



# Subcellular fractionation strategies for the proteomic analysis of frozen tissue samples

Jutta Korder, Christian Baumann, Sandra Steiner, and Thorsten Prinz\*  
Proteome Sciences R&D, Altenhöferallee 3, D-60438 Frankfurt/M., Germany, www.proteomics.com  
\* E-mail Thorsten.Prinz@proteomics.com

## Overview

- The nucleus and the cellular membranes contain many low-abundant proteins that are key to cell signaling and highly relevant to pharmaceutical research. Thus, a powerful enrichment of these compartments is the prerequisite for a successful proteomic analysis strategy of these subcellular fractions.
- Here we present the evaluation of different subcellular fractionation approaches using frozen mouse liver tissue. The nuclear fractions obtained with two different protocols were shown to be highly enriched in their corresponding proteins (46% and 54% nuclear proteins vs. 8% nuclear proteins in soluble fraction). The membrane fractions obtained with a third protocol contained 61% membrane proteins vs. 0% in the soluble fraction.
- Next steps include the evaluation of the robustness and the reproducibility of the here presented cell fractionation approaches.
- The subcellular fractionation strategies presented here are crucial steps to further enhance the application range of our ProteoSHOP toolkit of gel-based (2-DE) and gel-free (qPST) quantitative proteomics technologies. As demonstrated here they enable the analysis of lower abundant proteins in nuclear and membrane fractions.

## Introduction

In order for proteomics to reach its full analytical potential it is required to establish subcellular fractionation strategies for the individual compartments of an eukaryotic cell. The reason for this is mainly the low expression of certain proteins that play a key role in the metabolism. Two compartments are of special interest in the light of biomarker identification by proteomic methods: the cell nucleus and the cellular membranes. The nucleus as one of the most important organelles plays an important role in the organization of the genome, gene expression, ribosome biogenesis, and nuclear transport. A substantial portion of the entire cellular proteome resides in the nuclear compartment: in yeast nuclear proteins account for ~25% of the total proteins (Kumar et al., 2002). Usually, sample material has been stored frozen prior to the proteomic analysis. However, the enrichment of subcellular fractions from frozen human or animal material requires special protocols.

Here, we studied the effectiveness of two nuclear extraction protocols (method A and B) and our general fractionation protocol resulting in a total membrane and a total soluble fraction (method C) that were applied to frozen mouse liver tissue. Furthermore, the soluble protein fraction served as a control for the success of the enrichment of nuclear proteins in the other two experiments. Our results demonstrate that an efficient biological sample preparation enables us to characterize even low abundant proteins that reside either in the nucleus or the cellular membranes.

## Methods

### Preparation of subcellular fractions.

For the each fractionation experiment one liver was prepared from a Balb/C mouse. The general workflow of the three fractionation strategies is shown in figure 1.

Two nuclear extraction protocols were applied to two mice liver for the enrichment of nuclear proteins (Figs. 1 A and B). Both methods include the homogenization of mouse liver tissue and the isolation of a crude nuclear fraction by differential centrifugation. Finally, the nuclei are lysed in a high-salt buffer and the nuclear proteins are released to the supernatant.

One liver was subjected to our general cell fractionation protocol resulting in a soluble and a membrane protein fraction (Prinz et al., 2004 and Fig. 1C).

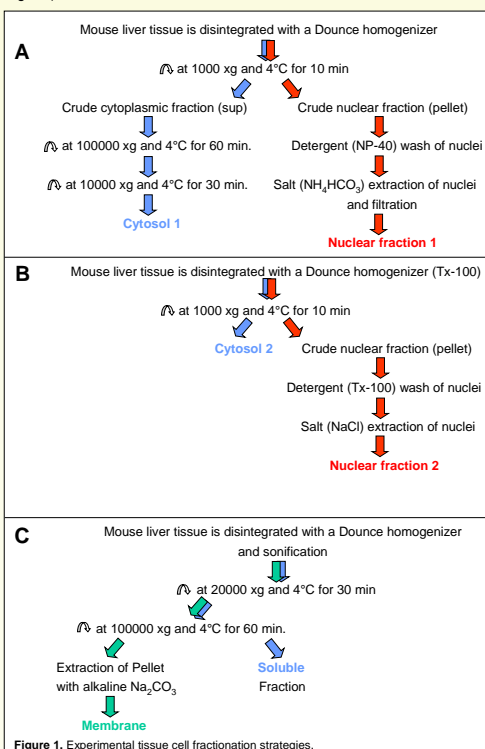


Figure 1. Experimental tissue cell fractionation strategies.

