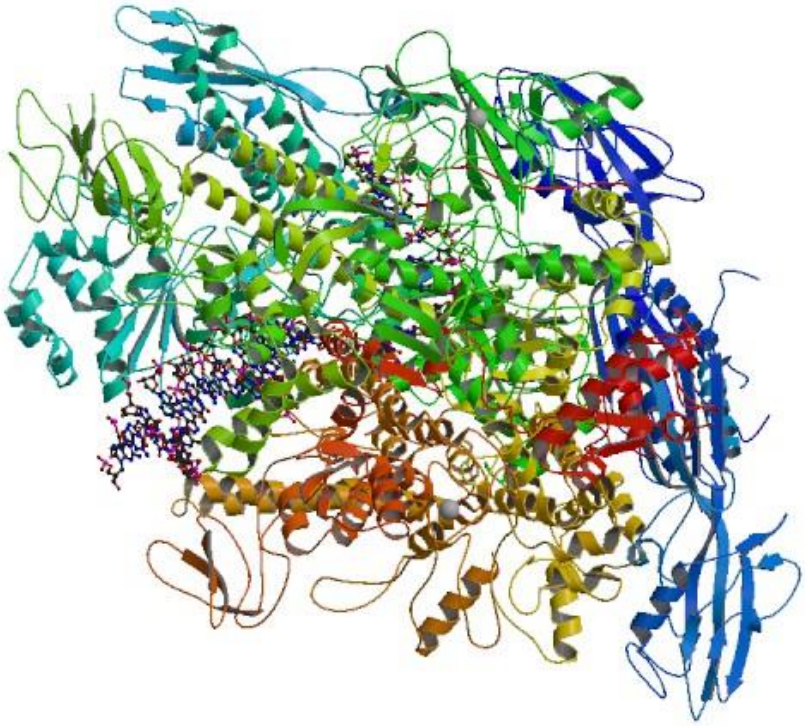
TRIALTUS BIOSCIENCE

High Purity | High Activity | High Yield

CL7/Im7 Expression & Purification Protocol

A comprehensive guide



Last date of revision

September 2019

trialtusbioscience.com

Table of Contents

Table of Contents 1

Introduction 2

1 Plasmid construction and preparation 3

2 Cell cultivation and lysis 3

3 Protein purification 4

3.1 DNA/RNA-binding proteins 4

3.1.1 Option 1- High salt loading 5
Table 3.1.1 5
3.1.2 Option 1- Example- Cas9 6
Figure 3.1 6
3.1.3 Option 2- DNAse treatment → high salt loading 7
Table 3.1.3 7
3.1.4 Option 2- Examples- ttRNAP and mtRNAP 8
Figure 3.2 9

3.2 Membrane proteins 9
3.2.1 Non-chromatographic step- Option 1- Ultra-centrifugation 10
3.2.2 Non-chromatographic step- Option 2- PEI precipitation 10
3.2.3 Examples 11
Figure 3.3 11
3.2.4 Chromatographic purification of MPs 12
Table 3.2 12
3.2.5 Examples 13
Figure 3.4 13

3.3 Other soluble proteins 14
3.3.1 Purification 14
Table 3.3.1 14
3.3.2 Examples 15
Figure 3.5 16

4 On-column proteolytic elution of a target protein 17

5 Cleaning and regenerating the Im7 column in gravity mode 18

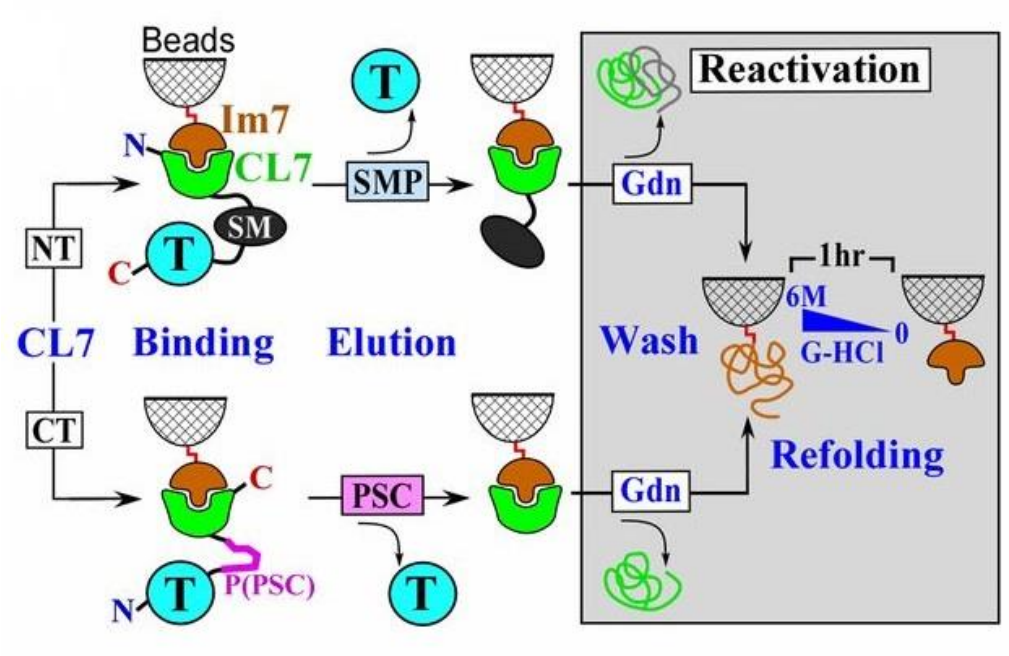
Table 5.1 18

Introduction

The CL7/Im7 method of HHH (high purity, activity, and yield) purification is based on the ultra-high-affinity interaction of Colicin E7 DNAse and its inhibitor Immunity Protein 7 (Im7). CL7, a variant of CE7 that has no DNA binding or DNAse activity, binds to Im7 with a K_d of ~10^{-14} – 10^{-17} M.

Recombinant proteins can be easily engineered to include a CL7 tag at their N or C terminal, either alone or in combination with other tags such as His. Im7 is immobilized to agarose beads via covalent cross-linking. The high affinity interaction between CL7 and Im7 allows for stable binding of the CL7 tag to the Im7 beads on a column in the first lysate loading step in high salt buffers.

