Isolation and molecular characterization of hemocyte sub-populations in kuruma shrimp Marsupenaeus japonicus

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1 **Isolation and molecular characterization of hemocyte sub-populations in kuruma shrimp**

2 ***Marsupenaeus japonicus***

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9

Abstract

Crustacean hemocytes, which have usually been classified morphologically based on dyeing methods such as Giemsa or May-Giemsa staining, have recently been categorized with monoclonal antibodies or marker genes. However, these techniques have not become widely used, resulting in the use of different classification methods for hemocytes among laboratories. Therefore, in this research, we aimed to develop a classification method that can be widely used. The method uses lectins and a magnetic-activated cell sorting (MACS) system to isolate sub-populations. Two lectins, wheat germ agglutinin (WGA) and tomato lectin (*Lycopersicon esculentum* lectin: LEL), characteristically bound to the hemocytes, which allowed them to be classified into three sub-populations. Furthermore, by using LEL and the MACS system, different sub-populations of hemocyte could be isolated. These sub-populations were characterized as non-granular and granular hemocytes, and the accumulation patterns of the gene transcripts were consistent with the results of a functional analysis reported previously. The lectin-based hemocyte isolation method developed in this study has good reproducibility.

Keywords

lectin staining; transcriptomics; hemocytes; magnetic-activated cell sorting system (MACS); shrimp; invertebrate

Introduction

Hemocytes of shrimp act as immune organs (Jiravanichpaisal et al. 2006; Tassanakajon et al. 2013; Söderhäll 2016). The classification of hemocytes is indispensable to analyze the biological defense mechanism in detail. So far, dyeing methods such as Giemsa or May-Giemsa staining, and antibody-based classification methods have been developed based on the leukocyte classification methods of mammals. The Giemsa or May-Giemsa staining method is excellent for staining the cytoplasmic granules of hemocytes, which contain anti-microbial peptides (Bachère et al. 2004; Rosa and Barracco 2010). Hemocytes can be roughly divided into three types morphologically, hyaline hemocytes (HCs), semi-granular hemocytes (SGCs) and granular hemocytes (GCs) by Giemsa or May-Giemsa staining (Söderhäll and Smith 1983; Johansson et al. 2000). However, the results of Giemsa and May-Giemsa staining are not always the same, and can be affected by pH, dyeing time, humidity and worker's degree of training. Therefore, these methods are not well-suited for quantitative experiments.

Ten kinds of monoclonal antibodies were produced using whole hemocytes of kuruma shrimp *Marsupenaeus japonicus* as antigens (Rodriguez et al. 1995). Similarly, eight kinds of monoclonal antibodies were produced using hemocytes or hemocyte lysate as antigens against hemocytes of black tiger shrimp *Penaeus monodon* (Sung et al. 1999; van de Braak et al. 2000; Sung and Sun 2002;

43 Winotaphan et al. 2005). As a result of immunological staining using these monoclonal antibodies, even
44 the same morphologically classified cells such as HCs, SGCs and GCs showed differences in reactivity to
45 their cell surface antigens, and due to the reactivity difference of the monoclonal antibodies, hemocytes
46 have been defined in more detail. More recently, monoclonal antibodies reactive to whiteleg shrimp
47 *Litopenaeus vannamei* hemocytes were developed (Lin et al. 2007; Zhan et al. 2008). Using these
48 antibodies, the isolating two sub-populations of *L. vannamei* hemocytes: agranulocytes and granulocytes
49 were succeeded (Xing et al. 2017). However, these monoclonal antibodies are not widely used for
50 classifying shrimp hemocytes because it is difficult to prepare identical monoclonal antibody-producing
51 clones in different laboratories and because few suppliers are interested in developing products for
52 crustaceans due to the small number of researchers. Therefore, it is also important to classify specific
53 hemocytes without relying on antibodies.

54 In other organisms especially in human, cells are classified based on sugar chains present on
55 the cell surface. Lectins are proteins that bind to sugar chains, and are used for staining and classification
56 of various cells, such as cancer cells, based on their sugar chains such as glycans (Kobata 1992;
57 Christiansen et al. 2014; Gabius et al. 2015). Until now, hemocytes of bees *Apis mellifera*, fly *Drosophila*
58 *melanogaster*, mosquito *Anopheles gambiae*, Pacific oyster *Crassostrea gigas* and Europe mussel *Mytilus*

edulis have been classified by lectins (Pipe 1990; Tirouvanziam et al. 2004; Rodrigues et al. 2010; Marringa et al. 2014; Jiang et al. 2016). In addition, cytoplasmic granules of hemocytes of ridgeback prawn *Sicyonia ingentis* and American lobster *Homarus americanus* have been reported to be stained by wheat germ agglutinin (WGA) (Martin et al. 2003). Furthermore, WGA, tomato lectin (*Lycopersicon esculentum* lectin: LEL) and peanut agglutinin (PNA) were found to bind to some of the GCs, SGCs and HCs of *L. vannamei* (Estrada et al. 2016). However, few studies have stained shrimp hemocytes with lectins, and molecular biological analyses of lectin-positive hemocytes have not been conducted.

In this study, we isolated two hemocyte sub-populations using LEL and a magnetic-activated cell sorting (MACS) system, and then predicted their functions by measuring the accumulation of mRNA transcripts by RNA sequencing (RNA-seq) and quantitative RT-PCT (qRT-PCR) analyses.

Materials and Methods

Shrimp samples

Apparently healthy kuruma shrimp *M. japonicus* weighing 20–25 g were obtained from farms in Okinawa and Miyazaki prefecture, Japan. Shrimps were kept in tanks provided with a water recirculating system maintained at 25 ^{0}C and 30-35 ppt. Shrimps were acclimatized for at least 3 days before the experiment.

