Aug 14, 2024

Optimized Cloning Protocol for Standard Curve Generation in qPCR of Environmental Functional Genes

DOI
dx.doi.org/10.17504/protocols.io.5qpvok2odl4o/v1

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Protocol Citation: Abul Bashar, Raju Podduturi, Amaru Miranda Djurhuus, H. M. Rakibul Islam, Mohammad Mahfujul Haque, Lars Hestbjerg Hansen, Niels O. G. Jørgensen 2024. Optimized Cloning Protocol for Standard Curve Generation in qPCR of Environmental Functional Genes. **protocols.io https://dx.doi.org/10.17504/protocols.io.5qpvok2odl4o/v1**

Protocol status: Working
We use this protocol and it's working

Created: August 09, 2024

Last Modified: August 14, 2024

Protocol Integer ID: 105064

Keywords: Standard curve, Cloning, Plasmid DNA, qPCR, Functional gene

Funders Acknowledgement:
Climate-friendly and climate-resilient prawn farming in Bangladesh
Grant ID: DANIDA Funded (21-01-KU)

Abstract

Due to the complex and varied biochemical requirements and physiological behaviors of certain environmental bacterial and archaeal groups that carry specific functional genes as potential biomarkers, it is challenging to extract and maintain pure cultures of these organisms. Therefore, generating standard curves for qPCR necessitates cloning gene sequences from environmental DNA samples.

This protocol was developed for cloning target genes from environmental samples to produce plasmid DNA, which was then used to prepare standard curves for quantifying functional genes, including mcrA, pmoA, amoA, and nosZ from water and soil samples. The procedure involves inserting amplified gene sequences into pGEM®-T Vectors and transforming them into JM104 E. coli competent cells. Testing this protocol yielded higher amounts of pure plasmid DNA, making it suitable for standard curve preparation for qPCR of the target genes.

Materials

Biological materials:

1. DNA extracted from water and soil
2. pGEM®-T Vectors (Promega Corporation)
3. JM104: E. coli competent cells (Promega Corporation)

Chemical and reagents:

1. Absolute ethanol
2. Dream Taq Polymerase (5 U/µL)
3. Dream Taq buffer (10X)
4. dNTPs (10 nM)
5. Nuclease-free Water
6. mcrA primer pairs (mlas: GGTGGTGTMGGDTTCACMCARTA and mcrA-rev: CGTTCATBGCGTAGTTVGGRTAGT)
7. pmoA primer pairs (A189F: GGNGACTGGGACTTCTGG and mb661R: CCGGMGCAACGTCYTTACC)
8. amoA primer pairs (amoA-1F: GGGGTTTCTACTGGTGGT and amoA-2R: CCCCTCKGSAAAGCCTTCTTC)
9. nosZ primer pairs (nosZ1F: WCSYTGTTCMTCGACAGCCAG and nosZ1R: ATGTCGATCARCTGVKCRTTYTC)
10. DNA Clean and Concentrator kit
11. AMPureXP beads
12. Ligation buffer
13. T4 DNA ligase
14. Luria-Bertani (LB) broth
15. Luria-Bertani (LB) media
16. SOC media
17. ampicillin (100 mg/µL)
18. Xgal (5-Bromo-4-Chloro-3-Indolyl-beta-D-Galactoside: 20 mg/uL)
19. IPTG (isopropyl beta-D-1-thiogalactopyranoside: 100 mM)
20. Cut smart buffer
21. EcoRI HF restriction enzyme
22. 1% agarose gel

Equipment:

1. Thermal cycler
2. Nanodrop spectrophotometer
3. Qubit fluorometer
4. Refrigerator
5. Freezer
6. Incubator

7. Shaker
8. Autoclave
9. Water bath
10. Centrifuge machine
11. Vortex machine
12. Gel documentation system

Step-1: Amplification of Target Gene Sequence

1 Amplify the target gene sequences from environmental DNA using a PCR reaction. The reaction mixture should include polymerase (0.5 µL), buffer (5 µL), dNTPs (5 µL), forward primer (1 µL), reverse primer (1 µL), template DNA (3 µL), and water (32.5 µL).

Note: The presence of humic acid or other contaminants may impact the efficiency of the PCR reaction. Therefore, it is recommended to assess the purity of DNA samples using a Nanodrop spectrophotometer.

2 **Thermal condition:**

[1]Initial Denaturation: 95 °C 3-5 min.
Denature: 95 °C 30sec.
[2]Cycles [3]Anneal: 55-60 °C 30 sec.
Extend: 72 °C 30 sec.
Extension: 72 °C 5 min.
Hold: 4 °C Indefinitely

[1]Initial Denaturation: The temperature and duration of the initial denaturation step depend on the sample type. DNA extracted from soil may require a longer denaturation time due to potential chemical contamination.

