**SYPRO Ruby fluorescent staining for protein gels**

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**Goal:** The goal of this protocol is to provide an alternate quantitation of protein in EVs from standard assays (BCA, microBCA, etc.), which can overestimate EV protein and/or be thrown off by lipids. This method quantitates protein from the entire lane of a gel, based on comparison to the BenchMark protein ladder. We have found this to frequently be a better estimate of EV protein and very useful for subsequent loading of Western blots and other applications like proteomics.

Materials:

* **BenchMark Protein Ladder** (#10747-012, Life Technologies)
* **SYPRO Ruby fluorescent stain** (#S-21900, Life Technologies)
* SYPRO Ruby fix solution (in H2O)
  + 40% Methanol
  + 10% Acetic Acid
* SYPRO Ruby destaining solution (in H2O)
  + 10% Methanol
  + 6% Acetic Acid
* 4X SDS sample buffer
* 8% Acrylamide Gel (PAGE)

**Procedure**

1. Prepare samples in 4X SDS sample buffer
   1. Mix 5ul of sample + 5ul of 4X SDS sample buffer
   2. Heat at 95 C for 5 min
2. Load 5 ul of BenchMark Protein Ladder standard of known protein concentration into the gel (1.7 μg/μl – 8.5ul total)
3. Load samples into the gel
4. Run gel for 30min at 100V, then change to 120V for \_\_\_\_\_\_\_\_ (variable)

\*All incubations should be done in the dark or a dark box

1. Fix the gel with SYPRO Ruby fix solution for 45-1h at RT on a shaker
2. Stain the gels with SYPRO Ruby fluorescent stain with 5-10 mL of SYPRO Ruby.
   1. Incubate for 2 h or overnight (prefer) on shaker at RT
   2. After incubation pour SYPRO Ruby solution into a container (SYPRO Ruby dye can be re-used several times)
3. Wash gel with SYPRO Ruby destaining solution for 30-45 mins twice
4. Image the protein gel using iBright
   1. Protein Gel > Protein Fluorescence > Smart Exposure > Capture
   2. A second image can be taken with a higher exposure time if needed

\*Colors can be inverted, and contrast can be changed

* 1. Safe a G2i file for analysis

1. ImageQuant software (Molecular Dynamics) or suitable densitometry-based analysis software, iBright analysis software has several options to quantify the image.

**iBright Analysis**

1. Load G2i image into iBright analysis software > Select file > click Next
2. Adjust Image
   1. Invert colors
   2. Edit image display enhancement to visualize bands clearly if necessary
   3. Straighten the image if necessary
3. Analyzed Image tab
   1. Change Automatic to Manual
   2. Adjust image display to visualize the bands on gel if necessary
   3. Don’t show saturation
4. Regions Tab
   1. Add regions
   2. Select rectangle > on the image make a rectangle over the latter/benchmark, make sure it covers everything
   3. Copy the rectangle and paste it over each of the sample lanes

\*The rectangle should not change sizes from lane to lane

* 1. Click Apply

1. Export > Analysis Report > Preview
   1. PDF file > Save

Protein quantification

1. A screenshot of a table

   Description automatically generatedFrom the last table of the PDF file copy and paste values from Vol. (Int.) and Local Bg. Corr. Vol. columns into Excel

\*Local Bg. Corr. Vol. shows the intensity of bands/columns with the background removed from Vol. (Int.)

1. Label each of the samples properly
   1. BenchMark Protein Ladder standard will be used to calculate sample concentration
2. Multiply values from Local Bg. Corr. Vol. by 8.5 (total concentration loaded in 5ul of the ladder) to calculate intensity per concentration (AU/ug)
3. Divide Local Bg. Corr. Vol. by AU/ug to calculate the total amount of protein loaded (ug/column)
   1. Each sample should be divided by the number obtained from the BenchMark Protein Ladder standard
4. Divide ug/column by 5 (ul loaded from each sample) to calculate concentration per ul (ug/ul)

\*Volume loaded from sample can changed adjust the last calculations