Please cite this article as: Puentes *et al.*, (2019). Isolation of PCR-quality Genomic DNA from Soils Impacted with Extra Heavy Crude Oil, *Bio-protocol* 9 (12): e3265. DOI: 10.21769/BioProtoc.3265.

Isolation of PCR-quality Genomic DNA from Soils Impacted with Extra Heavy Crude Oil

Laynet A. Puentes*, Yusibeska L. Ramos, Ysvic A. Inojosa, César E. Rivera and Angela De Sisto

Department of Energy and the Environment, Institute for Advanced Studies Foundation, IDEA, Caracas, Venezuela
*For correspondence: laynetpuentes@gmail.com

[Abstract] For the study of microbial communities in samples of soils impacted with extra heavy crude oil, it is necessary to perform molecular analyses. Due to the difficulty of oil matrix handling, there are very few protocols reported in writing. Also, one can only observe a very low concentration of DNA. That's why it is required to have an effective protocol to conduct studies in this type of matrix. This protocol includes steps of cell lysis by saline buffer with ionic/non-ionic detergents, and enzymatic digestion with lysozyme and proteases, complemented with organic extraction and alcohol precipitation. Additionally, it requires purification to eliminate the inhibitory substances of the extract that cause PCR inhibition. The method of DNA extraction proposed in this study is easy to handle and low cost. It allows the extraction of DNA from different bacteria and fungi, associated with soil contaminated with extra heavy crude.
Keywords: Metagenomic, Microorganisms, Crude Oil, Soil, Contamination, PCR

[Background] On bioremediation, the identification of helpful bacteria is not always easy. Kathiravan *et al.* (2015) developed a way of extracting total genomic DNA from samples of farmland. However, working with crude oil can be a little bit more problematic. The metagenomic DNA obtained must be purified, since humic acid contaminants and proteins have serious negative effects on the DNA polymerase (Wang *et al.,* 2013). In the same way, hydrocarbon-contaminated soils present an even greater challenge. Because, in addition to the usual soil contaminants, traces of organic substances and heavy metals remain in the DNA extracts and reduce PCR amplification efficiency, by degrading or capturing nucleic acids, or by deactivating DNA polymerase (Fortin *et al.*, 2004). Polymerase chain reaction (PCR), is used in order to detect genes involved in metabolic pathways of interest and obtain an analysis of soil microbial diversity. The PCR is a necessary study to remedy and restore oil contaminated soils through environmentally friendly biological technologies (Lozano-A *et al.*, 2008).

In order to bypass the PCR inhibitors in DNA extraction from soil, different methodologies are employed to eliminate them: washing steps, thermal shocks, chemical lysis with detergents, and an enzymatic digestion step that frequently employs lysozyme and proteinase K to quicken the process and increase the DNA yield. However, these steps tend to make DNA extraction protocols time-consuming, costly, tiresome and laborious.

The objective of this study was to evaluate the protocol of Kathiravan *et al.* (2015) modified to match a soil matrix highly contaminated with extra heavy oil. The protocol includes a cell lysis by means of saline buffer with ionic/non-ionic detergents, and enzymatic digestion with lysozyme and proteases, plus a purification step to remove the contaminants. The quality and purity of the DNA were validated by the

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

amplification efficiencies of several genes of interest for PCR. It was also tested if this DNA extraction proposed is easy to handle and low cost.

