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Short Communication

An Optimized Method for Protein Extraction from OCT-Embedded Human Kidney Tissue for Protein Quantification by LC-MS/MS Proteomics[S]

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ABSTRACT

The existing biobanks of remnant tissue from clinically indicated kidney biopsies are attractive potential reservoirs for quantification of clinically relevant human tissue proteins by quantitative proteomics. However, a significant caveat of this strategy is that the tissues are often preserved in optimal cutting temperature (OCT) medium. Although OCT is an effective method of preserving the morphologic and immunohistological characteristics of tissues for later study, it significantly impacts efforts to quantify protein expression by liquid chromatography–tandem mass spectrometry methods. We report here a simple, reproducible, and cost-effective procedure to extract proteins from OCT-embedded tissue samples. Briefly, the excess frozen OCT medium was scraped before thawing from the tissue specimens stored at –80°C for ~3 months. The tissue samples were homogenized and diethyl ether/methanol extraction was performed to remove the remaining OCT medium. The recovered protein was denatured, reduced, and alkylated. The second step of protein extraction and desalting was performed by chloroform/methanol/water extraction of denatured proteins. The resultant protein pellet was trypsin-digested and the marker proteins of various kidney cellular compartments were quantified by targeted selective reaction monitoring proteomics. Upon comparison of peptide signals from OCT-embedded tissue and flash-frozen tissue from the same donors, both individual protein quantities, and their interindividual variabilities, were similar. Therefore, the approach reported here can be applied to clinical reservoirs of OCT-preserved kidney tissue to be used for quantitative proteomics studies of clinically relevant proteins expressed in different parts of the kidney (including drug transporters and metabolizing enzymes).

Introduction

Twenty-six million Americans adults have chronic kidney disease (CKD) (http://www.niddk.nih.gov/health-information/health-statistics/Pages/kidney-disease-statistics-united-states.aspx), and people with CKD are at higher risk for cardiovascular and all-cause mortality (Tonelli et al., 2006). However, development of new diagnostic and therapeutic tools for CKD is hampered by our incomplete understanding of its underlying pathophysiology. In this direction, targeted mass spectrometry is a powerful tool, enabling simultaneous quantification of several prespecified proteins in biologic samples (Hood et al., 2012). Determining the abundance of protein components or targets of a given pathway using quantitative proteomics can generate a snapshot of the pathway status and activity within these samples. More recently, kidney tissue has gained notice as a more informative biologic sample for proteomics studies aiming to understand CKD pathophysiology. However, although kidneys play a critical role in the elimination of many drugs (Feng et al., 2012; Yacovino and Aleksunes, 2012; Moss et al., 2014), we have a limited quantitative understanding of the protein expression of drug transporters and metabolizing enzymes in the kidney tissue. Further, these data are scarcely available from CKD patients. Quantitative characterization of the transporters and enzymes in healthy as well as diseased tissues is indispensable for building pharmacokinetic and pharmacodynamic models to predict drug disposition and response. As such, both targeted and untargeted, or "shotgun," proteomics methods have been applied to blood and urine in attempts to gain insight into mechanisms underlying CKD (Rossing et al., 2008; Filip et al., 2014). However, lack of power of such studies owing to the limitation of sample size often leads to inconclusive outcomes.

To overcome the sample-size problem, an attractive potential reservoir for such tissue is the existing biobanks of remnant tissue from clinically indicated kidney biopsies. Unfortunately, a significant caveat of this strategy is that these clinically stored tissues are often preserved in the optimal cutting temperature (OCT) medium, which interferes with mass spectrometry signal. On the other hand, mass spectrometric analyses perform best on protein extracted from flash-frozen tissue. Here, we describe optimization and evaluation of a method for membrane or structural protein extraction and targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS), selective reaction monitoring (SRM) on OCT-embedded kidney tissue and assess the method's performance compared with the gold-standard samples preserved by flash freezing.

