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ELISA (enzyme-linked immunosorbent assay) to quantify SMN protein levels

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1. OBJECTIVE

ELISA is a method to quantify protein levels in a wide variety of samples such as cell cultures, or various tissues, including blood. In contrast to semiquantitative western blotting, ELISA circumvents the need to use housekeeping proteins to calculate target-protein amounts. The SMN ELISA Kit (Enzo Life Sciences; catalog # ADI-900-209, developed in collaboration with the SMA Foundation) allows for the quantification of human SMN protein levels in a variety of tissues including human peripheral mononuclear cells (PBMCs). In brief, samples are prepared and incubated in a SMN-antibody coated 96-well plate. Non-specific binding is eliminated by several washing-steps and followed by incubation with a 2nd anti-SMN-antibody. Again, samples are washed and then incubated with a HRP-conjugated 3rd antibody recognizing the 2nd anti-SMN-antibody. HRP-substrate is added and plates are read at 450nm after blocking the reaction by adding a stop solution. Absorbance at 450nm is proportional to the amount of bound SMN protein and allows calculation of initial SMN levels. For each assay, a standard curve is created by measuring absorbance of 7 standard dilutions containing concentrations between 3200 pg/ml and 50 pg/ml of recombinant SMN.

2. SCOPE AND APPLICABILITY

ELISA is a widely used method to quantify expression levels of a specific protein. The SMN ELISA is suitable for a variety of lysates from human tissues or tissues from animal models expressing human SMN genes. Users wishing to use the ELISA for the detection of homologous proteins of other species should first assess compatibility of the assay with their desired application. In SMA patients – although not the target tissue of the disease – peripheral blood is an accessible tissue. Peripheral Blood Mononuclear Cells (PBMC) can be repeatedly obtained. Comparison of different time points or a variety of conditions is possible since final results are absolute values allowing direct comparison of different measurements. In this SOP we describe, in addition to the general assay procedure for the SMN ELISA (please also refer to the SMN ELISA Kit manual for further information concerning reagents etc.), the isolation and preparation of PBMCs for quantification by the SMN ELISA.

3. CAUTIONS

Of note, the comparability between samples strongly relies on the accurate extraction, avoidance of possible contamination with non-target tissues and exact quantification of sample total protein.

Best results are obtained by whole blood samples collected into EDTA tubes and processed to PBMCs by Lymphoprep within 2 hours of collection to cell lysates, which can then be frozen. Freezing isolated PBMCs for subsequent lysis and SMN extraction typically results in decreased cell recovery and reduced SMN signals.

We recommend normalization of SMN amounts to total protein or DNA content of samples analyzed. In addition, performing cell count or measurement of tissue weight is also recommended.

For cell cultures or PBMCs, we recommend using a hemocytometer to count cells as well as trypan-blue to distinguish between dead and viable cells. Estimation of cell numbers solely based on the shape of cells will easily give false cell numbers. Alternatively accurate and rapid cell counts can be provided by automated cell counting systems, e.g. Countess Automated Cell Counter by Life Technologies or an equivalent instrument.

For PBMCs, Extraction Reagent 4 (ER4) is provided for cell lysis and the procedure is described below. For cell lysis/extraction of protein from cell culture and animal tissues, please refer to your standard protocols. Compatibility of reagents not provided with the SMN ELISA system should be established prior to use.

For animal tissues, e.g. varying degrees of contamination with surrounding tissue might impair comparability of individual samples. For blood, substantial variation in cell subpopulations in response to external factors such as infections needs to be considered.

To guarantee an exact quantification of SMN protein levels, it is essential that the level of protein be within the linear range of the standard curve. Higher or lower SMN protein concentrations than covered by the standard curve may lead to inadequate protein concentrations as there is no guarantee that these values would fall within the linear range of the curve. Samples falling outside the range of the standard curve range must be reanalyzed using an appropriate dilution.