Materials and Reagents

1. Pipette tips
2. Eppendorf tubes 1.5 ml
3. Porcelain Mortar and pestle 500 ml
4. Reusable Stainless Pellet Pestles, 1.5 ml (Thomas Scientific, catalog number: 749515-0000)
5. 2 mm 100 200 micron sand mesh ss oil size test slotted soil testing filter sieve/sediment sieves
6. Proteinase K (Invitrogen, catalog number: 25530-015)
7. Lysozyme (Sigma, catalog number: 235-747-3)
8. Hexadecyltrimethylammonium Bromide (CTAB) (Sigma, catalog number: 52366)
9. SDS (Sigma-Aldrich, catalog number: L3771)
10. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, catalog number: P3803)
11. Chloroform:Isoamyl Alcohol 24:1 (Sigma, catalog number: C0549)
12. Isopropanol (Sigma, catalog number: I0398)
13. Ethanol (Sigma, catalog number: V0T0041)
14. GoTaq Flexi DNA Polymerase (Promega, catalog number: M829)
15. dNTP Set (100 mM) Solution (Invitrogen, catalog number 10-297-018)
16. Agarose D-1 LE GQT (Scientific Trade Corp, catalog number: 8015)
17. FavorPrep Gel/PCR Purification mini kit (Favorgen, catalog number: FAGCK001-1)
18. 100 bp DNA Ladder (Promega, catalog number: G2101)
19. 1 kb DNA Ladder (Sigma-Aldrich, catalog number: D0428-1VL)
20. Na_2EDTA (Sigma-Aldrich, catalog number: 6381-92-6)
21. NaH_2PO_4 (J.T. Baker, catalog number: 10049-21-5)
22. NaCl (Sigma-Aldrich, catalog number: S-6191)
23. Tris-Base (1 M) (Promega, catalog number: 77-86-1)
24. Boric Acid AR/ACS (Loba Chemie, catalog number: 10043-35-3)
25. EDTA (Sigma-Aldrich, catalog number: E9884)
26. $MgCl_2$
27. Ethidium bromide
28. Tris-HCl
29. DNA extraction buffer (see Recipes)
30. Buffer TBE 5x (see Recipes)
31. Agarose Gel (see Recipes)

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

Equipment

1. Analytical balance (Ohaus adventurer AR2140, catalog number: 67850)
2. Eppendorf pipettes
3. Thermomixer (Eppendorf 5350, catalog number: 5350-000.013)
4. Vortex (Heidolph Reax Top, catalog number: 541-10000-01-0)
5. Centrifuge (Eppendorf 5415 D, catalog number: 5425-000.014)
6. Centrifuge (Eppendorf 5424, catalog number: 5424-000.614)
7. Concentrator (Eppendorf 5301, catalog number: 5301-000.016)
8. Mastercycler ep gradient (Eppendorf 5341, catalog number 5341-00-108)
9. Horizontal electrophoresis system (Mini-Sub Cell GT Cell, and Power Pac Basic Power Supply) (Bio-Rad, catalog number: 1640300)
10. Bio photometer (Eppendorf 6131, catalog number 6131-000.012)
11. Gel Documentation Systems (Bio-Rad Universal Hood II, catalog number: 1708195EDU)

Procedure

A. Soil pretreatment
   1. The soil sample was sieved through a 2 mm mesh to homogenize the size of the soil particles.
   2. Soil contaminated with extra heavy crude was ground in a mortar and sieved through a 10 mesh screen (2 mm opening).

B. DNA isolation
   1. Take 250 mg of soil sample impacted with hydrocarbon (previously homogenized), place it in a 1.5 ml Eppendorf tube (perform it in quadruplicate to obtain 1 g of soil).
   2. Mix with 270 µl of DNA extraction buffer (Recipe 1), 2 µl of proteinase K (10 mg/ml) and 2 µl of lysozyme (10 mg/ml).
   3. Incubate at 37 °C for 45 min at 800 rpm, vortexing at 32,000 *x g* every 5 min.
   4. Mechanically grind the soil for 1 min, by forcefully twisting by hand the pellet pestle against the inner surface of each 1.5 ml tube three times every 15 min.
   5. After incubation, add 30 µl of 20% SDS (w/v) and incubate at 65 °C for 2 h, vortexing every 10 min.
   6. Centrifuge at 16,100 *x g* for 10 min, and transfer the supernatant to a new 1.5 ml Eppendorf tube.
   7. Mix the supernatant with an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v), and centrifuge at 20,200 *x g* for 15 min at room temperature.
   8. Transfer the supernatant (upper “aqueous” phase) to a new 1.5 ml Eppendorf tube and mix with an equal volume of Chloroform:Isoamyl Alcohol (24:10, v/v).
   9. Centrifuge at 20,200 *x g* for 15 min at room temperature.

