Automated Cell-free Protein Expression and Purification for High-Throughput Screening using NEBExpress® Cell-free *E. coli* Protein Synthesis System and NEBExpress Ni-NTA Magnetic Beads

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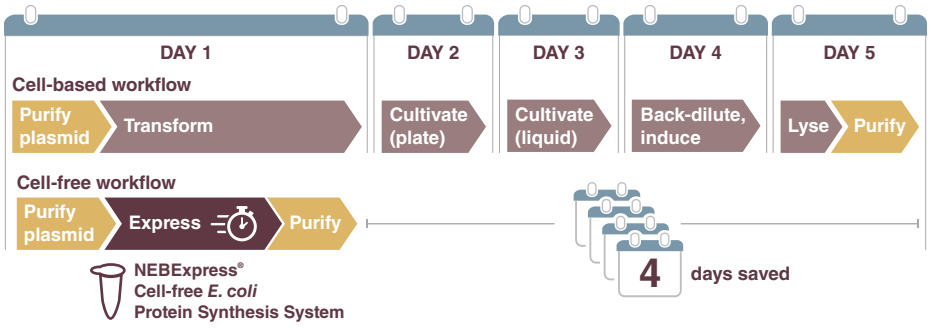
INTRODUCTION

The unparalleled access researchers have to efficient and cost-effective DNA assembly methods (e.g., NEBuilder® HiFi DNA Assembly, NEBridge® Golden Gate Assembly) has rendered traditional cell-based protein expression and purification the main hurdles to high-throughput screening efforts. Transforming recombinant vectors into specific expression strains and cultivating precise biomass to reproducibly induce expression is imprecise and difficult to automate. Furthermore, traditional column purification is single-stream, thus lacking high-throughput compatibility and limiting project scope. To overcome these issues, researchers have turned to cell-free protein synthesis methods and affinity purification with functionalized magnetic beads to streamline and automate high-throughput efforts to rapidly generate analytical amounts (~0.5 mg/ ml) of diverse target proteins in hours instead of days (Figure 1).

NEB offers a cell-extract based, cell-free protein synthesis kit - the NEBExpress® Cell-free *E. coli* Protein Synthesis System (NEB #E5360S/L). Central to this system is the cell extract, which is derived from an *E. coli* strain that has been engineered to synthesize analytical amounts of diverse proteins up to 230 kDa. The system employs an optimized T7 RNA Polymerase for increased and specific transcription, an RNase Inhibitor to reduce RNase carryover, and a formulated reaction buffer containing all the necessary cofactors and energy sources to drive coupled transcription and translation of a target gene. Gene products encoded in the form of linear DNA (e.g., PCR product), circular DNA (e.g., purified plasmid), or RNA are all compatible with the system; however, circular DNA templates typically provide the highest yield and sensitivity. Note: DNA templates require the presence of a T7 promoter upstream and T7 terminator downstream (Figure 2) (1-4). In cases where

FIGURE 1: **Timeline of traditional expression and purification vs. cell-free protein synthesis and automated Ni-NTA magnetic bead purification**



Cell-based workflow: A recombinant plasmid that directs expression is purified and transformed into an appropriate host strain and plated overnight. Colonies are picked the next day and grown overnight in liquid media. The overnight culture is back-diluted, grown to mid-log phase (OD = 0.4-0.8) and induced. The culture is pelleted the next day, lysed, and purified via column chromatography.

Cell-free workflow: A recombinant plasmid that directs expression is purified and mixed with NEBExpress Cell-free *E. coli* Protein Synthesis master mix, incubated for a few hours, mixed with NEBExpress Ni-NTA Magnetic Beads, and loaded onto a magnetic particle processor for automated purification.

