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PBRT 2.0 kit - *PCR based replicon typing – New replicons P1, N2, FIB-KN, FIB-KQ, X4*

REF **MBK0078**

22 **192 REACTIONS**

INTENDED USE

The PBRT 2.0 kit is intended for molecular typing of plasmid conferring drug resistance in *Enterobacteriaceae*.

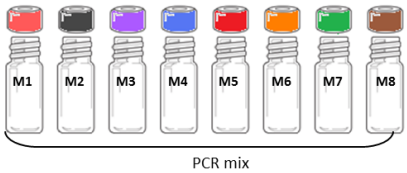
INTRODUCTION

The epidemiology of resistance plasmids is a major issue for the description of antimicrobial resistance diffusion. Plasmids can be categorized in families on the basis of their phylogenetic relatedness.
The molecular identification and classification of plasmids by PCR is currently possible and it is based on the detection of plasmid replication controls (replicons), namely the portion of large, naturally occurring plasmids encoding the replicase gene (*rep*) and *cis*-acting regulatory elements, controlling plasmid DNA replication and copy number. The majority of the replicons have been demonstrated to confer plasmid incompatibility (Couturier et al. 1989), therefore the detection of one of these replicons may be interpreted as a provisional molecular assignation of the plasmid to its respective Inc group. The PCR-Based Replicon Typing (PBRT), was an efficient method for plasmid identification and typing in Enterobacteriaceae, developed by Alessandra Carattoli, director of research at the Italian National Institute of Health in Rome, Italy (Carattoli et al. in 2005) and subsequently industrialized by Diatheva,. The 2005 PBRT scheme detected 18 replicons in 8 PCR reactions. Recently, the identification of novel replicons and plasmid types requested an update of the PBRT scheme. The PBRT 2.0 system is a PCR-based assay that allows fast and easy plasmid characterization and molecular identification through replicons detection. Together with a convenient format the system represents a valid tool to test a wide panel of bacterial strains and has been extensively used to classify plasmids, following the dissemination of resistance determinants in different countries and environments (Carattoli A. et al., 2009).

PRINCIPLE OF THE ASSAY

The PBRT 2.0 kit provides a novel set of 8 specific standard PCR assays optimized to perform 8 multiplex PCRs for the amplification of 30 replicons:
HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M and FIB-M, representative of major plasmid incompatibility groups and replicase genes identified on resistance plasmids circulating among *Enterobacteriaceae* (Carattoli et al. 2005, García-Fernández . et al. 2009. García-Fernández . et al. 2012, Villa et al. 2010).
A list of reference plasmids, one for each replicon detected by the PBRT kit is provided in the table below with the indication of the associated Inc group. Replicons that are not yet assigned to any known Inc group are reported in the table below as "not assigned".
Positive controls for all the respective replicons are included in the kit.

PCR mix	M1	M2	M3	M4	M5	M6	M7	M8
Target site (Amplicon lenght, bp)	HI1 (534) HI2 (298-308) I1α (159)	M (741) N (514) I2 (316) BO (159)	FIB(683) FIA(462) P1 (345) W (242)	L (854) X3 (284) I1γ (161)	T (750) A/C (418) FIIS(259-260) N2 (177)	U (843) X1 (370) R (248) FIIK (142-148)	FIB KN (631) X2 (376) FIB KQ (258) K (190)	HIB-M(570) FIB-M(440) FII (288-292) X4 (172)



PCR mix

KIT CONTENTS

1X PCR mix M1: 600 µl
1X PCR mix M2: 600 µl
1X PCR mix M3: 600 µl
1X PCR mix M4: 600 µl
1X PCR mix M5: 600 µl
1X PCR mix M6: 600 µl
1X PCR mix M7: 600 µl
1X PCR mix M8: 600 µl

1X positive control M1: lyophilized -15 µl
1X positive control M2: lyophilized -15 µl
1X positive control M3: lyophilized -15 µl
1X positive control M4: lyophilized -15 µl
1X positive control M5: lyophilized -15 µl
1X positive control M6: lyophilized -15 µl
1X positive control M7: lyophilized -15 µl
1X positive control M8: lyophilized -15 µl

DNA Polymerase (5U/µl): 40 µl (200U)

DNAaese free water: 1000 µl

OTHER SUPPLIES REQUIRED

- Disposable latex gloves.
- Precision pipettes.
- Sterile pipette tips.
- Sterile 1.5ml vials and sterile 0.2ml PCR vials.
- Tabletop centrifuge.