Lectin staining of hemocytes by LEL and WGA

Hemolymph was collected from each shrimp using a 23-gauge needle and syringe containing equal amount of anti-coagulant (0.45 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.6) (Söderhäll and Smith 1983), and then centrifuged to obtain hemocytes. The hemocytes were fixed with 4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.3) for 15 min at room temperature. One of two lectins, DyLight 488-conjugated LEL or FITC-conjugated WGA (both Vector Laboratories, Inc., USA), was added at a ratio of 2 µg to 10^6 fixed cells and reacted for 15 minutes at 4 ^{0}C in reaction buffer (0.5% BSA, 2 mM EDTA in PBS). After washing twice, hemocytes were analyzed by flow cytometry and observed under a fluorescence microscope. For the observation of flow cytometry, the fluorescent intensities of at least

5,000 DyLight 488- or FITC-stained hemocytes were analyzed by FACSCalibur (Becton-Dickinson, USA) using an FL-1 filter with Cell Quest Pro software ver. 5.2.1 (Becton-Dickinson). Simultaneously relative cell size and relative cell complexity were determined by FACSCalibur and Cell Quest Pro software ver. 5.2.1 using a forward-scatter (FSC) filter and a side-scatter (SSC) filter, respectively. For the observation of fluorescence microscope, nucleolus of lectin-stained hemocytes were stained by 10 μg/mL of Hoechst 33258 (Invitrogen, USA) for 15minnutes in PBS. The stained hemocytes were examined by bright- and fluorescent-field using upright microscope ELIPSE Ci (Nikon Co., Japan), and the images were analyzed by NIS-Elements (Nikon Co.) and ImageJ ver. 2.0.0. (Schneider et al. 2012). The assay was performed three times from three individual shrimps.

Double lectin staining

PFA-fixed hemocytes were prepared as described above. Both biotin-conjugated LEL (Vector Laboratories, Inc.) and FITC-conjugated WGA were added at a ratio of 2 μg each to 10^6 fixed cells and reacted for 15 minutes at 4^0C in reaction buffer. After the hemocytes were washed twice, DyLight 550-conjugated natural streptavidin protein (Abcam plc., U.K.) was added at a ratio of 0.4 μg to 10^6 fixed cells and reacted for 15 minutes at 4^0C in reaction buffer. After washing twice, the stained hemocytes were examined by bright- and fluorescent-field as described above. The assay was performed three times from

three individual shrimps.

Isolation of LEL^{Dim} and LEL^{Strong} hemocytes by MACS system

PFA-fixed hemocytes were prepared as described above. From the flow cytometry results, LEL- or WGA-stained hemocytes were classified into two sub-populations; stained weakly as WGA^{Dim}/LEL^{Dim} and stained strongly as $WGA^{Strong}/LEL^{Strong}$. For isolation of LEL^{Dim} hemocytes, PFA-fixed hemocytes were stained with biotin-conjugated LEL (Vector Laboratories, Inc.) at a ratio 1 µg to 10^6 fixed cells for 15 minutes at 4^0C in reaction buffer. After washing once, hemocytes were reacted with 10 µl of streptavidin microbeads (Miltenyi Biotec, Germany) in 90 µl of reaction buffer for 15 min at 4^0C. After washing once, hemocytes were separated by MACS using MS column (Miltenyi Biotec) and MiniMACS separator (Miltenyi Biotec) following the manufacturer's protocol. The negative fraction was collected as LEL^{Dim} hemocytes. For isolation of LEL^{Strong} hemocytes, PFA-fixed hemocytes were stained with biotin-conjugated LEL at a ratio 0.1 µg to 10^6 fixed cells for 15 minutes at 4^0C in reaction buffer. After washing once, hemocytes were reacted with 1 µl of streptavidin microbeads in 99 µl of reaction buffer for 15 min at 4^0C. After washing once, hemocytes were separated by MACS. The positive fraction was collected as LEL^{Strong} hemocytes. Total, LEL^{Dim} and LEL^{Strong} hemocytes were analyzed by flow cytometry. Five thousand (5,000) events of each sample were collected and then FSC and SSC analyses

were conducted by FACSCalibur with Cell Quest Pro software ver. 5.2.1 as described above. Two gates, R1 and R2, were established based on the FSC and SSC, and the percentage of dot plots in each gate were analyzed by Cell Quest Pro software. The assay was performed six times from six individual shrimps. Since the hemocytes stained with WGA could not be separated by MACS system, this isolation experiment could not be carried out on WGA-stained hemocytes.

May-Giemsa staining of total, LEL^{Dim} and LEL^{Strong} hemocytes

Total, LEL^{Dim} and LEL^{Strong} hemocytes were collected as described above. Each hemocyte suspension was spread on a glass slide in a cell collection bucket SC-2 (TOMY, Japan) at 100 g for 1 min. Glass slides were dried, stained for 3 min with 20% May-Grunwald stain solution (Wako, Japan) in 0.67 mM phosphate buffer (pH 6.6), washed with phosphate buffer, stained for 15 min with 4% Giemsa stain solution (Wako) in 0.67 mM phosphate buffer (pH 6.6), washed with tap water, dried, mounted with Malinol (Muto Pure Chemicals, Japan) and visualized with NIS-Elements software.

cDNA Library construction and RNA sequencing by Illumina Miseq

Total, LEL^{Dim} and LEL^{Strong} hemocytes were collected from six shrimps as described above. The PFA-fixed hemocytes were digested with proteinase K (Masuda et al. 1999). Total RNA was then extracted with a NucleoSpin RNA XS kit (Takara Bio Inc., Japan) following the manufacturer's protocol.

134 The total RNAs of each type of hemocyte were pooled. The concentration and purity of total RNA were

135 measured using a Qubit RNA HS Assay Kit and NanoDrop Lite (both Thermo Fisher Scientific Inc.,

136 USA). cDNA libraries were prepared with total RNA using a TruSeq stranded mRNA sample preparation

137 kit (Illumina Inc., USA) following the manufacturer's protocol. The libraries were amplified with 20

138 cycles of PCR and contained indexes within the adapters. The yields in the amplified libraries were

139 measured with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) and D1000 ScreenTape

140 System (Agilent Technologies, USA). The yields of LEL^{Dim}, LEL^{Strong} and total hemocytes were 1.9,

141 0.184 and 14.5 ng/μl, respectively, with average lengths of 293, 286 and 297 bp, respectively, indicating

142 concentrations 10.3, 1.05 and 77.5 nM, respectively. Six (6) pmol of each library was sequenced using

143 MiSeq (Illumina Inc.) and MiSeq reagent kit version 2 (Illumina Inc.) with 75 nt paired end reads.

144 ***De novo* assembly and identification of differentially expressed transcripts**

