Marker Gene Technologies, Inc

Instruction Manual

pNosdcGUS Plant GUS Expression Vector

Version 1.01

A plasmid expression vector for cloning and expression of GUS into plants.

Catalog no. M1390

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Important Information:

MSDS Sheets and product safety information are available by request from Marker Gene Technologies, Inc. and by accessing our web site at www.markergene.com.

Shipping and Storage

The pNosdcGUS plant GUS expression vector is shipped at room temperature. Store all samples of the vector at –20°C or below, once resuspended. Products are guaranteed for six months from date of shipment when stored properly.

Contents

Item	Concentration
pNosdcGUS plant GUS Expression Vector, lyophilized in TE buffer, pH 8.0	20μg

Quality Control

The pNosdcGUS plant GUS expression vector has been qualified by restriction endonuclease digestion. pNosdcGUS is further qualified by transformation using an appropriate *Agrobacterium* strain in culture into *Arabidopsis thaliana* plant species and verified for activity.

Accessory Products
Additional products that may be used with the pNosdcGUS Plant GUS Expression Vector are now available from Marker Gene.

Ordering information is provided below.

Product	Unit Size	Catalog no.
Expression of your recombinant fusion protein can be detected using:		
MarkerGene™ ß-Glucuronidase (GUS) Reporter Gene Activity Detection Kit	1 kit	M0877
4-Methylumbelliferyl ß-D-Glucuronide (MUGlcU)	25 mg	M0240
Carboxyumbelliferyl ß-D-Glucuronide (CUGlcU)	10 mg	M0256
Fluorescein di-ß-D-Glucuronide, di-methyl ester	5 mg	M0969
Inhibition of cloned GUS activity can be afforded using:		
Phenethyl-1-thio-ß-D-Glucopyranosiduronic Acid (PETGU)	10 mg	M0261

Methods

Overview

Description

This high copy number plant vector, pNosdcGUS, expresses the GUS gene under the control of the nopaline synthase (pNos) promoter. This vector may be used to monitor virus production and transfection efficiency. GUS expression can be easily detected by addition of X-Gluc to the medium or by using our MarkerGene™ ß-Glucuronidase (GUS) Reporter Gene Activity Detection Kit (M0877).

- pNosdcGUS expression vector also contains the kanamycin gene, which acts as a selection marker (@ 75μg/mL kanamycin resistance) in *Agrobacterium* host.

- The GUS gene can be excised using the 5' *XbaI* and 3' *HindIII* sites to allow the insertion of other genes to be expressed under the same regulatory elements in plants.

- For a map of pNosdcGUS, see page 10.

The pNosdcGUS Plant GUS Expression Vector System

The pNosdcGUS vector is a cloning vector that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest simply:

1. Clone your gene of interest into an entry vector to create an entry clone.

2. Perform an LR recombination reaction between the entry clone and a destination vector *(e.g.* pCMVβ, pGPTV-KAN, etc.) to generate the expression vector.

3. Transfect your expression clone into the cell line of choice for stable expression of your gene of interest.

Using pNosdcGUS Plant GUS Expression Vector

The pNosdcGUS plant GUS expression vector is supplied as a supercoiled plasmid. Although Marker Gene has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of this vector is NOT required to obtain optimal results for a downstream application.

Propagating pNosdcGUS :
If you wish to propagate and maintain pNosdcGUS, we recommend using *Agrobacterium tumefaciens* for transformation.

Entry Clone:

To recombine your gene into pNosdcGUS, you should have an entry clone containing your gene of interest.

Points to consider before recombining*:*

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). Other sequences are also possible, but the G or A at position –3 and the G at position +4 are the most critical for functional expression. If you wish to include the V5 epitope and 6xHis tag, your gene in the entry clone should not contain a stop codon. The gene should also be designed to be in frame with the C-terminal epitope tag after recombination. If you do NOT wish to include the V5 epitope and 6xHis tag, please be sure that your gene contains a stop codon in the entry clone.

Transfection

Introduction: This section provides general information for transfecting your expression clone into the plant of choice. We recommend that you include a positive control vector and a mock transfection (negative control) in your experiments to evaluate your results.

