eBook

Recombinant protein process development with Cytiva™ Protein Select™ resin

Principle, applications, and practical considerations



cytiva

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01

Introduction

Recombinant proteins: a family of very diverse molecules

The family of recombinant proteins includes very different types of molecules, such as enzymes, protein scaffolds, hormones, growth factors, etc. They often don't have an affinity binding partner to facilitate their purification. To be able to standardize and simplify the purification with an affinity step, one strategy has been to add a tag to the recombinant protein. Historically, using a tag has mainly been used for research purposes and not for biotherapeutic purification because the tags could not be removed without leaving traces on the target molecules.

With Cytiva™ Protein Select™ technology, you can now use a tag purification protocol with confidence that the protein will be automatically removed from the tag and eluted with no residual tag amino acids.

Here you'll find insights about process development for recombinant proteins and how you can standardize it using Cytiva Protein Select technology.

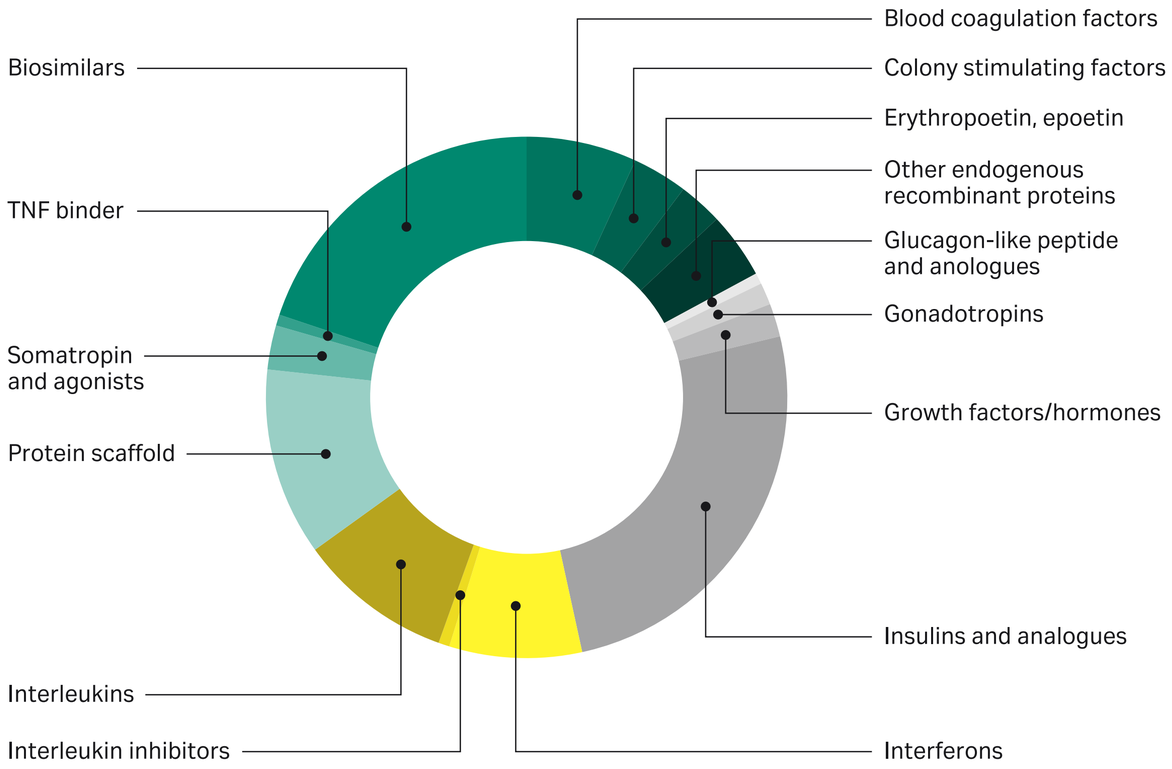


Fig 1. The diversity of molecules in preclinical development. Note: this chart does not show a large amount of unclassified molecules. Source: GlobalData [Online.] https://www.globaldata.com/. Accessed 2022.

02

Top challenges in recombinant protein purification process development

Insights from process developers

You might be one of the many process developers who are working on a diversity of molecules such as enzymes, peptides, scaffolds, and other recombinant proteins. This constantly evolving molecular diversity brings challenges in chromatography process development. In this article, we focus on the challenges in purifying recombinant proteins that lack affinity chromatography solutions.

We asked about your challenges, and you told us

In 2021, we asked 45 process developers, about the challenges they have purifying recombinant proteins that cannot be purified with affinity chromatography. The top three challenges most process developers experience are:

- Low purity in the first step
- Slow process development time
- Lengthy, multistep purification processes

Indeed, in contrast to traditional monoclonal antibodies (mAbs), which have well-established processing protocols, the newly evolving molecules often do not have set purification platforms. As a result, you end up having to develop a new purification method for each protein, as explained in this video.



Lack of an affinity solution can result in low purity after the capture step

Benefits of affinity capture steps

Process developers often use an affinity chromatography step as their first protein purification step, or "capture step." Affinity chromatography purification is favored because it is easy to implement and can give high purity in one step. If you have high purity after the capture step, you might need fewer chromatography polishing steps to get the purity you need. And fewer polishing steps mean higher yields and savings in time and cost during both process development and future manufacturing.

For example, in mAb production, protein A affinity chromatography is widely used for the capture step. Purity greater than 95% can be achieved after this step, which means that fewer steps are required before the final formulation steps.

There is no common affinity solution for all recombinant proteins

Many recombinant proteins do not have an existing affinity binding partner. Figure 2 shows that 64% of the survey respondents purifying non-mAbs frequently face problems with low purity in their first chromatography step. Also, when asked, more than 40% say that this low purity is a major issue in their work.

Therefore, these molecules need more chromatography steps to reach the needed purity.

Developing a new purification process for each new molecule takes time

Figure 3 shows that 53% of process developers in the survey frequently face slow process development challenges with recombinant proteins. Indeed, when a molecule cannot be purified with affinity chromatography, you need to develop a complex, multistep purification process for it because each new molecule behaves differently during chromatography.

Fig 2. Low purity in the first chromatography capture step is one of the main challenges faced by process developers working with a recombinant protein that cannot be purified with affinity chromatography.
Source: Survey performed in December 2021. n = 45 respondents.

