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Wolbachia isolation by fractioned centrifugation from *Dirofilaria immitis* for protein analysis

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Objective : *The biological roles of Wolbachia, a filarial intracellular bacteria, have promoted applied researches in lymphatic filariasis. Analysis of Wolbachia proteins will provide complementary information on the biology of Wolbachia, as well as the immune mechanisms against filarial nematodes. We therefore developed a Wolbachia isolation method from Dirofilaria immitis (dog heartworm) for protein analysis.*

Methods : *Wolbachia bacteria were homogenized and isolated by fractioned centrifugation by using 0.85% NaCl supplemented with varying concentration of Nonidet P-40 (NP-40) as the homogenization buffer. The purity of Wolbachia extracts were analyzed by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), and by quantitative polymerase chain reaction (qPCR) targeting the single copy of the Wolbachia surface protein (wsp) gene. The contaminations from filarial extracts were analyzed by qPCR targeting the filarial glutathione-S-transferase (gst) gene.*

Results : *We were able to isolate Wolbachia bacteria from D. immitis with high purity. The higher concentration of NP-40 could increase the yield of bacterial isolation and decrease the host parasite contaminations.*

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Conclusion : *The homogenization buffer which provided the highest Wolbachia protein concentration, but the lowest filarial contamination, was 0.85% NaCl + 0.08% NP-40. This technique is likely to provide the most suitable methods for Wolbachia isolation to facilitate the proteomic studies. However, our purification procedures do need validation in other applications, which require intact Wolbachia DNA materials, and/or viable cells.*

Keywords : *Wolbachia isolation, Dirofilaria immitis, Protein analysis.*

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วิวรพรรณ สรรประเสริฐ, ศิวะพงษ์ สังข์ประดิษฐ์, สุรางค์ นุชประยูร. การแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขโดยวิธีปั่นแยกลำดับขั้นสำหรับการศึกษาโปรตีน. จุฬาลงกรณ์เวชสาร 2553

วัตถุประสงค์ : *บทบาททางชีววิทยาของแบคทีเรียโวลบาเชียสนับสนุนให้มีการศึกษาวิจัยประยุกต์ในโรคเท้าช้าง การวิเคราะห์โปรตีนของแบคทีเรียโวลบาเชียจะให้ข้อมูลที่ดีในด้านชีววิทยาของแบคทีเรียโวลบาเชียรวมถึงกลไกการตอบสนองทางภูมิคุ้มกันต่อหนอนพยาธิฟิลาเรีย คณะผู้วิจัยจึงพัฒนาวิธีการแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขสำหรับการศึกษาวิเคราะห์โปรตีน*

วิธีการ : *แบคทีเรียโวลบาเชียถูกแยกโดยวิธีปั่นลำดับขั้น โดยใช้ 0.85% NaCl ผสมด้วย Nonidet P-40 (NP-40) ที่ความเข้มข้นต่าง ๆ เป็นสารบัฟเฟอร์ ความบริสุทธิ์ของแบคทีเรียโวลบาเชียที่สกัดแยกได้ถูกวิเคราะห์โดยวิธี sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) และวิธี quantitative polymerase chain reaction (qPCR) โดยใช้ยีนเป้าหมายเป็นยีนที่มีเพียงยีนเดียว ในเซลล์ของแบคทีเรียโวลบาเชียคือยีน Wolbachia surface protein (wsp) การปนเปื้อนของสารสกัดจากหนอนพยาธิฟิลาเรียถูกวิเคราะห์โดย qPCR โดยใช้ยีนเป้าหมายเป็นยีนที่มีเพียงยีนเดียวในเซลล์ของหนอนพยาธิฟิลาเรีย คือ ยีน glutathione-S-transferase (gst)*

ผลการทดลอง : *คณะผู้วิจัยสามารถแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขได้ด้วยความบริสุทธิ์สูง โดยความเข้มข้นของ NP-40 ที่เพิ่มขึ้นสามารถเพิ่มปริมาณแบคทีเรียโวลาเชียที่แยกได้เพิ่มขึ้น และสามารถลดการปนเปื้อนจากหนอนพยาธิซึ่งเป็นโฮสต์ได้*