145 The reads were assembled by Trinity v2.5.1 (Grabherr et al. 2011) using default parameters

146 (minimum assembled transcripts length 200) to obtain trinity-assembled transcripts. The sequenced

147 libraries were mapped back to the reference trinity-assembled transcripts using RSEM (Li and Dewey

148 2011) to quantify the read counts. Read counts were normalized by trimmed mean of M-values (TMM) to

149 account for differences in library size (Robinson and Oshlack 2010) and then normalized by transcripts

per million (TPM) to account for differences in transcript length. The differentially expressed transcripts between total, LEL^{Dim} and LEL^{Strong} hemocytes libraries were identified using EdgeR (Robinson and Oshlack 2010) including a p-value cutoff for false discovery rate of 0.001 and a minimum 16-fold change in expression. Blastx program (Altschul et al. 1997) was then used for homologous gene searching with an e-value cut-off of 0.05 in Penaeidae's 5,942 proteins in NCBI database (http://www.ncbi. nlm.nih.gov "Accessed 18 Oct 2018").

Quantification of transcripts of immure-related genes by qRT-PCR

Total, LEL^{Dim} and LEL^{Strong} hemocytes were extracted from three shrimps, then total RNAs were extracted as described above. cDNAs were synthesized from RNA of each sample using a High capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc.). After synthesis, cDNA samples were diluted five times with distilled water and 2 µl of samples were used for qRT-PCR. The set of primers were designed based on registered sequences or trinity-transcripts (Table 1). Elongation factor 1α (EF-1α: as an internal control) for qRT-PCR (Table 1). qRT-PCR was conducted using THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Japan) and condition was 95^0C for 1 min, 40 cycles of 95^0C for 15 secs and 60^0C for 1 min followed by dissociation analysis step. mRNA accumulation of each gene was calculated as ΔCT by comparing with CT value of EF-1α (as a reference gene). The statistical

significance between total, LEL^{Dim} and LEL^{Strong} hemocytes respectively was analyzed using t-test.

Lectin staining on hemocytes phagocyted micro beads

Shrimps were injected with 200 µl of 10% suspension of fluorescent beads (Fluoresbrite YO Cartoxylate Microspheres 1.0 µm: Polysciences, Inc., USA) in artificial seawater. Three (3) hours post injection, PFA-fixed hemocytes were prepared and stained by DyLight 488- conjugated LEL or FITC-conjugated WGA, respectively as described above. The stained hemocytes were examined by bright- and fluorescent-field as described above. The assay was performed three times from three individual shrimps.

Results

Lectin staining of total hemocytes

Both WGA and LEL showed reactivity to all hemocytes, however there were a difference in reactivity, and they could be classified into two subpopulations, WGA^{Dim}/WGA^{Strong} and LEL^{Dim}/LEL^{Strong}, respectively (Fig. 1). WGA reacted strongly with cells with relatively large and complex intracellular structure (Fig. 1d), whereas LEL reacted strongly with cells with relatively small and simple intracellular structure (Fig. 1h). WGA and LEL strongly reacted with the intracellular structure and the cell surface of hemocytes, respectively (Fig. 2). Dim-positive and strong- positive of each lectin hemocytes were also observed under fluorescent-field (Fig. 2).

Double lectin staining

Double lectin staining of total hemocytes by LEL and WGA was able to divide hemocytes into three sub-populations: LEL- positive, WGA-positive and LEL/WGA-positive hemocytes (Fig. 3). The ratio of LEL/WGA-positive hemocytes was 19% (n=3), and the fluorescent intensity of LEL/WGA-positive hemocytes was weaker than the other sub-populations. As with single staining, LEL well stained the cell surface and WGA well stained the intracellular structure of hemocytes.

Isolation of LEL^{Dim} and LEL^{Strong} hemocytes by MACS system

Using the MACS system and biotin-conjugated LEL, LEL^{Dim} and LEL^{Strong} hemocytes were isolated, respectively. May-Giemsa staining showed that LEL^{Dim} hemocytes (Fig. 4c) were relatively larger than LEL^{Strong} hemocytes (Fig. 4d), and unlike the latter, contained intracellular granules and a large cytoplasm compared to the nucleus. The granules of LEL^{Dim} hemocytes showed round shape, 0.4-0.6 μm in diameter and stained eosinophilic as purplish red (Fig. 4c). On both LEL^{Dim} and LEL^{Strong} hemocytes, cytoplasm were stained pale purple and had condensed chromatin (Fig. 4c, d). Regions 1 and 2 before separation of hemocytes were 45.8 ± 12.4% and 51.9 ± 12.0%, respectively, whereas after separation of LEL^{Dim} hemocytes, they were 11.0 ± 3.2% and 83.8 ± 6.0%, and after separation of LEL^{Strong} hemocytes, they were 86.7 ± 7.2% and 10.9 ± 6.6% (n=6). Fig. 5 showed an example dot plot analyses of total, LEL^{Dim} and LEL^{Strong} hemocytes from a shrimp.

Differentially expressed transcripts by RNA sequencing

All the sequences from total, LEL^{Dim} and LEL^{Strong} hemocytes with raw data archived at the DDBJ Sequence Read Archive under Accession DRA007926. The assembled transcripts contained 11,870 trinity-genes. The median trinity-gene length was 339 bp and the N50 (weighted median) was 539 bp. We identified 2,630 differentially expressed transcripts based on a p-value cut-off for FDR of 0.001 and a minimum 16-fold change in expression. In blastx searches, 163 trinity-genes matched Penaeidae proteins

206 with e-values less than 0.05 (Online Resource), 31 of which were immune-related (Fig. 6). The immune-

207 related trinity-genes fell into four clusters that were highly expressed in (1) only LEL^{Strong} hemocytes, (2)

208 both total and LEL^{Dim} hemocytes, (3) only total hemocytes and (4) only LEL^{Dim} hemocytes (Fig. 6).

