

Preparation of iPSCs for targeted proteomic analysis

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Abstract

Induced pluripotent stem cells have great potential as a human model system in regenerative medicine, disease modelling and drug screening. However, extensive analysis of iPSC are required before their therapeutic applications. With recent developments in mass spectrometry and proteomics, this technique can become a great alternative to traditional genomic approaches for iPSC analysis. Here, we describe preparation of iPSC for targeted proteomic analysis, and measurement of pluripotency markers allowing for classification into either pluripotent or non-pluripotent cells.

Keywords

Induced pluripotent stem cells, pluripotency, proteomics, mass spectrometry

Introduction

Induced pluripotent stem cells (iPS cells) are somatic cells which acquire properties similar to the embryonic stem cells¹. There are multiple ways to generate iPSCs: retroviral transfection of transcription factors OCT4, SOX-2, c-MYC and KLF-4², plasmids³, non-integrating viruses (Sendai virus)^{4,5}, drug-inducible systems⁶, transposition⁷, protein transduction⁸ or mature microRNAs⁹. Yet, despite different techniques available, the reprogramming process yields low number of iPS cells and there is a need for an assay able to distinguish between pluripotent and non-pluripotent cells. Several assays for iPSC quality control are available: visualization of colony-morphology using light microscopy or extracellular marker staining, embryoid body or teratoma formation, as well as assays based on gene expression, i.e. PluriTest¹⁰ or ScoreCard¹¹. However these tests have major drawbacks, such as long performance time and significant cost.

With recent advances in targeted mass spectrometry, this technique is now enabling fast, reliable and absolute quantitation of proteins of interest, and supports not only basic, but also clinical research^{12,13}. Taking advantage of that, and in order to increase the simplicity and efficiency of iPSCs characterization, we have developed a peptide based multiple reaction monitoring-mass spectrometry assay (MRM-LC-MS/MS)¹⁴. In this assay, iPS cell pellets are digested and resulting peptides are analyzed by UPLC-MS/MS (Figure 1). At the first stage of method development, the list of potential pluripotency biomarkers was determined from the literature and current pluripotency tests (staining markers and self-renewal markers included in ScoreCard assay). Then, after sample preparation and analytical method refinement, the expression of five proteins: OCT4, SOX-2, LIN28, PODXL and CD44 were demonstrated as being able to discriminate between pluripotent and non-pluripotent cells. The pluripotency signature measured by the mass spectrometry was confirmed by the generation of three-germ layer embryonic bodies and the successful terminal differentiation of these clones into neuronal cells^{15,16}.

To summarize, a simple and high-throughput measurement of pluripotency signature can be achieved using as little as 200,000 cells by means of targeted mass spectrometry. Moreover, this assay is significantly simpler and faster than conventional genomic tests. Our test can be implemented in any laboratory, which has access and expertise in using triple quadrupole mass spectrometers and this method can be easily amended for measuring other proteins of interest in iPSC pellets, based on literature search or mass spectrometry discovery experiments.

Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical and mass spectrometry grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

1. Standard peptides:
 - a. The list of quantotypic peptides selected for pluripotency test is given in Table 1. (Note 1)
 - b. 50:50 (v:v) acetonitrile (CH₃CN)/ H₂O
2. Standard isotope labelled (SIL) analog of standard peptide:
 - a. The list of SIL peptides selected for pluripotency test is given in Table 1. (Note 2)
3. iPS cell harvest
 - a. 0.5M EDTA pH 8.0, diluted to 0.5mM in PBS
 - b. PBS
4. Digestion:
 - a. Digestion buffer: 100 mM Tris HCl, 6M Urea, 2M Thiourea, 2% ASB-14.
 - b. DL-dithiothreitol (DTT): 30 mg/mL in 100 mM Tris, pH 7.8. (Note 3)
 - c. Iodoacetamide (IAA): 36 mg/mL in 100mM Tris, pH 7.8. Protect from light. (Note 4)
 - d. Sequencing grade modified trypsin: 0.1 µg/µl in 50mM ammonium bicarbonate buffer pH 7.8. Store at -20 before ready to use.
5. Solid Phase Exchange (SPE):
 - a. Commercially available SPE C18 100 mg/1mL column
 - b. 0.2% Trifluoroacetic Acid (TFA)
 - c. 0.1% TFA
 - d. 70% CH₃CN in 0.1% TFA
6. Chromatography and Mass Spectrometry:
 - a. Solvent A: 0.1% Formic Acid in dd H₂O.
 - b. Solvent B: 0.1% Formic Acid in CH₃CN.
 - c. Waters CORTECS UPLC C18 Column, 90 Å, 1.6 µm, 2.1 mm x 50 mm attached to a C18 VanGuard pre-column
 - d. Acquity UPLC system (Waters) coupled to a Xevo™ TQ-S triple quadrupole mass spectrometer (Waters) equipped with electrospray source.