### Validation of the sub-cellular fractionations

We have chosen two methods, the Multidimensional protein identification technology (MudPIT) and Western Blot using antibodies directed against RNA polymerase II (nucleus) and Hsp60 (mitochondria) in order to assess the degree of the fractionation of the liver cells.

### Multidimensional protein identification technology (MudPIT) analysis

The workflow of the here applied MudPIT procedure is shown in figure 2.

### Western blot

Hundred µg of each isolated fraction was separated on a 4-16% Tris-tricine gradient gel and, subsequently, blotted onto a PVDF membrane. After the immunodetection the chemiluminescence signals from the peroxidase-coupled secondary antibody were detected with a CCD camera (Versadoc 5000, Bio-Rad).

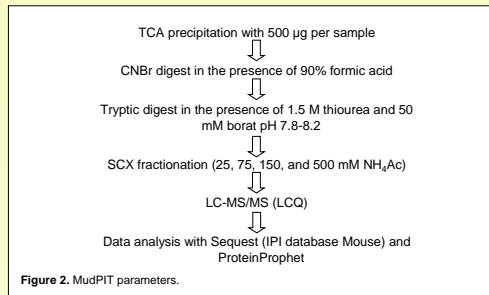


Figure 2. MudPIT parameters.

### Protein sequence analysis.

Proteins were initially annotated by similarity searches using BLAST against SWISSPROT/TrEMBL and their function was determined with Euclid and InterProScan. Characterisation of proteins concerning membrane relationships has been undertaken by SWISSPROT/TrEMBL, and by primary literature. Transmembrane and lipid-modified proteins were considered as integral membrane proteins. Otherwise, proteins were assumed to be soluble. The subcellular localisation information was extracted from data base annotations in SWISSPROT, TrEMBL, Refseq, or predicted by Psort. Molecular weight and pI values have been calculated by the pepstats program from the EMBOSS package.

## Results

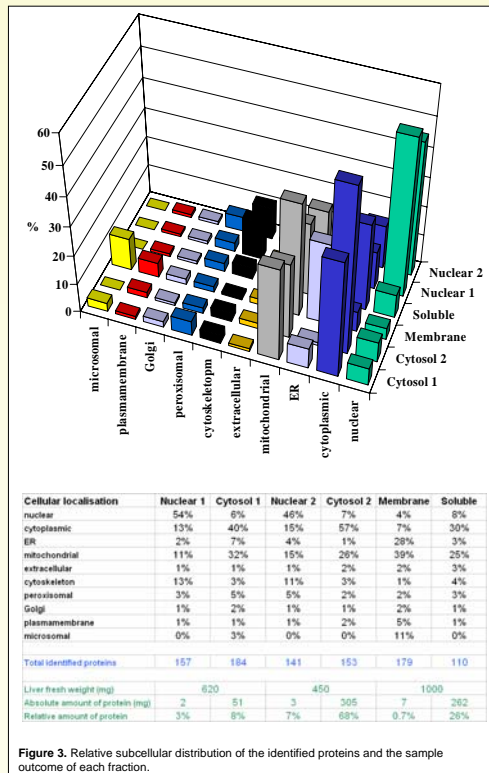


Figure 3. Relative subcellular distribution of the identified proteins and the sample outcome of each fraction.

