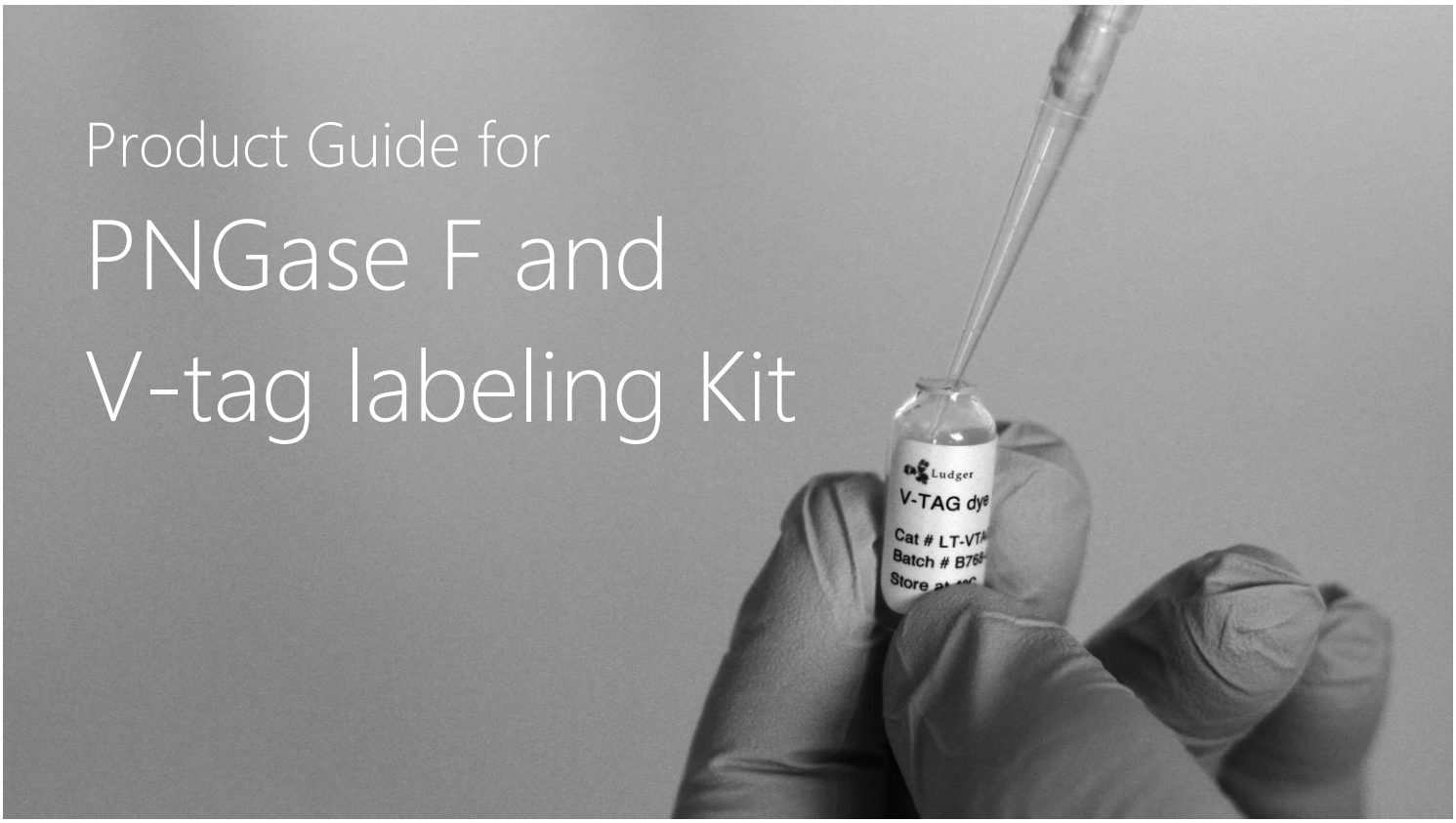
Ludger



Product # LT-VTAG-C30

Ludger Document # LT-VTAG-C30-Guide-v.1.0

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Contents

Specifications for LT-VTAG-C30

Application Release of aminoglycans from glycoproteins, subsequent fluorophore labeling and clean up

Description This kit contains the reagents needed for PNGase F release of aminoglycans, followed by fluorophore labeling and the specialised solid phase extraction (SPE) cartridges required for the purification of the labeled glycans.