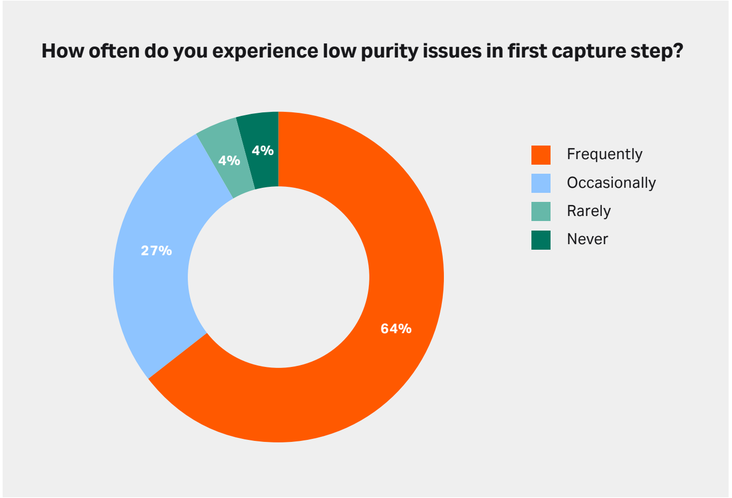
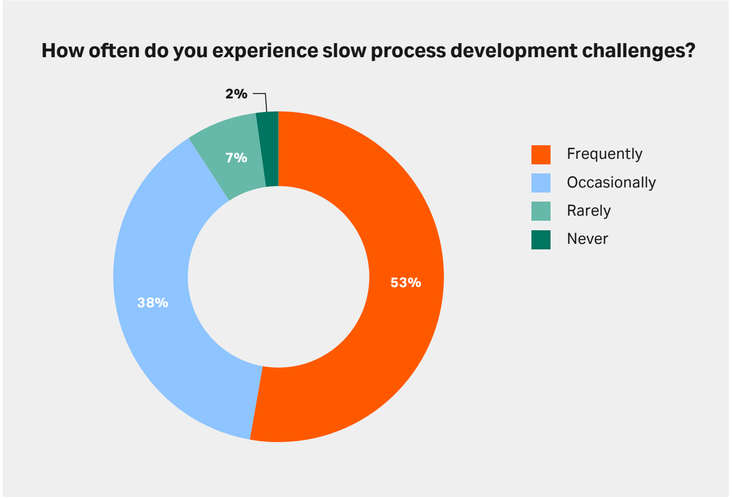


Fig 3. Slow process development is another challenge faced when working with a recombinant protein that cannot be purified with affinity chromatography.
Source: Survey performed in December 2021. n = 45 respondents.



To reach the required purity and yield, your purification process might require a combination of many steps, such as ion exchange chromatography, hydrophobic interaction chromatography, and multimodal chromatography. Optimizing these methods all take time.

The whole screening and optimization process might take several months, and you risk not meeting time constraints. The risk of having a long process development phase can cause you to abandon the molecule. You may even abandon it before it's up for consideration to begin the process development process.

Long chromatography processes generate increased cost

Almost half (43%) of survey respondents think that their chromatography process takes too long. When purifying a protein that cannot use affinity chromatography, you risk needing more chromatography steps in the process. A long chromatography process becomes a major contributor to your overall production time and costs and needs to be taken into account during early process development.

Now available! An affinity chromatography resin for purification of any recombinant protein

We heard you! Our research and development scientists developed Cytiva Protein Select resin that allows you to use standard affinity chromatography to purify any protein that does not have an affinity binding partner.

Using Cytiva Protein Select resin will make your process development:

- Easier
- Faster
- More efficient

Learn more about Cytiva Protein Select technology



03

What is Cytiva Protein Select resin

An affinity resin for purifying recombinant proteins

Cytiva Protein Select resin is an affinity chromatography resin for purifying recombinant proteins using the self-cleaving Cytiva Protein Select tag (Fig 4).

During an affinity step performed with Cytiva Protein Select resin, the protein self-cleaves from the tag and elutes with no residual tag amino acids.

This technology standardizes purification during process development and later phases of any protein that does not have an affinity binding partner.

Key features and benefits:

- Simple affinity purification protocol for recombinant proteins.
- One-step purification and tag cleavage without the need for proteases or other elution reagents.
- Delivers a highly pure, native protein with no remaining tag amino acids after tag cleavage.
- Can be processed under mild conditions, which means no exposure to conditions that cause protein aggregation or loss of biological activity.
- Flexible, enabling the use in multiple expression systems.

