PROJECT OF MEXICAN OFFICIAL NORM PROY-NOM-000-SAGARPA/ SEMARNAT-2017. Good practices for the genetic transformation of risk 2 microorganisms by conventional methods.

PREFACE

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0. Introduction

Currently, the broad and rapid development of modern biotechnology has provided important possible solutions to problems of public interest directly contributing to human well-being. However, these possibilities are often hampered by public concern about biosecurity or the possible adverse effects that the manipulation of microorganisms may have on human health and the environment.

Considering the little and ambiguous legislation in the country regarding the technical process of genetic transformation in a microorganism and recognizing the great positive impact that it would be to have access to these possibilities, it is considered of great importance to establish a regulated protocol that guarantees this effective genetic manipulation and biosecurity.

Genetic transformation is a complex molecular biology technique that can be carried out by different methods. However, there are already standardized protocols that promote this process in a safe and relatively simple way; the conventional methods.

1. Objective and field of application

1.1 This Official Mexican Norm establishes the general method for the genetic transformation by conventional methods of risk 2 microorganisms whose destiny is the commercialization of the same or derived products.

1.2 This Official Mexican Norm is mandatory in the National Territory for natural or legal persons who seek to perform this method for either personal or general benefit.

2. Fundaments

The method is based on the insertion of exogenous material to a microorganism of interest by conventional methods. The protocol mainly uses thermal shock as a transmembrane destabilizer allowing the entry of said material, while electroporation uses electricity to perform the same effect.

3. References

This Norm is complemented by the following:
NOM-092-SSA1-1994. Method for aerobic bacteria counts in a plate.

4. Definitions

For purposes of this standard, it means:

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

Competence: The temporary ability of a cell to internalize exogenous DNA from the environment. Within this Norm, Competence only refers to artificial competence.

Colony Forming Units (CFU): a term that must be used to report the count of plaque colonies, which may arise from a cell or from a cell cluster.

Genetic material: macromolecule of animal, vegetable or microbial origin that contains information of one or several genes in the form of nitrogenous bases and can be transmitted from generation to generation.

Exogenous material: genetic material that is foreign to the microorganism or cell in question.

Transformation: the process in which an insertion of an external DNA to a host cell is performed, genetically altering it.

Transformation vector: carrying molecule that brings within it an insert of genetic material of interest. The genetic engineering techniques, this molecule is introduced into a cell by cloning or expressing the vector.

5. Symbols and abbreviations

When reference is made to the following abbreviations and symbols in this standard, it means:

mM millimolar
µL microliters
mL milliliters
mm millimeters
g grams
pg picograms
Potential-pH of hydrogen
% percent
UFC colony-forming units
h hours
min minutes
OD_{600} optical density at 600 nanometers
LB Luria Bertani
CaCl2 Calcium Chloride
° C degrees Celsius
rpm revolutions per minute
kV kilovolt
ms microseconds

6. Reagents and materials

6.1 Reagents

The reagents that are mentioned below must be analytical grade.

6.1.1 Reagents for thermal shock

Water (hereinafter referred to as distilled water with pH close to neutrality)

Calcium Chloride 50mM

FORMULA

INGREDIENTS AMOUNTS

Water 1000 ml
Calcium Chloride 5.549 g

PREPARATION

Weigh the desired amount of CaCl2 respecting the established ratio.

Weigh the desired amount of CaCl2 respecting the established ratio.

Pour a bed of water into a volumetric flask to the final volume of the solution.

Add the CaCl2 avoiding the adherence of this to the walls.

Continue pouring the remaining volume of water to the afore line.

Invert three to six times the flask.

If necessary, fill the volume up to the limit.

Store at 4 ° C.

100mM Calcium Chloride

FORMULA

INGREDIENTS AMOUNTS

Water 1000 ml
Calcium Chloride 11,098 g

PREPARATION

Weigh the desired amount of CaCl2 looking up to the established ratio.

Pour a bed of water into a volumetric flask to the final volume of the solution.

Add the CaCl2 avoiding the adherence of this to the walls.

Continue pouring the remaining volume of water to the afore line.