Number of Samples 30 separate analytical samples per kit.

Amount of Sample As a guideline up to 100 µg of glycoprotein per sample.

Suitable Samples Glycoproteins and glycopeptides containing N-linked glycans.

Storage Store at 4°C. Protect from sources of heat and light.

Shipping The product should be shipped at 4°C.

Handling Ensure that any glass, plastic ware or solvents used with this item are free of environmental carbohydrates and contaminating enzymes. Use powder-free gloves for all sample handling procedures and avoid contamination with environmental carbohydrate.

Safety **For research use only. Not for human or drug use**
Please read the Safety Data Sheets (SDS's) for all chemicals used. All processes involving labeling reagents should be performed using appropriate personal safety protection – safety glasses, chemically resistant gloves (e.g. nitrile), lab coat, and when appropriate, in a laboratory fume cupboard.

Kit Contents

Each kit contains the following materials and reagents:

Cat. #	Item	Quantity
LZ-rPNGaseF-30	LudgerZyme™ rPNGase F release kit	1 kit (4 components)
	PNGase F (*Elizabethkingia miricola*) supplied in 50 mM NaCl 5 mM EDTA 20 mM Tris-HCl pH 7.5	1 vial of 30 µL
	10XGlyco Buffer 2 500 mM sodium phosphate (pH 7.5 at 1X dilution)	1 vial of 1.0 mL
	10X Glycoprotein Denaturing Buffer 5% SDS 400 mM DTT	1 vial of 1.0 mL
	10% NP-40 solution	1 vial of 1.0 mL
LT-VTAG-02	V-tag dye	1 ampoule (22 mg)
LT-DMSO-02	DMSO	1 ampoule (400 µL)
LC-VSPE-30	LudgerClean™ VSPE Cartridges	1 pack (30 cartridges)
LC-N-BUFFX40-30	ammonium formate Buffer, pH 4.4	1 bottle (3 mL)
LC-AA-BUFF-30	ammonium acetate Buffer	1 bottle (38 mL)

Some products may be classified as hazardous and are intended for use by professional laboratory personnel trained in the competent handling of such materials. Responsibility for the safe use of products rests entirely with the purchaser and user. The compiled Safety Data Sheets (SDS) for this product is available here: www.ludger.com/docs/products/lt/lt-vtag-c30/ludger-lt-vtag-c30-sds.pdf

Additional Reagents and Equipment Required

- Pure water: resistivity above 18 MΩ-cm, particle free (>0.22 µm), TOC <10 ppb.
- Polypropylene reaction vials with caps.
- Water bath, oven or heating block with constant temperature maintenance at 37°C.
- Vortex or shaker.
- Acetonitrile (UHPLC grade)
- Holder for the cartridges
- Vacuum manifold system that can take plates

Outline of Protocol

The LudgerTag™ V-tag labeling kit (LT-VTAG-C30) is designed for the fluorophore labeling of aminoglycans directly after PNGase F digestion. The fluorescent dye should be added to the digested glycans straight after PNGase F incubation is finished, as aminoglycans are likely to hydrolyse over time. V-tag labeled glycans are separated using the same HILIC mode HPLC or UHPLC chromatography that is used for N-glycans. The following columns can be used for separation LudgerSep N2 HILIC column (LS-N2-2.0X150) for HPLC or columns such as the Waters BEH Glycan 2mm x 150 mm for UHPLC. See later in the guide for HPLC/UHPLC separation details.

The outline labeling procedure is as follows:

I PNGase F glycan release
Release the N-Glycans from the protein using the PNGase F kit provided.

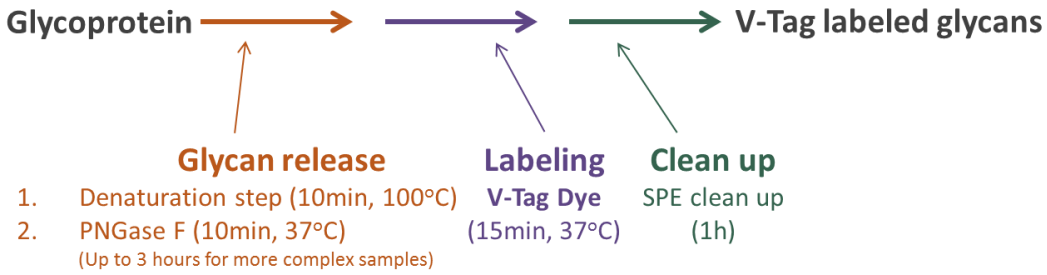
II Add V-tag dye to released N-aminoglycans inmediately after PNGase F release is completed.
Add the labeling solution to each sample.