Download the data file

Visit the web product page

Watch the animation

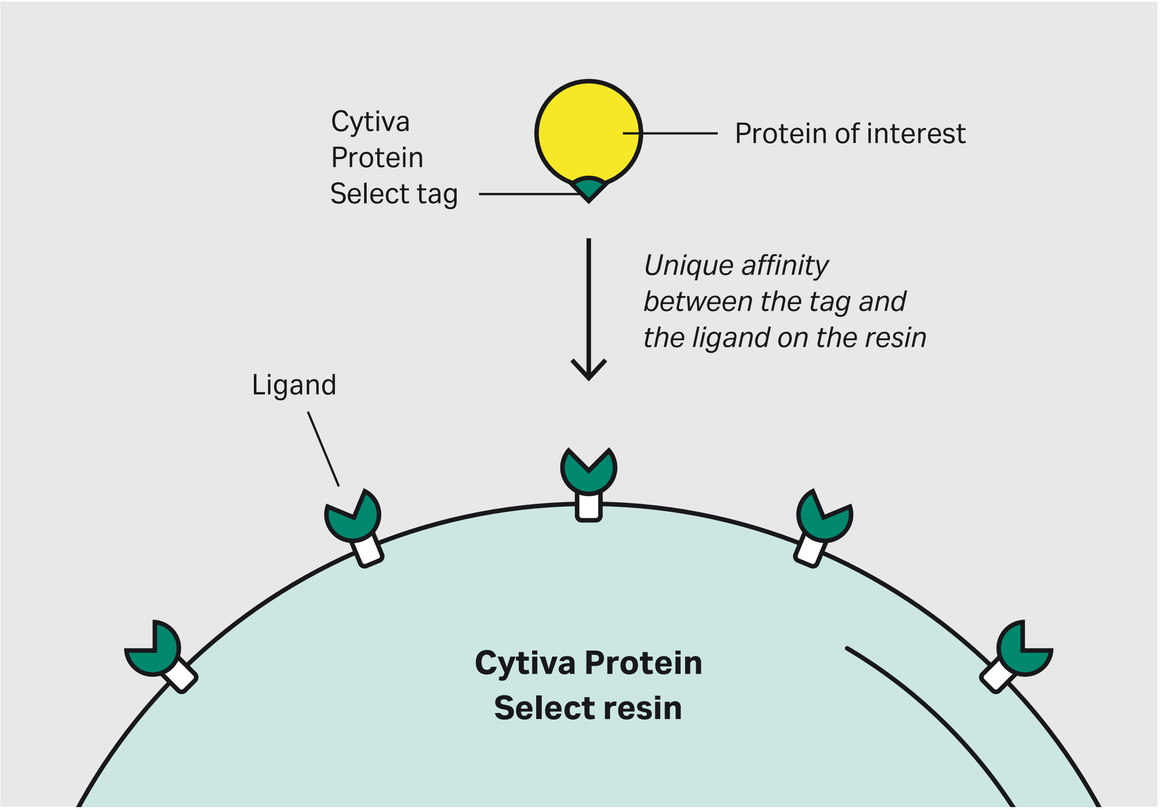


Fig 4. Cytiva Protein Select tag is recognized by the ligand on Cytiva Protein Select resin.

04

Using Cytiva Protein Select resin

A simple purification protocol

Purifying a protein tagged with Cytiva Protein Select tag is simple.

One chromatography step does the affinity purification, a traceless tag cleavage, and the tag removal. The pure protein obtained is in its native state with no traces of the tag amino acids.

The protocol does not require the use of a protease, and the same buffer can be used for equilibration, binding, wash, and elution.

Principle

Figure 5 describes how a recombinant protein is purified using Cytiva Protein Select resin and tag, from incorporating the tag to obtaining a pure protein.

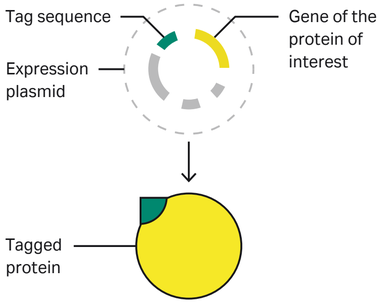
See FAQs for practical considerations

1. Protein design and expression

Incorporate the Cytiva Protein Select tag sequence and the gene of your protein of interest into your expression plasmid.

Express the protein in the suitable expression system.

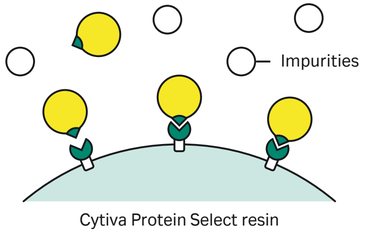
Collect the sample that contains the expressed protein.



2. Binding and wash

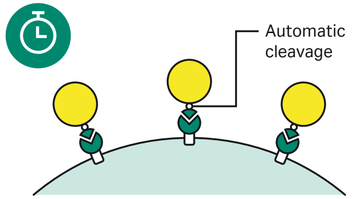
Load your sample onto the Cytiva Protein Select resin.

Your tagged protein binds to the ligand while impurities flow through during the wash.



3. Hold step

Pause the flow and initiate a hold step. Automatic cleavage happens when the ligand and the tag fold together.



4. Elution

Elute your pure, tag-free protein with no residual tag amino acids.

You do not have to change the buffer conditions, but you can if needed.

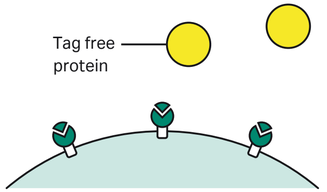


Fig 5. Overview of how Cytiva Protein Select technology works.

05

Cytiva Protein Select tag

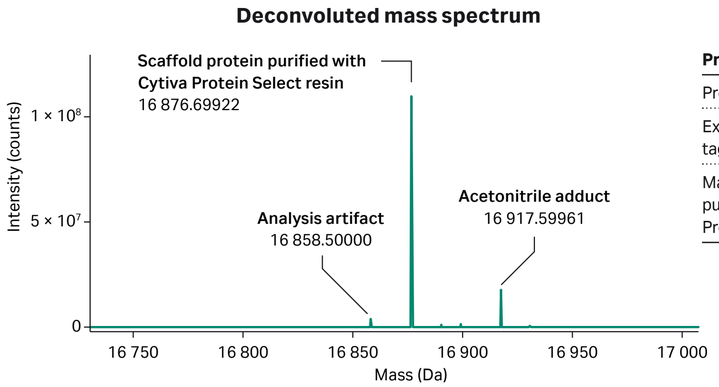
A tag that allows traceless self-cleavage

Tag characteristics

Cytiva Protein Select tag is a 36-amino acid tag that has a specific affinity to Cytiva Protein Select resin. The resin and the tag constitute the self-cleaving Cytiva Protein Select technology.

A completely traceless tag cleavage

After self-cleavage, Cytiva Protein Select tag is completely removed with no remaining amino acids left on the protein of interest. Figure 6 shows mass analysis of a scaffold protein purified using Cytiva Protein Select resin and tag. It shows that the mass of the protein obtained exactly equals the expected mass after cleavage, confirming that the protein is the pure, native state with no traces of the tag amino acids.



Protein	Mass
Protein with tag	22 141 Da
Expected mass after tag removal	16 876 Da
Mass measured after purification with Cytiva Protein Select resin	16 876.7 Da

Fig 6. Mass analysis shows that the mass of the protein matches the mass expected after tag cleavage. Mass error = 40 ppm. LC-MS analysis on Waters ACQUITY, C8- RPC.

Automatic cleavage site

The cleavage site is located between the last amino acid of the tag and the first amino acid of the target protein (Fig 7).

To produce your protein with Cytiva Protein Select tag:

- Get the tag sequence for free by registering here.
- Design your construct so that the tag is located on the N-terminus of your target protein.