209 **Differentially expressed transcripts by qRT-PCR**

210 In the qRT-PCR results, the ΔCT values of transcripts of two major anti-microbial peptides

211 (AMPs) (crustin and penaeidin- II) and c-type lysozyme were significantly lower in LEL^{Strong} hemocytes

212 than in total and LEL^{Dim} hemocytes, while the ΔCT values of transcripts of hemocyte transglutaminase

213 and prophenoloxidase (proPO) activation enzyme were significantly lower in LEL^{Dim} hemocytes than in

214 total and LEL^{Strong} hemocytes (Fig. 7). The trend was also seen in that the ΔCT values of transcripts of

215 Toll and integrin were lower in LEL^{Strong} hemocytes than in total and LEL^{Dim} hemocytes.

216 **Lectin staining of hemocytes phagocyted micro beads**

217 The fraction of hemocytes phagocyted micro beads was 5.6% (n=3). Both LEL-positive and -

218 negative hemocytes phagocyted micro beads (Fig. 8b, d), whereas only WGA-positive hemocytes

219 phagocyted micro beads (Fig. 8f, h). In addition, the fluorescent intensity of WGA-positive beads

220 phagocyted hemocytes tended to be weaker than other WGA-positive hemocytes.

221

Discussion

The stainability of hemocytes by two lectins, WGA and LEL, were different. This suggests that sugar chains on hemocytes are different depending on the type of hemocytes. Like the reports on the other crustacean (Martin et al. 2003; Estrada et al. 2016), WGA strongly stained the granules of hemocytes of kuruma shrimp *M. japonicus*. The flow cytometry data also showed a strong WGA signal in hemocytes with high SSC values, suggesting that WGA stains granules of hemocytes. The investigation of the existence of granules on hemocytes is important for characterization of hemocytes. However, it was unclear which hemocytes contained granules on dyeing methods such as Giemsa or May-Giemsa staining. Combination of WGA staining, microscopic observation and FCM analysis, it became easier to prove the existence of granules on hemocytes. In contrast to WGA, LEL appeared to bind to the cell surface and not cytoplasmic granules. Since LEL stained the cell surface, MACS system could be used.

May-Giemsa staining showed that LEL^{Dim} hemocytes contained a lot of cytoplasmic granules, while LEL^{Strong} hemocytes contained little or no granules. The flow cytometry data also showed that LEL^{Strong} hemocytes was smaller and had lower SSC value than LEL^{Dim} hemocytes. These results indicate that hemocytes could be divided into two sub-populations by LEL: LEL^{Strong} hemocytes that were agranulocytic and LEL^{Dim} hemocytes that were granulocytic. Kuruma shrimp hemocytes were classified

238 into 3 types (Kondo et al. 1992; Kondo et al. 1998) or 8 types (Kondo et al. 2014) by electron microscopy

239 observation or May-Grunwald staining. Since we used different sampling methods or anticoagulant

240 solution in this study, we could not observe the reported detailed granule structure, cytoplasmic structure

241 and dyeability. In addition, morphological changing especially degranulation were easily occurred even

242 when collected using anticoagulant (Kondo et al. 2012). The development of the optimal sampling

243 method and comparison with the existing report are future tasks.

244 The two populations, LEL^{Dim} and LEL^{Strong}, were associated with specific transcripts.

245 Transcripts of hemocyte transglutaminase, which is related to clotting of hemolymph (Maningas et al.

246 2013), were highly accumulated in LEL^{Strong} hemocytes in both the RNA-seq and qRT-PCR analyses.

247 Abundant transglutaminase transcripts were also reported on HCs (also called agranular hemocytes) in *L.*

248 *vannamei* (Yang et al. 2015). The transglutamase results also strongly suggest that LEL^{Strong} (i.e.,

249 agranular) hemocytes contribute to blood coagulation in kuruma shrimp. On the other hand, total and

250 LEL^{Dim} hemocytes highly accumulated transcripts of crustin, crustin-like, penaeidin-II and c-type

251 lysozyme, as shown by the RNA-seq and qRT-PCR analyses. AMPs and c-type lysozyme are also present

252 in cytoplasmic granules of hemocytes (Bachère et al. 2004; Rosa and Barracco 2010). Our RNA-seq

253 analysis also showed that LEL^{Dim} hemocytes had abundant transcripts of proPO activation enzymes and

254 serine proteases, which are also proPO-related enzymes (Hernández-López et al. 1996; Cerenius and
255 Söderhäll 2004). In many crustaceans, the proPO system is carried by granular hemocytes (Sung et al.
256 1998; Yang et al. 2015; Söderhäll 2016). Based on these previous reports and the present results, LEL^{Dim}
257 hemocytes (i.e. granulocytes) are responsible for the production of AMPs and c-type lysozyme, and
258 contribute to the proPO system, as reported previously.

259 The hemocytes which have the phagocytic activity vary greatly from species to species in
260 crustacean. In kuruma shrimp, strong phagocytic activity was observed in SGCs and GCs (Kondo et al.
261 1992). LEL^{Dim} hemocytes accumulate transcripts involved in foreign object recognition, such as integrin,
262 lectins, Toll and scavenger receptor (Arts et al. 2007; Yang et al. 2007; Han-Ching Wang et al. 2010;
263 Zhang et al. 2012; Lin et al. 2013; Wang and Wang 2013; Wang et al. 2014; Bi et al. 2015). Furthermore,
264 there was a correlation between WGA-positive hemocytes and phagocytosis, not LEL-positive hemocytes
265 (Fig. 8f, h), in this study. Together, these results indicate that kuruma shrimp granular hemocytes are the
266 main players in phagocytosis. Interestingly, LEL-positive not WGA-positive cells were reported to be
267 phagocytotic in Pacific oyster *C. gigas* (Jiang et al. 2016), which suggests that the composition and
268 function of cell surface glycans can differ in the same invertebrates.

269 Some hemocytes stained with both WGA and LEL. Lin and Söderhäll (2011) argue that GCs

and SGCs differentiate from HCs. In this study, both LEL- and WGA-positive hemocytes were present, but we were unable to analyze their functions. For example, both LEL- and WGA-positive hemocytes may be in transition from HCs to GCs or SGCs. By using a combination of LEL and WGA, it is now possible to more accurately classify the types, functions and life cycles of hemocytes.

