CERES NANO

Powering the Future of Diagnostics

Nanotrap® Sample Processing Protocol with On-Particle Digestion for Mass Spectrometry

For rapid and reliable fractionation and concentration of target analytes from complex biological matrices.

User Manual

Nanotrap® White Protein Particles
Nanotrap® Blue Protein Particles
Nanotrap® Azure Protein Particles
Product SKUs 33200, 33210, 33220, 33230

Protocol: APP-UM-002, Rev:1; Release Date: July 29, 2019

Nanotrap® Particle Sample Processing

Mass Spectrometry Applications

Nanotrap® White Protein Particles, Nanotrap® Blue Protein Particles, Nanotrap® Azure Protein Particles (Product SKUs 33200, 33210, 33220, 33230)

This protocol is specifically designed for use with serum, plasma, saliva, and urine upstream of mass spectrometry applications. For recommendations and protocols utilizing alternative sample types and analysis methods, contact *support@ceresnano.com*.

This document must be read in its entirety before using this product

FOR RESEARCH USE ONLY.

Ceres Nanosciences

9460 Innovation Drive
Manassas, VA 20110
www.ceresnano.com

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Contact Information

For information regarding Ceres Nanosciences' products, to contact technical support, or for general inquiries, please contact:

Ceres Customer Service & Scientific Support

Phone: +1-800-615-0418
Fax: +1-703-722-0731
Email: *support@ceresnano.com*

Introduction

The Nanotrap® particles[1] included in this kit are designed for the simple, rapid, and reliable purification and concentration of proteins, peptides, and low molecular weight compounds found in complex biological matrices upstream of mass spectrometry analysis.

Principle of Nanotrap® Technology

Nanotrap® particles are composed of cross-linked N-isopropylacrylamide (NiPAm) copolymers functionalized with chemical affinity baits which enable enrichment of proteins, peptides, nucleic acids, and pathogens.

The ability to trap and enrich a series of proteins or peptides based on tailored affinity and size characteristics simultaneously improves work flows, reduces sample processing time, and maximizes the potential to collect critical data from valuable and complex bio-fluid samples.

Sample Processing Workflow Overview

1. Nanotrap® Particle Capture
    - Nanotrap® particles are introduced to the sample and low abundance analytes are rapidly captured from complex biological matrices.
    - The sample is centrifuged, separating the Nanotrap® particles and captured analytes from the more abundant analytes within the sample.

2. Sample Digestion & Clean-up
    - The supernatant is carefully removed and the Nanotrap® particles are washed to remove any residual unbound molecules.
    - Captured peptides and proteins are trypsin digested and desalted.

3. Analysis
    - Samples can be processed for analysis on any mass spectrometer, however this method has been optimized for samples analyzed on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer coupled to an Easy-nLC 1000 Liquid Chromatography System (Thermo Fisher Scientific).

[1] If you are interested in using Nanotrap® particles to purify, concentrate, or preserve analytes that are larger than 60,000 Daltons, please contact Ceres Nanosciences for more information: *info@ceresnano.com*.

Kit Components

Nanotrap® particles

- CN2020 Nanotrap® Blue VSA CS particles, [5 mg/mL]
- CN2030 Nanotrap® White CS particles, [20 mg/mL]
- CN2050 Nanotrap® Purple VSA CS particles, [5 mg/mL]

Nanotrap® particles are packaged in 18.0 MΩ-cm purified water and should be stored at +4 ºC; do not freeze.

Additional Materials & Equipment

The following reagents and equipment are not provided by Ceres Nanosciences.

For Nanotrap® particle capture

- 50 mM Tris-HCl, pH 7.4
- 0.25 M sodium thiocyanate
- 18.0 MΩ-cm purified water
- Micro-centrifuge (must be able to reach speeds of 16,800 RCF)
- Micro-centrifuge tubes (1.5 mL, 2.0 mL)
- Vortex

For sample digestion & clean up

- 50 mM Ammonium bicarbonate (NH_4HCO_3) in 18.0 MΩ-cm purified water
- UA solution: 8 M urea (Sigma, U5128) in 100 mM NH_4HCO_3, pH 8.0
- 0.1 M DTT in UA solution

  Note: Must be freshly prepared and used within the same day.
- 0.5 M iodoacetamide (IAA) in UA solution

  Note: Store up to 1 week in the dark.
- MS Grade Trypsin (cat #V5113, Promega)
- 0.02% ProteaseMax (cat# V2071, Promega) in 50 mM NH_4HCO_3
- 80% Acetonitrile with 1% Formic Acid
- 0.1% Formic Acid
- SpeedVac concentrator

- C18 Spin tips (cat# 84850, Pierce™)

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the safety data sheet (SDS), which can be obtained from Ceres Nanosciences upon request by emailing *info@ceresnano.com*.