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

10. Transfer the supernatant (upper “aqueous” phase) to a new 1.5 ml Eppendorf tube, and precipitate the DNA by adding an equal volume of Isopropanol and placed at -20 °C O/N.
11. Centrifuge the samples at 16,100 *x g* for 20 min at room temperature, discarding the supernatant keeping the pellet.
12. Perform two washes with 70% ethanol through centrifugation at 16,100 *x g* for 15 min at room temperature.
13. Concentrate the pellets using a speed-vac concentrator system at 240 *x g* for 10 min at 30 °C to evaporate the residual ethanol.
14. Dry and resuspend the samples in 10 µl sterile distilled water, in this step the four samples are unified to a volume of 40 µl.
15. Once the protocol is completed, verify DNA integrity using a 0.8% agarose gel electrophoresis (Figure 1) and quantify the DNA obtained (Table 1) with the help of a bio photometer.
16. Stain the gel with ethidium bromide, and then observe in a transluminator with fluorescence for product identification. Gel purification needs to be carried out due to contaminants present in the sample.
17. The purification was made according to the FavorPrep Gel/PCR Purification mini kit, using an excision from the gel on the DNA band.

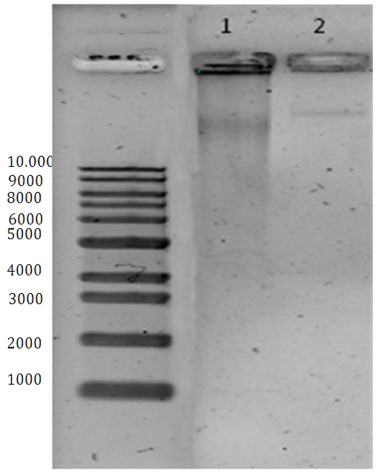

Figure 1. Photographic record of the 0.8% agarose gel of the metagenomic DNA isolation. Lane M: 1 Kb DNA Ladder. Lane 1: Genomic DNA obtained from the soil sample impacted by extra heavy crude. Lane 2: Genomic DNA purified from the extraction of the agarose gel band (used for subsequent PCR assays).

Table 1. Quantification of metagenomic DNA

	Concentration of DNA	Relation 260/280	Relation 260/230
Purified Metagenomic DNA	15 ng/µl	2.9	2.5

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

Note: For the amplification of the genes (16S rDNA, ITS, nahAc, sfp, rhlAB and dszA), the conditions of a 25 µl reaction were: 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 µM primers (Table 2), Taq polymerase 0.2 U/µl and 60 ng/µl of template DNA. The amplification reaction was performed in a thermocycler at 95 °C for 5 min; 95 °C for 30 s, annealing that depends on each gene and extension at 72 °C for 1 min 30 cycles, and final elongation at 72 °C for 10 min. The products were analyzed on a 1% agarose gel stained with ethidium bromide (Figure 2).

Table 2. Primers used for the detection of different metabolic pathways in metagenomic DNA.

Target gene	Primer	Sequence (5′-3′)	AT	bp	Reference
16S rRNA	U1 U2	5′ CCAGCAGCCGCGGTAATACG 5′ ATCGG(C/T)TACCTTGTTACGACTTC	60 °C	990	Weisburg *et al*., 1991
ITS	ITS1 F ITS4 R	5′ CGA CAC ACT GAC CTA CAG CA 5′ CCA GGT TCG CGG CGT GCA	56 °C	650	Gardes and Bruns, 1993 White *et al*., 1990
nahAc	F9 Rn	5′-CCC YGG CGA CTA TGT 5′CCT CRG GCA TGT CTT TTT C	50 °C	850	Moronta, 2006
sfp	sfp F sfp R	5′-CCC YGG CGA CTA TGT 5′CCT CRG GCA TGT CTT TTT C	50 °C	650	This study
rhlAB	Rhlab F Rhlba R	5′-CAG GCC GAT GAA GGG AAA TA 5′-AGG ACG ACG AGG TGG AAA TC	57 °C	749	Mathiyazhagan *et al*., 2011
dszA	dszA F dszA R	5′CGG CAA GTT CGA TCT GTT GTT 5′GM A CCA CCT GGT CGA CGA A	55 °C	903	This study