Methods of Transformation and Transfection:

Preparation of competent cells, transformation and recovery and floral dip with *Agrobacterium thumefaciens* protocols can be found in Weigel and Glazebrook's "Arabidopsis: A Laboratory Manual" (2002). For more information contact our Technical Assistance Staff (www.markergene.com or techservice@markergene.com).

Preparation of competent *Agrobacterium tumefaciens* cells:

1. Inoculate 250 mL of LB medium with 750 uL *Agrobacterium tumefaciens* (for example GV3101::pMP90).

2. Incubate at 28^{o}C (Dubenoff shaking water bath) with vigorous shaking for 11 hours until the OD is approximately 0.75.

3. Pellet the cells by centrifugation (5000 rpm).

4. Wash the pellet with 2 mL sterile TE.

5. Centrifuge at room temperature for 10 min at 5000 rpm.

6. Resuspend the pellet in 20 mL LB medium. Aliquot 250 uL of this suspension into 1.5 mL Eppendorf microcentrifuge tubes.

Transformation and recovery of *Agrobacterium tumefaciens* cells:

1. Add 20 uL of the **M1390** pNosdcGUS Plant GUS Expression Vector (5 ug) to the competent GV3101::pMP90 *Agrobacterium tumefaciens* sample (250 uL) iin LB medium from above.

2. Incubate on ice (0^{o}C) for 5 minutes.

3. Incubate in liquid nitrogen (-80^{o}C) for 5 min.

4. Incubate at 37^{o}C (water bath) for 5 min.

5. Add LB medium (1 mL) to each vial and incubate at room temperature (with rotation) for 4 hours.

6. Streak the bacteria onto LB Agar plates containing Kanamycin (50 ug/mL) and incubate at 28^{o}C for 3 days.

7. Pick one colony and re-streak onto an LB Agar plate containing Kanamycin (50 ug/mL) and incubate at 28^{o}C for 2 days.

Floral dip with *Agrobacterium thumefaciens:*

1. Prepare Infiltration Medium (250 mL) (see recipe below in Appendix)

2. Prepare MS Agar Gel for plant growth (see recipe below in Appendix)

3. Pipette 15 – 20 mL into 25 x 250 mm sterile test tubes for each plant to be grown after dip.

4. Grow up one colony of M1390 transformed bacteria (See step 7 from above) in LB media contain Kanamycin (50 ug/mL) (2 mL) overnight at 28^{o}C (Dubenoff shaking water bath) with vigorous shaking.

5. Inoculate 225 mL of LB medium with 1.25 mL of preculture from step 4 above. Incubate overnight at 28^{o}C (Dubenoff shaking water bath) with vigorous shaking.

6. Pellet the bacteria by centrifugation at 6000 rpm at 25^{o}C for 10 min.

7. Resuspend the bacteria in Infiltration Medium (50 mL) and swirl to ensure complete mixture of the bacteria.

8. Transfer the bacterial suspension to a sterile dipping box (the lid of a pipette tip box works well). This process is best done in a laminar flow hood under sterile conditions.

9. Dip the roots of plants in the bacterial suspension for 30 seconds.

10. After dipping, place the plants on agar plates (MS medium) and seal the lids with parafilm. Allow to set overnight at room temperature.

11. After overnight storage, rinse the plant roots with sterile water and transfer the transformed plants to sterile tubes containing the MS Agar Gel for growth.

12. Grow the plants under long-day light conditions (16 hour daylight, 8 hour darkness) for several weeks until flowering and seed pod production occurs.

13. Remove seed pods and dry seeds.

14. Replant seeds on MS Agar Gel plates and collect seeds as above. Regrowth of these plants will provide stably transformed GUS plants and seeds for use.

Positive Control:
We recommend the use of a positive control vector for plant transfection and expression which may be used to optimize recombinant protein expression levels in your particular plant. A vector that allows expression of a C-terminally tagged β-glucuronidase fusion protein that may be detected by Western blot or functional assay provides the easiest way to measure protein expression levels. Consult our technical assistance for more information about C-terminal fusion protein expression systems.

To propagate and maintain the plasmid:

1. Resuspend the vector in 20 μl sterile water to prepare a 1μg/μL stock solution and store at -20°C. Use the stock solution to transform *Agrobacterium tumefaciens*, or equivalent vehicle.