MATERIALS

- NEBExpress Cell-free *E. coli* Protein Synthesis System (NEB #E5360)
- Monarch® Plasmid Miniprep Kit (NEB #T1010)
- NEBExpress Ni-NTA Magnetic Beads (NEB #S1423)
- 2X IMAC Buffer (NEB #B1076SVIAL, part of NEB #S1423)
- 2M Imidazole (NEB #B1077SVIAL, part of NEB #S1423)
- Blue Protein Loading Dye (NEB #B7703)
- Color Prestained Protein Ladder, Broad Range (NEB #P7719)
- SimplyBlue™ SafeStain (Invitrogen #LC6060)
- KingFisher® Flex Purification System (Thermo Scientific® #5400630)
- KingFisher 96 deep-well plate (Thermo Scientific #95040450)
- KingFisher 96-well microplate (Thermo Scientific #97002540)
- Novex® Tris-Glycine Mini Protein Gels, 10-20% (Thermo Scientific #XP10200BOX)

OTHER MATERIALS

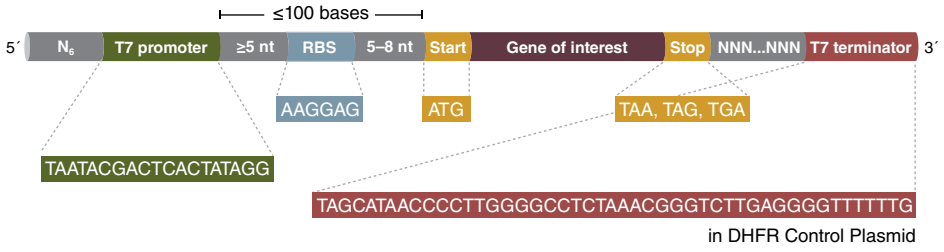
- NEBuilder® HiFi DNA Assembly Cloning Kit (NEB #E5520)
- NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)
- NEBridge® Golden Gate Assembly Kit (BsaI-HF® v2) (NEB #E1601)
- NEBridge Golden Gate Assembly Kit (BsmBI-HF® v2) (NEB #E1602)
- PURExpress® *In Vitro* Protein Synthesis Kit (NEB #E6800)
- NEBExpress GamS Nuclease Inhibitor (NEB #P0774)

linear DNA is used as template, NEBExpress GamS Nuclease Inhibitor (NEB #P0774S) is included to enhance synthesis yield. In addition to standard templates, utilizing rolling circle amplification and high-throughput assembly allows for an entirely *in vitro* workflow, enabling users to go from DNA to proteins in a single day (5,6).

The NEBExpress Cell-free *E. coli* Protein Synthesis System is used for diverse applications. For approaches like high-throughput protein engineering and screening that require further downstream analysis, the activities that persist in the cell extract can confound results. Researchers can employ workarounds that suppress these background activities, like focusing on thermophilic targets that survive heat shock or activity assays that employ non-standard conditions (e.g., high/low pH, high salt, etc.), but for many targets a purification step is required. We have previously described the purification of polyhistidine-tagged (His-tagged) from NEBExpress Cell-free *E. coli* Protein Synthesis reactions using NEBExpress Ni Spin Columns (NEB #S1427S/L) and standard immobilized metal affinity chromatography (IMAC) solutions (1). Such columns, however, do not take advantage of the high-throughput afforded by cell-free protein synthesis strategies.

NEB offers agarose-based, super-paramagnetic Ni-NTA beads - NEBExpress Ni-NTA Magnetic Beads (NEB #S1423S/L), for small-scale purification of His-tagged proteins. Since these beads can be easily dispensed into 96- well plates, they are particularly amenable to purifying synthesized His-tagged proteins from NEBExpress Cell-free *E. coli* Protein Synthesis reactions in a high-throughput manner. We aimed to take this one step further by automating the purification using a magnetic particle processor (MPP) as depicted in the workflow in Figure 3. The MPP automates the purification procedure by transferring the magnetic beads between plates containing purification buffers. In this case, His-tagged proteins bind to the magnetic beads, the MPP transfers the beads to plates containing wash buffer and then to a plate containing elution buffer to elute up to 96 purified proteins. Herein, we detail a workflow incorporating a magnetic particle processor, NEBExpress Ni-NTA magnetic beads, and NEBExpress Cell-free *E. coli* Protein Synthesis reactions, that enables users to go from DNA to purified protein in a single day with less hands-on time and greater reproducibility to facilitate high-throughput screening.

