EXTRACTMAN®: Rapid Yet Gentle Purification of Protein-Ligand Complexes

APPLICATION NOTE AN1007

APPLICATION BENEFITS

RNA-binding proteins (RBPs) are involved in many key cellular processes, but identification of the native binding partners (target RNAs) can be challenging. Many methods for isolating RBP-target complexes require crosslinking, which is inefficient, and/or harsh washes, which can disrupt native interactions holding the ribonucleoprotein complex together.

SOLUTIONS

EXTRACTMAN uses a novel magnetic apparatus and hydrophobic surface treatments to enable rapid and gentle ribonucleoprotein complex isolation. Biomolecules bound to paramagnetic beads are washed gently, facilitating capture of endogenous complexes that may be lost with harsher methods. Immunoprecipitation followed by EXTRACTMAN® purification was used to purify an RNA-binding protein and native ligands without crosslinking.

SAJA FAKHRALDEEN | University of Wisconsin; Madison, WI, USA | SCOTT BERRY & DAVID BEEBE | Salus Discovery; Madison, WI, USA

ABSTRACT

Specific RNA partners of an RNA-binding protein were easily captured using EXTRACTMAN® without the need for cross-linking. EXTRACTMAN enables rapid isolation of biomolecules bound to paramagnetic beads with gentle wash steps that facilitate capture of endogenous complexes that may be lost with conventional harsh methods.

INTRODUCTION

RNA-binding proteins (RBPs) are involved in many key cellular processes, including transcription, RNA processing, RNA stability, and translation. Deregulation of RBP expression is implicated in several human diseases, including cancer. The human genome encodes more than 1,500 different RBPs, but few have been fully characterized.[1]

> "...The process is rapid yet gentle, does not require protein cross-linking, and can be carried out with 90% less lysate..."

One challenge is identification of native binding partners (target RNAs). De novo prediction of RBP binding partners is difficult.[2] RBPs may rely on sequence and/or structure for recognition of their target RNA molecules. RNA structures can be complex, with single- and double-stranded regions, gaps, and stem-loops. Isolation of native RBP protein-target complexes from live cells is an area of active study.

Figure 1
EXTRACTMAN® enables rapid and gentle affinity purification of proteins, nucleic acids, and protein-target complexes.

GILSON®

Interactions between an RBP and endogenous RNA targets can be short lived, increasing the difficulty of isolating intact complexes. Many common methods for isolating these complexes require crosslinking, which is inefficient and artifact-prone, and/or a series of harsh washes, which can disrupt the interactions holding the ribonucleoprotein complex together.

EXTRACTMAN® offers a new method to overcome these challenges: Exclusion-based sample preparation (ESP™) technology reduces processing steps while enhancing performance. EXTRACTMAN employs surface tension and patterned hydrophobicity in conjunction with 'capture' and 'release' magnets to quickly attract, move, and disengage magnetic beads in an aqueous solution. The process is rapid yet gentle, does not require protein cross-linking, and can be carried out with 90% less lysate than is typically required for ribonucleoprotein immunoprecipitation. Moreover, the RNA targets isolated via ribonucleotide immunoprecipitation with ESP affinity purification are specific and biologically relevant.[3]

In this application note, we show that EXTRACTMAN can be used to efficiently and specifically enrich endogenously expressed ribonucleoprotein complexes.

MATERIALS AND METHODS

Mammalian Cell Culture

Cell lines were grown and maintained under standard conditions at 37°C with 5% CO_2 and controlled humidity. Cell viability and morphology were routinely assessed using standard techniques.

Ribonucleoprotein Immunoprecipitation

Cells (~1-2 x 10^6) were lysed in 500 µL polysome lysis buffer [10 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 100 mM KCl, 0.5% NP-40], which maintains RNA-protein interactions, containing freshly added DTT (1 mM), RNase inhibitor (100 U/ml), protease inhibitor, and phosphatase inhibitor (Thermo Fisher Scientific). Lysates were sonicated for 10 pulses at 4-5 W, cleared of debris by spinning 3x at 12,000 rpm for 30 min at 4 °C, then immediately subjected to immunoprecipitation using antibodies targeting RBP of interest: lysate (200 µL), 1.2 µg of primary antibody, and 5 µL of protein G-bound paramagnetic Dynabeads® (Life Technologies cat# 10003D). These immunoprecipitation suspensions (containing lysate, antibody and paramagnetic beads) were incubated overnight at 4°C on a rotator and then subjected to EXTRACTMAN®-based purification. Protein concentrations were determined using Bradford reagent (Sigma-Aldrich).

