I- Protein extraction (work on ice):

1. Crush the whole ant just after ovaries and gut removal in 24 µl of **lysis buffer**: TNEB (50 mM Tris–HCl, 2 mM EDTA, 100 mM NaCl, and 1%NP-40; pH 7.8) with added protease inhibitor cocktail (1%)
2. Centrifugate at 1400g for 15 min at 4°C
3. Take the supernatant. Save 3 µl for protein concentration measurement (I used Nanodrop), and re-suspend the rest in a DTT solution in order have a final concentration of 50 mM of DTT. At that point sample can be stored at -80°C, including sample for protein assay.

II- Oxyblot procedure:

Kit used: OxyBlot Protein Oxidation Detection Kit, Merck Millipore, ref: S7150; https://www.merckmillipore.com/DE/de/product/OxyBlot-Protein-Oxidation-Detection-Kit,MM_NF-S7150?client=safari&rls=en&q=oxyblot&ie=UTF-8&oe=UTF-8&gfe_rd=cr&ei=mI7eV5XjCdCP8QeSrI_gCw&bd=1)

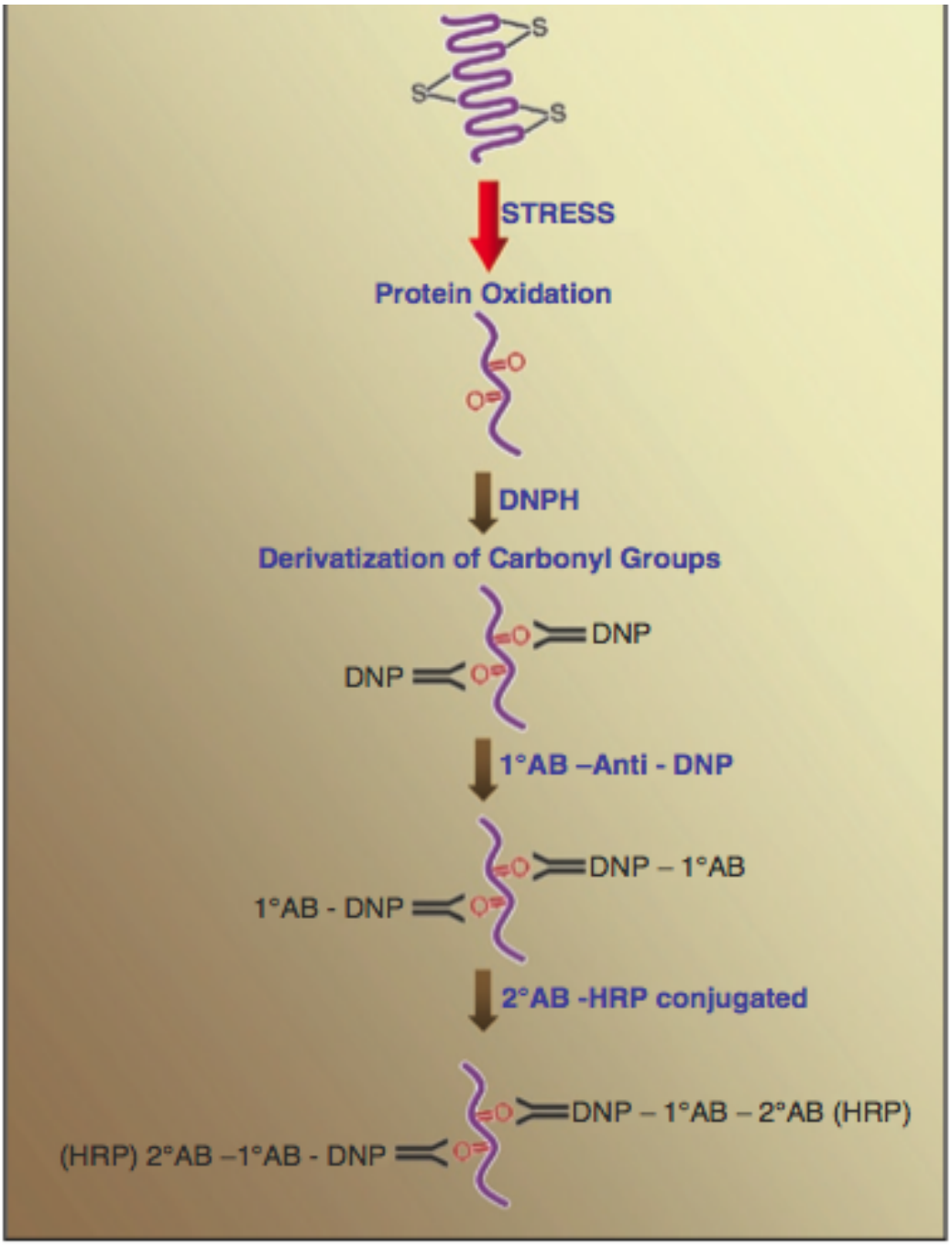

Fig. 1. Schematic of the derivatization of protein carbonyl groups. Cellular exposure to stress results in protein damage characterized by the formation of protein carbonyl groups (side chains). The OxyBlot methodology uses 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyl groups leading to the formation of a stable dinitrophenyl (DNP) hydrazone product. The primary antibody is directed against the DNP moiety of the protein. The secondary, HRP-conjugated antibody allows for the use of chemiluminescent reagent to visualize bands of oxidized proteins upon exposure to film.