Since our lectin-based hemocyte isolation method requires cell fixation, functional analysis was impossible. Therefore, further studies are needed to identify buffers that can make it possible to stain living hemocytes with lectins to conduct functional analysis or extract high quality RNAs. It is also necessary to identify the antigens of LEL and WGA to clarify how hemocytes are classified. Despite these problems, lectin-based hemocyte isolation uses easily available lectins and a relatively inexpensive MACS system, which should make it useful in many laboratories.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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402

403 **Table 1.** Primer sequences used in this study

Primer name	Sequence (5'-3')	Accession number
EF-1α F	ATT GCC ACA CCG CTC ACA	AB458256.1
EF-1α R	TCG ATC TTG GTC AGC AGT TCA	AB458256.1
Crustin F	AAC TAC TGC TGC GAA AGG TCT CA	AB121740-4.1
Crustin R	GGC AGT CCA GTG GCT TGG TA	AB121740-4.1
Penaeidin-II F	TTA GCC TTA CTC TGT CAA GTG TAC GCC	KU057370.1
Penaeidin-II R	AAC CTG AAG TTC CGT AGG AGC CA	KU057370.1
C-type lysozyme F	ATT ACG GCC GCT CTG AGG TGC	AB080238.1
C-type lysozyme R	CCA GCA ATC GGC CAT GTA GC	AB080238.1
Anti-lipopolysaccharide factor F	AGC CTC CTT TTC CTT TCC CCT	KX424931.1
Anti-lipopolysaccharide factor R	CAC AAT CCT GTC AGT TTT TCC GC	KX424931.1
C-type lectin F	ACG CTG GTG TGA TGC CCG	KJ175168.1
C-type lectin R	ACC GAG TCT GAG CCG CCT AA	KJ175168.1
Hemocyte transglutaminase F	GAG TCA GAA GTC GCC GAG TGT	DQ436474.1
Hemocyte transglutaminase R	TGG CTC AGC AGG TCG TTT AA	DQ436474.1
Transglutaminase F	TGA CTG CGA AGA ACA TGA GC	AB162767.1
Transglutaminase R	GTT CTT GGT TTC CCC GAC TC	AB162767.1
Prophenoloxidase activation enzyme F	ACC CGA CGA TGC CAG AAC	This study
Prophenoloxidase activation enzyme R	TGG GAA GAT TTG GGA TAA GAA GAC	This study
Prophenoloxidase activation factor F	TCA AGG AGG TGG CTC TCC CT	This study
Prophenoloxidase activation factor R	GAT ACC CGA ACC CGG TCT CC	This study
Prophenoloxidase F	CCG AGT TTT GTG GAG GTG TT	AB073223.1
Prophenoloxidase R	GAG AAC TCC AGT CCG TGC TC	AB073223.1
Toll F	ACT GGA ACG TGT TGG GAA GA	AB333779.1
Toll R	TGC AAG TCC AGA ACC TCC AA	AB333779.1
Integrin α F	GAC GAG CCA AGC CAT CTG A	LC114983.1
Integrin α R	TCC GTC GAG CAG TCT TCA TG	LC114983.1

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406 **Fig. 1.** Flowcytometry analysis of WGA- or LEL-stained hemocytes from a shrimp. The intensity of FL-1

407 signal of WGA-stained hemocytes (a) and LEL-stained hemocytes (e). Dotted line indicates negative

408 control of FL-1 value. Dot-plot analysis of total hemocytes (b and f), WGA^{Dim} hemocytes (c), WGA^{Strong}

409 hemocytes (d) LEL^{Dim} hemocytes (g) and LEL^{Strong} hemocytes (h). X- and Y-axes indicate FSC and SSC,

410 respectively.

411 **Fig. 2.** Lectin staining of total hemocytes from a shrimp. Hemocytes stained LEL (a-d) and WGA (e-h).

412 Bright-field (a, e). Nucleolus stained as blue by Hoechst 33258 (b, f). Each fluorescent lectin stained as

413 green, LEL (c) and WGA (g). Merged figure (d, h). Bars indicate 10 μm scale.

414 **Fig. 3.** Double lectin staining of total hemocytes from a shrimp. Hemocytes stained LEL and WGA.

415 Bright-field (a). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL as red and WGA

416 as green (b). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL as red (c).

417 Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by WGA as green (d). Bars indicate 10

418 μm scale.

419 **Fig. 4.** Bright field microscopic observation and May-Giemsa staining of hemocytes from a shrimp. Total

420 hemocyte observed under bright-field (a). Total hemocytes stained by May-Giemsa staining (b). Bright

421 field observation and May-Giemsa staining of LEL^{Dim} hemocytes (c) and LEL^{Strong} hemocytes (d). Bars

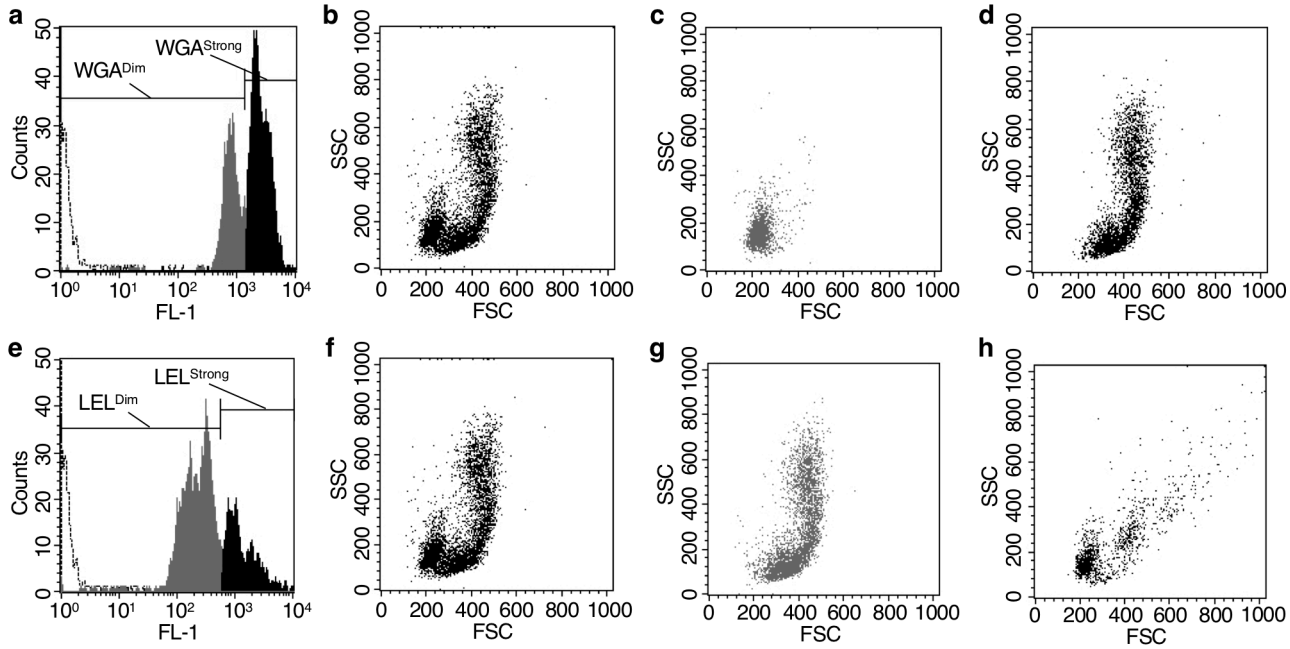
indicate 10 μm scale.

