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APPLICATION NOTE

Protein extraction efficiency of Omni Bead Ruptor Elite bead mill homogenizer and detection of total and phosphorylated proteins by HTRF immunoassay.

Omni Bead Ruptor Elite bead mill homogenizer

Summary

Protein extraction techniques from organs, tissues, and cells is a critical step for validation of protein expression in biomedical research, such as in drug efficacy and toxicity studies. Upstream of protein extraction, sample preparation is required to lyse cells within a sample, releasing analytes of interest into solution for downstream evaluation. Traditional lysis methods can be inefficient and time consuming, typically involving manual hand-held homogenizers or enzymatic techniques. The Omni Bead Ruptor Elite™ bead mill homogenizer, however, has been shown to be a quick and efficient method for sample preparation of various types like tissues and cells [1].

In this application note, MCF7 cells, an estrogen receptor positive breast cancer cell line, were cultured and treated with MAPK/ERK pathway inhibitors (U0126 and PD98059) or treated with a cell cycle (CDK4/6) inhibitor, Palbociclib. The cells or cell pellets harvested from the tissue culture plates were lysed using two methods: the Omni Bead Ruptor Elite bead mill homogenizer and standard cell lysing method described in the protein detection kits. The resulting cell lysate samples were tested for total cellular protein content and expression of total and phosphorylated ERK and Rb (retinoblastoma) proteins to determine the protein extraction efficiency of the two methods of lysate sample preparation, as well as the effects of inhibitor treatments. Results indicated that the Omni Bead Ruptor Elite bead mill homogenizer is a quick and effective sample preparation instrument when extracting proteins from cells.

For research use only. Not for use in diagnostic procedures.

This was demonstrated through the detection of total cellular protein, total and phosphorylated ERK and Rb proteins and the inhibition of phosphorylated ERK and Rb protein expressions in cells treated with inhibitors.

Materials and methods

Equipment

- Omni Bead Ruptor Elite bead mill homogenizer (Cat # 19-042E)
- Omni Bead Ruptor Elite 2 mL Tube Carriage (Cat # 19-373)
- 2 mL Hard Tissue Homogenizing Mix 2.8 mm Ceramic Beads (Cat # 19-628)
- EnVision® 2105 Multimode Plate Reader (Cat # 2105-0010)
- HTRF® Total-Rb Detection Kit (Cat # 64RBTPEH)
- HTRF Phospho-Rb (Ser780) Detection Kit (Cat # 64RBS780PEG)
- HTRF Total-ERK Detection Kit (Cat # 64NRKPEG)
- HTRF Advanced phospho-ERK (Thr202/Tyr204) Detection Kit (Cat # 64AERPEG)

Procedure

MCF7 culture, cell treatments, and protein extraction

MCF7 cells (ATCC, Cat # HTB-22) were cultured and maintained in T75 flasks with complete culture medium: EMEM (ATCC, Cat # 30-2002) containing 10 % FBS (ATCC, Cat # 30-2020), 1 % penicillin/streptomycin (ATCC, Cat # 30-2300) and 0.01 mg/mL human recombinant insulin (Santa Cruz Biotech, Cat # sc-360248). When the cells were > 80 % confluent, the cells were disassociated with 2 mL of 0.25 % trypsin (ATCC, Cat # 30-2101). 8 mL of fresh media was added to the flask and the cell solution was transferred into a sterile 15 mL centrifuge tube. The tube was spun, media was discarded, 1 mL fresh media was added, and the cells were dispersed by pipetting up and down. The number of viable cells was determined by trypan blue staining and a cell solution containing 250,000 (250K) cells/mL was prepared. A volume of 2 mL/well cell solution was dispensed into two 6-well tissue culture plates (Fisher Scientific, Cat # 08-772-1B), resulting in a cell density of 500K cells/well. The cell plates were incubated in a 37 °C, 5 % CO_2 incubator for 3 days. The cell media was replaced with or without 10 µM inhibitors and cultured for an additional 2 days. Control 1, 2, and 3 represents the vehicles used to solubilize the inhibitor U0126 (Thermo Fisher, Cat # J61246.MB), PD98059 (Gibco, Cat # PHZ1164), and Palbociclib (Sigma, Cat # PZ0383-5MG), respectively (Figure 1). The cells in plate 1 were lysed for 60 minutes using the method described in HTRF assay kit protocol. The cells in plate 2 were scraped and harvested.