SHIPPING CONDITIONS

Shipping at room temperature has no detrimental effect on the performance of this kit.

STORAGE

Store the kit at -20°C. Repeated freeze-thawing should be avoid. For discontinued use storage of mixes in working aliquots is recommended.

GENERAL PRECAUTIONS FOR PCR

The operator should always pay attention to:

- MAINTAIN STRICTLY SEPARATE WORKING AREAS FOR DNA EXTRACTION AND PCR SET-UP
- use pipette tips with filter;
- store positive material (specimens, controls and amplicons) separately from all other reagents and, if possible, add it to the reaction mix in a facility separated space;
- thaw all components and samples at room temperature before starting an assay;
- work on ice.

IMPORTANT NOTE

The PBRT 2.0 kit has been validated on a wide panel of bacterial strains isolated from various samples according to the manufacturer instruction. It has been demonstrated that the introduction of modifications in the protocol (e.g. use of less volume of mastermixes) can cause the failure of the amplification of some plasmids.

PROTOCOL

1.1 DNA ISOLATION

Total DNA must be obtained by :

- Boiling lysis method: Briefly, put 3-4 isolated colony in 0.1ml of distilled water and boil for 10 minutes. Lysate preparations must be clarified by centrifugation (typically 15000 x g for 10 minutes). Transfer clarified supernatant into a clean 1.5ml eppendorf tube.
- Commercial DNA Purification System (i.e. Wizard Genomic DNA Purification System, Promega). Start from 5 ml of Tryptone Soy Broth cultures without antibiotics and carry out the DNA isolation according to the protocol supplied from the manufacturer making sure that the extracted DNA is free from PCR inhibitors.
- Store the DNA at -20°C avoiding repeated freeze/thaw cycles.
- In the same separate area thaw the PBRT positive controls (lyophylized). Reconstitute each vial by adding 15µl of DNase free water water, vortex for 60 seconds and centrifuge briefly. Add 1µl of each positive control into the corresponding PCR vial containing amplification mix and vortex briefly.

1.2 PCR SET UP

- Thaw the PBRT Amplification Mixes, vortex for 20 seconds and centrifuge briefly.
- In 8 separated sterile 1.5ml vials, prepare the amplification reaction mixes (from M1 to M8) needed for each sample to be tested plus one (1 reaction will be used for the amplification of positive control) following the pipetting scheme below:

	For 1 sample *	**For 1 samples + 1 positive control**	**For 20 samples + 1 positive control**
PCR mix M1 HI1, HI2, I1-α	23.8 ul mix + 0.2ul DNA polymerase	47.6ul mix + 0.4ul DNA polymerase	499.8 ul mix + 4.2ul DNA polymerase
PCR mix M2 M, N, I2, B/O	The same as above	The same as above	The same as above
PCR mix M3 FIA, FIB, P, W	The same as above	The same as above	The same as above
PCR mix M4 L, X3, I1-γ	The same as above	The same as above	The same as above
PCR mix M5 T, A/C, FIIS, N2	The same as above	The same as above	The same as above
PCR mix M6 U, X1, R, FIIk	The same as above	The same as above	The same as above
PCR mix M7 FIB-KN, X2, FIB-KQ, K	The same as above	The same as above	The same as above
PCR mix M8 HIB-M, FIB-M, FII, X4	The same as above	The same as above	The same as above

* For the analysis of more than one sample, simply multiply the volumes of mix and DNA polymerase for the number of samples to be tested

- Mix each vial for 20 seconds and centrifuge briefly.
- Aliquot 24 µl of the Amplification mixes obtained according to the scheme above into each of the PCR vials.
- In a separate area, add the DNA samples to be tested (prepared as indicated in section 1.1) into the corresponding PCR vial containing amplification mixes and vortex:
    - 1µl of DNA obtained by boiling method or,
    - 1µl of DNA obtained by commercial kit (wizard), diluted to a final concentration of 50-200ng/µl
- In the same separate area thaw the PBRT positive controls and vortex for 30-40 seconds. Add 1µl of each positive control into the corresponding PCR vial containing amplification mix and vortex briefly.

1.3 PCR RUN

- Program the PCR thermal cycler with the following parameters:

1 cycle	95°C for 10min
25-30 cycles*	95°C for 60sec 60°C for 30sec 72°C for 60sec
1 cycle	72°C for 5min
	cool down to 4°C

* the cycle number should be set at 25 if you are using high performance thermal cycler, or at 30 if you are using a low performance thermal cycler.

