Proteomics Grade Trypsin

Catalog #204310

Protocol

Version A, January 2010

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Notices

Manual Part Number

Edition

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Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This document describes how to use Proteomics Grade Trypsin to prepare proteolytic peptides for mass spectrometric analysis.

If you have comments about this protocol, send an e-mail to feedback_lcms@agilent.com.

1 Before You Begin

This chapter contains information (such as required reagents and equipment) that you should read and understand before you start an experiment.

2 Procedures

This chapter describes how to prepare, digest and extract peptides using Proteomics Grade Trypsin.

3 Troubleshooting

This chapter contains instructions to troubleshoot this protocol.

Contents

Contents

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Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

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Kit contents

Table 1   Kit Contents

Product Name	Content
Proteomics Grade Trypsin	100 µg

Conditions

Store the lyophilized enzyme at -20°C. After the enzyme is resuspended, aliquot and store at -20°C for up to one month or at -80°C for long-term storage. Do not exceed five freeze/thaw cycles.

Required equipment and supplies

Table 2   Required Equipment

Description	Company and catalog number
Non-stick microcentrifuge tubes	VWR p/n 20170-315 or equivalent
Vacuum centrifugal concentrator	Savant SpeedVac or equivalent
Razor blade or scalpel	
Spatula	
Reverse-phase (C-18) purification tips	Agilent p/n 5188-5239

Required reagents

The reagents you need depend on whether you do an in-gel (Table 3) or in-solution (Table 4) digestion.

Table 3   For In-Gel Digestion

Reagent	Formula
Acetic Acid	CH_3COOH
Ammonium bicarbonate	NH_4HCO_3
Acetonitrile, HPLC-grade	CH_3CN
Iodoacetamide	ICH_2CONH_2
Formic acid	HCOOH
DTT (dithiothreitol)	
CaCl$_2$ (accelerated in-gel digestion protocol only)	

Table 4   For In-Solution Digestion

Description	Formula
Tris-HCl (pH8)	
Urea	
DTT (dithiothreitol)	
Ammonium bicarbonate	NH_4HCO_3
Formic acid	HCOOH

Overview

Mass spectrometry is a key technology to identify individual protein species in proteomics experiments.[*] Proteomics Grade Trypsin is used to prepare proteolytic peptides for mass spectrometric analysis. Porcine pancreas trypsin is modified by reductive methylation and other procedures to produce Proteomics Grade Trypsin, with enhanced specificity and resistance to autolytic cleavage, ideal for mass spectrometry applications. Protocols are included for both in-gel and in-solution digestion of protein samples.

Trypsin cleaves protein peptide bonds specifically on the carboxy side of lysine and arginine residues. For most cellular proteins, this cleavage specificity results in digestion products of the appropriate size range for mass spectrometric analysis. To identify the protein of interest, analysis of the set of proteolytic peptides observed in the mass spectrum can be compared to the proteolytic peptides that are predicted from protein sequence information.

The utility of trypsin in mass spectrometric proteomics experiments is dependent on the strict specificity of the protease. Native trypsin undergoes considerable autolysis, generating proteolytic peptides that can interfere with analysis of the protein of interest. In addition, autolysis of trypsin produces pseudotrypsin, a derivative with broadened cleavage specificity.

To overcome these problems, Agilent Proteomics Grade Trypsin is modified to achieve maximum specificity. First, the porcine pancreas trypsin is reductively methylated to produce an enzyme that retains activity on exogenous substrates but is highly resistant to autolysis. The modified enzyme preparation is then treated with TPCK, an inhibitor of the common contaminant chymotrypsin. Finally the modified, treated trypsin is affinity purified and lyophilized.

The Proteomics Grade Trypsin that results retains the ability to produce three autolytic fragments. These fragments correspond to monoisotopic masses (M+H)+ of 842.5099 Da, 1045.5642 Da and 2239.1359 Da, when the protease is subjected to typical reaction conditions. The presence of these peptides does not interfere with mass spectral analysis.