III Incubate
Incubate the samples to allow the labeling reaction to progress.

IV Post-labeling clean up
After labeling, the removal of excess V-tag dye is performed with the LC-VSPE-30 cartridges supplied.

V Store or analyse the labeled glycans
The labeled glycans are now ready for analysis or can be stored in a freezer until required.

Workflow



Labels the amino group of the released glycans

Part 1: PNGase F glycan release

Application

Peptide N-glycosidase F (PNGase F) (LZ-rPNGaseF-30) is suitable for release of N-linked glycans in solution, and from immobilized samples. The enzyme cleaves between the innermost GlcNAc of the oligosaccharide moiety at its attachment point to the asparagine residue on the protein and subsequently converts the asparagine into aspartic acid. Released glycans can be labeled using LudgerTag Velocity labeling technology for fluorescence detection.

Description

PNGase F (EC 3.5.1.52) is a recombinant glycosidase cloned from *Elizabethkingia miricola* and expressed in *E. coli*. The enzyme is supplied glycerol free (for optimal performance in HPLC intensive methods) along with Glyco Buffer, Denaturation Solution and NP-40 Solution for efficient de-glycosylation. The methods described in this document have been developed and validated at Ludger.

Specificity

PNGase F is suitable for release of all types (high-mannose, hybrid and complex) N-glycans from glycoproteins and glycopeptides. Xaa-Asn-Xaa sequence is the minimal peptide substrate for this enzyme. Note that some non-mammalian glycans from sources such as plants, insects and parasites carrying α1-3 linked core fucose will not be cleaved with PNGase F. For these samples PNGase A can be used.

No. of Samples

The kit contains 15,000 units of PNGase F at concentration of 500,000 units/ml. Sufficient for approximately 30 samples.

Heat Inactivation

PNGase F is inactivated after 10 minutes at 75°C.

Time Line for Procedure

Procedure	Approx. Time
Sample preparation	5 min
Protein denaturation	10 min
Addition of enzyme	5 min
Incubation	10min to 3h

Method

The method described here is for in-solution release of N-glycans from glycoproteins/glycopeptides under denaturing conditions. Typical reaction conditions are demonstrated. The exact amount of enzyme and incubation times should be determined empirically for each glycoprotein and may require further optimisation.

The de-glycosylation rate can be determined by analysis of the remaining protein moiety using SDS-PAGE or alternatively, MS analysis of digested peptides. Released N-glycans can be analysed using chromatographic and mass spectrometric techniques in order to obtain their structural information.

An IgG glycoprotein standard (#GCP-IGG-100U) for use as a positive control in glycan release protocols is available to order from Ludger.

Denaturing reaction conditions

For many glycoproteins, the conformation of the protein in its native form can create steric hindrance that restricts access of any PNGase F enzyme to certain glycosylation sites. For this reason we recommend denaturation of samples using SDS and DTT (which are components of the Denaturation Solution), prior to enzyme incubation to aid efficient de-glycosylation.

Step 1: Sample preparation

- Ensure that samples are free of other contaminating glycoproteins prior to N-glycan release. Use up to 100 µg of glycoprotein per replicate. Dry the samples down if the volume exceeds 9 µL.
    - Make up sample volume to 9 µL with ultrapure water.

Step 2: Kit preparation

- Vortex and briefly centrifuge all four components of the kit befor using it.

To ensure that the component are fully in solution, Denaturation solution and Glyco buffer-2 can be placed in the oven at 37°C for 5-10min before using it.

Step 3: Denaturation of the protein

- Add 1 µL of 10X Denaturation Solution to each glycoprotein sample. Close the reaction vials, vortex thoroughly to ensure the samples are completely dissolved and briefly centrifugeto ensure samples are at the bottom of the vials.
- Incubate the samples at 100°C for 10 minutes
- Cool the samples to room temperature and briefly centrifuge before proceeding to the next step.

