Modified U- tube for Ruling out Naked DNA Transfer during Conjugation and application in Antibiotic Resistance Genes Transfer Research

Ning Zhang^a, Xiang Liu^{a*}, Bing Li^b, Limei Han^a, Xuejiao Ma^{a,c}, Fanbin Meng^a, Miao Li^{a*}

Contents:

Text S1 Leakage tests of the modified U-tube

Text S2 Text S1 PCR procedure for traF and aphA

Text S3 Procedures for flow cytometry (FCM) and confocal laser scanning

microscopy (CLSM)

Figure S1 The filter sealing verification

Figure S2. traF gene sequence analyses of gel bands

Table S1 PCR primers and conditions

Table S2 Statistical test of donor and donor/receptor

References

Text S1

Leakage tests of the modified U-tube were performed using water and *P. putida* vs sterile LB medium, respectively. 80 ml distilled water was poured into the donor flask of the U-tube, then standing for 12 hours to observe the liquid leakage from the flange and filter. The compared images were presented in figure S1-A. Compared to Figure S1-A1, there was no liquid leakage from the U-tube. The volume of distilled water in the donor flask was still 80 ml. After the initial verification test, *P. putida* and sterile LB medium were injected into the donor and recipient flask, respectively. Pump was used to assist the exchange of liquid in the system. After 4 hours samples from the donor and recipient flask of the U-tube were inoculated on selective medium containing 50 mg/L kanamycin to observe the cell transfer. AsFigure S1-B shows, there was no colony on the plate, which mean the *P. putida* cannot pass from the donor flask to the other.

Text S2

PCR assays were conducted in a Bio-Rad C1000 gradient thermal cycler (Bio-Rad, USA) to verify whether plasmid RP4 (the presence of *tra*F and *aph*A genes) had been transferred into recipient. The total volume of PCR mixture was 20µL, containing 10µl PCR mix (TIANGEN, China), 7µL ddH₂O, 1µLof each 10µM primer, and 1µl DNA template. The PCR procedure for *tra*F and *aph*A gene amplification was: initial denaturation at 95°C for 3 min; 40 cycles of 30 s at 95°C, 30 s of annealing at a specified temperature for each gene, and extension for 1 min at 72°C; with a final

extension at 72° C for 5 min. The nucleotide sequence of PCR primers and annealing temperatures are shown in Table S1 [1]. The PCR products were determined by comparison with DNA standards (Marker Ladder, TIANGEN, China) on 1.5% agarose gels run for 30 min at 120 V.

Text S3

FCM and CLSM were used to sort and identify donor, recipient and transconjugant (or transformant) by fluorescence. In preparation for FCM, 1 mL samples were centrifuged (10000 g for 1 min), and then resuspended in PBS. Flow cytometric detection of cells was carried out using a FACS Calibur system (BD, USA). The excitation wavelengths of GFP and RFP were 488 nm and 561 nm, respectively [2]. A flow rate of 1 µl/s was chosen, and 10000 events/s were counted. Bacterial populations were gated according to GFP vs. RFP plots. P. putida and E. coli were used as positive and negative controls, respectively. BD FACS Diva software was used for data acquisition, and subsequent analysis was performed on FlowJo v.10 software. For CLSM, samples (100 µl) were performed on Olympus FV3000, Japan, which were added to the confocal plate and excited with a 488 nm and 561 nm diode-pumped solid-state laser. Fluorescence was observed using an Olympus UApo N 100×1.49 numerical aperture oil-immersion objective lens with an extra $3.0\times$ intermediate magnification lens with the same zoom and different Z dimensions. The images were processed using ImageJ software v.1.47 and Adobe Photoshop 7.0.

Figure S1



Figure S1. The filter sealing verification, A Liquid leakage, A1 original state after water poured, A2 state of the system after 12 hours; B plate counting of *P. putida*, B1-2 colony in recipient flask after 4 hours, B3-4 colonies in donor flask after 4 hours

Figure S2

Bands of gene *tra*F in samples from recipient flask of test 1# at 180 min, test 3# at 180 min and test 4# at 180 min were sent for sequence analysis. The sequences were corrected by blasting to Genbank. Sequence results were shown in Figure S2.