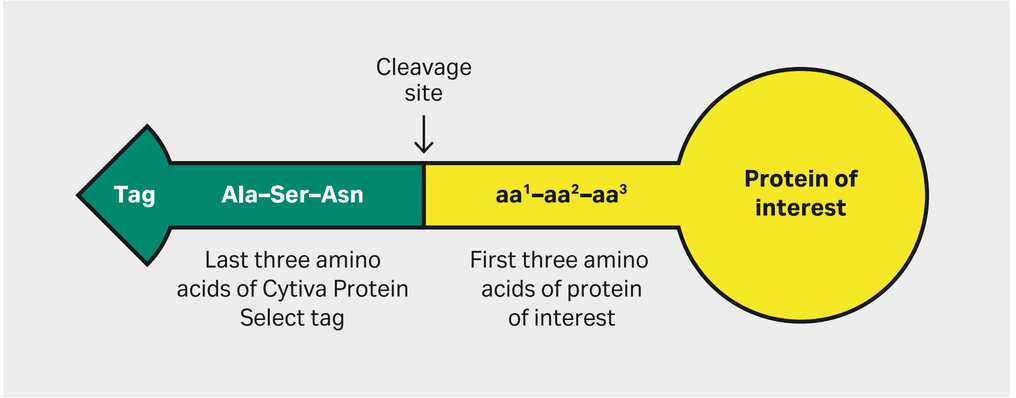


Fig 7. The cleavage site of the Cytiva Protein Select tag is between the last three amino acids of the tag and the first three amino acids of the target protein.

Receive the sequence of Cytiva Protein Select tag for free.
The sequence will be sent to you by email today.

06

A platform process with traceless tag affinity purification to accelerate process development

A standardized capture step and fewer polishing steps

As discussed in Chapter 2, when a protein has an affinity binding partner, the purification process can include an affinity capture step, which usually provides high purity level (~ 95%). One or two polishing steps are often sufficient to obtain the final expected purity. However, if a protein does not have an existing affinity binding partner, which is often the case for recombinant proteins, the first chromatography step will be less efficient. As a result, more polishing steps are usually needed, which requires time and effort during process development.

Adding Cytiva Protein Select tag to the recombinant protein enables the use of an affinity step with Cytiva Protein Select resin thereby standardizing and simplifying protein purification to obtain sufficient purity (Fig 8).

Process development gets easier, faster, more efficient. Fewer chromatography steps are required, which improves process time and cost.

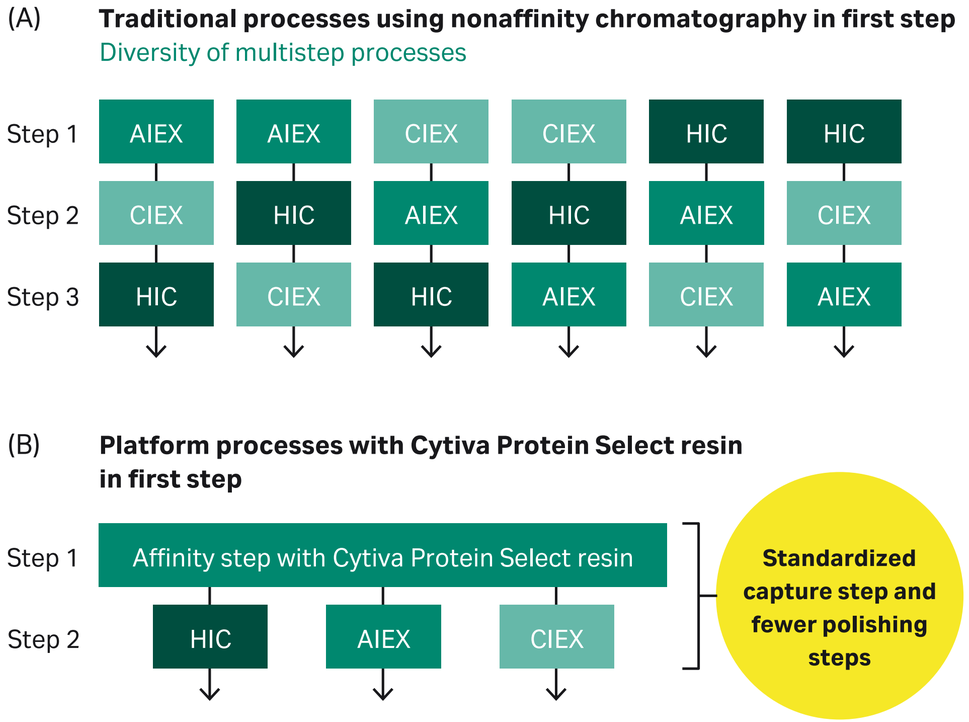


Fig 8. Comparing processes using non affinity method in first step (A) vs a method using Cytiva Protein Select resin (B) in first step. AIEX = anion exchange chromatography, CIEX = cation exchange chromatography, HIC = hydrophobic interaction chromatography.

07

Case study: comparing two process development approaches

Case study: purification of a scaffold protein expressed in *E. coli*

In this study, we developed two approaches to developing a process for purifying a recombinant protein that lacks an affinity binding partner — in this case, a thermostable scaffold protein (used as an antibody mimetic that binds to a desired ligand and is selected from a library of engineered variants) expressed in *E. coli.* Recombinant proteins that lack an affinity binder typically require extensive development to determine an optimal purification protocol, making their process development more costly and time-consuming.

Here we compared a conventional approach (referred to as the non-affinity process) to an alternative process based on Cytiva Protein Select tag and resin, designed to standardize capture of such proteins. We present the results of screening and transfer to columns suitable for scale-up studies, comparing development time, protein recovery, and protein purity between the non-affinity method and the affinity method using Cytiva Protein Select technology.

Part 1: Screening

We first performed screening in small scale (using HiTrap™ chromatography columns) to identify the techniques and resins to carry forward to larger scale. The requirements were: purity ≥ 95%, recovery ≥ 50%. Figure 9 shows an overview of the complexity and time requirements of each approach.