2. Select transformants on LB agar plates containing 50-100 μg/ml kanamycin.

3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Expression and Analysis

Expression of your gene of interest from the expression clone can be performed in transiently transfected cells or stable cell lines.

GUS Assay using Cell Lysates
Cell lysate from above step can be assay for GUS activity using the MarkerGene™ ß-Glucuronidase (GUS) Reporter Gene Activity Detection Kit, M0877 . For more detailed description of the MarkerGene™ Live Cell GUS Assay Kit please visit our website: http://www.markergene.com/kits.php

Detecting Recombinant Fusion Proteins:
To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen or Amersham Biosciences or an antibody to your protein of interest.

Assay for β-glucuronidase:
If you use a positive control vector, you may assay for β-glucuronidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). X-GlcU staining, or fluorescence detection are common methods of analysis. Marker Gene offers several reagents and kits for fast and easy detection of β-glucuronidase expression. See Accessory Products (page 3) for more information about these products.

Purification of Recombinant Fusion Proteins:
The presence of the C-terminal polyhistidine (6xHis) tag in your recombinant fusion protein allows for purification using a metal-chelating resin (available from Invitrogen). Note: Other purification methods may also be suitable including affinity chromatography.

Creating Stable Cell Lines:
The neomycin resistance gene can be cloned into the pNosdcGUS vector to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the plant cell line of choice and select for foci using Geneticin®. General guidelines are provided below.

To obtain stable transfectants, we recommend that you linearize your pNosdcGUS construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in plant cells. To linearize your construct, cut at a unique site that is neither located within a critical element nor within your gene of interest.

Geneticin® (G418) blocks protein synthesis in plant cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in plant cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from T*n*5, results in detoxification of Geneticin® (Southern and Berg, 1982).

Geneticin® Selection Guidelines:
Geneticin ® is available from GIBCO (Catalog no. 11811-023). Use as follows:
1. Prepare Geneticin® in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
2. Use 100 to 1000 μg/ml of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin®. Cells will divide once or twice in the presence of lethal doses of Geneticin® Selective Antibiotic, so the effects of the drug

takes several days to become apparent. Complete selection for positive clones of cells can take up to 2 to 3 weeks of growth in selection medium.

Appendix: Recipes

LB (Luria-Bertani) Medium and Plates Composition:
1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0
1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed. Store at room temperature or at +4°C.

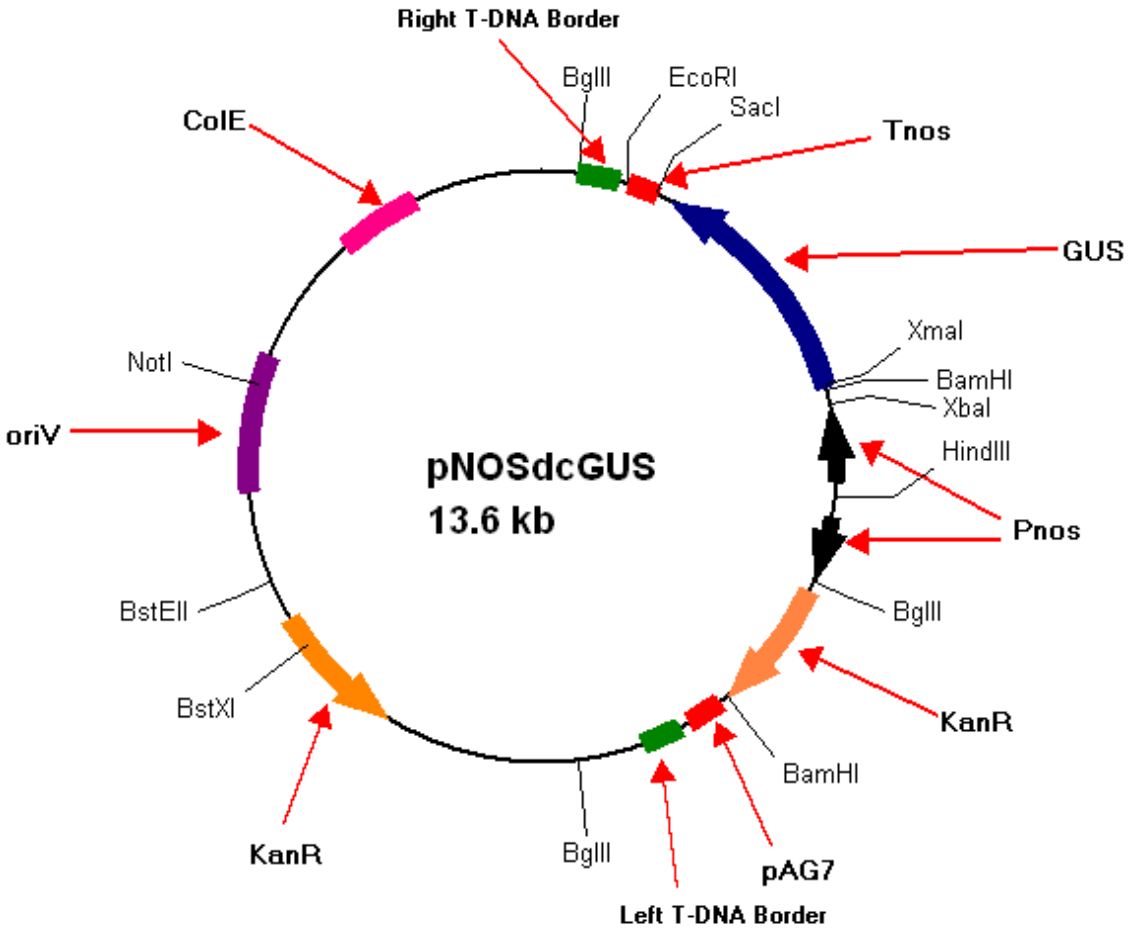
LB agar plates:
1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