สรุปผล : *สารบัฟเฟอร์ที่ให้ความเข้มข้นของโปรตีนของแบคทีเรียโวลบาเชียมากที่สุด แต่มีการปนเปื้อนจากโปรตีนของหนอนพยาธิน้อยที่สุดคือ 0.85% NaCl + 0.08% NP-40 วิธีการแยกที่ได้พัฒนาขึ้นนี้เหมาะสมสำหรับการแยกแบคทีเรียโวลบาเชียเพื่อการศึกษาโปรตีนทั้งหมดของแบคทีเรียโวลบาเชีย อย่างไรก็ตาม วิธีการแยกบริสุทธิ์นี้จำเป็นต้องพิสูจน์ความเหมาะสมสำหรับการประยุกต์ใช้ในงานอื่น ๆ ซึ่งต้องใช้สารพันธุกรรมที่สมบูรณ์ของแบคทีเรียโวลบาเชีย และ/หรือเซลล์ที่มีชีวิตต่อไป*

คำสำคัญ : *การแยกแบคทีเรียโวลบาเชีย, หนอนพยาธิหัวใจสุนัข, การวิเคราะห์โปรตีน*

The obligate intracellular bacteria of the genus *Wolbachia* has been discovered in arthropods and filarial parasites. *Wolbachia* is alphaproteobacteria belonging to the order Rickettsiales, and family Anaplasmataceae.[1] It is described by the general characteristics of rickettsiae; small (0.3-0.5 x 0.8-2.0 μm) gram-negative coccobacilli that is obligate intracellular bacteria of eukaryotic cells. Under electron microscopy studies, the bacteria typically reside within a vacuole covering with three layers of membranes in the cytoplasm of the cell. The outer layer is host-derived, followed by the outer cell wall of the bacteria, and the innermost layer consists of the plasma membrane of the bacteria.[2,3]

Wolbachia appears to play an important role in the development, viability and fertility of filarial nematodes. The significant biology functions of *Wolbachia* have promoted applied researches to use them as targets for a novel chemotherapy in filarial disease control and eradication.[4-6] *Wolbachia* also plays an important role in the pathogenesis of filarial diseases.[7,8] A relevant study has suggested that *Wolbachia*, rather than the lymphatic filarial parasite *Brugia malayi*, plays a major role in inducing inflammatory responses by mouse macrophages.[9] Host immune responses against *Wolbachia* are thought to be a major factor contributing to the progression of lymphatic filariasis, and drug-induced adverse reactions.[10-14]

Analysis of *Wolbachia* proteins will provide complementary information on the biology of this endosymbiont, and a basis molecular mechanism of the inflammatory response in lymphatic filariasis. Because *Wolbachia* is an intracellular bacterium, the *Wolbachia* isolation from filarial parasites has been impeded by the difficulty to obtain the highly purified and sufficient amount of *Wolbachia*. To harvest *Wolbachia* protein materials for protein analysis, we therefore developed a method to isolate *Wolbachia* from filarial nematodes for protein analysis.

All extraction methods for isolation of rickeettsia bacteria from host cells are not effective to isolate *Wolbachia* from *Drosophila* eggs.[15] The major contaminations are mitochondria.[15] Sun *et al* modified methods to purify mitochondria from *Drosophila* and isolate *Wolbachia* bacteria from adult worms of *B. malayi* by using 0.85% NaCl supplemented with 0.001% Nonidet P-40 (NP-40). This was a non-ionic detergent which was used as the homogenization buffer for determination of *Wolbachia* genome size as well.[16] However, this isolation method was not proved to be suitable for proteomic studies.

Detergents are known as surfactants which decrease the surface water tension, and solubilize membrane proteins by mimicking the lipid-bilayer environment.[17] Therefore, the higher percentage of non-ionic detergent should increase yield of the bacterial isolation as well as decrease host parasite contamination. In this study, we investigated the most appropriate concentration of NP-40 as homogenization buffer to isolate *Wolbachia* from *D. immitis*.

Therefore, we adjusted a proper condition for the *Wolbachia* isolation from *Dirofilaria immitis* by various concentrations of NP-40 ranging from 0.04% to 0.10% in the homogenization buffer.