Materials and Methods

Chemicals. The ProteoExtract Native Membrane Protein Extraction Kit was procured from Calbiochem/MerckMillipore (Darmstadt, Germany). The protein

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ABBREVIATIONS: CKD, chronic kidney disease; DOC, sodium deoxycholate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; OCT, optimal cutting temperature; OCT2, organic cation transporter 2; P-gp, P-glycoprotein.

quantification bicinchoninic acid (BCA) assay kit, sequencing-grade trypsin, iodoacetamide, and dithiothreitol were purchased from Pierce Biotechnology (Rockford, IL). Synthetic heavy peptides for all the surrogate peptides (Supplemental Table 1S) were obtained as internal standards from Thermo Fisher Scientific (Rockford, IL). Synthetic light peptides for the renal transporters OAT1, OAT3, OCT2, and P-glycoprotein (P-gp) were obtained as calibrators from New England Peptides (Boston, MA). Chloroform, ethyl ether, high-performance liquid chromatography (HPLC)-grade acetonitrile/methanol, and formic acid were purchased from Fischer Scientific (Fair Lawn, NJ). Ammonium bicarbonate (98% purity) and sodium deoxycholate (DOC, 98% purity) were obtained from Thermo Fisher Scientific (Rockford, IL) and MP Biomedicals (Santa Ana, CA), respectively.

Sample Procurement, OCT Removal, and Trypsin Digestion. Histologically normal sections of kidney tissue from healthy donors (n = 5, 3 male, 2 female, 53–67 years old) undergoing clinically indicated partial nephrectomy (e.g., removal of a kidney mass) were obtained after informed consent (Proteogenex, Culver City, CA). Specimens weighed 450–700 mg and were divided into two equal sections, with one section immediately frozen in liquid nitrogen and stored at –80°C. The second half was embedded in the OCT medium using a protocol followed by the University of Washington Department of Pathology; samples were subsequently transferred to –80°C. After 3 months of storage at –80°C, excess OCT around the tissue block was removed with a scalpel without compromising the embedded tissue. Two milliliters of extraction buffer I and 10 μl of protease inhibitor (Calbiochem ProteoExtract Native Protein Extraction Kit) were added to both sets of tissue samples. The samples were homogenized until completely suspended and then incubated on ice for 20 minutes on a rocker table to prevent the formation of cell clumps. The samples were then sonicated for 60 seconds and centrifuged at 16,000g, 4°C, for 15 minutes. The supernatant (the cytosolic fraction) was removed without disturbing the pellet and transferred to a new tube and set aside. One milliliter of extraction buffer II plus 5 μl of protease inhibitor was added to the pellet, which was then resuspended by pipetting up and down ten times or until the pellet was completely broken apart. The sample was incubated on ice for 30 minutes on a rocker table to prevent cell clumps from forming, then centrifuged at 16,000g, 4°C, for 15 minutes. The supernatant (membrane fraction) was removed without disturbing the pellet and transferred completely to a new tube. Total protein was quantified using BCA assay kit (Pierce Biotechnology) and the sample was diluted to 2 mg/ml before analysis.

To remove OCT contamination a previously developed method was modified (Weston and Hummon, 2013). First, 800 μl of diethyl ether and 200 μl of methanol were added to 50 μl of diluted protein sample (100 μg total protein). The sample was vortexed for 5 minutes and then centrifuged at 10,000g for 10 minutes. The upper and lower liquid layers were removed carefully so as not to disturb the interfacial pellet, and air dried. The pellet was resuspended in 80 μl of ammonium bicarbonate, 20 μl of DOC (3%), and 15 μl of 250 mM dithiothreitol and incubated for 5 minutes at 95°C. iodoacetamide (IAA) (18 μl of 500 mM) was added to the samples, and then they were incubated in the dark for 20 minutes. Beyond the previously reported method, a second cleaning step was added to decrease ion suppression in liquid chromatography–tandem mass spectrometry (LC-MS/MS) and to remove any contamination that could affect trypsinization. Five-hundred microliters of methanol, 100 μl of chloroform, and 400 μl of water were added and the samples were vortexed for 1 minute. The samples were centrifuged at 6000g, 4°C, for 5 minutes. The upper and lower liquid layers were removed without disturbing the interfacial layer. The pellet was washed with 1 ml of methanol followed by centrifugation at 6000g for 5 minutes. Then the methanol was completely removed and the sample was air dried. The pellet was completely resuspended in 20 μl of sodium DOC (3%) and 40 μl of 100 mM ammonium bicarbonate. Twenty microliters of 0.16 μg/μl trypsin was added to the sample, which was then incubated for 18 hours at 37°C, with gentle shaking to assist trypsin digestion. The reaction was quenched by adding 30 μl of heavy peptide cocktail in 80% acetonitrile containing 0.1% formic acid. The sample was centrifuged at 3000g for 2 minutes and 100 μl of the supernatant was transferred into LC-MS vials for analysis. The absolute quantification of OAT1, OAT3, OCT2, and P-gp was performed by using light surrogate peptides as calibrators per published protocol (Prasad et al., 2016).

