NSK-A-TS and NSK-B-TS

Application Note and Instructions for Use

Tandem Mass Spectrometer (MS/MS) Tuning Standards for Analysis of Amino Acids, Free Carnitine and Acylcarnitines

Introduction

Tandem Mass Spectrometer (MS/MS) Tuning Standards, NSK-A-TS and NSK-B-TS, have been developed to complement quality assurance and quality control (QA/QC) procedures in the laboratory and specifically address the need for amino acid and acylcarnitine tuning solutions as noted in Clinical and Laboratory Standards Institute's Newborn Screening by Tandem Mass Spectrometry: Approved Guide, NBS04-A, Vol. 30, No. 16 (7.1.1 Optimization Solution and 7.1.2 Check Solution).

Use MS/MS Tuning Standards to:

- Ensure MS/MS instrument is operating at peak sensitivity for analysis of amino acids and acylcarnitines prior to analysis.
- Monitor instrument sensitivity from analysis of the first dried blood spot (DBS) to the last, whether samples are from one or several microtiter plates, during and between analysis runs.
- Quickly locate the source of sensitivity loss during an analytical run or between batch analyses.
- Compare performance of multiple instruments within a laboratory or across many laboratories.
- Evaluate performance before and after instrument maintenance.
- Assess MS/MS performance in analysis of amino acid (AA) and acylcarnitines (AC) independent of DBS samples and their preparation.

Tuning Standards Description

Two sets of tuning standards (NSK-A-TS and NSK-B-TS) each contain a subset of the same standards used in a comprehensive MS/MS assay of amino acids (NSK-A) and acylcarnitines (NSK-B). Each set is provided in dry form in separate conical vials labeled **Tuning Standard for Labeled Amino Acids (NSK-A-TS, Tuning Set A)** and **Tuning Standard for Labeled Carnitine Standard Set B (NSK-B-TS, Tuning Set B)**. Components for each set are shown below. Tuning Set A (NSK-A-TS) contains stable isotope-labeled alanine (Ala-D_4), methionine (Met-D_3), phenylalanine (Phe-$^{13}C_6$), glutamic acid (Glu-D_3) and citrulline (Cit-D_2), while Tuning Set B (NSK-B-TS) contains stable isotope-labeled free carnitine (CN-D_9), propionylcarnitine (C3-D_3), octanoylcarnitine (C8-D_3), and palmitoylcarnitine (C16-D_3). After reconstitution in mobile phase, whether as free acids or derivatized as butyl esters, the tuning standards are stable in solution for up to 30 days when stored at 4˚C. The prepared solutions are ready for use *immediately*, whether for tuning the instrument as part of regular maintenance, for troubleshooting MS/MS instrument problems or for a quick daily check before each batch run (or as often as a protocol may require). These tuning standards are concentrated solutions and do not replace the stable isotope internal standards used in analysis of each DBS. As with any concentrated standard solution, blank samples or fresh mobile phase should be used prior to analysis of DBS samples.

Tuning Standard Components:

NSK-A-TS	
Amino Acid	Conc. (µM)*
L-Alanine (2,3,3,3-D_4)	25
L-Phenylalanine (ring-$^{13}C_6$)	25
L-Citrulline (5,5-D_2)	25
DL-Glutamic acid (2,4,4-D_3)	25
L-Methionine (methyl-D_3)	25

NSK-B-TS	
Carnitine	Conc. (µM)*
L-Carnitine-D_9 (free carnitine CN)	7.6
O-Propionyl-L-carnitine-D_3 (C3)	0.38
O-Octanoyl-L-carnitine-D_3 (C8)	0.38
O-Palmitoyl-L-carnitine-D_3 (C16)	0.76

**Concentration shown when reconstituted in 1 mL of solvent.*

Preparation of Tuning Standards

Procedure FA – Free Acid (nonderivatized) Tuning Standards:

1. Add 2 mL of the mobile phase used in your method to each tuning standard vial (Tuning Set A and Tuning Set B).
2. Vortex and shake for 30 minutes prior to use.
3. Store reconstituted tuning standards at 4°C.

(continued)

Procedure BE – Butyl Esterified Tuning Standards:

1. Add 1 mL of methanol to each tuning standard vial (Tuning Set A and Tuning Set B).
2. Vortex and shake for 15 minutes.

