AFFINITY PURIFICATION SYSTEM

# Profinity eXact™ Fusion-Tag System

FAQs

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## Product Description

### 1 What is the Profinity eXact fusion-tag system and how does it assist in the purification of recombinant proteins?

The Profinity eXact fusion-tag system using exact affinity cleavage technology is an innovative solution for a key dissatisfaction associated with the purification process of affinity-tagged proteins — cleavage of the affinity tag. It is an *E.coli*-based system for the expression, detection, purification, and on-column cleavage of affinity-tagged proteins, without the addition of a protease. The result of the entire process is true, single-step purification without the hassle and expense of cleavage enzymes, incubation times, or removal of reagents.

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### 2 Which products make up the Profinity eXact system?

The kits that are part of this system include pPAL7 vectors for protein expression, a Profinity eXact antibody detection reagent, and competent cells for both cloning and expression of the fusion construct, as well as purification resin, spin columns, and cartridges. There are 12 catalog numbers associated with these products.

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| **156-3000** | Profinity eXact cloning and expression starter kit, 20 reactions (contains 156-3001, 156-3002, and 156-3003) |
| **156-3001** | Profinity eXact RIC-ready expression vector, 20 reactions |
| **156-3002** | Profinity eXact supercoiled expression vector, 20 reactions |
| **156-3003** | BL21 (DE3) chemi-competent expression cells kit, 10 reactions |
| **156-3004** | Profinity eXact-affinity tag antibody |
| **156-3005** | Profinity eXact purification resin, 10 ml |
| **156-3006** | Profinity eXact mini spin purification starter kit, 10 pk |
| **156-3007** | Profinity eXact mini spin columns, 10 pk |
| **732-4646** | Bio-Scale™ Mini Profinity eXact cartridges, 2 x 1 ml |
| **732-4647** | Bio-Scale Mini Profinity eXact cartridges, 4 x 1 ml |
| **732-4648** | Bio-Scale Mini Profinity eXact cartridge, 1 x 5 ml |
| **156-3008** | Profinity eXact expression and purification starter kit (contains 156-3000 and 156-3006) |

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## Profinity eXact Technology

**1 What is the affinity tag and how does it bind to the resin?**

The tag in this system is the prodomain of the subtilisin protease, a 75-amino acid sequence that is fused to the N-terminus of the target protein of interest. The prodomain sequence and mature subtilisin protease sequences have been coengineered to produce a specific, high affinity interaction between the binding partners. As the ligand, the subtilisin protease is not only involved with binding and recognition of the tag, but upon application of fluoride elution buffer, also serves to quickly and precisely cleave the tag from the fusion protein and release the purified target protein. At the end of the purification process the tag remains tightly bound to the resin.

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**2 How does this system work with subtilisin? Isn't subtilisin a very aggressive, broad substrate protease?**

Dr Philip Bryan (University of Maryland) has engineered this enzyme by making a number of amino acid mutations that improve the stability of the subtilisin protein, change its substrate specificity to bind a defined amino acid sequence, and isolate the cleavage reaction from substrate binding. The result is a very stable enzyme that enables highly specific and tight binding to the Profinity eXact tag and a novel triggered cleavage of the tag to effect purification. Details of these mutations are described in Ruan et al. 2004, Biochemistry 43(46): 14539-46.

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**3 I read Biao Ruan's paper (2004, Biochemistry 43(46): 14539-46) that describes the general principles of this system and I'm confused by the cleavage recognition site on the affinity tag. Why does Bio-Rad literature refer to a different P4-P1 recognition site from Ruan's FKAM? And how do the 9 amino acid (EEDKLFKAL) P9-P1 residues affect binding and cleavage?**

The Profinity eXact tag's recognition site consists of two domains that ensure cleavage at the desired site. First is the EEDKLFKAL region, which has approximately 10× higher binding affinity to the Profinity eXact resin ($K_D$ = 1 µM ) than just an FKAL sequence ($K_D$ = 10 µM). The second domain is the entire 8 kD Profinity eXact tag, which increases binding affinity by four orders of magnitude ($K_D$ < 100 pM) and targets cleavage at the C-terminus of the affinity tag by facilitating placement of the Profinity eXact tag's cleavage site in the subtilisin's active site. The FKAM sequence described in Ruan's paper is part of a subtilisin variant with similar cleavage kinetics.



## General

**1 What is the solubility of the Profinity eXact fusion tag and how does it affect solubility of a sample protein?**

The Profinity eXact tag is highly soluble by itself. Overall, the tag does not affect the solubility of the sample protein in most cases.