Affinity Purification with EXTRACTMAN

Magnetic bead-based affinity purification was carried out using EXTRACTMAN, a small benchtop device for manual isolation of biomolecules in aqueous solutions. The device consists of a base, into which a collection plate (SLAS footprint) is placed; a sliding lid,[4] which contains a mechanism for capture of paramagnetic beads; and a release magnet positioning handle which, when moved forward or backward to the appropriate well column, actuates the magnetic mechanism and thereby 'captures' or 'releases' the magnetic beads.

RESULTS AND DISCUSSION

Immunoprecipitation suspensions were loaded into the input wells of an EXTRACTMAN® microplate. The sliding handle of EXTRACTMAN was positioned to capture the paramagnetic beads on the bead capture strip, then moved along the track to the next well column in order to transfer the paramagnetic beads to the meniscus of the first wash solution (100 µL). Beads were released into the wash solution, then recaptured and the handle was repositioned to transfer the paramagnetic beads to the next wash step. In the final step, beads are released into 100 µL of PBS containing 0.01% TWEEN-20.

Bound (immunoprecipitated with anti-RBP 1° Ab or anti-IgG 1° Ab) and unbound (material that remained unbound in the well following immunoprecipitation) fractions were run in parallel lanes of wells on EXTRACTMAN®. No cross-contamination between lanes on EXTRACTMAN was observed, consistent with previous reports.[5] The bound and unbound fractions were collected and a portion of each fraction was used for SDS-PAGE analysis to assess protein enrichment efficiency. The remaining volumes from the immuno-precipitation reactions were used for RNA isolation (RNeasy Mini Kit by Qiagen) and subsequent RNA analyses.

Figure 2
Schematic representation of experimental workflow.

Western Blotting

Ribonucleoprotein immunoprecipitation fractions were analyzed by SDS-PAGE, transferred to PVDF membranes, blocked in 5% milk in TBS-Tween and incubated with the 1° antibodies at 4°C overnight and 2° antibodies for one hour at room temperature. All antibodies were diluted in 5% milk in TBS-Tween. Primary antibodies used and dilutions: anti-RBP (Cell Signaling) at 1:1000; anti-vinculin (Millipore cat, PN 05-368) at 1:5000. Secondary (2°) antibodies used and dilutions: anti-mouse-HRP (Jackson Immunoresearch); anti-rabbit-HRP (Life Technologies) both at 1:5000.

RNA Analysis

Total RNA was isolated from ribonucleotide immunoprecipitation fractions using the RNeasy Mini Kit (Qiagen). RNA concentrations and quality were determined using a Nanodrop (Thermo Scientific).

RESULTS AND DISCUSSION

Ribonucleotide immunoprecipitation followed by purification using EXTRACTMAN® was used to efficiently and specifically enrich for RBP-containing ribonucleoprotein complexes. EXTRACTMAN was used to isolate endogenous ribonucleoprotein complexes from two different subtypes of human breast cancer cells: MCF7 and MDA-MB-231.

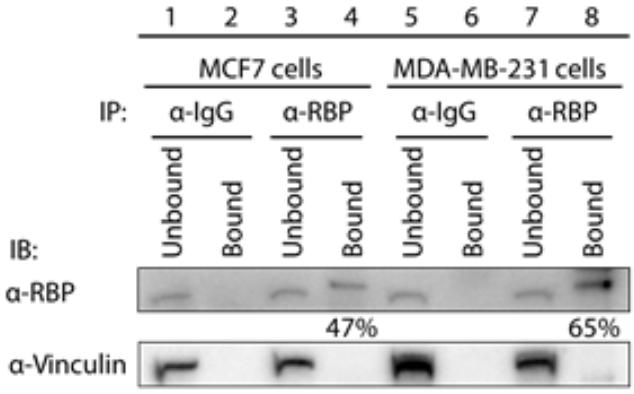

Figure 3
Western Blot Analysis of Proteins Purified from Crude Lysates using EXTRACTMAN.

