



Certificate of Analysis

Certified Reference Material

ALNP-1

Anionic Lipid Nanoparticles Certified Reference Material

The ALNP-1 certified reference material (CRM) is a stable and homogeneous dispersion of anionic lipid nanoparticles of known provenance intended for methods development, inter-laboratory comparisons, verification of instrument performance, quality control, and general laboratory use to increase the confidence of size measurements of lipid-based as well as other soft nanoparticle formulations by dynamic light scattering. A single ALNP-1 vial contains minimum 2 mg of anionic lipid nanoparticles dispersed in 1 mL of 0.5x Dulbecco phosphate buffered saline (PBS) 15% sucrose.

Certified values

The sphere-equivalent intensity-weighted harmonic-mean hydrodynamic diameter commonly known as the Z-average diameter, \bar{x}_{DLS} , and the corresponding expanded uncertainty, U_{DLS} , values are presented below as the certified values. The certified values are those for which National Research Council Canada (NRC) has the highest confidence and that all known and suspected sources of bias have been taken into account and are reflected in the stated expanded uncertainties. The certification measurements were conducted by two analysts each using a different Zetasizer Nano ZS (Malvern) dynamic light scattering nanoparticle size analyzer.

Table 1: Certified sphere-equivalent intensity-weighted harmonic-mean hydrodynamic diameter (Z-average diameter), \bar{x}_{DLS} , and expanded uncertainty values.

\bar{x}_{DLS}	123.2 nm
U_{DLS}^a	1.5 nm

^a The 95 % confidence level expanded uncertainty includes both Type A and Type B contributions estimated according to the JCGM 100:2008 Guide.¹ U_{DLS} is intended to account for every significant contribution to the uncertainty of the measurement.

The certified values are traceable to SI unit of length expressed in nanometers. The combined uncertainty value of the hydrodynamic diameter is 0.75 nm and the effective degrees of freedom is 218.

The hydrodynamic diameter (Z-average) is a method defined measurand, as described in the ISO 22412 2017 international standard.² Therefore, this certification is only valid for comparison with measurements conducted using a sample thermally equilibrated at 25 °C and the dispersion medium viscosity and

¹ JCGM 100:2008; Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (JCGM) (2008).

² International Standard ISO 22412 2017 Particle size analysis — Dynamic light scattering (DLS).

refractive index values set at 0.911 mPa·s and 1.334, respectively. The viscosity and refractive index values were estimated for the purpose of this certification using the ISO/TR 3666:1998 international standard³ and literature data,^{4,5} and should be considered as model parameters. For measurements conducted not at 25 °C, the dispersion medium viscosity value must be adjusted accordingly.

Certification validity

The certification of the ALNP-1 \bar{x}_{DLS} (Z-average) value is valid, within the stated expanded uncertainty range, until 31 March 2025, provided the storage, handling, and use instructions specified in this certificate are followed.

Maintenance of certification

NRC will monitor ALNP-1 CRM and if any technical changes affecting the validity of the certification occur before the expiration date, NRC will promptly notify the purchaser.

Shipping and storage

ALNP-1 is long-term stored frozen at (–80 to –70) °C temperatures. It is shipped to a purchaser thermally insulated on dry ice. NRC normally ships ALNP-1 in thermal insulation boxes that can maintain temperatures below –70 °C for a minimum of three (3) days; should a longer transit time be anticipated to the final destination, the purchaser must notify NRC and arrange for an alternative shipping method. Upon receiving, the purchaser must immediately unpack the vials, examine the shipping box to ensure that a sufficient amount of dry ice remained to maintain the temperature at or below –70 °C, and transfer the vials to a –70 °C freezer for storage.

Handling and use

Prior to a measurement, an ALNP-1 vial should be thawed and thermally equilibrated at room temperatures (20 °C to 25 °C), preferably positioned horizontally on an open space flat surface. Alternatively, for accelerated thawing and thermal equilibration, an ALNP-1 vial can be partially immersed in room temperature water. Before opening, the vial should be repeatedly inverted, ten or more times, to ensure the uniformity of the nanoparticle dispersion. Any dispersion that remains in the bottom conical part of the vial should be removed by flicking the inverted vial with a finger or shaking vertically. To prevent contamination, the vial must remain capped at all times except when drawing test aliquots.

For measurements, disperse 10 µL aliquot of ALNP-1 formulation in 500 µL of 1x Dulbecco PBS, thoroughly mix the dispersion by repeatedly pipetting in and out making sure that no air bubbles are injected, and insert the sample cell into a sample compartment ensuring thermal equilibration at the measurement temperature. Any air bubbles noted in the cell should be removed prior to the insertion by tapping the cell against a hard surface or flicking it with a finger. The homogeneity of ALNP-1 has not been evaluated for aliquots smaller than 10 µL. Therefore, the certified values might not be valid for test quantities of less than 10 µL.