(A)

Gene sequence:

CGGGGTTTGTCCTTGTTGCTCGCCGGCGCGCGGCCTATCTCGCCGGCGCGAAG GTCAACACCACCAAAAGCATTCCGGTCGGCCTGTACTGGAAATCGAATGC GCCGGTGGAGAAGGGGGGCTTTACGTCATGTTCTGCCCGCCGCAAGTCGGC

GTGTTTTCGGACGCCAAGGAGCGTATCTGGCGCCAGACGCAG

NCBI blasting:

Plasmid RP4 (from Pseudomonas aeruginosa) essential transfer protein (traF) gene, complete cds Sequence ID: <u>M94366.1</u> Length: 580 Number of Matches: 1

Rang	e 1: 3	334 to 496	GenBank	Graphics				Vext Match	A Previous Match
Scor	e		Expect	Identities		Gaps		Strand	
296 b	oits(1	60)	2e-76	163/164(99	%)	1/164(0%)		Plus/Minus	
Query	11	CCTTGTTGCT		CTATCTCGCCGGCGC	GAAGGTCA	CACCACCAAAAGCA	70		
Sbjet	496	CCTTGTTGCTO	CGCCGGCGCGGG	CTATCTCGCCGGCGC	GAAGGTCA	CACCACCAAAAGCA	437		
Query	71	TTCCGGTCGG		ATCGAATGCGCCGGT	GGAGAAGGO	GGCTTTACGTCATG	130		
Sbjet	436	TTCCGGTCGG	CCTGTACTGGAA	ATCGAATGCGCCGGT	GGAGAAGGG	GGCTT-ACGTCATG	378		
Query	131	TTCTGCCCGC	CGCAAGTCGGCG	TGTTTTCGGACGCCA	AGGAGCG	174			
Sbjet	377	TTCTGCCCGC	CGCAAGTCGGCG	TGTTTTCGGACGCCA	AGGAGCG	334			

(B)

Gene sequence:

CTCCGATGGAGGCCTTGCTCGCCGGCGCGCGGCCTATCTCGCCGGCGCGAAGG TCAACACCACCAAAAGCATTCCGGTCGGCCTGTACTGGAAATCGAATGCGC CGGTGGAGAAGGGGGGCTTTACGTCATGTTCTGCCCGCCGCAAGTCGGCGT GTTTTCGGACGCCAAGGAGCGGAGGGGGGGCAGATGTGATCGA

NCBI blasting:

Plasmid RP4 (from Pseudomonas aeruginosa) essential transfer protein (traF) gene, complete cds Sequence ID: <u>M94366.1</u> Length: 580 Number of Matches: 1

Rang	e 1:	333 to 491	L <u>GenBank</u>	Graphics		🔻 Next Match 🔺	Previous Match
Scor	e		Expect	Identities	Gaps	Strand	
289 l	oits(1	.56)	3e-74	159/160(99%)	1/160(0%)	Plus/Minus	
Query	15	TTGCTCGCCG	GCGCGGCCTAT	CTCGCCGGCGCGAAGGTCAA	CACCACCAAAAGCATTCCG	74	
Sbjet	491	TTGCTCGCCG	GCGCGGCCTAT	CTCGCCGGCGCGAAGGTCAA	CACCACCAAAAGCATTCCG	432	
Query	75	GTCGGCCTGT	ACTGGAAATCG	AATGEGEEGGTGGAGAAGGG	GGCTTTACGTCATGTTCTG	134	
Sbjet	431	GTCGGCCTGT.	ACTGGAAATCO	AATGCGCCGGTGGAGAAGGG	GGCTT-ACGTCATGTTCTG	373	
Query	135	CCCGCCGCAA	GTCGGCGTGTT	TTEGGAEGEEAAGGAGEGG	174		
Sbjet	372	CCCGCCGCAA	GTCGGCGTGTT	TTCGGACGCCAAGGAGCGG	333		

(C)

Gene sequence:

