Immunoprecipitation using Thermo Fisher Dynabeads on the OT-2

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ABSTRACT

Automated protocols developed to perform magnetic bead-based immunoprecipitation (IP) to process up to 96 samples with minimized hands-on-time were tested on the robotic liquid handler Opentrons OT-2. The samples were prepared by diluting recombinant GAPDH protein in lysis buffer or by using HEp-2 cell lysates and then processed for IP using Thermo Fisher Dynabeads™ conjugated with Protein G or with Protein A, which binds to soluble antibodies to facilitate subsequent binding to the target protein, GAPDH. Protein analysis results showed that the target protein was successfully extracted from the sample solutions with desirable consistency, demonstrating the ability of the OT-2 to automate IP experiments.

INTRODUCTION

Immunoprecipitation (IP) is a common method that uses antibody to capture a target protein within a mixture of molecules. The antibody can be pre-attached to a solid-phase substrate such as agarose resins or superparamagnetic beads before being exposed to its target protein or directly added into the sample together with such solid-phase substrate. The incubation period is optimized for target capture which depends on the affinity of the antibody to its target protein. The antibody-protein-bead complex is precipitated and collected by centrifugation or by magnetism if superparamagnetic beads are used.

Dynabeads™ (Thermo Fisher Scientific, Waltham, MA, USA) are superparamagnetic beads that can be modified to have different functionalities. For instance, recombinant Protein G or Protein A can be covalently attached to the bead surface. Both Protein G and Protein A are bacterial proteins that exhibit high affinity to the Fc region of monoclonal or polyclonal IgG-type of antibodies. The Dynabeads™-Protein G or Dynabeads™-Protein A serve as the solid-phase support for the antibodies. Using the Opentrons OT-2, robust automated Dynabeads™-mediated IP protocols were generated for targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for up to 96 samples per experiment, with excellent reproducibility between samples.

METHODS

The procedure consists of: Part 1 for preparation of a mixture of beads, antibody, and sample; an antibody/target protein incubation period determined by the user; Part 2 for washing and elution steps **(Figure 1)**. Part 1 and Part 2 are conducted on the OT-2 with a GEN2 Magnetic Module to achieve the separation of magnetic beads from the solution and a GEN2 Temperature Module for preparation of denatured protein eluates for SDS-PAGE. The incubation is carried out on a shaker. The OT-2 deck layouts for Part I and Part 2 of the IP protocol are presented in **Figure 2**. This Reagent-In-Tube protocol was designed to transfer reagents directly from 15 mL conical tubes to the working plate where the bead separation was conducted. Alternatively, the Reagent-In-Plate protocol **(Figure 3)** uses prefilled reagent plates prepared by running a reagent plate preparation protocol on the OT-2 and can reduce runtime **(Figure 4)**. The binding of the antibody to its target protein is the most critical step for the success of IP, which is highly dependent on the affinity of the antibody. For capture of proteins with low binding affinity or low abundance, preincubation of the antibody in the sample solution is recommended.

Figure 1: This is an overview of the 3 major steps of protein purification on the OT-2.

