Evaluation of common protein extraction reagents in mammalian and bacteria lysates, by infrared (IR) based quantification and Western blotting

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Abstract

The quality of sample preparation ultimately impacts the quality of the downstream analyses. For example, selecting the "wrong" lysis conditions may preclude subsequent detection of the desired biomarker. If conditions are too aggressive, the marker may be denatured or destroyed. If not sufficiently stringent, the marker may be lost in the insoluble fraction. Using the "right" combination of extraction reagents and inhibitors during sample preparation is critical to maximizing recovery of "active" biomarker. Further, downstream detection and analysis methods often rely on proper determination of total protein content, a measurement that can be biased by the presence of residual reducing agents or detergents.

In this work, we have evaluated several buffers and reagents commonly used for protein extraction from mammalian and bacterial lysates. Differences in the recovery of protein(s) and other biomolecules between the buffer formulations have been determined by a novel infrared (IR)-based biomolecule detection system, which is less influenced by reducing agents and detergents than either BCA or Bradford assays. An accurate determination of total protein concentration, along with additional information from the IR analysis, allowed well-resolved protein separation by electrophoresis followed by Western blotting analysis of some of the proteins using a new, rapid Western blotting method. Together, these innovations for the protein sample preparation workflow have not only improved sample confidence but also compressed the processing time from lysis to immunodetection to a few hours.

Introduction

Many commercial and homebrew formulations for lysis buffers are available for use with biological samples. Selection of the "best" lysis buffer depends on the type of protein being investigated, its structure, stability and solubility. In addition, the choice of sample preparation method is frequently dictated by the downstream analysis method. Further, the total protein and lipid content in cell and tissue lysates can influence their downstream analysis. In the past, the quantitation of proteins and lipids has been tedious, required large sample volumes, and in many cases, the results were impacted by interfering substances. The Direct Detect™ spectrometer, an IR-based biomolecule quantitation system, has enabled rapid analysis of total protein and lipid content, substantially improving sample handling. The accurate concentration and composition information facilitates optimization of subsequent steps of the analysis (gel electrophoresis and immunodetection). Also, the process of immunodetection has been compressed to 30 minutes by using the SNAP i.d.® 2.0 protein detection system. Using a range of sample types, from bacterial culture through cancerous cell lines to breast cancer tissue, we have demonstrated the importance of properly optimized sample preparation for efficient downstream analysis.

Methods

Bacterial lysate preparation: Recombinant *E.coli* polyhistidine-tagged c-reactive protein (6X HIS-CRP), grown in LB medium, was harvested by centrifugation (20 minutes at 5,000 rpm). The pellet was lysed by either BugBuster® Protein Extraction Reagent or Homebrew buffer (20 mM Tris-HCl pH 8, 137 mM sodium chloride, 10% glycerol, 1% NP40). Both lysates were supplemented with protease inhibitors (Cat. No. 539138). To pellets of certain cultures, other reagents, such as Benzonase® Nuclease (Cat. No. 71205-3) and rLysozyme™ (Cat. No. 71110-3) were also added. Samples were spun at 16,000 x g for 20 minutes and the supernatants were used for further analysis.

Cell lysate preparation: MCF-7 (ATCC® HTB-22™) breast cancer cell line and T47D P17 (ATCC® HTB-133™) ductal breast carcinoma cell line were grown to confluency. Total cell number was determined using the Scepter™ 2.0 cell counter (Cat. No. PHCC20060). The cells were washed with PBS and separated into 2 equal samples for the lysis. Cells were lysed with 1 mL of either RIPA (radioimmunoprecipitation assay) buffer (Cat. No. 20-188) or CytoBuster™ Protein Extraction Reagent (Cat. No. 71009-50mL), containing inhibitor cocktail, homogenized for few seconds with handheld homogenizer and spun at 15,000 x g for 10 minutes. Supernatant was used for biomarker analysis.

Tissue lysate preparation: A frozen surgical breast ductal carcinoma tissue sample obtained from Analytical Biological Services Inc. was divided into 2 equal samples of 115 mg each. Tissue was covered with 2 mL of RIPA buffer or CytoBuster™ Protein Extraction Reagent, both supplemented with the inhibitor cocktail, and disrupted with a glass tissue homogenizer. Samples were spun at 10,000 x g for 10 minutes, the top layer (fat fraction) was separated and the bottom layer was transferred to a clean microcentrifuge tube for a second spin (15,000 x g for 10 minutes). The resulting protein fraction was analyzed for biomarker content.