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

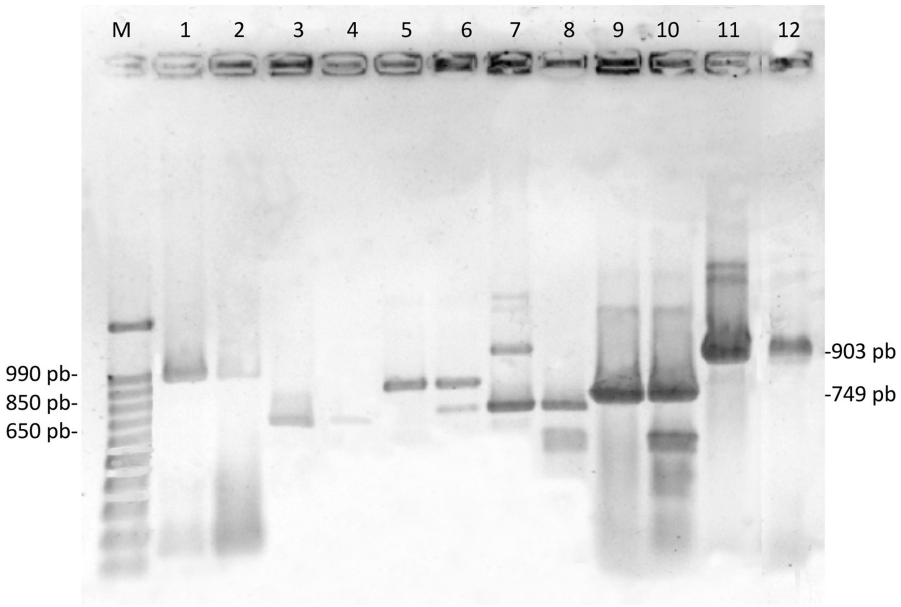

Figure 2. Photographic record of the 1% agarose gel of the PCR amplified products of genes of interest using the metagenomic DNA isolated. M: Molecular weight marker 100 bp; Lane 1: PCR 16S C+ (990 bp); Lane 2: PCR 16S Metagenomic DNA; Lane 3: PCR ITS C+ (650 bp); Lane 4: PCR ITS metagenomic DNA; Lane 5: PCR gene *nahAc* C+ (850 bp); Lane 6: PCR gene *nahAc* metagenomic DNA; Lane 7: PCR gene *sfp* C+ (650 bp); Lane 8: PCR gene *sfp* metagenomic DNA; Lane 9: PCR gene *rhlAB* C+ (749 bp); Lane 10: PCR gene *rhlAB* metagenomic DNA; Lane 11: PCR gene *dszA* C + (903 bp); Lane 12: PCR gene *dszA* metagenomic DNA.

Data analysis

The metagenomic DNA obtained by the above-mentioned protocol showed a high level of contamination by the extra heavy crude oil (which was visualized in the electrophoretic run through a brown spot), making it necessary to purify the genomic DNA band obtained from the agarose gel (Young *et al.*, 1993). A purified DNA yield of 15 ng/µl was obtained, and 260/280 and 260/230 ratios close to a value of 2, indicating low contamination of proteins, humic acids and phenolic compounds.

