



LONG RANGE PCR: PROMISING TOOL FOR ALLELIC MINING IN WILD POTATO SPECIES



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Introduction

Late Blight (LB) is the most devastating disease that affects potato yield (Agrios, 1997), causing multi-billion dollar losses each year worldwide (Kamoun, 2001). One of the several approaches to control LB is the development of resistant cultivars. *Solanum bulbocastanum*, a diploid Mexican wild potato species, has been shown to be resistant to LB (Helgeson *et al.*, 1998). Recently, the *RB* gene, a functional *LR* resistance gene conferring resistance against all known *LR* races, was cloned from *S. bulbocastanum* genotype PT29 using a traditional map-based approach in combination with Long Range-PCR (LR-PCR) (Song *et al.*, 2003). The successful cloning of the *RB* resistant allele suggests LR-PCR could be a reliable method to isolate functional alleles in diverse populations of this wild species or related species. However, technical optimization is required for large-scale LR-PCR application.

Here , we optimized 3 critical factors for LR-PCR application:

- Quality of Genomic DNA template
- Efficiency and Fidelity of Amplification, and
- Primer design.

Quality of Genomic DNA template

- Template of sufficient integrity and purity is needed.
- 5 commercial genomic DNA extraction kits were compared based on ease, cost, and yield/purity.
- Besides DNA quality, length and concentration, preparations were checked for LR-PCR performance using the protocol of Song *et al.* (2003).

Efficiency and Fidelity of Amplification

- Here, we compare commercial LR-PCR Taq preparations for amplification efficiency and sequence fidelity at the *RB* locus.
- 4 systems were compared with a positive control (Takara + Song *et al.* (2003) protocol).
- All 4 systems followed manufacturer's recommended conditions.

Primer design

- Like many other plant R-genes, *RB* gene is a member of a multigene cluster composed of gene copies with high sequence similarity (79.6-85.9%) (Song *et al.*, 2003).
- LR-PCR, at this locus, requires a specific primer design.

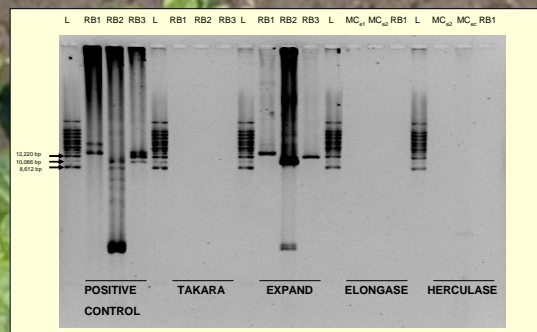
Table 1. Effectiveness of LR-PCR performance using Kit-extracted DNA template and LR-PCR conditions of Song *et al.* (2003).

DNA Extraction Kits	Product size		
	5 Kb	12 Kb	15 Kb
Wizard Magnetic 96, Promega ¹	Yes	No	Yes
GenElute, Sigma (1 st elute)	Yes	Yes	Yes
GenElute, Sigma (2 nd elute)	Yes	No	Yes
DNeasy, Qiagen (1 st elute)	Yes	Yes	Yes
DNeasy, Qiagen (2 nd elute)	Yes	Yes	Yes
Easy-DNA, Invitrogen	No	No	No
Nucleon Phytopure, Amersham Bio	Yes	No	No
Tanksley extraction	No	No	No
CsCl-purified DNA	Yes	Yes	Yes

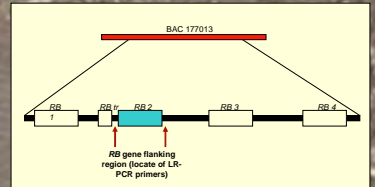
¹ Protocol was adapted due to the lack of magnetic equipment

- DNeasy Plant Mini Kit (Qiagen) and GenElute Plant Genomic DNA Extraction Kit 1st elute (Sigma) generated consistent LR-PCR amplification results under our research conditions.
- Results are comparable with those obtained with the control template, CsCl-purified DNA template.

