

DISPOSABLE LABTUBE CARTRIDGES FOR AUTOMATED PROTEIN PURIFICATION IN STANDARD LAB CENTRIFUGES

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ABSTRACT

The protein purification process is often the bottleneck for the efficient production of a large number of different proteins. Automation of these procedures is often the crux of the matter and is frequently a trade-off between efficiency and cost. Using our novel disposable LabTube cartridges we demonstrate how the process of His-tagged protein purification can be automated in standard laboratory centrifuges. LabTube cartridges include prestored reagents which are sequentially applied to a Ni-NTA purification matrix by an integrated ballpen mechanism actuated by acceleration changes of the centrifuge. Fully automated runs demonstrated similar yield and purity compared to manual purifications with sample addition as the only manual handling step. Thus, the user is available for parallel tasks during 95 % of the overall process time (33 min). In contrast manual processing requires the user to be present for 18 minutes out of the 33 minute overall process time. Since LabTube automation requires no investment in a special lab automation device, this platform lowers the market entry barrier for lab automation.

KEYWORDS: Lab-on-a-chip platform, centrifugal microfluidics, protein purification, assay automation

INTRODUCTION

In systems biology, functional genomics or drug discovery, production of large numbers of different recombinant proteins in microgram quantities is the basis for screening studies and process controls. Here, the protein purification process is critical in terms of quality and time [1,2]. Since the purification of tagged proteins creates a high amount of routine work, automation of these procedures is the ultimate goal for every researcher. Commercial systems such as the QIAcube (Qiagen, ≤ 12 samples per run) or the Maxwell 16 (Promega, ≤ 16 samples per run) are not attractive to many labs because they require extensive investment in extra lab equipment, additional lab space and practice in using new equipment.

At the last MicroTAS we introduced the LabTube as a novel, generic Lab-on-a-Chip platform for the automation of biochemical assays in standard laboratory centrifuges [3]. LabTube cartridges use changes in centrifugal acceleration in combination with integrated ballpen mechanism to perform all essential operations (see Figure 1). Since LabTube cartridges are developed for processing in standard laboratory centrifuges, no special lab instruments are required. Thus LabTube based automation is cost-effective at even lowest number of samples. In this study, we demonstrate the use of LabTube cartridges for automated purification of His-tagged proteins from cell lysates.

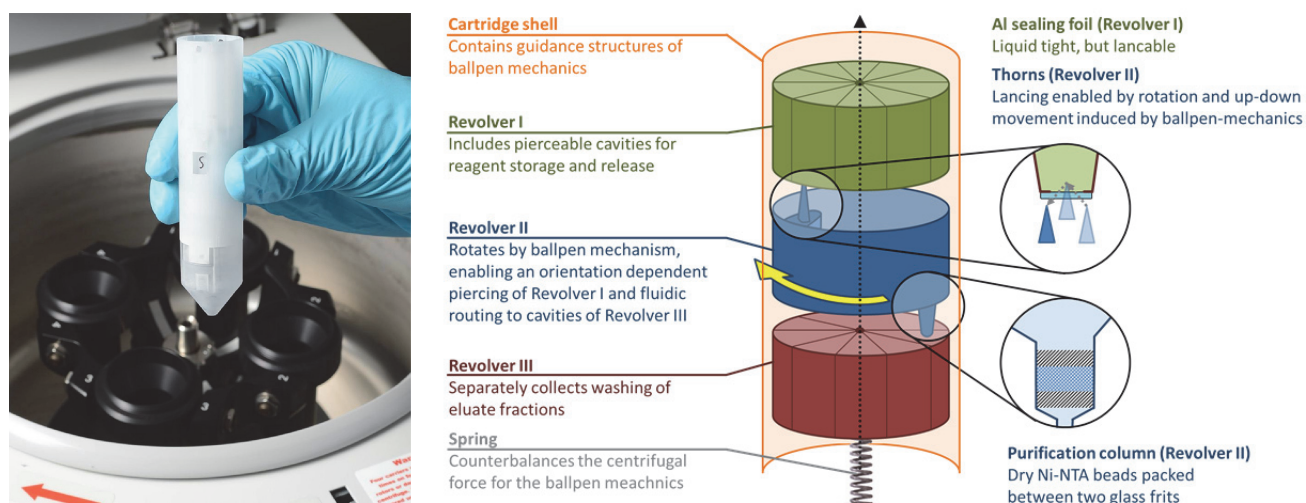


Figure 1: a) LabTube cartridge for automated processing in standard lab centrifuges.
b) Illustration of LabTube cartridge equipped for protein purification based on a Ni-NTA SpinColumns.

