

***Ichthyophthirius multifiliis* Membrane Protein Isolation Protocol** (Laboratory of Dr. H.W. Dickerson)

Reagents

10 mM tris-HCl (pH7.5)

ice cold acetone

ethanol

10mM/ml PMSF in isopropanol (phenyl-methyl-sulfonyl-fluoride is a protease inhibitor)

Triton X-114 extraction buffer (2.0% v/v triton X-114, 300 mM NaCl, 20 mM tris-HCl (pH7.5))

Sucrose Cushion (6.0% (w/v) sucrose, 150 mM NaCl, 0.06% (v/v) Triton X-114, 10 mM tris-HCl (pH 7.5))

Note: save samples from phases for SDS gel: Insoluble pellet, membrane protein, SUPP from extraction

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1. Concentrate a sample of theronts. Keep on ice.
 - a. When using fresh theronts ($0.5-1 \times 10^6$) strain through nylon filter (~35 micron in diameter). Pellet at 1,000 RPM for 1 minute. Re-suspend pellet in 2 ml of 10mM Tri-HCl (pH7.5).
 - b. When using frozen samples (*frozen samples are previously concentrated theronts which have been resuspended in 100 ul of 10 mM tris (pH 7.5) and 20 ul of 1mg/ml PMSF, then stored at -80C*) Thaw about 15 pellets. Add 100 ul of 10mM Tris-HCl (pH7.5) to each pellet. Transfer samples to a 30 ml Corex centrifuge tube. Wash original tubes with 500 ul of 10mM Tris-HCl (pH7.5) starting with first tube and transferring to remaining tubes. Add washes to Corex tube. (Total volume should be approximately 2 ml).
 2. Add equal volume (2 ml) of ice cold Triton X-114 Extraction Buffer.
 3. Add 1/100 Volume (20 ul) of 1M PMSF to the solution (final conc. = 10mM). Reserve 50ul for protein concentration determination.
 4. Vortex sample for one minute or use douncer. Incubate for 5 minutes on ice.
 5. Repeat step 4.
 6. Centrifuge at 16,000g for 10 minutes at 4 °C. Pour off SUPP (supernatants) and reserve SUPP on ice. Add 1ml of Triton X-114 Extraction Buffer to pellet.
 7. Vortex for one minute. Incubate for 5 minutes on ice.
 8. Repeat step 7.
 9. Centrifuge at 16,000g for 10 minutes at 4 °C. Pour off SUPP and reserve SUPP on ice. Resuspend insoluble pellet in laemlli lysis buffer and boil.
 10. Combine both SUPPs, discard pellet. Warm SUPP in a 30 °C water-bath for 5 minutes.
 11. Determine the volume of combined SUPPs. Put an equal volume of sucrose cushion into a separate Corex tube. Layer the combined SUPPs onto the sucrose cushion, and warm in 30 °C water-bath for 5 minutes.
 12. Centrifuge at 300g (1,200 rpm on tabletop centrifuge) for 3 minutes at room temperature (RT). (Cloudy pellet = detergent phase). Remove the aqueous phase to another tube. Save the detergent phase on ice.
 13. Dilute the supernatant 1:3 (Triton X-114 Extraction Buffer : SUPP).
 14. Vortex for one minute and incubate 5 minutes on ice.
 15. Repeat step 14.
 16. Warm for 5 minutes in 30 °C water-bath. Layer SUPP over the original sucrose cushion. Centrifuge at 300g for 3 minutes at RT. Discard SUPP and sucrose cushion.
 17. Quantitate protein and prepare sample (SUPP and membrane protein) for SDS gel.
- Optional: Do these steps to dry and remove detergent for long term storage.
17. Add 9 volumes ice-cold acetone to pellet. Incubate 30 minutes ~ 1hr on ice. Store overnight at -20°C.
 18. Centrifuge at 16,000g for 15 minutes at 4 °C. Remove acetone. Vacuum dry the pellet and re-suspend in 100 ul of 10mM Tris-HCl (pH7.5). Fast freeze in a dry ice ethanol bath or liquid nitrogen. Store at -70 °C.

(Reference: H.W. Dickerson, T.G. Clark and R.C. Findly. *Ichthyophthirius multifiliis* Has Membrane-Associated Immobilization Antigens, The Journal of Protozoology, 1989, Vol. 36, No2 March-April, pp. 159-164.)

