LSBio
an Absolute Biotech Company

Rat AGRP ELISA Kit (Competitive EIA)

User Manual

Catalog No. LS-F27858

It is important that you read this entire manual carefully before starting your experiment.

SAMPLE ONLY

This kit is for **Research Use Only**.
This kit is not approved for use in humans or for clinical diagnosis.

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ASSAY SPECIFICATIONS

Target: AGRP

Synonyms: AGRP, agouti related protein homolog (mouse), Agouti (mouse) related protein, Agouti-related protein, Agouti-related transcript, AGRT, ASIP2

Specificity: This kit is for the detection of Rat AGRP. No significant cross-reactivity or interference between AGRP and analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs.

Sample Types: This kit is recommended for use with Rat Plasma and Serum. Use with other sample types is not supported.

Detection: Colorimetric - 450nm (TMB)

Detection Range: 50–1000 pg/ml

Sensitivity: Typically less than 1 pg/ml

Performance: Intra-Assay CV (<10%); Inter-Assay CV (<10%)

Measurement: Quantitative

Limitations: This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

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ASSAY PRINCIPLE

This assay is based on the competition ELISA principle. Each well of the supplied microtiter plate has been pre-coated with an antigen-specific capture antibody. Standards or samples are added to each well as well as a fixed quantity of Horseradish Peroxidase (HRP)-conjugated target antigen. The free antigen in the sample competes with the HPR-conjugated antigen for binding to the capture antibody. Unbound antigen is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm. The OD of an unknown sample can then be compared to an OD standard curve generated using known antigen concentrations in order to determine its antigen concentration. In contrast to typical Sandwich ELISA assays in competition assay the greater the amount of antigen in the sample the lower the color development and optical density reading.

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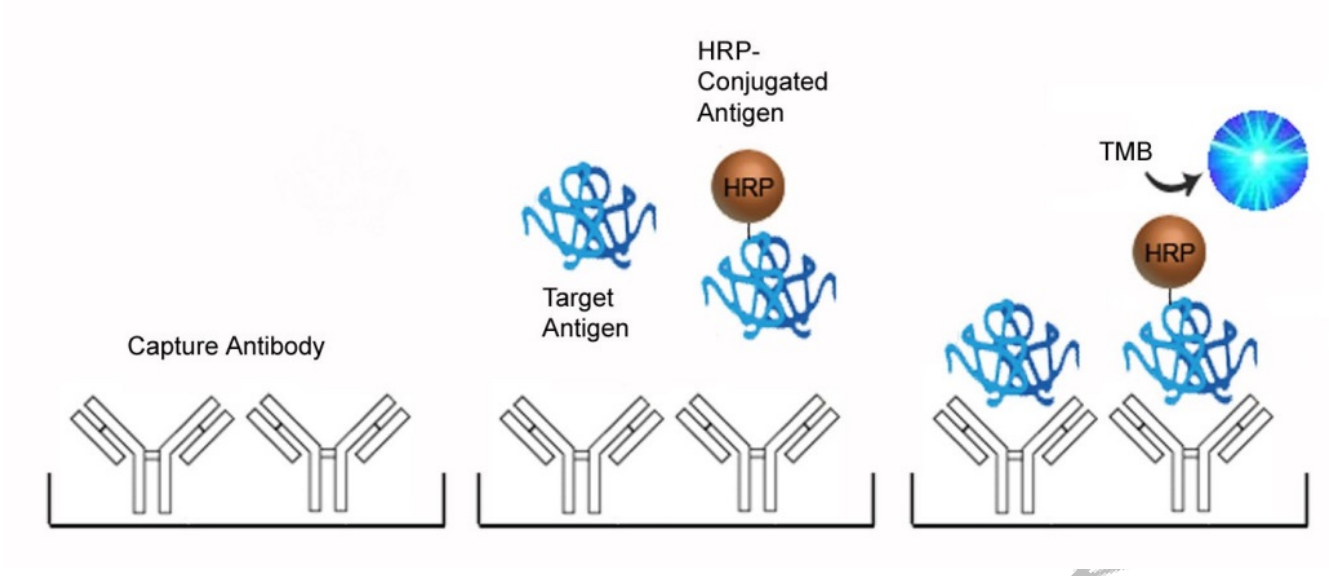
Assay Principle Image