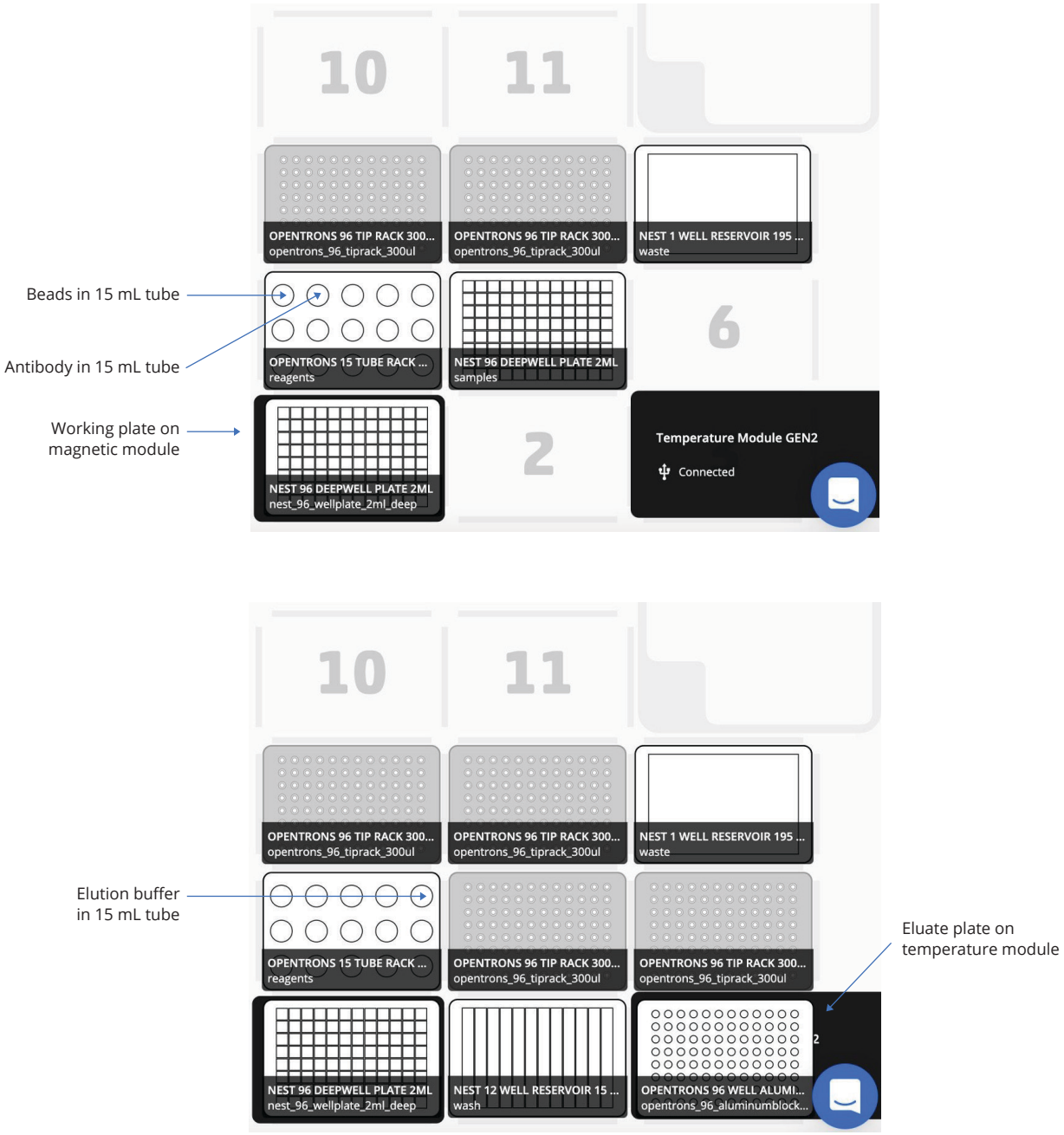


Figure 2: Schematic representation of the labware arrangements on the OT-2 (Reagent-In-Tube).