4X SDS-PAGE Sample Buffer:
1. Combine the following reagents:
0.5 M Tris-HCl, pH 6.8, 5 ml
Glycerol (100%), 4 ml
β-mercaptoethanol, 0.8 ml
Bromophenol Blue, 0.04 g
SDS, 0.8 g
2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Infiltration Medium:
½ MS salts (MS salts: 1650.00 mg/L ammonium nitrate, 332.02 mg/L calcium chloride anhydrous, 180.70 mg/L magnesium sulfate anhydrous, 1900.00 mg/L potassium nitrate, 170.00 mg/L potassium phosphate monobasic, 6.20 mg/L boric acid, 0.025 mg/L cobalt chloride·$6H_20$, 0.025 mg/L cupric sulfate·$5H_20$, 37.26 mg/L Na^{2-} EDTA, 16.90 mg/L manganese sulfate-H_20, 0.250 mg/L molybdic acid sodium salt, 0.83 mg/L potassium iodide, 27.80 mg/L ferrous sulfate·$7H_20$, 8.60 mg/L zinc sulfate·$7H_20$) (Sigma, St. Louis, M0654)
1X Gamborg's B5 Vitamin (Sigma, St. Louis, G1019)
5% Sucrose (w/v)
0.044 μM 6-benzylaminopurine (BAP) (stock solution 1mg/mL DMSO)
0.05% Silwet L77 (Lehle Seeds)
1. For 250mL Infiltration media, mixed 12.5mL 10X MS salts, 0.25mL Gamborg's B5 Vitamin 1000X, 12.5mg Sucrose, 2.5μL 1mg/mL stock solution of Benzylaminopurine (BAP), 12.5μL Silwet L77, 224.73mL dH_20. Store at +4°C.

MS Agar Plant Growth Medium:
1. Autoclave 10.6g Murashige & Skoog (MS) Medium and 250 mL dH_20 on the liquid cycle for 20 minutes at 15 psi.
2. After autoclaving, cool to ~55°C, adjust to pH 5.7 with 1N NaOH.
3. Autoclave solution, again, on the liquid cycle for 20 minutes at 15 psi.
4. After autoclaving, cool to ~55°C, and pour into 10 cm plates.
5. Let harden, store at 25°C.

Map and Features of pNosdcGUS:
The map below shows the elements of pNosdcGUS. The GUS gene can be excised using the 5' *XhoI* and 3' *NotI* sites to allow the insertion of other genes to be expressed under the same regulatory elements in plant cells.



Restriction Digests of pNosdcGUS give the following sizes (kb):

EcoRI 13.6

BglII 8.2, 3.2, 2.7

HindIII 13.6

Technical Service

Please visit our website at www.markergene.com
Or write to techservice@markergene.com

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