Using separate microtiter plates, prepare each tuning standard as follows:

1. Pipette 50 µL aliquots into a microtiter plate (20 wells, 10 x 2).
2. Add another 1 mL aliquot of methanol to each original tuning standard vial, vortex and shake for 15 minutes.
3. Pipette 50 uL aliquots into the same 20 wells, 10 x 2 for a total of 100 µL per well for 20 wells.
4. Dry under a gentle stream of nitrogen using a microtiter plate dryer.
5. Derivatize these 20 wells using the standard protocol in your lab for butyl esterification. This procedure was developed using 50 µL of 3N HCl in *n*-butanol (Regis Technologies, Inc.) per well and incubated for 15 minutes at 65ºC.
6. After this incubation procedure, dry the contents of each well under a gentle stream of nitrogen using a microtiter plate dryer.
7. Reconstitute each well with 100 µL of mobile phase used in your current method.
8. Combine the contents of each well into a new vial (or the original standard vial if washed appropriately) to produce a final volume of approximately 2 mL of butyl esterified standards.
9. Store reconstituted tuning standards at 4ºC.

Instrument Optimization

The reconstituted tuning standards can be used for optimization of MS/MS instruments. The general principles described below can be applied to any instrument.

General Principles:

Syringe pump setup

- A syringe pump that can deliver a constant flow of 10 µL per minute of either Tuning Set A or B is required for periodic optimization of the instrument or for major troubleshooting of the MS/MS instrument operation.
- Syringes are typically 1 mL in size and are loaded with 300-600 µL of tuning standard depending on the length of time the tuning process will be performed.
- At 10 µL per minute, the tuning process will last approximately 30-60 minutes.
- The syringe should be cleaned with methanol followed by mobile phase.
- A 50 µL volume of tuning standard should be drawn into the syringe and discarded to ensure an undiluted sample.
- After use, the syringe should be rinsed in methanol.

Separate tubing for concentrated solution

- Connections from the syringe pump syringe to the capillary inlet of the ion source should be made with PEEK tubing or deactivated fused silica.
- Generally, a short length of Teflon is connected to a union such that one end can slip over the needle while forming a tight seal. The other end of the union is connected to the PEEK or capillary tubing using the appropriate fittings.
- The tubing that flows from the liquid pump system is disconnected and not used in order to prevent contamination with high concentrations of tuning standards.
- Connect the special tubing and syringe as described above for infusion and optimization only.

General Suggestions:

- All suggested procedures can be modified to suit the requirements of your laboratory and instrument protocols. These procedures do not replace the instrument optimization of the engineers, and are not used for instrument calibration.
- It is recommended that the tuning procedure be performed after a regular cleaning and maintenance by an engineer to compare source and instrument parameters, as well as peak shape and peak sensitivity.
- It is also strongly recommended that amino acid Tuning Set A (NSK-A-TS) solution be used first as it contains the highest molar concentrations of analytes and thus will have the highest potential ion counts.

Tuning for Amino Acids:

- The target of tuning is to optimize the sensitivity of the amino acid D_3-methionine as this is the most challenging metabolite for the analysis of amino acids.
- Optimized parameters are applicable to all other amino acids except the basic amino acids, for which D_2-citrulline is used.
- Changing source parameters with m/z value for full scan neutral loss acquisition of amino acids is not necessary.

Tuning for Carnitines:

- Acylcarnitines are optimized using short-, medium- and long-chain acylcarnitines (C3, C8 and C16), as well as free carnitine (L-carnitine, CN).
- The ion source voltages at the orifice or cone (entrance to the MS) and the collision energy can vary greatly between short- and long-chain acylcarnitines. Therefore, these parameters should be ramped (increased) from low to high m/z values for full scan analysis based on the values found during tuning using the tuning standards.
- Free carnitine is generally acquired as a selected ion scan, and its optimum values are determined separately. In fact, for any metabolite that is acquired selectively as an SRM (also known as MRM) instrument parameters are assigned specifically for each m/z value, i.e. basic amino acids such as citrulline are often acquired using an SRM.

Quick Tuning Check

The most common use of the tuning standards will be use as an instrument quality-control sample. How it is used will be based on your laboratory's QA/QC protocol. It is designed to be used at the beginning of a multiple-plate run. The goal is to check for sufficient sensitivity (generally at its maximum) prior to running 200-400 samples (2-4 plates) in succession. It may also be useful as a quick check of the instrument if there is a perceived loss of sensitivity, as well as after a cleaning to check if that sensitivity is restored. The ideal aspect of this tuning standard is that it is ready to be used (after being prepared approximately once a month) and is already derivatized (if butyl esters).

The use of this standard has two helpful features.

- First, it provides confidence that the instrument is performing above specifications so that a run of 200-400 samples will likely remain above these specifications.
- Second, if there are problems during the run, such as a loss of sensitivity, the tuning check allows a quick analysis to determine whether the problem is related to the sample preparation of a batch of samples (a plate) or to the instrument or injector. If the tuning check shows poor sensitivity then the problem is the instrument and or injector (flow).
  - Flow can be checked by visual inspection and pressure examination.
  - If further information is needed, the instrument can be isolated from the injector by *infusing* the tuning solution.
  - All of this can be achieved in 30 minutes, saving time and providing valuable information that can be used by laboratorians to further troubleshoot or, alternatively, provided to an engineer.