# Technical Data Sheet

## Avanti Ceramide Synthase (CerS) Assay Kit

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|---|---|
| CerS1/S4 Assay Kit | Avanti Number 640011 |
| CerS2 Assay Kit | Avanti Number 640012 |
| CerS3 Assay Kit | Avanti Number 640013 |
| CerS5/S6 Assay Kit | Avanti Number 640014 |

**Background:**

Ceramide synthases (CerSs) acylate sphingoid long-chain bases to form (dihydro)ceramides[1]. Mammals contain six distinct CerSs, each of which uses a specific subset of fatty acyl-coenzyme As to produce various subspecies of (dihydro)ceramides[1]. CerSs have become of considerable interest due to the growing involvement of this enzyme family in cell regulation and the pathology of human disease[1]. The CerS Assay Kit provides a simple and high-throughput assay for measuring CerS activity in biological samples. This assay uses NBD sphinganine and a 18:0 (CerS1/4), 16:0 (CerS5/6), 24:1 (CerS2) or 26:0 (CerS3) coenzyme A as the substrates for the enzyme. Upon acylation the lipid product, NBD (dihydro)ceramide, is separated from the reaction mixture using SPE column chromatography in a convenient 96-well plate format, and the formation of the fluorometric product is monitored using a standard multiwell plate reader. The CerS Assay Kit offers the advantage of a short assay time, requires only small amounts of biological material, and eliminates the use of TLC as a separation technique, which has been well documented to result in the degradation of NBD sphinganine[1].

**Reagents Supplied:**

| | |
|---|---|
| Fatty acyl-CoA* | 0.3 mg |
| NBD Sphinganine | 100 µg |

*CerS1/CerS4 Assay Kit includes 18:0 fatty acyl-CoA
*CerS2 Assay Kit includes 24:1 fatty acyl-CoA
*CerS3 Assay Kit includes 26:0 fatty acyl-CoA
*CerS5/CerS6 Assay Kit includes 16:0 fatty acyl-CoA
Note: The kit is sufficient for ~275 reactions.
The kit should be stored at -20°C

**Reagents and equipment required but not supplied:**

- Fatty Acid Free Bovine Serum Albumin (BSA) (Sigma A6003)
- HEPES Buffer, pH 7.2 (Protocol for preparation found in the Reagent Preparation section below)
- Protease Inhibitor Cocktail (Sigma P8340)
- Ethanol
- Water (Fisher HPLC grade, W5-4)
- Methanol (Fisher HPLC grade, A452-4),
- Chloroform (Fisher HPLC grade, C606-4)
- Formic Acid (EMD, FX0440-5)
- Ammonium Acetate (Fluka, 73594)
- 96-well plate vacuum manifold and Sealing Mat (Phenomenex, AH0-8950)
- Strata® C18-E, 15 mg/well, 96- well plates (Phenomenex, 8E-S001-BGB)
- Nunc 96-well polypropylene plates, black (Nunc, 267342)
- Vacuum pump
- Fluorescence multiwell plate reader
- Assay tubes (The assay may be performed in any tube; however, we suggest the MicroAMP 8-tube strip, 0.2 mL or a 96-well PCR plate)
- Adjustable pipettes

**Reagent Preparation:**

### CerS *In Vitro* Assay

- HEPES Buffer, pH 7.2

| Reagent | Amount to Add (for 1L) | Final Concentration |
|---|---|---|
| HEPES | 4.77 g | 20 mM |
| KCl | 1.84 g | 25 mM |
| Sucrose | 85.6 g | 250 mM |
| $MgCl_2$ | 0.41 g | 2 mM |

Dissolve in ~ 800 mL of deionized water, adjust the pH to 7.2 using HCl or NaOH, add deionized water to 1L. HEPES buffer can be stored refrigerated for several weeks.