As with many other affinity tags, the tag may yield more soluble fusion protein for some proteins or it may also reduce the solubility of the fusion protein. We have found some examples of both in our testing.

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**2 Can the Profinity eXact fusion tag function as an internal tag?**

Yes. One can try fusing a solubility-enhancing partner (for example MBP) to the N-terminus of the Profinity eXact tag to purify and recover an otherwise insoluble or predominantly insoluble tag-free protein. There are many other applications that can utilize this feature.

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**3 What are the details of the Profinity eXact tag-MBP control lysate — molecular weight, $A_{280}$ to concentration conversion factor, etc.?**

The molecular mass of the Profinity eXact-tag MBP fusion protein is 48.6 kD and that of the purified MBP is 40.3 kD. To calculate a tag-free MBP concentration from an $A_{280}$ spectrophotometer measurement, use the conversion. 1.0 $A_{280}$ = 0.62 mg/ml. (The Profinity eXact tag-fusion protein has a 1.0 $A_{280}$ = 0.71 mg/ml conversion factor.)

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**4 Why can't I get good western blots of the 8 kD Profinity eXact fusion tag?**

The tag is small and has a very high pI (9.06); consequently, it does not transfer well in the standard Towbin buffer system used during protein transfer from the gel to a blotting membrane. Use a CAPS buffer for better results. See manual provided with Bio-Rad transfer cells.

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**5 What is the ligand density of subtilisin on the resin?**

The ligand density is about 7 mg subtilisin/ml resin.



## 6 Why does the Profinity eXact fusion tag (8 kD) run higher than the 10 kD standard in the Precision Plus Protein™ SDS-PAGE standards? Why does it seem to run correctly when compared to the lysozyme (14 kD) in the SDS-PAGE broad range standards?

How a protein migrates through an SDS-PAGE gel may be affected by a variety of factors that are not completely mitigated by the negative charge the SDS detergent is expected to provide. Certain proteins, either due to structure or size, are not completely coated by SDS compared to other proteins. Consequently, a protein's pI, amino acid length, and amino acid composition may have a larger than expected role on migration. For example, the pH of a Tris-Glycine SDS-PAGE gel is around 9.0. Examining the pI of the different proteins in question, one sees that the Profinity eXact tag and lysozyme would maintain a more neutral charge at pH 9; whereas the 10 kD protein in the Precision Plus Protein standard has a more basic charge and may behave differently in an SDS-PAGE gel.

- pI of the Profinity eXact tag = 9.0.
- pI of the 10 kD protein in the Precision Plus Protein standard (161-0363) = 5.2.
- pI of the lysozyme in the SDS-PAGE broad range standard (161-0317) = 9.3.

Mass spectrometry is a more accurate method for determining molecular weight and our mass spectrometry data confirm that the MW of the tag is 8 kD.

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## 7 You mention maximum binding capacities in all your literature. Is it an accurate term?

You are correct. We really mean to say target protein yield, since the binding capacity values that are indicated are derived from the target protein collected in the elution fraction.



## Cloning and Expression

**1 What does RIC stand for?**

Restriction independent cloning. It is an easy way to clone without having to worry about whether your target gene has restriction sites that would interfere with cloning into the vector's sites.

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**2 I am cloning by RIC, but I don't have any way to perform the 12°C T4 DNA polymerase/dGTP reaction on my PCR product. What can I do?**

The easiest method to achieve 12°C for treatment of your PCR product is to use your PCR machine to accurately obtain the desired reaction temperature. An alternative is to set a water bath or heating block to 12°C in a refrigerator or in a cold room.

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**3 I am having trouble cloning with the RIC vector. I am not obtaining any positive clones. I've rechecked my PCR primer sequences; I obtain a large amount of PCR product, but my ligation is still not working. What's wrong?**

Are you sure that 5'-phosphorylated PCR primers were ordered? Primers must be phosphorylated at the 5' end because the RIC-ready vector had been alkaline phosphotase-treated.

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**4 I incubated my RIC ligase reaction mix for 5 minutes and it didn't work. Are there any tips for ligase use?**

Because the SapI-generated overhang of the RIC vector has only three bases, using a quick ligase for only five minutes does not allow enough time for proper ligation. Try incubating the quick ligase reaction mix for 20 minutes at room temperature.

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**5 Why are the Thr-Ser codons listed for the primer sequences recommended in the system manual the sequences generated from different from the SpeI imprecise fusion (Thr-Ser linker) cloning site?**

The Thr-Ser codons suggested for the primer sequences utilize the more commonly used serine codon by *E. coli*; however, no differences in expression levels have been observed for *E. coli* expression when using ACTTCT or ACTAGT.

