Journal of Biotechnology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Short communication

# Reduced quenching and extraction time for mammalian cells using filtration and syringe extraction

Juan A. Hernández Bort[a,1], Vinoth Shanmukam[a,b,1], Martin Pabst[c], Markus Windwarder[c], Laura Neumann[c], Ali Alchalabi[d], Guido Krebiehl[d], Gunda Koellensperger[a,c], Stephan Hann[a,c], Denise Sonntag[d], Friedrich Altmann[a,c], Christine Heel[e], Nicole Borth[a,b,*]

[a] ACIB GmbH, Austrian Centre of Industrial Biotechnology, Vienna, Austria
[b] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
[c] Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria
[d] Biocrates Life Sciences, Innsbruck, Austria
[e] Sandoz GmbH, Tirol, Austria

ARTICLE INFO

*Article history:*
Received 16 October 2013
Received in revised form 8 April 2014
Accepted 11 April 2014
Available online xxx

*Keywords:*
Metabolomics
Quenching
Metabolite extraction
Fast filtration
CHO cells

ABSTRACT

In order to preserve the *in vivo* metabolite levels of cells, a quenching protocol must be quickly executed to avoid degradation of labile metabolites either chemically or biologically. In the case of mammalian cell cultures cultivated in complex media, a wash step previous to quenching is necessary to avoid contamination of the cell pellet with extracellular metabolites, which could distort the real intracellular concentration of metabolites. This is typically achieved either by one or multiple centrifugation/wash steps which delay the time until quenching (even harsh centrifugation requires several minutes for processing until the cells are quenched) or filtration.

In this article, we describe and evaluate a two-step optimized protocol based on fast filtration by use of a vacuum pump for quenching and subsequent extraction of intracellular metabolites from CHO (Chinese hamster ovary) suspension cells, which uses commercially available components. The method allows transfer of washed cells into liquid nitrogen within 10–15 s of sampling and recovers the entire extraction solution volume. It also has the advantage to remove residual filter filaments in the final sample, thus preventing damage to separation columns during subsequent MS analysis. Relative to other methods currently used in the literature, the resulting energy charge of intracellular adenosine nucleotides was increased to 0.94 compared to 0.90 with cold PBS quenching or 0.82 with cold methanol/AMBIC quenching.



## 1. Introduction

In recent years, different sophisticated protocols have been developed for the quantification of intracellular metabolites (Bennett et al., 2008; Buchholz et al., 2001; Dietmair et al., 2010; Neubauer et al., 2012; Pabst et al., 2010; Sellick et al., 2011) due to the increasing interest in more rational metabolic engineering and control tools for the optimization of cell lines and processes (Dietmair et al., 2012a,b; Martínez et al., 2013; Murabito et al., 2009; Schilling et al., 2000; Sheikh et al., 2005). The use of reconstructed genome-scale models requires sufficient and reliable experimental data to predict the cellular needs of high-producer cell lines (Chung et al., 2010; Licona-Cassani et al., 2012; Selvarasu et al., 2012) and to assist with rational prediction of necessary changes in media composition, feeding strategy and process

*Abbreviations:* ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; UTP, uridine-5′-triphosphate; UDP, uridine-5′-diphosphate; UMP, uridine-5′-monophosphate; GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; GMP, guanosine-5′-monophosphate; UDP-Gal, uridine-5′-diphosphate galactose; UDP-Glc, uridine-5′-diphosphate glucose; UDP-HexNAc, uridine-5′-diphosphate N-acetylhexosamine.

* Corresponding author at: Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria. Tel.: +43 1 47654 6232; fax: +43 1 47654 6675.
*E-mail address:* nicole.borth@boku.ac.at (N. Borth).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jbiotec.2014.04.014

control (D'Huys et al., 2012; Martínez et al., 2013; Selvarasu et al., 2009, 2012; Zamorano et al., 2012).

To ensure that the measured data are as close to the real *in vivo* values as possible, an efficient quenching protocol should: (i) ensure a fast and complete blockage of any intracellular metabolic reaction, as most intermediates have high conversion rates (Canelas et al., 2008; de Koning and van Dam, 1992; Weibel et al., 1974), (ii) avoid contamination of the sample with extracellular metabolites present in the supernatant, (iii) lose none of the intracellular substances through leaky membranes. It has proven very difficult to achieve all of these requirements to perfection, so that most protocols try to keep a reasonable balance of preventing all three reasons of metabolite loss or contamination.

Several authors (Pabst et al., 2010; Sellick et al., 2009) designed quenching protocols in which the cell broth was directly quenched using cold solvent mixtures after sampling, as their lower freezing points allow using them below −20 °C. Even though these methods were successfully implemented for yeast and bacteria (de Jonge et al., 2012; Gonzalez et al., 1997; Moritz et al., 2000) their direct application to mammalian cells provokes some controversies due to higher leakage of intracellular metabolites, as mammalian cells lack a cell wall. Sellick (Sellick et al., 2009) and later Sengupta (Sengupta et al., 2011) used different additives to the cold methanol quenching solvent showing an increase in metabolite recovery. However, these results proved difficult to transfer between labs (Dietmair et al., 2010) and indeed, as expected, resulted in membrane damage and consequently uncontrolled leakage of intracellular metabolites.

A less aggressive quenching method uses direct dilution of the cell broth with ice-cold PBS (Pabst et al., 2010), which prevents leakage while at the same time diluting extracellular contaminants, however the final mixture does not achieve temperatures below 0 °C, which does not ensure quenching of all enzymatic reactions and it cannot be excluded that molecules from the supernatant contaminate the intracellular concentrations analyzed.