Materials and Methods

Preparation of adult worm extracts

This study has been approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Adult worms of *D. immitis* were collected from hearts of naturally infected dogs at necropsy. Worms were washed with normal saline, and chopped up on ice with a sterile scalpel blade. The chopped-up worm tissue fragment of about 200 mg were homogenized in pestle homogenizer in ice-chilled with 1.2 ml of 0.85% NaCl supplemented with 0.04%, 0.06%, 0.08%, or 0.10% NP-40 (Sigma, St. Louis, MO) as the homogenization buffer.

Wolbachia isolation

The purification procedures for filarial *Wolbachia* were modified from the protocol previously described.[16] After incubation on ice for 15 min *Wolbachia* cells were isolated by differential centrifugation (Figure 1). To pellet the worm debris and worm nuclei, the homogenates were centrifuged at 350 *xg* at 4°C for 40 minutes. To pellet *Wolbachia* bacteria, the supernatant was further centrifuged at 16,100 *xg* at 4°C for 20 minutes. The *Wolbachia* pellet was resuspended with the homogenization buffer. After three washes with the homogenized buffer, enriched *Wolbachia* pellet was collected, and examined under a light microscope.

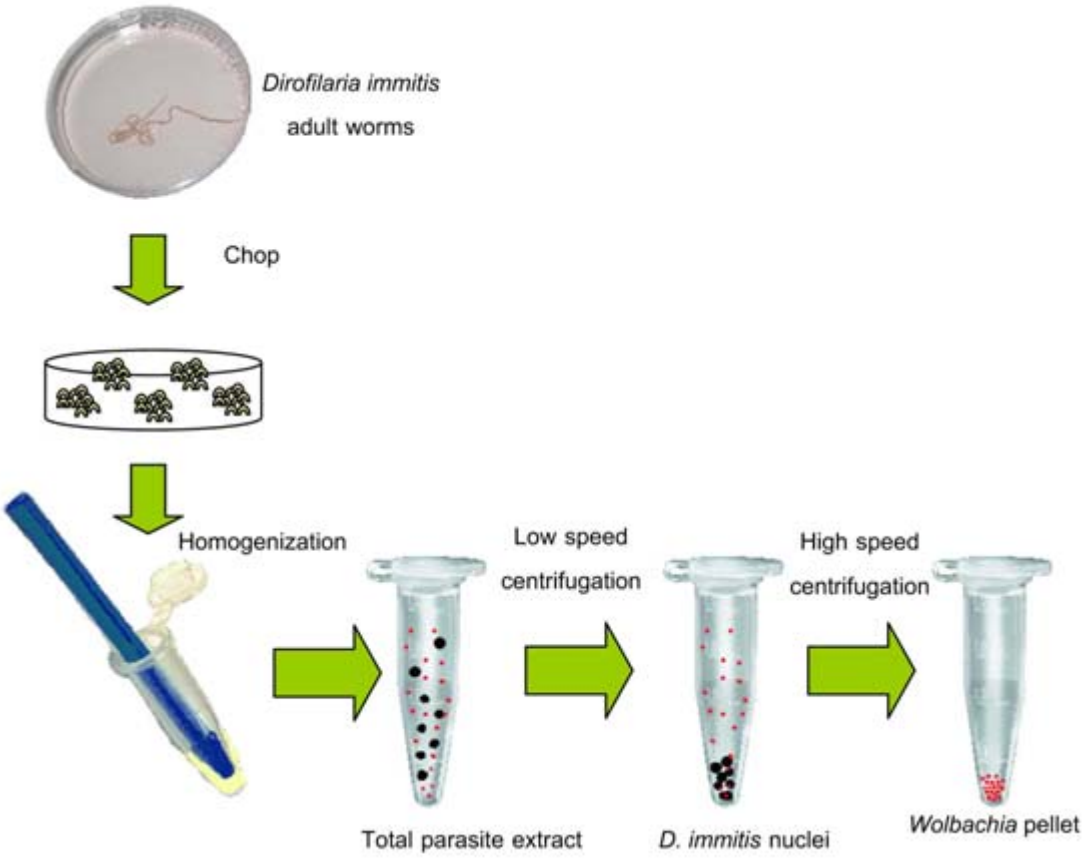


Figure 1. The schematic diagram of the process used for *Wolbachia* isolation from *Dirofilaria immitis* adult worms.