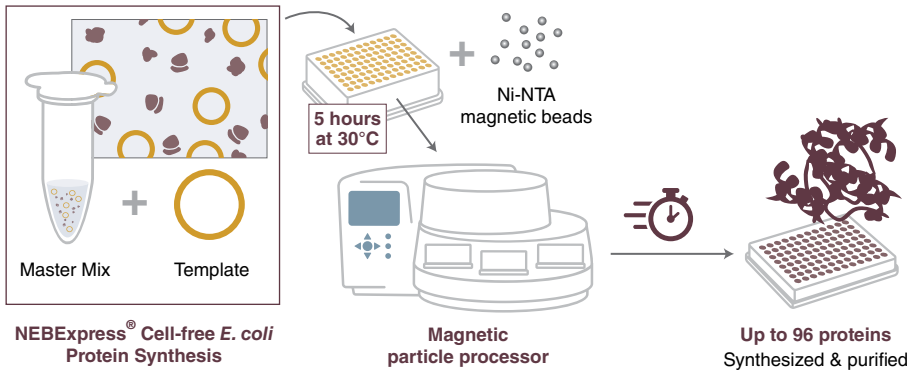
FIGURE 2: **Required elements for template DNA**



In addition to the desired protein coding sequence (in-frame), the template DNA must contain the following elements:

- Start codon (ATG)
- Stop codon (TAA, TAG, or TGA)
- T7 promoter (20 to 100 nucleotides upstream of the coding sequence)
- Ribosome binding site (RBS, also known as a Shine-Dalgarno sequence) upstream, approximately 6-8 nucleotides, of the start of translation
- Spacer region 6 base pairs downstream from the stop codon (for PCR products)
- T7 terminator downstream from the stop codon (recommended for all templates)

FIGURE 3: **Semi-automated workflow incorporating cell-free protein synthesis, magnetic beads and a magnetic particle processor**



NEBExpress Cell-free *E. coli* Protein Synthesis master mix is assembled and dispensed to a 2 mL 96-well plate. DNA or mRNA template encoding His-tagged protein is added to each well, and the reactions are incubated for 5 hours at 30°C with agitation. NEBExpress Ni-NTA Magnetic Beads are then added to each well, and the plate is placed in the magnetic particle processor, which performs the binding, wash, and elution steps in approximately 1 hour, yielding up to 96 purified proteins.

