Protocol to amplify T-DNA Flanking Sequence Tags (FST) from rice

Martine Bès, Christophe Sallaud, Emmanuelle Bourgeois,
Pietro Piffanelli, Christophe Périn and E. Guiderdoni
(adapted to rice from Devic et al., 1997 and Balzergue et al., 2001)

contact: Martine Bès (bes@cirad.fr) or Pietro Piffanelli (Piffanelli@cirad.fr)

Starting material: DNA isolated from rice young leaves using the QIAGEN Dneasy 96 plant Kit - Ref. 69181 – this extraction procedure yields 200 µl of genomic DNA at a concentration of approx. 33 ng /µl.
The protocol is divided in the following four steps:

I) Restriction-ligation (RL)

Rice genomic DNA is cut with a frequent cutter restriction enzyme giving blunt ends (*DraI* or *SspI*) and the obtained genomic fragments are ligated to a suitable adaptor.

Note: the same protocol can be applied to other restriction enzymes e.g. EcoRV, NaeI, ECl136

Preparation of adaptor stock (25 µM):

This step of hybridisation of the two primers constituting the adaptor is carried out just before setting up the restriction-ligation reaction.

Adpr1 (100µM)	8,3 µl
Adpr2 (100 µM)	8,3 µl
Buffer4 10X (Gibco BRL)	3,3 µl
H_2O	13,3 µl
TOTAL	33,2 µl

PCR conditions:

95°C, 5 min
28°C, 5 min

The adaptor is kept on ice until utilisation. For one 96-well plate 33 µl of adaptor are needed.

Restriction-Ligation (RL) reaction

Rice genomic DNA is cut with *DraI* or *SspI* and ligated to the adaptor in the same reaction. This step is carried out in 96-well plates

Note: the use of the *Sst2* enzyme in the restriction-ligation, which cuts in the binary vector next to the T-DNA left border, limits the amplification of integrated binary vector.

RL DraI or SSPI	**Quantity/well**	**Volume/well**
DNA (33 ng/µl)	82,5 ng	2,5 µl
DraI or SspI (10 U/µl)	1 U	0.1 µl
Sst2 (10U/µl)	0,5U	0,05 µl
T4 Ligase (20 U/µl)	0,24 U	0.012 µl
T4 ligase buffer10X	1 X	1 µl
Adaptator (4µM)	375 nM	0.15µl
H_2O	to final10µl	6,188 µl

The RL samples are incubated at 25°C overnight (16 hours)
Following incubation the RL samples are diluted - 40 µl H_2O are added to each well
The RL samples can be stored at -20°C until their utilisation.

II) PCR1

This step enables to amplify genomic DNA fragments between the adaptor and the T-DNA. This step is carried out in 96-well plates

	Quantity/well	**Volume/well**
Diluted RL reaction	16,5 ng	10 µl
Taq Buffer10X	1X	2 µl
dNTPs (1,875mM each)	75 µM	0.8 µl
AP1 primer 100 µM	0,2 µM	0,04 µl
Hyg7 primer100 µM	0,2 µM	0,04 µl
Taq (2 U/µl)	2 U	1 µl
H_2O	to final 20 ul	6,12 µl

PCR1 program

94°C	3 mn	1 cycle
92°C	30 sec	30 cycles
67°C	45 sec	
72°C	2mn30sec	
72°C	5 mn	1 cycle
15°C	storage	

The PCR1 reaction is diluted 50 times before proceeding to the next step.
To a volume of 2 µl of PCR1 reaction, 98 µl of H_2O are added

II) PCR2:

This step increases the specificity of amplification of rice genomic DNA between the adaptor and the T-DNA.

	Quantity/well	**Volume/well**	
Diluted PCR1	2 µl	2 µl	
Taq buffer 10X	1X	10 µl	
dNTPs (1,875mM each)	75µM	4 µl	
AP2 primer (100µM)	0.2 µM	0,2 µl	
Hyg8 primer (100µM)	0,2 µM	0,2 µl	
Taq (2U/µl)	4U	2 µl	
H_2O	to final 100 µl	81,6 µl	

PCR2 program

94°C	3 mn	1 cycle
92°C	30 sec	35 cycles
67°C	45 sec	
72°C	2mn30sec	
72°C	5 mn	1 cycle
15°C	storage	

IV) PCR2 analysis and sequencing

3 µl of PCR2 reaction are analysed by gel eletrophoresis on an agarose gel 1,2%.
The obtained PCR bands are directly sequenced using the CAMB6 primer.

V) Solutions and primers

1) Taq buffer10X

670 mM Tris-HCl (pH 8.8)
160 mM $(NH4)_2$ SO_4
0.1% Tween 20
17mM MgCl2
pH to 8,8 with HCl
sterilise by filtration (0.2 µm)

2) Primer sequences

ADPR1	5′ CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGGAGGT 3′
ADPR2	5′P ACCTCCCC 3′ NH2
AP1	5′ GGATCCTAATACGACTCACTATAGGGC 3′
AP2	5′ CTATAGGGCTCGAGCGGC 3′
Hyg7	5′ GTCGATGCGACGCAATCGTCCGATC 3′
Hyg8	5′ GTCTGGACCGATGGCTGTGTAGAAG 3′
CAMB6	5′ CGCTCATGTGTTGAGCATAT 3′