LC-MS/MS Analysis. LC-MS/MS was performed to quantify 23 structural proteins expressed in different sections of the nephron, the functional unit of the kidney (Table 1). The surrogate peptides of the markers of various cellular compartments of kidney were quantified using triple-quadrupole LC-MS instruments [Xevo TQ-S coupled to ACQUITY UPLC (Waters, Milford, MA)] in positive electrospray ionization mode. Approximately 10 μg of the trypsin digest (5 μl) was injected onto the column (Waters 2.1 μm, C18 100A; 150 × 2.1 mm; Phenomenex, Torrance, CA) and eluted at 0.3 ml/min. A mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) was employed. A flow

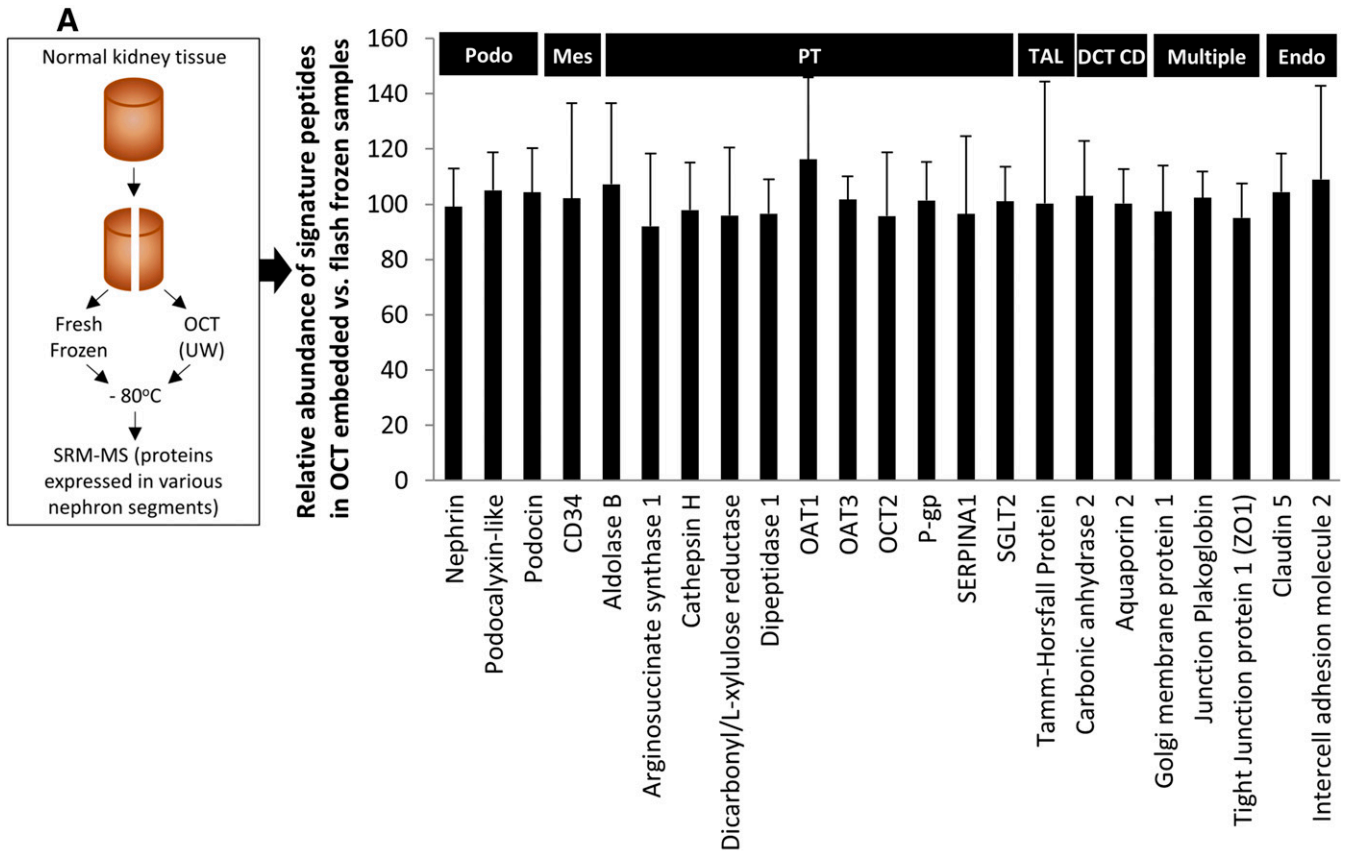
TABLE 1

Proteins and peptides quantified in the kidney tissue

Protein	Tissue Localization[a]	Detected Peptide
Nephrin	Podo	ELVLVTGPSDNQAK
Podocalyxin-like	Podo	LGDQGPPEEAEDR
Podocin	Podo	LPAGLQHSLAVEAEAQR and SLTEILLER
CD34	Mes, Glom endo	LGILDFTEQDVASHQSYSQK and SWSPTGER
Aldolase B	PT	ELSEIAQSIVANGK
Arginosuccinate synthase 1	PT	APNTPDILEIEFK