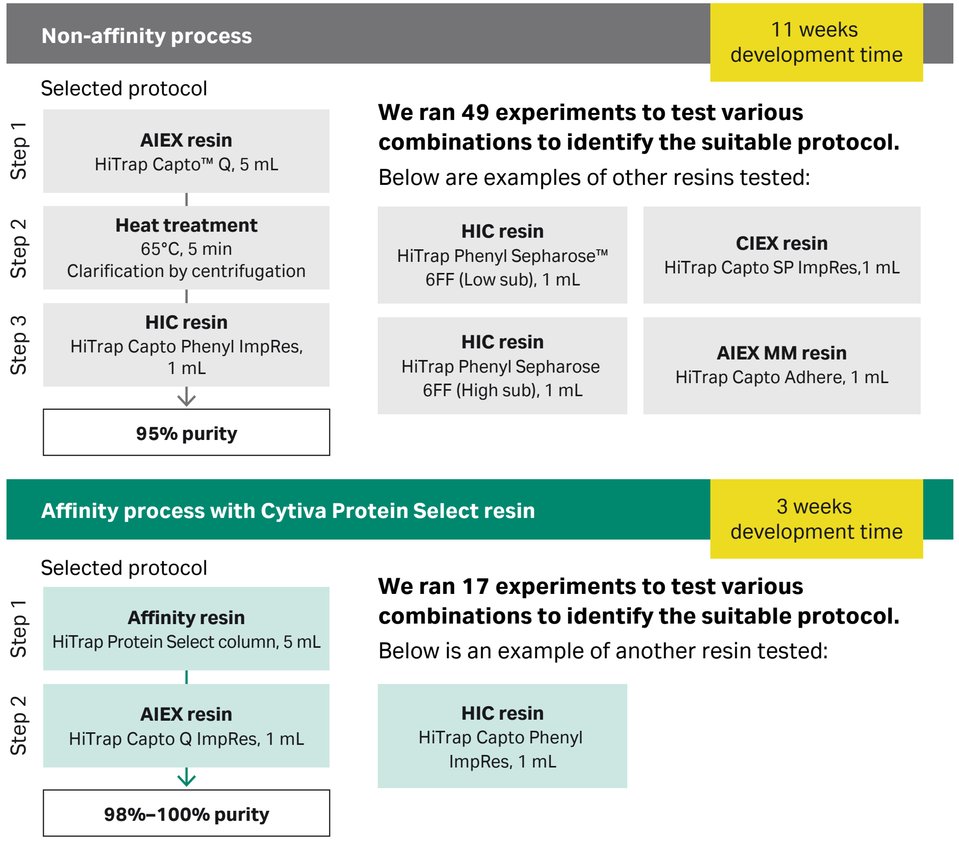
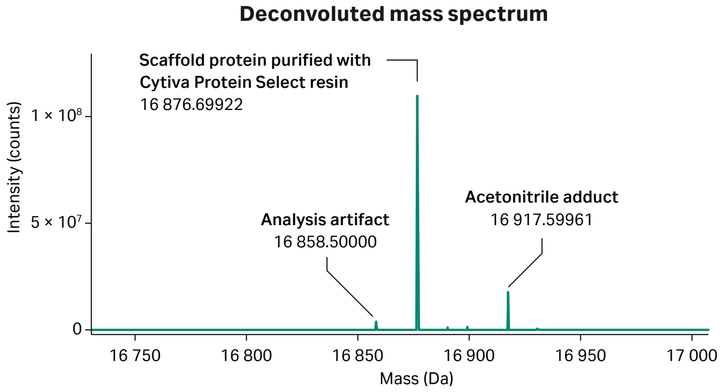


Fig 9. Development of the small-scale affinity-based process using Cytiva Protein Select resin took less time (3 weeks vs 11 weeks for non-affinity process) and was less complex (2 steps vs 3 steps).

AIEX = anion exchange chromatography; CIEX = cation exchange chromatography; MM = mixed mode chromatography; HIC = hydrophobic interaction chromatography

Results of screening

- **Non-affinity process:** We ran 49 experiments testing various combinations (buffers, techniques, and resins) to identify a protocol. This required 11 weeks of development time. For the protocol we chose, purity after the last step (HIC) was 95% (Fig 9) according to analytical SEC at 214 nm, which was our goal for the screening. However, for applications such as production of therapeutic molecules, purity of 95% would be too low, thus requiring either more optimization later in development or addition of an extra polishing step before a final process optimization. Either scenario would require additional development time.
- **Affinity process using Cytiva Protein Select resin**: We ran 17 experiments to identify a suitable protocol, and this required 3 weeks of development time. The protocol we selected delivered a purity level close to 100% according to analytical size-exclusion chromatography (SEC) (Fig 9). Figure 10 shows the results of mass analysis of the scaffold protein purified with Cytiva Protein Select resin and tag. The mass of the purified protein equals the expected mass after cleavage, confirming that the protein is pure and in native state with no traces of the tag amino acids.



Protein	Mass
Protein with tag	22 141 Da
Expected mass after tag removal	16 876 Da
Mass measured after purification with Cytiva Protein Select resin	16 876.7 Da

Fig 10. Mass analysis of the purified protein after tag cleavage. Mass error = 40 ppm. LC-MS analysis on Waters ACQUITY, C8- RPC.

Part 2: Process performance at 10 cm bed height

We transferred the best-performing processes from the screening phase (Part 1) to 10 cm bed-height columns and compared their performance in terms of purity and recovery. Figure 11 shows an overview of the process steps and results.

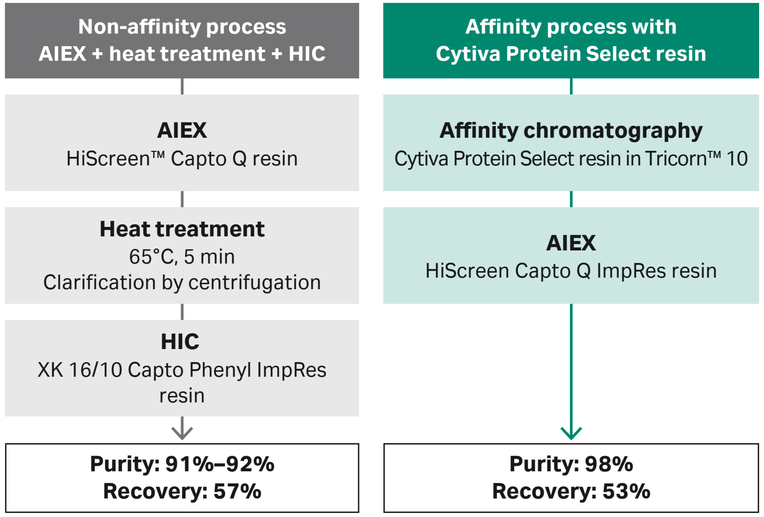


Fig 11. Comparison of process performance at 10 cm bed height.

Note that to facilitate recovery analysis of full-length fusion protein in crude cell extracts, we added a 6×His epitope tag upstream of the Cytiva Protein Select tag. We used the same protein construct for both processes to enable a fair comparison (Fig 12).