Kit Components and Storage

Component	Quantity
Coated 96-well Strip Plate	1
Standard A (0 pg/ml)	1 vial
Standard B (50 pg/ml)	1 vial
Standard C (100 pg/ml)	1 vial
Standard D (250 pg/ml)	1 vial
Standard E (500 pg/ml)	1 vial
Standard F (1000 pg/ml)	1 vial
Balance Solution	1 vial x 3 mL
HRP-Conjugate	1 vial x 6 mL
Wash Buffer (100x)	1 vial x 10 mL
Substrate A	1 vial x 6 mL
Substrate B	1 vial x 6 mL
Stop Solution	1 vial x 6 mL
Adhesive Plate Sealers	4
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Kit Storage

The unopened kit can be stored at 2°C to 8°C through the expiration date. Once opened the kit can be stored at 2°C to 8°C for 1 month. Unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air.

Other Required Supplies

- Microplate reader with 450 nm wavelength filter.
- High-precision pipette and sterile pipette tips
- Eppendorf tubes
- 37°C incubator
- Deionized or distilled water
- Absorbent paper

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Experimental Layout

The following is an example of how to layout a study. A dilution series of the positive control Standard should be run in duplicate or triplicate with the last well in each series being the negative control blank. Samples should also be run in duplicate or triplicate. Unknown samples should be run as a dilution series in order to identify the optimal dilution that produces an OD reading within the OD range of the positive control Standard dilution series.

Example 1: Standard Curve and dilution series of an unknown sample.

	1	2	3	4	...
A	Standard A	Standard A	Sample (1:1)	Sample (1:1)	...
B	Standard B	Standard B	Sample (1:10)	Sample (1:10)	...
C	Standard C	Standard C	Sample (1:100)	Sample (1:100)	...
D	Standard D	Standard D	Sample (1:1k)	Sample (1:1k)	...
E	Standard E	Standard E	Sample (1:10k)	Sample (1:10k)	...
F	Standard F	Standard F	Sample (1:100k)	Sample (1:100k)	...
G	Blank	Blank	Sample (1:1,000k)	Sample (1:1,000k)	...
H	Blank	Blank	Sample (1:10,000k)	Sample (1:10,000k)	...

Example 2: Standard Curve and samples run in duplicate.

	1	2	3	4	...
A	Standard A	Standard A	Sample A	Sample E	...
B	Standard B	Standard B	Sample A	Sample E	...
C	Standard C	Standard C	Sample B	Sample F	...
D	Standard D	Standard D	Sample B	Sample F	...
E	Standard E	Standard E	Sample C	Sample G	...
F	Standard F	Standard F	Sample C	Sample G	...
G	Blank	Blank	Sample D	Sample H	...
H	Blank	Blank	Sample D	Sample H	...

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Sample Collection

This assay is recommended for use with Rat Plasma and Serum. Use with other sample types is not supported. The following are a collection of sample collection protocols for your reference.

Breast Milk - Centrifuge samples for 20 minutes at 1,000 × g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cell Lysates – Adherent cells should be detached with Trypsin. Collect and pellet the cells by centrifugation and remove the supernatant. Wash the cells three times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication four times. Centrifuge at 1,000 × g for 15 minutes at 2°C to 8°C to remove cellular debris. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Erythrocyte Lysates - Centrifuge whole blood for 20 minutes at 1,000 × g to pellet the cells and remove the supernatant. Wash the cells three times with PBS* then resuspend in PBS*. Freeze the cells to -20°C and thaw to room temperature three times. Centrifuge at 5,000 × g for 10 minutes at 2°C to 8°C to remove cellular debris. Collect the supernatant for assaying. Erythrocyte lysates must be diluted with Sample Diluent before running. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1,000 × g at 2°C to 8°C within 30 minutes of collection. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Platelet-Poor Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1,000 × g at 2°C to 8°C within 30 minutes of collection. It is recommended that samples should be centrifuged for 10 minutes at 10,000 × g for complete platelet removal. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Sperm and Seminal Plasma – Allow semen to liquefy at room temperature or 37°C. After liquefaction, centrifuge at 2,000 × g for 10 to 15 minutes. Collect seminal plasma supernatant for assaying. Wash the precipitated protein three times with PBS* then resuspend in PBS*. Lyse

