Diagnostics
Diagnostic

Rep-PCR tests for identification of bacteria

Specific scope

This standard describes how to perform rep-PCR tests for identification of bacterial isolates.

Specific approval and amendments

Approved as an EPPO Standard in 2010-09.

Introduction

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Louws *et al.*, 1994; Rademaker *et al.*, 1998; Watts *et al.*, 2001). Three families of repetitive sequences have been identified, including the 35–40 bp repetitive extragenic palindromic (REP) sequences, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.*, 1994). These sequences appear to be located in distinct, intergenic positions around the genome. Primers designed from these sequences can, after PCR amplification, distinguish distinct genomic regions located between REP, ERIC or BOX elements. The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint. The method presented here, yielding reproducible results over the years, is based on those published by Smith *et al.* (2001) and Versalovic *et al.* (1994).

The procedure for performing the DNA isolation and the PCR is described in the Appendix.

Acknowledgements

This test description was originally drafted by Janse J (Dutch General Inspection Service, NL).

References

Louws FJ, Fulbright DW, Stephens CT & de Bruijn FJ (1994) Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**, 2286–2295.

EC (1998) COUNCIL DIRECTIVE 98/57/EC of 20 July 1998 on the control of *Ralstonia solanacearum (Smith) Yabuuchi et al.* Official Journal of the European Communities L 235/1

Versalovic J, Schneider M, De Bruijn FJ & Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based polymerase chain reaction. *Methods in Molecular Cell Biology* **5**, 25–40.

Rademaker JLW, Louws FJ & de Bruijn FJ (1998) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. Molecular Microbial Ecology Manual 3.4.3/1–3.4.3/27 Kluwer Academic

Smith NC, Hennesy J & Stead DE (2001) Repetitive sequence-derived PCR profiling using the BOX-A1 primer for rapid identification of plant pathogen *Clavibacter michiganensis* ssp. *sepedonicus*. *European Journal of Plant Pathology* **107**, 739–748.

Watts JL, Lowery DE, Teel JF, Ditto C, Horng JS & Rossbach S (2001) Phylogenetic studies on *Corynebacterium bovis* isolated from bovine mammary glands. *Journal of Dairy Science* **84**, 2419–2423.

Appendix

Isolation of DNA

1 Suspend 1/3 loopful of bacteria from a 48–72 h culture on nutrient agar (NA) or yeast peptone glucose agar (YPGA) in 100 µL R/DNAse free water in a 1.5 mL Eppendorf vial (approximately 10^9 cells mL^{-1}). Other suitable non-selective media can be used. Once a medium and method have been selected and proven to function in the laboratory, rep-PCR should be strictly standardized following the methods selected, to obtain reproducible results and reliable libraries of rep-PCR fingerprints.
2 Vortex to acquire a homogeneous suspension

3 Lyse bacteria and extract DNA, preferably using a commercially available extraction kit such as the Roche High Pure PCR Template Preparation Kit or Biorad's Chelex 100 kit. Extraction procedures for both kits are presented in this protocol. Extraction of DNA is also possible by heating the bacterial suspension for 15 min at 95°C, followed by quickly cooling on ice and pelleting down debris by centrifugation for 5 min at 7000 *g* and using the supernatant.

3a) Roche High Pure PCR Template Preparation Kit

Principle

– Bacterial cells are lysed during a short incubation with lysozyme in proteinase K and all nucleases are inactivated by guanidine-HCl.
– Nucleic acids bind selectively to glass fibres in the High Pure purification filter tube.
– Bound nucleic acids are washed with inhibitor removal buffer in order to remove PCR-inhibitory components.
– Bound nucleic acids are washed with wash buffer to remove salts, proteins and other cellular contaminants.
– Purified nucleic acids are recovered from the glass fibre using a low-salt elution buffer.
– Purified DNA can subsequently be used for (rep)PCR, restriction digestion or amplified fragment length polymorphism (AFLP).

Buffers, etc.