METHODS

Cell-free protein synthesis reaction assembly, dispensing, and SDS-PAGE analysis

NEBExpress Cell-free *E. coli* Protein Synthesis System reactions were assembled (Table 1) for seven His-tagged target proteins and a no template control (Table 2) in 100 µl total volume, which is twice the volume of a typical reaction. This reaction volume was used based on our established protocols. NEBExpress Cell-free *E. coli* Protein Synthesis reactions are scalable from 10 µl up to 2 ml; however, it is important to note the maximum recommended volume for the specific plates and magnetic particle processor to be used. Six replicates of each sample were included to determine reproducibility, resulting in 48 total reactions. A master mix of NEBExpress Cell-free *E. coli* Protein Synthesis System components excluding template DNA was assembled for 55 reactions (48 rxns + 15% overage) in a 5 ml tube. Importantly, NEBExpress Cell-free *E. coli* Protein Synthesis System master mixes assembled without template are stable on ice for 2 hours with no appreciable loss in protein synthesis, affording some flexibility during reaction setup. The master mix was bulk dispensed (90 µl/well) into 48 wells (A1-H6) of a KingFisher 96 deep-well plate (Thermo Scientific #95040450). Note: Other instrument platforms, including Integra Biosciences® VIAFLO 96/384, Beckman Coulter® Echo® 525, Formulatrix® Mantis®, Formulatrix Flo i8®, Opentrons® OT-2, Eppendorf Repeater®, and the Rainin® E4™, Integra VIAFLO or Eppendorf Xplorer® multichannel electronic pipettes have all been used successfully to bulk dispense/transfer NEBExpress Cell-free *E. coli* Protein Synthesis System master mixes (results not shown). Purified plasmid DNA encoding each of the seven His-tagged target proteins was obtained by standard methods using the Monarch® Plasmid Miniprep Kit (NEB #T1010S/L). 500 ng/well plasmid DNA or H_2O was multi-dispensed across the 6 columns using an 8-channel repeat pipettor. The deep-well plate was sealed with a Microseal B Film (Bio-Rad #MSB1001) and incubated in a ThermoMixer® C (Eppendorf® #2231001005) for 5 hours at 30°C with 500 rpm shaking. Prior to purification, representative samples were retrieved (2 µl), mixed with 1X Blue Protein Loading Dye (NEB# B7703S) supplemented with 42 mM DTT and run on a 10-20% Tris-Glycine SDS-PAGE gel stained with SimplyBlue SafeStain (Invitrogen #LC6060) for visualization. The Color Prestained Protein Standard (NEB# P7719S/L) was used as an electrophoretic mobility standard and gel images were captured with a LI-COR Odyssey® M imager to visualize target synthesis prior to automated affinity purification (Figure 4, page 4).

TABLE 1: **NEBExpress Cell-free *E. coli* Protein Synthesis system master mix assembly**

NEBEXPRESS CELL-FREE *E.COLI* PROTEIN SYNTHESIS REACTION COMPONENTS	STANDARD RXN (µl)	2X RXN (µl)	55X MASTER MIX (µl)
Template (100 ng/µl)	5	10	
Protein Synthesis Buffer (2X) (#B0864SVIAL)	25	50	2,750
NEBExpress S30 Synthesis Extract (#P0864SVIAL)	12	24	1,320
RNase inhibitor, Murine (#M1018AAVIAL)	1	2	110
T7 RNA Polymerase (#M1019AAVIAL)	1	2	110
RNase-free water	6	12	660
Total Volume (µl):	**50**	**100**	**4,950**

TABLE 2: **His-tagged target proteins**

SAMPLE	PROTEIN	MOLECULAR WEIGHT (kDa)
1	neg. ctrl	–
2	CALM	16.7
3	Myokinase	25.5
4	vGFP	26.9
5	GluRS	54.9
6	LysRS	75.0
7	EndoS	89.0
8	β-Gal	100

Automated protein purification using Ni-NTA magnetic beads and a magnetic particle processor