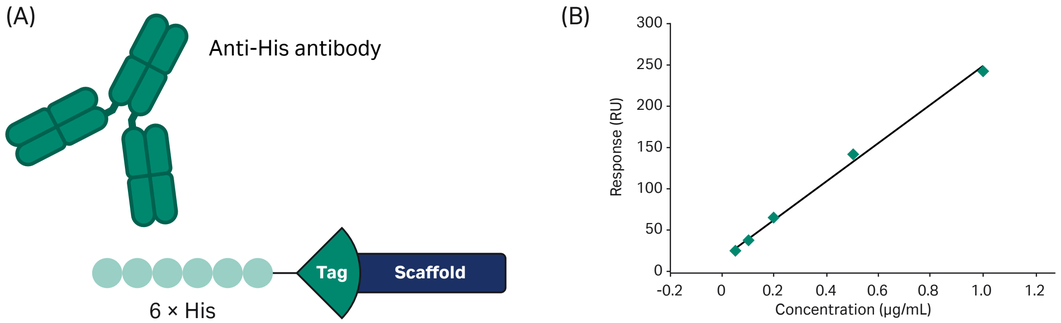


Fig 12. (A) Protein construct with upstream 6×His tag allowing Biacore™ SPR analysis using anti-His antibodies. (B) Calibration curve using purified fusion protein as standard for recovery calculations.

Results of performance testing

- **Non-affinity process:** The purity obtained after the HIC step was 91% to 92% (Fig 14), and the recovery was 57% over the process (data not shown). Furthermore, a low absorption coefficient at 280 nm made pooling criteria difficult in the first step. Therefore, extensive analytical work was necessary to identify the fractions to be pooled for the next step.

- **Affinity process using Cytiva Protein Select resin:** The purity obtained after the AIEX step was 98% (Fig 14) and the recovery was 53% over the process (data not shown). (Note: a single buffer, PBS pH 7.4, was used in the affinity step.) Furthermore, by looking at both 1st steps on Fig 13, we can see that the pool from the affinity step (right) is much easier to identify compared to the pool from the non-affinity step (left), which reduced the amount of analytical work required.

Figure 14 shows the results from analytical SEC. The different retention times reflect the uncleaved or cleaved scaffold fusion obtained in the processes.

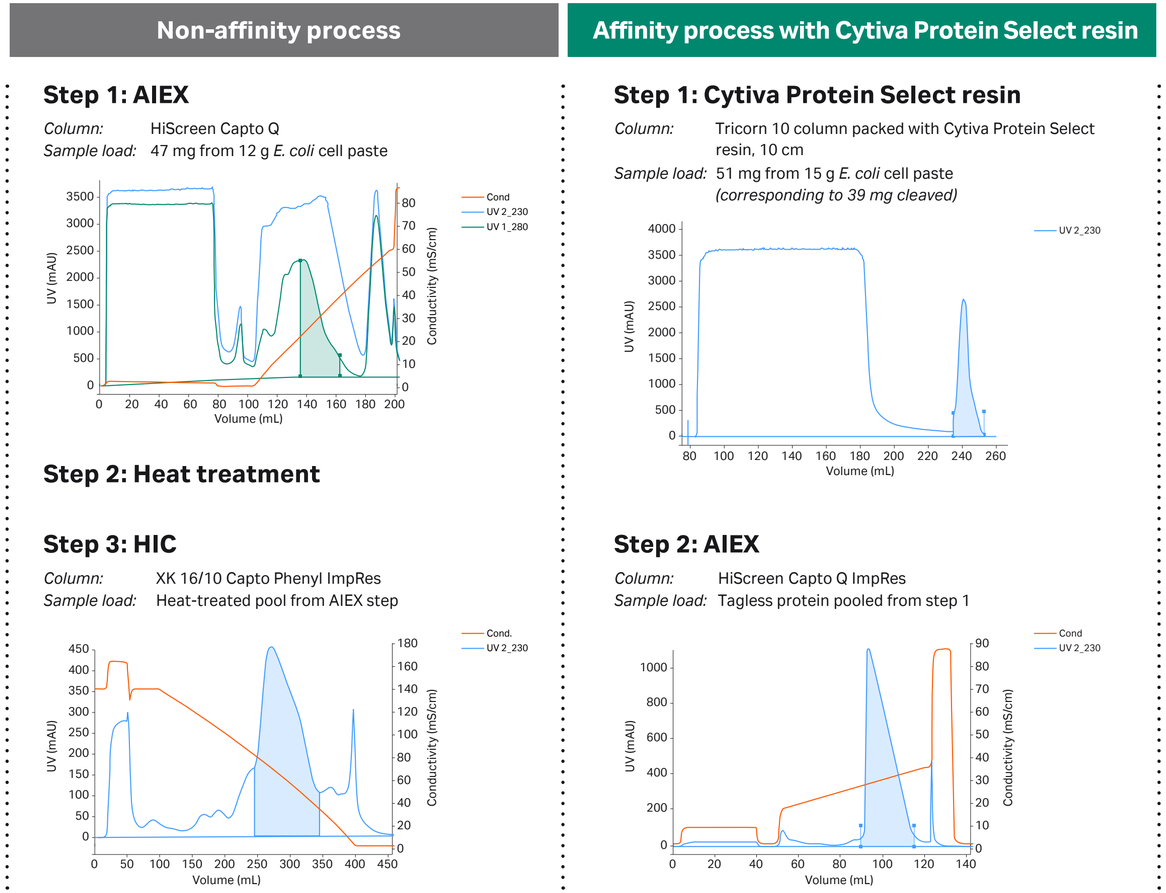
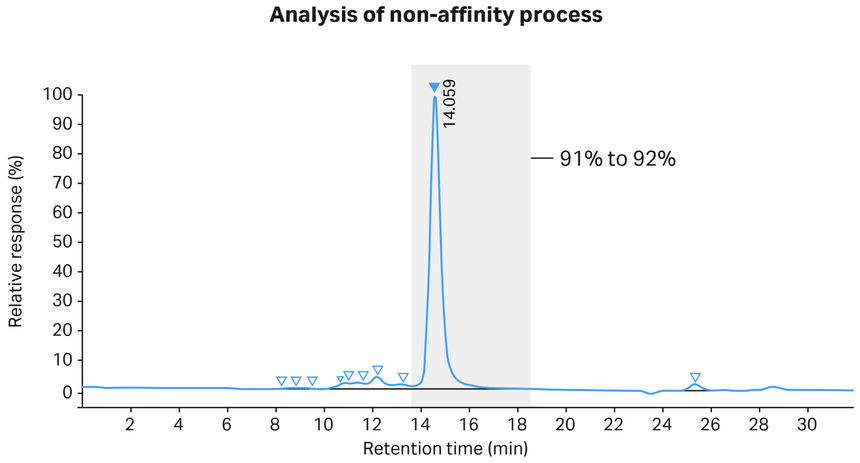


Fig 13. Chromatograms obtained from the different processes. The shaded areas correspond to the pooled fractions.