Step 4: Incubation

- Add 2 µL of 10xGlycobuffer to each glycoprotein sample.
- Add 2 µL of 10% NP-40 solution.

PNGase F is inhibited by SDS, therefore it is essential to have NP-40 in the reaction mixture when you have used denaturing conditions. Failure to include NP-40 into the denaturing protocol will result in loss of enzymatic activity.

- Adjust the reaction volume to 20 µL by adding 6 µL of water.
- Add 1 µL of PNGase F. Close the reaction vials, mix gently and briefly centrifuge.
- Incubate the samples at 37°C for 1 hour.

Different glycoprotein classes as well as heavily glycosylated proteins may require different incubation time typically varying from 10 minutes up to 3 hours. Make sure total incubation time does not exceed 3 hours as this may lead to aminoglycans to hydrolysed.

Part 2: V-tag labeling of glycans

Application For the fluorophore labeling of aminoglycans.

Description This kit contains the reagents needed for the conjugation of V-tag dye (LT-VTAG-02) to the aminoglycans, and the specialised solid phase extraction (LC-VSPE-30) cartridges required for the purification and enrichment of the labeled glycopeptides.

Dye Properties Mass = 434.42 (resulting in a glycopeptide mass increase of 319.33 Da.)
Fluorescence, λ_{ex} = 250 nm, λ_{em} = 360 nm.

Labeling Selectivity One V-tag label for every aminoglycan

Time Line for Labeling

Procedure	Time
Addition of dye to samples	5 min
Incubate samples	15 min
Clean up using SPE cartridges	1.5 hours

Method

Step 1: Dissolve the V-tag dye in DMSO

- The V-tag dye is supplied as a solid. Add 330 µL of DMSO to the dye provided and dissolve by pipette action until the dye is completely dissolved to make the final labeling reagent.
  Once the V-tag dye is used the spare solution can be re-frozen and re-used. As with many fluorescent dyes, care should be taken to minimise exposure to light as it will degrade the dye over time.

Step 2: Add V-tag dye to samples

- Add 10 µL of the V-tag dye directly to each enzyme digested sample. Vortex and briefly centrifuge the samples.

Step 3: Incubate

- Place the reaction vials in a heating block or dry oven set at 37°C and incubate for 15 minutes.

Step 4: Centrifuge and cool

- After the incubation period, briefly centrifuge the micro-tubes and allow them to completely cool to room temperature.

Step 5: Prepare the SPE solution for clean up: 90% Acetonitrile/10% 50mM Ammonium formate pH = 4.4

Part 1 - Preparation of the 50mM Ammonium formate, pH 4.4

- Add 117 mL of MiliQ water to the 3mL of LC-N-BUFFx40-96 and mix. The 3 ml of x40 buffer will make 120 ml of LS-N buffer.
  Note: Shake the LC-N-BUFFx40-30 bottle before use.

Part 2 - Preparation of 90% Acetonitrile/10% 50mM Ammonium formate

- Measure 225mL of acetonitrile into a graduated cylinder and pour then into a bottle.
- Measure 25mL of the 50mM Ammonium formate solution into a graduated cylinder, and pour into the bottle with the acetonitrile to mix

Step 6: Prepare the SPE solution for elution: 400mM Ammonium Acetate/5% ACN

- Add 2mL of Acetonitrile to the 38mL of LC-AA-BUFF-30 and mix.
  Note: Shake the LC-AA-BUFF-30 bottle before use.

Step 7: Prepare the LC-VSPE-30 cartridges

- Place a LudgerClean™ VSPE cartridge for each sample into the cartridge holder, and position onto a vacuum manifold. For a more in-depth description of how to set up the cartridges on the vacuum manifold see the LudgerVelocity SPE vacuum manifold system Guide found on our website www.ludger.com

- Prime each LudgerClean™ VSPE cartridge by adding the following solutions, applying a slow vacuum to drain and discarding the flow-through.

Reagent	Volume (ml)
1st wash:Water	1
2nd wash: 90% Acetonitrile/10% 50mM Ammonium formate	1

Note: Ensure that the LC-VSPE-30 are at RT before use them. Once you have open the seal bag, store the remaining cartridges at RT.