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**6 What is the benefit of including a Thr-Ser spacer between Profinity eXact tag and target protein?**

Some proteins exhibit significant N-terminal structure that may affect binding to the resin or their first two amino acids (P1' and P2') resulting in non-ideal cleavage kinetics. These challenges are easily addressed at the cloning stage by utilizing a two amino acid linker (Thr-Ser linker) between the Profinity eXact tag and the target protein — generating an imprecise fusion.

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**7 When using a PCR primer that contains the HindIII site, why do I need to add specific extra bases between HindIII and my target protein?**

The unique HindIII site in pPAL7 is located within the C-terminus leucine of the Profinity eXact tag. For the tag to cleave, this leucine must be restored by including the TG bases immediately downstream of the HindIII sequence in the forward primer.



**8 Are additional amino acids left after cleavage if using the HindIII or SapI sites?**

No amino acids are left after the cleavage if the insert is cloned into the HindIII site or the SapI site. Cleavage occurs at the C-terminus of the EEDKLFKAL site. The SapI site is engineered into the cleavage site, which also contains the HindIII site. The insert is cloned either at the HindIII site (when using supercoiled vector) or at the Sap/EcoRI site when using the pre-digested RIC vector.

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**9 Are sequencing primers available to confirm the sequence of the clones that are picked prior to expression?**

We do not have sequencing primers available that are specific to the tag (for the forward primer). We recommend using the T7 promoter and T7 terminator primers as sequencing primers. One would have to synthesize primers complementary to the region near the tag cleavage site to allow for greater sequence data around the target protein.

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**10 Can the tag be placed in alternate vectors?**

The Profinity eXact tag can be placed from the pPAL7 vector into a customer's vector of choice. Simply isolate the tag's DNA from the pPAL7 vector by using NdeI and a downstream restriction enzyme corresponding to a site in the multiple cloning site (e.g. NcoI, BamHI, EcoRI, XhoI, or NotI). Another option is described in Bulletin 5813A. The complete vector sequence is available to download from the Bio-Rad website.

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**11 I would like to express Profinity eXact tag fusions in a mammalian system. Would a codon-optimized Profinity eXact tag be better?**

Yes. Our initial studies show that a human codon-optimized Profinity eXact tag yields 3-4× higher expression levels than the tag from pPAL7. It is not an available product yet.

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**12 Is the N-terminal methione of the Profinity eXact tag removed during expression?**

Mass spectrometry data confirm that the methionine is removed when expressed in *E. coli* and HeLa cells.



## Purification

### 1 Which buffers may be used with the Profinity eXact fusion-tag system?

While many different buffers and pH ranges may be used (see the Profinity eXact system manual), the lysis, bind/wash, and elution buffers included in the mini purification starter kit are recommended. The composition of the buffers are as follows:

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| Lysis: | 100 mM sodium phosphate, pH 7.2 (or bacterial lysis and extraction reagent) |
| Bind/Wash: | 100 mM sodium phosphate, pH 7.2 |
| Elution: | 100 mM sodium fluoride, 100 mM sodium phosphate, pH 7.2 |
| Regeneration: | 0.1 M phosphoric acid, pH 1.8 |
| Storage: | 100 mM sodium phosphate, 0.02% sodium azide, pH 7.2 |

The system is compatible with a broad range of additives commonly used to purify proteins. A complete list can be found in the manual. Highest purification yields are achieved by eliminating $Cl^-$ ions from all lysis and wash buffers. Chloride ions from additives like NaCl, KCl, and Tris-HCl act as slower cleavage/elution-triggering anions. When using the Profinity eXact purification resin, sodium acetate (NaOAc) and potassium acetate (KOAc) are recommended substitutes for NaCl or KCl.

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### 2 I'm confused by the discussion of P1'-P2' amino acids and how they affect my purification. I just want to know, "Will my protein purify?"

If the first amino acid of your target protein (immediately downstream of the Profinity eXact cleavage site), or P1' amino acid, is:

- **Proline** — fusion will not cleave; introduce a Thr-Ser spacer.
- **Cysteine** — fusion will most likely cleave during sample loading and resin washing steps; pre-chill the lysate and use cold wash buffers.
- **Aspartate** — fusion will cleave very slowly; perform an overnight elution incubation at room temperature or introduce a Thr-Ser spacer.
- **Anything else** — you shouldn't have a problem.

If the second amino acid of your target protein is a proline, you may want to consider using a Thr-Ser spacer.