The system outperforms His-trap and other traditional methods in several areas. CL7/Im7 allows for the single-step purification of many difficult-to-purify proteins unable to be isolated by traditional methods. There is a distinct increase in purity levels while maintaining or improving upon activity of the protein product. Furthermore, CL7/Im7 excels because of its lack of affinity for untagged cellular components and lack of sensitivity to other protein purification reagents such as PEI.



1. Plasmid construction and preparation

The commercial pET28a expression vector (Invitrogen) was used to produce a set of the CL7-tagged template vectors. Vectors contain either a N- or C-terminal location of the CL7-tag, or they are dual-tagged (His/CL7) vectors. The coding nucleotide sequences may be inserted in these vectors using a standardized set of restriction sites. See each plasmid's respective Genebank files on their product page. The resulting expression plasmids may be transformed into the BL21 (DE3) (Invitrogen) competent cells or their derivatives.

Note 1.1. It might be essential to design a target gene NA sequence with the codons optimized for *E. coli* and with a reduced G/C content of ~43-45%. In some cases, in our vectors with the N-terminal CL7-tag, we observed an abundant expression of the tag only, with little or no full-length protein, if the original (non-optimized for *E. coli* codons) NA sequences of the target genes were fused.

Note 1.2. We recommend using the BL21 Star (DE3) (Invitrogen) competent cells since in many cases they show notably better expression levels of the target proteins than those of the BL21 (DE3) cells.

2. Cell cultivation and lysis

Grow colonies on agar Petri plates overnight (37^0C; ~17 h). Plate enough culture to have 100-200 colonies on the plate. The next day, scrape all of the colonies from the plate using a spreader and add to a flask with TB media. Use a small amount of additional media to rinse and scrape any remaining culture off the agar and add to the flask. Use 1 Petri plate with colonies for 0.5L TB media (2 plates for 1L, etc.). Grow bacteria at 37^0C, 200 rpm for ~2-2.5 h, until the OD_{560} of the culture reaches ~0.7-0.8. Then reduce the temperature to 18^0C or 20^0C, and when the OD_{560} reaches ~1, induce over-expression by addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). At these conditions, grow the cells overnight for ~20-24 h. Centrifuge the culture at 4,000 g for ~30 min and freeze the cell pellets at -80^0C.

For purification, suspend the frozen cell pellet in the respective lysis buffers based on the type of protein being purified (see buffer tables) (1 g cells → 10 ml buffer) and then disrupt it at 4^0C using a high-pressure homogenizer at ~15,000 PSI for ~3 min (for ~3 g cells).

Alternatively, one may use sonication. With the Fisher Sonic Dismembrator Model 500 (500 W power) we may recommend the following parameters:

- 4 g cells suspended in 40 ml lysis buffer in a Falcon tube on ice
  - 58% power amplitude, 5 sec pulse/15 sec pause for 20 min
- 100 g cells suspended in 1 L lysis buffer on ice
  - 58% power amplitude, 5 sec pulse/15 sec pause for ~110 min
  - Use a magnetic mixer to uniformly cool the lysate during sonication.

Centrifuge the lysates at 40,000 x g for 20 min and filter through a 45 μm filter.

Note 2.1. The above protocol produced the best results in terms of the expression levels and amount of cells grown in ~90% of cases in our experience. However, in a few cases, when poor expression or cell growth is observed, some modifications to this protocol might be essential. For example, the temperature for over-expression might be increased to 30-37^0C, an IPTG concentration of 1 mM may be used, or both.

3. Protein purification

There are roughly three categories of proteins:

- ***(i)*** DNA/RNA-binding proteins
- ***(ii)*** Membrane proteins
- ***(iii)*** All other soluble proteins

Each has distinct functional properties that impose specific requirements on the approaches to their purification. Loading the lysate on the column is the major purification step during which most impurities should be eliminated. Otherwise, there is a high probability of obtaining a significantly contaminated target protein at the end. This may happen because the impurities, which are accumulated, concentrated, and likely aggregated in a relatively small column volume during loading, are difficult to remove during the washing steps, regardless of the washing conditions you use.

3.1. DNA/RNA-binding proteins

The strong non-specific affinity of this category of proteins to nucleic acids (NA) may result in major contamination by NA and respectively, by NA-binding cellular proteins.

To avoid these potential impurities, two major options of lysate preparation/loading may be used:

- ***(i)*** High salt lysate/loading buffer
- ***(ii)*** DNAse treatment of the lysate in low salt buffer followed by loading in high salt buffer

In place of the DNAse treatment, polyethyleneimine treatment of the lysate may be used followed by loading in high salt buffer.

3.1.1. Option 1- High salt loading

Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
1-2.5 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1 mM PMSF Inhibitory tablet(s)*	1-2.5 M NaCl 20 mM Tris pH 8 5% Glycerol	2 M NaCl 20 mM Tris pH 8 5% Glycerol	0 M NaCl 20 mM Tris pH 8 5% Glycerol	0.2-0.5M NaCl 20 mM Tris pH 8 5% Glycerol 0.2 mM EDTA Elution Protease**	6M Gdn*** pH 8 OR 0.1M Gly*** pH 3	0.5M NaCl 20 mM Tris pH 8 5% Glycerol

Table 3.1.1. Buffers for high salt loading.

* - add 1 tablet of inhibitory cocktail (Roche) for 30-40 ml of lysate.

** - the details of the proteolytic elution are provided in Section 4.

*** - Gdn - Guanidine Hydrochloride; Gly – Glycine.

Protocol

1). Lysis – As detailed in Section 2. Dilute the lysate 2 times with Loading Buffer A.

2). Loading (Buffer A) – Load lysate on the Im7 column using a flow rate of 1/10 – 1/4 of the column volume (CV).

I.e. with ~0.2-0.3 ml/min for the 1 ml column, ~0.5-1.2 ml/min for the 5 ml column, ~2-4 ml/min for the 20 ml column, etc.

In gravity mode, where it might be difficult to control the actual flow rate, one may load the lysate repeatedly 2-3 times to obtain better binding capacity. For large lysate volumes (long loading time), it might be useful to alternate the loading with the high salt washing steps. For example, alternate after each 30 min of loading to avoid accumulation of impurities on the column.

3). High salt washing (Buffer A1) – Wash the Im7 column with 6-8 CV of the high salt Buffer A1.

4). NO salt washing (Buffer A2) – Wash the Im7 column with 6-8 CV of the NO salt Buffer A2.

5). Washing cycles – Repeat Steps 3 and 4 above 2-3 times. On a chromatography system, the flow rate may be as high as allowed by the column pressure limits.