Reagent Preparation

Nanotrap® particles

For initial analysis, 100 µL of each particle type is recommended to capture and elute protein and peptide biomarkers for mass spectrometry (MS) applications. Higher or lower amounts of particles can be used to optimize yields for MS analysis. *See the Alternative Conditions section for more details.*

Best Practices

Supernatant removal

- Carefully pipette off the supernatant from the side opposite the pellet to avoid risk of aspirating the pellet. Video *here*.
- After removing the majority of the supernatant, a short (≤1 minute) centrifugation at max speed should be performed to maximize the supernatant removal.

Particle resuspension

- To re-suspend the particle pellet most efficiently, pipette the solution up and down to break up the pellet into smaller clumps. Continue to pipette the suspension until the pellet is completely re-suspended. The particles can be vortexed to mix after the pellet is resuspended.

- Do not use the pipette tip to poke at the pellet in an attempt to break it up, as the Nanotrap® particles may stick to the tip. Centrifugation times may be reduced if the particles become difficult to resuspend.
- Avoid introducing air bubbles into the solution as this may cause foaming.

Sample Preparation

Nanotrap® particles are designed to capture and enrich proteins and other molecules directly from biological matrices like serum, plasma, saliva, and urine. For initial analysis, 100 µL of serum, plasma, or saliva is recommended for Nanotrap® processing, while urine volumes between 500 µL and 1 mL are recommended. Larger or smaller sample volumes can be utilized to optimize yields for analysis by mass spectrometry. See the *Alternative Conditions* section for more details.

Best Practices

Sample centrifugation

- Visually inspect each sample to be analyzed. If the sample appears turbid, centrifuge at 16,800 RCF for 10 minutes to remove aggregates or debris. Use sample supernatant for processing and incubation with Nanotrap® particles.

Sample dilution

- Serum, plasma, and saliva should be diluted 3-fold in Tris-HCl, pH 7.4, prior to Nanotrap® particle addition and incubation to allow for efficient mixing.

  Example calculation for Prepared Sample:

  100 µL (Serum, plasma, or saliva) + 200 µL (Tris-HCl) = 300 µL Prepared Sample

- Urine can be processed "neat" without additional dilution prior to incubation with Nanotrap® particles.

Protocol

The following instructions are for processing a single sample with a single Nanotrap® particle type. Repeat these steps as needed to process additional samples.

Nanotrap® particle capture

Methods

1. Briefly vortex the Nanotrap® particle stock vial to ensure homogenous suspension.
2. Pipette 100 µL of each Nanotrap® particle type into a separate microcentrifuge tube.
3. Add the prepared sample to the Nanotrap® particles following *Best Practices*.
4. Allow the Nanotrap® particles to incubate in the sample for 30 minutes at room temperature, with gentle shaking to allow for complete and thourough mixing (eg. on a slow shaking speed).
5. Centrifuge the particle-sample suspension at 16,800 RCF for 10 minutes at room temperature.
6. Carefully remove the supernatant and transfer to a clean microcentrifuge tube and discard the supernatant according to *Best Practices.*
7. Quickly wash the particles in 500 µL of 0.25 M sodium thiocyanate. Alternatively, a less aggressive wash may be performed with 50 mM Tris-HCl.
8. Centrifuge the sample at 16,800 RCF for 10 minutes at room temperature and discard all supernatant.
9. Resuspend the particle pellet in 1 mL of 18 MΩ-cm water to wash according to *Best Practices*.
10. Centrifuge the particle-water suspension at 16,800 RCF for 10 minutes at room temperature and discard all supernatant.
11. Repeat steps 9 and 10 for a total of two water washes following the brief sodium thiocyanate wash (Step 7).

Sample digestion & clean-up

Methods – sample digestion

1. Resuspend the post-capture particle pellet in 100 µL of 50 mM ammonium bicarbonate (ABC).
2. Add 10 µL of 100 mM DTT in UA solution and briefly vortex to mix. Allow to incubate at room temparature for 30 minutes.