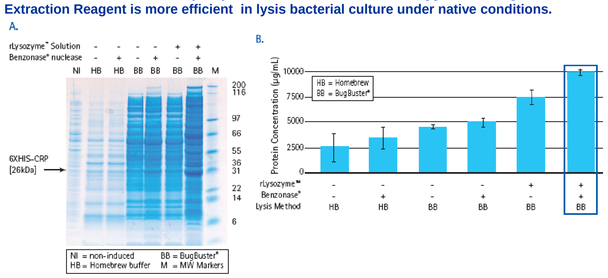
Protein concentration: Total protein concentration and relative fat content was determined using the Direct Detect™ spectrometer (Cat. No. DDHW000-10-WW). For some samples, a second aliquot was taken comparative analysis using the Pierce® bicinchinonic acid (BCA) protein assay.

Electrophoresis and Western Blotting: Samples were separated by electrophoresis using 4-12% NUPAGE® 20 well gels (Invitrogen Cat. No. WG1402Box10), and transferred to Immobilon-P® membrane (Cat. No. IPVH08130), using semidry blotting system. Membrane blots were processed using the SNAP i.d.® 2.0 protein detection system (Cat Nos. SNAP2BASE, Midi Frame SNAP2FRMD02 and Midi Blot Holders SNAP2BHMD0100) using specific antibodies against breast cancer markers: anti-cathepsin D (Cat. No. 06-114), anti-cyclin D (Cat. No. 04-1151), anti-estrogen receptor (Cat. No. 04-824) and anti-cytokeratin 18 (Cat. No. MAB3234). Samples were detected by chemiluminescence after 5 minutes incubation with Luminata™ Forte Western HRP Substrate (Cat. No. WBLVF0500) or chromogenically using TMB insoluble reagent (Cat. No. 613548).

Immunoprecipitation: Isolation of cathepsin D and cyclin D from MCF-7 cells was performed using PureProteome™ protein A magnetic beads (Cat. No. LSKMAGA10).

Results

Comparison of the protein content in six *E. coli* lysates prepared with the homebrew method *versus* BugBuster® Protein Extraction Reagent, in the presence or absence of Benzonase® Nuclease and rLysozyme™ solutions. The results suggest that BugBuster® Extraction Reagent is more efficient in lysis bacterial culture under native conditions.



Total protein content liberated by BugBuster® Extraction Reagent was much higher than the amount produced by homebrew method. Also, the addition of Benzonase® Nuclease and rLysozyme™ solutions had a significant impact on overall yield. (A) *E. coli* lysates (5 µL of 1 mL total lysate) from various lysis protocols were fractionated and analyzed by SDS-PAGE. A band corresponding to 6XHIS-CRP is prominently visualized in the BB +/+ lane. (B) Cleared cell lysates (2 µL of 1 mL total) were spotted on assay cards and quantified using the Direct Detect™ spectrometer. In each case, bars represent the average of 3 independent samples.

Protein concentration in two breast cancer cell lysates prepared with CytoBuster™ Protein Extraction Reagent and RIPA buffer. Values obtained by BCA assay and the Direct Detect™ spectrometer.

			BCA assay		Direct Detect™ spectrometer	
Cell line	Scepter ™ total cell count	Estimated No. of cells per sample	CytoBuster ™ solution [mg/mL]	RIPA [mg/mL]	CytoBuster ™ solution [mg/mL]	RIPA [mg/mL]
MCF-7	2.8 X 10^6	1.4 x 10^6	2.4	3.4	2.7	4.6
T47D P17	8.0 x 10^6	4.0 x 10^6	Not determined	Not determined	4.6	5.8

The total protein content estimated by the Direct Detect™ spectrometer is slightly different from the results obtained by BCA assay. The differences observed in measured protein concentration are most likely due to the fact that the colorimetric assays are influenced by the detergents present in both buffers. By contrast, the Direct Detect™ system is not.

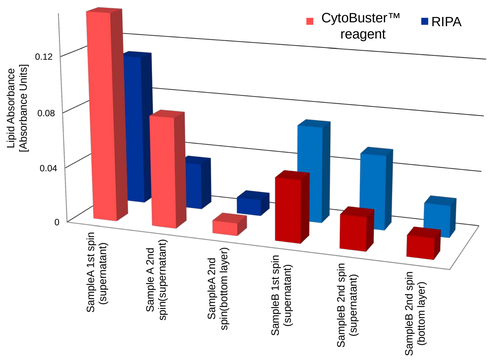
Protein concentration and lipid profiles from breast cancer tissue lysates prepared in two different lysis buffers. Values obtained by the Direct Detect™ spectrometer.