6). Proteolytic elution (Buffer A3) – Equilibrate the column with 2-3 CV of the Buffer A3. Add the elution protease to Buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in Section 4.

7). Cleaning (Buffer A4) – Wash the Im7 column with 8-10 CV of the denaturing Buffer A4 to remove the bound CL7-tag. On a chromatography system, when using Gdn, the flow rate may be as high as allowed by the column pressure limits.

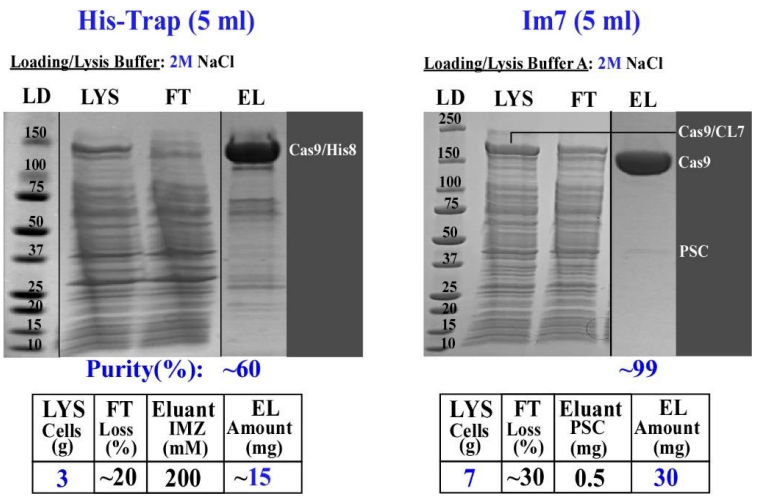
The low pH, Gly Buffer elutes the bound CL7-tag quite slowly and non-uniformly. In this case, we recommend a flow rate of 0.5-1 ml/min and the whole process may take up to 1-1.5 h. In gravity mode, follow the Protocol described in Section 5.

8). *Regeneration (Buffer A4 & Buffer B)* – On a chromatography system, exchange the denaturing Buffer A4 with Buffer B using a 1 h gradient option (flow rate – 1 ml/min; gradient volume – 60 ml). In gravity mode, follow the protocol described in Section 5.

9). *Final Wash (Buffer B)* – Wash the regenerated Im7 column with 3-4 CV of Buffer B.

3.1.2. Option 1 – Example

1). Cas9 enzyme (MW ~ 144 kDa) - A key element of the CRISPR gene editing technology.



LYS Cells (g)	FT Loss (%)	Eluant IMZ (mM)	EL Amount (mg)
3	~20	200	~15

LYS Cells (g)	FT Loss (%)	Eluant PSC (mg)	EL Amount (mg)
7	~30	0.5	30

LD – ladder; **LYS** – lysate; **FT** – flow through; **EL** – eluate; **PSC** – PreScission protease; **IMZ** - imidazole

Fig. 3.1. Gels compare His-trap and Im7 purification of Cas9 enzyme.

The purity of this enzyme is directly coupled with its editing efficiency. Cas9 is particularly crucial for gene therapy trials of *in vivo* editing of the genes in living human cells, for which an extra purity is required. Previously, the extra-pure samples could be obtained only using 4-5 different chromatographic steps. With the CL7/Im7 technology, we were able to obtain an extra-pure (99%) sample of Cas9 in one step with activity that was practically identical to that of the samples purified by the conventional multi-step techniques (Fig. 3.1**)**. For comparison, the His-Trap run with essentially the same lysis/loading conditions provided only ~60% sample purity (Fig. 3.1).

3.1.3. Option 2- DNAse treatment → high salt loading

Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
0.1 M NaCl 20 mM Tris pH 8 5% Glycerol DNAse I* 10 mM $MgCl_2$ 0.5 mM $CaCl_2$ 0.1 mM PMSF Inhibitory tablet(s)*	1-2.5 M NaCl 20 mM Tris pH 8 5% Glycerol	2 M NaCl 20 mM Tris pH 8 5% Glycerol	0 M NaCl 20 mM Tris pH 8 5% Glycerol	0.2-0.5M NaCl 20 mM Tris pH 8 5% Glycerol 0.2 mM EDTA Elution Protease**	6M Gdn*** pH 8 OR 0.1M Gly*** pH 3	0.5M NaCl 20 mM Tris pH 8 5% Glycerol

Table 3.1.3. Buffers for DNAse treatment with high salt loading.

* - add 1 tablet of inhibitory cocktail (Roche) and ~120 μg DNAse Grade I (Roche) for 30-40 ml of lysate.

** - the details of the proteolytic elution are provided in Section 3.4.

*** - Gdn - Guanidine Hydrochloride; Gly – Glycine.

Protocol

1). *Lysis* – As detailed in Section 2. Incubate the cell lysates for ~1.5-2 h at 4^0C in the DNAse-containing lysis buffer with addition of 0.05 mM PMSF after each 30 min during incubation. Then dilute the lysate 2 times with 2-fold concentrated loading buffer to match the high salt concentration of the Loading Buffer A.

2). *Loading (Buffer A)* – Load lysate on the Im7 column using a flow rate of 1/10 – 1/4 of the column volume (CV).

I.e. with ~0.2-0.3 ml/min for the 1 ml column, ~0.5-1.2 ml/min for the 5 ml column, ~2-4 ml/min for the 20 ml column, etc.

In gravity mode, where it might be difficult to control the actual flow rate, one may load the lysate repeatedly 2-3 times to obtain better binding capacity. For large lysate volumes (long loading time), it might be useful to alternate the loading with the high salt washing steps. For example, alternate after each 30 min of loading to avoid accumulation of impurities on the column.

3). *High salt washing (Buffer A1)* – Wash the Im7 column with 6-8 CV of the high salt Buffer A1.

4). *NO salt washing (Buffer A2)* – Wash the Im7 column with 6-8 CV of the NO salt Buffer A2.

5). *Washing cycles* – Repeat Steps 3 and 4 above 2-3 times. On a chromatography system, the flow rate may be as high as allowed by the column pressure limits.

6). *Proteolytic elution (Buffer A3)* – Equilibrate the column with 2-3 CV of Buffer A3. Add the elution protease to Buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in Section 4.