Fig. 5. Dot plot analyses of total, LEL^{Dim} and LEL^{Strong} hemocytes from a shrimp. Total hemocytes (a), LEL^{Dim} hemocytes (b) and LEL^{Strong} hemocytes (c). Each region was established based on characteristic cell plots. X- and Y-axes indicate FSC and SSC, respectively.

Fig. 6. Hierarchical clustering analysis of immune-related trinity-transcripts extracted as differentially expressed in total, LEL^{Dim} and LEL^{Strong} hemocytes. Each column is the TMM-TPM value. Relatively highly expressed trinity-genes are shown in red, relatively weakly expressed trinity-genes are shown in green.

Fig. 7. qRT-PCR analyses of 12 transcripts. ΔCt values analyzed by qRT-PCR. Higher ΔCT value indicates higher accumulation of transcript of mRNA. Each bar indicates the average value. Double asterisk (**) and an asterisk (*) on the bars indicates the ΔCt values were significantly different between each sub-population. ** = $P < 0.01$; *=$P < 0.05$.

Fig. 8. LEL and WGA staining on hemocytes phagocyted microbeads. Microscopic observation under bright-field (a, c, e, g) and under fluorescent-field (b, d, f, h). Nucleolus stained by Hoechst 33258 as blue, hemocytes stained by LEL (b, d) or WGA (f, h) as green and phagocytized beads as red. Bars indicate 10 μm scale.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SSC
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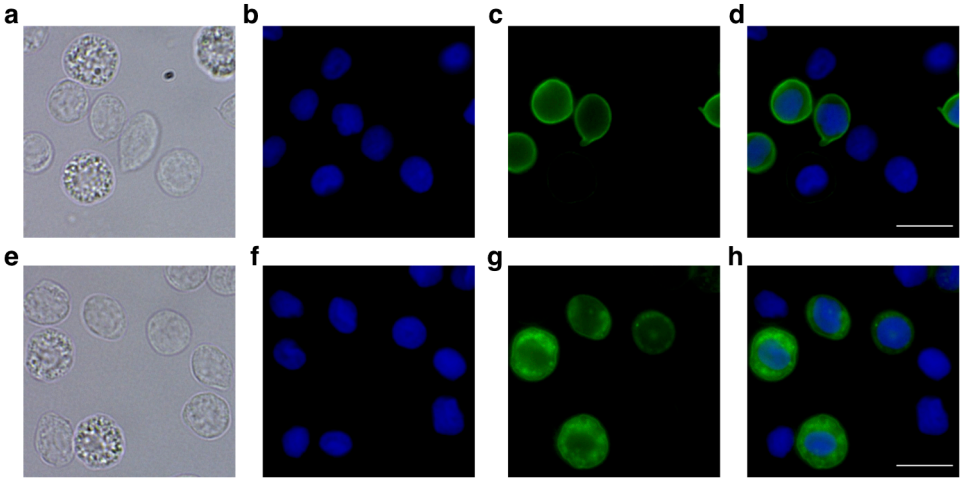
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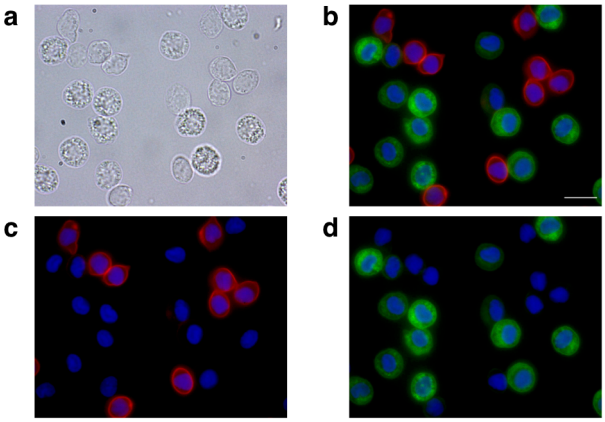
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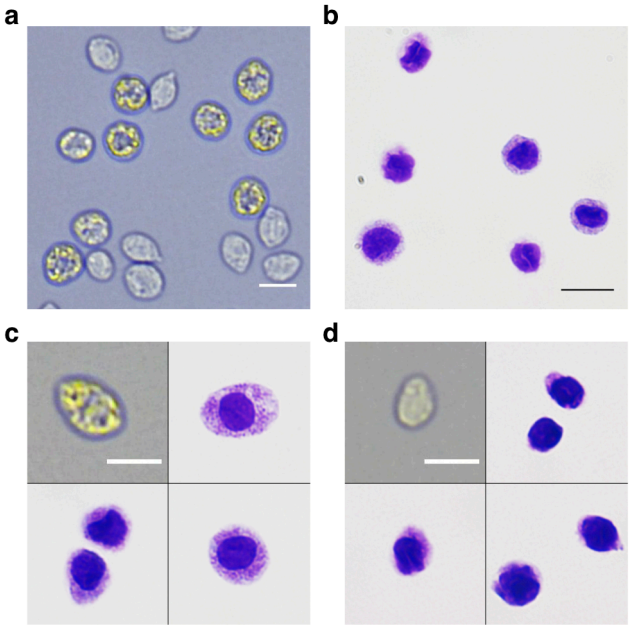
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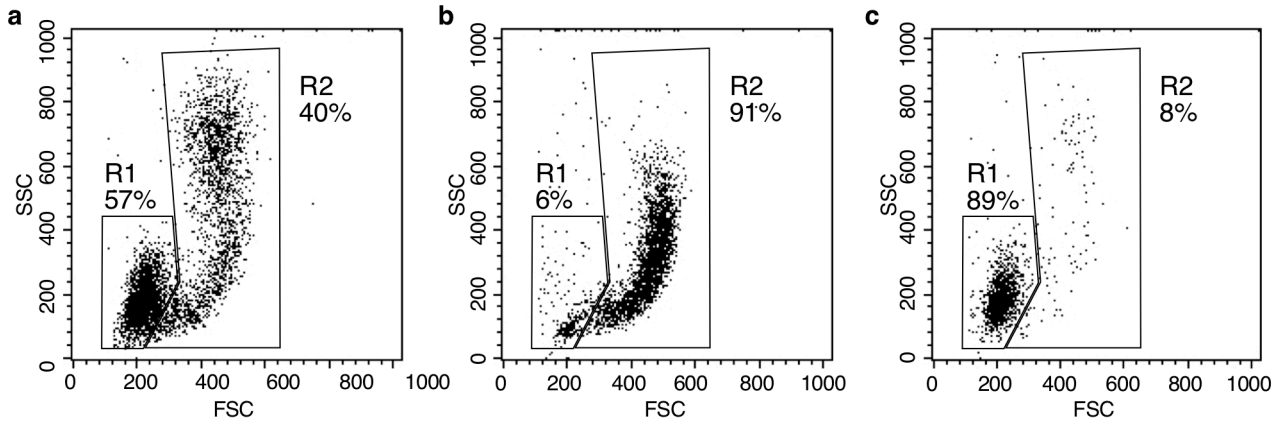
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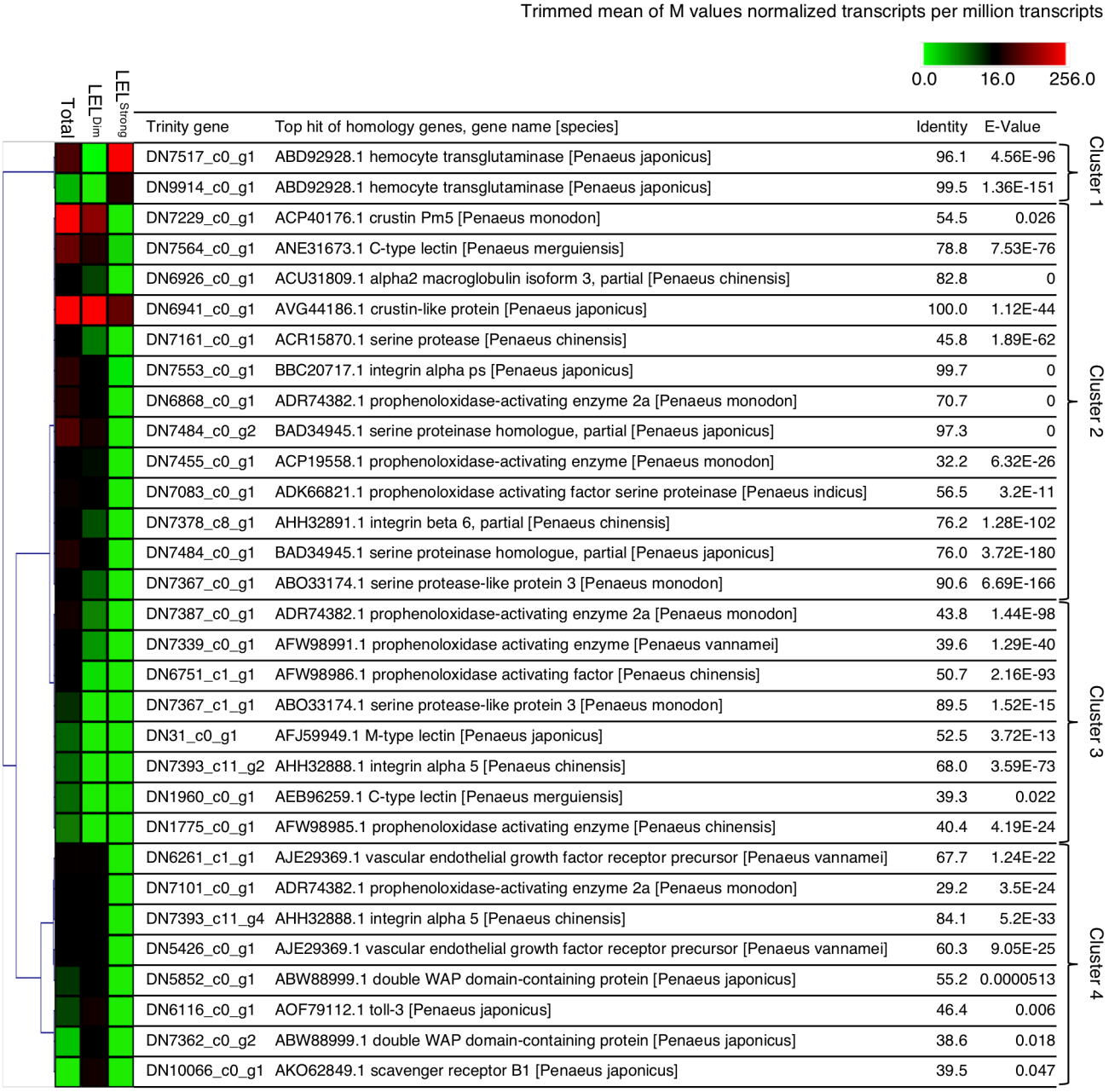
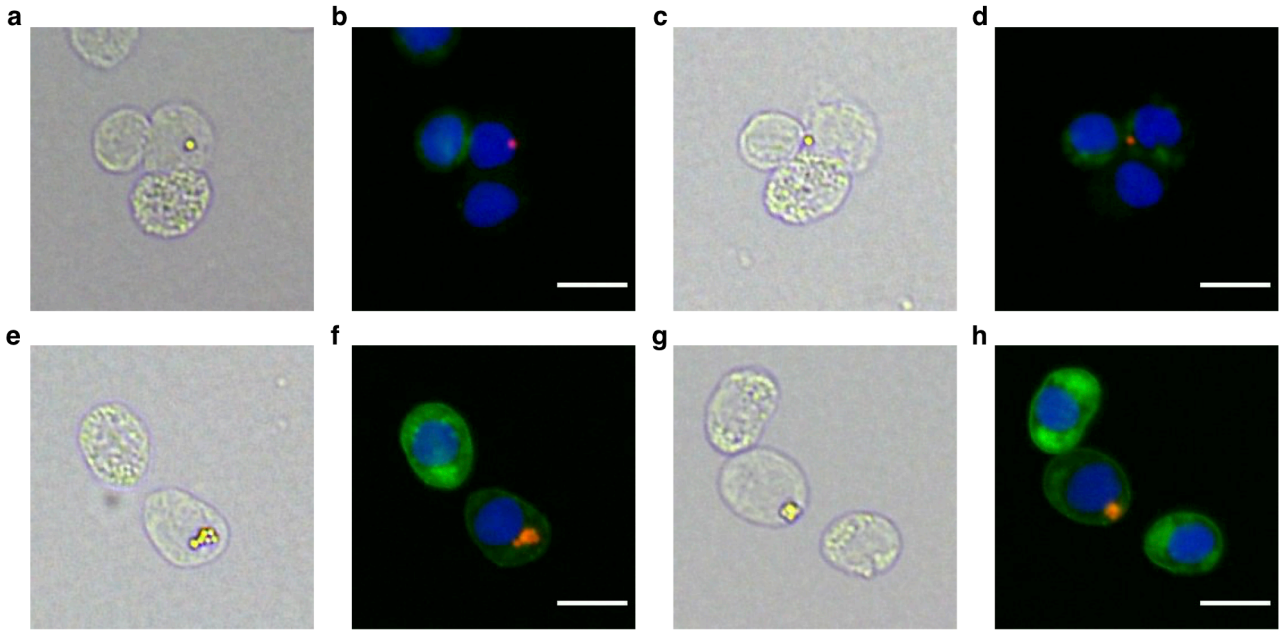


