**SDS-PAGE and Western blot protocol for Rubisco (RBCL, 55 kDA)**

 All of my protein samples are dissolved in 1x Laemmli “sample” buffer, meaning that you just need to add a little (~4-8 µl) bromophenol blue (in fridge) prior to loading. This protocol will use BioRad’s Mini-Protean TETRAD system. Two gels will always be run; one will be blotted and the other stained with SYPRO® Ruby (Bio-Rad) or Coomassie blue, according to the manufacturer’s recommendations, to visualize protein quality.

1. Cast the separating gels: 12% bis-acrylamide (15 ml for two gels).
	1. 4.50 ml acrylamide solution (40%, in a 50 ml tube in Dr. Hsiao’s refrigerator)
	2. 5.6 ml 1 M Tris-HCl (pH 8.8, made fresh on 6-26-11) See page 3.
	3. 4.71 ml H20
	4. 150 ul 10% SDS (made fresh on 6-26-11)
	5. 10 ul TEMED (stored in Dr. Chen’s refrigerator)
	6. 100 ul 10% ammonium persulfate (APS, stored @ -20°C in my reagent box)
	7. Quickly use 1 ml pipet to fill mold with about 7.5 ml solution.
2. Overlay with 100% cold ethanol and allow to polymerize for 25-45 min.
3. While waiting, remove 7-9 samples from the freezer and thaw proteins on ice.
4. Remove aliquot representing 20-30 µg and transfer to a new 1.5-ml microcentrifuge tube.
5. Add 4-8 µl bromophenol blue and boil in dry bath for 3-5 min @ 100°C.
6. Spin @ 12,000 x g for 10 min @ 4°C to pellet insoluble material.
7. During this spin, prepare the 4% stacking gel (6 ml for two gels).
	1. 600 µl acrylamide solution (40%)
	2. 3.8 ml H20
	3. 1.5 ml 0.5 M Tris-HCl (pH 6.8, made fresh on 6-26-11)
	4. 15 µl 50% SDS
	5. 6 µl TEMED
	6. 30 µl 10% APS
8. Decant ethanol over separating gel and quickly add ~2 ml stacking gel solution until reaching the top of the mold.
9. Insert comb. Make sure it is the same thickness as the mold, usually 1 or 1.5 mm.
10. Allow to polymerize for 15-20 min and move dock into Bio-Rad Mini-Protean TETRAD chamber.
11. Fill with running buffer (made fresh [10x] on 6-26-11) and place entire unit on ice in portable cooler.
12. Remove comb and load pre-stained protein ladder (5 ul) into first well. Remove chunks of acrylamide with a syringe if they are blocking the wells.
13. Slowly add supernatants from step 6 to remaining wells.
14. Run @ 75 V until sample dye front reaches top of separating gel (typically 60 min).
15. Increase voltage to 130 V for run for 90 min (until the dye front is near the bottom of the gel. Basically, we want the target protein, Rubisco, to move halfway down the gel, so look for the marker closest to 55 kDa.
16. Turn off voltage and recycle running buffer (except running buffer immediately next to gels, which can be discarded).
17. For one gel, fix in 10% methanol and 7% acetic acid and proceed w/ SYPRO® Ruby protocol, staining either overnight or with microwave protocol.
18. Wet PVDF membrane in 100% methanol for 10 s and then transfer buffer for 5 min.
19. Make gel sandwich with the second gel. Remember that proteins will migrate from (-) to (+) because SDS will confer a negative charge on them.
20. Transfer @ 100 V for 75 min at 4°C in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS [made fresh on 7-15-11]). Put unit on ice.
21. Disassemble gel sandwich, and stain PVDF membrane with Ponceau S (red) for several minutes to make sure that proteins transferred.
	1. If protein concentration on the membrane looks low, abandon blotting and continue with 2nd gel to check protein quality.
	2. If protein concentration looks high, destain with water for 10 min (perform 3-4 washes) and proceed with protocol.
22. Block membrane in Tris-buffered saline (TBST; 100 mM Tris base, 150 mM NaCl, 0.05% Tween-20, pH 7.6) with 5% skim milk for 1 hr on a shaker table at 70 rpm.
	1. For HSP70 WBs, used 10 mM Tris-HCl, pH 8, instead of above.
23. Remove blocking buffer and incubate membranes overnight at 4°C (or RT for 2 hrs) in 10 ml of a 1:2000 dilution (in TBST with 5% skim milk) of an RBC antibody.
24. Decant antibody into Falcon tube (to recycle it for next time) and perform three membrane washes with TBST.
25. Incubate membrane in 15 ml of a 1:10,000 dilution (in TBST) of the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) at RT for 1 hr at 70 rpm.
26. After decanting the secondary antibody, wash membranes three times with TBST at RT for 5 min.
27. Overlay membranes on plastic wrap, coat with 400 µl SuperSignal® West Pico Chemiluminescent Substrate ECL solution (Thermo-Scientific), and incubate for several minutes.
28. Use Fusion gel doc to visualize instead. Use auto-exposure or 2-2.5 min.
29. Take picture of ladder, as well, and merge with chemiluminescent image.