Methods

Carry out all procedures at room temperature unless stated otherwise. Always wear gloves to reduce keratin contamination.

1. Preparation of standard peptides and SIL analogs of standard peptides:
 - a. Resuspend synthetic peptides to 1 mg/ml stock concentration according to manufactures instructions. By default if instructions are not available, resuspend peptides in 50:50 (v:v) acetonitrile (CH₃CN)/ H₂O.
 - b. Prepare the 1:10 dilutions of the peptide from the stock concentration and pool 1000 pmoles of each peptide into a low binding microcentrifuge tube.
 - c. Prepare several pools of 1000pmols of SIL analogs of standard peptides.
 - d. Dry down in a speed-vac concentrator the final pool and store at -20 °C. Prepare several pools for future use.
 - e. Resuspend an aliquot of pooled 1000 pmol peptides in digestion buffer to obtain concentrations of 10 pmol/µl.
2. iPS cell harvest
 - a. Warm 0.5mM EDTA to 37°C

- b. Aspirate media from almost confluent well of iPS cells and add PBS to wash cells then immediately aspirate
 - c. Add warmed 0.5mM EDTA to cover surface of dish and incubate at 37°C for 5 minutes
 - d. Aspirate EDTA carefully to leave cells attached to dish, pipette PBS around surface of dish approximately 3 times to detach cells
 - e. Collect detached cells and centrifuge at 300 x g, carefully remove PBS from cell pellet.
3. Digestion:
- Typical assay workflow is given in Figure 1.*
- a. Dissolve harvested cell pellet (~2 million cells/pellet) in 100 µL of digestion buffer (Note 5). Vortex well and sonicate for 3 minutes.
 - b. Add 1 µL of 10 pmol/µl SIL analog of standard peptides with trypsin tag. Incubate 1h.
 - c. Add 7.5 µL of 30 mg/mL DTT. Incubate for 1h.
 - d. Add 15 µL of 36 mg/mL IAA. Incubate for 30 min in dark. (Note 4)
 - e. Add 750 µL of dd H₂O.
 - f. Add 58 µL of 0.1 µg/µl trypsin.
 - g. Complete with H₂O to a final volume of 1000 µL.
 - h. Incubate in water bath at 37 °C for 12-16h.
4. Purification of iPS cell digests using SPE.
- a. Take 500 µL of digest and mix it thoroughly with 500 µL 0.2% TFA.
 - e. Prime the SPE column with 2 mL of 70% CH₃CN in 0.1% TFA.
 - f. Prime the SPE column with 2 mL of 0.1% TFA.
 - b. Apply the sample on the column and collect the flow-through.
 - c. Apply the flow-through on the column.
 - d. Wash the column with 1 mL of 0.1% TFA.
 - g. Elute peptides with 500 µL of 70% CH₃CN in 0.1% TFA.
 - e. Freeze-dry the peptides.
 - f. Resuspend peptides in 50 µL of Solvent A.
5. LC-MRM Assay.
- Typical chromatographic results are given in Figure 2.*
- a. Transfer purified peptide digests into glass insert vials.
 - b. Set up analytical UPLC conditions: the flow rate 0.8 mL/min, and a 7-min linear gradient starting at 100% solvent, ramping to 40% solvent B in 3.5 min, then to 99.9% Solvent B over 0.25 min (held for 1.5 min) and finally decreased to 100% solvent A in 0.25 min and reconditioned for 1.5 min.
 - c. Set up mass spectrometry conditions: positive ion mode, dynamic multiple reaction monitoring with a dwell time set up to automatic, and minimum 8 data points per peak. Transition list for peptides selected for pluripotency test are given in Table 1.
 - d. Set up sequence of analysed samples, including standard curve, quality controls (QC) and blanks.
 - e. Inject 10 µL of sample per analysis.
6. Standard curve:
- Representative standard curves for the pluripotency LC-MRM assay are given in Figure 3.
- a. Digest and purify one pooled 1000 pmol aliquot of standard peptides spiked with SIL. After purification, freeze-dry and resuspend peptide standards in Solvent A.
 - b. Dilute digested standards in Solvent A to the following concentrations: 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 pmol/µL.
 - c. Transfer diluted standards into glass insert vials.
 - d. Inject 10 µL of standard per analysis.
 - e. Analyse standard peptides using the same LC-MRM method as for samples.
7. Quality control:

- a. Pool 10 μL of each sample and spike in your standard peptides, then transfer into glass insert vial.
 - b. Before analysing samples, inject 3 QC samples to ensure good sensitivity of the instrument.
 - c. Inject QC samples in intervals between samples, i.e. between every 10 samples.
8. Data analysis:
- a. Use Waters MassLynx and TargetLynx V4.1 software to analyse the raw data.
 - b. Set up a method to ratio integrated peaks to SIL peptide analogs.
 - c. Verify the automatic integration to ensure accuracy.
 - d. Interpolate response values from the standard curve.