CGCATCGTCTCCTTGTTGCTCGCCGGCGCGCGGCGCGAAG GTCAACACCACCAAAAGCATTCCGGTCGGCCTGTACTGGAAATCGAATGCG CCGGTGGAGAAGGGGGGCTTTACGTCATGTTCTGCCCGCCGCAAGTCGGCG

TGTTTTCGGACGCCAAGGAGCGGGGCTACATCGCCGGCGGTT

NCBI blasting:

Plasmid RP4 (from Pseudomonas aeruginosa) essential transfer protein (traF) gene, complete cds Sequence ID: <u>M94366.1</u> Length: 580 Number of Matches: 1

Rang	e 1: 3	314 to	496	5 <u>Ge</u>	nBa	nk	Gr	aph	ics									۷	Next	t Mat	tch	A P	revio	ous N	1atch
Score	e			Ex	pect	t	I	de	ntit	ies				(Gaps				Strar	nd					
333 b	its(1	80)		1e-	87		1	83/	184	4(99	9%)			1	/184	(0%)		-	Plus/I	Minus	s				
Query	11	CCTTG	ITGCT(CGCCO	GCGC		CTAI	гстс 	6000 	GCG	CGAA	(GGT)		ACC.	ACCAA	AAGCA	70								
Sbjet	496	ĊĊŦŦĠ	TIGCT	ĊĠĊĊ	GCGC	ĊĠĠĊ	ĊŤĂŢ	icto	śććo	GCG	ĊĠĂĂ	GGT	CAAC	ÁĊĊ.	ACCAA	AAGCA	437								
Query	71	TTCCG	GTCGG	CCTG	TACT(GGAA	ATCO	GAAT	GC GC		TGG#	GAA	GGGG	GCT	TTACG	TCATG	130								
Sbjet	436	TTCCG	STCGG	ĊĊŤĠ	TACTO	GGAA	ÁTC (JAAT	ĠĊĠĊ	cccc	ŤĠĠĂ	GAA	ŝĠĠĠ	ĠĊŦ	Í-ÁĊĠ	TCATG	378								
Query	131	TTCTG		CGCAJ	AGTC(GGCG	TGTI 		GGAC	CGCC.	AAGO	AGC	GGGG(CTA	CATCG	CCGGC	190								
Sbjet	377	TTCTG	ccccc	ĊĠĊĂJ	AGTCO	ĠĠĊĠ	İĞİI	rttc	GGAC	GCC.	ÅÅĠĠ	AGC	ŚĠĠĠ	ĊŤÁ	CATCG	ĊĊĠĠĊ	318								
Query	191	GGTT	194																						
Sbjet	317	GGTT	314																						



Table S1

Primer	Target	Sequence (5'-3')	PCR annealing temp	Amplicon size	
	1		(°C)	(bp)	
aphA-FW	anh	GGCTTCGTGATGCCTGCTT	()	109	
aphA-RV	apnA	CATTCCTGGCCGTGGTTCT	02	198	
traF-FW	tu a F	CTCCGATGGAGGCCGGTAT	541	106	
traF-RV	irdF	GGGAATGCCATCTGCCTTGA	34.1	190	

Table S1 PCR primers and PCR conditions

Table S2

Table S2 Statistical test of donor and donor/receptor

Test		Donor		Donor/receptor					
	mean value	square deviation	P-value	mean value	square deviation	P-value			
7#	5786.16	332265.3	7.050.04	10.06	0.90	5 05E 05			
8#	2306	102529	/.93E-04	0.33	0.003	3.93E-03			

References

[1] Wang, Q.; Lu, Q.; Mao, D.; Cui, Y.; Luo, Y. The horizontal transfer of antibiotic resistance

genes is enhanced by ionic liquid with different structure of varying alkyl chain length. *Front Microbiol*, 2015, 6, 864.

[2] Dunny, G.M.; Craig, R.A.; Carron, R.L.; Clewell, D.B. Plasmid transfer in Streptococcus faecalis: production of multiple sex pheromones by recipients. *Plasmid*. 1979, 2, 454-465.