– Roche High Pure PCR Template Preparation Kit (Catalog no. 1 796 828)
– Lysozyme solution (10 mg mL^{-1} lysozyme in 10 mM Tris–HCl, pH 8.0)
– Isopropanol
– R/DNAse free water

Procedure

Note: Pre-warm elution buffer to 70°C

1. Pipette 200 µL extract of bacterial suspension in R/DNAse free water in a 1.5 mL vial.
2. Add 5 µL lysozyme solution (10 mg mL^{-1} lysozyme in 10 mM Tris–HCl, pH 8.0) and incubate 15 min at 37°C.
3. Add 200 µL binding buffer and 40 µL proteinase K, mix immediately and incubate 10 min at 70°C.
4. Add 100 µL isopropanol and mix well.
5. Pipette the sample into the upper reservoir of a combined filter tube–collection tube assembly
6. Centrifuge at 8000 rpm for 1 min in a microcentrifuge.
7. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µL inhibitor removal buffer.
8. Centrifuge at 8000 rpm for 1 min.
9. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µL wash buffer.
10. Centrifuge at 8000 rpm for 1 min.
11. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µL wash buffer.
12. Centrifuge at 8000 rpm for 1 min.
13. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube
14. Centrifuge at 14 000 rpm for 10 s to remove residual wash buffer.
15. Insert the filter tube into a clean 1.5 mL reaction tube.
16. Add 200 µL elution buffer that has been pre-warmed to 70°C.
17. Centrifuge at 8000 rpm for 1 min.
18. Discard the filter tube. The flowthrough in the reaction tube contains the DNA.
19. The DNA solution can be used directly or stored in a freezer at −20º or −80°C

Remarks

– Vials with lysozyme solution and proteinase K should be kept on ice to avoid diminution of enzyme activity.
– Lysozyme solution is stored in 1-time use portions at −20°C. Non-used solutions are discarded.

3b) Chelex 100 kit

Resuspend 1.5 g Chelex 100 (BioRad 142-2832) in 25 mL R/DNAse free water (not Tris–EDTA buffer) to make a 6% solution, which can be used directly or stored after autoclaving. A 1 mL pipette is used to aliquot the Chelex, which is maintained in suspension under agitation at moderate speed.

1. Prepare 1 mL cell suspensions to between OD_{650} 0.1–0.2 and micro-centrifuge for 5 min at 10 000 *g*.
2. Discard the supernatant carefully using a pipette.
3. Resuspend the pellet in 300 µL of Chelex suspension (see above) by vortexing, and incubate in a water bath at 56°C for 20 min.
4. Vortex at high speed for 10 s. Place the microfuge tube in a heated block at 100°C for 8 min. Note: Fix the caps using a weight to avoid the caps opening (explosively) during heating.
5. Vortex tubes at high speed for 10 s, and immediately chill on ice.
6. Centrifuge the tube for 5 min at 14000 *g*. Transfer 200 µL of the supernatant carefully to a new micro-centrifuge tube.
7. Use 2 µL aliquot of the supernatant as template DNA.

PCR conditions

B1) PCR conditions BOX-PCR

Primers
Reference: Smith *et al.* (2001) and Versalovic *et al.* (1994)
Forward: BOXA1R 5′-CTA.CGG.CAA.GGC.GAC.GCT.GAC.G-3′
Reverse: Forward = Reverse
Product: 100–3500 bp

BOX-PCR conditions: initial denaturation at 95°C 7 min followed by 30 cycles (94°C 1 min, 53°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C.

Mastermix

For reaction volume of 25 µL	Per reaction (µL)	Reaction (µL)	Endconc.
R/DNAse-free water	17.55	17.55	
Reaction buffer (10×, Invitrogen)	2.50	2.50	1×
$MgCl_2$ (50 mM, Invitrogen)	0.75	0.75	1.5 mM
dNTP mix (10 mM each, Promega)	0.50	0.50	0.2 mM
BOX AIR (20 µM)	2.50	2.50	2 µM
PlatinumTaq (5 U μL^{-1}, Invitrogen)	0.20	0.20	1 U
DNA extract	2.00	–	
Total	25.00	23.00	

B2) PCR conditions REP-PCR

REP-PCR
Primers
Reference: Smith *et al.* (2001); Versalovic *et al.*, 1994.[1]
Forward: REP1R-1 5′-III ICG ICG ICA TCI GGC-3′
Reverse: REP2-1 5′-ICG ITT ATC IGG CCT AC-3′

I = Inosine.