- 100 µM Fatty Acid Free Bovine Serum Albumin (BSA). Dissolve 33 mg of BSA in 5 mL of HEPES buffer. Store refrigerated.
- Fatty acyl-CoA (supplied as powder)
  Prepare a 5 mM stock solution in EtOH:Water (1:1)

| Fatty acyl-CoA | Dissolution Volume for 0.3 mg |
|---|---|
| 16:0 | 57 µL |
| 18:0 | 55 µL |
| 24:1 | 51 µL |

0.2 µL of this stock solution will be used per reaction. Store at -20°C.

- NBD Sphinganine (supplied as powder)
  Prepare a 1 mM stock solution in EtOH:Water (1:1)
  Dissolve 100 µg of NBD sphinganine in 208.5 µL of EtOH:Water (1:1).
  0.3 µL of this stock solution will be used per reaction. Store at -20°C.

Note: NBD Sphinganine may precipitate out of solution following storage at -20°C. If precipitates are visible, incubate at 37°C and/or sonicate briefly to facilitate re-dissolution.

**Lipid Separation using 96-Well Columns requires the following solvent solutions**

- Methanol containing 1% Formic Acid
- Water containing 1% Formic Acid
- NBD Sphinganine Elution Solvent
  30:14:6:1(Methanol:Water:Chloroform:Formic Acid) + 10 mM Ammonium Acetate
- NBD Ceramide Elution Solvent
  30:14:6:1 (Methanol:Chlorom:Water:Formic Acid) + 10 mM Ammonium Acetate

Note: The use of HPLC grade solvents is recommended for all solutions above.

**Procedure:**

## CerS *In Vitro* Assay

1. Harvest cells/tissues according to established protocols. Re-suspend the cell pellet in HEPES buffer containing protease inhibitors, and determine the protein concentration.

- Samples should be maintained on ice, and all procedures should be performed in the absence of lysis buffer.
- For example, HEK293 cells in a 10 cm tissue culture dish were harvested in PBS following 48 hrs of transfection. The pellet was re-suspended and mechanically homogenized in 400 µL of HEPES buffer containing protease inhibitors prior to protein concentration determination.

2. Set up the Sample Tubes according to the following scheme:

| Reagent | Amount (µL) |
|---|---|
| Cell Homogenate | X* |
| HEPES Buffer | 15.5-X |

* The amount of cell homogenate added is determined by the protein concentration.

- Different cell types and tissues vary in the expression of the CerS enzymes; therefore, the amount of protein required must be determined empirically. See suggested conditions below.
- Final volume for the Sample Tubes should not exceed 15.5 µL
- Two or more Sample Tubes containing 0 µL of protein should be prepared for assay blanks.
- Sample Tubes should be kept on ice.

Suggested Conditions:

| | Protein Amount (µg) |
|---|---|
| HEK293 cells transfected with CerS2, 3, or 4 | 30-70 |
| HEK293 cells transfected with CerS1, 5, or 6 | 1-10 |
| HEK293 cells (non-transfected) | 30-70 |
| Tissue | 50-150 |

Note: These HEK293 cells were transfected with human CerS1-6 and collected 48 hrs post transfection.

3. Set up the Master Reaction Mix in a separate tube according to the following scheme.

| Reagent | Amount (µL) |
|---|---|
| 100 µM BSA in HEPES Buffer | 4 |
| 1 mM NBD Sphinganine | 0.3 |
| 5 mM Coenzyme A | 0.2 |

- 4.5 µL of the Reaction Mix is required for each Sample Tube.
- Multiply the amount of each reagent by the number of samples tubes to create one Master Reaction Mix tube.
- Prepare a small excess of the Master Reaction Mix (i.e., 3-5 additional tubes) in order to ensure adequate volume.
- Vortex the Master Reaction Mix well.

4. Start the reaction by adding 4.5 µL of the Master Reaction Mix to each Sample Tube. Incubate at 37°C with gentle agitation. The use of a water bath is recommended in order to ensure an even distribution of heat.

- Different cell types and tissues vary in the expression of the CerS enzymes; therefore, the optimal reaction time must be determined empirically.