4. MATERIALS

Chemicals:

- SMN-ELISA-Kit (Enzo Life Sciences, catalog # ADI-900-209)

- Deionized water
- Trypan Blue 0.4% (Sigma #T8154) or equivalent
- 1xPBS (phosphate buffered saline without Ca^{++} and $Mg^{++,}$ Gibco #10010-023 or equivalent)
- Lymphoprep (Accurate Chem and Scientific #1114545)
- Erythrocyte lysis buffer (Qiagen #79217) or equivalent
- Protease inhibitor cocktail (PIC, Sigma #P8340) or equivalent
- Phenylmethylsulphonyl fluoride (PMSF, Sigma #P7626) or equivalent
- Protein quantitation kit (Biorad DC protein assay, Thermoscientific protein analysis kit #23225 or equivalent)
- DNA quantitation kit (Cyquant Cell Proliferation assay #35007 or equivalent)

Equipment:

- Repeater pipets for dispensing 100µl
- Microplate shaker
- Adsorbent paper for blotting
- Centrifuges for blood tubes (Beckman CS-6R or equivalent)
- Microcentrifuges for clarifying lysates
- Microplate reader capable of reading at 450nm (e.g. Safire², Tecan or equivalent plate reader)
- Hemocytometer + cover slip for cell counts or automated cell counter
- For PBMC isolation
    - Vacutainer EDTA tubes (Becton Dickinson #367861) (2mL, 4mL or 8mL tubes filled to capacity with whole blood)

5. METHODS

(DESCRIPTON OF ASSAY PROCEDURE TAKEN IN PART FROM KIT´S MANUAL [SEE REFERENCE] – ADDITIONAL NEW INFORMATION PROVIDED IN NOTES IN RESPECTIVE SECTION BELOW).

NEW OPTIMIZED WHOLE BLOOD COLLECTION, PBMC ISOLATION, CELL LYSATE DENSITIES AND SMN PROTEIN STANDARD FORMULATIONS ARE DESCRIBED BELOW.

5.1. Human PBMC Lysate Preparation

5.1.1. Peripheral Blood Mononuclear Cell (PBMC) Collection

1. Collect blood samples using standard venipuncture into BD Vacutainer™ EDTA tubes (2mL, 4mL or 8mL tubes, Becton Dickinson #367861 or equivalent, filled completely). Invert tubes 8 to 10 times to mix anticoagulant additive with blood and store upright at **room temperature** until further processing. Blood samples **should be processed to PBMCs within two hours** of blood collection.

   Processing samples with delays of up to 24 hours produce PBMC yields and SMN signals that are relatively stable but care should be taken to ensure that the same processing delays are used with each collection.

   Note:

   Blood collection tubes have been changed to EDTA. Extended waiting periods of greater than 24 hours before centrifugation and/or further processing will lead to gradual and near complete loss of cell and protein recovery (for further information concerning tube performance refer to manufacturer's website).

2. Dilute blood samples 1:2 using room temperature PBS (without Ca^{++} and Mg^{++}) and the diluted blood layered onto 3 ml of Lymphoprep solution in a 15 ml polypropylene centrifuge tube. No more than 6.0 ml of diluted blood is layered onto a single 15 ml tube with 3.0 ml of Lymphoprep.

3. Centrifuge the layered sample at 1800 x G at 20°C for 20 minutes in a Beckman CS-6R centrifuge with the brake off. Following centrifugation harvest PBMCs from the interface by pipetting the buffy coat into a separate 15 ml centrifuge tube.

4. Add ice cold PBS to a final volume of 15 ml and mix by inversion. Following mixing, collect cells by centrifugation at 650 x G in a Beckman CS-6R centrifuge at 4°C for 10 minutes with the brake on low.

5. Remove supernatant by aspiration without disturbing the cell pellet and re-suspend cells in 5 ml of ice cold erythrocyte lysis buffer (EL) and incubate on ice for 5 minutes. Following incubation collect cells by centrifugation in a Beckman CS-6R centrifuge at 650 x G set at 4°C for 10 minutes with the brake on low.

6. Resuspend cells in 5mL PBS and remove a 50uL sample for counting using a hemocytometer (see section 5.1.2). Cell viability is determined using trypan blue dye exclusion.

7. Cells are centrifuged in a Beckman CS-6R centrifuge at 650 x G for 10 minutes at 4°C with the brake on low.

8. Lyse cells using Extraction Reagent 4 (ER4 supplied with the SMN ELISA kit) containing protease inhibitor cocktail **at a final cell density of 1 X 10^7 cells/mL**.

   Note: This lysis density will provide the best SMN signal. If cell counts are low, lysis and extraction can be done at a density of 10^6 cells/mL.

9. Clarify lysates by centrifugation at 14,000 rpm in a microcentrifuge. Lysates can be frozen at -80°C until the time of assay.

