

ProteoSolve-TD2 PrEP: Membrane Protein Recovery from Metastatic Ovarian Tumors Using ProteoSolve-TD2 and Pressure Cycling Technology (PCT)

Introduction

Integral membrane proteins play key biological roles in cell signaling [1-5], membrane transport [6-7] as well as pathogen invasion [8-10]. Integral membrane proteins, particularly G-protein coupled receptors (GPCRs), are the biological targets for 49-52% of all the small molecule pharmaceuticals on the market today [11-13]. As such, membrane proteins also play key clinical roles in drug efficacy and resistance, and should have a larger role in clinical diagnostics and personalized medicine. However, quantitative clinical assays for this critical class of proteins (e.g., immunosorbant assays) remain elusive, and are generally limited to monitoring serum-soluble extracellular fragments [14-15], or indirect measurement of their mRNAs [16-17]. Yet, genetic assays are unable to detect potentially clinically-relevant post-translational modifications, such as: glycosylation, phosphorylation, acetylation, ubiquitination, and editing. Furthermore, it is well established that measurements of mRNA levels, which are produced transiently, do not correlate well to protein levels, which accumulate over time [18-19].

Classically, detergents [20] and organic solvents [21] and strong chaotropic agents [22-23] are used to extract membrane proteins from biological tissues. Alternatively proteolytic digestion of the non-transmembrane portions of integral membrane proteins has been used in global proteomic strategies [24-25]. However, Immunoaffinity analyses or purifications of proteins from these samples are nearly impossible because the structure of the protein is significantly distorted [26], or the agent necessary for membrane protein dissolution also destroys the activity of immunoglobulins. Such agents are also typically incompatible with chromatographic and mass spectrometric analyses [27-29].

Heretofore, clinical exploration of integral membrane proteins has been limited by an inability to recover this class of proteins in a form suitable for immunoaffinity quantification and rapid proteomic characterization. Here we describe the use of the ProteoSolve-TD2 buffer system in combination with pressure cycling technology (PCT) for the recovery of integral membrane proteins from solid metastatic ovarian tumor samples.

PCT Sample Preparation System (PCT SPS)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) uses rapid cycles of hydrostatic pressure between ambient and ultra high levels to control biomolecular interactions. The PCT SPS can be used to disrupt tissues, cells, and cellular structures to extract proteins and nucleic acids [30, 31, and 32]. In addition, the PCT SPS can also be used to accelerate enzymatic reactions such as trypsin digestion [33]. The PCT SPS uses a small, semi-automated bench top instrument (Barocycler NEP3229 or the NEP2320) in combination with single-use sample processing containers called FT500 PULSE Tubes (Pressure BioSciences, Inc. South Easton, MA).

PCT Sample Preparation

Membrane proteins are solubilized by the thermodynamic changes induced by high pressure in the cell membrane fluidity, protein conformation and in the activity of the buffer ingredients, weakening molecular interactions which keep the cellular components together. Upon return to normal pressure, the extracted membrane proteins are then fully solubilized in the ProteoSolve-TD2 buffer system.

Pre-processing of the solid tissue to obtain high surface-to-volume ratios is essential for efficient protein recovery. This was done by cryogenic grinding under liquid N₂ as described in the ProteoSolve-TD2 user manual. The tumor samples used in this study proved intractable to efficient protein extraction with ProteoSolve-TD1 under the same conditions.

The ground tissue sample (200mg) was processed as described in the ProteoSolve-TD2 User Manual in 1.3 mL of ProteoSolve-TD2 buffer, including treatment with micrococcal nuclease, clarification by centrifugation and dilution with 4x-NuPAGE LDS Sample buffer (Invitrogen). The insoluble pellets recovered after centrifugation were re-suspended in 1 mL of ProteoSolve-TD2 to which 300 μ L of 4X-NuPAGE LDS Sample buffer was added, and the sample was heated at 95C for 15 min. Aliquots (22.5 μ L) of each sample were run on pre-cast 4-12% Bis-Tris NuPAGE mini-gels (Invitrogen). Proteins were blotted to PVDF membranes at 65V for 2 hours using a transfer buffer consisting of 20mM TRIS, 160 mM glycine, and 0.04% SDS. Primary antibodies were obtained from Santa Cruz Biotechnology (EDG4, LAMP-3, NRP1, and KDR), Cell Signaling (FAS), and Abcam (EDG2). The blocking buffer consisted of either 100mM phosphate buffered saline with 0.05% Tween, 0.01% Thimerosal, and 10% non-fat milk (FAS, NRP1, KDR, LAMP-3) or 25 mM Tris, 0.15 M NaCl, 0.1% Tween-20, 0.01% thimerosal at pH 7.4 containing 2% non-fat milk (EDG2 and EDG4). Species-appropriate secondary antibodies (Cell Signaling) were used with Supersignal West Femto substrate (Thermo-Fisher) following the manufacturer's recommendations to develop the blots. The chemiluminescent images were collected using a Fluorochem SP gel imager (Alpha Innotech) and inverted.