Suggested Conditions:

| | Reaction Time |
|---|---|
| HEK293 cells transfected with CerS2, 3, or 4 | 20-40 minutes |
| HEK293 cells transfected with CerS1, 5, or 6 | 5-10 minutes |
| HEK293 cells (non-transfected) | 20-40 minutes |
| Tissue | 20-40 minutes |

Note: These HEK293 cells were transfected with human CerS1-6 and collected 48 hrs post transfection.

5. Stop the reaction by adding 20 µL of methanol containing 1% formic acid.

## Lipid Separation using 96-well SPE Column Plate

1. Set up the 96-well vacuum manifold and the 96-well SPE column plate according to manufacturer's instructions.
- A waste collection plate or other waste collection vessel can be used under the 96-well SPE column plate during Steps 2-7 , as these priming, washing, and elution steps do not remove the analyte of interest, NBD Ceramide, from the column.
- For best results, the vacuum gauge on the manifold should read in the range of 5-7 inHg throughout the separation procedures.

2. Prime the necessary number of columns (one per Sample Tube plus one additional to be used as a fluorescence blank) by washing with 150 µL of methanol containing 1% formic acid followed by 150 µL of water containing 1% formic acid.
- Add solutions to the center of the column well in order to ensure that the wash solution does not get stuck on the column wall.
- Place the sealing mat over the 96-well SPE column plate, and allow each wash solution to elute under vacuum for ~ 30 sec in order to ensure complete removal from the column.
- Break the vacuum and remove the sealing mat.

3. Add 110 µL of water containing 1% formic acid to each Sample Tube. Mix up and down 5 times, and add the contents of the Sample Tube to the center of the column well.
- Add 150 µL of water to a separate column well to be used as the fluorescence blank.
- Place the sealing mat over the 96-well SPE column plate, and allow each sample solution to move through the column well under vacuum for ~ 30 sec.
- Break the vacuum and remove the sealing mat.

4. Add 150 µL of water containing 1% formic acid to the center of each column well (both the Sample column well(s) and the Blank column well).
- Place the sealing mat over the 96-well SPE column plate, and allow the wash solution to move through the column well under vacuum for ~ 30 sec. This wash is intended to facilitate to removal of salts and other water soluble components found in the Sample Tubes allowing only the lipid species to remain associated with the column.
- Break the vacuum and remove the sealing mat.

5. Repeat Step 4.
- Place the sealing mat over the 96-well SPE column plate, and allow the wash solution to move through the column well under vacuum for ~ 30 sec.
- Following the second 30 sec wash, allow the wash solution to move through the column well under vacuum for an additional ~ 2 min. The additional time is designed to ensure that all of the wash solution is removed from the column.
- Break the vacuum and remove the sealing mat.

6. Add 150 µL of NBD Sphinganine Elution Solvent to the center of each column well (both the Sample column well(s) and the Blank column well).
- Place the sealing mat over the 96-well SPE column plate, and allow the elution solvent to move through the column well under vacuum for ~ 30 sec.
- Break the vacuum and remove the sealing mat.

7. Repeat Step 6 twice.
- Place the sealing mat over the 96-well SPE column plate, and allow the elution solvent to move through the column well under vacuum for ~ 30 sec.
- Following the third 30 sec wash, allow the elution solvent to move through the column well under vacuum for an additional ~ 2 min. The additional time is designed to ensure the complete elution of NBD Sphinganine from the column.
- Break the vacuum and remove the sealing mat.

8. Remove the waste collection plate or other waste collection vessel from beneath the 96-well column plate. Place a 96-well polypropylene plate (black) under the 96-well SPE column plate.
- In order to minimize the distance between the bottom of the column wells and the plate, stack 1-2 additional 96-well polypropylene plates beneath the plate used for collection. The decreased distance between the column well and the collection plate is intended to minimize splashing during NBD Ceramide elution and prevent contamination between neighboring wells.

9. Add 150 µL of NBD Ceramide Elution Solvent to the center of each column well (both the Sample column well(s) and the Blank column well).
- Place the sealing mat over the 96-well SPE column plate, and allow the elution solvent to move through the column well under vacuum for ~ 30 sec.
- Break the vacuum and remove the sealing mat.