CARTRIDGE DESIGN FOR PURIFICATION OF HIS-TAGGED PROTEINS

LabTube cartridges for purification of His-tagged proteins include prestored buffers, ballpen-mechanism, a purification matrix and separate cavities for collection of flow-through, washing and elution fractions (see Figure 1). The integrated ballpen mechanism is actuated via changes in centrifugal load and induces a rotation of Revolver II against the other revolvers as well as a simultaneous up-down movement of Revolver II with respect to Revolver I. This way, thorns of Revolver II lance the sealing foil at the bottom of Revolver I enabling sequential reagent release. In Revolver II, the released liquids are guided towards and centrifuged through the purification matrix and subsequently get transferred into the corresponding collection cavities within Revolver III.

EXPERIMENTAL

For demonstration, all parts except Revolver I were fabricated in a synthetic resin material by stereolithography or Scan-LED (Teufel Prototypen, Germany and 3D-Labs, Germany). The final product will be fabricated by injection molding, enabling cartridge mass production at low cost. Revolver I is realized as an injection molded polypropylene prototype. Revolver I is sealed at the bottom with a PP-specific, pierceable 20 µm aluminum foil including a 5 µm layer of heat seal lacquer. After sealing, the assay specific buffers are filled into Revolver I as given in Table 1 which is subsequently sealed from top with an adhesive foil (HJ Bioanalytik, no. 900320). The purification column placed in Revolver II corresponds to the frit and the dry resin of a commercial spin column (Ni-NTA Spin Columns, Qiagen). In this demonstration, histidyl tRNA synthetase expressed in insect cells was used as target protein.

For the expression of histidyl-tRNA synthetase, the baculovirus/insect cell system was used [4]. cDNA encoding the histidyl-tRNA synthetase was cloned into a transfer vector. Recombinant baculoviruses were generated by homologous recombination of the transfer vector with linearized baculovirus DNA in *Spodoptera frugiperda* (Sf9), identified and cloned by plaque assays. Isolated baculoviruses were propagated and used to infect Sf9 cells cultured in spinner flasks at 27 °C. After 68 h the infected cells were harvested by centrifugation; the pellets were washed in PBS and frozen for storage at -70 °C. Cell lysates were prepared by resuspending the frozen cell pellet in extraction buffer (20 mM HEPES, pH 8.0; 0.2 % Triton X-100, 1M NaCl) and stirring for 30 min on ice. Cell debris was separated from soluble proteins by centrifugation (20 min; 40000 g).

To demonstrate LabTube automated purifications, the lysate was added into a sample cavity of Revolver I (see Figure 1). Subsequently the LabTube was transferred into the centrifuge (Z326K, Hermle Labortechnik) for automated processing (see Table 1 for centrifugation protocol). The centrifuge used in these studies allows for parallel processing of four LabTube cartridges, others allow for up to 16 simultaneous tests (e.g. Rotina 380R, Hettich Lab Technology). Manual reference purifications were performed following the original protocol provided by Qiagen, but 500 µl volumes were reduced according to the available cavity volumes of the current LabTube cartridge as indicated in Table 1. Pretests showed equal purification performance between the original and the volume adjusted manual protocol.

Table 1. Overview of process protocols used for manual and LabTube based protein purifications.

Process step	Manual processing		LabTube processing	
	Volume / µl	Centrifugation	Volume / µl	Centrifugation *
Equilibration**	350 µl	2 min @ 890 g	350 µl	2 min @ 890 g
Lysate addition	450 µl	15 min @ 270 g	3x 150 µl	3x 5 min @ 100-2000 g
Washing 1,2 **	350 µl	2 min @ 890 g	350 µl	2 min @ 100-2000 g
Washing 3 **	300 µl	2 min @ 890 g	300 µl	2 min @ 100-2000 g
Elution 1-3 ***	100 µl	2 min @ 890 g	100 µl	2 min @ 100-2000 g
*	During LabTube processing centrifugal force is increased step by step within the listed limits.			
**	Washing and equilibrium buffer: 20 mM HEPES, pH 8.0 with 5 mM imidazole			
***	Elution buffer: 20 mM HEPES, pH 8.0 with 500 mM imidazole			