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the cells by ultrasonication then centrifuge at 2,000 × g for 10 to 15 minutes. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at approximately 1,000 × g. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Tissue Homogenates–Because preparation methods for tissue homogenates vary depending upon tissue type, users should research tissue specific conditions independently. The following is one example only. Rinse 100 mg of tissues in PBS* to remove excess blood and weigh before homogenization. Finely mince tissues and homogenize them in 1 mL of PBS*with a glass homogenizer on ice. Lyse the cells by ultrasonication or freeze the cells to -20°C and thaw to room temperature two to three times. Centrifuge homogenate at 1,500 × g for 15 minutes. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day, mid-stream, voided directly into a sterile container. Centrifuge to remove particulate matter and collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cell culture supernatants, cerebrospinal, follicular, and lung lavage fluids, saliva, sweat, tears, and other biological fluids - Centrifuge samples for 20 minutes at 1,000 × g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

* 1xPBS (0.02 mol/L pH 7.0-7.2)

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Sample Collection Notes

1. LSBio recommends that samples are used immediately upon preparation.
2. Avoid repeated freeze/thaw cycles for all samples.
3. In the event that a sample type not listed above is intended to be used with the kit it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18°C to 25°C) before performing the assay without the use of extra heating.
7. Sample concentrations should be predicted before being used in the assaying. If the sample concentration is not within the range of the standard curve users must determine the optimal sample dilutions for their particular experiments.
8. LSBio is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer supplied samples use with the kit.

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Reagent Preparation

Bring all reagents to room temperature (18°C to 25°C) before use.

1x Wash Buffer: If crystals have formed in the concentrate warm to room temperature and mix gently until crystals have completely dissolved. Prepare 1,000 mL of Working Wash Buffer by diluting the supplied 10 mL of 100x Wash Buffer Concentrate with 990 mL of deionized or distilled water. Wash Buffer can be stored at 4°C once prepared.

All other reagents are ready-to-use.

Reagent Preparation Notes

1. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
2. Reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
3. All solutions should be gently mixed prior to use.
4. To minimize imprecision caused by pipetting ensure that pipettes are calibrated. Pipetting volumes of less than 10 µL is not recommended.
5. Substrate Solution is easily contaminated so sterility precautions should be taken. Substrate Solution should also be protected it from light.
6. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
7. Due to the antigen specificity of the antibodies used in this assay, native or recombinant proteins from other manufacturers may not be detected by this kit.

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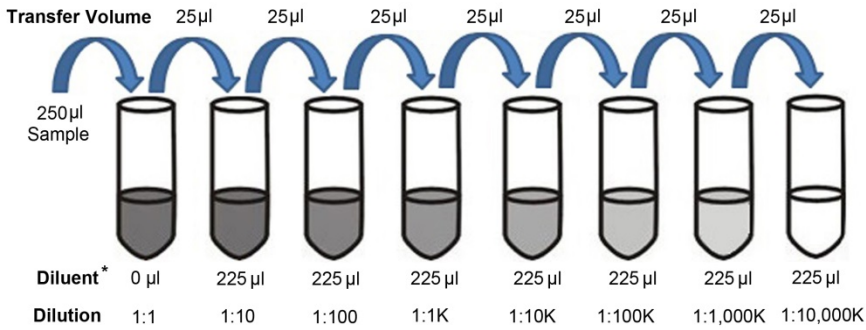
SAMPLE PREPARATION

The resulting Optical Density (OD) values of your sample must fall within the OD values of the standard curve in order for the calculated antigen concentration to be accurate. In many cases samples will need to be diluted in order to lower the antigen concentration to sufficient levels. Information about antigen concentrations within various sample types may be available from the published literature; however it is often necessary to run a dilution series of each sample type. The following will prepare sufficient volumes to run the Sample dilution series in triplicate. In the case of small volume samples, a preliminary step dilution, such as 1:5 or 1:10, can be made using PBS (0.02 mol/L pH 7.0-7.2) as the diluent. ***The final dilution should always be made using the same buffer that is used to dilute the Standards, and/or generate the Standard Curve.** Running duplicate or triplicate wells for each sample is recommended.



STANDARDS PREPARATION

The Standards supplied with this kit are ready-to-use and no preparation is necessary.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature without additional heating and mix thoroughly by gently swirling before pipetting (avoid foaming). Prepare all reagents, working standards, and samples as directed in the previous sections.