Invert three to six times the flask.

If necessary, fill the volume up to the limit.

Store at 4 ° C.

Culture Medium

Lennox Broth

FORMULA

INGREDIENTS AMOUNTS

Water 1000 ml
Triptone 10 g
Yeast extract 5 g
Calcium Chloride 5 g

PREPARATION

In case of using the components, suspend these in a liter of water and boil until completely dissolved.

Distribute in sterilizable glass containers in a way that does not exceed three-quarters of the volumetric capacity of the same.

Sterilize in an autoclave at 121 ± 1.0 ºC, for 15 minutes. The final pH of the medium should be 7.0 ± 0.2 at 25 ° C.

If the culture medium is used immediately, cool to 45 ° C ± 1.0 ° C in a water bath and keep it at this temperature until before use. The medium should not melt more than once.

In case of dehydrated media follow the manufacturer's instructions.

Culture Medium

Lennox Broth Agar

FORMULA

INGREDIENTS AMOUNTS

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

PREPARATION

In case of using the components, suspend these in a liter of water and boil until completely dissolved.

Distribute in sterilizable glass containers in a way that does not exceed three-quarters of the volumetric capacity of the same.

Sterilize in an autoclave at 121 ± 1.0 ºC, for 15 minutes. The final pH of the medium should be 7.0 ± 0.2 at 25 ° C.

If the culture medium is used immediately, cool to 45 ° C ± 1.0 ° C in a water bath.

Distribute the volume, approximately 20 - 25 mL, in Petri dishes.

In case of dehydrated media follow the manufacturer's instructions.

6.1.2 Reagents regarding Electroporation

Ultrapure water

Culture medium

LB (See section 6.1.1 for formulation and preparation)

Agar LB (See section 6.1.1 for formulation and preparation)

6.2 Materials

6.2.1 Materials needed for thermal shock

Crushed ice
Sterile conical tubes 50 ml
Serological pipettes 10 ml
Sterile microtubes 1.5 ml
Petri dishes.
Glass elbows for plate extension.

6.2.2 Materials required for Electroporation

Crushed ice
Sterile microtubes 1.5 ml
Electroporation cell 2 mm

7. Apparatus and instruments

7.1 Regarding Thermal Shock

Centrifuge with cooling that allows revolutions greater than 3000 rpm.

Spectrophotometer capable of reading at a wavelength of 600 nanometers.

Biological safety hood equipped with an ultraviolet light for sterilization.

Incubator with agitation and thermostat that avoids variations greater than ± 1.0 ºC, provided with a calibrated thermometer.

A water bath that maintains the temperature up to 45 ± 1,0 ° C.

Micropipette that allows the taking of a volume that is in the range of between 20 - 200 µL.

Micropipette that allows taking a volume that is in the range of .2 - 20 µL.

7.2 Regarding Electroporation

Electroporator that allows discharges up to 3.0 kV and pulse selection in a range of 1.0 to 4.0 ms with an accuracy of ± .1 ms.

Biological safety hood equipped with an ultraviolet light for sterilization.

Micropipette with sampling range of between 20 - 200 µL.

Micropipette with sample collection range between 0.2 - 20 µL.

8. Preparation of the sample (Chemically Competent Cells)

8.1 Inoculate 15 mL of LB medium with a starter culture of the microorganism that is desired to transform, free of antibiotic resistance. Incubate overnight at 37 ° C under constant agitation at 225 rpm.

8.2 Preheat half LB in a water bath at 37º C.

8.3 Inoculate 40 mL of the preheated medium with 1.5 mL of the starter culture obtained in step 1.

8.4 Grow in a shaker at 37° C and 225 rpm.

8.5 Measure OD_{600} every 30 minutes until a value within the range of 0.4 to 0.6 is reached, putting immediately the cells on ice. Remain incubated for 20 min.

