ELISA PRODUCT INFORMATION & MANUAL

SMN

NBP2-62175

Enzyme-linked Immunosorbent Assay for quantitative detection of Human, Mouse SMN.

For research use only.

Not for diagnostic or therapeutic procedures.

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Product Manual

Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs.

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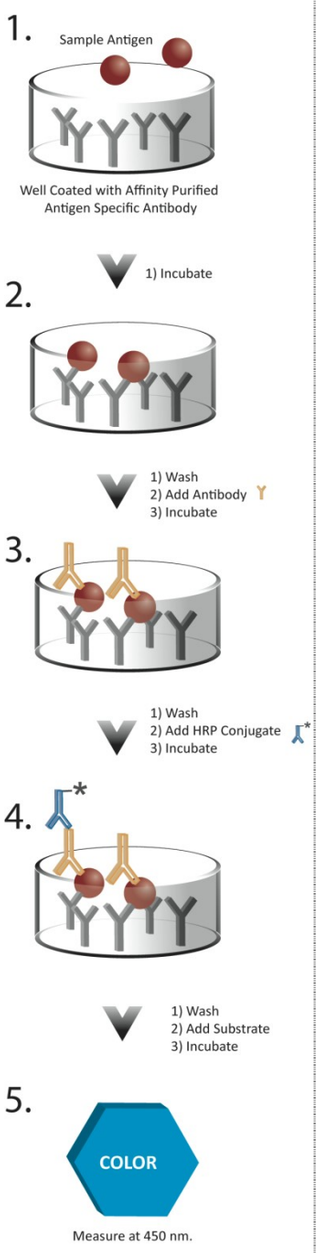


INTRODUCTION

The SMN ELISA kit is a complete kit for the quantitative determination of SMN in samples of human and mouse origin. The kit was developed in collaboration with the SMA Foundation (New York, NY). Please read the complete kit insert before performing this assay.

Survival Motor Neuron (SMN) is a ~38 kDa protein produced chiefly by the SMN1 gene, located on the telomeric portion of chromosome 5q[1-4]. A nearly identical centromeric copy of the gene (SMN2) also produces a small amount of full-length SMN protein, but due to a translationally silent C→T transition that results in alternative splicing of the pre-mRNA, most of the resulting SMN is truncated, causing reduced protein stability and lower overall SMN levels[5-6]. Deletion or mutation of the SMN1 gene results in a reduced level of full-length SMN protein and manifests as a range of neuromuscular phenotypes in humans as the disease spinal muscular atrophy (SMA). SMA is characterized by muscle weakness and atrophy, functional disability and is the most common lethal genetic disease of infants and toddlers. Approximately one in 35 adults is a carrier of the SMN1 mutation. The incidence of SMA is 1 in 6,000 to 1 in 10,000 live births[7].

SMN protein is present in the cell cytoplasm, and also in the nucleus where it is concentrated in “gem” structures associated with Cajal bodies[8-9]. SMN protein is a constituent of Gemin-containing complexes, and is thought to participate in many aspects of RNA metabolism. SMN complexes have been shown to mediate the assembly of uridine-rich small nuclear ribonucleoproteins (snRNPs), which in turn act as critical components of spliceosomes[10].

Protected by US Patent no. US. 6,080,577.

PRINCIPLE

1. Samples and standards are added to wells coated with a monoclonal antibody specific for SMN. The plate is then incubated.
2. The plate is washed, leaving only bound SMN on the plate. A yellow solution of rabbit polyclonal antibody to human SMN is then added. This binds the SMN captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of HRP conjugate is added to each well that binds to the rabbit polyclonal SMN antibody. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450nm. The amount of signal is directly proportional to the level of SMN in the sample.

MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the kit's expiration date.

1. **SMN Microtiter Plate, One Plate of 96 Wells,**
   A plate using break-apart strips coated with a monoclonal antibody specific to SMN.
2. **SMN Antibody, 10ml**
   A yellow solution of rabbit polyclonal antibody to human SMN.
3. **Assay Buffer 13, 100ml**
   Tris buffered saline containing proteins and detergents.

Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

4. **SMN Conjugate, 10ml**
   A blue solution of goat anti-rabbit IgG conjugated to horseradish peroxidase.

The standard should be handled with care due to the known and unknown effects of the antigen.

5. **Wash Buffer Concentrate, 100ml**
   Tris buffered saline containing detergents.
6. **SMN (human) Standard**
   Two vials each containing 3200pg of recombinant human SMN.
7. **TMB Substrate, 10ml**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.

Protect substrate from prolonged exposure to light.

8. **Stop Solution 2, 10ml**
   A 1N solution of hydrochloric acid in water. Keep tightly capped.

Stop solution is caustic. Keep tightly capped.

9. **Extraction Reagent 4, 100ml**

   Tris buffered saline containing salts, detergents, enzyme and glycerol.
10. **SMN Assay Layout Sheet, 1 each**
11. **Plate Sealer, 3 each**

Reagents require separate storage conditions.

STORAGE

All components of this kit, **except the Standards**, are stable at 4°C until the kit's expiration date. The Standards should be stored at or below -20°C upon receipt.

MATERIALS NEEDED BUT NOT SUPPLIED

1. Deionized or distilled water.
2. Precision pipets for volumes between 10µl and 1,000µl.
3. Disposable polypropylene test tubes for dilution of samples and standards.
4. Repeater pipets for dispensing 100µl.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A microplate shaker.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450nm.
10. Graph paper for plotting the standard curve or, preferably, an immunoassay software package capable of per-forming a 4 parameter logistic curve fit.
11. Hemocytometer for cell counts.
12. Cover slip for hemocytometer.
13. Trypan Blue 0.4%
14. Mechanical homogenizer or manual dounce homogenizer
15. Phosphate buffered saline.
16. Protease inhibitor cocktail (PIC)
17. Phenylmethlysulphonyl fluoride (PMSF)

SAMPLE HANDLING

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

Samples must be stored frozen at or below -20° to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.

The Novus Biologicals SMN ELISA Kit is compatible with human and mouse SMN samples. Prior to assay, frozen samples should be brought slowly to 4°C (on ice) and centrifuged, if necessary, to isolate residual cell debris.
Samples diluted sufficiently into the assay buffer can be read directly from a standard curve. A minimum 1:4 dilution is recommended for cell lysates and 1:8 dilution is recommended for tissue extracts. This is the minimum recommended dilution to remove matrix interference in the assay.

Dilutional Linearity

The minimum required dilution for several common samples was determined by serially diluting samples into the assay buffer and identifying the dilution at which linearity is observed.

Dilution	Human cell lysate	Mouse Brain Tissue	Mouse Muscle Tissue	Mouse Spinal Cord Tissue
Neat	---	---	---	---
1:2	---	>LOD	74%	65%
1:4	98%	>LOD	84%	82%
1:8	102%	>LOD	90%	88%
1:16	105%	>LOD	99%	89%
1:32	100%	94%	100%	97%
1:64	---	100%	<LOD	106%

Spike and Recovery

After diluting each sample matrix to its minimum required dilution, recombinant human SMN was spiked at high, medium, and low concentrations. The recovery of the standard in spiked samples was compared to the recovery of identical spikes in the assay buffer. The mean and range of percent recovery at the three concentrations are indicated below for each matrix.