Wolbachia protein analysis

Wolbachia proteins were characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the density values of the bands were analyzed by Quantity One Software version 4.6 (Bio-Rad Laboratories, Inc., Hercules, CA).

Wolbachia purification

The purity of the *Wolbachia* was determined by the ration of *wsp/gst* which characterized by quantitative real-time PCR. The method for *Wolbachia* detection using qPCR was modified from the protocol previously described.[18] The *Wolbachia* pellets were suspended in 5 μl lysis buffer (0.05% SDS and 0.025N NaOH). The samples were incubated at 95°C for 15 min, spun down, and resuspended in 195 μl sterile deionized water. All DNA extractions were stored at -20 °C until use. The quantitative real time PCR analysis was performed using specific *D. immitis Wolbachia* surface protein (*wsp*) primers, *wsp*Diro-F (5' CTT TAG TGG CGT TGC TAA GT 3') and *wsp*Diro-R (5' ACC TTC AAT ATC AAC CCT AAT ATC G 3') (Invitrogen, Carlsbad, CA). The amplification program included an initial denaturation step for 1 cycle at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10s, annealing at 60 °C for 10s, and extension at 72 °C for 12s. Plasmids containing inserts of the amplified single copy of *D. immitis wsp* gene sequence were prepared for use as standards in the quantitative PCR. Specific *D. immitis* glutathione-S-transferase *(gst)* primers, *gst* Diro-F (5' GAA TGG TGA AAA TAA TGC GGA AAC 3') and *gst*Diro-R (5' ATA AGC CTC ATA GAT CAT TCT TGT G 3') (Invitrogen, Carlsbad, CA) were designed from a comparison among *D. immitis, W. bancrofti,* and *B. malayi*'s *gst* mRNA (accession number U14753, AY195867, and Y12788, respectively) using CLUSTAL X program. The amplification program included an initial denaturation step for 1 cycle at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10s, annealing at 60 °C for 10s, and extension at 72 °C for 5s. Plasmids containing inserts of the amplified single copy of *D. immitis gst* gene sequence were prepared for use as standards in the quantitative PCR. Sample detection was based on the SYBR Green I dye incorporation with the intended PCR products. The specificity of amplification was confirmed by melting curve analysis.

Results

After varying conditions for extraction, we could enrich *Wolbachia* cells from *D. immitis*. Under a microscope, we found a lot of shinny bacteria-like cells in the preparation. The cocco-bacilli, with pleomorphic shapes of *Wolbachia*, were most arranged in single and double cells (Figure 2).

Protein profiles of *Wolbachia* enriched extracts on the Coomassie blue stained-SDS-PAGE gel showed that using higher NP-40 concentration provided higher protein concentration in pellet, but lower protein concentration in supernatant (Figure 3). The homogenization buffer which provided the highest ration of *Wolbachia* protein/total parasite extract protein was 0.85% NaCl + 0.08% NP-40. However, the *Wolbachia* protein concentration was dramatically decreased when using 0.85% NaCl + 0.10% NP-40 as the homogenization buffer.

The *Wolbachia* quantity and the parasite contamination were quantified by real-time PCR. The higher concentration of NP-40 could increase the yield of bacterial isolation and decrease the host parasite contaminations. The homogenization buffer provided the highest ration of *wsp*/*gst* was 0.85% NaCl + 0.08% NP-40. However, the *wsp/gst* ration was dramatically decreased by using 0.85% NaCl + 0.10% NP-40 as the homogenization buffer.