7). *Cleaning (Buffer A4)* – Wash the Im7 column with 8-10 CV of the denaturing Buffer A4 to remove the bound CL7-tag. On a chromatography system, when using Gdn, the flow rate may be as high as allowed by the column pressure limits. The low pH, Gly Buffer elutes the bound CL7-tag quite slowly and non-uniformly. In this case, we recommend a flow rate of 0.5-1 ml/min and the whole process may take up to 1-1.5 h. In gravity mode, follow the protocol described in Section 5.

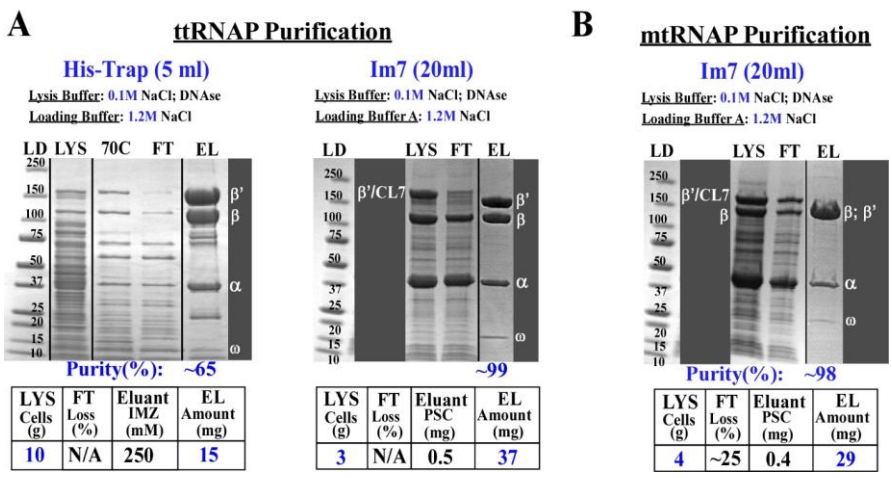
8). *Regeneration (Buffer A4 & Buffer B)* – On a chromatography system, exchange the denaturing Buffer A4 with Buffer B using a 1 h gradient option (flow rate – 1 ml/min; gradient volume – 60 ml). In gravity mode, follow the protocol described in Section 5.

9). *Final Wash (Buffer B)* – Wash the regenerated Im7 column with 3-4 CV of the Buffer B.

3.1.4. Option 2 – Examples

1). The core enzyme of RNA polymerase (RNAP) from T. thermophilus (ttRNAP) - A complex of five protein subunits (α_2,β,β',ω; MW ~ 400kDa). The purity of the enzyme is essential for both its activity and crystallization. Multi-subunit RNAPs have at least 5 different DNA/RNA-binding domains and, therefore, possess strong non-specific affinity to cellular nucleic acids (NA). This property usually results in significant contamination of the samples by NA and NA-binding cellular proteins during purification by conventional techniques. Traditionally, crystallization quality RNAP samples could be obtained only using 4-5 distinct chromatographic steps. With the CL7/Im7 technology, we were able to obtain an extra-pure (99%) sample of ttRNAP in one step with high activity that is suitable for successful crystallization trials (Fig**.** 3.2A). For comparison, the His-Trap run with essentially the same lysis/loading conditions provided only ~65% sample purity, even though the lysate was heated at 60^{o}C prior to the His-Trap run to eliminate most of the *E. coli* proteins (Fig. 3.2A).

2). The core enzyme of RNAP from M. tuberculosis (mtRNAP) - A functional and structural analog of ttRNAP (α_2,β,β',ω; MW ~ 400kDa). Despite the functional and overall structural similarity between mtRNAP and ttRNAP, the fine structural and surface properties of these two multi-subunit enzymes are quite distinct. Though similar to ttRNAP, high purity samples of mtRNAP could be also obtained using 4-5 distinct chromatographic steps, but the set of the chromatographic techniques is distinct between these two proteins. With the CL7/Im7 technology, we were able to obtain an extra-pure (~98%) sample of mtRNAP in one step using a protocol that is nearly identical to that of ttRNAP (Fig. 3.2B).



LD – ladder; **LYS** – lysate; **70C** – heat 70°C; **FT** – flow through; **EL** – eluate; **PSC** – PreScission protease; **IMZ** – imidazole

Fig. 3.2. Gel comparing His-trap and Im7 purification of ttRNAP, and gel showing mtRNAP purification with Im7.

3.2. Membrane proteins

The hydrophobic nature of membrane proteins (MPs) is a major purification problem for this category of proteins. MPs may form strong, non-specific hydrophobic contacts with each other and soluble cytoplasmic proteins or nucleic acids. In fact, successful purification of these proteins directly from cell lysates is difficult to achieve. Therefore, an ultra-centrifugation is used as a standard pre-chromatographic step in order to precipitate the MP fraction and thereby purify it from most nucleic acids and soluble cytoplasmic proteins. The MP fraction pellet is then solubilized for the chromatographic runs using high concentrations of a non-denaturing detergent, which also removes/exchanges the lipids naturally bound on the surface of the MPs. However, if a strong over-expression of a target MP is achieved, the ultra-centrifugation sometimes doesn't work, resulting in little or no MP fraction pellet. In our experience, polyethyleneimine (PEI) precipitation may be efficiently used to purify the MPs from the nucleic acids and soluble cellular proteins. This step may provide initial samples of the same or even better purity than that of the ultra-centrifuged ones.

Thus, two major option**s** may be used at the initial, non-chromatographic step of the MP purification:

- ***(i)*** Ultra-centrifugation
- ***(ii)*** PEI precipitation

3.2.1. Non-chromatographic step - Option 1- Ultra-centrifugation

Protocol

1). *Ultra-centrifugation (Lysate)*– As detailed in Section 2. Ultra-centrifuge the lysate at 120,000 g for 1.5 h.

2). *Solubilization* – Dissolve the pellet containing the membrane fraction in a Solubilization Buffer with a volume equal to ~1/5-1/2 of the lysate volume so that the total protein concentration is roughly ~2-4 mg/ml. Ultra-centrifuge again at 120,000 g for 30 min and discard the pellet.

Solubilization Buffer 1: 0.2-0.9 M NaCl, 20 mM Tris pH 8.0, 5% Glycerol, 0.1 mM PMSF, 1.5% DDM

3.2.2. Non-chromatographic step - Option 2- PEI precipitation

Protocol

1). *Dilution (Lysate)* – As detailed in Section 2. Dilute the lysate with an appropriate Buffer (with no detergent) to reach OD_{260} ~25 and salt (NaCl) concentration of ~0.3-0.35 M.