Separately collected flow-through, wash and eluate fractions were analyzed by SDS-PAGE and subsequent Coomassie blue staining for analysis of yield and purity. For SDS-PAGE 15 µl of a 1:1 mixture of sample and Laemmli sample buffer (161-0737, Bio-Rad) with addition of DTT (0.1 mM) were applied to precast gels (no. 456-9033, Bio-Rad) and operated in a Mini-PROTEAN Tetra cell electrophoresis system (100 V, ~80 minutes, Bio-Rad). Coomassie staining was performed using the Blue R Staining Kit of Serva. For size confirmation and identification of His-tag proteins also western blots were performed using Trans-Blot Turbo transfer packs (no. 170-4159, Bio-Rad) in the Trans-Blot Turbo system (Bio-Rad). AntiHis monoclonal antibodies (no. 34660, QIAGEN), rabbit anti-mouse IgG-Fc-AP antibodies (no. 31332, Pierce) and BioFX® BCIP/NBT Purple AP Membrane Substrate (Sur Modics) were used for His-tag specific staining.

For densitometric quantification of Coomassie stained gels and western blots, line plots of the color intensity were calculated from scanned images using the freely available image processing software Image J (<http://rsbweb.nih.gov/ij/>).

The intensity peak around 50 kD corresponding to the target protein (size confirmed by western blots) was integrated. The line plot peak of the molecular weight marker (no. 161-0374, Bio-Rad) at 50 kD was used as reference to calculate relative intensities of the eluate fractions and to enable comparison of different gels. Additionally, the total protein content was determined using the BCA protein assay (Novagen).

RESULTS AND DISCUSSION

Figure 2 shows typical results observed via SDS-PAGE for manual and LabTube-automated purifications of recombinantly produced human Histidyl-tRNA synthetase. The profile of unprocessed lysate shows prominent bands at two distinct molecular sizes (about 50 and 55 kD). The larger protein is also visible in flow-through and wash fractions, whereas the 50-kD protein is not substantially present in the wash but in the elution fractions. The 50 kD protein was confirmed to be the His-tag specific target protein by western blots with anti-His monoclonal antibodies. This clearly demonstrates the desired purification effect for both purification protocols, since only the target protein is specifically bound and eluted from the column matrix.

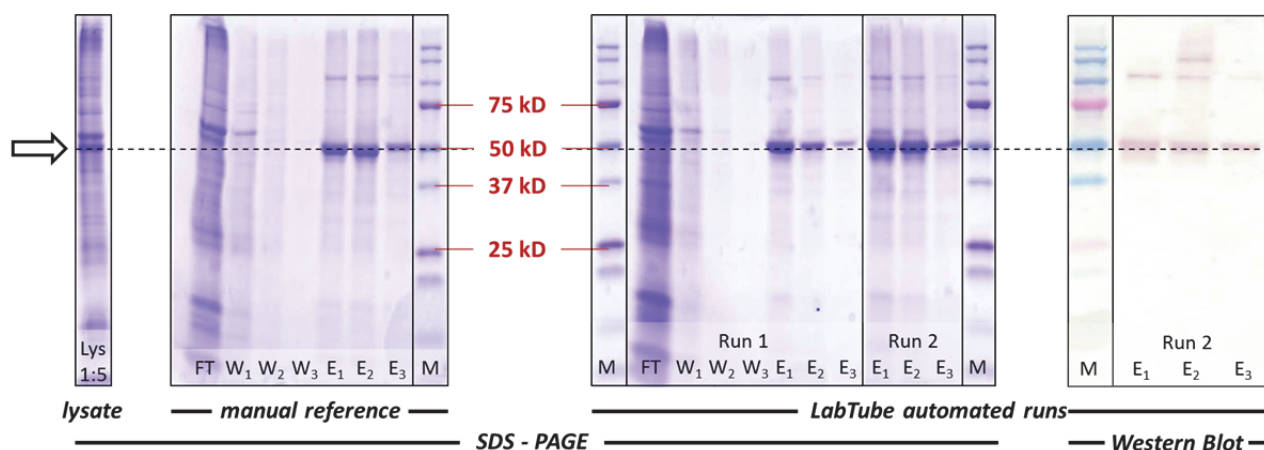


Figure 2: Comparison of manual and LabTube based purification of human Histidyl-tRNA synthetase results with 3x washing and 3x elution. Gels from SDS-PAGE show unprocessed lysate (Lys, diluted 1:5), molecular weight marker and the collected flow-through (FT), washing (W_1 - W_3) and elution fractions (E_1 - E_3) of manually processed and LabTube based purifications (eluates of 2 different LabTube runs). For purification run 2, also Western Blot results are shown for the elution fractions E_1 - E_3 . Dashed line illustrates the molecular weight of the target protein.

