A two-tube, nested PCR detection method for AHPND bacteria

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Background

The AP3 method for detection of the small AHPND toxin gene (AHPND ToxA) released at the NACA website in June 2014 (A new and improved PCR method for detection of AHPND bacteria) has proven effective for detection of bacteria that cause acute hepatopancreatic necrosis disease (AHPND), but it has the disadvantage in being a 1-step PCR method that lacks the sensitivity to detect very low levels of AHPND bacteria. It could not be adapted into an effective nested PCR method. Thus, the method required a bacterial pre-enrichment culture step prior to DNA extraction and PCR testing for such samples.

To overcome this problem of relatively low sensitivity (approx. 10 pg bacterial DNA) and need for enrichment culture, we have developed a new two-step (nested-PCR) method called AP4 for detection of AHPND-bacteria with higher sensitivity (approx. 100 fg bacterial DNA). This method targets a 1269 bp portion of the AHPND toxin plasmid sequence that includes the full *ToxA*gene plus a large portion of the *ToxB* gene sequence.

In purified form, the Pir-like toxins(ToxA and ToxB)work together to cause the hepatopancreatic necrosis characteristic of AHPND {Sirikharin, 2015 #8283}. The toxin genes are located very close together (separated by 12 bp) on a plasmid (VpA1) of approximately 69 kbp that is carried by isolates of *Vibrio parahaemolyticus* that cause AHPND (VP_{AHPND}) {Yang, 2014 #8265}. The AP4 target sequence consists of a chimeric DNA fragment comprising the full *ToxA* gene sequence plus the 12 bp linker plus 70% of the succeeding *ToxB* gene sequence for a total of 1269 bp out of the whole ToxA and ToxB region (1665 bp) on the 69 kbp VpA1 plasmid (Fig. 1).For the first-step PCR reaction, the outer primers AP4-F1 and AP4-R1(Fig. 1, bold underlined text) target a 1269 bp portion of the region that includes the full *ToxA* gene and a large portion of the *ToxB* gene sequence (921/1317 bp = 70%). The AP4-F1 primer is equal to the AP3-Fprimer

of the AP3 method. For the second-step PCR reaction, a portion of the final solution from the first-step PCR reaction is used as the template with the inner (nested) primers AP4-F2 and AP4-R2 (Fig. 1, text in grey outline). The target is a 230 bp portion of the sequence that includes 209 bp of the *ToxA* gene sequence plus the 12 bp spacer sequence plus 9 bp of the succeeding *ToxB* gene sequence. At high concentrations of target DNA, additional, bands for amplicons may occur as the product of residual primer AP4-F1 working with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

The advantage of the AP4 method over the AP3 method is that the it has 100 times higher sensitivity than the AP3 method. Because of its higher sensitivity, the bacterial culture enrichment step needed when using the AP3 with low levels of AHPND bacteria may be omitted. However, the AP4 method should not be considered as a replacement for the AP3 method but simply as an alternative choice for the users to choose should they need a more sensitive detection method.

The AP4 method has been tested with the same 104 bacterial isolates that were used for validating the AP3 detection method, and the results were identical, i.e., 100% specificity and sensitivity with the 104 isolates but at 100x lower template levels.

As with the previous announcements in this series, the AP4 method is provided for free use in the detection of AHPND bacteria. A positive control plasmid for the AP4 method will be sent out to those who are already on our mailing list as recipients of plasmids for our previous AP methods to detect AHPND bacteria. For those not already on our list, the plasmid will also be provided upon request to: Dr. Kallaya Sritunyalucksana <kallaya@biotec.or.th>.

Details of the PCR method

PCR primers

Primers	5′-3′	Length	%GC	Tm	Ta	Expected amplicon
AP4-F1 (= AP3-F)*	ATGAGTAACAATATAAAACATGAAAC	26	23	49	55	1269 bp
AP4-R1	ACGATTTCGACGTTCCCCAA	20	50	52		
AP4-F2	TTGAGAATACGGGACGTGGG	20	55	54	55	230 bp
AP4-R2	GTTAGTCATGTGAGCACCTTC	21	48	52		

*Please note that primer AP4 F1 is identical to primer AP3-F from the AP3 method.

First PCR reaction conditions

Components	µl	Final Conc.	Protocol	
10x PCR mix (Invitrogen)	2.5	1X	Denature	94°C, 2 min
50 mM $MgCl_2$	1.5	3 mM	30 cycles	
10 mM dNTPs	0.5	0.2 mM	Denature	94°C, 30 sec
10 µM AP4-F1	0.5	0.2 µM	Annealing	55°C, 30 sec
10 µM AP4-R1	0.5	0.2 µM	Extension	72°C, 90 sec
5U/µlTaq DNA polymerase	0.3	1.5 U	Final	72°C, 2 min
DNA template (50 ng/ µl)	2.0			
Sterile water	17.2			
Total	**25.0**			