8.6 Collect cells by centrifugation at ~ 3,000 rpm for 15 minutes at 4° C.

8.7 Discard the supernatant and gently resuspend the tablet in 20 mL of ice-cold 50 mM $CaCl_2$.

8.8 Put the cells back to incubate on ice for 20 minutes.

8.9 Collect cells by centrifugation at ~ 3,000 rpm for 15 minutes at 4 ° C.

8.10 Decant the supernatant and gently resuspend the tablet in 4 mL of ice-cold 100 mM $CaCl_2$.

8.11 Once the tablet is completely resuspended, transfer 50 µL mix with cells to a 1.5 mL microtube. Repeat this depending on the number of transformations that you want to perform considering that by the vector, normally, a microtube is necessary.

Once this protocol is completed, the cells should be used immediately or up to 24 hours later.

9. Heat shock transformation

9.1 Add 1 µL (10 pg / µl) of DNA in 50 µL of competent cells in a 1.5 mL microtube by gently mixing with flicks.

9.2 Place the competent cell / DNA mixture on ice for 30 minutes.

9.3 Heat shock each transformation tube by placing it in a water bath at 42 ℃ for 45 seconds.

9.4 Incubate the tubes on ice for 5 minutes.

9.5 Add 950 µL of LB medium and grow in an incubator with shaking at 37 ℃ (225 rpm) for 1 hour.

10. Preparation of the sample (Electrocompetent Cells)

10.1 Inoculate 15 mL of LB medium with a starter culture of the microorganism that it is desired to transform free of antibiotic resistance. Incubate overnight at 37° C under constant agitation at 225 rpm.

10.2 Preheat LB medium in a 37° C water bath.

10.3 Inoculate 40 mL of the preheated medium with two-hundredths of the starter culture obtained in step 1.

10.4 Grow in an agitator at 37° C and 225 rpm.
10.5 Measure OD_{600} every 30 minutes until a value within the range of 0.4 to 0.6 is reached, immediately putting the cells on ice. Remain incubated for 20 min. llñpoo

10.4 Collect cells by centrifugation at ~ 12,000 rpm for 15 minutes at 4 ° C.

10.6 Decant the supernatant and gently wash the tablet with Ultrapure water. The volume of water should remain the same as the decanted one, in this case, 40 mL.

10.7 Repeat the two previous steps once more.

10.8 Collect cells by centrifugation at ~ 12,000 rpm for 15 minutes at 4° C.

10.9 Decant the supernatant and resuspend the tablet with 4 mL of Ultrapure water.

10.10 Once the tablet is the resuspended, transfer 50 µL mix with cells to a 1.5 mL microtube.

11. Transformation by electroporation

11.1 Add 1 µL (10 pg / µl) of DNA in 50 µL of competent cells in a 1.5 mL microtube by gently mixing with flicks.

11.2 Retrieve all the DNA / competent cells mixture and place in a 2 mm electroporation cell.

11.3 Place the cells in the electroporator and run the program. The configuration of this equipment must vary depending on the microorganism that you want to transform.

11.4 Add 1 mL of LB medium to the cell and resuspend the contents.

11.5 Recover the volume of the cell and place again in a 1.5 mL microtube. Grow in an incubator with shaking at 37 ℃(225 rpm) for 1 hour.

12. Obtaining Results

Regardless of the conventional method, proceed with the following steps:

12.1 Perform a drop size test of the transformation mixture to be inoculated on the LB agar.

12.1.1 Extend 5, 15, 25 and 50 µL of the transformation mixture on an individual LB agar plate. The technique is specified in NOM-092-SSA1-1994. Method for aerobic bacteria counts in a plate.

12.1.2 Incubate the plates at 37 ℃for 18-24 hours.

12.1.3 Wait for the time to pass and select that plate where you can identify isolated CFUs.

13. Results report

The results report must be issued based on the provisions of the PROJECT OF THE MEXICAN OFFICIAL STANDARD PROY-NOM-164-SEMARNAT / SAGARPA-2012, WHICH ESTABLISHES THE CHARACTERISTICS AND CONTENT OF THE RESULTS REPORT OF THE OR THE RELEASES MADE BY ORGANISMS GENETICALLY MODIFIED, IN RELATION TO THE POSSIBLE RISKS TO THE ENVIRONMENT AND BIOLOGICAL DIVERSITY AND, ADDITIONALLY, TO ANIMAL, VEGETABLE AND AQUACULTURE HEALTH.

