CERES NANO

Powering the Future of Diagnostics

Nanotrap® Sample Processing Protocol 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-001, V:1; Release Date: 11 July 2018

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.

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Contact Information

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Introduction

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

Principle of Nanotrap® Technology

Nanotrap® particles do not rely on specific antibodies; and are instead comprised of cross-linked N-isopropylacrylamide (NiPAm) copolymers functionalized with chemical affinity baits which enable enrichment of target 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. Capture
    - Nanotrap® particles are introduced to the sample and low abundance target analytes are rapidly captured from complex biological matrices.
    - The sample is centrifuged, separating the Nanotrap® particles and captured analytes from the larger, and more abundant, proteins and molecules within the sample.

2. Elution
    - The supernatant is carefully removed and the Nanotrap® particles are washed to remove any residual unbound molecules. An elution buffer is then applied to the Nanotrap® particles to extract the concentrated target analytes.
    - The sample is centrifuged, separating the Nanotrap® particles and eluted analytes for downstream analysis.

3. Analysis
    - Using standard protocols, the eluted target analytes are prepared for downstream analysis by mass spectrometry.

[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® capture and elution

- 50mM Tris-HCl, pH 7.4
- 18.0 MΩ-cm purified water
- Elution buffer: 70% Acetonitrile, 10% Ammonium hydroxide by volume
- Micro-centrifuge (must be able to reach speeds of 16,800 RCF)
- Micro-centrifuge tubes (1.5 mL, 2.0 mL)
- Vortex

For mass spectrometry analysis

- N_2 evaporator
- 8 M urea
- 1 M DTT
- 0.5 M iodoacetamide
- Ammonium bicarbonate (NH_4HCO_3)
- Trypsin
- Acetic Acid
- Zip-tip (0.6 µL C18, resin, Millipore)

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 at *info@ceresnano.com*.

Reagent Preparation

Nanotrap® particles

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

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

1. Briefly vortex the Nanotrap® particle stock vials to ensure homogenous suspension.
2. Pipette 100 µL of each Nanotrap® particle type in a separate microcentrifuge tube.
3. Centrifuge at 16,800 RCF for 5 - 10 minutes at room temperature and discard the supernatant. Retain the particle pellets for use in Capture Step 1 of the Protocol section below.

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. This “quick spin” should be done prior to elution to ensure maximum enrichment.

Particle resuspension

- To re-suspend the particle pellet most efficiently, pipette the solution up and down to break up 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.
- 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 desired molecules directly from biological matrices including 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 and 1 mL are recommended. Higher sample volumes can be utilized to optimize yields for mass spec analysis. 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 2-fold in Tris-HCl, pH 7.4 prior to incubation with Nanotrap® particles to allow for efficient mixing.

Example calculation for Prepared Sample:

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

- Urine can be processed “neat” with no additional dilution prior to incubation with Nanotrap® particles.

Prepared samples should be set aside for use in Capture Step 1 of the Protocol section below.

Elution Buffer Preparation

The standard elution buffer recommended in this protocol is comprised of 70% acetonitrile (ACN) and 10% ammonium hydroxide (NH_4OH) by volume. Concentrated ammonium hydroxide can be purchased as 30% volume by water. **The elution buffer must be prepared fresh prior to every experiment** and utilized in Elution Step 1 of the Protocol section below.

Example calculation for 1 mL of elution buffer:

700 µL ACN + 300 µL (30% NH_4OH in water) = 1mL Elution Buffer

Other elution buffers may be used with this protocol depending on the downstream mass spectrometry processing required. See the *Alternative Conditions* section for more details.

Protocol

Capture

Methods

1. Add the prepared sample to the Nanotrap® particle pellet. Resuspend each Nanotrap® particle pellet following *Best Practices*.
2. Allow the Nanotrap® particles to incubate in the sample for 30 minutes at room temperature, with gentle shaking to allow for Nanotrap® particles to fully mix with the sample (eg. on a slow shaking speed).
3. Centrifuge the particle-sample suspension at 16,800 RCF for 10 minutes at room temperature.
4. Carefully remove the supernatant and transfer to a clean microcentrifuge tube if downstream analysis of the supernatant is desired; otherwise discard the supernatant.

5. Resuspend the particles in 1 mL of 18 MΩ-cm water to wash. Resuspend each Nanotrap® particle pellet following *Best Practices*.
6. Centrifuge the particle-water suspension at 16,800 RCF for 10 minutes at room temperature and discard all supernatant unless needed for downstream analysis.