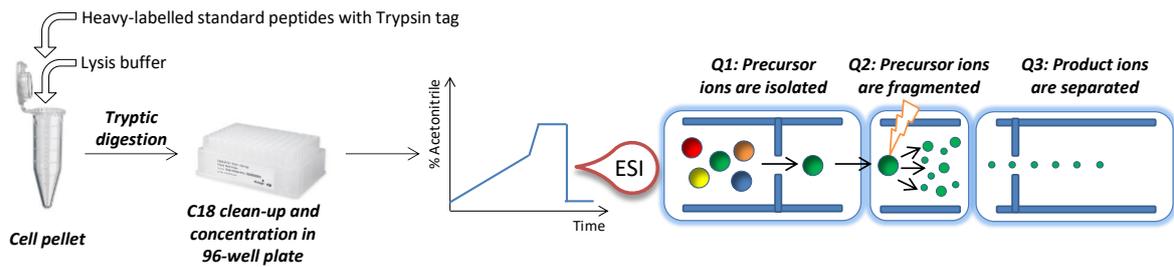


Figure 1. Typical LC-MRM pluripotency assay workflow. Adapted from¹⁴.

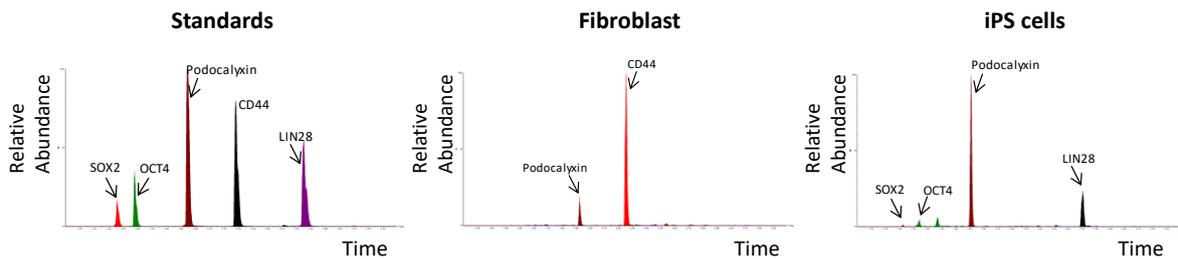


Figure 2. Typical chromatographic results of the LC-MRM pluripotency assay. Adapted from¹⁴.