To extract proteins from the cell pellets from plate 2 (Figure 1) using the Omni Bead Ruptor Elite bead mill homogenizer, 1 mL of cell lysis buffer from the detection assay kit was added to cell pellets and the solution was transferred to 2 mL reinforced tubes containing 2.8 mm ceramic beads. The tubes were placed into the homogenizer and the cell pellets were homogenized at 4.5 m/s for 20 seconds. Following homogenization, the samples were transferred to pre-chilled Eppendorf tubes (VWR, Cat # 80077-226) and centrifuged at 4000 RPM for 10 min at 4 °C using an Eppendorf refrigerated centrifuge (VWR, Cat # 97058-928). The supernatants (top portion) were transferred to 96-well sample storage plate that were then sealed and stored at -80 °C until the assays were performed.

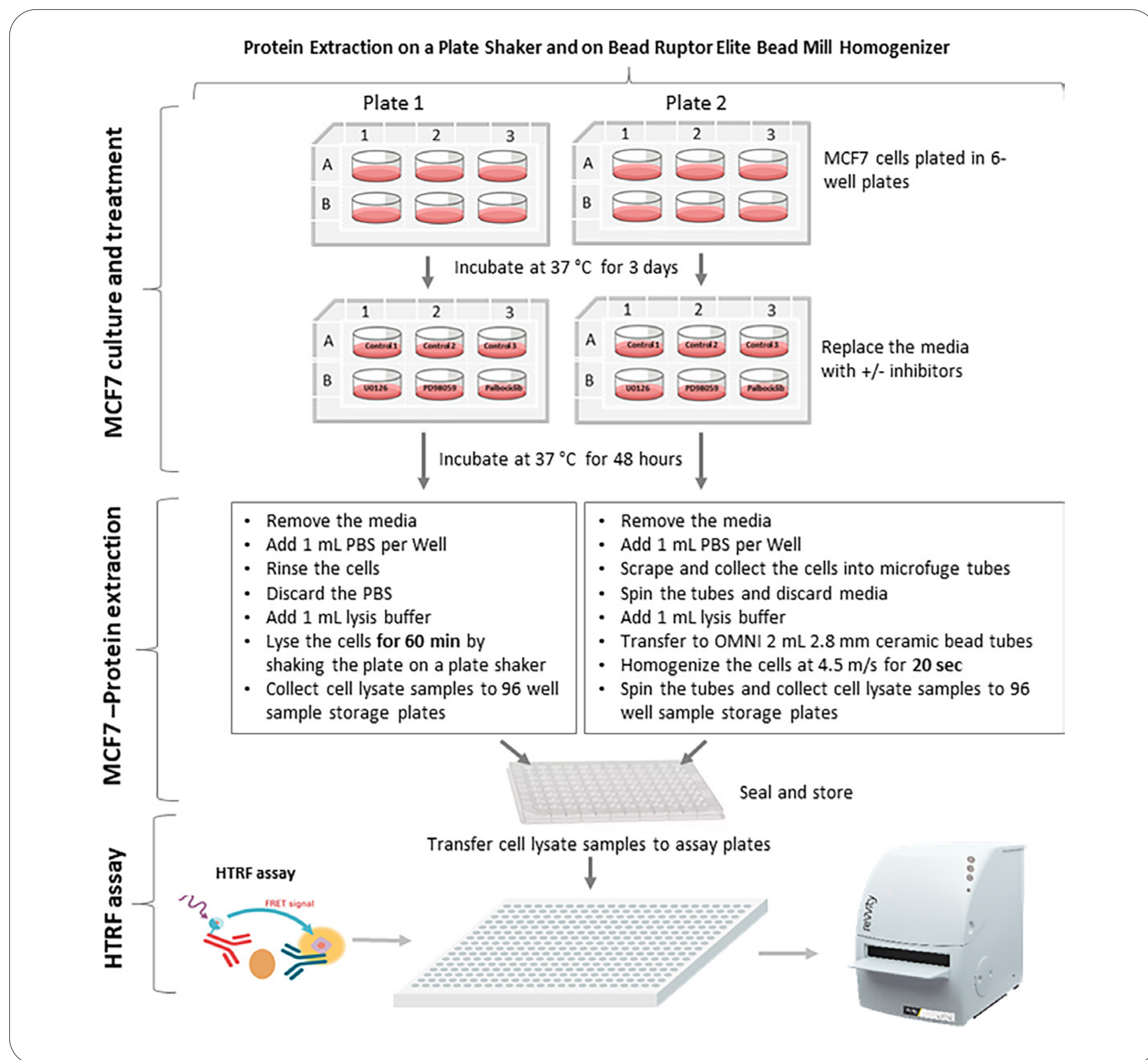

Figure 1: Experimental workflow and HTRF assays [2].

BCA total protein, HTRF total and phosphorylated ERK and Rb assays

The assays were performed by following the kit protocol. The cell lysate sample plates were removed from -80 °C freezer and thawed for 60 min at room temperature. The plates were then centrifuged at 4000 rpm for 10 min at 4 °C. The required volumes of cell lysate samples were carefully transferred to the appropriate assay plates and the assay reagents were added as recommended in each kit protocol for BCA total protein (Thermo Scientific, Cat # A53226), Total Rb, phosphorylated-Rb, Total ERK and phosphorylated-ERK. The plates were gently tapped, sealed, and incubated as instructed in the kit manual. After the final incubation, the top seal (Cat # 6050185) was removed if the plates were sealed, and the plates were then read on Revvity EnVision 2105 Multimode Plate Reader.

Data analysis