To avoid metabolite leakage, fast filtration was proposed as a washing step prior to quenching with liquid $N_2$ (Volmer et al., 2011a,b). Using an in-house made filter module, a determined amount of cells were passed through a filter and rapidly washed with 0.9% (w/w) NaCl. The entire procedure took between 30 and 60 s and allows obtaining cells free from any contaminating metabolites present in the cultivation media. Quenching with liquid $N_2$ has the advantage to rapidly freeze samples below −100 °C, thus avoiding further metabolic conversion (Buziol et al., 2002; Sellick et al., 2011). However, the system so far required individually made equipment and thus necessitated fine tuning of the individual components to identify optimal conditions of handling and use, making it difficult to transfer to other labs.

Despite the fast quenching step, the subsequent extraction of metabolites from cells captured on a filter is time consuming, as the filters have to be cut to fit into a tube, to be centrifuged together with the extraction solvent, and the final liquid extract is separated from the filter by decantation. The filter retains some fluid which makes it difficult to recover the entire volume added for the extraction. This can be partially resolved by using a suction system coupled to a column (Volmer et al., 2011a,b).

It was reported, that several methods for the intracellular metabolite extraction using solvents like hot methanol (Shryock et al., 1986), cold methanol (de Jonge et al., 2012; Volmer et al., 2011b), boiling ethanol (Gonzalez et al., 1997), chloroform/methanol (de Koning and van Dam, 1992), acid (Ryll and Wagner, 1991), alkali (Nissom et al., 2006) and hot water (Bolten et al., 2007) were successful. Though, to attain the maximum recovery of metabolites, cold methanol (Cao et al., 2011; Shin et al., 2010; Volmer et al., 2011a) and cold acetonitrile (Dietmair et al., 2010) are more preferred in mammalian cells.

All in all, the selection of a tailored protocol will depend on the cell characteristics, the metabolites required for the study or analytical equipment availability. In this work, we developed a modified protocol for fast filtration, washing and quenching using readily available parts. The entire procedure routinely takes less than 15 s to transfer washed cells into liquid nitrogen. For subsequent extraction either immediately after quenching or after storage, different cold solutions were compared and evaluated by LC–ESI-MS and FIA-MS–MS analyses of nucleotides and amino acids. The method is benchmarked against other quenching and extraction methods described in the literature and found to preserve the energy charge of AMP/ADP/ATP at the highest value (0.94 vs 0.90 and 0.82).

## 2. Materials and methods

### 2.1. Cell line and cultivation media

CHO-K1 suspension cell lines adapted to growth in glutamine free media (Bort et al., 2010) were cultivated in 500 ml spinner flasks using CD CHO media (Gibco, Invitrogen, Carlsbad, CA, USA) under 7% $CO_2$ and incubated at 37 °C with constant stirring at 50 rpm. Viability and cell counts were measured on a Vi-Cell analyzer (Beckman Coulter Inc., Fullerton, CA) based on the trypan-blue dye exclusion method.

### 2.2. Filtration and quenching

For each sample, after humidification of the filter membrane (PALL A/D Glass Fibre, 47 mm, New York, USA) with 10 ml of 0.9% (w/w) NaCl, a total of $10^7$ cells harvested from a suspension culture were filtered using a standard filtration holder (Millipore, MA, USA) and immediately washed with 30 ml 0.9% (w/w) NaCl solution under controlled vacuum at 10 mbar (Controller CVC 3000 with W-B 6C, Vacuumbrand, Germany) to remove extracellular contaminating substances. The filter membrane with the captured cells was transferred into a 20 ml syringe barrel (20 ml BD™ Syringe with Luer-Lock™ connection, Becton Dickinson, NJ, USA) closed with a cap (Fig. 1). At this step a standard solution could be added (see f.i. Neubauer et al., 2012), before freezing of samples in liquid nitrogen, followed either by storage at −80 °C or immediate extraction.

Cold PBS quenching (Pabst et al., 2010) and cold methanol/AMBIC quenching (Sellick et al., 2009) were carried out exactly as published and compared to fast filtration by analysis of nucleotides and sugar nucleotides.

### 2.3. Metabolites extraction

Following quenching in liquid $N_2$, 8 ml of 50% (v/v) cold methanol were added into the syringe barrel. The plunger was carefully reinserted into the syringe barrel after removing the Luer-Lock™ cap and slightly pushing on the plunger to remove the excess air inside. The syringe barrel was relocked with a Luer-Lock™ cap and vortexed for 15 s. The cap was removed and a 0.45 μm filter membrane unit (Millex HV-Durapore PVDF filter) was attached to the syringe. The syringe plunger was squeezed to transfer the extraction solution into a fresh tube. The entire extracted volume (8 ml or as specified), was dried with a Thermo Savant SPD121P Speed Vac Concentrator equipment (Thermo Fischer Scientific, MA, USA) and stored at −80 °C for further quantification of metabolites.

### 2.4. LC–ESI-MS analysis for nucleotides

The sample extraction and analysis of the nucleotides and nucleotide sugars were performed as described in Pabst et al. (2010) and Taschwer et al. (2012). The data were interpreted using

Please cite this article in press as: Hernández Bort, J.A., et al., Reduced quenching and extraction time for mammalian cells using filtration and syringe extraction. J. Biotechnol. (2014), http://dx.doi.org/10.1016/j.jbiotec.2014.04.014