Surrounding residues have a limited influence on proteolytic cleavage by trypsin at arginine and lysine residues. The presence of a proline residue adjacent to the susceptible bond (on the carboxy side of arg or lys) confers

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[*]   Lahm, H. W. and Langen, H. (2000) Electrophoresis. 21(11):2105-14
      Patterson, S. D. and Aebersold, R. H. (2003) Nat Genet. 33 Suppl:311-23.

resistance to cleavage at that bond. To a lesser extent, the presence of an acidic residue on either side of the arg or lys residue decreases cleavage at that site.
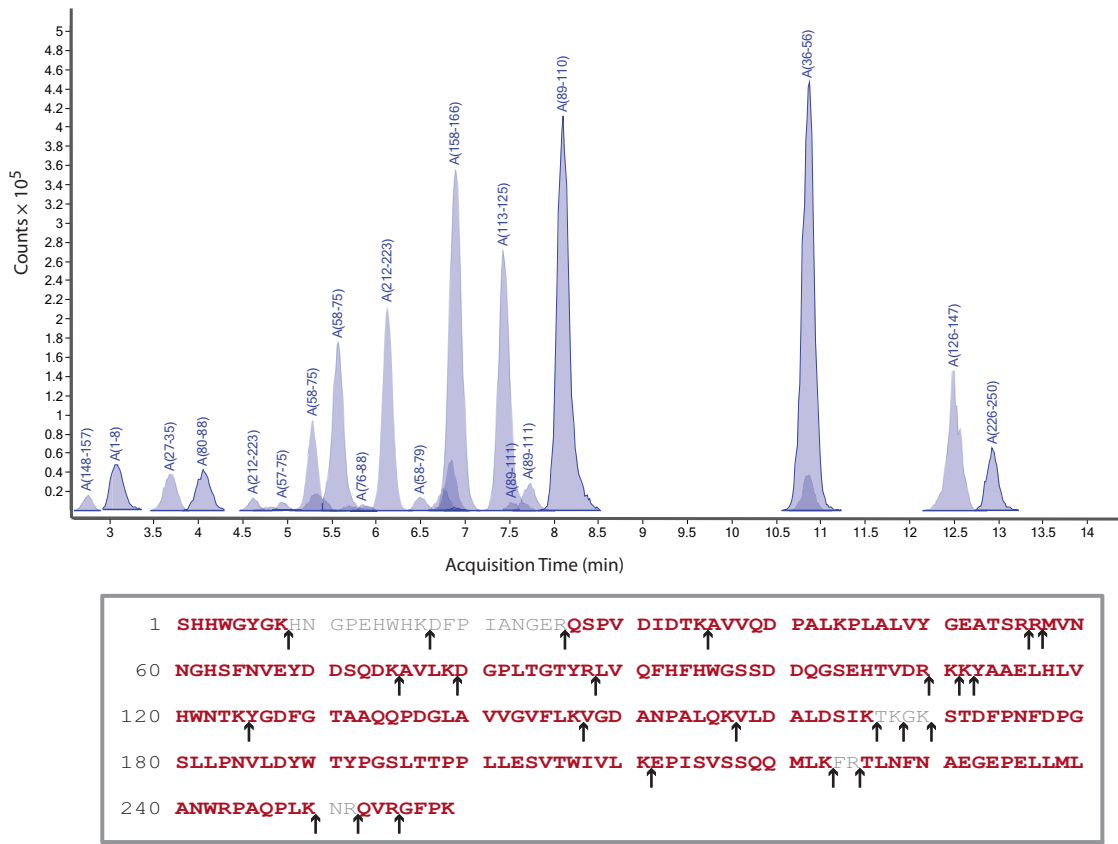
Figure 1   Total ion chromatogram and peptide coverage map of bovine carbonic anhydrase II digested with Agilent Proteomics Grade Trypsin and analyzed using Agilent's 6520 Accurate Mass Q-TOF with the HPLC-Chip interface.
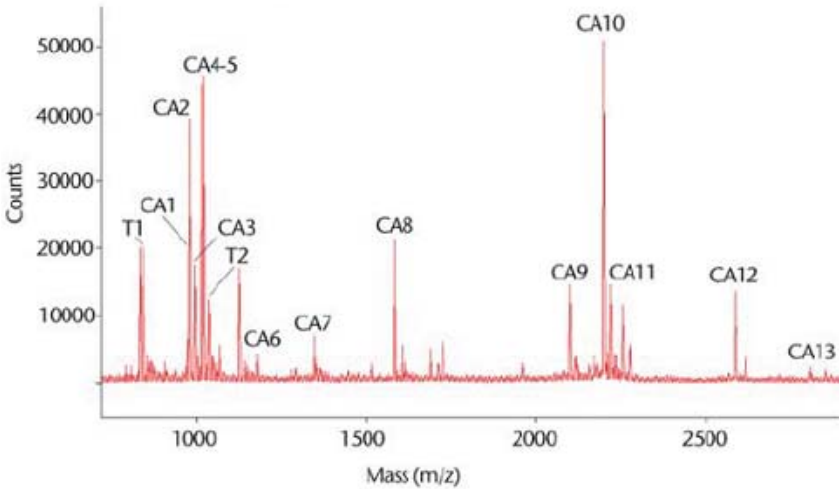
Label	Fragment size
CA1	973.54
CA2	979.48
CA3	1001.47
CA4	1013.45
CA5	1018.49
CA6	1141.54
CA7	1346.70
CA8	1581.82
CA9	2099.88
CA10	2198.22
CA11	2253.18
CA12	2584.19
CA13	2852.49
T1	842.51
T2	1045.48