2). *PEI Precipitation* – Add 10% PEI stock solution to lysate in three aliquots to a final concentration of 0.06%. At each step, gently mix the suspension for ~5-10 min. After the final step, centrifuge the suspension at 3,000-5,000 g for 15 min.

3). *Solubilization* – Wash the final PEI pellets with the Solubilization Buffer for 10-15 min. Use the volume of the Solubilization Buffer equal to ~1/5-1/2 of the original lysate volume so that the total protein concentration in a supernatant is roughly ~2-4 mg/ml. Centrifuge the suspension at 3,000-5,000 x g for 15 min and discard the pellet.

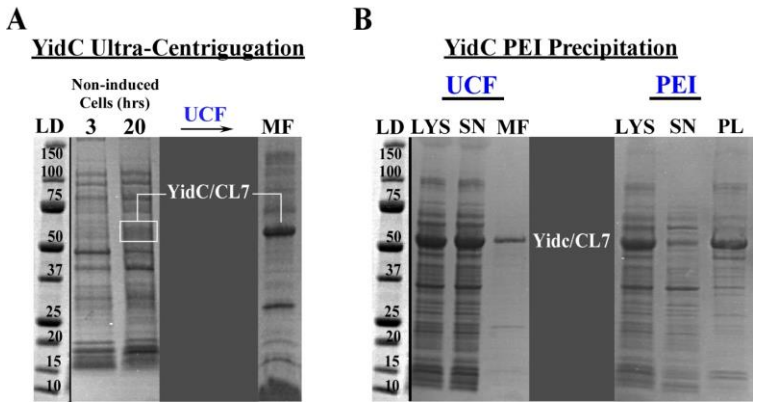
Solubilization Buffer 2: 0.6-0.8 M NaCl, 20 mM Tris pH 8.0, 5% glycerol, 1.5% DDM

To optimize the conditions, it might be useful to try the PEI treatment with small (1-2 ml) aliquots of the lysate using different salt concentrations and/or washing volumes. For example, you may use 0.2M, 0.3M, 0.35M NaCl concentrations at the precipitation step and 0.6M, 0.8M NaCl concentrations at the washing (solubilizing) step, load the supernatants from each experiment on a gel and analyze the results to select the best working conditions.

3.2.3. Examples

1). Option 1 - B. halodurans YidC membrane integrase (non-induced cells) - An "all"-membrane protein, without bulky extra-membrane domains. The gene sequence was adjusted for *E. coli* codons and cloned in the pET28a vector with the C-terminal CL7 tag. To mimic modest over-expression typical for MPs, we have cultivated the cells with no IPTG induction since the promoter in this vector is slightly leaky. In this case, ultra-centrifugation of the lysate provided a large pellet corresponding to the membrane fraction (MF). Solubilization of the MF with 1.5% DDM resulted in a pool of cellular MPs with a prominent YidC band on a gel (Fig. 3.3A).

2). Option 2 – YidC (induced cells) - Optimization of the YidC gene sequence for the *E. coli* codons in combination with the CL7 tag greatly improved the protein over-expression when it was induced with IPTG. In this case, however, ultra-centrifugation provided very little membrane fraction, with almost all YidC and other MPs remaining largely in solution. Therefore, polyethyleneimine (PEI) precipitation was used for the initial YidC purification (Fig. 3.3B).



UCF – ultra-centrifugation; **PEI** - polyethyleneimine precipitation; **LD** – ladder; **LYS** – lysate; **SN** – supernatant after UCF or PEI; **MF** – membrane fraction; **PL** – supernatant of washed PEI pellet.

Fig. 3.3. Gels comparing ultra-centrifugation and PEI precipitation methods of YidC.

3.2.4. Chromatographic purification of MPs

Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
0.2-0.5 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1 mM PMSF Inhibitory tablet(s)*	0.2-1 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1% DDM**	1 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1% DDM	0 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1% DDM	0.2-0.5M NaCl 20 mM Tris pH 8 5% Glycerol 0.1% DDM 0.2 mM EDTA Elution Protease**	6M Gdn**** pH 8 OR 0.1M Gly**** pH 3	0.5M NaCl 20 mM Tris pH 8 5% Glycerol

Table 3.2. Buffers for lysis and chromatographic purification of the MPs.

* - add 1 tablet of inhibitory cocktail (Roche) for 30-40 ml of lysate.

** - DDM - *n-Dodecyl-β-D*-Maltopyranoside. Other popular detergents might be used, of course.

*** - the details of the proteolytic elution are provided in Section 4.

**** - Gdn - Guanidine Hydrochloride; Gly – Glycine.

Protocol

1). *Dilution* – Dilute the lysate ~8-10 times with Buffer A to yield a detergent concentration of ~0.25%. In our experience, though such dilution increases purification time, it may also substantially improve binding capacity of the column towards a target MP.

2). *Loading (Buffer A)* – Load lysate on the Im7 column using a flow rate of 1/10 – 1/4 of the column volume (CV).

I.e. with ~0.2-0.3 ml/min for the 1 ml column, ~0.5-1.2 ml/min for the 5 ml column, ~2-4 ml/min for the 20 ml column, etc.

In gravity mode, where it might be difficult to control the actual flow rate, one may load the lysate repeatedly 2-3 times to obtain better binding capacity. For large lysate volumes (long loading time), it might be useful to alternate the loading with the high salt washing steps. For example, alternate after each 30 min of loading to avoid accumulation of impurities on the column.

3). *High salt washing (Buffer A1)* – Wash the Im7 column with 6-8 CV of the high salt Buffer A1.

4). *NO salt washing (Buffer A2)* – Wash the Im7 column with 6-8 CV of the NO salt Buffer A2.

5). *Washing cycles* – Repeat Steps 3 and 4 above 2-3 times. On a chromatography system, the flow rate may be as high as allowed by the column pressure limits.

6). *Proteolytic elution (Buffer A3)* – Equilibrate the column with 2-3 CV of Buffer A3. Add the elution protease to Buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in Section 4.

7). *Cleaning (Buffer A4)* – Wash the Im7 column with 8-10 CV of the denaturing Buffer A4 to remove the bound CL7-tag. On a chromatography system, when using Gdn, the flow rate may be as high as allowed by the column pressure limits. The low pH, Gly Buffer elutes the bound CL7-tag quite slowly and non-uniformly. In this case, we recommend a flow rate of 0.5-1 ml/min and the whole process may take up to 1-1.5 h. In gravity mode, follow the protocol described in Section 5.