[2]PCR Cycles: Perform 30 cycles for the mcrA gene, 35 cycles for the pmoA and nosZ genes, and 40 cycles for the amoA gene.

[3]Annealing Temperature: Set the annealing temperature to 55°C for mcrA and nosZ, 60°C for pmoA, and 57°C for amoA.

3 Primer dimers and other PCR components often form during the reaction and should be removed. This can be done using DNA Clean and Concentrator kits according to the manufacturer's instructions or by using AMPure XP beads.

Note: When using beads for cleanup, add 9 µL of beads to a 15 µL sample. After washing with ethanol, ensure all ethanol is completely removed by pipetting and allowing the sample to air dry.

Step 2: Ligation to Vector

4 Thaw the ligation buffer, vector (pGCM T vector), PCR products, and T4 DNA ligase on ice.

5 In a 1.5 mL Eppendorf tube, combine 2.5 µL of ligation buffer, 0.5 µL of vector (pGCM T vector), 2.0 µL of cleaned PCR products, and 0.5 µL of T4 DNA ligase. Gently mix the reaction and

incubate at room temperature (22–23°C) for 5 minutes. It is recommended to include a negative control to check for uncut plasmid contamination.

Note: The cloning reaction can be stored at −20°C until further use.

Step-3: Transformation

6 After removing the competent cells (JM104: E. coli) from the freezer (-80°C), place them on ice for 20 minutes. Mix 3 µL of the cloning mixture (including the negative control) with 50 µL of competent cells in a 1.5 mL tube.

7 For heat shock, keep the reaction on ice for 30 minutes, then transfer it to a 42°C water bath for 45 seconds. Immediately transfer the tube back to ice for 2 minutes.

Note: To optimize the transformation efficiency, it is crucial to maintain precise temperature and timing. It is recommended to set the water bath to 42°C before starting the heat shock to avoid any delays.

8 Add 300 µL of LB media (without antibiotics) or SOC media to the reaction and incubate at 37°C for 90 minutes.

Note: It is highly recommended to place the reaction on a shaker during incubation.

Step-4: Culture and isolation of pure transformed cell

9 Prepare plates with solid LB media containing yeast extract (10 g/L), NaCl (5 g/L), tryptone (10 g/L), and bacteriological agar (15 g/L). To inhibit the growth of non-competent cells, add 1 mL of ampicillin (100 mg/µL) to the media. For blue-white colony screening to differentiate between recombinant and non-recombinant colonies, add 4 mL of X-gal (20 mg/µL) and 5 mL of IPTG (100 mM) during media preparation.

Note: The pH of the media should be maintained around 7. Store the plates in the refrigerator until use.

10 Label the plates accordingly, then pipette 100 µL and 200 µL of the transformed reactions onto separate plates. Spread the mixture evenly using a sterile spreader. Incubate the plates at 37°C overnight.

11 In 50 mL falcon tubes, prepare 10 mL of LB-amp broth containing 5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, and 1 mL/L ampicillin (100 mg/µL). From the blue and white colonies, select pure white colonies (indicating successful transformation) and transfer a single colony from each plate into the LB-amp media. Place the falcon tubes in a shaking incubator at 37°C overnight.

Note: LB-amp media can be prepared in advance and stored at room temperature in a sealed condition.

Step-5: Plasmid DNA extraction and verification

12 Centrifuge the cultures at 8,000–9,000 rpm for 5 minutes and carefully remove all the supernatant. Use the resulting pellets for plasmid DNA extraction following the QIAprep Spin Miniprep kit manufacturer's instructions. For additional details, refer to the protocol documented by Kodackattumannil et al.[1].

13 Determine the purity and quantity of the plasmid DNA using a NanoDrop spectrophotometer and a Qubit fluorometer, respectively.

Note: If the quality of the plasmid DNA is suboptimal, consider cleaning it using a DNA Clean and Concentrator kit or beads.

14 Verify successful transformation through Sanger sequencing and diagnostic restriction digestion followed by gel electrophoresis. For the latter, mix 1 µL of plasmid DNA (300-500 ng/µL), 5 µL of CutSmart buffer, 1 µL of EcoRI HF restriction enzyme, and 43 µL of water. Incubate the mixture at 37°C for 5 minutes. Running the reaction on a 1% agarose gel should yield two distinct bands, representing the vector DNA and the target genes, confirming successful insertion of the target gene sequence into the vector.

15 Calculate the number of plasmid DNA copies using the following formula:

Number of copies=(ng×[6.022×1023])/(length ×[1×109]×650)

16 Store the plasmid DNA at -20 °C for downstream usage including for generating standard curve.

Protocol references

1. Kodackattumannil, P. *et al.* Protocol for the High-quality Plasmid Isolation from Different Recalcitrant Bacterial Species: Agrobacterium spp., Rhizobium sp., and Bacillus thuringiensis. *Bio Protoc***13**, (2023).