10. Repeat Step 9.
- Place the sealing mat over the 96-well SPE column plate, and allow the wash solution to move through the column well under vacuum for ~ 30 sec.
- Break the vacuum and remove the sealing mat.

11. Add 50 $\mu$L of NBD Ceramide Elution Solvent to the center of each column well (both the Sample column well(s) and the Blank column well).

- Place the sealing mat over the 96-well SPE column plate, and allow the elution solvent to move through the column well under vacuum for ~ 30 sec.
- Allow the elution solvent to move through the column well under vacuum for an additional ~ 2 min. The additional time is designed to ensure the complete elution of the NBD Ceramide from the column.
- Break the vacuum and remove the sealing mat.

12. Remove the 96-well SPE column plate from the vacuum manifold. Cover the 96-well polypropylene plate (black), and immediately measure the fluorescence intensity (NBD $\lambda$ex = 465/ $\lambda$em = 535 nm) using a multiwell plate reader.

- For a more quantitative analysis, a standard curve may be prepared using the following scheme.

| Vial | Volume of Diluent ($\mu$L, EtOH) | Volume of NBD-Sphinganine ($\mu$L) | Con. (pmol/ $\mu$L) | Amount of NBD Sphinganine in well (pmol) |
|---|---|---|---|---|
| A | 38 | 2 of NBD Sa 1 mM stock | 50 | 250 |
| B | 30 | 20 from vial A | 20 | 100 |
| C | 20 | 20 from vial B | 10 | 50 |
| D | 40 | 10 from vial C | 2 | 10 |
| E | 20 | 20 from vial D | 1 | 5 |
| F | 15 | 0 | 0 | 0 |

- To prepare wells for analysis of the standard curve, add 350 $\mu$L of NBD Ceramide Elution Solvent to 12 wells (duplicate analysis of standards) of a 96-well polypropylene plate (black).
- Add 5 $\mu$L of each standard to its respective well.
- Measure fluorescence intensity (NBD $\lambda$ex = 465/ $\lambda$em = 535 nm) using a multiwell plate reader in the manner in which the samples eluted from the 96-well SPE column plate were measured.

**Reference:**

[1]Tidhar, R., K. Sims, E. Rosenfeld-Gur, W. Shaw, and A.H. Futerman. (2015). A rapid ceramide synthase activity using NBD-sphinganine and solid phase extraction. *J Lipid Res* 56:193-9.

**Tips and Troubleshooting:**

Dissolution of Lipid Reagents, NBD Sphinganine and Coenzyme A:

The complete dissolution of the lipid reagents is imperative for experimental reproducibility. Water sonication and slight heating (~40°C) may be necessary to facilitate complete dissolution. Following dissolution of the lipid reagents in the provided ampoule, it is recommended that the stock solutions be transferred to a small glass serum or screw cap vial (ideally 1 mL or less). Minimizing the empty volume/head space of the vial will help to maintain a constant concentration of the lipid species; however, small alterations in the stock concentration may occur over time as the vial is opened and closed due to the volatility of the organic solvent used to prepare the stock solutions.

Following storage at -20°C, allow the lipid reagents to reach room temperature prior to performing the assay. It is not uncommon for the lipid reagents to precipitate out of solution following storage at -20°C. Warming to room temperature, a short sonication, and/or slight heat should facilitate to redissolution of the lipid reagents after storage at -20°C.

Performing the CerS *In Vitro* Assay:

It is recommended that duplicate or triplicate analysis be performed on all samples during the assay. Each column is single use.

Performing the Lipid Separation using the 96-well SPE Column Plate:

A steady, consistent vacuum is crucial for the success of the kit. Adjusting the vacuum pressure settings on the pump and/or manifold is the primary way to begin troubleshooting incomplete elution from the column wells. It may be useful to secure the vacuum pump tubing to the manifold using a strong tape in order to ensure a good seal and enable a strong vacuum. The vacuum may be controlled on the vacuum manifold by manipulating the gold knob on the left and the silver valve to the right.

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