Chapter 10

PLASmid TAXonomic PCR (PlasTax-PCR), a Multiplex Relaxase MOB Typing to Assort Plasmids into Taxonomic Units

Raquel Cuartas, Teresa M. Coque, Fernando de la Cruz, and M. Pilar Garcillán-Barcia

Abstract

Plasmids transmissible by conjugation are responsible for disseminating antibiotic-resistance genes, making plasmid detection relevant for pathogen tracking. We describe the use of a multiplex PCR method for the experimental identification of specific plasmid taxonomic units (PTUs) of transmissible plasmids. The PCR primers were designed to target conserved segments of the relaxase MOB gene of PTUs encoding adaptive traits for enterobacteria (antimicrobial resistance, virulence, and metabolism). In this way, PlasTax-PCR detects the presence of these plasmids and allows their direct assignation to a PTU.

Key words Plasmid taxonomic units, Bacterial conjugation, Horizontal gene transfer, Relaxase, MOB family, Plasmid typing

1 Introduction

Plasmids are critical vehicles in disseminating antimicrobial resistance (AMR) [1]. Thus, their detection and classification are crucial for molecular epidemiology to track AMR beyond the boundaries of specific bacterial clones. Plasmids transmissible by conjugation have a distinctive characteristic, they encode a MOB relaxase that recognizes a cognate plasmid sequence, the *nic* site within the origin of transfer (*oriT*) [2]. Nine MOB relaxase classes are currently described [3]. Still, just five of them comprise more than 95% of conjugative relaxases present in plasmids hosted in the order Enterobacterales [4]. To detect transmissible plasmids, a PCR-based method, Degenerate-Primer MOB typing (DPMT), was developed [5, 6] (Fig. 1). Applied to collections of clinical and environmental isolates of enterobacteria [8–12], DPMT detected plasmids with relaxase sequences identical or nonidentical

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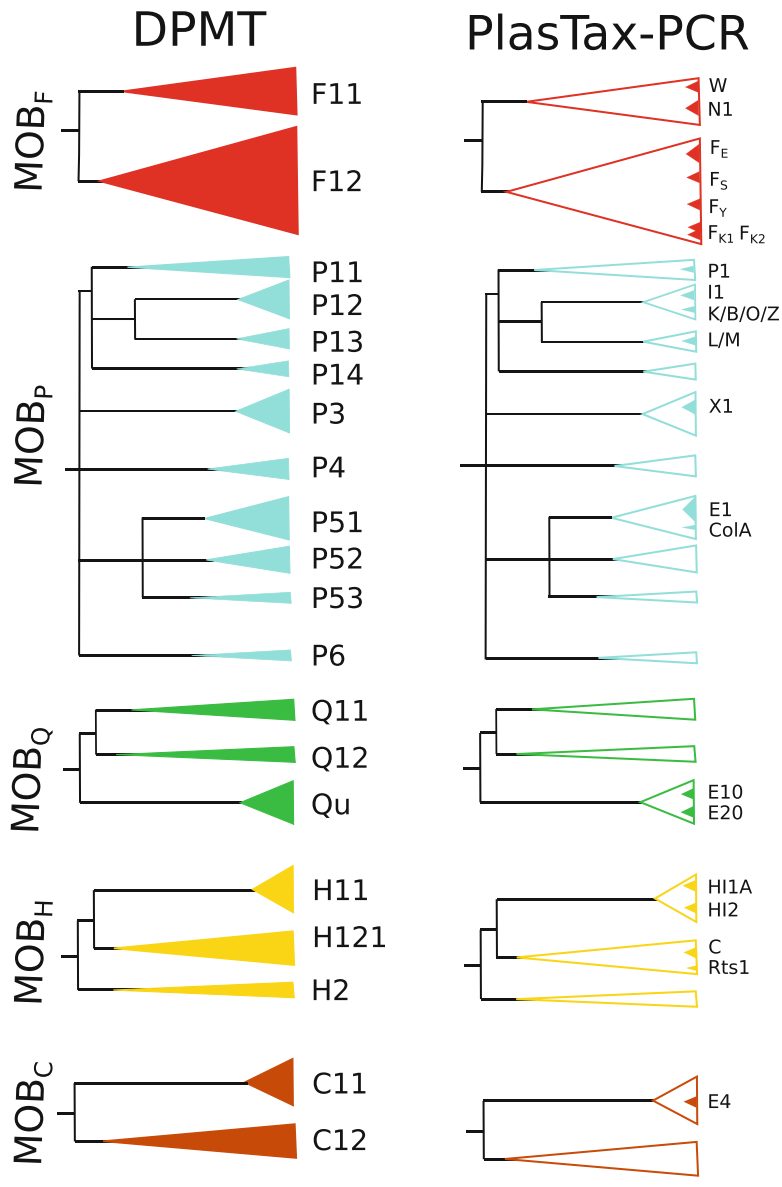

Fig. 1 DPMT and PlasTax-PCR scheme concepts. Phylogenetic tree representations of the five relaxase MOB classes prevalent in Enterobacterales are schematized. Families into each MOB class with members in Enterobacterales are represented by colored triangles. Those identified by the DPMT Scheme [6, 7] are indicated in the left panel. At the right panel, the same families are depicted, but only the tips contain colored triangles corresponding to the PTUs included in the PlasTax-PCR scheme

to those previously known and classified them into broad MOB families.