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### 3 Can I purify inclusion bodies containing Profinity eXact fusion-tagged protein with urea? Most of the available affinity tag systems are so limiting because they do not tolerate urea.

Yes, as described in the manual, you can use urea to solubilize and purify an inclusion body. The Profinity eXact system functions in up to 4 M urea; therefore, we recommend solubilizing your protein in 8 M urea, diluting the urea-solubilized protein down to 2–4 M, and then loading the diluted lysate on to the resin. While up to 1 M guanidine-HCl may be used in the elution buffer, remember that Gu-HCl must be avoided in lysis and wash buffers. If a protein does not stay soluble in 4 M urea, the Profinity eXact system is unaffected by nonionic and zwitterionic detergents. These detergents may be added to enhance solubility.



## 4 How might I improve protein binding to the resin?

Allow lysate to incubate with resin for up to an hour at 4°C or for 30 minutes at room temperature; lysates with fusion proteins >75 kD often benefit from a longer incubation period.

## 5 My protein is not binding. What is wrong?

When a protein is not binding, there can be two different observed phenomena. Is the fusion protein not binding or is cleavage of the fusion protein occuring during sample loading and washing? Determine whether full length fusion protein is observed in the flowthrough (indicating the former case) or cleaved target protein is present (suggesting the latter case). Appropriate suggestions will depend on which phenomenon is observed (described in the Profinity eXact system manual).

## 6 What general purification strategies can one employ to minimize premature cleavage of the target protein?

- **Fusion Protein** — Insert Thr-Ser spacer between Profinity eXact fusion tag and target protein by cloning into pPAL7's SpeI site
- **Lysis** — For maximum yields, do not use lysis buffers and reagents that contain triggering ions, specifically $Cl^-$ or $F^-$. Maintain lysate at 4°C prior to loading. Shorten incubation time. Utilize lysis buffer with pH <7.0
- **Wash** — Prechill wash buffer to 4°C prior to use

Do not use lysis buffers and reagents that contain triggering ions, specifically $Cl^-$ or $F^-$. When preparing wash buffer, substitute sodium acetate (NaOAc) for NaCl; do not use HCl to adjust pH — phosphoric or acetic acid should be used. Utilize wash buffer with pH <7.0.

## 7 Why do I observe some intrinsic cleavage with lysates prepared from the bacterial lysis and extraction reagent, which was not observed with lysates prepped using the provided bind/wash buffer of the Profinity eXact mini spin purification starter kit?

The slightly higher pH of the lysis and extraction reagent (pH 7.5) may result in intrinsic cleavage that was not previously observed with a lower pH lysate, for some proteins inherently susceptible to such cleavage (P1': Ser, Ala, Gly, His, Arg). Intrinsic cleavage is significantly minimized by loading chilled lysate to the resin.

## 8 I see cleaved target protein in my wash fractions. What's wrong?

Be certain that your wash buffer does not contain chloride ions. Are you using Tris-HCl and/or NaCl? If using Tris-HCl, instead use Tris base and adjust pH with acetic (or phosphoric) acid or the appropriate Tris conjugate. If using NaCl, use a similar concentration of sodium acetate to replace the sodium chloride.



**9 I still am hung up about not being able to use sodium chloride in my wash buffer. Why is it okay to use sodium acetate — I'm afraid it will not work as well as NaCl.**

In almost all situations where NaCl is used in a wash buffer, the compound is used because higher salt concentrations help to minimize electrostatic interactions between contaminant proteins and the resin. Thus, almost any other anion — such as acetate — may be used instead of chloride to reduce these nonspecific interactions. That is also why just increasing the buffer concentration is an effective alternative to using sodium chloride. Just remember the other general chromatography principle: do not use too high a salt concentration (e.g., >0.5 M). This can then promote nonspecific hydrophobic interactions.

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**10 How do I purify or elute uncleaved fusion protein?**

If you want to obtain purified, **tagged** fusion protein, skip the fluoride (or azide) elution and elute with 0.1 M phophoric acid. You may want to neutralize the acid eluate to prevent long term acid hydrolysis of the target protein.

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**11 Why would I consider using azide instead of fluoride as the cleavage triggering ion?**

A much lower concentration of azide (10 mM or 0.05%) may be used compared to fluoride (100 mM). Therefore, often the purified protein in the eluate can be directly used in the desired downstream application without the need for desalting or buffer exchange.