Figure 2   MALDI-TOF mass spectrum of bovine carbonic anhydrase II digested with Agilent Proteomics Grade Trypsin.
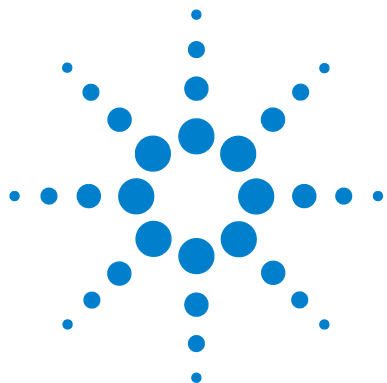
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Procedures

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Preparation

To prepare trypsin

CAUTION   Do not freeze and thaw the trypsin more than five times after the lyophilized powder is resuspended, or you can have loss or reduction of activity.

1 Dissolve the lyophilized proteomics grade trypsin in 100 µL of 50 mM acetic acid, to a final concentration of 1 µg/µL.

2 Mix by gently pipetting up and down.

3 Keep the trypsin solution on ice and transfer the solution into 10 aliquots of 10 µL each.

   Store the aliquots at -20°C for up to one month or at -80°C for long term storage.

During use

1 Thaw the reconstituted proteomics grade trypsin at room temperature.

2 Place immediately on ice.

3 Remove the amount of enzyme required for the experiment and then refreeze the unused portion. Do not exceed five freeze-thaw cycles.

To prepare reagents and supplies

• Wash non-stick (low protein binding) microcentrifuge tubes with 100% ethanol. Allow the tubes to dry completely.

• Use only ultrapure H_2O ($\geq$18 MΩ) for reagent preparation.

To prepare gel for In-Gel Digestion protocol

Each in-gel digestion reaction is designed for the preparation of proteolytic peptides from a protein band or spot after 1-D or 2-D electrophoretic separation of protein samples.

1 Do SDS-PAGE using polyacrylamide gels that are 1 to 1.5 mm thick.

2 Stain the gels with Coomassie blue stain or silver stain. (For silver stained gels, you need to do a mass spectrometry-compatible silver destaining procedure before you do the gel core destaining protocol.)[*]

Best results are achieved when the amount of protein in the excised band is $\geq$ 1 pmol. This approximately corresponds to the sensitivity of Coomassie stain-based detection.