Plasmids have been recently assorted into taxonomic units (PTUs), which gather members with a common genomic backbone

[4]. One thousand seven hundred and seventy out of the 2535 plasmids hosted in the order Enterobacterales were included in 83 PTUs. Out of them, 55 PTUs did not correspond to any known incompatibility (Inc) group. Nevertheless, 50 out of these 83 PTUs were MOB^+, according to MOBscan [3]. Besides, each PTU was characterized by a single relaxase MOB type, while replication functions within a given PTU showed considerable variation [4]. These facts endorse the use of the MOB relaxase sequences to classify transmissible plasmids into their corresponding PTUs. Here, we describe a PCR-based method, Plasmid Taxonomic PCR (PlasTax-PCR), to detect plasmid relaxases from PTUs circulating in clinical enterobacteria. This method targets specifically the MOB relaxases corresponding to 19 PTUs, and of two additional groups without PTU assignment (IncT and ColA-like colicin plasmids) (Fig. 1). For each group, the coding sequences of the N-terminal relaxase domain (5′ 900 nucleotides) were aligned and conserved specific blocks were chosen to design the primers. As proof of principle, this method was applied to detect transmissible plasmids in a series of *E. coli* ST131 clinical isolates, a polyclonal cluster able to harbor a high variability of plasmids (Fig. 2).

2 Materials

2.1 Solutions (See Notes 1 and 2)

1. InstaGene™ Matrix (Bio-Rad).
2. DNA polymerase and its corresponding reaction buffer, e.g., BioTaq™ DNA polymerase (Bioline) (*see* **Note 3**).
3. 50 mM $MgCl_2$.
4. 10 mM dNTP.
5. PCR-grade H_2O (*see* **Note 4**).
6. 100 μM solutions for each primer. PlasTax-PCR primers are listed in Table 1 (*see* **Notes 5** and **6**).
7. Agarose basic, DNAse free.
8. 10× TBE (Tris/Borate/EDTA) buffer, pH 8.3.
9. Intercalating agent, e.g., SafeView™ Classic (ABM) (5 μL stock/100 mL gel).
10. Ladder for DNA electrophoresis, e.g., HyperLadder™ 1 kb (Bioline).
11. DNA loading buffer: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue.

2.2 Equipment

12. UV-visible spectrophotometer, e.g., NanoDrop™ 2000 (Thermo Scientific).
13. A thermocycler to carry out the PCR reactions.