- Set the reaction volume to **25μl.**
- Perform the PCR run.

When the run is completed, proceed immediately to the next step or store the reaction at +4°C or at –20°C for a longer time.

1.4 AGAROSE GEL ELECTROPHORESIS

- Add 5μl of DNA loading buffer directly to amplified samples.
- Load 5μl of amplicons on a 2.5% agarose gel containing ethidium bromide or any other stain gel agent, in the presence of a DNA standard specific for the low range (100-1000 bp).
- To correctly identify all PCR products obtained, run the gel as long as all amplicons generated by multiplex reactions are well distinguishable each other as shown below (**Fig.1**).

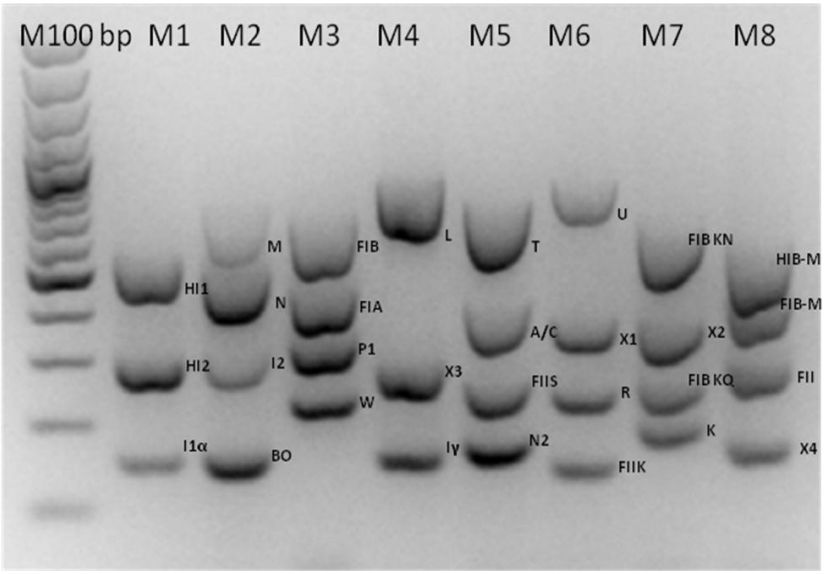


Fig.1: Representative agarose gel electrophoreses analysis of multiple amplicons obtained through the amplification of positive controls using all PCR mixes from M1 to M8
Lane 1: molecular weight marker 100bp gene ruler (MBI Fermentas)
Lane 2-9: PCR products for M1; M2; M3; M4; M5; M6; M7** and M8 respectively
** Please note: Extra bands generally appear in M7 but they do not invalidate the reaction result.

1.5 INTERPRETATION OF RESULTS

Small plasmids (2-15 kb) that do not control copy number and replication, do not contain replicons and are not detected by PBRT.
Bacteria may contain more than one plasmid species within the same cell and plasmids may contain more than one replicon (multireplicon plasmids). An assessment of bacterial plasmid content can be obtained using total genomic DNA purified from isolates under study. Replicon content may be a useful bacterial typing tool for epidemiological investigations.
IncF family: IncF multireplicon plasmids may show different combinations of the FIA, FIB, and/or FII replicons. The size of the amplicon obtained by FII PCRs may vary of 3-

16 bp with respect to the control, depending on the DNA sequence of the FII replicon under investigation (Villa et al., 2010). The FIIS PCR recognizes a divergent FII replicon identified on *Salmonella virulence plasmids*. The FIIK PCR recognizes a divergent FII replicon identified on plasmids from *Klebsiella* spp (Villa et al. 2010). FIB KN and FIB KQ are useful to identify and distinguish the two most common IncFIIk plasmids in *Klebsiella pneumoniae*: FIB KN recognizes the pKPN3- like plasmids; FIB-KQ recognize the pKpQIL-like plasmids (García-Fernández . et al. 2012).

IncHI family: three types of IncHI-plasmids are detected by PBRT 2.0: HI1, HI2 and a novel, not assigned HI-like group. The latter is detected by the FIB-M and HIB-M PCRs, two novel replicons identified for the first time in the pNDM-MAR plasmid (Villa et AL., 2012). The size of the HI2 amplicon may vary depending by the number of iterons present in the replicon under investigation.