3 Excise a gel core (~1 mm × 4 mm) that contains the protein spot or band of interest from the polyacrylamide gel.

4 Carefully cut the gel using a clean scalpel or razor blade. Cut as close as possible to the stained protein band or spot. To facilitate gel core excision, place the gel on a glass plate in a puddle of H_2O before you cut the gel.

5 Clean the blade and the glass plate with ethanol before use.

6 Cut the gel core into ~1 mm^3 pieces and put the gel pieces in non-stick 1.5-mL microcentrifuge tubes (see "To prepare reagents and supplies" on page 14). Use a clean spatula or the edge of a razor blade.

7 Spin the tube in a microcentrifuge at 14K rpm for 10 seconds to collect the gel pieces at the bottom of the tube.

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[*]   Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal Chem. 68(5):850-8.

Protocol 1: In-Gel Digestion

Step 1. Gel core destaining, protein reduction and alkylation

1 Prepare gel cores. See "To prepare gel for In-Gel Digestion protocol" on page 15.

Volumes of reagents in this step are appropriate for gel cores of ~1 mm × 4 mm × 1 mm. If the gel core is significantly larger than 4 mm^3, increase the volumes of reagents accordingly.

2 Destain silver-stained gels with a mass spectrometry-compatible silver destaining protocol.

3 Add 200 µL of wash solution (50% CH_3CN, 50 mM NH_4HCO_3) to the sample tube.

4 Mix the sample on a vortex mixer continuously for 10 minutes at room temperature.

5 Remove and discard the liquid using a pipette. Repeat two more times for a total of three washes.

6 Add 200 µL of acetonitrile (CH_3CN) to the gel core.

7 Mix on a vortex briefly, then incubate the tube at room temperature for 5 minutes.

8 Remove and discard the liquid. The gel pieces will appear shrunken and can appear opaque at the end of this treatment.

9 Remove the residual acetonitrile from the destained gel core sample with a heated vacuum centrifugal concentrator for 5 minutes.

10 Prepare a solution of 10 mM DTT in 50 mM NH_4HCO_3.

NOTE   Prepare fresh iodoacetamide and DTT solutions just before use.

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11 To reduce the protein sample, add 100 µL of the freshly prepared DTT solution to the dried gel core and incubate the tube at 55°C for 1 hour. Remove and discard the liquid.

12 Prepare a solution of 55 mM iodoacetamide in 50 mM NH_4HCO_3.

13 To alkylate the protein sample, add 100 µL of the freshly prepared iodoacetamide solution to the sample tube and mix on a vortex.

14 Incubate the tube in the dark at room temperature for 45 minutes. Remove and discard the liquid.

15 Add 200 μL of wash solution (50% CH_3CN, 50 mM NH_4HCO_3) to the sample tube. Mix the tube on a vortex continuously for 10 minutes at room temperature.

16 Remove and discard the liquid. Repeat two additional times for a total of three washes.

17 Add 200 μL of acetonitrile (CH_3CN) to the gel core.

18 Mix briefly on a vortex mixer, then incubate the tube at room temperature for 5 minutes.

19 Remove and discard the liquid.

20 Remove the residual acetonitrile from the destained gel core sample with a heated vacuum centrifugal concentrator for 5 minutes.

Step 2. In-gel digestion with trypsin and peptide extraction

You have two options to do this step.

In the first option (Conventional Digestion Protocol), the in-gel digestion is done overnight at 37°C. In the second option (Accelerated Digestion Protocol), the in-gel digestion is done for 30 minutes at 55°C. The first option produces more complete protein digestion. Use it when you need to obtain maximum sequence coverage or when you are analyzing proteins that are refractory to digestion by trypsin.

Option 1: Conventional Digestion Protocol

This protocol requires approximately 12 hours (digestion is done overnight).