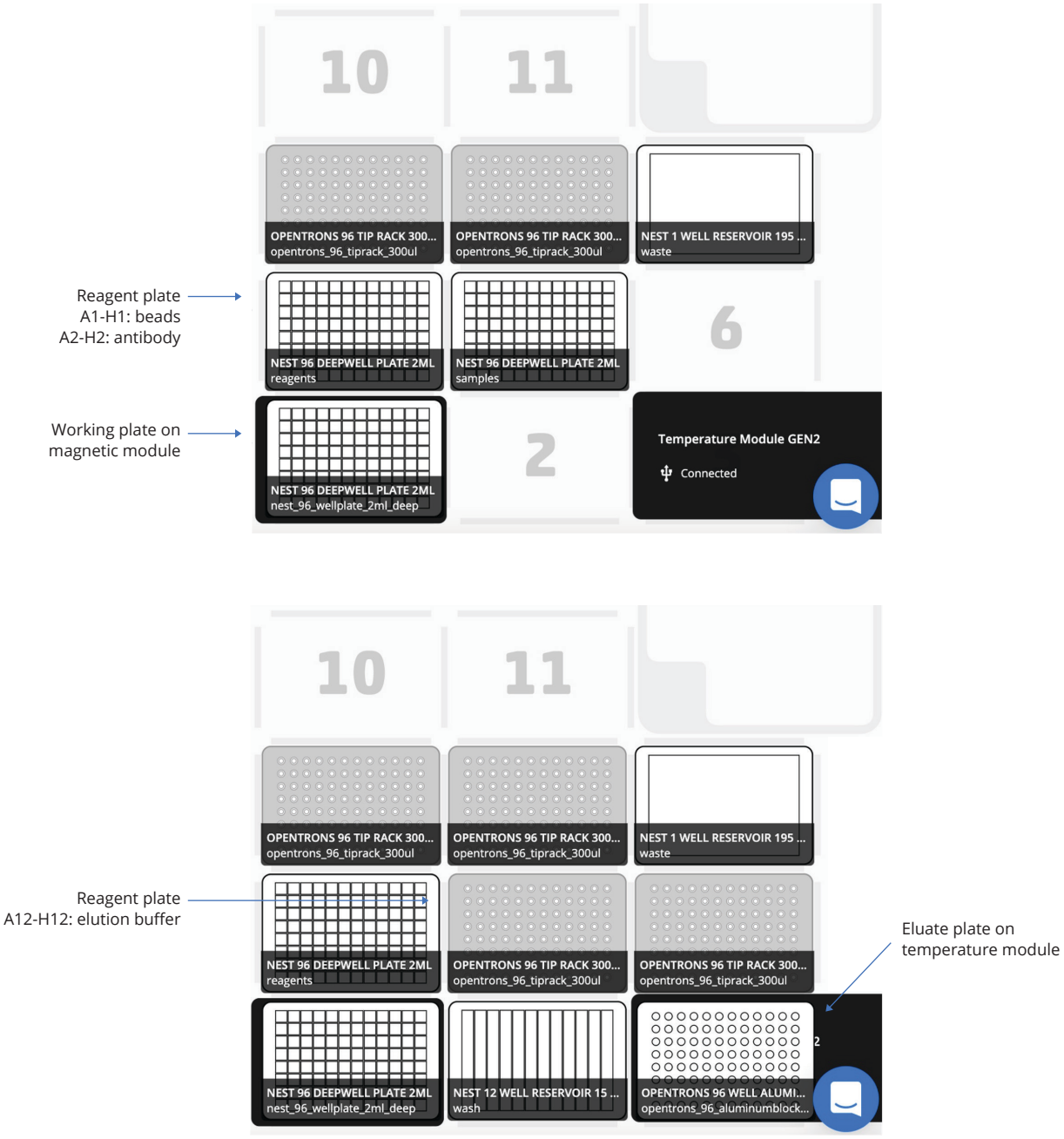
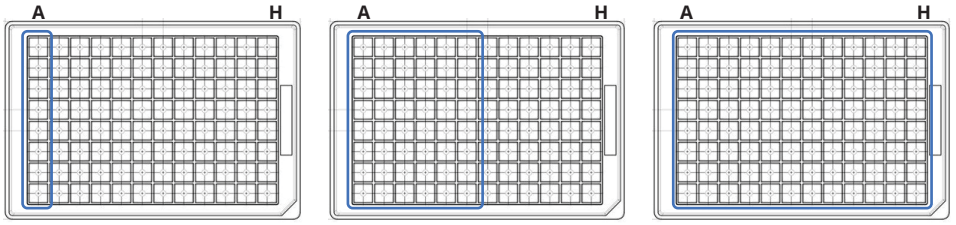


Figure 3: Schematic representation of the labware arrangements on the OT-2 (Reagent-In-Plate).



		8 Samples		48 Samples		96 Samples	
Protocol		Runtime (min)	Tip boxes	Runtime (min)	Tip boxes	Runtime (min)	Tip boxes
Reagent-In-Tube	Part 1	7	1	20	1	37	2
	Part 2	15	2	39	3	68	4
	Sum	22	3	59	4	105	6
Reagent-In-Plate	Part 1	4	1	12	1	20	2
	Part 2	13	2	34	3	64	4
	Sum	17	3	46	4	84	6

Figure 4: Runtimes and tip boxes required for Reagent-In-Tube and Reagent-In-Plate protocols.

RESULTS

Rabbit IgG has high affinity for both Protein G and Protein A, and to confirm antibody interaction with Dynabeads™, we used anti-GAPDH rabbit polyclonal IgG (Proteintech, Rosemont, IL, USA). Briefly, 50 µL bead slurry was mixed in 200 µL PBS containing 2.5 µg GAPDH antibody on the OT-2 and agitated on an 800 rpm shaker at room temperature for 10 minutes. The protocol proceeded on the OT-2 to perform 3 washes with phosphate-buffered saline with 0.1% Tween 20 (PBS-T). The bound GAPDH antibody was eluted using Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) at 70°C for 10 minutes, subjected to SDS-PAGE, and probed with a near-infrared fluorescent-dye (IRDye 680RD) conjugated, goat anti-rabbit IgG antibody (LI-COR Biosciences, Lincoln, NE, USA). Results from Western Blot analysis showed that both Protein G and Protein A beads supported immobilization of the GAPDH antibody in a reproducible manner when performed in quadruplicate **(Figure 5A)**.