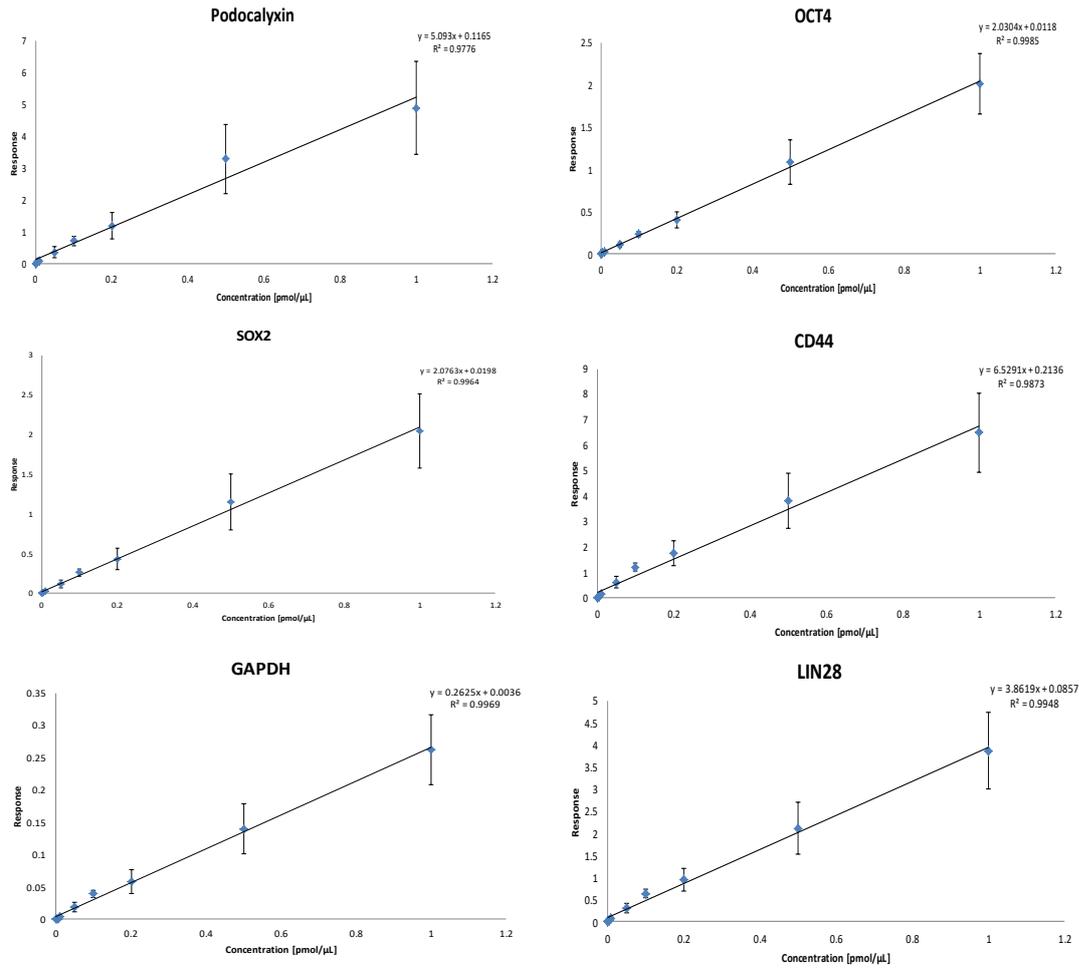


Figure 3. Representative standard curves for the pluripotency LC-MRM assay.

Table 1. List of peptides and transitions selected for the pluripotency test.

Protein	Peptide	Transition	Cone Voltage	Collision Energy
LIN28	AAEEAPEEAPEDAAR	778.39>1084.28	43	20
LIN28	AAEEAPEEAPEDAAR	778.39>729.24	43	20
LIN28	MGFGFLSMTAR	609.41>882.40	58	18
LIN28	MGFGFLSMTAR	609.41>678.29	58	16
LIN28 SIL	MGFGFLSMT[A(13C3;15N)]RAGVA	611.44>886.61	17	22
LIN28 SIL	MGFGFLSMT[A(13C3;15N)]RAGVA	611.44>686.45	17	20
CD44	LVINSGNGAVEDR	672.47>904.52	39	26
CD44	LVINSGNGAVEDR	672.47>566.43	39	20
CD44	YGFIEGHVVIPR	462.82>612.52	15	14
CD44	YGFIEGHVVIPR	462.82>510.39	15	14
OCT4	AETLVQAR	444.34>687.62	34	10
OCT4	AETLVQAR	444.34>586.32	34	14
OCT4	WVEEADNNENLQEICK	645.33>847.18	14	15
OCT4	WVEEADNNENLQEICK	645.33>677.33	16	15
OCT4 SIL	AETL[V(13C5;15N)]QAR	447.39>693.57	17	22
OCT4 SIL	AETL[V(13C5;15N)]QAR	447.39>479.36	17	22
PODXL	LASVPGSQTVVVK	643.05>914.66	43	20
PODXL	LASVPGSQTVVVK	643.05>457.87	43	20
PODXL	ATFNPAQDK	496.28>819.56	43	16
PODXL	ATFNPAQDK	496.28>558.36	43	18

SOX-2	SEASSPPVVTSSSHSR	568.06>743.49	2	10
SOX-2	SEASSPPVVTSSSHSR	568.06>577.38	2	16
SOX-2	DMISMYLPGAEVPEPAAPSR	711.1>824.43	34	22
SOX-2	DMISMYLPGAEVPEPAAPSR	711.1>598.33	34	22
SOX-2 SIL	SEASSPP[V(13C5;15N)]VTSSSHSR	569.94>623.82	2	16
SOX-2 SIL	SEASSPP[V(13C5;15N)]VTSSSHSR	569.94>580.31	2	16

Notes

Note 1. Marker peptide should be unique to the protein of interest (proteotypic) and representing the quantitative abundance of the protein (quantotypic). Standard marker peptides can be custom synthesized by various commercial companies.

Note 2. In order to monitor for digestion efficiency, a trypsin tag should be added to the standard peptide sequence. The trypsin tag of 3-5 amino acids can be determined from the sequence of the protein, found in the Uniprot database. SIL peptides with trypsin tag can be custom synthesized by various commercial companies.

Note 3. DTT should be prepared fresh on the day and kept on ice until use.

Note 4. Iodoacetamide is sensitive to light. Protect the stock solution of iodoacetamide and samples containing iodoacetamide from light by wrapping tubes in aluminium foil and keeping them in dark cabinet. Try to manipulate quickly in order to limit exposition to light. The stock solution should be prepared fresh on the day.

Note 5. If 2 million cells are not available, the protocol can be adjusted to the lesser amount of cells, by decreasing the volumes of added reagents during digestion. The minimum of cells required is 200,000.

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