1 Reconstitute the proteomics grade trypsin. See "To prepare trypsin" on page 14.

2 Dilute an appropriate volume of the 1 µg/µL trypsin to 20 ng/µL in 50 mM NH_4HCO_3.

NOTE   Store the unused portion of concentrated trypsin at -20°C for up to one month or at -80°C for long-term storage. Divide into small enough aliquots to avoid multiple freeze-thaw cycles.

3 Add 15 µL of the 20 ng/µL trypsin solution to the dried gel core.

4 Incubate the sample for 1 hour at 30°C to allow gel rehydration.

5 Add sufficient digestion buffer (50 mM NH_4HCO_3/10% CH_3CN) to the sample tube to completely cover the gel pieces. Make sure that the tube is capped tightly to prevent evaporation.

6 Incubate the tube at 37°C overnight.

7 Add 50 µL of ultrapure (≥18 MΩ) H_2O to the in-gel digestion mixture.

8 Mix the tube on a vortex mixer continuously for 10 minutes at room temperature.

9 Remove and keep the liquid in a fresh, non-stick microcentrifuge tube.

10 Add 50 µL of 50% CH_3CN/5% (v/v) formic acid to the gel pieces.

11 Incubate the tube for 60 minutes at room temperature. Mix frequently on a vortex mixer.

12 Spin the tube briefly in a microcentrifuge and then collect the liquid with a pipet. Add the liquid to the liquid collected in step 9.

13 Reduce the volume of the pooled liquid from the previous step. Use a centrifugal concentrator (at room temperature), until the appropriate volume (typically 10 to 20 μL) is reached.

14 If you want to desalt or concentrate the sample before mass spectrometry, use a reverse-phase C-18 cleanup pipette tip (Agilent p/n 5188-5239) to purify the peptides.

If online desalting will be used as part of an LC-MS method, then the extracted peptide solution may be used directly after centrifugal concentration.

Option 2: Accelerated Digestion Protocol

You need approximately one hour to complete this protocol.

1 Reconstitute the proteomics grade trypsin. See "To prepare trypsin" on page 14.

2 Dilute an appropriate volume of the 1 μg/μL trypsin to 100 ng/μL in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$.

NOTE   Store the unused portion of concentrated trypsin at -20°C for up to one month or at -80°C for long-term storage. Divide into small enough aliquots to avoid multiple freeze-thaw cycles.

3 Add 3 μL of the 100 ng/μL trypsin-digestion buffer solution to the dried gel core.

4 Incubate the sample for 5 minutes at room temperature.

NOTE   If you want to reduce the size of the autolytic trypsin fragment peak (m/z = 842.5099) in the mass spectrum, reduce the amount of trypsin to 1.5 to 2 μL.

5 Add 17 μL of freshly-prepared, room temperature digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$) to the sample tube.

6 Incubate the tube at 55°C for 30 minutes. The digestion buffer should just cover the gel pieces.

To limit condensation in the sample tube, use a dry 55°C incubator instead of a water bath.

7 Extract the peptides from the gel core: Add 15 µL of 5% (v/v) formic acid to the tube that contains the gel pieces, enzyme and digestion buffer. Mix the tube on a vortex mixer continuously for 15 minutes at room temperature.

8 Briefly spin the tube in a microcentrifuge.

9 If sample desalting or concentration is desired prior to performing mass spectrometry, purify the peptides using a reverse-phase C-18 cleanup pipette tip.

10 If online desalting will be used as part of an LC-MS method, then the extracted peptide solution may be used directly. In this case, spin the sample tube at 14K rpm for 1 minute and then remove a suitable volume of the supernatant for LC-MS analysis.

Protocol 2: Protein Digestion in Solution

Step 1. Protein reduction and denaturation

Complete digestion requires reduction of disulfide bonds and denaturation of the protein. For partial digestion of native proteins, the following steps may be omitted. Ensure that the native protein sample is in a buffer with pH 7–9 before proceeding to the Digestion with Trypsin (In Solution) section.

