Measurement of Hemoglobin

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[Abstract] This protocol allows to measure the levels of intratumoral hemoglobin from human or rodent fresh samples but also frozen tumors. The advantage of this method is to use very few microliters of biological material for hemoglobin and the protocol is carried out quickly.

Materials and Reagents

1. Fresh or frozen tumor tissues
2. Extraction buffer (Life Technologies, catalog number: FNN0011)
3. Human hemoglobin (Sigma-Aldrich, catalog number: H7379)
4. Drabkin's reagent (Sigma-Aldrich, catalog number: D5941)
5. Brij 35 Solution 30% (Sigma-Aldrich, catalog number: B4184)
6. Antifoam Y-30 Emulsion (Sigma-Aldrich, catalog number: A5758)
7. BCA protein quantification kit (Interchim, catalog number: MP2920)
8. Extaction buffer

Equipment

1. Homogenizer such as Precellys (Ozyme BER1011S, France) or ultraturax
2. 96 wells plates (DUTSCHER SCIENTIFIC, catalog number: 047632)
3. Luminoskan (Thermo Fisher Scientific, catalog number: 5210470)
4. Centrifuges

Procedure

1. 20 mg of fresh or frozen tumor tissues were resuspended in 200 µl of extraction buffer, on ice. The extaction buffer contains protease inhibitors.

2. Tissues were mechanically ground using a homogenizer such as an ultraturax or a Precellys. With ultraturax an antifoam solution (Sigma-Aldrich - antifoam Y-30 Emulsion) is used.
3. The homogenate was centrifuged for 10 min at 6,000 rpm, at 4 °C.
4. The supernatant was recovered. 10 µl supernatant is used to determinate the protein concentration using a protein assay such as BCA. The sample can be stored at -80 °C.
5. Reconstitute a vial of Drabkin's reagent in 1 L of water, add 0.5 ml of Brij 35 solution 30%. This solution can be stored at room temperature for 6 months, protected from light.
6. In parallel, resuspend hemoglobin (concentration of stock solution is 10 mg/ml) in the extraction buffer.
7. Achieve an eight point standard curve using 2-fold serial dilutions of hemoglobin, in extraction buffer, and a high standard of 2,000 pg/µl is recommended. To obtain the concentration of 2,000 pg/µl, the working solution (10 mg/ml) must be diluted 5 times.
8. Add 10 µl of sample or standards per well in triplicate.
9. Add 100 µl of Drabkin’s reagent/Brij 35 solution 30%.
10. Incubate 15 min at room temperature. The reaction is stable a couple of hours.
11. Determine the optical density of each well, using a microplate reader set to 540 nm.
12. Average the triplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
13. Create a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a curve through the points on the graph.
14. Hemoglobin concentration of samples must be divided by the protein concentration determined by BCA to obtain a result in microgram per micro liter per microgram of protein.

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