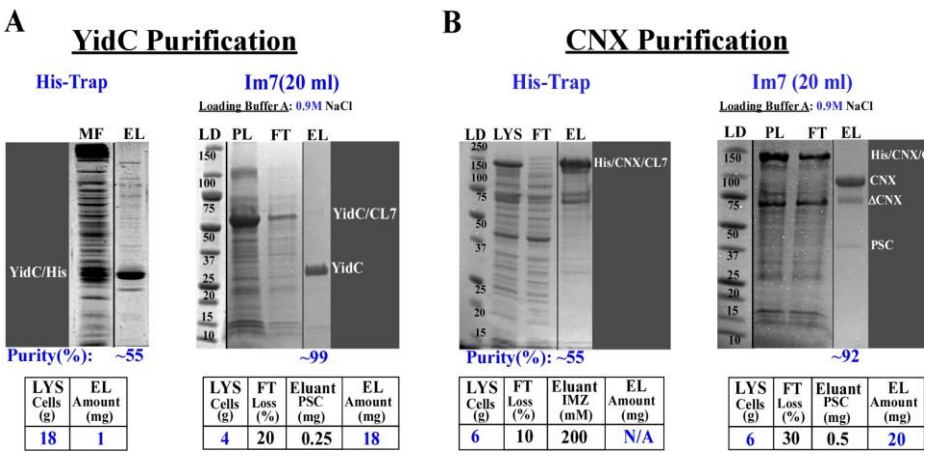
8). *Regeneration (Buffer A4 & Buffer B)* – On a chromatography system, exchange the denaturing Buffer A4 with Buffer B using a 1 h gradient option (flow rate – 1 ml/min; gradient volume – 60 ml). In gravity mode, follow the Protocol described in Section 5.

9). *Final Wash (Buffer B)* – Wash the regenerated Im7 column with 3-4 CV of Buffer B.

3.2.5. Examples

1). YidC (MW ~ 32 kDa; induced cells) - YidC lysate in a Lysis Buffer containing 0.3M NaCl was precipitated with 0.06% PEI. The PEI pellet was washed with a Solubilizing Buffer containing 0.6M NaCl and 1.5% DDM. The resulting supernatant was diluted 10 times with a high salt buffer with no detergent to yield a final NaCl concentration of 0.9M and DDM of 0.15%, and then loaded onto a 20 ml Im7 column with a flow rate of 2 ml/min. A single Im7 purification run provided an extra-pure (~99%) sample of YidC. In contrast, His-Trap purification resulted in only ~55% sample purity (Fig. 3.4A).

2). Calnexin (CNX; MW ~65 kDa; induced cells) - A human, trans-membrane chaperon. CNX contains a ~35 residue-long trans-membrane segment and two soluble domains on either side of the membrane. A full-length protein has never been expressed in *E. coli,* and no purification was reported. The CNX protein was purified in essentially the same manner as YidC. After PEI precipitation, a single Im7 purification run provided a high-purity (~92%) sample, whereas only ~55% could be achieved with the His-Trap approach under similar purification conditions (Fig. 3.4B).



LD – ladder; **MF** – membrane fraction; **PL** – supernatant of washed PEI pellet; **FT** – flow through; **EL** – eluate; **PSC** – PreScission protease.

Figure 3.4. Gels comparing His-trap and Im7 purification of YidC and CNX proteins.

3.3. Other soluble proteins

Assuming that the proteins from this category have no significant non-specific affinity to nucleic acids and/or hydrophobic patches on the surface, their purification with the CL7/Im7 approach is straightforward and may be carried out in a nearly identical manner. To avoid impurities, we still recommend loading the proteins in a salt (NaCl) concentration of at least 0.5M unless there are specific reasons to use lower salt concentrations. The major problem with this type of protein may arise on the upstream (expression) stage if the protein (alien to *E. coli*) is poorly folded in, or toxic to, bacteria, or intrinsically prone to aggregation at high concentrations. We note that in many cases, the CL7-tag alone or in combination with other expression/solubilizing protein tags such as thioredoxin and/or SUMO domain apparently increases solubility and assists proper folding of the target proteins. With no tag, they are expressed in *E. coli* only in the insoluble state as inclusion bodies.

3.3.1. Purification

Lysis Buffer	Loading Buffer A	Washing Buffer A1	Washing Buffer A2	Elution Buffer A3	Cleaning Buffer A4	Refolding Buffer B
0.2-1 M NaCl 20 mM Tris pH 8 5% Glycerol 0.1 mM PMSF Inhibitory tablet(s)*	0.2-1 M NaCl 20 mM Tris pH 8 5% Glycerol	1-2 M NaCl 20 mM Tris pH 8 5% Glycerol	0 M NaCl 20 mM Tris pH 8 5% Glycerol	0.2-0.5M NaCl 20 mM Tris pH 8 5% Glycerol 0.2 mM EDTA Elution Protease**	6M Gdn*** pH 8 OR 0.1M Gly*** pH 3	0.5M NaCl 20 mM Tris pH 8 5% Glycerol

Table 3.3.1. Buffers for purification of other soluble proteins.

* - add 1 tablet of inhibitory cocktail (Roche) for 30-40 ml of lysate.

** - the details of the proteolytic elution are provided in the Section 4.

*** - Gdn - Guanidine Hydrochloride; Gly – Glycine.

1). <u>Lysis</u> – As detailed in Section 2. Dilute the lysate 2 times with Loading Buffer A.

2). <u>Loading (Buffer A)</u> – Load lysate on the Im7 column using a flow rate of 1/10 – 1/4 of the column volume (CV), i.e. with ~0.2-0.3 ml/min for the 1 ml column, ~0.5-1.2 ml/min for the 5 ml column, ~2-4 ml/min for the 20 ml column, etc. In gravity mode, where it might be difficult to control the actual flow rate, one may load the lysate repeatedly 2-3 times to obtain better binding capacity. For large lysate volumes (long loading time), it might be useful to alternate the loading with the high salt washing steps. For example, alternate after each 30 min of loading to avoid accumulation of impurities on the column.

3). <u>High salt washing (Buffer A1)</u> – Wash the Im7 column with 6-8 CV of the high salt Buffer A1.