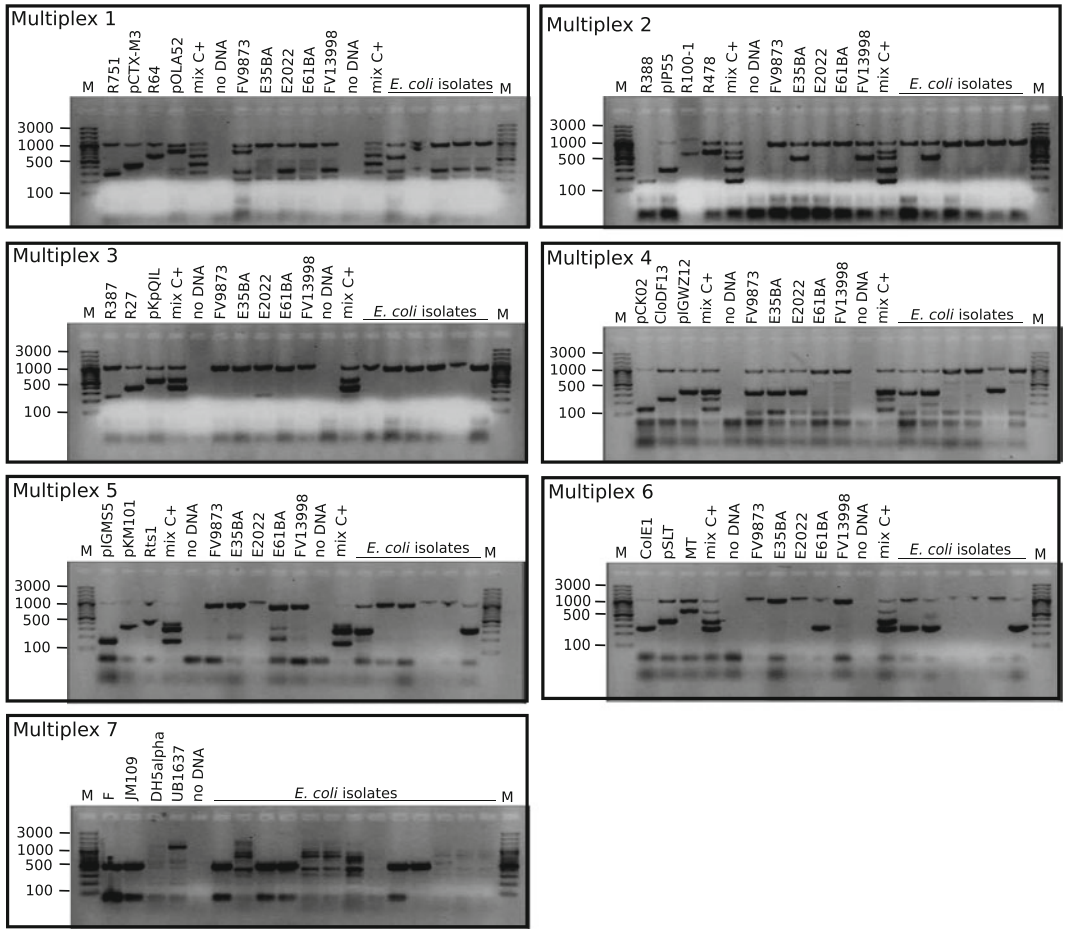

Fig. 2 Analysis of PlasTax-PCR reactions. For each multiplex, a 2% agarose gel is shown. M: HyperLadder™ 1Kb. Positive controls (*see* Table 1): individual plasmids, indicated above the corresponding lane, and a mix of the individual plasmids in the same PCR sample (mix C+). Negative control: no DNA. Samples: PCRs from clinical *E. coli* ST131 isolates, whose genomes are fully sequenced (strains FV9873, E35BA, E2022, E61BA, and FV13998 from [14]) or not (*E. coli* isolates from [15, 16])

14. An agarose electrophoresis system.
15. A gel imaging system to visualize fluorescent nucleic acid stains.

3 Methods

3.1 Preparation of the PlasTax-PCR DNA Template

Total DNA was extracted from *Escherichia coli* cultures bearing the plasmids listed in the second column of Table 1 using InstaGene™ Matrix (Bio-Rad) and following the manufacturer's recommendations (*see* **Note 7**).

1. Centrifuge 50 μL of a saturated bacterial culture at 13,800 rcf for 1 min.

Table 1
Targets, primers, and conditions for PlasTax-PCR

Multiplex	MOB family/PTU/plasmid prototype	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon size (bp)	T_{ann} (°C)
1	MOB_{P11}/PTU-P1/R751	CTGGCGACCCAGCACACGA	GTAGTCGCGCTCCAGGGCC	297	62
	MOB_{P13}/PTU-L/M/pCTX-M3	AAAATCATTGAGGGCCGAAGGG	CATACTGGTGATCGGACATGCCC	436	
	MOB_{P12}/PTU-I1/R64	CATGAAGGACGGCTGCGAATG	ATTTTCCGTCCATCTGAGACAGG TACA	629	
	MOB_{P3}/PTU-X1/pOLA52	AGTCATCAGAAAATGGTCGTAAG TCAGCT	CTTTCCCGCGAACATAAGTCCC	823	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	
2	MOB_{F11}/PTU-W/R388	CATAGGGCGGGCTGCAAGC	GTGGCCCTCGCCGATATTCC	169	62
	MOB_{H12}/PTU-C/pIP1202	GTATGCCGCGTTTGTTCACCTG	GCGCCACCGGATAAAGTAACG	340	
	MOB_{F12}/PTU-F_E/R100 [b]	GTTGAGTTTCAGCGTTGTTAAA TCGG	AGCACGTTCTCGGAAAATCCG	567	
	MOB_{H11}/PTU-HI2/R478	ATCTCAGGAGAATGATGCAACCTC TG	AAGACATACCGGGTTTAGGA TTCGC	712	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	
3	MOB_{P12}/PTU-B/O/K/Z/R387	CGGACAAAGCTGTTTTTTCCCGTA	TCCTGGCTCCAGGAAAGCCAG	253	62
	MOB_{H11}/PTU-HI1A/R27	TGAGCAGTCTATTCTTTCGTTG TACAGAGAC	GGCGCCCATGTTAATGTTTCACTC	405	
	MOB_{F12}/PTU-F_{K1} – PTU-F_{K2}/pKpQIL [c]	GGATAAGGCGCTGTTTACGGAAC TG	GGTCCTGCAGCCTCCCTGACC	600	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	
4	MOB_{P51}/-/ColA, pCK02	GGGGGCGAAACATCCGACT	ACCGTTCGTGCTCGCTGAAT	142	64
	MOB_{C11}/PTU-E4/CloDF13	CGCACCGGCTGGCCG	TGACCTGCGTCGCCCGG	270	
	MOB_{Q41}/PTU-E10/pIGWZ12	CCCCTGCCTGGTGTACGAACC	TATGAACGATGGTCTATCTCTTCC TGATAAC	394	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	

(continued)

Table 1
(continued)

Multiplex	MOB family/PTU/plasmid prototype	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon size (bp)	T_{ann} (°C)
5	MOB_{Q4}/PTU-E20/pIGMS5	CCCGTGAGATTTCCCGCGAG	CTTTCCTGCGAATCCCGTTTCC	176	65
	MOB_{F11}/PTU-N1/R46	CTTGATATAACCACGATTACCCGCC	CTTCACGCACAGCAGCGGC	355	
	MOB_{H12}/-/Rts1	AGCTGAATTGGGTTTTCCGGC	CCGCTCAATCTTTTCAGTTTCGG	460	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	
6	MOB_{P51}/PTU-E1/ColE1	AAAAGGCCGTCAGGATGTGATTCA	GCGTCTCTGATTTTCGTCTCG TTTG	280	64
	MOB_{F12}/PTU-F_S/pSLT	GGCGGCAACAAACACCGC	AACCTTCTCTTTCAGCACCGCG	411	
	MOB_{F12}/PTU-F_Y/pMT	CGGACTCAGGACGGGGCG	CCACTCGGCCATGCGCTG	630	
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	
7	MOB_{F12}/PTU-F_E/F [b]	GCTGGGCAGCATGGGAGAAC	CAATCTGATTAGCGTACACATTC TCAATG	509	62
	16S rRNA [a]	CTCCTACGGGAGGCAGCAG	GACGGGCGGTGTGTRCA	1034	

