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Short communication

A multiplex bead-based assay for immune gene expression analysis in shrimp

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ABSTRACT

Here, we developed a 9-plex bead-based array as a tool to evaluate molecular effects on transcription levels of immune-related genes in the black tiger shrimp (*Penaeus monodon)*. The bead array technology allows simultaneous detection of multiple target genes in a single sample, reducing time, labor and cost. The oligonucleotide probes were designed to target eight immune-related genes that involve in antimicrobial activity, melanization, pathogen pattern recognition proteins, lysozyme and one housekeeping gene as an internal control. The nine probes were coupled to carboxylated-magnetic bead sets. The 9-plex PCR primers were designed and optimized for conditions to allow multiplex detection. The specificity of the assay was validated and the sensitivity was determined to be 10^3 copies/μL for all target genes. The 9-plex immune gene expression assay was applied to determine transcript levels in gills of *P. monodon* under exposure to a shrimp pathogen, *Vibrio harveyi,* and gene expression patterns were consistent to patterns observed under a traditional realtime PCR method. While realtime PCR method gave a better sensitivity but limited multiplexity, our 9-plex immune gene expression assay was able to simultaneously measure expression of multiple target genes, providing useful alternative assay in the need of higher-throughput gene expression analysis such as evaluation of immune stimulatory effects in different feed additives under various dosages and time points in shrimp.

Due to a declining trend of natural resources, fisheries production must rely on aquaculture to meet global consumer demands (FAO, 2016). The black tiger shrimp (*Penaeus monodon)* is one of the most economically important farmed marine shrimp. Nonetheless, domestication of the black tiger shrimp has been facing high disease susceptibility leading to difficulties to achieve sustainable farming (Thitamadee et al., 2016). Thus, it is essential to develop safe and effective strategies to promote shrimp immunity to prevent losses caused by infectious diseases. Particularly, antibiotics and chemical applications in shrimp farming have raised increasing concerns on food safety issues of these compounds, forcing the industries to seek other alternatives for disease controls (Xiong et al., 2016). Stimulating shrimp immune system via feed additives such as mannan-oligosaccharides and β-glucans has been shown to increase survival of shrimp upon a presence of pathogens (Rungrassamee et al., 2014; Smith et al., 2003; Yogeeswaran et al., 2012). The immunostimulants provide promising alternative approach to improve the black tiger shrimp survival in farming environments (Wang et al., 2017).

To efficiently evaluate immunostimulatory effects of candidate compounds, it is crucial to identify their mode of action at a molecular level to shrimp immune response system. The protective mechanisms are commonly evaluated based on enzymatic activity assays and host immune gene expression analysis (Wang et al., 2017), in which the latter method provides a better sensitive approach to examine immunostimulatory responses. The standard immune gene expression assays are based on a gene-by-gene expression approach using RT-PCR or realtime PCR, and they can be labor intensive and time consuming when analyzing multiple target genes (Yang et al., 2001). Among the emerging DNA-based detection platforms, a Luminex system (using xMAP™ technology) can be applied for high throughput gene expression analysis (Dunbar, 2006; Naciff et al., 2005; Spierings and Dunbar, 2013). In this platform, there are different microsphere magnetic bead sets with different internal dye ratio between red and infrared, hence, enabling bead identification. Here, we applied this bead array platform to develop multiplex immune gene expression analysis in the black tiger shrimp. Each gene-specific probe was immobilized on a magnetic bead set (Fig. 1), and the bead was used to specifically bind to biotin-labeled DNA products amplified from a sample of interest. Finally, fluorescent signals could be detected through streptavidin and R-phycoerythrin reaction with biotin-labeled DNA and bead complex.

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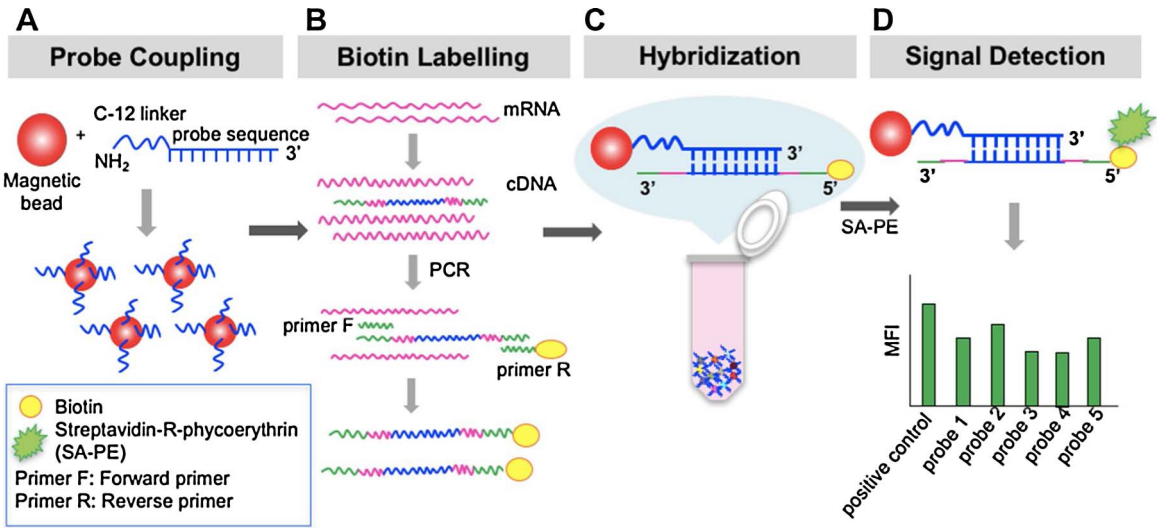