5.1.2. Cell counts with Hemocytometer

1. Transfer 50 µL of cell suspension to a solution containing 75 µL PBS and 125 µL trypan-blue. Vortex the trypan-blue cell solution.
2. With the cover slip in place, transfer a small amount of trypan blue-cell suspension to a chamber on the hemocytometer. Ensure that the entire area under the cover slip contains the staining solution before removing any excess staining solution from the edge of the cover slip.
3. Both chambers of the hemocytometer must contain staining solution before performing cell counts.
4. Place hemocytometer on the microscope and count the number of trypan-blue excluding (viable) cells in the 4 outer squares. If there are less than 10 cells or more than 100 cells per square, repeat the procedure adjusting to an appropriate dilution factor.
5. Calculate the cell concentration as follows:

Cell concentration per ml = Total cell count in 4 squares x 2500 x 5 (dilution factor)

Total cell count = Cell concentration per ml x 2.0 mL (cell suspension)

6. Centrifuge cell suspension for 10 minutes at 300 x G, 4°C. Aspirate supernatant without disturbing cell pellet.
7. Proceed to cell lysis immediately.

5.2 Sample normalization and handling

5.2.1 Evaluate total soluble protein or DNA content using a BCA protein assay or DNA quantification kit according to manufacturer instructions.

5.2.2 If cell lysates were frozen prior to assay, the frozen lysate samples should be brought slowly to 4°C (on ice) and, if residual precipitate is present, centrifuge to isolate residual cell debris.

5.2.3 Samples must be diluted at least 1:4 to remove matrix interference in the assay, the recommended dilution is 1:8.

5.3 Reagent preparation

5.3.1 Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

5.3.2 Addition of Inhibitors to Extraction Reagent 4

Add protease inhibitors cocktail (PIC) to ER4 prior to use. PIC contains 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and a mixture of protease inhibitors at 0.5 µl/mL ER4 from Sigma # P8340 (consisting of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin with broad specificity for the inhibition of serine, cysteine, aspartic proteases and aminopeptidase). **Do not freeze ER4 pre-mixed with protease inhibitors!!!**

5.3.3 Human SMN Standards

Standard assay buffer should contain the same 1:4 or 1:8 dilution of ER4 as the PBMC lysates. Henceforth the term ‘standard assay buffer’ will refer to assay buffer material containing ER4 for the purpose of formulating the human SMN standard curve.

- Allow the 160 ng/mL human SMN standard to thaw prior to use.
- Label seven disposable 12 x 75 mm polypropylene plastic tubes, or equivalent, #1 through #7. Standards can be prepared at room temperature and are stable up to 60 minutes. Pipet 490 µL standard assay buffer (delivered with the kit) into tube #1.
- Pipet 250 µL standard assay buffer into tubes #2 through #7. Remove 10 µL from the stock vial and add to tube #1, vortex thoroughly. Remove 250 µL from tube #1 and add to tube #2, vortex thoroughly. Continue this serial dilution for tubes #3 through #7.

5.4 Assay Procedure

Determine the number of wells to be used and put any remaining wellstrips with the desiccant back into the foil pouch and seal the bag. Store unused wellstrips at 4°C.

1. Pipet 100 µL of standard assay buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
3. Pipet 100 µL of the Samples into the appropriate wells. Use a minimum of duplicates per Sample to detect any pipetting/handling errors.
4. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
5. Empty the contents of the wells and wash by adding 300 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of yellow Antibody solution (delivered with the kit) into each well, except the Blank.
7. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
8. Wash as above (Step 5).
9. Add 100 µL of blue Conjugate solution to each well, except the Blank.
10. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
11. Wash as above (Step 5). Pipet 100 µL of Substrate Solution into each well.
12. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Pipet 100 µL Stop Solution to each well.

14. Zero the plate reader against the Blank wells, read the optical density at 450 nm. If the plate reader is not able to automatically subtract blank well values from each well, manually subtract the mean optical density of the Blank wells from all the readings.

6 EVALUATION AND INTERPRETATION OF RESULTS

Several options are available for the calculation of the concentration of SMN in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. Alternatively, the concentration of SMN can be calculated as follows (e.g. using standard statistic software such as Excel):

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample. Average Net OD = Average OD - Average Blank OD
2. Using linear graph paper, plot the Average Net OD for each standard versus SMN (human) concentration in each standard. Approximate a straight line through the points. The concentration of SMN in the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range need to be reanalyzed using a different dilution.

7 REFERENCES

1. SMN EIA Kit Protocol, Catalog # ADI-900-209, Revision 03/08/2010, Enzo Life Sciences (formerly Assay Designs)
2. www.bd.com