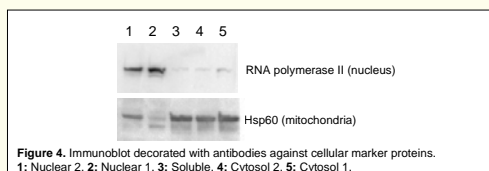


Figure 4. Immunoblot decorated with antibodies against cellular marker proteins. 1: Nuclear 2, 2: Nuclear 1, 3: Soluble, 4: Cytosol 2, 5: Cytosol 1.

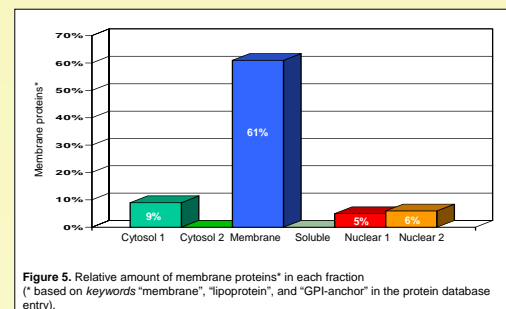


Figure 5. Relative amount of membrane proteins\* in each fraction (\* based on keywords "membrane", "lipoprotein", and "GPI-anchor" in the protein database entry).

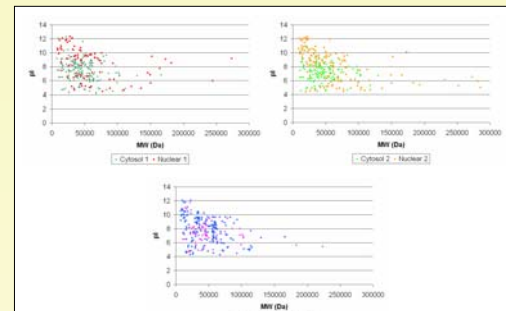


Figure 6. Distribution of the identified proteins concerning their molecular weight (MW) and pI.

Table 1. Selection of proteins that are typical for each fractions.

Nuclear 1
Small nuclear ribonucleoprotein Sm D1
Splicing factor 3B subunit 1
Nucleolar protein Nop56
Splicing factor 3A subunit 3
Nuclear matrix protein SNEV
Cell division cycle 5-like
CDK17 protein
Nuclear protein Hcc-1
Splicing factor, argonaute-associated 5 (Srafl0, HRS)
Mitotic checkpoint protein BUB3
Lamina-associated polypeptide 2 isoforms alpha/beta
Nuclear 2
Lamin A
Small nuclear ribonucleoprotein E (snRNP-E)
Splicing factor 3b, subunit 3, 130kDa
Lamin C2 (Fragment)
Nucleolin
Nucleolar protein Nop56 (Nucleolar protein 5A)
MYB binding protein (P160) 1a
Fibrillarin (Nucleolar protein 1)
Apoptotic chromatin condensation inducer in the nucleus (Acinus)
DNA-repair protein XRCC1
Membrane
Solute carrier family 2 (Glucose transporter type 2, liver)
Band 3 anion transport protein
Basigin precursor
Dipeptidyl peptidase IV (EC 3.4.14.5)
Voltage-dependent anion-selective channel protein 1 (VDAC-1)
Sodium/bile acid cotransporter (Bile-salt acid cotransporter)
Estradiol 17-beta-dehydrogenase 2 (EC 1.1.1.62)
ADP/ATP carrier protein, heart/skeletal muscle isoform T1
Potassium-transporting ATPase alpha chain 1 (EC 3.6.3.10)
Ras-related protein Rab-14

## Conclusions

- Frozen tissue is amenable to sub-cellular fractionation prior to proteomics analysis.
- Sub-cellular fractions typically have 40 - 60% organelle specific proteins (Figs. 3, 4, and 5).
- A normal distribution of pI and molecular weight proteins are seen by LC-MS/MS (Fig. 6).
- Mitochondrial protein leakage is an issue for fractionation of liver cells (Figs. 3 and 4).
- These protocols provide highly sensitive detection of even low abundant markers of disease, toxicity, drug efficacy etc. (Table 1).
- Future experiments will expand the reproducibility and robustness of the presented subcellular fractionation strategies, e.g. by quantitative immunoblots and two-dimensional electrophoresis.

## References

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