4). *NO salt washing (Buffer A2)* – Wash the Im7 column with 6-8 CV of the NO salt Buffer A2.

5). *Washing cycles* – Repeat Steps 3 and 4 above 2-3 times. On a chromatography system, the flow rate may be as high as allowed by the column pressure limits.

6). *Proteolytic elution (Buffer A3)* – Equilibrate the column with 2-3 CV of Buffer A3. Add the elution protease to Buffer A3 and perform proteolytic elution for ~1.5-2.5 h according to the Elution Protocol described in Section 4.

7). *Cleaning (Buffer A4)* – Wash the Im7 column with 8-10 CV of the denaturing Buffer A4 to remove the bound CL7-tag. On a chromatography system, when using Gdn, the flow rate may be as high as allowed by the column pressure limits. The low pH, Gly Buffer elutes the bound CL7-tag quite slowly and non-uniformly. In this case, we recommend a flow rate of 0.5-1 ml/min and the whole process may take up to 1-1.5 h. In gravity mode, follow the protocol described in Section 5.

8). *Regeneration (Buffer A4 & Buffer B)* – On a chromatography system, exchange the denaturing Buffer A4 with Buffer B using a 1 h gradient option (flow rate – 1 ml/min; gradient volume – 60 ml). In gravity mode, follow the protocol described in Section 5.

9). *Final Wash (Buffer B)* – Wash the regenerated Im7 column with 3-4 CV of Buffer B.

3.3.2. Examples

Below are the examples of the HHH-purification of five functionally significant and therapeutic proteins (biologics/biosimilars) that were performed in one Im7 step and under essentially identical conditions. Notably, the CL7 fusion to a SUMO domain allowed us to obtain 100% natural amino acid sequence of the target proteins upon the on-column elution with SUMO Protease (SMP). This is especially critical for purification of biosimilars that serve as drugs in the treatment of various human diseases.

1). HIV Nef protein (MW ~25 kDa) - Essential for HIV infection, a target for drug design (Fig. 3.5A).

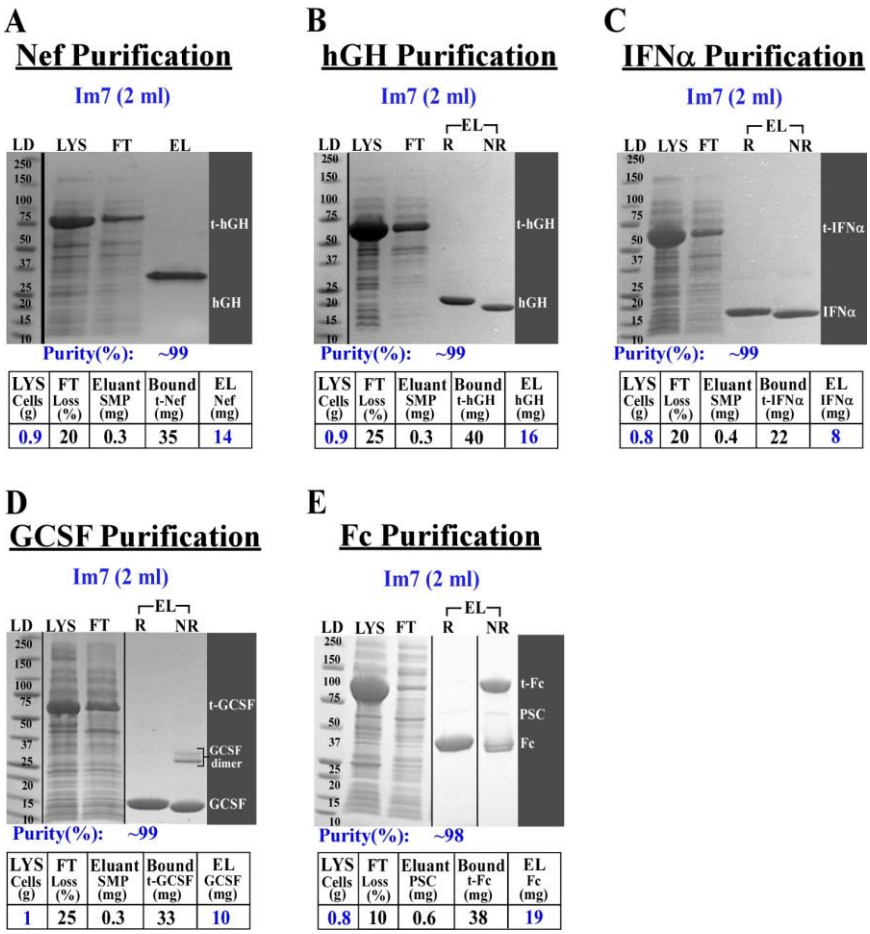
2). Human Growth Hormone (hGH) (MW ~22.1 kDa) – Clinically used for the treatment of hGH deficiency and stimulates weight gain (Drug – Somatropin; FDA approved). hGH contains the two internal disulfide bonds (SS-bonds) (Fig. 3.5B).

3). Human Interferon α (IFN-α) (MW ~19.2 kDa) – Clinically used for the treatment of viral infections and certain cancers (Drug – Pegasys; FDA approved). IFN-α contains two internal SS-bonds (Fig. 3.5C).

4). Granulocyte-Colony Stimulating Factor (GCSF) (MW ~18.7 kDa) - Clinically used to reduce the risk of infection after some types of cancer treatment (Drug – Neulasta; FDA approved). GCSF contains two internal SS-bonds (Fig. 3.5D).

5). Human Antibody Invariable Region (Fc) (MW ~26.1 kDa; forms dimer MW ~ 52.1 kDa) - Fc contains four internal SS-bonds and two inter-molecular SS-bonds that mediate dimer formation (Fig. 3.5E).

Note 3.3.2.1. The internal SS-bonds often cause misfolding of the proteins expressed in E. coli. Consistently, all proteins in Examples 2-5 above are insoluble when expressed in *E. coli* with no tag. In contrast, expression with the CL7-containg tag produced large amounts of the fully soluble protein suitable for a single-step HHH Im7 purification (Fig. 3.5B-E).



LD – ladder; **LYS -** lysate; **FT** – flow through; **EL** – eluate; **SMP** – SUMO protease; **PSC** – PreScission protease; **R/NR** – reduced/non-reduced gel samples; **t-[Nef/hGH/IFNa/GCSF/Fc]** – respective proteins with the CL7-containing tag.