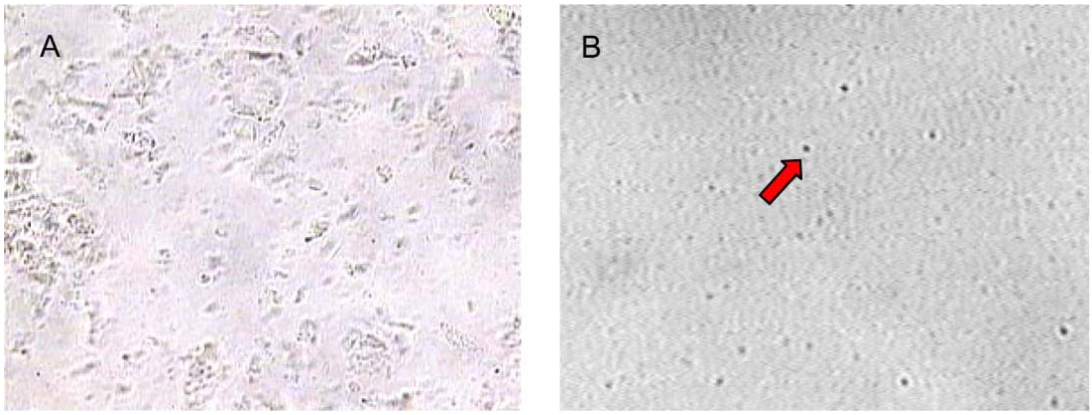
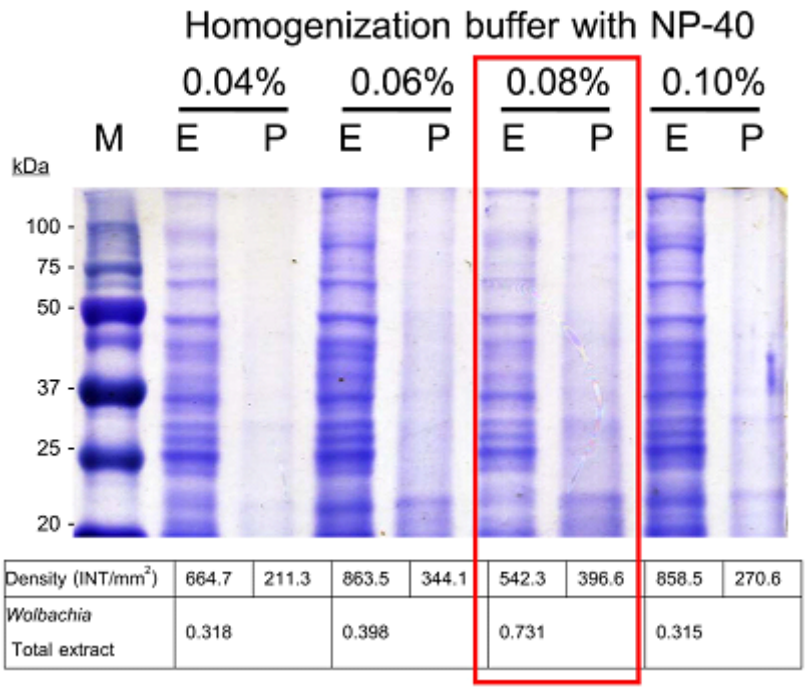


Figure 2. Wet-mount preparation of *Wolbachia* pellet from *Dirofilaria immitis* adult worms (A) Total parasite extract; (B) *Wolbachia* cells.



Density (INT/mm^2)	664.7	211.3	863.5	344.1	542.3	396.6	858.5	270.6
Wolbachia Total extract	0.318		0.398		0.731		0.315	

Figure 3. Protein analysis of *Wolbachia* purification from *Dirofilaria immitis* by various concentrations of NP-40 (0.04-0.10%) by SDS-PAGE. M: Markers; E: parasite extracts; and P: *Wolbachia* pellets

