the strategy proposal to customize a PAM sequence adapted only to the target DNA site desired through the alteration of the amino acids that interact with responsible PAM, through what is established in the risk assessment section.

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Jian-Hua Zhang,Poorni Adikaram,Mritunjay Pandey,Allison Genis & William F. Simonds. Pages 166-174 | Received 13 Apr 2016, Accepted 09 May 2016, Published online: 24 Jun 2016
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15. Observance of the norm

The monitoring of compliance with this Standard corresponds to the Secretary of Agriculture, Livestock, Rural Development, Fisheries and Food and the Secretary of Environment and Natural Resources.

16. Validity

This Mexican Official Norm will enter into force as a mandatory requirement within 30 days of its publication in the Official Gazette of the Federation.