[a]16S rDNA primers are 357F and 1391R, described by [13]
[b]PTU-F_E presents low conservation in the gene repertoire of its members [4], and thus two different primer pairs were designed to target this group
[c]This is the only case in which a PlasTax-PCR primer pair targets plasmids from two different PTUs. Plasmids from the related group PTU-F_{K3} are not targeted

2. Add a volume of 200 μL of InstaGene™ Matrix to the pellet.
3. Incubate at 56 °C for 15–30 min.
4. Vortex at high speed for 10 s.
5. Incubate at 100 °C for 8 min.
6. Repeat **step 4**.
7. Centrifuge at 13,800 rcf for 3 minutes and recover the supernatant.
8. Repeat **step 7** to eliminate traces of the matrix.
9. Quantify the DNA (*see* **Note 8**).
10. Store the supernatant at −20 °C (*see* **Note 9**).

3.2 PlasTax-PCR for Enterobacterial Plasmids

1. **Preparation of the reaction mixture for the multiplex PCRs**. For a reaction of 50 μL, add 100 ng of genomic DNA (*see* **Notes 8** and **10**), 5 μL of 10× reaction buffer, 1.5 μL of 50 mM $MgCl_2$ (final concentration 1.5 mM), 1 μL of 10 mM dNTP (final concentration 0.2 mM), 0.5 μL of 100 μM primers (final concentration 1 μM), 1 U of BioTaq polymerase (Bioline), and ddH_2O up to 50 μL (*see* **Note 4**).
2. **PCR running** (*see* **Note 11**). The amplification program includes an initial denaturation step 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C + 30 s at annealing temperature (*see* Table 1) + 30 s at 72 °C, and a final extension at 72 °C during 7 min.
3. **Visualization of the amplicons**. Load 10 μL of the PCR reaction and 2 μL of the DNA loading buffer onto a 2% agarose gel containing an intercalating agent. Load at least one lane of DNA ladder in each gel (*see* **Note 12**). Separate by electrophoresis at room temperature at voltage 100 V for 40 min. Visualize under ultraviolet light (*see* **Note 13**).

4 Notes

1. Pipette all reagents and samples using filter tips. The preparation of the reactions before the DNA amplification should be ideally carried out in a clean area (pre-PCR) to reduce the chance of contamination.
2. Aliquot reagents to avoid multiple freeze-thaw cycles and the contamination of master stocks.
3. No need to use extremely low error-rate DNA polymerases if amplicons are not going to be sequenced.
4. The use of commercially available nuclease-free water is preferred.

5. A thorough process of primer design is recommendable. A nucleotide multiple sequence alignment of the relaxase gene portion encoding the N-terminal relaxase domain is the basis to detect high identity stretches for primer designing. It is useful to design several primer pairs to detect a single PTU, which render amplicons of different sizes, in order to ease their combination in multiplex reactions. Sequences that produce intra- or inter-oligonucleotide secondary structures should be avoided. The melting temperature (Tm) of the primers should be as close as possible, favoring Tm > 60 °C to avoid nonspecific targets. The primer pair selected should have good performance in both the individual and the multiplex PCR reaction.
6. The individual amplicons included in a multiplex reaction should differ in size, ideally 150–200 bp, as to be clearly distinguished in a 2% agarose gel. Take it into account when designing the primers.
7. A purification method rendering PCR-quality template DNA is advised, instead of picking and adding a bacterial colony directly into the PCR master mix.
8. We prepared aliquots of template DNA at 20 ng/μL to add the same volume to each PCR reaction.
9. DNAs extracted using InstaGene Matrix can be stored at −20 °C for a month without degradation.
10. When a mix of template DNAs is used in the reaction, 100 ng of each DNA sample is added.
11. Positive controls for each reaction should be used in parallel. A list of GenBank accession numbers of plasmids that can be targeted by each primer pair and can thus be used as positive controls, according to the criteria explained in the footnote to Table 2, is provided:

    For multiplex 1:

    PTU-P1: NC_013176.1, NC_005088.1, NC_017908.2, NC_019263.1, NC_019264.1, NC_019283.1, NC_016968.1, NC_016978.1, NC_004956.1, NC_006830.1, NC_021077.1, NC_019312.1, NC_001735.4, NC_024998.1, NC_007353.2, NC_010935.1, NC_019320.1, NZ_CP017760.1, NC_020994.1, NZ_CP015373.1, NZ_CP009797.1, NZ_CP014846.1, NZ_CP019238.1, NC_014911.1, NC_008766.1, NC_014641.1, NC_007337.1, NZ_CP021650.1, NC_008385.1.

