MethodsX 5 (2018) 466–474

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

Tools and protocol for quantification of myosin phosphorylation with MRM-MS

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A B S T R A C T

The phosphorylation of myosin regulatory light chain (LC20) at Thr18 and Ser19 is positively correlated with tension development in smooth muscle tissue, and the molar stoichiometry of LC20 phosphorylation is commonly profiled as a measure of smooth muscle contractility. We provide details for a newly applied multiple reaction monitoring (MRM)-mass spectrometry (MS) method for the quantification of LC20 phosphorylation at Thr18 and Ser19. This MRM-MS method provides a robust alternative to antibody-based detection systems (such as Phos-Tag SDS-PAGE) for the quantification of LC20 phosphorylation.

A R T I C L E I N F O

Method name: MRM-MS myosin phosphorylation assay

Keywords: Smooth muscle, Myosin regulatory light chain, LC20, MLC, Artery, Intestine, Targeted proteomics, Quantitation, Stoichiometry, Phosphorylation, Serine 19, Threonine 18

Article history: Received 6 April 2018; Accepted 11 May 2018; Available online 17 May 2018

Specifications Table

Subject area	Biochemistry, Genetics and Molecular Biology
More specific subject area	Targeted proteomics
Method name	MRM-MS Myosin Phosphorylation Assay
Name and reference of original method	Not applicable
Resource availability	Skyline v3.7 program – https://skyline.ms/ Skyline Panorama Public Repository – https://panoramaweb.org

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https://doi.org/10.1016/j.mex.2018.05.004

Materials

Skyline software for targeted mass spectrometry environment

- Skyline is a freely-available, open-source Windows client application for building targeted quantitative proteomic methods and analyzing the resulting mass spectrometry data [1,2]. The Skyline v3.7 program is available for download from the MacCross Laboratory website: https://skyline.ms/project/home/software/Skyline/begin.view
- Panorama open-source repository server application for targeted proteomic assays that integrate into Skyline MRM-MS workflows [3]. Panorama is also available via the MacCross Laboratory website: https://panoramaweb.org.

In vitro *generation of phosphorylated LC20 protein*

- Smooth muscle myosin light chain (LC20), myosin light chain kinase (MLCK), and calmodulin proteins were purified from chicken gizzard as previously described ([4–6], respectively).
- HEPES buffer: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (200 mM) Dissolve in dH_2O and adjust pH to 7.4.
- $MgCl_2$ (100 mM) and $CaCl_2$ solutions (100 mM). Dissolve in dH_2O.
- ATP: adenosine 5'-triphosphate (100 mM). Dissolve in Tris-(hydroxymethyl) aminomethane hydrochloride (TRIS-HCl, 25 mM, pH 8.0). Neutral ATP solutions stored frozen at −20 °C are stable for at least one year.
- EDTA/EGTA quenching buffer: prepared by mixing 336 μl of 0.2 M EDTA and 192 μl of 0.2 M EGTA to give a final stock solution of 126 mM EDTA & 73 mM EGTA.

Preparation of smooth muscle tissue extracts

- TCA/DTT/acetone: 10% (w/v) trichloroacetic acid, 10 mM dithiothreitol in ice cold acetone.
- DTT/acetone: 10 mM dithiothreitol in ice cold acetone
- Lyophiliser- Freeze Dry System (Labconco, Freezone 6)
- Tissue extraction buffer: 50 mM ABC, pH 8.2, 50 mM NaCl, 1 M urea, 2% (w/v) sodium deoxycholate, 1 mM DTT and cOmplete protease inhibitor cocktail (Millipore-Sigma). LC20 is primarily associated with the insoluble fraction during isolation of muscle proteins with centrifugation, so we include of 2% (w/v) sodium deoxycholate and 1 M urea to enhance the solubilization efficiency.
- Micro ground-glass, Potter-Elvehjem tissue homogenizer
- Vortex shaker
- Refrigerated micro-centrifuge

Tryptic digestion

- ABC: 50 mM ammonium bicarbonate, pH 8. Make fresh by dissolving 0.039 g ammonium bicarbonate in 10 ml dH_2O. The ABC solution should have a pH of approximately 8. The pH does not need to be further adjusted.
- BSA: 10 mg/ml bovine serum albumin. Make fresh by dissolving in 50 mM ABC.
- IAA: 200 mM stock iodacetamide. Make fresh by dissolving 0.037 g iodoacetamide in 1 ml of 50 mM ABC.
- DTT: 1 M stock dithiothreitol, dissolve 0.154 g in 1 ml pure H_2O. Stored at −80 °C in small aliquots. Discard and do not refreeze once thawed. The DTT is not soluble in ice-cold ABC, and the solution must be warmed sufficiently to fully dissolve DTT.
- Mass spectrometry grade trypsin: 0.5 mg/ml (Promega). Solution made according to the manufacturer's manual. In brief, add 40 μl of the supplied resuspension buffer to one vial of trypsin, swirl the