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**12 When using a cartridge, either manually or with the BioLogic DuoFlow™ system, sometimes I immediately observe cleaved target protein in the collected eluate as the one column volume of the elution buffer is applied — before any cleavage incubation has occurred. What gives?**

Fluoride diffuses very, very quickly throughout the cartridge. For highest yields, when the elution buffer is first pumped onto the resin, one may capture the first 1.5 ml of eluate (in 3 × 0.5 ml fractions) from a 1-ml cartridge. This collection is suggested in the system manual's protocol for use with a syringe. To be clear, we are referring to the fraction collected after the wash step is completed and elution buffer is first added — before the cleavage incubation — when one typically expects to collect wash buffer from the cartridge's void volume.



## Resin Storage and Cleaning

**1 How should the resin be regenerated?**

The Profinity eXact resin can be regenerated with 0.1 M $H_3PO_4$ by stripping off the cleaved Profinity eXact tag from the mutant subtilisin ligand. Use of the acid cleaning solution also effectively removes contaminants from the resin. Immediately after cleaning, the resin should be re-equilibrated with bind/wash buffer or storage buffer (100 mM sodium phosphate, 0.02% sodium azide, pH 7.2) to prevent loss of activity. Residual contaminants may also be removed by washing the resin with 0.1 M NaOH as the resin is base stable. After cleaning with NaOH, the resin should be equilibrated with bind/wash buffer. The Profinity eXact tag must then be removed using the aforementioned $H_3PO_4$ regimen. Long-term (> 1hr) storage in 0.1M $H_3PO_4$, 0.1M NaOH, or water is not recommended

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**2 How stable is the subtilisin protein? Should I worry that I'm doing my cartridge or column purification at room temperature, even though you recommend storing the cartridge at 4°C?**

The subtilisin mutant used in the Profinity eXact resin is very, very stable. It has been tested at 60°C for over 30 minutes without any adverse effect. Similarly, it is unaffected by 37°C incubation for over five days. The 4°C storage temperature is recommended to ensure maximum shelf life of your resin.

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**3 I forgot to store my cleaned cartridge/resin in the recommended storage buffer. Is my cartridge still good?**

So long as the regeneration solution has been neutralized, the cartridge is most likely still fully functional. The storage buffer contains 0.02% azide to prevent bacterial growth. However, the acid cleaning step also helps to kill many microorganisms, providing some safety margin to resins stored in the absence of azide.

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**4 What other storage buffers may be used to store my resin?**

Resins have also been stored in phosphate buffer (pH 7.2) containing either 20% ethanol or 2% butyl alcohol. However, Bio-Rad does not have long-term shelf-life data with these alternative storage buffers.



## Protocols

**1 How can I use the Profinia™ protein purification system to purify Profinity eXact fusion-tagged protein?**

Profinity eXact purification methods are preprogrammed into the Profinia system. Older instruments may require a flash firmware update. Contact technical service for assistance.

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**2 After elution buffer is pumped into the cartridge, how do you set up the cleavage incubation on the BioLogic DuoFlow system?**

To program the cleavage incubation, water is pumped for 28 minutes through the lines and out through the purge valve to prevent water from contacting the cartridge. Steps are then added to purge the water of the line and fill the lines with elution buffer before proceeding to the elution collection; this purge step adds a total of 2 minutes. Therefore, the resin experiences a 30 minute cleavage incubation.

Alternatively, the pump may be stopped for 30 minutes. Another option is to pump elution buffer at 0.1 ml/min for a 1 ml cartridge.

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**3 Should I cap the spin columns during each step?**

The spin columns may be spun with the top caps on. However, if the spin columns are rotated during the loading steps — and not used during the wash — some unwashed resin and residual flowthrough may be retained in the cap; if the cap is then used during elution incubation, the eluate may contain impurities from the cap's underside.

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**4 What speed should I centrifuge the spin columns at?**

The speed should be 1,000 x g and is listed in point #2 of the Protocol Notes section of the system manual.

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**5 What is the best method to break off the end cap from a spin column?**

Do not try to twist off the end cap; instead, snap it in one direction, and then completely snap it off in the opposite direction.

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**6 When using a syringe, how quickly should I push my buffers through a cartridge?**

Generally, there are 20-24 drops per ml; therefore, for a 1 ml/min flow rate, one would push buffer through at a rate of ~24 drops/min; for 5 ml/min, ~120 drops/min. Practically, one can push buffer through as quickly as the syringe and cartridge will allow with little effect on purification performance.



Purification and preparation of fusion proteins and affinity peptides containing at least two adjacent histidine residues may require a license under U.S. patents 5,284,933 and 5,310,663, including foreign patents (assignee: Hoffmann-La Roche).

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