Cathepsin H	PT	VNHAVLAVGYGEK
Dicarbonyl/L-xylulose reductase	PT	TQADLDSLVR
Dipeptidase 1	PT	VASLIGVEGGHSIDSSLGVLR
OAT1	PT	TSLAVLGK
OAT3	PT	TVLAVFGK
OCT2	PT	SPGVAELSLR and LNPSFLDLVR
SERPINA1	PT	DTEEEDFHVDQVTTVK
SGLT2	PT	LEDISEDPSWAR
P-gp (MDR1)	PT, Mes	NTTGALTTR
Tamm-Horsfall protein	TAL	DWVSVVTPAR
Golgi membrane protein 1		DTINLLDQR
Aquaporin 2	CD	QSVELHSPQSLPR and SLAPAVVTGK
Carbonic anhydrase 2	DCT, CD	YDPSLKPLSVSYDQATSLR
Tight junction protein 1 (ZO1)	Podo, TAL, DCT, CD	DNPHFQSGETSIVISDVLK
Plakoglobin	PT, TAL, DCT, CD	LLNQPNQWPLVK
Claudin 5	Endo	EFYDPSVPVSQK and VYDSVLALSTEVQAAR
Intercellular adhesion molecule 2	Endo	ILLDEQAQWK

Podo, glomerular podocytes; Mes, glomerular mesangial cells; PT, proximal tubule; TAL, thick ascending limb of the loop of Henle; DCT, distal convoluted tubule; CD, collecting duct; Endo, endothelial cells

[a]Ernest et al., 1997; Kwon et al., 1998; Miyanaka et al., 1998; Motohashi et al., 2002; Reyes et al., 2002; Higgins et al., 2004; Kuusniemi et al., 2004; Aires et al., 2013; Motohashi et al., 2013; Fujinaka et al., 2014.