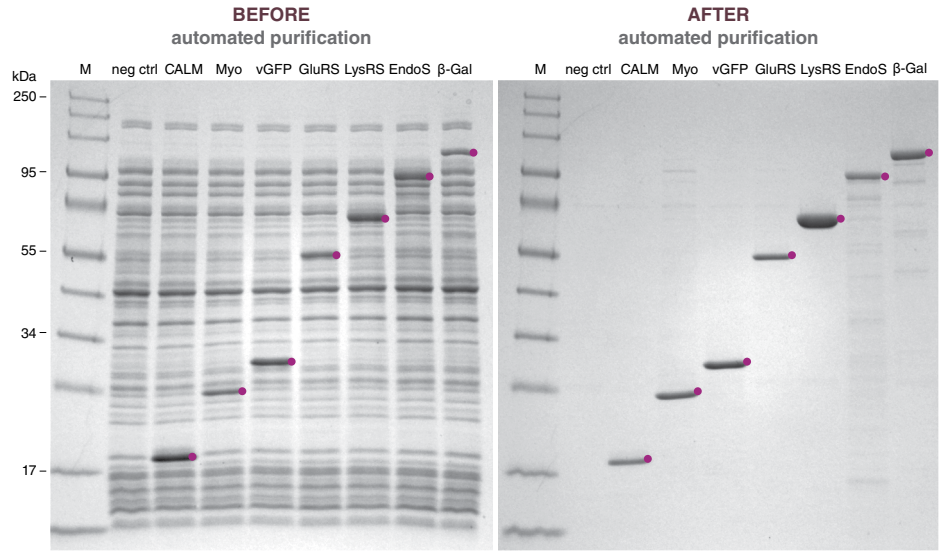
For purification of proteins from the 48 NEBExpress Cell-free *E. coli* Protein Synthesis reactions, 5 ml of NEBExpress Ni-NTA Magnetic Beads were equilibrated and resuspended in 5 ml of 2X IMAC Buffer (NEB #B1076SVIAL, 40 mM Sodium phosphate pH 7.4, 600 mM NaCl). The equilibrated beads were diluted with 10 ml of 1X IMAC Buffer (20 mM Sodium phosphate pH 7.4, 300 mM NaCl) supplemented with 40 mM imidazole, bulk dispensed with a tip-based liquid handler to each sample well (300 µl beads/well, wells A1-H6) of the reaction plate, and the target proteins were purified using the Thermo Scientific KingFisher Flex System. This instrument couples a magnetic pinning tool to an automated turntable consisting of 8 decks, each holding individual plates that enable defined mixing, transfer, and elution protocols with programmable times and speeds. For the KingFisher procedure, equilibrated beads were mixed with the NEBExpress Cell-free *E. coli* Protein Synthesis System reactions for 30 min (Plate #1), washed twice for 5 min in 500 µl/well 1X IMAC Buffer supplemented with 20 mM imidazole (Plates #2-3), and eluted for 10 min in 75 µl/well of 1X IMAC Buffer supplemented with 250 mM imidazole (Plate #4). All steps were performed at room temperature, employed slow, continuous agitation, and utilized deep-well plates apart from the elution step (Plate #4), which employed a KingFisher 96-well microplate (Thermo Scientific #97002540). Purity and yield of the purified protein targets were determined by analyzing 5 µl representative samples by SDS-PAGE as described above (Figure 4). Sample concentration and purity were additionally quantified on the Revvity® LabChip® GXII Touch using the high-sensitivity protocol.

RESULTS

NEBExpress Cell-free *E. coli* Protein Synthesis System master mixes were successfully assembled and bulk dispensed to 48 wells of the KingFisher 96 deep-well plates using a tip-based liquid handler. Replicate columns received normalized (50 ng/µl) plasmid DNA encoding the following targets: human calmodulin-like protein 3 (CALM), *E. coli* adenylate kinase or myokinase (Myo), Venus fluorescent yellow protein (vGFP), *E. coli* glutamate-tRNA ligase (GluRS), *E. coli* lysine-tRNA ligase (LysRS), *S. pyogenes* endoglycosidase (Endo S) and *E. coli* β-galactosidase (β-Gal). Following incubation, synthesis of all seven templates was observed in representative samples by SDS-PAGE (Figure 4), with no synthesis detected for the no template control reaction. Synthesis appeared to be construct-dependent and not correlated to target size nor host origin. The observed synthesis of CALM, vGFP and β-Gal templates was consistent with previous results (3). Overall, these results demonstrate that the NEBExpress Cell-free *E. coli* Protein Synthesis System can be dispensed using standard tip-based liquid handlers into 96-well plates, thereby decreasing hands-on time and increasing throughput and reproducibility, and the reactions resulted in the expected target proteins synthesized from template DNA.