For the red samples (adult corals): use 1:2,000 dilution of RBC (fresh) with 1:5,000 secondary antibody dilution (anti-rabbit).

For the green samples: used 1:2,000 dilution of RBC with 1:10,000 dilution secondary antibody with 75 V for 45-60 min and 130 V for 90 min run times, transferring at either 100V for 75 min (first two gels) or 75 V for 130 min (3rd gel, run on 7-11-11).

For the yellow samples (adult *S. hystrix* samples), try 1:5000 RBC primary antibody dilution and 1:15,000 secondary antibody dilution (anti-rabbit) with new antibody (arrived on \_\_\_\_).

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| *Pocillopora damicornis* (adults) long-term temperature manipulation study, t = 6 months samples |
| Sample | Concentration(ng/ul) | ul to reach 20 ug (total for 2 gels) | ul bromophenol blue for 2 gels | ul/well |
| **C24T1S1** | **779** | **25.7 (51.4)** | **5** | **27** |
| **C24T1S2** | **655** | **30.5 (61)** | **6** | **32** |
| **C24T2S1** | **2267** | **8.8 (17.6)** | **2** | **9** |
| **C24T3S1** | **721** | **27.7 (55.4)** | **5** | **29** |
| **H24T1S1** | **598** | **33.4 (66.8)** | **6** | **35** |
| **H24T2S1** | **2284** | **8.8 (17.6)** | **2** | **9** |
| **H24T2S2** | **2321** | **8.6 (17.2)** | **2** | **9** |
| **H24T3S1** | **484** | **41.3 (82.6)** | **7** | **43** |
| **H24T3S2** | **493** | **40.6 (81.2)** | **7** | **42** |
| *Pocillopora damicornis* (larvae) pCO2 x temperature study (April 2010) |
| 10-17 | 795 | 25.2 (50.4) | 2-3 | 27.2 |
| 10-18 | 547 | 36.6 (73.2) | 3-4 | 39.6 |
| 10-19 | 1179 | 17 (34) | 2 | 19 |
| 10-20 | 1273 | 15.7 (31.4) | 2 | 17.7 |
| 10-21 | 619 | 32.3 (64.6) | 3 | 35.3 |
| 10-22 | 1056 | 18.9 (37.8) | 2 | 20.4 |
| 10-23 | 1334 | 15 (30) | 2 | 17 |
| 10-24 | 1043 | 19.2 (38.4) | 2 | 21.2 |
| 10-25 | 1616 | 10-12.4 (24.8)\* | 1-2 | 13.4 |
| 10-27 | 793 | 25.2 (50.4) | 2-3 | 27.2 |
| 10-28 | 937 | 21.4 (42.8) | 2 | 23.4 |
| 10-29 | 871 | 23 (46) | 2 | 25 |
| 10-30 | 967 | 20.7 (41.4) | 2 | 22.7 |
| 10-31 | 1212 | 16.5 (33) | 2 | 18.5 |
| 10-32 | 807 | 24.8 (49.6) | 2-3 | 26.8 |
| *Seriatopora hystrix* (adult) laboratory-based reciprocal transplant study (June 6-15, 2010) |
| HBH variable colony 1 | 652 | 31 (62) | 6 | 33 |
| HBH variable colony 2 | 628 | 32 (64) | 6 | 34 |
| HBH variable colony 3 backup | 760 | 26.5 (53) | 5 | 28.5 |
| HBH variable colony 4 | 1008 | 20 (40) | 4 | 22 |
| HBH variable colony 5 | 1068 | 19 (38) | 4 | 21 |
| HBH variable colony 6 | 996 | 20 (40) | 4 | 22 |
| HBH stable colony 1 backup | 612 | 33 (66) | 6 | 35 |
| HBH stable colony 2 backup | 540 | 37 (74) | 7 | 39 |
| HBH stable colony 3 | 796 | 25 (50) | 5 | 27 |
| HBH stable colony 4 | 660 | 30 (60) | 6 | 32 |
| HBH stable colony 5 | 780 | 25.5 (51) | 5 | 27.5 |
| HBH stable colony 6 | 724 | 27.5 (55) | 5 | 29.5 |
| HWN variable colony 1 | 1012 | 20 (40) | 4 | 22 |
| HWN variable colony 2 | 1020 | 19.5 (39) | 4 | 21.5 |
| HWN variable colony 3 | 1044 | 19 (38) | 4 | 21 |
| HWN variable colony 4 | 1504 | 13.5 (27) | 3 | 15.5 |
| HWN variable colony 5 | 1284 | 15.5 (31) | 3 | 17.5 |
| HWN variable col. 6 backup | 536 | 37.5 (75) | 7 | 39.5 |
| HWN stable colony 1 | 1112 | 18 (36) | 3 | 20 |
| HWN stable colony 2 backup | 792 | 25.5 (51) | 5 | 27.5 |
| HWN stable colony 3 | 1720 | 11.5 (23) | 2 | 13.5 |
| HWN stable colony 4 | 596 | 33.5 (67) | 6 | 35.5 |
| HWN stable colony 5 | 1080 | 18.5 (37) | 4 | 20.5 |
| HWN stable colony 6 | 1452 | 14 (28) | 3 | 16 |