Elution

Methods

1. Resuspend the post-capture particle pellet in 100 µL of elution buffer. Resuspend each Nanotrap® particle pellet following *Best Practices*. See the *Alternative Conditions* section for other elution buffers and volume recommendations.
2. Incubate particle-elution buffer suspensions at room temperature for 5 minutes with gentle shaking.
3. Centrifuge the particle-elution buffer suspension at 16,800 RCF for 10 minutes at room temperature.
4. Carefully remove the supernatant and transfer to a clean microcentrifuge tube for downstream mass spectrometry analysis.
5. Perform a second round of elution by repeating steps 1-3. Combine eluates from both elution steps.
6. Proceed with downstream mass spectrometry preparation and analysis.

Analysis

Methods

1. Dry the combined eluates using an N_2 evaporator manifold at room temperature. See the *Alternative Conditions* section for other recommendations.
2. Use a Zip-tip (0.6 µL C18, resin, Millipore) to desalt the sample.
3. Perform mass spectrometry analysis of the peptides.

Optional trypsin digestion method

1. Dry the combined eluates using an N_2 evaporator manifold at room temperature.

2. Add 40 µL of 8M urea and 2 µL of 1 M DTT to the dried sample and Incubate at 37 °C for 30 minutes.
3. Add 12 µL 0.5 M iodoacetamide to the mixture and incubate in the dark for 20 minutes at room temperature.
4. Prepare 150 µL of trypsin solution using the following recipe:

   100 µL dH2O + 48 µL NH_4HCO_3 + 2 µL trypsin (1 mg/ml)
5. Add 150 µL of the trypsin solution to the sample and incubate the trypsin digestion reaction overnight (approximately 15 hr) at 37 °C.
6. Following overnight digestion, add 5 µL of concentrated acetic acid to the sample to stop the digestion.
7. Use a Zip-tip (0.6 µL C18, resin, Millipore) to desalt the sample.
8. Perform mass spectrometry analysis of the sample.

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. Certain collaborators 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 used. ***Note:*** Increasing or decreasing particle volume impacts centrifugation time (50 µL of particles should require 3-5 minutes of centrifugation 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.
Sample preparation	Volume ▪ Increasing the starting sample volume can increase the enrichment of certain 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 2-fold to 6-fold could improve capture efficiency.
Elution buffer preparation	Alternative Elution Buffers: See associated references for experimental use ▪ 1% Rapigest (Waters, Milford, MA) in 50mM ammonium bicarbonate with 10mM TCEP. Reference: *doi: 10.1016/j.nano.2017.11.020* ▪ 1:1 TFE: H2O (Trifluoroethanol); 0.1% TFA (Trifluoroacetic acid) Reference: *Ceres MS Biomarker Whitepaper 08MAR2018* ▪ 0.5% TFA Trifluoroacetic acid: 200 µL per every 100 µL of serum/plasma/saliva or 500 µL of urine. A total of 2 elutions, the first for 1 h, and the second for 20 minutes with gentle shaking. ▪ For SDS-PAGE applications upstream of mass spectrometry; resuspend particles post-capture with 40 µL of Laemmli sample buffer, boil for 5 minutes then load particle-buffer sample directly to the gel and electro-elute analytes. Reference: *https://doi.org/10.1371/journal.pone.0004763*.

Capture Methods

STEP	RECOMMENDATIONS & ALTERNATIVE METHODS
Capture incubation time	Capture incubation times can be lengthened or shortened to optimize and streamline workflow while ensuring efficient capture of target analytes.

Elution Methods

VARIABLE	RECOMMENDATIONS & ALTERNATIVE METHODS
Elution buffer volume	Elution buffer volumes can be increased to optimize elution efficiency.
Elution incubation time	Elution incubation times can be lengthened or shortened to streamline workflow while ensuring efficient elution.

Analysis

VARIABLE	RECOMMENDATIONS & ALTERNATIVE METHODS
Sample eluent drying	▪ Eluates containing an organic component can also be lyophilized.
Sample reconstitution	▪ 40 µL of dH_2O can be added to reconstitute the sample prior to trypsin digestion.

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 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.
Particles are not resuspending well in the Elution buffer.	▪ After attempting to resuspend particles following *Best Practices*, try vortexing particles to resuspend, then sonicate for 2 minutes.
There are other high abundant and or contaminating 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

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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.

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