2nd (Nested) PCR reaction conditions

Components	µl	Final conc.	Protocol	
10x PCR mix (Invitrogen)	2.5	1X	Denature	94°C, 2 min
50 mM $MgCl_2$	1.5	3 mM	25 cycles	
10 mM dNTPs	0.5	0.2 mM	Denature	94°C, 20 sec
10 µM AP4-F2	0.375	0.15 µM	Annealing	55°C, 20 sec
10 µM AP4-R2	0.375	0.15 µM	Extension	72°C, 20 sec
5U/µlTaq DNA polymerase	0.3	1.5 U	Final	72°C, 2 min
First PCR product	2.0			
Sterile water	17.45			
Total	**25.0**			

Figure 1.Diagram of the target sequences for the AP4 primers on the VpA1 plasmid in the region of the total nucleic acid sequence of the AHPND *ToxA* and *ToxB* genes plus the 12 nucleic acid spacer that links them together (spacer underlined text in grey background, i.e., included in the AP4-R2 primer)(total length 1665 bp of the fragment). The outer primers AP4-F1 and AP4-R1(bold underlined text) target a 1269 bp portion of the sequence that includes the full *ToxA*gene sequence and a large portion of the *ToxB* gene sequence (921/1317 bp = 70%). The inner (nested) primers AP4-F2 and AP4-R2 (text in grey outline) target a 230 bp portion of the sequence that includes 209 bp of the *ToxA* gene sequence plus the 12 bp spacer sequence and 9 bp of the succeeding *ToxB* gene sequence.

ATGAGTAACAATATAAAACATGAAACTGACTATTCTCACGATTGGACTGTCGAACCAAACGGAGGCGTCACAGAAGT
AGACAGCAAACATACACCTATCATCCCGGAAGTCGGTCGTAGTGTAGACATTGAGAATACGGGACGTGGGGAGCTTA
CCATTCAATACCAATGGGGTGCGCCATTTATGGCTGGCGGCTGGAAAGTGGCTAAATCACATGTGGTACAACGTGAT
GAAACTTACCATTTACAACGCCCTGATAATGCATTCTATCATCAGCGTATTGTTGTAATTAACAATGGCGCTAGTCG
TGGTTTCTGTACAATCTATTACCACTAAGAAGGTGCTCACATGACTAACGAATACGTTGTAACAATGTCATCTTTGA
CGGAATTTAACCCTAACAATGCTCGTAAAAGTTATTTATTTGATAACTATGAAGTTGATCCTAACTATGCTTTCAAA
GCAATGGTTTCATTTGGTCTTTCAAATATTCCTTACGCGGGTGGTTTTTTATCAACGTTATGGAATATCTTTTGGCC
AAATACGCCAAATGAGCCAGATATTGAAAACATTTGGGAACAATTACGTGACAGAATCCAAGATTTAGTAGATGAAT
CGATTATAGATGCCATCAATGGAATATTGGATAGCAAAATCAAAGAGACACGCGATAAAATTCAAGACATTAATGAG
ACTATCGAAAACTTCGGTTATGCTGCGGCAAAAGATGATTACATTGGTTTAGTTACTCATTACTTGATTGGACTTGA
AGAGAACTTTAAGCGCGAGCTAGACGGTGATGAATGGCTTGGTTATGCGATATTGCCTCTATTAGCAACAACTGTAA
GTCTTCAAATTACTTACATGGCTTGTGGTCTGGATTATAAGGATGAATTCGGTTTCACCGATTCTGATGTGCATAAG
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CTCTTACAATAATGCAAACCAAGATAACGTGTATGATGAAGTGATGGGTGCTCGTAGTTGGTGTACGGTTCACGGCT
TTGAACATATGCTTATTTGGCAAAAAATCAAAGAGTTGAAAAAAGTTGATGTGTTTGTTCACAGTAATTTAATTTCA
TATTCACCTGCTGTTGGTTTTCCTAGTGGTAATTTCAACTATATTGCTACAGGTACGGAAGATGAAATACCTCAACC
ATTAAAACCAAATATGT**TTGGGGAACGTCGAAATCGT**ATTGTAAAAATTGAATCATGGAACAGTATTGAAATACATT
ATTACAATCGCGTAGGTCGACTTAAACTAACTTATGAAAATGGGGAAGTGGTAGAACTAGGCAAGGCTCATAAATAT
GACGAGCATTACCAATCTATTGAGTTAAACGGCGCTTACATTAAATATGTTGATGTTATTGCCAATGGACCTGAAGC
AATTGATCGAATCGTATTTCATTTTTCAGATGATCGAACATTTGTTGTTGGTGAAAACTCAGGCAAGCCAAGTGTGC
GTTTGCAACTGGAAGGTCATTTTATTTGTGGCATGCTTGCGGATCAAGAAGGTTCTGACAAAGTTGCCGCGTTTAGC
GTGGCTTATGAATTGTTTCATCCCGATGAATTTGGTACAGAAAAGTAG

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