\*Insufficient quantity to load 20 ug/gel

Common solutions, reagents, and buffers

1. 1 M Tris (pH 8.8)
	1. Weigh out 24.2 g Tris base and add to ~170 ml ddH20.
	2. Adjust pH to 8.8 with concentrated HCl and top off to 200 ml.
2. 1M Tris (pH 6.8)
	1. Weigh out 24.2 g Tris base and add to ~140 ml ddH20.
	2. Adjust pH to 6.8 with concentrated HCl and top off to 200 ml.
3. 10% sodium dodecyl sulfate (SDS)
	1. Weigh out 5 g pure SDS powder (Dr. Hsiao’s cabinet).
	2. Add to 50 ml ddH20 in either small glass bottle or 50 ml Falcon tube.
4. 1x Laemmli reducing “sample” buffer (15 ml)
	1. 10% glycerol, 5% B-mercaptoethanol, 2% SDS, 0.0625 M Tris-HCl (pH 6.8)
	2. Fill 15 ml Falcon tube with ~ 7 ml ddH20.
	3. Add 3 ml 50% glycerol, 3 ml 10% SDS and 938 ul 1 M Tris (pH 6.8).
	4. Vortex and top off to just over 14 ml with ddH20.
	5. Store at 4C.
	6. When ready to dissolve proteins, add 750 ul B-mercaptoethanol and vortex.
5. 10x SDS-PAGE running buffer (1 L)
	1. 10x concentrations: 0.25 M Tris, 1.92 M glycine, 1% SDS
	2. 1x concentrations: 0.025 M Tris, 0.192 M glycine, 0.1% SDS
	3. Add 500 ml ddH20 to a 1 L bottle.
	4. Weigh out 30.3 g Tris base and 144.2 g glycine and add to bottle.
	5. Weigh out 10 g SDS powder (weigh mask) and add to bottle.
		1. Alternatively, add 50 ml of 20% SDS or 100 ml 10% SDS.
	6. Vortex and store at room temperature (no pH adjustment required).
6. SDS-PAGE transfer buffer (2 L)
	1. Add 200 ml 10X running buffer to 400 ml methanol (100%, NOT industrial grade!).
	2. Vortex and add 1400 ml ddH20.
	3. Store at 4C.
7. Tris-buffered saline with Tween-20 (“TBST,”1 L)
	1. 1x concentrations: 0.1 M Tris, 0.15 M NaCl, 0.1% Tween-20.
	2. Dissolve 12.2 g Tris base in 200 ml NaCl solution (5X [already made]).
	3. Add 1 ml Tween-20 and top off to 1 L with ddH20.
	4. Adjust pH to 7.6 with concentrated HCl.