REP-PCR conditions: initial denaturation at 94°C 1 min followed by 35 cycles (95°C 7 min, 40°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C.

Mastermix

For reaction volume of 25 µL	Per reaction (µL)	Reaction (µL)	Endconc.
R/DNAse-free water	15.05	15.05	
Reaction buffer (10×, Invitrogen)	2.50	2.50	1×
$MgCl_2$ (50 mM, Invitrogen)	0.75	0.75	1.5 mM
dNTP mix (10 mM each, Promega)	0.50	0.50	0.2 mM
ERIC1R	2.50	2.50	2.0 µM
ERIC2	2.50	2.50	2.0 µM
PlatinumTaq (5 U μL^{-1}, Invitrogen)	0.20	0.20	1 U
DNA extract	2.00	–	
Total	25.00	23.00	

B3) PCR conditions ERIC-PCR

Primers
Reference: Smith *et al.* (2001) and Versalovic *et al.* (1994)
Forward: ERIC1R 5′-ATG TAA GCT CCT GGG GAT TCA C-3′
Reverse: ERIC2 5′-AAG TAA GTG ACT GGG GTG AGC G-3′

ERIC-PCR conditions: initial denaturation at 95°C 7 min followed by 30 cycles (94°C 1 min, 52°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C.

Mastermix

For reaction volume of 25 µL	Per reaction (µL)	Reaction (µL)	Endconc.
R/DNAse-free water	15.05	15.05	
Reaction buffer (10×, Invitrogen)	2.50	2.50	1×
$MgCl_2$ (50 mM, Invitrogen)	0.75	0.75	1.5 mM
dNTP mix (10 mM each, Promega)	0.50	0.50	0.2 mM
REP1R-1 (20 µM)	2.50	2.50	2.0 µM
REP2-1 (20 µM)	2.50	2.50	2.0 µM
PlatinumTaq (5 U μL^{-1}, Invitrogen)	0.20	0.20	1 U
DNA extract	2.00	–	
Total	25.00	23.00	

Electrophoresis

1. Prepare a 2% agarose gel of minimum 20 cm long.
2. Add 6 g agarose to 300 mL 1×TBE buffer in a 0.5–1 L flask. Melt the agarose in a microwave.
3. Place the gel tray in the casting system and choose appropriate combs.
4. Cool dissolved agarose under running tap water to hand-warm.
5. Pour agarose solution in the 15 cm gel tray, remove air bubbles with a disposable pipette tip and place the combs.
6. Clean the flask/Erlenmeyer immediately with hot water to remove residual agarose.
7. Leave agarose to solidify (minimum 20 min).
8. Remove the combs when gel has been formed. Clean combs carefully with hot water.
9. Submerge the gel in electrophoresis unit in 1×TBE buffer.
10. Mix a 6–10 µL PCR sample with 1–2 µL loading buffer on a piece of Parafilm and load the gel; load the 1 kb ladder (diluted Invitrogen 2 µL ladder and 4 µL water) in a similar amount.
11. Run the gel in a cold room at 90 V (for approximately 2.5 h), constant voltage. This corresponds to 6 V cm^{-1}, measured at the distance between the electrodes.
12. Stain the gel for 40 min in an ethidium bromide solution of 0.6 mg mL^{-1} in approximately 400 mL 0.5 × TBE, and destain for 30 min in distilled water or in 0.5 × TBE (for less background).
13. Visualize and document the bands on the gel under UV light, using suitable photographic gel documentation equipment.
14. Fingerprints (band patterns) can be compared by eye and checked for similarity, but patterns can also be transformed into peak patterns and compared using a computer software

program such as Bionumerics (Applied Maths NV, Belgium) or a comparable program, for more sophisticated pattern analysis and comparison of strains. These programs generally also enable the creation of rep-PCR fingerprint libraries. Identification should take place on the basis of similarity to patterns of control/reference strains analysed in the same run and in other runs, and/or with library entries.