1. Add 100 µL of PBS (0.02 mol/L pH 7.0-7.2) to a Blank well.
2. Add 10 µL of **Balance Solution** to each 100 µL sample of cell culture supernatant or tissue homogenate if applicable, and mix well. Do NOT add to plasma or serum samples, standards, or the Blank.
3. Add 100 µL of **Standard** or **Sample** per well.
4. Add 50 µL of **1x HRP-conjugate** to each well (excluding the Blank well), gently agitate to ensure thorough mixing, cover with a plate sealer, and incubate for 1 hour at 37°C.
5. Aspirate the liquid from each well and wash five times. Wash by adding approximately 350 µL of **1x Wash Buffer** using a squirt bottle, multi-channel pipette, manifold dispenser, or automated washer. Allow each wash to sit for 15 seconds before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.
6. Add 50 µL of **Substrate A** and 50 µL of **Substrate B** to each well (including the Blank), gently agitate to ensure thorough mixing, and incubate **in the dark** for 15-20 minutes at 37 °C.
7. Add 50 µL of **Stop Solution** to each well. The blue color will change to yellow immediately. If color change does not appear uniform gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as was the substrate solutions.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

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ASSAY PROCEDURE NOTES

1. **ELISA Plate**: Keep appropriate numbers of strips for one experiment and remove extra strips from microtiter plate. Removed strips should be placed in a sealed bag containing desiccant and stored at 4°C.
2. **Solutions**: To avoid cross-contamination change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Applying Solutions**: All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
4. **Assay Timing**: The interval between adding sample to the first and last wells should be minimized. Delays will increase the incubation time differential between wells which will significantly affect the experimental accuracy and repeatability. For each step in the procedure total dispensing time for addition of reagents or samples should not exceed 10 minutes.
5. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
6. **Washing**: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.
7. **Controlling Substrate Reaction Time**: After the addition of the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. Excessively strong color will result in inaccurate absorbance reading.
8. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings remove any

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residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.

9. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.
10. **Stop Solution**: The Stop Solution contains an acid therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
11. **Mixing**: During incubation times the use of a micro-oscillator at low frequency is recommended. Sufficient and gentle mixing is particularly important in producing reliable results.
12. Kits from different batches may be a little different in detection range, sensitivity, and color developing time. Please perform the experiment exactly according to the supplied instructions.
13. Due to inter- and intra-assay variability it is recommended that appropriate carry-over controls be included between assays.
14. Prior to running valuable samples LSBio recommends that the user run a preliminary experiment using the supplied controls in order to validate the assay.
15. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, and incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.
16. The kit should not be used beyond the expiration date on the kit label.

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ASSAY PROCEDURE SUMMARY

Prepare all reagents, samples and standards.

Add 100 µL of PBS as a **Blank** well.

Add 10 µL of **Balance Solution** to each 100 µL sample of cell culture supernatant or tissue homogenate sample if applicable.

Add 100 µL of **Standard** or **Sample** to each well.

Add 50 µL of **1x HRP-conjugate** to each well (excluding the blank) and incubate for 1 hour at 37°C.

Aspirate and wash five times.

Add 50 µL of **Substrate A** and 50 µL **Substrate B** to each well and incubate for 15-20 minutes at 37°C.

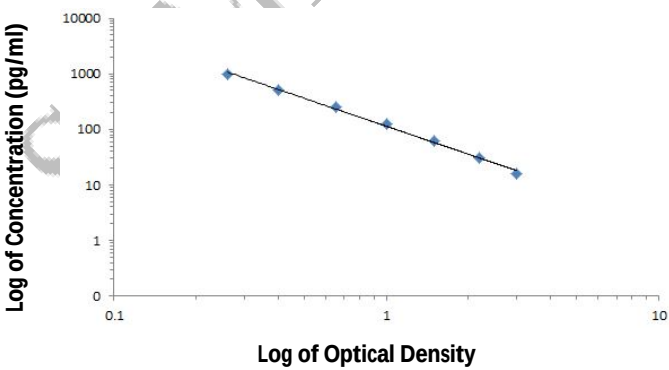
Add 50 µL of **Stop Solution**.

Read immediately at 450 nm.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average optical density of blank. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the target antigen concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. The use of a commercial software program such as CurveExpert is recommended for performing this calculation. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted the concentration read from the standard curve must be multiplied by the dilution factor.

Typical Data: The following standard curve is an example only and should not be used to calculate results for tested samples. A new standard curve must be generated for each set of samples tested.



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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat.

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Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of detector too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper washing instructions. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution not added	Stop solution should be added to each well before measurement.

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Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

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