(A)



(B)

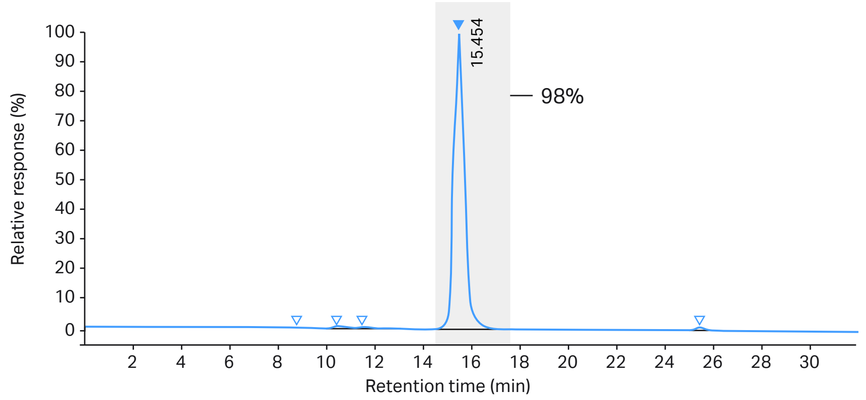


Fig 14. Analytical size exclusion at 214 nm on a Superdex™ 75 Increase 10/300 GL column. The non-affinity process (A) resulted in a protein purity of 91% to 92%, whereas the method using Cytiva Protein Select resin (B) resulted in protein purity of 98%.



Conclusion

Cytiva Protein Select technology standardizes purification of proteins that do not have an affinity binding partner. Our results (summarized in Table 1) show that this system enables:

- Predictable transfer to larger bed heights
- Faster method development than a conventional non-affinity method
- Higher-purity product in fewer steps
- Increased molecule throughput in the lab, as shorter process development time can help increase the number of candidate molecules going to preclinical studies.

Table 1. Recap of comparison of both methods

Scale	AIEX + heat treatment + HIC	Cytiva Protein Select resin + AIEX
Small scale (HiTrap format)	**Purity:** 95%	**Purity:** 98% to 100%
10 cm bed height columns	**Purity:** 91% to 92% **Recovery:** 57%, with most of the loss occurring in the first capture step	**Purity:** 98%, confirmed in analytical SEC, RPC, electrophoresis, and LC-MS analysis **Recovery:** 53%
Convenience	• Developing a HIC chromatography step required extensive screening and optimization. • The process required the addition of a non-chromatography step (heat treatment). • More stringent pooling criteria or an additional purification step are needed to reach > 95% purity.	• The affinity chromatography does not require optimization. • No need for buffer screening. Only 1 buffer (PBS pH 7.4) needed in affinity step. • 6 h of cleavage.
Development time	Developing the full small-scale process was significantly faster when we used Cytiva Protein Select resin in the first step.	

08

Try it now

Ready to try Cytiva Protein Select technology?

Contact us today to request a sample, a free consultation, or a quote

Explore related products

- Affinity capture resin: Cytiva Protein Select resin
- Ion exchange chromatography resins
- Hydrophobic interaction chromatography resins
- Mixed mode chromatography resins
- Bioprocess filters
- ÄKTA avant™ chromatography system



09

Frequently asked questions

FAQ

Scientists who are interested in learning more about Cytiva Protein Select technology have sent us questions covering a range of topics, from principles to protocols. Their questions and our answers are collected here.

Click on a topic to jump to the corresponding FAQ section. You can also use the search function to find questions or answers that include a given term.

- Additives
- Applications
- Binding capacity
- CIP/regeneration
- Cleavage mechanism
- Large-scale production and column packing
- Licensing
- Ligand
- Ligand leakage
- Multiple tags
- Pre-cleavage
- Protein constructs
- Purification conditions
- Purified proteins
- Resin
- Signal peptide
- Tag
- Upstream
- Viral clearance data

Visit the web version of this FAQ to see recently added questions or to browse related information.

Click to read the online FAQ



Additives

Back to FAQ topics

	Question	Answer
1	Are there any reagents that will prevent purification if they are present in the sample?	High concentrations of denaturing substances will prevent purification. Other than that, we've tested many common buffers, salts, and excipients, including EDTA, DTT, and divalent metal ions, and we haven't seen purification problems with them.
2	Is it possible to purify samples that contain detergents for membrane proteins?	**Yes.** n-Decyl-β-D-maltoside (DM) can be used in buffer and sample. Lauryldimethylamine oxide (LDAO) can also be used, although its use has coincided with some decrease in target protein yield. However, we have not yet purified a membrane protein ourselves. We used a CelLytic Express lysis kit for extraction of target proteins produced intracellularly in *E. coli*, and subsequent purification was successful. The kit does not say exactly what the detergents are but mentions a blend of detergent and enzymes.
3	One of the proteins I want to express with the system would need to be purified totally under denaturing conditions. Can the whole purification process (including binding, cleavage, etc.) be done in 4–8 M urea?	**No.** Purification cannot be run under denaturing conditions. However, a low concentration of urea (such as 1 M) can be present in the sample and buffer during purification.
4	Our protein has a tendency to oligomerize via S-S bridges. How does Cytiva Protein Select technology deal with this phenomenon? Is adding antioxidants to the buffer compatible with the technology?	For the excipients, you can use reducing agents; antioxidants and ascorbate would work.
5	We are working with insoluble protein and therefore using 8 M urea. Would this work with Cytiva Protein Select resin?	**No.** 8 M urea in the binding buffer will not work, because the tag needs to bind to the ligand on the resin and the complex needs to fold, which will be difficult to achieve in the presence of 8 M urea. Low concentrations of urea, such as 1 M, can be used.
6	Is the resin compatible with buffers containing amino acids, for example 50 mM to 1 M arginine (to prevent aggregation) or 10 mM histidine buffer?	**Probably.** We haven't tested this, but based on what we know, the resin should be compatible with amino acid buffers. A pH between 6 and 9 and ionic strength corresponding to 0–1 M NaCl works.
7	Can the eluate from an immobilized metal chelate affinity chromatography (IMAC) purification be directly applied to the Cytiva Protein Select column (i.e., without removing imidazole)?	**Yes.** You can apply samples containing imidazole to Cytiva Protein Select resin.
8	Is it possible to use the tag system in the presence of reducing agents?	**Yes,** you can use reducing agents (e.g., DTT) during purification.