Step 8: Prepare the glycans samples and apply to the LC-VSPE-30 cartridge

- Pipette 350 µL of 100% acetonitrile into the fluorophore labeled sample (typically the volume of the fluorophore labeling mix + glycan sample is 31 µL). Gently mix the sample by pipette action and immediately load each sample onto a primed cartridge. Wait 15 minutes and then apply a slow vacuum (taking approximately one minute) to drain the LC-VSPE-30 cartridge.

Note: In order to avoid sample precipitation, the addition of acetonitrile should be performed just before applying the sample onto the cartridge. Acetonitrile should be added one sample at a time.

Step 9: Wash the LC-VSPE-30 cartridges

- Wash the cartridges with 1 mL 90% Acetonitrile/10% 50mM Ammonium formate by applying the solution to the top of the cartridge and allow a slow vacuum to pull the solution through over a period of a few minutes. Repeat with 2 additional washes of 1 mL 90% Acetonitrile/10% 50mM Ammonium formate. In each case discard the flowthrough.

Step 10: Elute the labeled glycans

- Remove the waste reservoir from the vacuum manifold and replace with a collection plate. Without applying a vacuum, elute the labeled glycans by adding 0.5 mL 400mM Ammonium Acetate/5% ACN to the top of each cartridge and allow about 15 minutes for the solution to pass through the cartridge and drain into the collection plate. After the 15 minutes, apply a slow vacuum taking approximately one minute to completely elute the samples. Repeat with 1 additional elution of 0.5 mL 400mM Ammonium Acetate/5% ACN.
- The samples are now ready for HPLC/UHPLC analysis.

Analysis of released N-glycans

LudgerTag™ V-tag labeled glycans are analysed by HPLC/UHPLC.

U/HPLC analysis

LudgerTag™ V-tag labeled glycan mixtures are separated and analysed by a variety of HPLC/UHPLC (high pressure liquid chromatography) systems and columns. For the best resolution results we recommend the use of UHPLC separation. Detailed below are the suggested UHPLC conditions which were used for V-tag labelled glycans release from IgG and Fetuin.

UHPLC sample preparation:

- 24 µL of LC-VSPE-30 eluted glycans in 400mM Ammonium Acetate/5% ACN plus 76 µL ACN.
- Sample injected: 20 µL
- Injection mode: partial or full loop
- Eluent A: 50 mM Ammonium Formate, pH = 4.4
- Eluent B: Acetonitrile.
- Temperature: 60 °C
- Detection: Fluorescence, λ_{ex} = 250 nm, λ_{em} = 360 nm

Time (min)	Flow Rate (mL/min)	%A	%B
Initial	0.4	24	76
53.5	0.4	45	55
54.5	0.2	100	0
57.5	0.2	100	0
59.5	0.2	24	76
65.5	0.2	24	76
66.5	0.4	24	76
70.0	0.4	24	76
70.1	0.0	24	76

Table 1: Chromatography 70 min gradient used for a BEH Glycan 2.1 x 150 mm column (Waters) on a Thermo U3000 UHPLC.

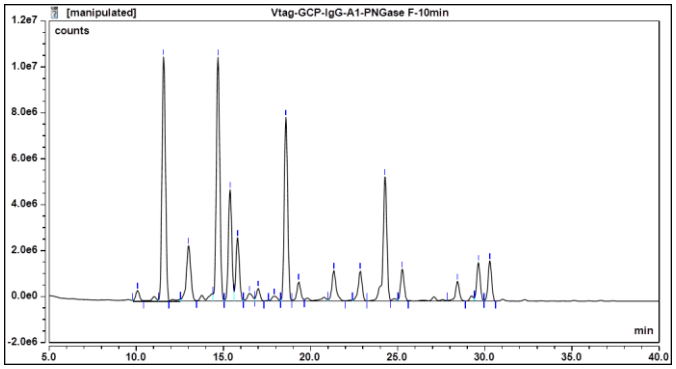


Figure 1: Chromatogram of V-tag labelled glycans released from IgG after 10 minutes PNGase F digestion. Analysis performed on a Thermo U3000 UHPLC using a BEH Glycan 2.1 x 150 mm column (Waters) with a 70 minute gradient.