    PTU-L/M: NC_004464.2, NC_005246.1, NC_011641.1, NC_019063.1, NC_019154.1, NC_019344.1, NC_019346.1, NC_019368.1, NC_019889.1, NC_021078.1, NC_021488.1, NC_023027.1, NC_024997.1, NC_025134.1,

Table 2
PlasTax-PCR efficiency for each targeted PTU

Multiplex	PTU	Number of PTU members (RefSeq84)[a]	Number of targeted PTU members[b]
1	P1	32	29
	L/M	58	56
	I1	120	119
	X1	49	24
2	W	4	4
	C	92	87
	F_E	198	52
	HI2	33	29
3	B/O/K/Z	20	14
	HI1A	8	8
	F_{K1} - F_{K2}	68/32	37/29
4	E4	40	39
	E10	23	21
5	E20	9	8
	N1	60	60
6	E1	51	45
	F_S	56	43
	F_Y	38	10
7	F_E	198	94

[a]Data are taken from Supplementary Table S4 [4]
[b]Number of plasmids that are potentially targeted by the corresponding primers. Take into account that not all plasmids from a PTU contain a MOB relaxase. Plasmids are considered targeted if the primers potentially anneal to their sequence producing a fragment with the size indicated in Table 1. Hybridization mismatches are allowed, except if they occur in the 3′ 12 nucleotides of the primers, in which case the plasmid was ruled out. According to these criteria, a list of plasmids that can be used as positive controls is provided in **Note 11**

NZ_CP007733.1, NZ_CP009852.1, NZ_CP009857.1, NZ_CP010365.1, NZ_CP011593.1, NZ_CP011599.1, NZ_CP011609.1, NZ_CP011614.1, NZ_CP011632.1, NZ_CP011640.1, NZ_CP014298.1, NZ_CP014698.2, NZ_CP015071.1, NZ_CP015075.2, NZ_CP016927.1, NZ_CP017282.1, NZ_CP017288.1, NZ_CP017853.1, NZ_CP017932.1, NZ_CP017936.1, NZ_CP018315.1, NZ_CP018342.1, NZ_CP018449.1, NZ_CP018452.1, NZ_CP018461.1, NZ_CP018669.1, NZ_CP018690.1, NZ_CP018700.1, NZ_CP018706.1, NZ_CP018712.1, NZ_CP018717.1, NZ_CP018723.1, NZ_CP018736.1, NZ_CP018974.1, NZ_CP019841.1, NZ_CP020844.1, NZ_CP021742.1, NZ_CP022147.1, NZ_CP022150.1, NZ_CP022153.1, NZ_CP022826.1, NZ_KX118608.1.

PTU-I1: NC_013120.1, NC_014383.1, NC_019044.1, NC_019043.1, NC_019061.1, NC_019097.1, NC_002122.1, NC_022885.1, NC_023326.1, NC_024980.1, NC_023915.1, NC_025140.1, NC_025144.1, NC_025147.1, NC_025180.1, NC_025198.1, NC_025142.1, NC_025143.1, NC_025176.1, NC_024975.1, NC_024976.1, NC_024977.1, NC_024979.1, NC_024978.1, NC_024955.2, NC_019123.1, NC_019131.1, NC_019137.1, NC_019099.1, NC_005014.1, NC_015965.1, NC_019111.1, NC_023899.1, NC_023900.1, NC_019104.1, NC_023329.1, NC_022267.1, NC_023275.1, NC_023276.1, NC_023290.1, NC_020991.1, NC_022742.1, NC_018659.1, NZ_CP019220.1, NC_011419.1, NC_016904.1, NC_017637.1, NC_017665.1, NC_017642.1, NZ_CP009580.1, NZ_CP006641.1, NZ_CP010317.1, NZ_CP012627.1, NZ_CP015161.1, NZ_CP015916.1, NZ_CP015996.1, NZ_CP015838.1, NZ_CP018116.1, NZ_CP018122.1, NZ_CP018110.1, NZ_CP010130.1, NZ_CP010233.1, NZ_CP019215.1, NC_017675.1, NC_011081.1, NC_017718.1, NC_011077.1, NC_021811.1, NC_021813.2, NZ_CP009566.1, NZ_CP012039.1, NZ_CP012835.1, NZ_LN890525.1, NZ_CP012923.1, NZ_CP012936.1, NZ_CP013224.1, NZ_CP012929.1, NZ_CP013221.1, NZ_CP014662.1, NZ_CP016516.1, NZ_CP016520.1, NZ_CP016533.1, NZ_CP016522.1, NZ_CP016568.1, NZ_CP016572.1, NZ_CP016585.1, NZ_CP016387.1, NZ_CP016407.1, NZ_CP016411.1, NZ_CP016413.1, NZ_CP016409.1, NZ_CP019205.1, NZ_CP019207.1, NZ_CP010142.1, NZ_CP019173.1, NZ_CP018946.1, NZ_CP018975.1, NZ_CP018993.1, NZ_CP014972.2, NZ_CP014622.1, NZ_KX443694.1, NZ_LT838199.1, NC_032100.1, NZ_CP018774.2, NZ_CP021208.1, NZ_CP021533.1, NZ_CP021693.1, NZ_CP021739.1, NZ_CP021841.1, NZ_CP021845.1, NZ_CP021882.1, NZ_CP018625.1, NZ_CP020494.1, NZ_CP019691.1, NZ_CP022456.1, NZ_CP014096.1, NZ_CP010831.1, NZ_CP014494.1, NZ_CP007651.1.