		CytoBuster ™ Protein Extraction Reagent		RIPA	
Sample	Spin condition and fraction collected	Protein content [mg/mL]	Lipid absorbance [AU]	Protein content [mg/mL]	Lipid absorbance [AU]
Sample A (1st extraction)	1st spin (top fatty fraction)	5.0	0.162	14.0	0.110
	2nd spin sample I (top fatty fraction)	2.7	0.080	20.0	0.034
	2nd spin sample II (bottom layer)	5.0	0.009	17.0	0.012
Sample B (2nd extraction)	1st spin (top fatty fraction)	3.3	0.045	3.6	0.070
	2nd spin sample I (top fatty fraction)	2.1	0.024	5.0	0.054
	2nd spin sample II (bottom layer)	2.8	0.015	5.0	0.023

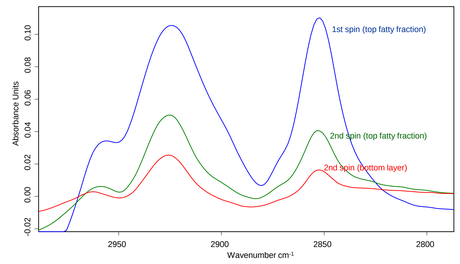
A new feature in the Direct Detect™ spectrometer software allows for simultaneous quantification of protein and relative measurement of lipid content in the same sample. This new capability permits monitoring of the sample during the fat removal process.

Change in the lipid content during the preparation of the tissue lysate.

Relative lipid levels in three different fractions of breast cancer tissue lysates prepared with CytoBuster™ Protein Extraction Reagent and RIPA buffer

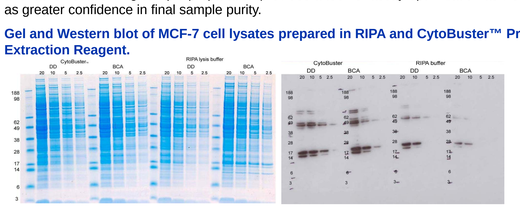


Comparison of lipid IR spectra from three fractions of breast cancer tissue lysate detected by the Direct Detect™ spectrometer.



IR spectra collected by the Direct Detect™ spectrometer showed gradual removal of a fatty fraction from the sample. The graph above highlights a "lipid" region (wavenumbers 3000 to 2800 cm^{-1}) in the infrared spectra collected from breast cancer tissue lysate in RIPA buffer. Total protein was quantified using the Amide I region of the same spectra (not shown in the graph above). A similar profile has been observed for lysate prepared using CytoBuster™ solution. The ability to simultaneously monitor protein concentration and fat removal during sample preparation provides a tool for assay optimization as well as greater confidence in final sample purity.

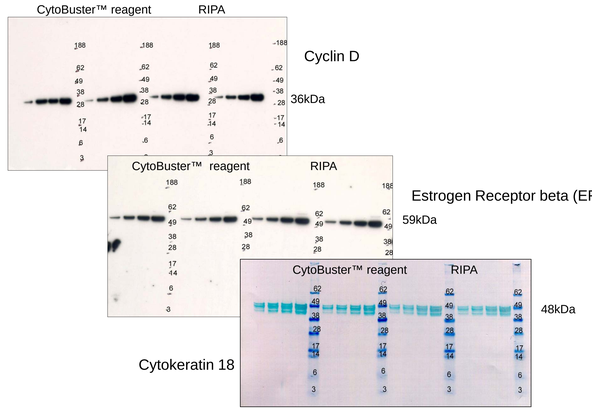
Gel and Western blot of MCF-7 cell lysates prepared in RIPA and CytoBuster™ Protein Extraction Reagent.



MCF-7 pellets were lysed with two different lysis buffers. Samples were then separated by electrophoresis, transferred to Immobilon P® membrane and processed in the SNAP i.d.® 2.0 system. The blot was probed with anti-Cathepsin D.

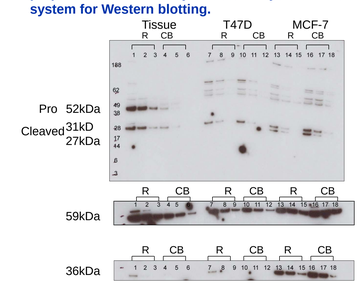
Cathepsin D is an aspartic endopeptidase present in most mammalian cells. Over-expression of this protease has been associated with the progression of several human cancers including gastric carcinoma, melanoma, and ovarian cancer. Cathepsin D has been shown to be an independent marker of poor prognosis for breast cancer patients [1].

SNAP i.d.® 2.0 immunodetection of breast cancer markers in MCF-7 cell lysates prepared with RIPA and CytoBuster™ reagents.