3. Centrifuge the samples at 16,800 RCF for 10 minutes at room temperature, then carefully remove the supernatant.
4. Resuspend the particles in 100 µL of 50 mM ABC.
5. Add 10 µL of 500 mM iodoacetaide (IAA) in UA solution and incubate at room temperature in the dark for 30 minutes.
6. Centrifuge samples at 16,800 RCF for 10 minutes at room temperature and remove the supernatant.
7. Resuspend particles in 500 µL 50mM ABC to wash residual DTT and IAA.
8. Centrifuge sample at 16,800 RCF for 10 minutes and remove supernatant.
9. Add ProteaseMax at 0.02% and MS Grade Trypsin at a ratio of 1:50 to cover the particles.
10. Incubate the tyrpsin digestion reaction overnight (16 – 18h) at 37 °C.
11. Centrifuge sample at 16,800 RCF for 10 minutes and transfer supernatant to a clean microfuge tube.
12. SpeedVac the digestion supernatant to a concentrated sample volume of ~5 µL.

Methods – sample clean-up

13. Equilibrate C18 spin tips with 20 µL of 1% Formic Acid in 80% Acetonitirile.
14. Centrifuge the spin tips for 1 minute at 1,000 RCF and transfer to a clean collection tube.
15. Add 0.1% Formic Acid to the concentrated sample from step 12 to a final volume of 50 µL and vortex to mix.
16. Add samples to the spin tips and centrifuge for 1 minute at 1,000 RCF.

    Note: Larger volumes may require longer centrifugation times to completely flow through the tip.
17. Re-apply the sample flow-through to the C18 spin tip 3 additional times to increase sample binding.
18. Wash the spin tips by adding 20 µL of 0.1% Formic Acid and centrifuging at 1,000 RCF for 1 minute. Repeat one additional time.
19. Transfer spin tips to a clean microcentrifuge tube.

20. Elute with 20 µL of 0.1% Formic Acid in 80% Acetonitrile and centrifuge at 1,000 RCF for 1 minute. Repeat one additional time for a total volume of 40 µL eluted sample.
21. Speed vac sample to near dryness and resuspend with 20 µL of 0.1% Formic Acid for LC-MS analysis.

Analysis

Methods

The above methods have been optimized for samples analyzed on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer coupled to an Easy-nLC 1000 Liquid Chromatography System (Thermo Fisher Scientific) through a nanoelectrospray ion source. Details on LC gradients, data acquisition, and analysis can be found in the application note "Nanotrap® Particles for Mass Spectrometry: Enrichment of low abundance, low molecular weight proteins from human plasma samples" located on the Ceres Nanoscienes website at:
http://www.ceresnano.com/literature/app-note-nanotrap-particles-for-mass-spectrometry

Alternative Conditions

The alternative conditions described below can be implemented independently or in any combination in the effort to improve analyte capture and elution efficiency.

Reagent Preparation

STEP	RECOMMENDATIONS & ALTERNATIVE METHODS
Particle preparation	Particle amount ▪ Increasing or decreasing particle volume can improve efficiency of capture. Some customers have had success with as few as 50 µL of particles, while others have seen improvements with higher volumes. This is one variable that can be optimized for each Nanotrap® particle type and sample matrix used. ***Note:*** Increasing or decreasing particle volume impacts centrifugation time (50 µL of particles should require 3-5 minutes of centrifugation time to pellet, whereas more than 200 µL of particles may require 10-15 minutes to pellet). Centrifugation times may also vary based on Nanotrap® particle type.

Sample preparation	Sample Volume ▪ Increasing the starting sample volume can increase the enrichment of some analytes. For sample volumes larger than 1 mL, please contact support@ceresnano.com for specific recommendations and alternative protocols. Sample Dilution ▪ Increasing the dilution of the starting sample in Tris-HCl from 3-fold to 6-fold might improve capture efficiency.

Capture Methods

STEP	RECOMMENDATIONS & ALTERNATIVE METHODS
Capture incubation time	Incubation times for the analyte capture portion of the method can be lengthened or shortened to optimize and streamline workflow while ensuring efficient capture of analytes.

Troubleshooting

ISSUE	SOLUTION
What should I do if the Nanotrap® particles do not pellet after the centrifugation step?	The recommended centrifugation time is dependent on the amount of Nanotrap® particles used in the sample (50 µL of particles should require 3-5 minutes of centrifugation time to pellet, whereas more than 200 µL of particles may require 10-15 minutes to pellet). Centrifugation times may also vary based on particle type. ***The centrifuge must be able to reach a maximum speed of at least 16,800 RCF.*** ▪ If a diffuse pellet forms or the pellet is not visible following centrifugation, spin the samples for an additional 2 – 7 minutes at 16,800 RCF or increase centrifuge speed to 21,100 RCF. ○ If you are using CN2030 Nanptrap® particles, be aware that it may be difficult to see the white particle pellet. Try viewing the pellet against a light source. ▪ If using a sample volume greater than 1.00 mL, increase the centrifugation time to 15 – 20 minutes at 16,800 RCF.