Data analysis was performed using Microsoft Excel and GraphPad Prism. For HTRF total and phosphorylated ERK and Rb assays, the ratios of HTRF signals (665 nm/620 nm*10,000) were calculated and summarized. The BSA standard curve of BCA assay were plotted using GraphPad Prism. Concentrations (µg/mL) of total protein in the cell lysate samples were obtained by interpolating sample signals to the BSA standard. HTRF ratios of total and phosphorylated ERK and Rb assay results were normalized to the corresponding total cellular protein measured by the BCA assay. The HTRF ratios of total and phosphorylated protein were divided by the concentration of the total cellular protein and multiplied by 1000 (Total Rb HTRF ratio/total cellular protein*1000) to obtain HTRF ratio per milligram of total cellular protein.

Results

The results from the BCA assay show that the levels of total cellular protein detected in cell lysate samples did not change with or without inhibitor treatments. Therefore, the protein concentration from each tissue culture plate was averaged to demonstrate the efficiency of total protein extraction from the cells lysed with shaking on a plate shaker for 60 min versus the cell pellets homogenized with Omni Bead Ruptor Elite bead mill homogenizer for 20 seconds. As presented in Figure 2, the total protein extraction efficiency of shaking on a plate shaker for 60 min (Shaking 60 min) is comparable to that of homogenizing the cell pellets for 20 seconds (Omni 20 sec) using the Omni Bead Ruptor Elite bead mill homogenizer. These results indicate that the Bead Ruptor Elite bead mill homogenizer is an efficient instrument and requires only 20 seconds to breakdown the cell pellets to release cellular proteins.