1 Dissolve the protein in sample buffer that contains 50 mM Tris-HCl (pH 8), 5 mM DTT, and 8 M urea to a final protein concentration of approximately 1 mg/mL.

2 Incubate the dissolved protein sample at 60°C for 60 minutes. After the incubation period, allow the sample to cool to room temperature.

3 Add a sufficient amount of dilution buffer [50 mM NH_4HCO_3 (pH 7.8)] to bring the urea concentration to less than 1M.

Step 2. Digestion with trypsin (in-solution)

1 Add the appropriate volume of MS grade trypsin (1 µg/µL stock) such that the ratio of trypsin:protein in the sample is between 1:20 and 1:100 (w/w).

2 Incubate the digestion reaction at 37°C for >1 hour. Digestion times will vary. Some proteins require up to 24 hours for complete digestion.

3 To monitor the extent of protein digestion, remove an aliquot of the sample and analyze with an appropriate method (e.g. gel electrophoresis or reverse-phase HPLC). If protein digestion is incomplete, return the sample to 37°C for the appropriate amount of time to allow further digestion.

4 Terminate the digestion reaction by adding formic acid to a final concentration of 5% (v/v). Remove a 10 to 20 µL aliquot that contains 1 to 2 µg protein, and separate the protein from urea and other contaminants using a reverse-phase C-18 cleanup pipette tip (Agilent p/n 5188-5239).

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Troubleshooting

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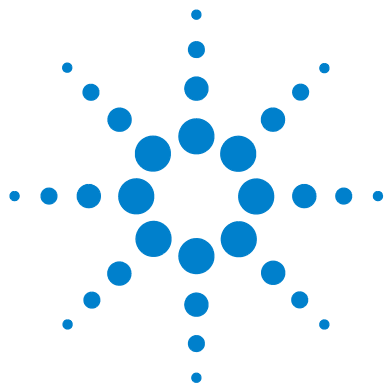
If peaks are completely absent in the mass spectrum

Failure to observe any peptide peaks may indicate that one or more protocol steps were not completed properly.

✔ Protein losses may have occurred during storage. If eluted peptides must be stored prior to analysis, use non-stick or low-protein-binding tubes (e.g. VWR, catalog #20170-315).

✔ Check that the correct reagents and reaction conditions (i.e. incubation temperature and duration) were used throughout the protocol.

If too many peaks are in the mass spectrum

The sample may be contaminated with keratin or other exogenous proteins.

✔ Wear gloves throughout the procedure and take care to use implements (razor blades, spatulas and glass plates) that have been freshly cleaned with ethanol.

If you get low-quality mass spectrum

The quantity of protein in the gel may be too low.

✔ Repeat the 1-D or 2-D electrophoresis and load a greater amount of the protein sample. Use a gel slice containing $\geq$ 1 pmol of protein (an amount that can typically be visualized after staining with Coomassie dye). Use a narrower pH gradient for the first dimension of a 2D electrophoresis experiment if necessary.

✔ Check that the gel slice is cut into 1 mm^3 pieces before you start the protocol. Peptide extraction is inefficient from large gel slices.

✔ Do not freeze and thaw the reconstituted trypsin multiple times. Limit the number of freeze-thaw cycles to five.

✔ Protein losses may have occurred during storage. If eluted peptides must be stored prior to analysis, use non-stick or low-protein-binding tubes (e.g. VWR, catalog #20170-315).

If you fail to determine protein identity after data mining

The protein that was isolated and analyzed may not be included in the database being queried.

If protein is identified with low confidence or low apparent sequence coverage

✔ Few proteolytic peptides were generated and available for analysis due to very low protein abundance in the sample. See "If you get low-quality mass spectrum" on page 25 for suggestions.

3   Troubleshooting
If protein is identified with low confidence or low apparent sequence coverage

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In This Book

This document describes how
to use Proteomics Grade
Trypsin to prepare
proteolytic peptides for mass
spectrometric analysis.

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