TP-095

Quantification performance of non-invasive LC-MS/MS analysis and evaluation of undifferentiated state of human iPS cells

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1. Introduction

Pluripotent stem cells (PSCs) have an unlimited self-renewal capacity, and can differentiate into any cell type in the body. They consequently hold great promise as a source of cells for applications in regenerative medicine and drug discovery. For these applications, development of technology for the mass production of high quality pluripotent stem cells is essential. To contribute to development and commercialization of regenerative medicines and drug discovery, we have aimed to establish a method for the evaluation of undifferentiated state of PSCs without cell disruption. We have developed a simultaneous analysis method for 95 compounds in basal medium and secreted metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Last year, we have reported that some biomarker candidates in cell culture supernatant identified by this non-invasive LC-MS/MS analysis approach can be used for the evaluation of undifferentiated state of PSCs (Table I). However, it is generally known that LC-MS/MS quantitative results are influenced by matrices such as other metabolites and salts. In this study, to evaluate the influence of matrices on LC-MS/MS quantification performance, the concentration of biomarker candidates in the culture supernatant was quantitated with external standard method and standard addition method.

Table I. List of biomarker candidates

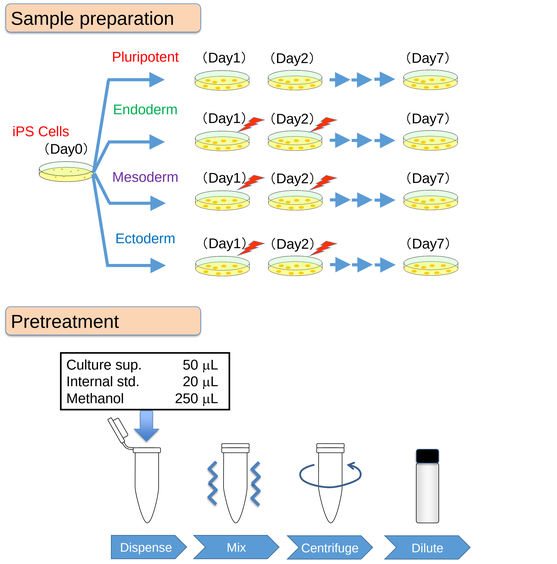
	Compound Name	Cell State	Comment
1	2-Aminoadipic acid	Ectoderm	Secreted metabolite
2	Alanine	Pluripotent	Medium component
3	Arginine	Pluripotent	Medium component
4	Cystathionine	Pluripotent	Secreted metabolite
5	Dexycytidine	Endoderm, Mesoderm	Secreted metabolite
6	Hypoxanthine	Ectoderm	Medium component
7	Kynurenine	Pluripotent	Secreted metabolite
8	Ornithine	Pluripotent	Secreted metabolite
9	Tryptophan	Pluripotent	Medium component
10	Hexose(Glucose)		Medium component
11	Glutamine	Cell growth	Medium component
12	Lactic acid		Secreted metabolite

2. Materials and Methods

Total experimental procedures were illustrated in Figure 1. We used iPS cell line PFX#9 derived from umbilical cord bloods. Cells were maintained in TeSR-E8 medium on vitronectin. Appropriate cytokines were added to each medium at next day of seeding to induce three germ layers. Medium exchange was performed every 24 hours. The spent medium was collected and stored at -80°C until use.

2-Isppropylmalic acid solution (20 μL), which is an internal standard (IS), was added to the supernatant (50 μL) , and methanol (250 μL) was also added to the mixture to precipitate proteins. Precipitated proteins were removed by centrifugation (15,000 rpm for 15 min). Then the supernatant was diluted with ultrapure water and used as the sample for LC-MS/MS. The samples were analyzed in triplicates.

The concentration of marker candidates in each medium was quantitated by external standard method (at least 4 point calibration) and standard addition method (4 point calibration including no addition sample).



LCMS-8050

LC/MS/MS Method Package For Cell Culture Profiling (including all the parameters necessary for LC-MS/MS analysis for 96 compounds including IS).

Figure 1 Experimental flow

3. Results and Discussion

Firstly, we tested linearity of candidate biomarkers using each standard. The results were summarized in Table II. We also calculated theoretical concentration in the sample prepared from fresh medium based on the medium components described in references 1 and 2. Six compounds out of 12 candidates are included in the fresh medium. And theoretical concentration of these six compounds was in the linearity range.