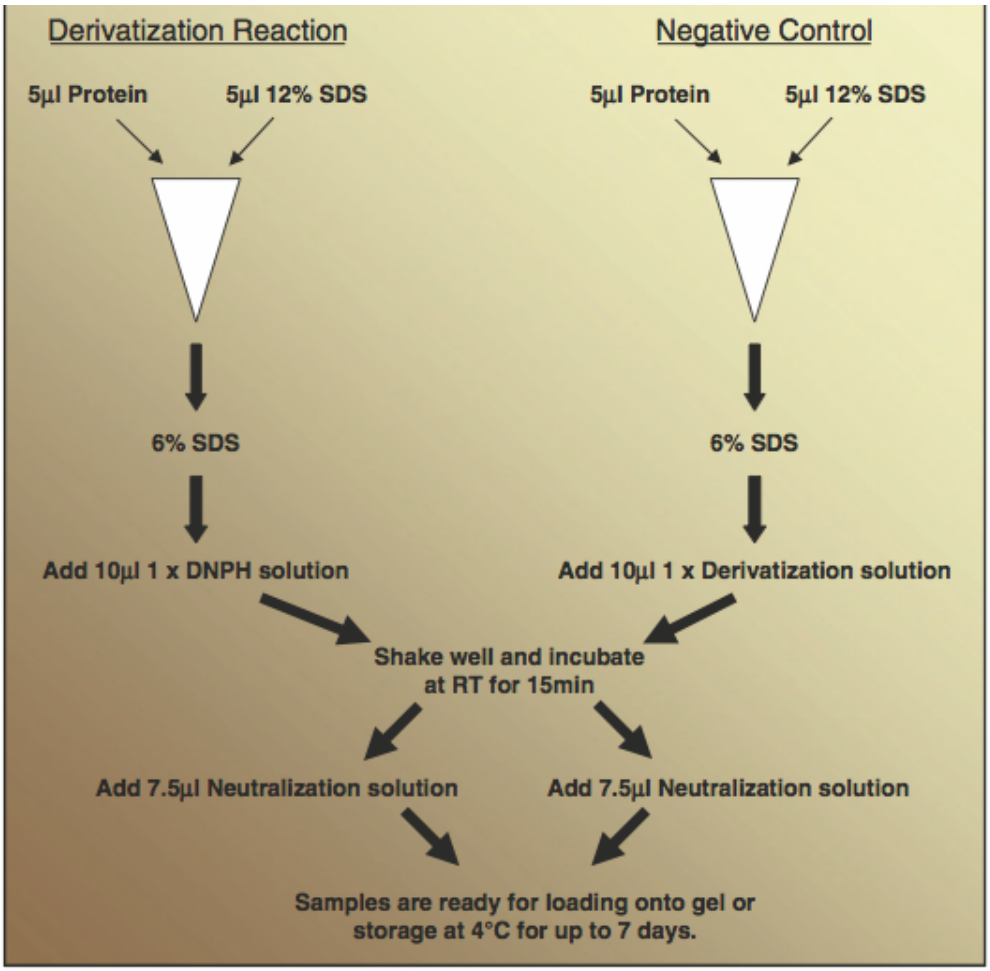

Fig. 2. OxyBlot methodology flow chart. 5 µl sample are added to two prelabeled 1.5 ml Eppendorf tubes, of which one is designated for the derivatization reaction (DR) and the second one for the negative control (NC). 5 µl of 12% SDS are added to each sample to obtain a final concentration of 6% SDS. 10 µl of 1× DNPH or 1× derivatization control solution should be added to samples designated for the DR or NC, respectively. Samples are allowed to incubate for 15 min at room temperature, after which 7.5 µl neutralization solution should be added to each sample. Samples are ready to be processed using gel electrophoresis.

1) Concentration adjustment: Dilute in TNEB (50 mM Tris–HCl, 2 mM EDTA, 100 mM NaCl, and 1%NP-40; pH 7.8) with added protease inhibitor cocktail (1%), protein samples in order to obtain similare concentration for every samples (to be able to compare Western Blot profiles)
2) Prepare the SDS solution 12%
3) Follow rigorously instructions presented on the Figure 2. All components figuring on the figure 2 but not in the text are provided in the kit (OxyBlot Protein Oxidation Detection Kit, Merck Millipore, ref: S7150)
4) Add in every tubes, Derivatization Reaction (DR) and Negative Control (NC), 9.2 µL of Weigh marker
5) Load the Gel (Precast Gels for SDS-PAGE, Precast Polyacrylamide Gels, Sigma, ref :PCG2009) with 30 µL of sample solution. Add the marker solution mixed with lysis buffer and protein standard mixture (marker solution: 15 µL of TNEB with added protease inhibitor cocktail, 5 µL of marker solution, 2.5 µL of Protein standard mixture)
6) Run the gel at 120V for 45 min

7) Transfer the proteins from the gel to nitrocellulose membrane via western blot (130 mA for 90 min for 2 gels 10X8 cm = **0.8 mA.cm^{-2}**)
8) Blocking and saturation of the membrane with non-specific protein (Blocking solution 1% BSA/PBS-T) leaving it incubate overnight at 10 °C in the shaker (or 1h at RT).
   Note: PBS-T: PBS (Phosphate buffered saline, pH 7.2-7.5) containing 0.05% Tween-20
9) Incubate with the primary antibody at RT for 2 h or overnight at 5°C
   Note: Dilute the Antibody stock 1:150 in Blocking solution (1% BSA/PBS-T)
10) Washing with PBS-T solution (Rinse the membrane 2 times with PBS-T, then once for 15 min, and twice for 5 min)
11) Incubate with secondary antibody
   Note: Dilute the Antibody stock 1:300 in Blocking solution (1% BSA/PBS-T)
12) Washing with PBS-T solution (repeat step 10)
13) Addition of chemo-luminescent reagent and expose to a film.