Fig. 1. Schematic representation of the bead array assay for multiplex gene expression analysis. A) coupling of gene-specific oligonucleotides as capture probes to a magnetic bead surface, B) The biotin was labelled to cDNA obtained from an RNA sample through PCR amplification with biotinylated primers, C) the target biotinylated sample was hybridized to mixed multiplex bead sets, and D) streptavidin and R-phycoerythrin was added to generate fluorescent signals, which was detected under Luminex xMAP system.

In this study, the multiplex bead array was developed for 9 target genes, which were 8 immune-related genes that involve in anti-microbial activity, melanization, pathogen pattern recognition proteins, lysozyme and a house-keeping gene as an internal control of the assay (Table 1). The 50-base unique sequences were designed as capture probes for 9 target genes (Table 1). To reduce DNA hybridization biases, each oligonucleotide was chosen to have a similar range of melting temperature (70 ± 4 °C) and minimal secondary structures predicted by Primer Quest and Oligo Analyzer tools (Integrated DNA Technologies, US). The capture probes were synthesized with 5′-amino

Table 1
List of immune-related genes in the black tiger shrimp and oligonucleotides used in multiplex gene expression assay.

Classification	Gene Name	Accession Number	Gene Abbreviation	Function	Sequence (5′-3′)	Amplicon Size (bp)
Antimicrobial peptides	anti-lipopolysaccharide factor1	BI784448	*alf1*	probe	/NH_2-C12/ ATACTGAATAGTAAATTCTCACGGAATTTCTCAGAAGTGCCAGCTCGGAC	207
				primer	alf1_F: CTGGCCGATGTGAGTGATTTA	
					alf1_R: GTCTTCCTCCGTGATGAGATTAC	
	anti-lipopolysaccharide factor3	EF523559	*alf3*	probe	/NH_2-C12/ GTGGGAGGCTGTGGCAGCGGCCGTCGCCAGCAAGATCGTAGGGTTGTGGA	169
				primer	alf3_F: AGTTTAGAAGATGCGTGTGTCC	
					alf3_R: GTGGCCGAGAAGTTCAGTTT	
	penaedin	FJ686016	*pen3a*	probe	/NH_2-C12/ AAACTGACTTCACAATGTATTAATCAGTTGTGAAGAAAGTGCAACCCTGA	215
				primer	pen3a_F: TATGGTTGATGGAGAAGACAA	
					pen3a_R: ACCTGCTGGGACAAATAC	
	crustin*Pm7*	EF654658.1	*crusPm7*	probe	/NH_2-C12/ ACACGCGCAGGATAAAGGCAATGCCGATACTCGCTTCCTAGGTGGAGTTG	132
				primer	crus_F: TTATCCGTTGTCGCTGTGG	
					crus_R: GAATCCACCTCCAACTCCAG	
Melanization and adhesion	prophenoloxidase	AF099741	*proPO1*	probe	/NH_2-C12/ AGAACACGGACCGGAGTTCTCTGGCACTCATCTCAAGCCAGAACACAGAA	223
				primer	proPO1_F: TCGTCATCCTGAGAAAGAA	
					proPO1_R: TAGTCCTCTCGCCAGTAA	
Pathogen pattern recognition proteins	C-type lectin	DQ078266	*lec*	probe	/NH_2-C12/ TGCTTCTATCTGAGCAAGGTCAAGCTGAACTGGAACCAAGCGCGACAGTA	153
				primer	lec_F: GCTCTATGTCCCTACCCGTATAA	
					lec_R: GACGAAGGACTTGAGAGCATAG	
	β-1,3-glucan binding protein	JN415536	*LGBP*	probe	/NH_2-C12/ ACTGGCCCTACGGACTTTGGCCAGCCAGCGGCGAGATTGACATTCTGGAG	200
				primer	LGBP_F: GGATGTTGCCTCGTAACT	
					LGBP_R: GGCGAAGGAACCTGTATT	
Enzymes	lysozyme	GQ478702	*lyz*	probe	/NH_2-C12/ GCACAGACTACGGTATCTTCCAGATCAACAACAAGTATTGGTGCGGCAGC	204
				primer	lyz_F: GCTGTTGGAAACGAGGTA	
					lyz_R: AGATCGGAACATGGGATTC	
Internal control	Elongation factor-1 alpha	DQ021452	*EF1a*	probe	/NH_2-C12/ ATCTGGCGACTCCTGCATCGTAAAAGATGGTTCCCAGCAAGCCCATGTGT	123
				primer	EF1a_F: CAGGCGTACTGGTAAGGAACTGG	
					EF1a_R: AGAGGAGCATACTGTTGGAAGGTCTC	