There are other high abundance and or interfering molecules in my sample post-Nanotrap® processing.	Particle washing conditions; ▪ Repeat *Capture Step 5-6* for a total of two washes with 18 MΩ-cm water to remove contaminating proteins. ▪ Alternatively, particles can be washed with 0.25 M sodium thiocyanate (500 µL) during the 1st washing step to remove any unwanted large molecules like albumin. This wash should be followed by a total of two water washes (perform Capture Method Step 5 twice). Eluent Collection Tubes ▪ Some microcentrifuge tubes require cleaning to remove contaminants or plasticizers that can interfere with data acquisition. Rinse microfuge tubes with 20% Acetonitrile prior to collecting eluents from *Elution Methods Step 4*.

For additional information and further troubleshooting assistance, visit our support site at www.ceresnano.com.

References

1. **Tamburro, D. et al. Multifunctional Core–Shell Nanoparticles: Discovery of Previously Invisible Biomarkers.** *Journal of the American Chemical Society* **2011** *133* (47), 19178-19188. **DOI:** 10.1021/ja207515j**.**
2. Longo, C. et al. **Core-shell hydrogel particles capture, concentrate and preserve labile low abundance biomarkers**. *PLoS One* 4, (2009). https://doi.org/10.1371/journal.pone.0004763.
3. Steinberg HE. et al. **Toward detection of toxoplasmosis from urine in mice using hydro-gel nanoparticles concentration and parallel reaction monitoring mass spectrometry.** *Nanomedicine*. 2018 Feb;14(2):461-469. DOI: 10.1016/j.nano.2017.11.020.
4. Fredolini, C. et al. **Concentration and Preservation of Very Low Abundance Biomarkers in Urine, such as Human Growth Hormone (hGH), by Cibacron Blue F3G-A Loaded Hydrogel Particles**. *Nano Res*. 1, 502–518 (2008). DOI:10.1007/s12274-008-8054-z.
5. Luchini, A. et al. **Smart hydrogel particles: Biomarker captureing: One-step affinity purification, size exclusion, and protection against degradation**. Nano Lett. 8, 350–361 (2008). DOI: 10.1021/nl072174l.
6. Bishop, B. M. *et al.* **Bioprospecting the American alligator (Alligator mississippiensis) host defense peptidome**. *PLoS One* **10,** 1–17 (2015). https://doi.org/10.1371/journal.pone.0117394.
7. Juba, M. L. et al. **Large Scale Discovery and de Novo-Assisted Sequencing of Cationic Antimicrobial Peptides (CAMPs) by Microparticle Capture and Electron-Transfer Dissociation (ETD) Mass**

Spectrometry. *J. Proteome Res*. 14, 4282–4295 (2015). DOI: 10.1021/acs.jproteome.5b00447.

Terms & Conditions

Product Use

Nanotrap® particles are manufactured by Ceres Nanosciences, Inc. ("Ceres"). This product conforms to specifications indicated for the intended use. (See complete terms at http://www.ceresnano.com/ceres-terms-of-sales-and-use .)

Warranty

Ceres does not guarantee the performance of our particle technology for specific applications. Nanotrap® particles conform to physical and performance criteria for sample processing for the duration of the stated shelf life. Ceres' obligation under this warranty is limited to replacement, at Ceres' expense, of any product which is deemed defective in manufacture. Defective product must be returned to Ceres with proof of such defect. Claims resulting from merchandise damaged during shipping and delivery should be directed to the carrier. This warranty does not apply to any products that have been altered, improperly stored or misused. ALL OTHER WARRANTIES, EXPRESSED, IMPLIED OR STATUTORY, ARE HEREBY SPECIFICALLY EXCLUDED, INCLUDING BUT NOT LIMITED TO WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Ceres' maximum liability is limited in all events to the price of the products sold by Ceres in each instance of a claim. IN NO EVENT SHALL CERES NANOSCIENCES BE LIABLE FOR ANY SPECIAL, INCIDENTAL OR CONSEQUENTIAL DAMAGES. Some states do not allow limits on warranties, or on remedies for breach in certain transactions. In such states, the limits set forth above may not apply, however such limits as otherwise codified by such state law are hereby incorporated by reference to the maximum benefit of such disclaimer on behalf of Ceres.

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Intellectual Property Disclaimer

Ceres will not be responsible for violations or patent infringements that may occur with the use of our products.

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