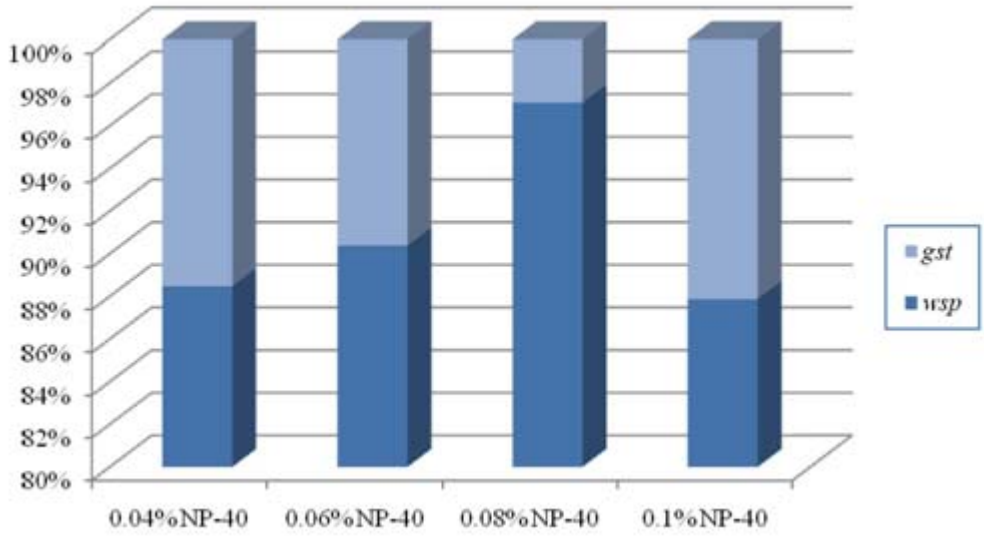


Figure 4. The relative concentration of *wsp* (*Wolbachia* gene) and *gst* (*Dirofilaria immitis* gene) copies in *Wolbachia* pellets extracted from *D. immitis* by using various concentrations of NP-40 quantified by real-time PCR

Discussion

Wolbachia have been implicated as novel drug targets for treatment of human filarial parasitic infections, and as the organism triggering the immunopathogenesis.[19, 20] Potential drug targets as well as candidate immunomodulatory/proinflammatory/antigenic molecules of the bacteria can be identified by analyzing the filarial *Wolbachia* genome.[21] However, a selection process is still needed to validate the protein expression levels. The nature of *Wolbachia* residing in host vacuole makes limitation on obtaining these purified strictly intracellular bacteria with host free materials, and has hampered progress in the detailed study of their biology. To harvest *Wolbachia* protein materials for analysis, the isolation protocol for filarial nematode *Wolbachia* was optimized.

The purification method of rickettsia bacteria from their host cells depends on the isopycnic density gradient centrifugation.[22] However, this technique does not effectively purify *Wolbachia* from the host materials.[15] The modified method using 0.1-1% Lubrol detergent and 3-μm-pore-size membrane filtration membranes is reported to isolate *Wolbachia* bacteria from *Drosophila* eggs.[15] The detergent removes host vacuolar materials from *Wolbachia* but it also generates the membrane aggregates from the host materials.[15] Therefore, the host material contaminations are found in the *Wolbachia* fractions after the filtration. The major contaminations are mitrochondria. The further modification of the *Wolbachia* isolation method is reported based on the purification of *Drosophila* mitochondria.[16] In this study, they used fractioned centrifugation method by using 0.85% NaCl supplemented with 0.001% of NP-40 detergent in the homogenization buffer. The lower concentration of detergent decreases *Wolbachia* aggregation with the worm tissue fragments, but results in the lower yield of *Wolbachia*.[15] Lubrol and NP-40 are non-ionic detergents. Lubrol detergent causes the DNA degradation, while NP-40 is the most common

used detergent in cell lysis buffer.[17] In this study, we therefore modified the fractioned centrifugation method by using 0.85% NaCl supplemented with varying concentration of NP-40 as the homogenization buffer to find the appropriate concentration of NP-40 which can provide the highest yield but the lowest host material contamination.

From this study, the modified protocol led to massive enrichment of *Wolbachia* proteins from the total parasite extracts. The use of 0.85% NaCl + 0.08% NP-40 as the homogenization buffer was found to facilitate the proteomic studies. However, *Wolbachia* bacteria broke up when 0.85% NaCl + 0.1% NP-40 is used as homogenization buffer.

However, like purification of other strictly intracellular bacteria, there are co-purified host proteins, which have nature major in cytoplasm of eukaryotic cells.[23,24] During the procedures of the *Wolbachia* isolation, we detected, for example, paramyosin, heat-shock protein, and beta-tubulin of *D. immitis* by using quantitative real time PCR (data not shown). The proteins may be closely associated with that of the bacteria by the fact that some of them can function in maintaining the cytoskeleton architecture.[25,26] Therefore, they are difficult to trade-off during purification. The *Wolbachia* prepared from this isolation method would be an optimal starting material for further extensive purification such as an antibody-affinity chromatography.

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