Cyclin D and Erβ were detected by chemiluminescence using Luminata™ Forte reagent while Cytokeratin 18 was detected by chromogenic methods using TMB insoluble reagent.
Cyclin D is a nuclear protein that regulate the growth of estrogen responsive tissues by activating the estrogen receptor (ER). Cyclin D has been strongly implicated as a proto-oncogene, and is amplified in 15% of all breast cancers [2] and 45-50% of primary ductal carcinomas [3]. **ERβ** is a nuclear protein, and member of the steroid/thyroid hormone receptor superfamily. It is involved in the regulation of normal function of reproductive tissues and has been implicated in supporting the growth of about 50% of the primary breast cancers [4].
Cytokeratin 18 is a structural marker protein specific for epithelial cells. It has been observed to be downregulated in lobular and ductal carcinomas [5] and is proposed as a useful biomarker for clinical trials [6].

Differences in the expression of Cathepsin D, Estrogen receptor β and Cyclin D in breast ductal carcinoma tissue and two breast cancer cell lines. Cell lysates were prepared in RIPA and CytoBuster ™ lysis buffers and detected using the SNAP i.d.® 2.0 system for Western blotting.



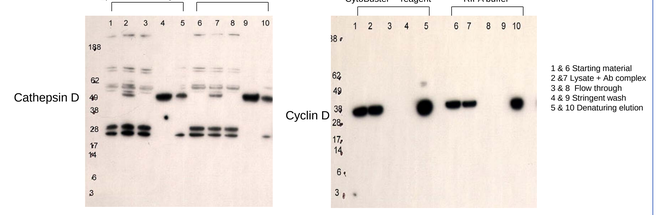
Lysates prepared in RIPA (R) and CytoBuster™ (CB) protein extraction reagent

Cathepsin D: Stronger signals of procathepsin D and cleaved product were observed in the tissue compared to the cell lines.

ERβ is expressed in all the samples (tissue and cells), but is less prominent in the T47D cell line.

Cyclin D was strongly expressed in the MCF-7, but to a lesser extent in tissue and in the T47D cell line.

Immunoprecipitation of Cathepsin D and Cyclin D in MCF-7 cell lysates using PureProteome™ protein A magnetic beads.



Although RIPA buffer seemed to yield more "total protein" in the lysis, immunoprecipitation revealed that more of the relevant biomarker was liberated using CytoBuster™ Protein Extraction Reagent (lane 10) than with RIPA buffer (lane 5).

Conclusions

- Bacterial cultures lysed with BugBuster® Protein Extraction Reagent supplemented with Benzonase® Nuclease and rLysozyme™ Solution liberated significantly more recombinant CRP than homebrew lysis buffer.
- Selection of the optimal lysis buffer is not as simple as selecting the one that liberates the most total protein. Factors such as cell type, sample format (tissue or cells), and fat content can impact sample performance.
- The Direct Detect™ spectrometer permits protein quantitation and qualitative assessment of fat content from a single sample. This feature enables sample monitoring during the optimization process.
- When used in tandem, the Direct Detect™ spectrometer and SNAP i.d.® 2.0 immunodetection system not only expedite the protein detection workflow, but also provide greater reliability in sample integrity and end results.

Summary

Successful biomarker analysis requires optimizing the sample preparation method not only for the liberation of the marker but also for the downstream analytical method. Evaluation of several lysis buffers for the detection of breast cancer markers revealed that no single system was optimal for all samples. In one case, RIPA buffer generated higher "total protein" content when applied to the lysis of breast cancer tissue, while CytoBuster™ Extraction Reagent was more efficient for the liberation of the desired biomarker. Application of the Direct Detect™ spectrometer and SNAP i.d.® 2.0 system allowed fast turnaround of the experiments, enabling rapid screening and optimization of sample preparation.

References

1. G. Jacobson-Raber, I. Lazarev, V. Novack et al. *Oncology Letters*,(2011) 2(6):1183–1190.
2. P. G. Roy and A.M. Thompson. *The Breast* (2006) 15:718-727.
3. R.L. Sutherland, E. A. Musgrove. *Breast Cancer Research* (2001) 4(1):1-4.
4. C. Palmieri, G.J. Cheng, S. Saji, et. al *Endocrine-Related Cancer* (2002) 9: 1-13.
5. U. Woelfle, G. Sauter, S. Santjer et. al. Clinical Cancer Research (2004) 10:2670-2674 .
6. M. Hagg Olofsson, T. Ueno, Y. Pan, et.al. *Cli Cancer Res* (2007) 13:3198 – 3206.

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