The similar protein distribution observed for wash and eluate fractions from manual and LabTube-automated purifications indicates a similar purification yield. This visual impression of a comparable target-protein yield is confirmed by the results of densitometric analysis (see experimental section) and protein concentrations measurement by BCA-assay shown in Table 2. Moreover, the intensities of the background intensity are comparable for manual and LabTube-automated purifications (see Figure 2) indicating a comparable purity.

Table 2. Quantified protein yield results obtained by three different analytical methods. Data is given as mean value of the three collected elution fractions per run and a minimum of four parallel purification runs.

Analytical method	SDS-PAGE densitometry	Western blot densitometry	BCA-assay
Yield compared to manual reference	105 % $\pm$ 17 %	107 % $\pm$ 39 %	114 % $\pm$ 28 %

In addition, this cartridge can be used to analyze or optimize purification conditions by elution with a step-gradient of increasing imidazole concentrations (Figure 3). In this example, significant elution of the protein requires imidazole concentrations of > 250 mM. Furthermore, the purity of the 250 mM fraction is higher than at 500 mM, which can also be a critical criterion.

Reduction of hands-on-time is a significant advantage of automation (Table 3). Since the sample loading and product removal are the only manual steps during LabTube-automated processing (1 minute hands-on-time), the user can make use of a 32 minute walk-away time while the LabTube cartridges are in the centrifuge. Manual processing includes 8 handling sequences (20-30 s hands-on-time each) consisting of reagent addition – centrifugation – liquid removal and requires additional time for preparation of the purifications (arrangement of instruments, disposables and buffers, about 2 min). Although with 5 minutes the pure handling time is also low for manual processing, the big difference is that the

short off-times (7x 2 minute centrifugation steps) in between the handling steps do not allow the user to walk away and follow further tasks.

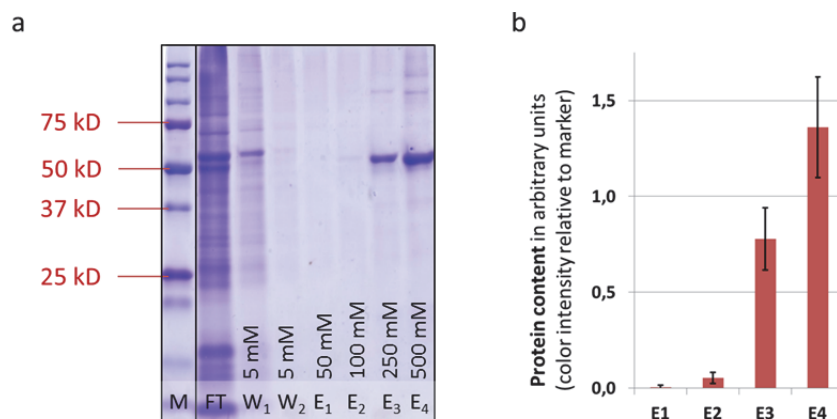


Figure 3: Purification of Histidyl tRNA synthetase expressed in insect cells with 2x washing and 4x elution at increasing imidazole concentrations. a) Purification results analyzed via SDS-PAGE and Coomassie blue staining. b) Protein content of the according eluate fractions indicated by densitometric analysis of Coomassie stained SDS-gels.

Table 3. Evaluation of manual and LabTube automated processing times

Purification method	Overall process time	Hands-on-time	Walk-away time (percentage of the overall time)
Manual processing	33 min	5 min	1 x 15 min (45 %)
LabTube automated	33 min	1 min	32 min (95 %)

CONCLUSION

The application of LabTube cartridges enables reliable automation of protein purifications at comparable yield and purity to manual purifications. Furthermore, the LabTube enables user-specific choices of column material and/or buffer systems, which offers the opportunity of a fast and simple optimization of protein purification conditions. Most importantly, LabTube cartridges offer the potential to significantly reduce the proportion of routine work load in protein related screening studies, without the need for investment in expensive, dedicated and specialized automation instruments.

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