15. When using simpler image-acquisition equipment (e.g. Kodak DC290), images should be saved as TIF files with a degree of resolution adequate to enable sharing the fingerprint with other laboratories holding fingerprinting libraries or platforms such as Bionumerics for databasing and analysis of these data.

Buffers

Tris-borate-EDTA (TBE) buffer according to Sambrook & Russell, 2001

5× stock solution, 1 L	
Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 mL
pH 8.3	

Autoclaving or filtering by 0.22 or 0.45 µm filter delays precipitation. 10×TBE is more likely to precipitate. pH of stock solution will be around pH 8.3 (do not adjust pH). If precipitate occurs, place bottle in hot water until precipitate has dissolved.

0.5×TBE solution: 45 mM Tris-borate
1 mM EDTA

Preparation of loading buffer

Bromophenol blue (10% stock solution)	
Bromophenol blue	5 g
Distilled water (bidest.)	50 mL
Loading buffer	
Glycerol (86%)	3.5 mL
Bromophenol blue solution	300 µL
Distilled water (bidest.)	6.2 mL

(From EU Directive 98/57/EC on *Ralstonia solanacearum*)

or:

0.25% bromophenol blue

40% w/v sucrose in H_2O

According to Sambrook & Russell, 2001

Reference

Sambrook J. and Russell D.W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA

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Erratum

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/100 (1)

Diagnostics
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PM 7/100 (1) Rep-PCR tests for identification of bacteria

This Standard (OEPP/EPPO, 2010) was published in December 2010 in the Diagnostic Protocol section of the *EPPO Bulletin*.

The volumes of water included in the Mastermix tables for BOX PCR, REP PCR and ERIC PCR were incorrect.

The modified figures are written in bold below. In addition the headings for the tables have been modified in line with the revised instructions for authors.

The correct volumes per reaction are included below. The EPPO Bulletin would like to apologize for this error.

B1) BOX-PCR

Mastermix

For reaction volume of 25 µL	Working concentration	Volume per reaction (µL)	Final concentration
R/DNAse-free water	**N/A**	**16.55**	
Reaction buffer (Invitrogen)	**10 ×**	2.50	1 ×
$MgCl_2$ (Invitrogen)	**50 mM**	0.75	1.5 mM
dNTP mix (Promega)	**10 mM each**	0.50	0.2 mM
BOX AIR	**20 µM**	2.50	2 µM
PlatinumTaq (Invitrogen)	**5 U μL^{-1}**	0.20	1 U
DNA extract	**N/A**	**2.00**	
Total		**25.00**	

B2) REP-PCR

Mastermix

For reaction volume of 25 µL	Working concentration)	Volume per reaction (µL)	Final concentration
R/DNAse-free water	**N/A**	**14.05**	
Reaction buffer (Invitrogen)	**10 ×**	2.50	1 ×
$MgCl_2$ (Invitrogen)	**50 mM**	0.75	1.5 mM
dNTP mix (Promega)	**10 mM each**	0.50	0.2 mM
ERIC1R	**20 µM**	2.50	2.0 µM
ERIC2	**20 µM**	2.50	2.0 µM
PlatinumTaq (Invitrogen)	**5 U μL^{-1}**	0.20	1 U
DNA extract	**N/A**	**2.00**	
Total	**25.00**	**25.00**	

B3) ERIC-PCR

Mastermix

For reaction volume of 25 µL	Working concentration)	Volume per reaction (µL)	Final concentration
R/DNAse-free water	**N/A**	**14.05**	
Reaction buffer (Invitrogen)	**10 ×**	2.50	1 ×
$MgCl_2$ (Invitrogen)	**50 mM**	0.75	1.5 mM
dNTP mix (Promega)	**10 mM each**	0.50	0.2 mM
REP1R-1	**20 µM**	2.50	2.0 µM
REP2-1	**20 µM**	2.50	2.0 µM
PlatinumTaq (Invitrogen)	**5 U μL^{-1}**	0.20	1 U
DNA extract	**N/A**	**2.00**	
Total	**25.00**	**25.00**	

Reference

OEPP/EPPO (2010) EPPO Standard PM 7/100(1) Rep-PCR tests for identification of bacteria. *Bulletin OEPP/EPP Bulletin* **40**, 365–368.