PTU-X1: NC_010378.1, NC_010421.1, NC_010422.1, NC_010860.1, NC_011204.1, NC_011739.1, NC_013503.1, NC_015472.1, NC_016036.1, NC_019013.1, NC_019046.1, NC_019067.1, NC_019088.1, NC_019096.1, NC_019106.1, NC_019256.1, NC_024961.1, NZ_CP011431.1, NZ_CP012734.1, NZ_CP014974.1, NZ_CP019180.1, NZ_CP020088.1, NZ_CP020341.1, NZ_CP020836.1.

For multiplex 2:

PTU-W: NC_010643.1, NC_009982.1, NC_010716.1, NC_028464.1.

PTU-C: NC_008612.1, NC_008613.1, NC_009139.1, NC_009140.1, NC_012690.1, NC_012692.1, NC_012693.1, NC_016974.1, NC_016976.1, NC_017645.1, NC_018994.1, NC_019045.2, NC_019065.1, NC_019066.1, NC_019069.1, NC_019107.1, NC_019116.1, NC_019118.1, NC_019121.1, NC_019153.1, NC_019158.1, NC_019375.1, NC_019380.1, NC_020180.1, NC_021667.1, NC_021815.1, NC_022372.1, NC_022377.1, NC_022522.2, NC_022652.1, NC_023291.1, NC_023898.1, NC_023908.1, NZ_CP003998.1, NZ_CP006661.1, NZ_CP007486.1, NZ_CP007636.1, NZ_CP008790.1, NZ_CP009409.2, NZ_CP009560.1, NZ_CP009562.1, NZ_CP009564.1, NZ_CP009567.1, NZ_CP009570.1, NZ_CP009868.1, NZ_CP010373.2, NZ_CP010391.1, NZ_CP011429.1, NZ_CP011540.1, NZ_CP011622.1, NZ_CP012682.1, NZ_CP013324.1, NZ_CP014295.1, NZ_CP014658.1, NZ_CP014775.1, NZ_CP014978.1, NZ_CP015139.1, NZ_CP015394.1, NZ_CP015835.1, NZ_CP016013.1, NZ_CP016036.1, NZ_CP017055.1, NZ_CP017987.1, NZ_CP018318.1, NZ_CP018689.1, NZ_CP018698.1, NZ_CP018704.1, NZ_CP018710.1, NZ_CP018716.1, NZ_CP018722.1, NZ_CP018817.1, NZ_CP018956.1, NZ_CP019441.1, NZ_CP020049.1, NZ_CP020056.1, NZ_CP021206.1, NZ_CP021551.1, NZ_CP021709.1, NZ_CP021719.1, NZ_CP021835.1, NZ_CP021853.1, NZ_CP021936.1, NZ_CP021952.1, NZ_CP021956.1, NZ_CP022126.1, NZ_CP022359.1, NZ_LT904892.1.

PTU-F_E: NC_019424.1, NC_019095.1, NC_019090.1, NC_019072.1, NC_019071.1, NC_019057.1, NC_018998.1, NC_017630.1, NC_016039.1, NC_013727.1, NC_013655.1, NC_013542.1, NC_013175.1, NC_011812.1, NC_011749.1, NC_009133.1, NC_007941.1, NC_005327.1, NC_002134.1, NZ_CP018125.1, NZ_CP018119.1, NZ_CP018113.1, NZ_CP018107.1, NZ_CP014496.1, NZ_CP017287.1, NZ_CP015072.1, NZ_CP014523.1, NC_008460.1, NZ_CP020340.1, NZ_CP014493.1, NZ_CP012139.1, NZ_CP021938.1, NZ_CP021871.1, NZ_CP021180.1, NZ_CP020117.1, NZ_CP019009.1, NZ_CP018982.1, NZ_CP018954.1, NZ_CP018952.1, NZ_CP016035.1, NZ_CP015140.1, NZ_CP015077.1, NZ_CP015070.1, NZ_CP010882.1, NZ_CP009860.1, NZ_CP009579.1, NZ_CP008715.1, NC_013951.1,

NC_025177.1, NZ_CP021690.1, NZ_CP014498.1, NZ_CP010239.1.

PTU-HI2: NC_005211.1, NC_009838.1, NC_010870.1, NC_012555.1, NC_012556.1, NC_019114.1, NC_024983.1, NZ_CP008825.1, NZ_CP008899.1, NZ_CP008906.1, NZ_CP011062.1, NZ_CP011601.1, NZ_CP012170.1, NZ_CP012931.1, NZ_CP015833.1, NZ_CP016526.1, NZ_CP016764.1, NZ_CP016838.1, NZ_CP019214.1, NZ_CP019443.1, NZ_CP019559.1, NZ_CP019647.1, NZ_CP020493.1, NZ_CP021177.1, NZ_CP021209.1, NZ_CP022165.1, NZ_CP022533.1, NZ_CP022696.1, NZ_CP023143.1.

For multiplex 3:

PTU-B/O/K/Z: NZ_CP023144.1, NZ_CP015141.1, NZ_CP013024.1, NZ_CP009107.1, NC_025138.1, NC_022996.1, NC_022992.1, NC_022371.1, NC_018995.1, NC_014843.1, NC_007365.1, NZ_CP005999.1, NC_011754.1, NZ_CP018772.1.