For purification of synthesized proteins, diluted and washed NEBExpress Ni-NTA Magnetic Beads were added directly to the reactions via tip-based bulk dispense. The overall assembly and dispensing of the bead solution and wash/elution plates required approximately 10 minutes of hands-on time. From protein binding to elution, the entire purification scheme was performed on the KingFisher Flex in 55 minutes. Representative samples of the resulting purifications were visualized via SDS-PAGE (Figure 4). All seven purified target proteins displayed remarkable improvements in purity relative to the crude reactions. Additionally, all purified target proteins displayed protein yields that correlated with their synthesis amounts. The Endo S sample exhibited heightened background relative to all other targets; however, we believe this background is specific to Endo S stability and not a product of non-specific carryover from Ni-NTA magnetic bead purification. Notably, Endo S is naturally secreted, so its instability may arise from incompatibility with one or more cytoplasmic elements present in the *E. coli* cell extract. Altogether, these purification results demonstrate the ability to purify His-tagged proteins synthesized in NEBExpress Cell-free *E. coli* Protein Synthesis reactions in approximately 1 hour by taking advantage of the high specificity of the NEBExpress Ni-NTA Magnetic Beads for His-tagged proteins. Additionally, these results show the compatibility of these beads with a magnetic particle processor to automate protein purification in a high-throughput manner, making protein synthesis and purification a one-day experiment rather than a multi-day experiment as with traditional cell-based workflows.

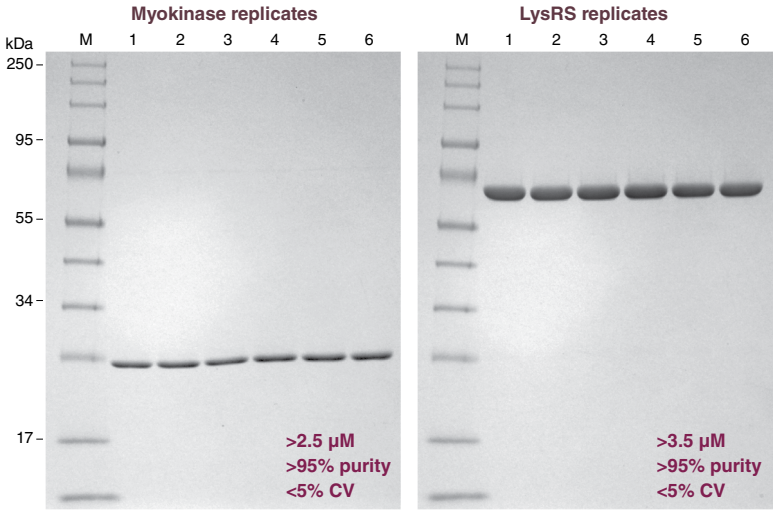
FIGURE 4: **Proteins synthesized in NEBExpress Cell-free *E. coli* Protein Synthesis reactions and purified using NEBExpress Ni-NTA Magnetic Beads and a magnetic particle processor**



Synthesized proteins in NEBExpress Cell-free *E. coli* Protein Synthesis reactions before and after automated purification. The purple circle indicates the specific protein of interest. M = Color Prestained Protein Standard, Broad Range (NEB# P7719S/L). Representative synthesis and purified samples were run for the following templates: no template (neg ctrl), CALM (16.7 kDa), Myokinase (25.5 kDa), vGFP (26.9 kDa), GluRS (54.9 kDa), LysRS (75.0 kDa), Endo S (89.0 kDa), and β-Gal (100.0 kDa).

To determine the reproducibility between experimental replicates, six replicates for Myokinase and LysRS were visualized by SDS-PAGE (Figure 5). Both gel images show that high-quality protein of nearly identical yield and purity was purified for all six replicates. The LabChip data indicated an average purity of >90% was achieved for all targets (excluding Endo S) and an average concentration of 2 µM or ~100 ng/µl isolated, with a maximum of 250 ng/µl for LysRS. Given a 75 µl elution volume, an average of 150 pmol of protein per well, or a maximum of 18.75 µg total, was purified. These results clearly demonstrate the high degree of reproducibility and protein purity achieved using a workflow consisting of the NEBExpress Cell-free *E. coli* Protein Synthesis System, NEBExpress Ni-NTA Magnetic Beads, and a magnetic particle processor.