14. Risks evaluation

This Mexican Official Standard explains in a timely and general way the genetic transformation by thermal shock and electroporation seeking the regulation of the creation of Genetically Modified Organisms (hereinafter "OGM"). It should be noted that, although these methods are considered conventional, they must meet requirements to regulate the process as biosecure for its application:

Because it works at the molecular level it is necessary that the person who develops the technique has a theoretical and practical domain that allows you to make decisions based on his/her knowledge. At all times, this protocol must be performed in a biosafety level 2 laboratory within a sterile environment. The latter is characterized by complying with a specific training of personnel in the management of pathogens. As for the facilities, although they are not necessarily isolated, their access is restricted especially when work is being done.

Considering the possibility to work with pathogens or other toxic agents/ compounds, these laboratories must comply with certain equipment and work areas; biosafety hood for gas extraction and handling of microorganisms, centrifuges, incubators of microorganisms, punches, laundry, extinguishers, tables and surfaces of easy decontamination, personal protection equipment, etc.

One of the most imminent fears when talking about genetic modification is resistance to antibiotics. This fear is justified with the reasoning that if a GMO is transformed by a plasmid vector whose selection gene confers the ability to grow in an environment with the presence of antimicrobial compounds, this ability can be transmitted naturally to other bacteria or microorganisms once the GMO is free interacting in the environment. To avoid this problem, the creation of a GMO whose final intention is the release to the environment should only be through the insertion of a vector of cloning or expression with positive selection. The basis of these vectors is the presence of a lethal or suicidal gene that can substitute an antibiotic resistance gene supplemented by a reporter gene as fluorescence (hereinafter "RFP gene"). The coding sequence of this alternate contains multiple cloning sites so that only if the gene is interrupted by the ligation of the desired insert, the host cell will be able to grow. Otherwise, as the insertion of the gene of interest does not happen, the suicide gene will inhibit the growth of the bacteria. Usually, the lethal genes used are sacB and ccdB that code for the enzyme levan and the toxin ccdB respectively.

Finally, the microorganisms that undergo genetic transformation must be only those that are considered within the classification of risk 1 or risk 2 given by the American Biological Safety Association (hereinafter "ABSA") and whose pathogenicity is not directed towards the environment and being alive with which I will be in direct contact. In the opposite case of this last requirement, the biosecurity must be verified within the experimental development of the GMO.

15.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. The present NOM was carried out in accordance with these principles when listing within its objectives the general method of transformation of a microorganism of risk 2.

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 its a scientific procedures, and in our project we use the means of inserting a cloning vector or expression with positive selection, which is considered a solid scientific procedure.

III.- According to the fifth paragraph of Article 16 of the Protocol states that the Parties must "determine 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, since the concern is mentioned that genetic manipulation can be made resistant to antibiotics, and that is why this NOM specifically establishes the means to be developed, this being the "means of inserting a vector of cloning or expression with positive selection". They must also "adopt the appropriate measures for the treatment of those living modified organisms or specific traits", which is also fulfilled according to the provisions of the Risk Assessment section since it establishes that the place where it will be carried out this insertion of a cloning vector, must be restricted, as well as having certain equipment and work areas, as well as certain parts of the procedure of preparation and transformation of the samples.

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 both from the introduction that makes clear the obligation of every person to perform the "Method of genetic transformation of risk microorganisms 2 by conventional methods" to carry out a certain method in an effective and biosecure The same establishes in its section of "Risk assessment" the problems or fears that the manipulation of the genetic material presents, and establishes the necessary materials and spaces to handle the GMOs in a careful and responsible manner.

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, as established in the first paragraph of the "Risk Assessment" which states that "because it is working at the molecular level it is necessary that the person developing the technique has a theoretical and practical domain that allows you to make decisions based on your knowledge". It also complies with the sixth general principle of the Protocol, given that the risk assessment of this NOM specifically talks about the prevention and management of genetic material when carrying out the conventional method for the genetic transformation of microorganisms at risk 2.

16. Bibliography

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17. Observance of the norm

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

18. State of being in force

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