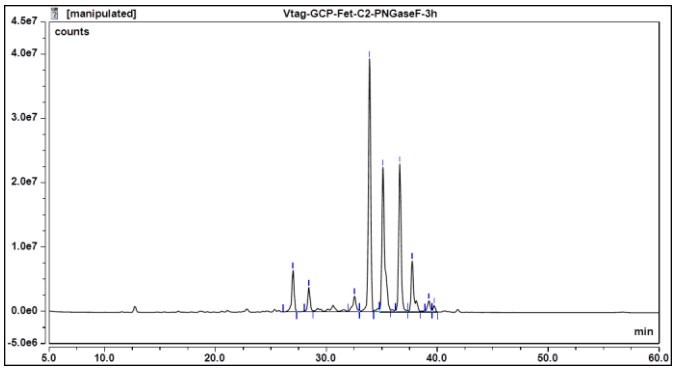


Figure 2: Chromatogram of V-tag labeled glycans released from bovine fetuin after 3 hours PNGase F digestion. Analysis performed on a Thermo U3000 UHPLC using a BEH Glycan 2.1 x 150 mm column (Waters) with a 70 minute gradient.

Appendix 1: Troubleshooting Guide

The following is a guide to the most likely problems associated with the use of the PNGase F kit for the release of glycans from glycoproteins and glycopeptides.

The positive control gives negative results.

The enzyme became inactive

Long-term storage of the PNGase F at a temperature different from that recommended can result in loss of enzymatic activity. For the best performance, store the kit components at 4°C.

Following the protein denaturation step ensure that the sample is cooled to room temperature before addition of the enzyme. Adding the enzyme to solution which has not been cooled down completely may cause enzyme denaturation and a decrease in release efficiency.

Post-release sample processing resulted in glycan loss

Make sure that your post-release glycan processing (including glycan clean-up methods) did not result in glycan loss or precipitation. For glycan preparation for chromatography and mass spectrometric applications refer to Ludger Guides (www.ludger.com/products).

The glycan release was not efficient.

The glycoproteins are not dissolved

If the solubilisation of glycoproteins is insufficient the glycan release will be incomplete. To ensure sample is dissolved properly, vortex sample longer or make up the release solution in a larger volume of reaction mixture.

The sample contained contaminants that interfered with PNGase F activity

Please ensure that the glycoprotein solution is free from contaminants before glycan release. PNGase F is compatible with wide range of buffers, however, some buffers additives can impact enzyme activity. Also avoid high ionic strength buffers in your sample as they can alter pH of the reaction mixture. Keep the pH of final reaction mixture within the PNGase F activity range (pH 6-10).

The incubation condition was incorrect

Ensure that the oven or heating block is equilibrated to the incubation temperature and that the reaction tube is subjected to this temperature for the entire period.

There was less starting glycoprotein material than was originally estimated

Please ensure sufficient amount of sample is used.

Skewing of the results was observed.

PNGase F incubation time was not sufficient

Some glycoforms or glycosylation sites of the protein can be less prone to de-glycosylation with PNGase F and for those, glycan release can occur with lower speed. Ensure that de-glycosylation time has been adjusted to your specific glycoprotein and its glycosylation level. Note that release will typically take longer under non-denaturing conditions.

Reagents were added in inadequate proportions

Ensure that appropriate proportion of reagents was used in the reaction. Failure in addition of Denaturation Solution may result in higher rate of sialylated glycans over neutrals being released, however, excessive amount of SDS will greatly impact enzymatic activity. Ensure that NP-40 (which stabilises the enzyme in the presence of denaturant) is present in the reaction mixture during PNGase F incubation under denaturing conditions.

Sample contains contaminating glycoproteins

PNGase F enzyme will remove N-glycans from all the proteins present in the reaction mixture. If you are interested in a specific glycoprotein, ensure that effective purification methods have been applied. Protein purity can be determined using SDS-PAGE analysis.

Warranties and liabilities

Ludger warrants that the above product conforms to the attached analytical documents. Should the product fail for reasons other than through misuse Ludger will, at its option, replace free of charge or refund the purchase price. This warranty is exclusive and Ludger makes no other warrants, expressed or implied, including any implied conditions or warranties of merchantability or fitness for any particular purpose.
Ludger shall not be liable for any incidental, consequential or contingent damages.

This product is intended for *in vitro* research only.

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