Mean Spike & Recovery Results			
Sample Matrix (# of samples)	**Minimum Required dilution**	**Spike Concentration (pg/mL)**	**Avg %Recovery (Range)**
Human PBMC lysate (n=5)	1:4	1667	100% (88-116%)
		667	100% (88-116%)
		267	99% (79-134%)
Mouse brain extract (n=2)	≥1:8[a]	1250	84% (83-84%)
		250	86% (85-87%)
		50	104% (96-112%)
Mouse muscle extract (n=2)	≥1:8[b]	1250	79% (76-81%)
		250	88% (85-90%)
		50	125% (103-146%)
Mouse spinal cord extract (n=2)	≥1:8[c]	1250	68% (66-69%)
		250	68% (67-68%)
		50	44% (39-48%)

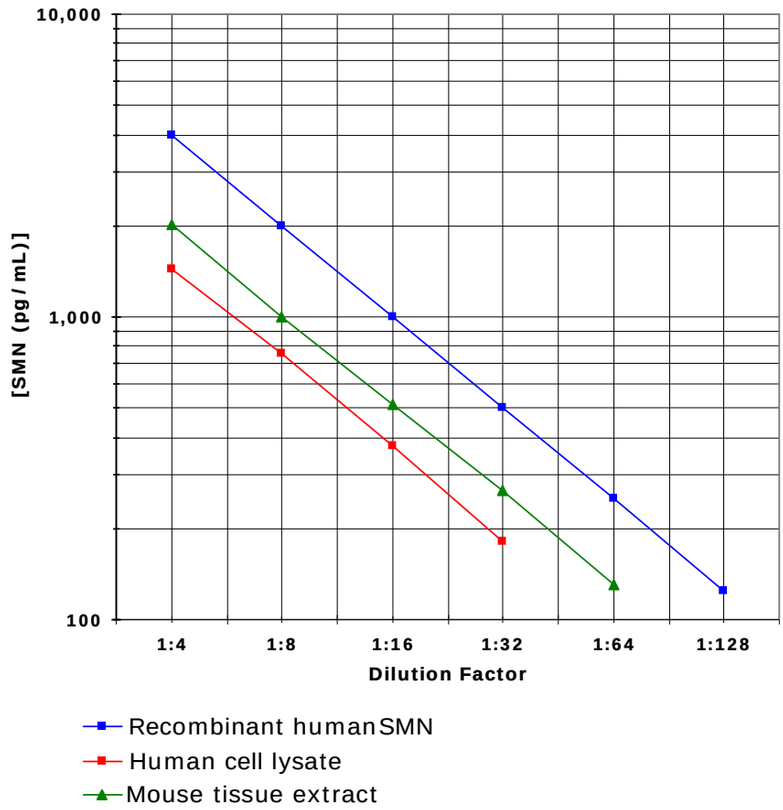
a – Dilute mouse brain tissue extract such that the final protein concentration in the assay sample is 25µg/mL with a minimum dilution of 1:8.

b – Dilute mouse muscle tissue extract such that the final protein concentration in the assay sample is 50µg/mL with a minimum dilution of 1:8.

c – Dilute mouse spinal cord tissue extract such that the final protein concentration in the assay sample is 100µg/mL with a minimum dilution of 1:8.

Parallelism

A parallelism experiment was carried out to determine if the recombinant human SMN standard accurately determines SMN concentrations in biological matrices. To assess parallelism, values for human PBMC lysate and mouse tissue extract was obtained from a standard curve using four parameter logistic curve fitting. The observed concentration was plotted against the dilution factor. Parallelism of the curves demonstrates that the antibody binding characteristics are similar enough to allow the accurate determination of analyte levels in diluted samples.



Human PBMC Lysate Preparation

Peripheral Blood Mononuclear Cell (PBMC) Collection

1. Collect blood samples using standard venipuncture into BD Vacutainer™ CPT™ tubes, Becton Dickinson #362760 or equivalent. Invert tubes 8 to 10 times to mix anticoagulant additive with blood. Blood samples should be centrifuged within two hours of blood collection.
2. Centrifuge tube/blood samples at room temperature (18–25°C) for 20 minutes at 1500 to 1800 RCF.
3. After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Immediately process the PBMCs, by aspirating approximately half of the plasma without disturbing the cell layer.

