Phospholamban TOTAL/Phosphorylation Western Assays (Kobra' s protocol/Kranias lab.)

A. Mouse Cardiac Homogenates' Homogenization Buffer:

Final	Stock	for 10 mL
Lysis buffer	(from Cell Signaling)	1 ml
0.3 mM	100 mM PMSF (/methanol) -20ºC	30 uL
Protease inhibitor	(from Roche/Sigma)	1 tablet
PPI Cocktail I and II	(from Millipore or any other company)	50 ul of each
H2O		8.87 mL

Homogenization:

-Powder the frozen heart in cold mortar and pistil (cooled down with liquid nitrogen) in cold room.
- Homogenize mouse hearts in 1 mL of the homogenization buffer.
[Tekmar Tissumizeror a regular drill with Teflon probes, (10 sec spin + 20 sec rest) x 4 times on ice in cold room]
-Quantitate the protein level and aliquot the homogenate (25-50 uL/tube), freeze them in liquid N2, and store them at -80ºC

B. Western Blotting:

1. Load cardiac homogenates (solubilized with an equal volume of 2X SDS sample buffer at 37ºC for 5 min or room temperature for about 20 minutes) to slots of Tris-glycine SDS-Polyacrylamide (12%) mini-gels fill center of gels with loading buffer
2. Linear range of the standard: around 20, 15, 10, 5 ug. **Note:** load 10-15 ug of each sample for serine-16 phosphorylation and 40 ug for each sample threonine-17 phosphorylation detection, adjust linear range accordingly. The dilution range of the standards should not be greater than one order of magnitude. Otherwise, the higher concentrations are in non-linear range.
3. Run the mini gels at 120 V for 15 min at 4ºC.
4. Increase the voltage to 150 V and keep running at 4ºC until the dye front is about 1 cm from the bottom of the gels (~1:30 min).
5. Equilibrate the gels, filter paper, sponges, and membranes (nitrocellulose, 0.05-0.1 um) in transfer buffer for 30 min at room temperature (R.T.).

6. Transfer the proteins with equilibration of the gels in transfer buffer at 200 mA for 1:30 hrs at 4ºC.
7. Block the membranes in 5% non-fat dry milk dissolved in TBS for 1 hr at R.T.
8. Discard the blocking solution and incubate the membranes with the primary Ab (1:2500 pSer16 Catalog #010-12 Badrilla [4 uL/10 mL 5% non-fat dry milk and 1:2500 pThr17 Catalog #010-13]) for O/N at 4 degree C
9. For total PLN (1:5000, Thermo-Fisher) 3 hr at r.t or O/N at 4 degree C.
10. Rinse the membranes twice with TBS, and wash them 3 times (10-15 min each).
11. ECL: Incubate the membranes with the secondary Ab (1/5000 Amersham's horseradish peroxidase-conjugated anti mouse or rabbit IgG [2 uL/10 mL "0.5%" non-fat dry milk]) for 1 hr at R.T.
.12. Rinse the membranes twice with TBS and wash them 3 times (10-15 min each).
13. Incubate the membranes with equal volumes of ECL reagents for 1 min at R.T.
14. Drain off the reagent, and wrap membranes in Saranwrap or heat seal plastic.
15. Put film on the top of the blot and expose the film for 30 sec at first.
16. Develop the film.
17. If necessary, re-expose longer or shorter (usually around 1 min works). If the standard of the lowest concentration is only just visible on the film, the signals from the rest of the standards should be in the linear range of the film.

NOTE: or use fluorescence congregated secondaries with LiCor method to see the bands.

Note: The Badrilla phosphorylation antibodies come in the lyophilized form, only dilute in 20 uL d.i. H2O, store at -80ºC.
Note: Phospholamban is seen as a monomer and pentamer, to migrate all monomer boil homogenate samples for 5 minutes and immediately set on ice after boiling and prior to loading the mini-gel!
Also with boiling, stop the electrophoresis just prior to dye front exiting the gels. Without boiling the majority usually appears in pentamer, migrating around the 30 kDa marker in the broad ranger protein standard (Bio-rad).
Note: We usually strip the P-PLN from the membrane and probe it with T-PLN (1:5000, Thermos- Fisher)
Also, we freeze the primary antibodies in -20 C freezer after first use and reuse it for a few times before discarding it by tweaking with small amount of the specific antibody at each use.

Loading sample buffer:
Tris-glycine SDS-Polyacrylamide Gel loading buffer 2 X: Final concentration:
100 mM Tris-HCl (pH 6.8),
4% SDS
2% B -mercaptoehtanol
20% glycerol
0.004% bromophenol blue dd H2O