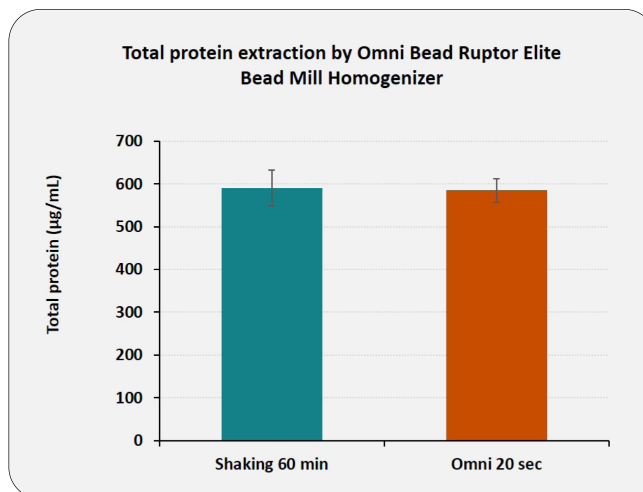

Figure 2: Total protein extraction efficiency of Omni Bead Ruptor Elite bead mill homogenizer, comparing to lysing the cells for 60 min on a plate shaker. Total protein levels were measured using BCA protein assay kit.

The results of measuring total ERK showed that total ERK protein levels also did not change with inhibitor treatments or the methods of protein extraction (Figure 3A). However, as expected, the levels of phosphorylated ERK (pERK) were significantly changed in cells treated with inhibitors (Figure 3B and 3C). Compared to the controls, U0126 and PD98059 treatment reduced the pERK expression while Palbociclib increased the pERK expression in the lysate samples prepared using both methods of protein extraction (Figure 3B and 3C). The reduced expression of pERK in cancer cell lines treated with U0126 and PD98059 is widely reported in the literature and the increased expression of pERK in cell treated with Palbociclib is also reported [3]. These results demonstrate that the effect of drug treatments on ERK phosphorylation can be determined for the cell lysate samples prepared using Omni Bead Ruptor Elite bead mill homogenizer.

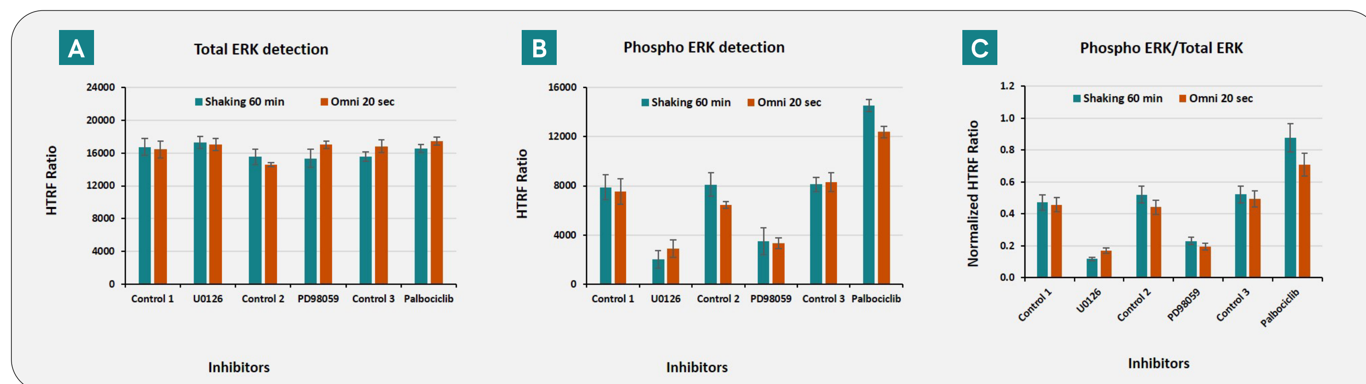

Figure 3: A. Total ERK and B. phosphorylated ERK levels in MCF7 cells treated with inhibitors. C. Phosphorylated ERK normalized to Total ERK (phosphorylated ERK/Total ERK). Comparing shaking 60 min to the Omni 20 sec. Total and phosphorylated ERK protein levels were measured by HTRF kits.

The results of total and phosphorylated Rb detection are shown in Figure 4. Compared to the controls, a small reduction of total Rb protein was observed in both methods of protein extraction when the cells were treated with U0126 and PD98059, and a greater reduction of total Rb protein was seen in the cells treated with Palbociclib (Figure 4A). Similarly, all three inhibitors reduced the levels of phosphorylated Rb protein expression in the samples (Figure 4B and 4C). These results harmonize with the data reported on the HTRF total and phosphorylated Rb detection kit manuals and the data reported on the paper published by Fang et al. [3] in which both total and phosphorylated Rb expression were reduced dramatically in cells treated with Palbociclib.