4. Collect the cell layer and transfer to a 15ml conical centrifuge tube with cap.
5. Add PBS to the PBMCs to bring the volume to 15ml. Cap tube and invert to mix cells.
6. Centrifuge tube for 15 minutes at 300 RCF, 4°C. Aspirate supernatant without disturbing the cell pellet.
7. Resuspend cell pellet in residual PBS by gently vortexing or tapping tube with index finger.
8. Add PBS to resuspended pellet to bring volume to 10ml. Cap tube and invert to mix cells.
9. Centrifuge tube for 15 minutes at 300 RCF, 4°C. Aspirate supernatant without disturbing the cell pellet.
10. Repeat washing steps 8 and 9 for a total of 3 washes.
11. Assay immediately or freeze down in freezing media and store in liquid nitrogen. We recommend using the Nalgene® “Mr. Frosty” Container (or equivalent) and the manufacturer’s instruction manual.

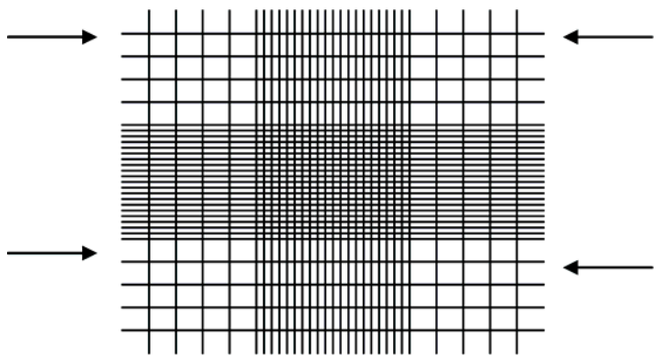
PBMC Thawing

1. Remove vials containing frozen cells from liquid nitrogen and place in 37°C water bath.
2. Remove vials from water bath when no ice crystals remain.
3. Transfer cell solution to 15ml conical centrifuge tube with cap.
4. Add PBS to the PBMCs to bring the volume to 15ml. Cap tube and invert to mix cells.
5. Centrifuge tube for 15 minutes at 300 RCF, 4°C. Aspirate supernatant without disturbing the cell pellet.
6. Resuspend cell pellet in 2ml PBS for performing cell counts.

Cell Counts with Hemocytometer

1. Transfer 50µl of cell suspension to a solution containing 75µl PBS and 125µl 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 milliliter = Total cell count in 4 squares x 2500 x 5 (dilution factor)

   Total cell count = Cell concentration per milliliter x 2.0ml (cell suspension)
6. Centrifuge cell suspension for 10 minutes at 300 RCF, 4°C. Aspirate supernatant without disturbing cell pellet.
7. Proceed to cell lysis immediately.

Cell Lysis

1. Resuspend cell pellet in Extraction Reagent 4, containing protease inhibitors. Add 1ml of extraction reagent per 10^8 cells. See Reagent Preparation Section for addition of protease inhibitors to Extraction Reagent 4.
2. Incubate cell suspension on ice for 30 minutes for complete lysis.
3. Transfer cell lysis to 1.5ml centrifuge tube. Centrifuge cell lysates for 10 minutes at 14,000 RCF, 4°C.
4. Clarified lysates may be assayed immediately, or aliquoted and stored at -70°C.
5. No degradation of SMN in cell lysate was observed after 2 freeze-thaw cycles.

Cell Lysate Sample Handling

1. 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. Samples diluted sufficiently into the assay buffer can be read directly from a standard curve. A minimum 1:4 dilution is recommended for cell lysates to remove matrix interference in the assay.

Mouse Tissue Homogenization

1. Prepare Extraction Reagent 4 with protease inhibitors. Recommended protease inhibitors are 0.5µl of PIC8340 per ml of reagent and PMSF to a final concentration of 1mM.
2. Transfer tissue sample to appropriate sized tube for homogenization with 1mL of prepared Extraction Reagent 4.
3. For mechanical homogenizer, disrupt the tissue with three pulses of 3-4 seconds each. For manual dounce homogenizer, complete a minimum of 5 passes of the pestle past the buffer/tissue volume, or until tissue appears completely homogenized. Keep samples on ice while completing all preparations.
4. Pellet out tissue/cellular debris via centrifugation at 14000g for 10 minutes at 4°C and transfer supernatant to a clean tube.
5. Measure the protein content of the supernatant using the biorad DC protein assay (with reagent S).
6. Prepare tissue homogenates for use in the SMN assay by diluting the extracted samples in assay buffer. Samples must be diluted at least 1:8. Dilute brain, muscle, and spinal cord tissue samples to final assay protein concentrations of 25µg/mL, 50µg/mL and 100µg/mL, respectively.