FIGURE 5: **Experimental replicates of automated Ni-NTA magnetic-bead-purified NEBExpress Cell-free *E. coli* Protein Synthesis reactions**



Six independently assembled and purified replicates for Myokinase (25.5 kDa) and LysRS (75.0 kDa).

CONCLUSION

Automated purification of proteins synthesized in NEBExpress Cell-free *E. coli* Protein Synthesis reactions has the potential to revolutionize research by enabling rapid, reproducible, and high-throughput production of highly pure and diverse proteins. The NEBExpress Cell-free *E coli* Protein Synthesis System is well-suited for protein screening since it offers high expression levels across a wide range of protein molecular weights, is scalable, tunable, reproducible, compatible with all common purification tags, able to synthesize proteins that exhibit toxicity *in vivo*, and cost-effective. Additionally, the system supports additives such as the PURExpress® Disulfide Bond Enhancer (NEB #E6820S), which promotes proper disulfide bond formation and greatly enhances the activity of disulfide bond-containing proteins.

While the presented approach is semi-automated, it can be universally integrated with any upstream liquid handling device and can be adapted to other commercially available magnetic particle processers, like the Accuris isoPURE™ 96/Mini or GenScript® AmMag® SA Plus automated systems or a manual magnetic pinning tool, like the V&P Scientific MagPin®.

Alternative methods and conditions

In the example shown here, cell-free protein synthesis reactions were performed at 30°C for 5 hours. While robust synthesis was observed, note that the optimal incubation temperature and duration is target specific and should be determined empirically for the protein(s) of interest. Similarly, while sodium phosphate was used as the buffering agent in the IMAC solutions, phosphate is potentially inhibitory in some activity assays. Tris-HCl, HEPES-NaOH, or MOPS can also serve as the buffering agent of IMAC solutions, but keep in mind that buffers influence the binding capacity and strength of different affinity interactions and should be thoroughly tested. If protein refolding is necessary, purifications can also be performed under denaturing conditions, as NEBExpress Ni-NTA Magnetic Bead purifications are compatible with 8 M urea or 6 M guanidinium chloride. Taken together, there are a variety of conditions that can be used to enhance protein synthesis yield and purification, so careful consideration of such conditions is recommended.

Alternative magnetic beads

The NEBExpress Cell-free *E. coli* Protein Synthesis system is compatible with other downstream purification strategies enabled by a variety of purification tags and their cognate magnetic protein purification beads: Chitin Magnetic Beads (NEB #E8036S), Amylose Magnetic Beads (NEB #E8035S) , Anti-MBP Magnetic Beads (NEB #E8037S), Protein A Magnetic Beads (NEB #S1425S), and Protein G Magnetic Beads (NEB #S1430S).

Removing imidazole

Downstream characterization of IMAC-purified protein can be complicated by the elevated levels of imidazole used for elution, which can interfere with spectrophotometric concentration determination and also inhibit some activity measurements. Fortunately, alternative methods for elution are available, including protonation or chelation of the Ni-NTA magnetic beads. These alternatives provide the user options in defining the final elution buffer. In instances where a defined endpoint buffer is required, products like Pierce® Zeba® Spin Desalting Plates (Thermo Scientific #PI89807) or Microdialysis Plates (Thermo Scientific #A50462) can be used. In some cases, it is worthwhile to test whether elution is required at all. As immobilized enzymes gain traction in the field, one should test whether the protein of interest can remain active when bound to Ni-NTA magnetic beads. If so, washed beads can be transferred to the desired buffer, bypassing the elution step, and the resulting solution can be used directly as "purified" protein. Immoblized enzymes are also easy to remove from reactions, making downstream analysis that requires removal of protein (e.g., liquid chromatography-mass spectrometry) simpler and faster. Lastly, when constructs containing cleaveable tags are employed with similarly-tagged proteases, on-bead digestion in a defined buffer can be an elegant solution to the problem at hand.

References

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