The DNA obtained was used as a template to perform different PCR reactions with genes of metabolic interest for the remediation of crude contaminated soils. The tests yielded the following results, in their respective lanes: 1) Detection of the rRNA 16S gene present in bacteria; 2) Detection of the ITS region present in fungi; 3) Detection of the *nahAc* gene (coding for the enzyme Naphthalene dioxygenase); 4) Detection of the *spf* gene (which encodes for the production of surfactin); 5) Detection of the *rhlAB* gene (which encodes for the production of mono ramnolipids); and 6) Detection of the *dszA* gene (gene that participates in the 4S bio desulfurization pathway). In Figure 2, the amplification to the expected sizes for the different genes tested was successful, showing that the DNA obtained can be used in PCR reactions without inhibitions, and that it also

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

contains a high representation of the microorganisms present in the sample of soil contaminated with extra heavy crude oil.

Notes

It is advisable to carry out the molecular analysis in a short time after the extraction of genomic DNA, in order to avoid possible degradation of the DNA. In case it will not be used right away, the DNA can be refrigerated at -20 °C.

Recipes

1. DNA extraction Buffer

Tris-HCl	100 mM
Na_2EDTA	100 mM
NaH_2PO_4	100 mM
NaCl	1.5 M
CTAB	1% (p/v)

2. Buffer TBE 5x (1 L)

Tris-Base (1 M)	54 g
Boric acid (900 mM)	27.5 g
EDTA (25 mM, pH 8)	4.68 g

3. Agarose Gel

	0.8%	1%
Agarose	0.8 g	1 g
Buffer TBE (0.5x)	100 ml	100 ml

Acknowledgments

This study received financial support from the Environmental Sanitation Project carried out in IDEA (Development, evaluation and validation of new clean technologies for sanitation on hydrocarbon contaminated soils. Science mission N 2007001401). The authors thank Meralys Gonzalez and Arneida Tarache for their valuable assistance in the selection and homogenization of contaminated soil with extra heavy crude taken adjacent to a trench of the Petropiar El Tigre plant, Anzoategui state, Venezuela.

Competing interests

The authors declare no conflict of interest.

Bio-protocol 9(12): e3265.
DOI:10.21769/BioProtoc.3265

References

1. Mathiyazhagan, N., Danashekar, K. and Natarajan, D. (2011). Amplification of biosurfactant producing gene (rhlB) from *Pseudomonas aeruginosa* isolated from oil contaminated soil. *International Journal of Pharma and Bio Sciences* 2(1): B-504.
2. Fortin, N., Beaumier, D., Lee, K. and Greer, C. W. (2004). Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. *J Microbiol Methods* 56(2): 181-191.
3. Gardes, M. and Bruns, T. D. (1993). ITS primers with enhanced specificity for *basidiomycetes*--application to the identification of mycorrhizae and rusts. *Mol Ecol* 2(2): 113-118.
4. Kathiravan, M. N., Gim, G. H., Ryu, J., Kim, P. I., Lee, C. W. and Kim, S. W. (2015). Enhanced method for microbial community DNA extraction and purification from agricultural yellow loess soil. *J Microbiol* 53(11): 767-775.
5. Lozano-A, L., Bautista, M., Dussan-G, J. and Vives-Flores, M. (2008). DNA extraction from heavy oil contaminated microcosms and *rpoB* gene PCR amplification. *Actual Biol* 30(88): 7-14.
6. Moronta, Felix. (2006). Estudio comparativo de los genes de la naftaleno dioxigenasa en bacterias degradadoras de crudos extra-pesados. UCV.
7. Wang, Y., Feng, J., Wang, G., Lv, X. and Wang, X. (2013). A modified protocol for microbial DNA extraction and purification in crude oil-spilled marsh soil. *Applied Mechanics Materials* 340: 353-357.
8. Young, C. C., Burghoff, R. L., Keim, L. G., Minak-Bernero, V., Lute, J. R. and Hinton, S. M. (1993). Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl Environ Microbiol* 59(6): 1972-1974.
9. Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173(2): 697-703.