REAGENT PREPARATION

Bring all reagents to room temperature for at least 30 minutes prior to opening.

1. Wash Buffer

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

2. Addition of Inhibitors to Extraction Reagent 4

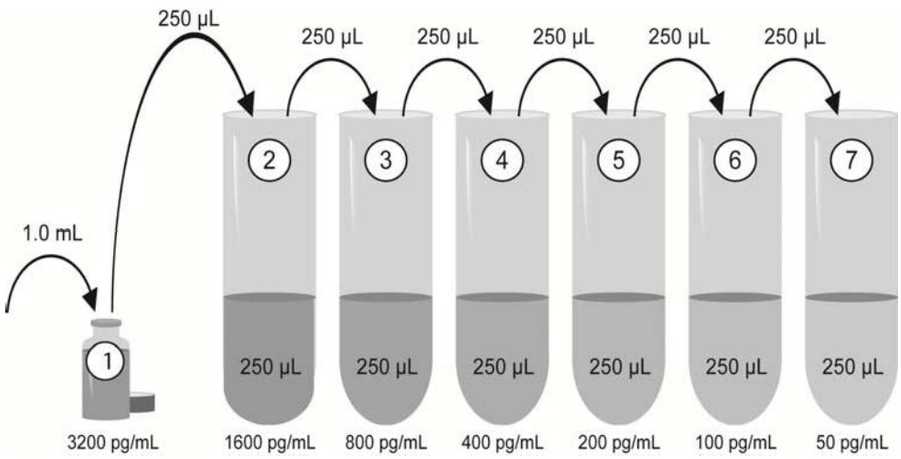
Add protease inhibitors to extraction reagent prior to use. Add 0.5µl of PIC per ml of extraction reagent and add PMSF to a final concentration of 1mM. **Do not store extraction reagent with protease inhibitors.**

Standards must be made up in plastic tubes.

3. Human SMN Standards

Allow the 3200pg human SMN standard to warm to room temperature. Reconstitute one vial of standard with 1mL of assay buffer for a 3200pg/mL stock vial. Vortex thoroughly, wait 5 minutes and vortex again prior to use. Label the vial standard#1.

Label six disposable 12 x 75 mm polypropylene plastic tubes, or equivalent, #2 through #7. Standards can be prepared at room temperature and are stable up to 60 minutes. . Pipet 250µl assay buffer into tubes #2 through #7.. Remove 250µl from standard #1 and add to tube #2. Vortex thoroughly. Continue this for tubes #3 through #7.



Standards can be used at room temperature and should be used within 60 minutes of preparation. The concentration of SMN in the tubes is labeled above.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to 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.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the standards and samples to the bottom of the wells.

Pipet the reagents to the sides of the wells to avoid possible contamination.

1. Pipet 100µl of assay buffer into the S0 (0pg/mL standard) wells.
2. Pipet 100µl of Standards #1 through #7 into the appropriate wells.
3. Pipet 100µl of the Samples into the appropriate wells.
4. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500rpm.
5. Empty the contents of the wells and wash by adding 300µl 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 100µl of yellow Antibody solution into each well, except the Blank.
7. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500rpm.
8. Wash as above (Step 5).
9. Add 100µl 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 ~500rpm.
11. Wash as above (Step 5). Pipet 100µl of Substrate Solution into each well.
12. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500rpm.
13. Pipet 100µl Stop Solution to each well.
14. Zero the plate reader against the Blank wells, read the optical density at 450nm. 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.

CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

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. If data reduction software is not readily available, the concentration of SMN can be calculated as follows:

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

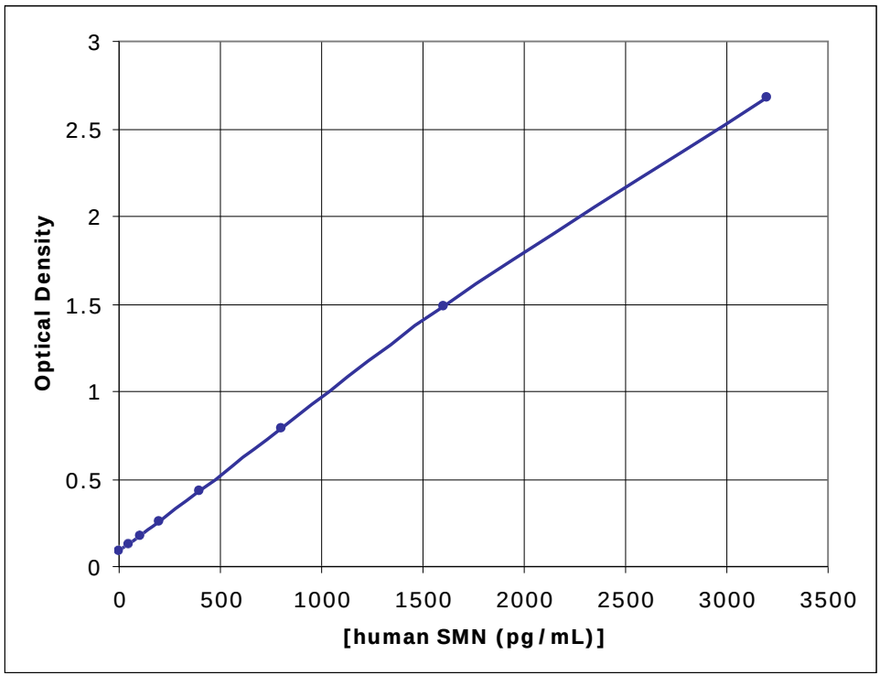
2. Plot the Average Net OD for each standard versus SMN (human) concentration in each standard. Approximate a straight line through the points. The concentration of SMN in the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	(pg/mL)
S0	0.088	0
S1	2.679	3,200
S2	1.482	1,600
S3	0.789	800
S4	0.434	400
S5	0.258	200
S6	0.170	100
S7	0.124	50



PERFORMANCE CHARACTERISTICS

Specificity

This kit detects SMN protein of both human and mouse origin. Other species have not been tested.

Sensitivity

The sensitivity of the assay, defined as the concentration of SMN measured at 2 standard deviations from the mean of 20 replicates of zero standard along the standard curve, was determined to be 50pg/mL.

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing SMN in a single assay.

pg/mL	%CV
928	0.8
322	1.0
122	3.2

Inter-assay precision was determined by measuring buffer controls (n=12) of varying SMN concentrations in multiple assays over several days.

pg/mL	%CV
983	7.1
378	8.9
134	11.4

REFERENCES

1. Sumner, C.J. (2007) J Child Neurol. **22**, 979-989.
2. Brzustowicz, L.M., et al. (1990) Nature **344**, 540-541.
3. Melki, J., et al. (1990) Lancet **336**, 271-273.
4. Lefebvre, S., et al. (1995) Cell **80**, 155-165.
5. Lorson, C., et al. (1998) Nat Genet **19**, 63-6.
6. Burnett, B.G., et al. (2009) Mol Cell Biol. **29**, 1107-1115.
7. Pearn, J. (1978) J Med Genet. **15**, 409-413.
8. Liu, Q., et al. (1997) Cell **90**, 1013-1021.
9. Cavalho T., et al. (1999) J Cell Bio **147**, 715-28.
10. Fischer, U., et al. (1997) Cell **90**, 1023-9.