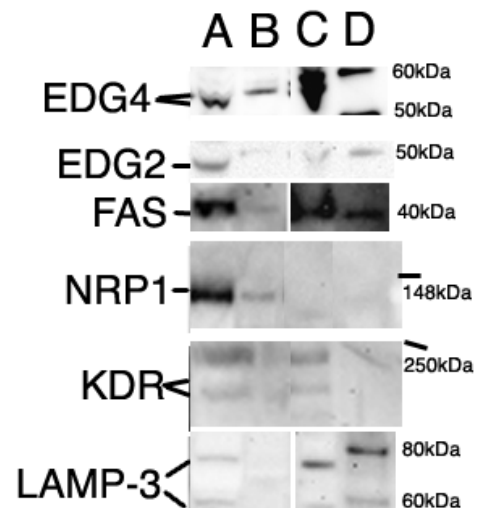


Figure 1. A composite of Western blots that shows a range of different membrane proteins can be recovered from metastatic ovarian tumor tissue using ProteoSolve-TD2 and PCT. Lane A: Clarified ProteoSolve-TD2 extract. Lane B: Equivalent amount of the recovered ProteoSolve-TD2 insoluble pellet. Lane C: Antibody positive control. Lane D: Immunoreactive molecular weight markers.

Western Blot Results

Figure 1 (Page 1) is a composite of Western blots that shows that a range of different membrane proteins can be extracted from metastatic ovarian tumor using ProteoSolve-TD2 and PCT. Direct comparison can be made between the amount of protein recovered in the clarified PCT extract (Lane A) to that left behind in the insoluble pellet (Lane B) and subsequently recovered in boiling SDS. With the exception of EDG4 (with better than 50% recovery), recovery of all the membrane proteins in ProteoSolve-TD2 using PCT was nearly quantitative.

Lysophosphatidic acid receptor 1 (EDG2) and Lysophosphatidic acid receptor 2 and (EDG4) are both G-protein coupled receptors, each with 7 transmembrane sequences that are present at low concentrations in the tumor sample. CD63 (Lamp-3) has 4 transmembrane helices. Both the mature (80kDa) form of Lamp-3 and the immature, less-glycosylated, 53kDa form were quantitatively recovered. The recoveries of three other single transmembrane proteins are also shown in Figure 1: vascular endothelial growth factor receptor 2 (KDR), vascular endothelial growth factor receptor 165 (NRP1), and tumor necrosis factor receptor super-family member 6 (FAS). FAS, the smallest of the three single transmembrane proteins (40kDa), was recovered nearly quantitatively. The immature 72kDa form of NRP1 was not detected in either the sample extract or pellet. Most of the mature form of NRP1 (148kDa) was recovered in the ProteoSolve-TD2 extraction. A slightly truncated form (140kDa) appears in the cell line control (lane C) at very low abundance. KDR is the largest of the three single transmembrane proteins tested with immature (150kDa), intermediate (200kDa), and mature (230kDa) glycoforms. Nearly quantitative recovery of both the intermediate and mature forms was achieved. The immature (150kDa) form of KDR was not detected.

Discussion

ProteoSolve-TD2 in combination with PCT opens the prospect for highly efficient membrane protein recovery from recalcitrant solid tissues. The ProteoSolve-TD2 PCT extracts can be visualized directly on SDS-PAGE gels and used in standard Western blotting protocols. ProteoSolve-TD2 is directly compatible with LC-MS/MS workflow. When diluted 1:8 in ProteoSolve™-TDiluent, also available from Pressure BioSciences, the buffer is compatible with immunoaffinity methods (separate application note is available) and many other standard proteomic techniques. Pressure Cycling Technology and the ProteoSolve-TD1 and TD2 buffer systems open the door to more complete proteomic study of this important class of proteins.

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