Trinity gene	Top hit of homology genes, gene name [species]	Identity	E-Value	
DN7517_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	96.1	4.56E-96	Cluster 1
DN9914_c0_g1	ABD92928.1 hemocyte transglutaminase [Penaeus japonicus]	99.5	1.36E-151	
DN7229_c0_g1	ACP40176.1 crustin Pm5 [Penaeus monodon]	54.5	0.026	Cluster 2
DN7564_c0_g1	ANE31673.1 C-type lectin [Penaeus merguiensis]	78.8	7.53E-76	
DN6926_c0_g1	ACU31809.1 alpha2 macroglobulin isoform 3, partial [Penaeus chinensis]	82.8	0	
DN6941_c0_g1	AVG44186.1 crustin-like protein [Penaeus japonicus]	100.0	1.12E-44	
DN7161_c0_g1	ACR15870.1 serine protease [Penaeus chinensis]	45.8	1.89E-62	
DN7553_c0_g1	BBC20717.1 integrin alpha ps [Penaeus japonicus]	99.7	0	
DN6868_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	70.7	0	
DN7484_c0_g2	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	97.3	0	
DN7455_c0_g1	ACP19558.1 prophenoloxidase-activating enzyme [Penaeus monodon]	32.2	6.32E-26	
DN7083_c0_g1	ADK66821.1 prophenoloxidase activating factor serine proteinase [Penaeus indicus]	56.5	3.2E-11	
DN7378_c8_g1	AHH32891.1 integrin beta 6, partial [Penaeus chinensis]	76.2	1.28E-102	
DN7484_c0_g1	BAD34945.1 serine proteinase homologue, partial [Penaeus japonicus]	76.0	3.72E-180	
DN7367_c0_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	90.6	6.69E-166	
DN7387_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	43.8	1.44E-98	Cluster 3
DN7339_c0_g1	AFW98991.1 prophenoloxidase activating enzyme [Penaeus vannamei]	39.6	1.29E-40	
DN6751_c1_g1	AFW98986.1 prophenoloxidase activating factor [Penaeus chinensis]	50.7	2.16E-93	
DN7367_c1_g1	ABO33174.1 serine protease-like protein 3 [Penaeus monodon]	89.5	1.52E-15	
DN31_c0_g1	AFJ59949.1 M-type lectin [Penaeus japonicus]	52.5	3.72E-13	
DN7393_c11_g2	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	68.0	3.59E-73	
DN1960_c0_g1	AEB96259.1 C-type lectin [Penaeus merguiensis]	39.3	0.022	
DN1775_c0_g1	AFW98985.1 prophenoloxidase activating enzyme [Penaeus chinensis]	40.4	4.19E-24	
DN6261_c1_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	67.7	1.24E-22	Cluster 4
DN7101_c0_g1	ADR74382.1 prophenoloxidase-activating enzyme 2a [Penaeus monodon]	29.2	3.5E-24	
DN7393_c11_g4	AHH32888.1 integrin alpha 5 [Penaeus chinensis]	84.1	5.2E-33	
DN5426_c0_g1	AJE29369.1 vascular endothelial growth factor receptor precursor [Penaeus vannamei]	60.3	9.05E-25	
DN5852_c0_g1	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	55.2	0.0000513	
DN6116_c0_g1	AOF79112.1 toll-3 [Penaeus japonicus]	46.4	0.006	
DN7362_c0_g2	ABW88999.1 double WAP domain-containing protein [Penaeus japonicus]	38.6	0.018	
DN10066_c0_g1	AKO62849.1 scavenger receptor B1 [Penaeus japonicus]	39.5	0.047	

Relative mRNA expression
ΔCT value Target-EF-1α
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
LEL Strong
LEL Dim
Total
Integrin α
Toll
Prophenoloxidase
Prophenoloxidase activation factor
Prophenoloxidase activation enzyme
Transglutaminase
Hemocyte Transglutaminase
C-type lectin
Anti-lipopoly-saccharide factor
C-type lysozyme
Penaeidin-II
Crustin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