Ref. 1：Ludwig TE., *et al., Nat. Methods* **8**, 637-646 (2006)
Ref. 2：Ludwig TE., *et al., Nat. Biotechnol.* **2**, 185-187 (2006)

Table II. Linearity of biomarker candidates

	Compound Name	Linearity	R^2	Theoretical Concentration in the Sample Prepared from Fresh Medium
1	2-Aminoadipic acid	0.1 - 10 μM	1.000	0
2	Alanine	2.5 - 100 μM	0.998	4.4 μM
3	Arginine	5 - 250 μM	0.998	17.5 μM
4	Cystathionine	0.05 - 5 μM	0.998	0
5	Dexycytidine	0.05 - 5 μM	0.999	0
6	Hypoxanthine	0.25 - 25 μM	0.999	0.38 μM
7	Kynurenine	0.05 - 5 μM	1.000	0
8	Ornithine	0.5 - 50 μM	0.999	0
9	Tryptophan	0.5 - 50 μM	1.000	1.1 μM
10	Hexose(Glucose)	100 - 1000 μM	0.993	438 μM
11	Glutamine	10 - 200 μM	0.999	93.8 μM
12	Lactic acid	200 - 2000 μM	0.996	0

We tested recovery rate of biomarker candidates during pretreatment process. Standard compounds were added to the TeSR-E8 medium (this medium contains only negligibly small amount of proteins). The mixture was treated as described in Materials and Methods (Test sample) or diluted with water (Control sample). We also tested recovery rate using protein containing medium, mTeSR1. In the case of the mTeSR1 medium, the standard compounds were added to the treated sample (Control sample). Test sample was prepared as following. The standard compounds were added to the mTeSR1 medium, and then pretreated as described in Materials and Methods. Good recovery rate (>85%) was observed for all the candidate compounds in both protein containing and not containing media (Figure 2).

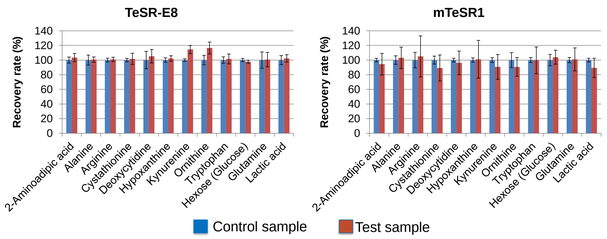


Figure 2 Recovery rate during pretreatment process

We compared time course of biomarker candidates in culture supernatant among undifferentiated iPS cells (Pluripotent state) and their differentiated counterparts. Figure 3 shows consumption of main carbon source (Glucose) and secretion of its waste product (Lactate). Glucose was gradually consumed, on the other hand lactate was gradually accumulated. These trends were observed in all culture samples, suggesting undifferentiated iPS cells and their differentiated counterparts were proliferated through culture process. Difference was observed in concentration of glucose between standard addition method and external standard method. This might be resulted due to glucose is contained at high concentration in fresh medium.

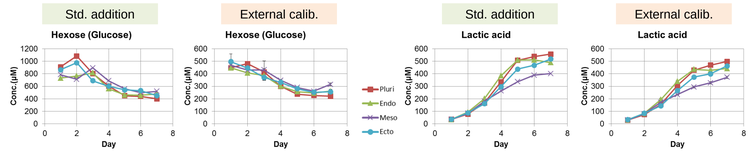


Figure 3 Time course of markers for cell growth

Time course of other biomarker candidates showed similar results between two quantitation methods (Figure 4). These results suggested the matrices effects in the culture supernatant samples were quite small, and the differences in concentration of biomarkers between pluripotent iPS cells and its differentiated counterparts reflected actual concentration change in the culture supernatant.

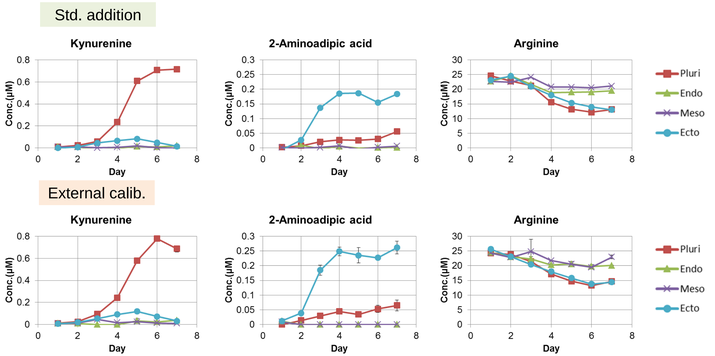


Figure 4 Time course of biomarker candidates

4. Conclusions

- Good linearity was confirmed and the range of linearity was matched to the theoretical concentration and quantitated results. These results showed our LC-MS/MS method is suited for simultaneous analysis of culture supernatant.
- Matrices effects on the LC-MS/MS analysis were quite small in our application. We concluded that the difference in concentration of biomarker candidates between pluripotent iPS cells and its differentiated counterparts caused from actual concentration change through differentiation process.
- Our method has the potential to be an effective means to evaluate the undifferentiated status of PSCs without cell disruption.