³ International Standard ISO/TR 3666:1998 Viscosity of Water.

⁴ Y.-S. Liu, Y.-F. Hu, Q.-C. Hao, X.-M. Zhang, Z.-C. Liu, and J.-G. Li, *J. Chem. Eng. Data* **54**(3), 739-744, (2009), Viscosity and Density of the System NaCl + LaCl₃ + H₂O and Its Binary Subsystems at Different Temperatures.

⁵ J. F. Swindells, C. F. Snyder, R. C. Hardy, and P. E. Golden, Technical Report No. 463432 (Supplement to National Bureau Standards Circular 440) (1958), Viscosities of sucrose solutions at various temperatures: Tables of recalculated values.

The certified Z-average value is only valid for the contents of the vial used the same day as thawed and first opened, within 6 hrs of the removal from $-70\text{ }^{\circ}\text{C}$ storage.

Note: Care should be taken to thoroughly disperse the ALNP-1 aliquot diluted for measurements. Occasionally, a significant number of dimers and small agglomerates may remain in the cell following sample preparation, resulting in an apparent Z-average value exceeding the certified value by a few nanometers correlated with an apparent polydispersity index (Pdl) value increase as compared with the reference value shown below. Should it be suspected to take place, ALNP-1 dispersion should be enhanced by additional pipetting and the measurement repeated. In most cases, additional sample pipetting is sufficient to disperse the residual agglomerates. If the repeated measurements yield excessively large apparent Z-average values, the sample may have been contaminated; the sample should be discarded and a new one prepared.

Reference values

Reference values are those for which not all uncertainty contributions may have been fully accounted for, metrological traceability has not been provided, or other factors prevented NRC to establish the highest confidence. The values provided below are suitable for method development, inter-laboratory comparisons, quality control, and other general laboratory use. The reference values are provided for information only and therefore, NRC cannot guarantee future support of their maintenance.

Polydispersity index. The best estimates of the ALNP-1 Pdl and the associated expanded uncertainty are 0.041 and 0.008, respectively. For formulations stored at $4\text{ }^{\circ}\text{C}$ for extended usage, Pdl will decrease with time to approximately 0.035 after two weeks and remain unchanged for several weeks thereafter.

Hydrodynamic diameter of ALNP-1 stored at $4\text{ }^{\circ}\text{C}$ for extended period of time. Once thawed ALNP-1 remains stable for up to several months when stored at $4\text{ }^{\circ}\text{C}$ with only a small increase of hydrodynamic diameter value proportional to the cube root of the $4\text{ }^{\circ}\text{C}$ storage time. For ALNP-1 immediately transferred to $4\text{ }^{\circ}\text{C}$ storage following the first use and tested at later times, the hydrodynamic diameter value provided in this certificate should be corrected as follows

$$\bar{x}_{DLS}(t) = \bar{x}_{DLS} + \alpha \sqrt[3]{t}, \quad \text{Eq. 1}$$

where t is the time elapsed since the vial removal from $-70\text{ }^{\circ}\text{C}$ storage and α is the size growth coefficient, average value of which is $1.0\text{ nm}\cdot\text{day}^{-1/3}$. It is recommended that the extended use of $4\text{ }^{\circ}\text{C}$ stored ALNP-1 is limited to 14 days. Since the size growth coefficient values vary between vials and may also depend on the number of vial openings generally ranging $(0.8 - 1.2)\text{ nm}\cdot\text{day}^{-1/3}$, further use of $4\text{ }^{\circ}\text{C}$ stored ALNP-1 beyond 14 days falls under user's responsibility.

Hydrodynamic diameter of ALNP-1 repeatedly thawed and refrozen at $-70\text{ }^{\circ}\text{C}$. Vials can be refrozen following the first use and thawed for measurements at later times. In such cases, the hydrodynamic diameter value provided in this certificate should be corrected using Eq. 1 with t being the total time vial contents spent in an unfrozen state between repeated thawing and freezing. It is recommended that the freeze/thaw cycle is not repeated more than 5 times and the total time t for all cycles does not exceed 24 hrs. ALNP-1 usage has not been tested beyond these limits and therefore falls under user's responsibility.

Quality Management System (ISO 17034, ISO/IEC 17025)

This material was produced in compliance with the NRC Metrology Quality Management System, which conforms to the requirements of ISO 17034 and ISO/IEC 17025.

The Metrology Quality Management System supporting the NRC Calibration and Measurement Capabilities, as listed in the *Bureau international des poids et mesures* (BIPM) Key Comparison Database (<http://kcdb.bipm.org/>), has been reviewed and approved under the authority of the Inter-American Metrology System (SIM) and found to be in compliance with the expectations of the *Comité international des poids et mesures* (CIPM) Mutual Recognition Arrangement. The SIM approval is available upon request.

This document should be cited as

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This Certificate is only valid if the corresponding material was obtained directly from the NRC or an Authorized Reseller.

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