PTU-HI1A: NC_002305.1, NC_003384.1, NC_009981.1, NC_013365.1, NC_016825.1, NC_023289.2, NZ_CP022495.1, NZ_LT904879.1.

PTU-F_{K1} - F_{K2}: NC_019390.1, NC_024992.1, NC_020132.1, NC_021654.1, NC_009649.1, NC_022078.1, NZ_CP007729.1, NZ_CP008800.1, NZ_CP008829.1, NZ_CP008930.1, NZ_CP009777.1, NZ_CP010393.1, NZ_CP010574.1, NZ_CP011577.1, NZ_CP011977.1, NZ_CP011990.1, NZ_CP015386.1, NZ_CP015823.1, NZ_CP018355.1, NZ_CP018365.1, NZ_CP018424.1, NZ_CP018430.1, NZ_CP018434.1, NZ_CP018441.1, NZ_CP018460.1, NZ_CP018693.1, NZ_CP019773.1, NZ_CP020072.1, NZ_CP020109.1, NZ_CP020838.1, NZ_CP020842.1, NZ_CP021540.1, NZ_CP021544.1, NZ_CP021713.1, NZ_CP021752.1, NZ_CP021834.1, NZ_CP022145.1, NC_009650.1, NZ_CP015395.1, NZ_LT216438.1, NZ_CP022698.1, NZ_CP022693.1, NZ_CP019774.1, NZ_CP018992.1, NZ_CP018989.1, NZ_CP015824.1, NZ_CP014765.1, NZ_CP014669.1, NZ_CP014650.1, NZ_CP011991.1, NZ_CP011986.1, NZ_CP009875.1, NZ_CP009115.1, NZ_CP008833.1, NZ_CP008830.1, NZ_CP007730.1, NC_025187.1, NC_025167.1, NC_025166.1, NC_023906.1, NC_023905.1, NC_023904.1, NC_023903.1, NC_019165.1, NC_019155.1, NC_014016.1.

For multiplex 4:

MOB_{P51}/-/ColA, pCK02: NC_009794.1, NC_001373.1, NC_016151.1.

PTU-E4: NC_002119.1, NC_009793.1, NC_018953.1, NC_019156.1, NC_019159.1, NC_020182.1, NC_021666.1, NC_022083.1, NZ_CP006928.1, NZ_CP007728.1, NZ_CP008828.1, NZ_CP008832.1, NZ_CP009772.1, NZ_CP009778.1, NZ_CP009873.1, NZ_CP009877.1, NZ_CP010394.1, NZ_CP011979.1, NZ_CP011982.1, NZ_CP011984.1, NZ_CP011988.1, NZ_CP011993.1, NZ_CP014648.1, NZ_CP015384.1, NZ_CP015391.1, NZ_CP018353.1, NZ_CP018425.1, NZ_CP018431.1, NZ_CP018433.1, NZ_CP018445.1, NZ_CP019776.1, NZ_CP020112.1, NZ_CP020840.1, NZ_CP021543.1, NZ_CP021717.1, NZ_CP021779.1, NZ_CP021837.1, NZ_CP022576.1, NZ_LT216440.1.

PTU-E10: NZ_CP016041.1, NZ_CP019024.1, NZ_CP018996.1, NZ_CP018972.1, NZ_CP018942.1, NC_019134.1, NZ_CP011432.1, NZ_CP011140.1, NZ_CP018980.1, NZ_CP019648.1, NZ_CP015142.1, NZ_CP012629.1, NC_010885.1, NZ_CP018966.1, NZ_CP018208.1, NZ_CP010878.1, NZ_CP006634.1, NC_011411.1, NZ_HG941720.1, NC_010486.1, NZ_CP018941.1.

For multiplex 5:

PTU-E20: NC_010883.1, NC_011977.1, NC_012882.1, NC_020412.1, NC_022585.1, NC_023325.1, NZ_CP014098.1, NC_013367.1.

PTU-N1: NC_003292.1, NC_007682.3, NC_009131.1, NC_009132.1, NC_009980.1, NC_011383.1, NC_011385.1, NC_011617.1, NC_014208.1, NC_014231.1, NC_014368.1, NC_015599.1, NC_019033.1, NC_019082.1, NC_019087.1, NC_019098.1, NC_019124.1, NC_019888.1, NC_020086.1, NC_020088.1, NC_021622.1, NC_021660.2, NC_021664.2, NC_022374.1, NC_022375.1, NC_023909.1, NC_023910.1, NC_024967.1, NC_024974.1, NC_025019.1, NC_025183.1, NC_025186.1, NC_032101.1, NZ_CP008901.1, NZ_CP008908.1, NZ_CP009853.1, NZ_CP009858.1, NZ_CP009862.1, NZ_CP009864.1, NZ_CP009867.1, NZ_CP009874.1, NZ_CP009881.1, NZ_CP011589.1, NZ_CP014524.1, NZ_CP017725.1, NZ_CP018442.1, NZ_CP018945.1, NZ_CP018959.1, NZ_CP018963.1, NZ_CP018977.1, NZ_CP019006.1, NZ_CP019026.1, NZ_CP020059.1, NZ_CP020119.1, NZ_CP021899.1, NZ_KX062091.1, NZ_KX154765.1, NZ_KX276209.1, NZ_KX397572.1, NZ_LT838197.1.