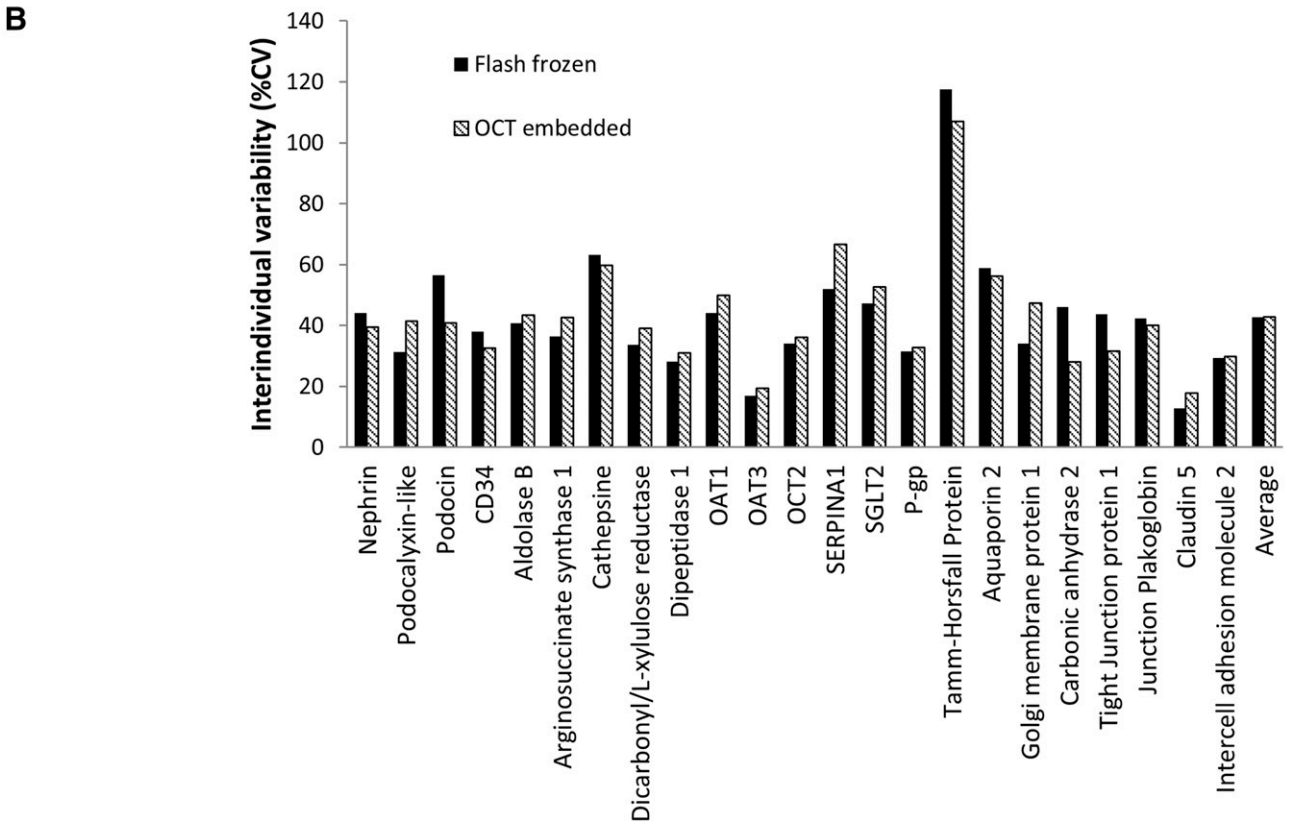


Fig. 1. Expression levels of selected proteins, shown as percent expression detected in OCT-embedded sample versus expression detected in flash frozen samples (A). The protein signals in OCT-embedded samples were measured at 101.1 ± 5.3% of the signal in flash frozen samples, indicating good recovery of proteins. Interindividual variability (%CV) of tissue protein expression determined in flash-frozen and OCT-embedded samples (B)

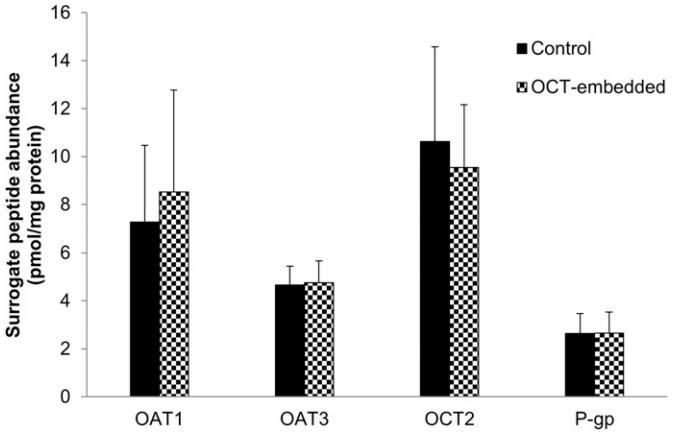


Fig. 2. Absolute protein abundance of OAT1, OAT3, OCT2, and P-gp in control (flash-frozen, solid black) and OCT-embedded (black and white pattern) human kidney samples (n = 5). The protein abundance is expressed as pmol/mg total membrane protein (mean ± standard deviation).

rate of 0.3 ml/min was used in a gradient manner (Supplemental Table 1S). MS/MS analysis was performed by monitoring the surrogate peptides and the internal standards using instrument parameters provided in Supplemental Table 1S.