IncX family: four types of IncX plasmids are detected by PBRT 2.0, as defined by Johnson et al. 2012

IncI family: PBRT detects I1-alpha, I1-gamma, I2, B/O, K, L and M within the IncI complex. Since the high level of DNA identity among the members of this plasmid family K plasmids may give a double positive result with both M2 and M7 PCRs, while B/O plasmids normally give a positive result only when their own B/O primers are used in M2. It may depend by the template concentration and PCR conditions since these two replicons are highly related. However the results can be easily deduced: when the K amplicon is obtained in M7 the plasmid is IncK even if it reacts with the B/O PCR in M2, when the B/O amplicon is obtained in M2, and M7 is negative the plasmid is IncB/O.

Other Inc families: PBRT 2.0 detect the replicon associated with IncN, IncN2, IncA/C, Inc W, IncT, IncU. Among the IncP family only the IncP1-alpha is detected, being the most frequent variant circulating in Enterobacteriaceae. PBRT also detect the R replicon that was not formally assigned to any known Inc group (García-Fernández . et al. 2009).

Replicons detected by PBRT 2.0

Replicon	Amplicon bp	Reference plasmid	GenBank	Inc Group
HI1	534	*Salmonella typhi* R27	AF250878	IncHI1
HI2	298-308	*Serratia marcescens* R478	BX664015	IncHI2
HIB-M	570	*K. pneumoniae* pNDM-MAR	JN420336	Not assigned
FIB-M	440	*K. pneumoniae* pNDM-MAR	JN420336	Not assigned
I1α	159	*S. enterica* Typhimurium R64	AP005147	IncI1α
I1γ	161	*S. enterica* Typhimurium R621a	NC_015965	IncI1γ
I2	316	*Escherichia coli* R721	AP002527	IncI2
M	741	*Citrobacter freundii* pCTX-M3	AF550415	IncM
L	854	*Klebsiella pneumoniae* pOXA-48	KM406491	IncL
K	190	*Escherichia coli* R387	M93063	IncK
B/O	159	*Escherichia coli* p3521	GU256641	IncB/O
A/C	418	*Aeromonas hydrophila* pRA1	FJ705807	IncA/C
N	514	*Escherichia coli* R46	NC_003292	IncN
N2	177	*Escherichia coli* P271A	JF785549	IncN2
W	242	*Escherichia coli* R721	AP002527	IncW
P1	345	pBS228	BN000925	IncP1-alpha
T	750	*Proteus vulgaris* Rts1	AP004237	IncT
U	843	*Aeromonas hydrophila* pRA3	DQ401103	IncU
R	248	*Klebsiella pneumoniae* pK245	DQ449578	Not assigned
X1	370	*Escherichia coli* pOLA52	EU370913	IncX1
X2	376	*Escherichia coli* R6K	M65025	IncX2
X3	284	*K. pneumoniae* pIncX-SHV	JN247852	IncX3
X4	172	*Escherichia coli* pUMNF18_32	CP002895	IncX4
FIA	462	*Escherichia coli* Plasmid F	AP001918	IncF
FIB	683	*Escherichia coli* Plasmid F	AP001918	IncF
FII	288-292	*Escherichia coli* NR1	DQ364638	IncFII
FIIS	259-260	*S. enterica* Typhimurium pSLT	AE006471	IncFIIS
FIIk	142-148	*Klebsiella pneumoniae* pKPN3	CP000648	IncFIIk
FIB KN	631	*Klebsiella pneumoniae* pKPN-IT	JN233704	IncFIIK
FIB KQ	258	*Klebsiella pneumoniae* pKpQIL-IT	JN233705	IncFIIK

Observation	Possible cause	Suggested solutions
No PCR product	Missing components (e.g. template or DNA	Check the assembly of the reaction

TROUBLESHOOTING

	Polymerase)	
	Missing or incorrect essential step in the cycler protocol	Check the cycler protocol
	Poor quality template	DNA extraction should be carried out according to manufacturer suggestions of recommended extraction kit (as indicated above)
	Insufficient starting template	Increase starting template
	Degraded reagents	Store reagents at -20°C. Avoid multiple freeze thaw cycles
	Pipetting mistake	Check pipetting and repeat the test
	Your plasmid is not typable by this scheme or the strain does not contain any plasmid	This scheme targets the major replicons circulating in *Enterobacteriaceae*, but broad host range plasmids from other bacterial genera or new plasmid types could emerge and be undetectable.
Extra bands on gel	Too much starting template	Reduce DNA starting template
	High performance of your PCR platform	Reduce to 20-25 the number of PCR cycles
	Cross-reaction with non-target sequences	Increase gel concentration from 2.5 to 3% agarose and run amplicons for a longer time in order to distinguish possible non-specific amplicon from target amplicons

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