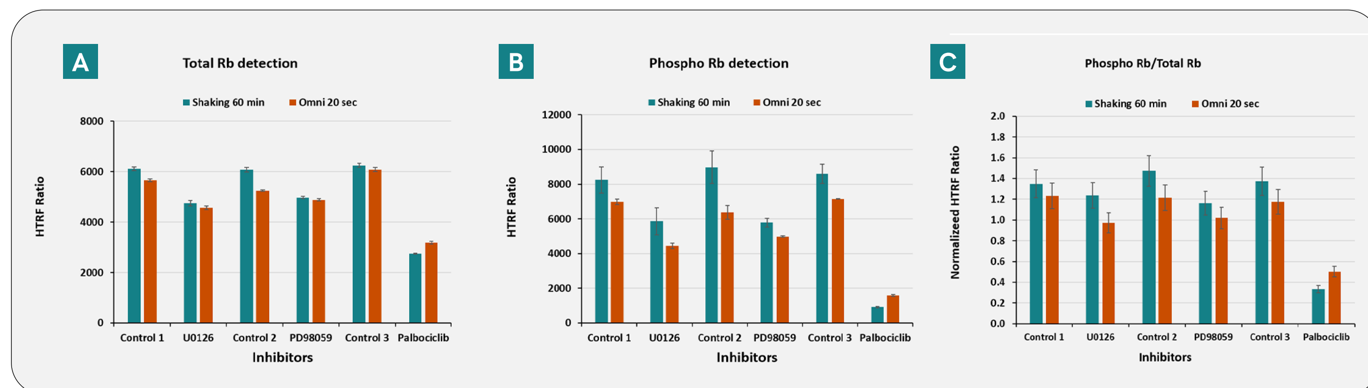

Figure 4: A. Total Rb and B. phosphorylated Rb protein levels in MCF7 cells treated with inhibitors. C. Phosphorylated Rb normalized to Total Rb (phosphorylated Rb/Total Rb). Comparing shaking 60 min to the Omni 20 sec. Total and phosphorylated Rb protein levels were measured by HTRF kits.

Conclusions

Our results demonstrate that the Omni Bead Ruptor Elite bead mill homogenizer can effectively break down MCF7 cell pellets to release intracellular proteins in lysate samples. The effectiveness of the homogenizer is shown by the detection of total cellular protein using BCA protein assay kit and HTRF total and phosphorylated ERK and Rb detection kits. The levels of total cellular protein in the lysate samples prepared using Omni Bead Ruptor Elite bead mill homogenizer for 20 seconds is essentially equal to those in lysate samples prepared using the 60-minute cell lysing protocol on a plate shaker. The reduction of phosphorylated ERK and Rb protein expression in cells treated with inhibitor indicate that Omni Bead Ruptor Elite is suitable for preparing the cell lysate samples for detection of cellular proteins.

In conclusion, the Omni Bead Ruptor Elite bead mill homogenizer is a quick, easy, and efficient instrument to prepare cell lysate samples from cell pellets. HTRF total and phosphorylated detection kits in combination with the EnVision Multimode Plate Reader, can be used to rapidly measure cell signaling proteins, and other important intracellular targets present in lysates to evaluate drug efficacy. When used together these two technologies can significantly reduce the effort and time needed to prepare samples for high-throughput applications.

References

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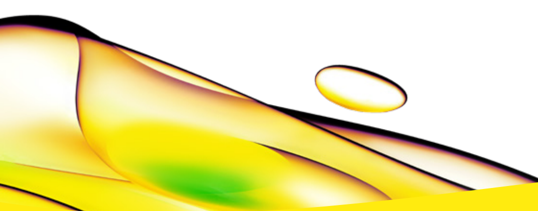

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