MOB_{H12}/-/Rts1: NC_003905.1.

For multiplex 6:

PTU-E1: NC_001371.1, NC_002809.1, NC_003079.1, NC_003457.1, NC_005019.1, NC_005970.1, NC_008488.1, NC_009791.1, NC_010485.1, NC_010672.1, NC_011214.1, NC_011407.1, NC_011799.1, NC_013363.1, NC_014235.1, NC_017321.1, NC_017636.1, NC_017654.1, NC_017655.1, NC_017661.1, NC_017662.1, NC_017721.1, NC_018997.1, NC_019076.1, NC_019078.1, NC_019102.1, NC_019136.1, NC_019250.1, NC_019357.1, NC_019982.1, NC_020251.1, NC_025004.1, NC_025026.1, NC_025178.1, NZ_CP010175.1, NZ_CP012628.1, NZ_CP014198.2, NZ_CP015917.1, NZ_CP016039.1, NZ_CP016513.1, NZ_CP016519.1, NZ_CP016584.1, NZ_CP017845.1, NZ_CP018943.1, NZ_CP019025.1.

PTU-F_S: NC_002638.1, NC_003277.2, NC_006855.1, NC_007208.1, NC_012124.1, NC_013437.1, NC_014476.2, NC_016855.1, NC_016858.1, NC_016861.1, NC_016864.1, NC_017054.1, NC_017720.1, NC_019001.1, NC_019108.1, NC_019109.1, NC_019112.1, NC_022570.1, NZ_AP014566.1, NZ_CP007489.1, NZ_CP007582.1, NZ_CP008745.1, NZ_CP012345.1, NZ_CP012348.1, NZ_CP013721.1, NZ_CP014050.1, NZ_CP014357.1, NZ_CP014359.1, NZ_CP014537.1, NZ_CP014577.1, NZ_CP014968.1, NZ_CP014970.1, NZ_CP014973.1, NZ_CP014976.1, NZ_CP014980.1, NZ_CP015158.1, NZ_CP016390.1, NZ_CP017618.1, NZ_CP017729.1, NZ_CP020923.1, NZ_CP022137.1, NZ_LN999012.1, NZ_LT855377.1.

PTU-F_Y: NC_006323.1, NC_009378.1, NZ_CP006746.1, NZ_CP006749.1, NZ_CP006752.1, NZ_CP006756.1, NZ_CP006760.1, NZ_CP006779.1, NZ_CP009714.1, NZ_CP010248.1.

For multiplex 7:

PTU-F_E: NZ_LT838198.1, NZ_CP017726.1, NZ_CP021847.1, NZ_CP021843.1, NZ_CP018994.1, NZ_CP014489.1, NZ_CP012636.1, NC_025106.1, NC_019089.1, NZ_CP010223.1, NZ_CP010215.1, NZ_CP019007.1, NZ_CP018455.1, NZ_CP017981.1, NZ_CP017633.1, NZ_CP014273.1, NZ_CP014271.1, NZ_CP011496.1, NZ_CP010158.1, NC_025175.1, NC_025139.1, NC_024956.1, NC_019073.1, NC_004998.1, NC_002483.1, NZ_CP012113.1, NZ_CP010316.1, NZ_CP010192.1, NC_013362.1, NC_011747.1, NZ_CP013026.1, NC_010409.1, NZ_CP015914.1, NZ_CP012683.1, NZ_CP012626.1, NZ_CP012928.1, NZ_CP011916.1, NZ_CP006001.1,

NC_018966.1, NC_018954.1, NC_017627.1, NC_014382.1, NC_011076.1, NC_006671.1, NZ_CP021880.1, NZ_CP021733.1, NZ_CP021289.1, NZ_CP021204.1, NZ_CP019028.1, NZ_CP018990.1, NZ_CP018958.1, NZ_CP016498.1, NZ_CP010372.1, NZ_CP010232.1, NC_023315.1, NC_020271.1, NC_014615.1, NC_010558.1, NC_010488.1, NZ_CP011019.1, NZ_CP010184.1, NZ_CP010181.1, NZ_CP019561.1, NZ_CP021737.1, NZ_CP011064.1, NZ_CP018978.1, NZ_CP013833.1, NZ_CP013832.1, NZ_CP011493.1, NZ_CP010138.1, NC_019037.1, NC_014384.1, NC_007675.1, NZ_CP012632.1, NZ_CP013027.1, NC_019122.1, NC_019117.1, NZ_CP022166.1, NZ_CP020934.1, NZ_CP019909.1, NZ_CP019018.1, NZ_CP018775.2, NZ_CP015239.1, NZ_CP010141.1, NZ_CP010123.1, NZ_CP006635.1, NZ_CP005931.1, NC_025179.1, NC_017659.1, NC_012944.1, NC_009837.1, NC_010720.1, NZ_CP009167.1, NC_017640.1.

12. For gels with more than ten lanes, include the DNA ladder in several lanes at different positions.
13. The amplicons obtained in a multiplex PCR can be confirmed by repeating the corresponding simplex PCR. Its product can be purified and sequenced by standard Sanger sequencing to verify the result.

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