A representative Western blot is shown in Figure 3. Proteins that a) were captured during immunoprecipitation (IP) with the antibody indicated and b) were recognized by the antibody indicated on Western Blots will show up as a dark band (immunoblot, IB). Vinculin served as an internal loading control. An anti-IgG immunoprecipitation was performed as a control for the specificity of the immunoprecipitation procedure. This blot compares levels of proteins in RBP-enriched bound fractions and their respective unbound fractions from two human breast tumor cell lines. Lanes 4 and 8 show that EXTRACTMAN purification following immunoprecipitation with an anti-RBP antibody successfully enriched for capture of RBP. The percentage of enrichment [(µg bound material)/(µg bound + µg unbound material)]) in lanes 4 and 8 are indicated. Enrichment depends on the avidity and specificity of the antibody, amongst other factors.

To illustrate that the RBP-containing complexes isolated by immunoprecipitation and EXTRACTMAN® purification contained RNA as predicted, we subjected the isolated complexes to RNA extraction and quantification. Table 1 shows that RNA in the bound fractions was significantly enriched in samples incubated with the anti-RBP antibody compared to the anti-IgG control. In further work we demonstrated that the RNA species isolated via ribonucleoprotein immunoprecipitation followed by ESP™ affinity purification are specific and biologically relevant, as assessed by microarray analysis and RT-PCR.[3]

Table 1:
RNA Quantification of EXTRACTMAN-Purified Fractions

	FRACTION	TOTAL RNA (MG)	RNA ENRICHMENT
MCF-7	Unbound	14.6	-
α-IgG	Bound	0.02	0.2%
MCF-7	Unbound	14.9	-
α-RBP	Bound	0.4	2.6%
MDA-MB-231	Unbound	7.9	-
α-IgG	Bound	0.13	1.7%
MDA-MB-231	Unbound	11.5	-

REFERENCES

1. S. Gerstberger, M. Hafner, T. Tuschl. A census of human RNA-binding proteins. *Nat. Rev. Genet.*, 15, 829-845 (2014).
2. J. Si, J. Cui, J. Cheng, R. Wu. Computational prediction of RNA-binding proteins and binding sites. *Int. J. Mol. Sci.* 16: 26303-26317 (2015).
3. S. Fakhraldeen, Ph.D. thesis, University of Wisconsin-Madison (2015).
4. B.P. Casavant, D.J. Gluckenberger, D.J. Beebe, S.M. Berry. Efficient sample preparation from complex biological samples using a sliding lid for immobilized droplet extractions. *Anal. Chem.*, 86, 6355-6362 (2014).
5. Application Note AN1007 (gilson.com)

ACKNOWLEDGMENTS

Saja Fakhraldeen would like to thank her thesis advisor, Caroline Alexander (University of Wisconsin-Madison), for her support during this project. The authors wish to thank Karen Kleman (Gilson) for her helpful discussions.

SUMMARY

- Endogenous RNA-binding protein and native ligands were purified via immunoprecipitation followed by EXTRACTMAN® purification, without the need for crosslinking or harsh washes.
- EXTRACTMAN can be used to replace lengthy conventional cross-linking procedures in isolating RNA-binding partners of a ribonucleoprotein.
- EXTRACTMAN uses a novel magnetic apparatus and hydrophobic surface treatments to enable rapid and gentle affinity purification of biomolecules.

Trademarks

All product and company names are trademarks™ or registered® trademarks of their respective holders. Use of the trademark(s) in this document does not imply any affiliation with or endorsements by the trademark holder(s).

Exclusion-based sample preparation (ESP™) is a trademark of Salus Discovery.

Dynabeads®is a registered trademark of Life Technologies

Nanodrop™ is a trademark of ThermoScientific