Fig. 3.5. Gels of biosimilar proteins purified with the Im7 system.

4. On-column proteolytic elution of a target protein

Elution protease (SUMO (SMP) or PreScission (PSC)) is normally added to ~1.5-3% of the expected amount of a bound target protein, i.e., ~0.5 mg protease for ~30-40 mg of a target. We recommend that the inhibitory tablet(s) (Roche) is added to the stock samples of the purified elution proteases for safety. We add ~1 tablet to a ~400 mg protease stock, assuming that the protease is of high (98+%) purity. Alternatively, a small piece of a tablet can be added to protease diluted in Elution Buffer A3 just before its use for on-column cleavage.

Chromatography mode – Dilute the required amount of protease in 3-4 CV of Elution Buffer A3 (the exact volume depends on the volume/length of the tubing through which the protease is loaded on a column). First, fill the loading tubing with the protease at a fast flow rate (4-5 ml/min). Then reduce the flow rate to 0.1-0.3 ml/min (depending on the CV) and run the protease through the column slowly until the OD_{260} peak corresponding to an eluted target protein ends. To increase the concentration of an eluted target, in some cases, it might be worth making a couple of pauses for 20-30 min when the OD_{260} peak stops increasing.

For example, in our system, we often use the 5 ml Im7 cartridge and ~7 ml tubing for loading a protease sample. If we expect that the amount of bound protein is ~30-40 mg, we use ~0.5 mg protease and dilute it in ~20 ml of Elution Buffer A3. First, we load 7 ml of the protease at 5 ml/min to fill the tubing. Then we reduce the speed to 0.2 ml/min and run the protease sample through the column for ~1.5 – 2.5 hours to elute all target protein. To improve the efficiency of cleavage or to increase the concentration of an eluted target, we sometimes interrupt protease loading for 20-30 min to give the protease more time to better complete cleavage on the column.

Gravity mode – After equilibration of the column with Elution Buffer A3, drain the column but don't dry the beads entirely. Dilute the required amount of protease in 0.5-1 CV of the Elution Buffer. Add the protease to the beads and incubate for ~1.5 – 2.5 h at 4^{o}C, mixing the beads gently every 5-7 min. To monitor the elution process, elute a 0.3-0.4 ml aliquot from the column each 20-30 min and check the sample concentration with a Nano Drop spectrophotometer. Return the aliquot to the column. When the protein concentration no longer significantly increases, the elution is complete. Note that columns typically have a "dead" volume (with no beads) at the bottom beneath the bottom frit where no cleavage would occur. In order to avoid measuring the "dead" volume in your concentration measurements, return the first eluted aliquot to the column and take another aliquot for the measurements.

Note 4.1. There might be several reasons why an elution protease doesn't elute a target protein efficiently.

(1). Remaining contamination (in particular, nucleic acids) may largely inhibit the elution proteases during the on-column cleavage. In many cases, loading the lysates in a higher salt Loading Buffer A may help to achieve better purification and subsequently higher cleavage efficiency.

(2). Steric hindrance of a protease cleavage site on column by the target protein molecules. This may happen for large, oligomeric proteins. In this case, using larger amounts of protease than usual (3-4-fold) may help, but then an additional purification step (for example, size exclusion, GST or His-Trap) might be required to remove the protease from the purified target protein.

(3). An untagged (cleaved from a tag) target protein aggregates/precipitates. In this case, optimizing the Elution Buffer conditions to improve solubility of the target protein may help. If it doesn't, the problem likely exists at, and should be resolved at, the upstream (expression) stage.

To further elucidate what is an actual cause of a poor proteolytic elution, one may strip a column with the denaturing Buffer A4 (6M Gdn), measure its concentration by a Nano Drop, and load the sample on a gel. If concentration measurements show an $OD_{(260/280)}$ ratio of 0.75 (or higher), or several protein bands are observed on a gel, then contamination ***(1)*** is a likely cause of poor elution efficiency. An $OD_{(260/280)}$ ratio of 0.7 (or less) in combination with a single dominant band on a gel corresponding to either uncleaved or cleaved target protein will be indicative of steric hindrance ***(2)*** or aggregation ***(3)***, respectively. Note that before loading the sample on a gel, Gdn should be exchanged for Urea by dialysis, because Gdn precipitates when mixed with SDS.

5. Cleaning & regenerating the Im7 column in gravity mode

Cleaning - Remove Elution Buffer A3 from the beads, but don't dry the beads entirely. Add 4 CV of the denaturing Buffer A4 (6M Gdn) to the beads, equilibrate it for 4-5 min, remove it and then repeat this step twice with no equilibration. In the case of a low pH, Gly Buffer, increase equilibration time for 10-12 min, remove the buffer and repeat the procedure 5-6 times.

Regeneration – At the end of cleaning, leave 1-2 CV of Buffer A4 on top of the beads (there will be in total 2-3 CV including the beads volume) and then perform 10 steps of 10% dilutions of Buffer A4 with Buffer B. For this, elute or pick up by pipette 10% of the total volume of Buffer A4 at the first step and add the same amount of Buffer B. Repeat the 10% dilution 10 times, equilibrating the column for 5-6 min between the dilution steps. This procedure will totally exchange Buffer A4 with Buffer B at the end after ~50-60 min. Note that the volume you should remove/add to achieve 10% dilution will differ (increase) from step to step according to the following formula:

$$\boldsymbol{V_{ra}(N) = V_{tot}\left(1 - \frac{1 - 0.1 * N}{1 - 0.1 * (N - 1)}\right)}$$

Where **N -** Step #; $\mathbf{V_{tot}}$ – Total Buffer Volume; $\mathbf{V_{ra}(N)}$ – Volume to Remove/Add at the Step number N.

Example – Assuming that the bead volume is 2 ml and the initial volume of Buffer A4 in the column ($\mathbf{V_{tot}}$) before regeneration is **4 ml** (including the beads volume) you should perform 10 dilutions, removing/adding the buffer volumes [$\mathbf{V_{ra}(N)}$] according to Table 5.1.

N	0	1	2	3	4	5	6	7	8	9	10
$\mathbf{V_{ra}(N)}$ (ml)	0.000	0.400	0.444	0.500	0.571	0.667	0.800	1.000	1.333	2.000	4.000
Gdn (%)	100	90	80	70	60	50	40	30	20	10	~0

Table 5.1. Example case of buffer volumes to remove/add if total volume is 4 ml.