To perform IP for GAPDH, samples containing recombinant human GAPDH protein (rhGAPDH, Thermo Fisher Scientific, Waltham, MA, USA) were processed on the OT-2. GAPDH antibody bound to Dynabeads™-Protein G and Dynabeads™-Protein A were tested in parallel. Western Blot results showed the presence of precipitated GAPDH and confirmed the ability of the OT-2 to automate IP **(Figure 5B)**.

The procedure was also evaluated for automated IP for endogenous GAPDH in cultured cells. HEp-2 cells were cultured in Gibco™ minimum essential medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin and at 5% CO_2 and 37°C. To prepare cell lysate for protocol testing, 4 x 10^6 HEp-2 cells were harvested and lysed in 200 µL Pierce™ IP lysis buffer supplemented with protease inhibitor cocktail, both obtained from Thermo Fisher Scientific. IP was performed by using Dynabeads™-Protein A on the OT-2 platform with a GEN2 Magnetic Module and a GEN2 Temperature Module. To allow sufficient time for antibody-target protein binding, the mixture was agitated on an 800 rpm shaker at 4°C overnight. The results further demonstrated successful IP of endogenous GAPDH with excellent reproducibility, as quantified by Western blot **(Figure 6)**. It is also worthwhile to note that the protocol was set to process 96 samples, with cell lysates for IP loaded in the last column of the 96-well sample plate, and the rest of the plate filled with lysis buffer only.

CONCLUSION

This work has demonstrated the capacity and usefulness of the OT-2 for automating IP using Dynabeads™ in medium- to high-throughput workflows. The protocols we have developed replicate the manual procedure with reduced hands-on time and provide excellent reproducibility.

									*
Bead / Pro A	+	+	+	+	-	-	-	-	-
Bead / Pro G	-	-	-	-	+	+	+	+	-
GAPDH Ab (rabbit IgG)	+	+	+	+	+	+	+	+	+

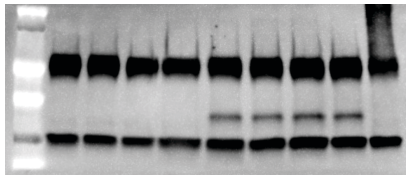


Figure 5A: Protein G and Protein A beads supported immobilization of rabbit antibody with desirable consistency of sample handling in quadruplicate.

									*
Bead / Pro A	+	+	+	+	-	-	-	-	-
Bead / Pro G	-	-	-	-	+	+	+	+	-
GAPDH Ab (rabbit IgG)	+	+	+	+	+	+	+	+	-
rhGAPDH	+	+	+	+	+	+	+	+	+

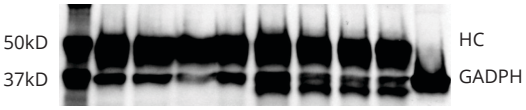


Figure 5B: Western Blot results showed the presence of precipitated GAPDH and confirmed the ability of the OT-2 to automate IP.

					CV =10%			*
Bead / Pro A	+	+	+	+	+	+	+	-
AB	+	+	+	+	+	+	+	-
endoGAPDH	+	+	+	+	+	+	+	+
rhGAPDH	-	-	-	-	+++	++	+	-

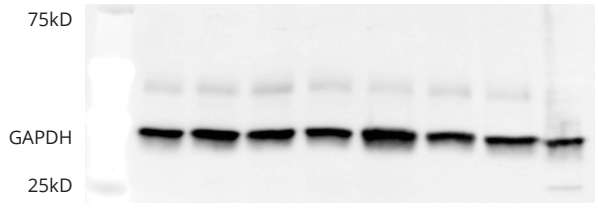


Figure 6: Western Blot results showed the presence of precipitated GAPDH and confirmed the ability of the OT-2 to automate IP. Relative protein levels of endogenous GAPDH (1, 1.26, 1.1, 1.1) were determined by densitometric analysis and CV calculated.