Data Analysis. LC-MS/MS data were processed by integrating the peak areas generated from the reconstructed ion chromatograms for the surrogate peptides and the internal standards using MassLynx 4.1 (Waters, Hertfordshire, UK). The peak response for two to three transitions from each peptide was averaged for quantification of samples or standards. Paired Student's t test was used to compare peptide recovery between flash-frozen and OCT-embedded samples.

Results

The results of LC-MS/MS quantification of 23 structural proteins expressed in different sections of the nephron are presented as % expression of each protein in the OCT-embedded samples compared with the paired flash-frozen control sample (Fig. 1A). When comparing peptide signal from OCT-embedded tissue to flash frozen tissue, we observed a cumulative peptide signal recovery of 101.1 ± 5.3%. Individual protein quantities in OCT were 92.1–116.2% of that in flash-frozen tissue (Fig. 1A). Interindividual variability of all the targeted proteins was also comparable in OCT-embedded versus flash-frozen tissue (42.7% and 42.9%, respectively; Fig. 1B). At absolute level, OAT1, OAT3, OCT2, and P-gp expression (pmol/mg total protein) was 7.26 ± 3.21, 4.67 ± 0.79, 10.62 ± 3.96, and 2.64 ± 0.83 (control) versus 8.53 ± 4.25, 4.76 ± 0.92, 9.54 ± 2.63, and 2.66 ± 0.87 (OCT-embedded), respectively (Fig. 2). The latter indicates 100% recovery of the proteins from the OCT-embedded kidney samples.

Discussion

The method reported here yields comparable protein quantification by targeted mass spectrometry from OCT-embedded tissue versus the gold standard of flash-frozen tissue and enables the use of clinical stores of OCT-preserved kidney tissue for quantitative proteomics studies. Methods have been reported for protein extraction and mass spectrometry from formalin-fixed and paraffin-embedded (FFPE) tissue; however, a limited number of reports exist on mass spectrometry proteomic analysis of OCT embedded tissue samples (Azimzadeh et al., 2010; Tian et al., 2011; Quesada-Calvo et al., 2015; Zhang et al., 2015). These published reports are mainly discovery proteomics data, which focuses on qualitative appearance of peptide signals rather than absolute peptide quantitation.

OCT and FFPE media are not ideal matrices for mass spectrometry quantification. OCT is mainly composed of polyvinyl alcohol (PVA), polyethylene glycol (PEG), and nonreactive ingredients, which serve as cryopreservative medium and support matrix for tissue sectioning. Unless completely removed, PVA and PEG can suppress ionization of the targeted peptides (Schwartz et al., 2003). Starting from recently developed methods for OCT removal (Tian et al., 2011; Johnson and White, 2014; Zhang et al., 2015), we optimized a simple and cost-effective method to extract proteins from OCT-embedded kidney tissue samples for quantitative mass spectrometry applications. This method can be applied to clinical reservoirs of OCT-preserved kidney tissue for use in quantitative proteomics studies of clinically relevant proteins expressed in different parts of the kidney (including drug transporters and metabolizing enzymes). Differential interindividual variability of proteins was observed, indicating unique regulation (e.g., mediated by individual genetic and epigenetic factors) of these proteins. The highest variability was observed for Tamm-Horsfall protein, consistent with reported values (Fu et al., 2016). However, as the main aim of this report was methodological, unique interindividual variability has no effect on our conclusions. Quantitative characterization of the clinically relevant proteins, including transporters and enzymes, in healthy as well as diseased kidney tissues is indispensable for building better pharmacokinetic and pharmacodynamic models to predict drug disposition and response.

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Performed data analysis: Vrana, Afkarian, Prasad.
Wrote or contributed to the writing of the manuscript: Vrana, Goodling, Afkarian, Prasad.

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