Amplification efficiency for four Taq systems.



- All LR-PCR reactions utilized CsCl-purified template (*RB* lanes) or manufacturer-supplied control DNA (*MC* Lanes).
- L* : High Molecular weight DNA markers. *RB1*: *RB* fragment using primer pair #16; expected size 11,819 bp. *RB2*: *RB* fragment using primer pair #17; expected size 8,439 bp. *RB3*: *RB* fragment using primer Ma3; expected size 10,592 bp. *MC*: manufacturer-supplied controls. *MC_{el1}* & *MC_{el2}*: Elongase controls. *MC_{ec}*: Expand control.
- The Expand 20 Kb^{PLUS} PCR system (Roche) was efficient for the amplification of the three tested product sizes.



- Primers selected using the Primer 3 program ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi)) were tested using Takara LA Taq + amplification conditions of Song *et al.* (2003).

- Amplification products (8.5 kb-fragment) from Takara and Expand systems were cloned for sequencing into pSMART vector (Lucigen corporation).

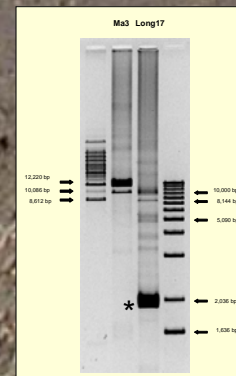
- Resulting sequence contigs (DNA Star SeqMan software) were compared with the susceptible BAC clone 177013 (Genebank #AY303171) and the resistant cDNA (Genebank # AY336128) sequences of the *RB* locus to determine amplification fidelity (DNA star MegAlign software).

Table 2. Alignment of single pass Expand LR-PCR sequence with direct cloning

	Expand <i>RB</i> vs. <i>RB</i> cDNA (Genebank #AY336128)	Expand <i>rb</i> vs. BAC <i>rb</i> (Genebank #AY303171)	Takara <i>RB</i> vs <i>RB</i> cDNA (Genebank #AY336128)
IDENTITY	99.3% (3617/3642)	99.3% (4873/4909)	99.9% (8566/8569)
ERRORS:			0.1% (3/8569)
• Transition/Transversion	0.08% (3/3642)	0.08% (4/4909)	
• Sequence Skips* [e.g. TCCCT instead of TCCT, etc.]	0.16% (6/3642)	0.16% (8/4909)	
• Unsolved sequences* [e.g. N (any nt.), K (G or T, etc.)]	0.44% (16/3642)	0.48% (24/4909)	

* Sequences skips and unresolved sequences may be the result of the sequencing procedure (we report here results of single pass sequencing) and not of LR-PCR.

Ma3 vs. Long17



- We designed and tested 3 primer sets for amplification of the *RB* gene and compared results with primer pair Long17 (Song *et al.*, 2003). Shown are results from the primer pair Ma3 (expected size: 10 592 bp) vs. Long17 (expected size 8.439 bp).

- Primer Long17 consistently amplifies an uncharacterized fragment of approximate 2 000 bp (*) in addition to the reported fragment.

- In contrast, primer Ma3 reliably amplifies *RB* fragments without many non-specific bands.

Conclusions

- The DNeasy Plant Mini Kit (Qiagen) and GenElute (first elute) Plant Genomic DNA extraction kit (Sigma) are easy, efficient and inexpensive alternatives to CsCl purification to produce DNA template for LR-PCR application.
- The Expand 20 Kb^{PLUS} PCR (Roche) system efficiently produces amplification of long products (8.5-10.5 kb) with a low error rate (0.7%) among the tested amplification systems.
- The Ma3 primer pair appears to be more specific than Long 17 and should result in reliable gene amplification at the *RB* locus.

The optimized LR-PCR broadens the scope of this method to multi-genotype applications and may ultimately prove useful for isolation of functional genes in other plant systems.

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References

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