Applications

Back to FAQ topics

	Question	Answer
1	What do you think of using Cytiva Protein Select in food applications such as enzyme purification?	Cytiva Protein Select resin can be used for enzyme purification in food applications. More information will be provided (together with the regulatory support file [RSF]) in the near future.

Binding capacity

	Question	Answer
1	What is the binding capacity of Cytiva Protein Select resin?	Since the tagged protein will cleave once bound to the resin, it is not possible to measure the traditional dynamic binding capacity (Q_{B10}) using UV signal. Yield will depend on protein design and cleavage time (hold time). We have measured yields (the amount of cleaved target protein in the eluate) up to 20 mg protein per mL resin.
2	How do I measure binding capacity or recovery?	You can add a detection tag or epitope tag and use that to analyze tagged protein in starting material, flowthrough, wash, and CIP fractions during operation. You can analyze eluted tagless protein by methods such as analytical size exclusion chromatography.

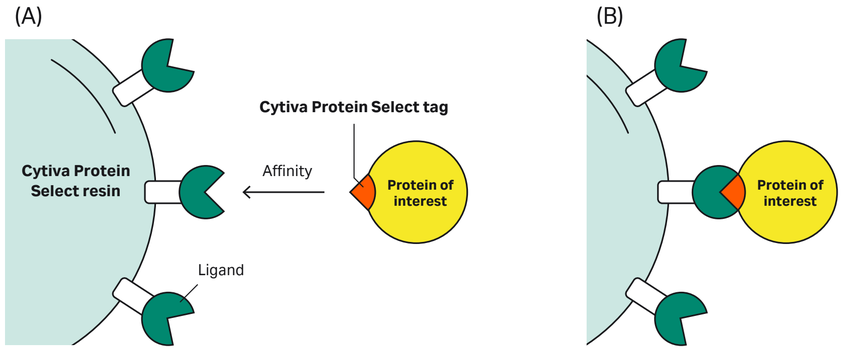
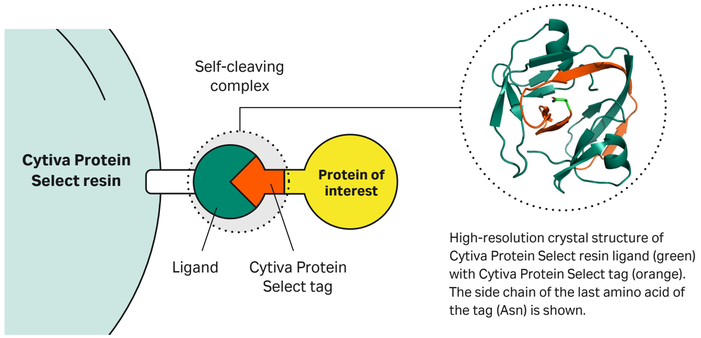
CIP/regeneration

Back to FAQ topics

	Question	Answer
1	How do I regenerate the resin?	To effectively regenerate/CIP the resin between purification cycles, we recommend using one of the following solutions: • 6M Urea with 50 mM NaOH • 4 M urea with 100 mM NaOH, or • 4 M guanidine hydrochloride with 100 mM NaOH. Alternatively, you may be able to use 30% isopropanol with 100 mM NaOH depending on sample composition.
2	Is it okay to regenerate the resin only with denaturants or NaOH?	**No.** Chaotropic agents (urea or guanidine-HCl) alone or NaOH alone will NOT effectively regenerate/CIP the resin. To effectively regenerate/CIP the resin between purification cycles, one needs a combination of chaotropic agents (urea or guanidine-HCl) and NaOH.
3	For the sanitization of the resin, it is indicated to use 4 M urea in 0.1 M NaOH. What is the stability of urea at this pH (~12)?	Urea solutions should always be freshly prepared and used, as solutions of urea may develop cyanate ions upon standing, especially at higher temperature. For small-scale purifications, we prepare aliquots of the regeneration solutions and store them at -20°C.
4	Can you strip the tag and reuse the resin?	**Yes.** Regeneration and CIP is possible with a combination of chaotropic agents (urea or guanidine-HCl) and NaOH.
5	How many times can the resin be reused?	We tested 20 purification cycles of *E. coli* lysates with a CIP/regeneration solution of 4 M urea and 100 mM NaOH, and measured yield (amount of eluted protein) for each cycle. After 20 cycles the resin retained 80% of its initial yield. We also tested a CIP/regeneration solution of 4 M guanidine HCl and 100 mM NaOH. The resin retained more than 65% of its initial yield after 50 purification cycles with *E. coli* lysates, and more than 86% of its initial yield after 20 purification cycles using a HEK293 feed. Different proteins were used in the CIP/regeneration experiments.

Cleavage mechanism

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	Question	Answer
1	What is the mechanism of self-cleavage?	**1. Binding of tag to resin** The unique affinity between Cytiva Protein Select tag and its corresponding ligand on Cytiva Protein Select resin causes strong binding between them.  **2. Formation of a self-cleaving complex** Once bound, a folding event takes place between the Cytiva Protein select tag and the ligand on the resin, after which a self-cleaving complex is formed.  High-resolution crystal structure of Cytiva Protein Select resin ligand (green) with Cytiva Protein Select tag (orange). The side chain of the last amino acid of the tag (Asn) is shown.

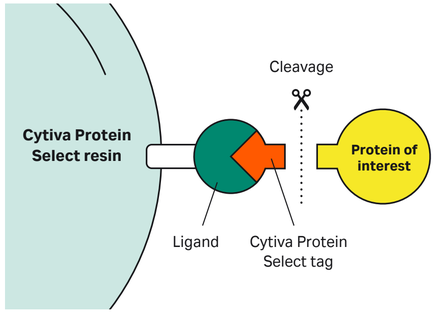
Cleavage mechanism *cont.*

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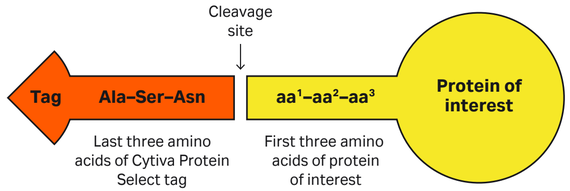
3. Tag cleavage

The cleavage reaction is spontaneous and does not require additional excipients or stimuli.

The last amino acid (Asn) of the Cytiva Protein Select tag becomes positively charged, which causes breaking of the peptide bond between the Asn and the first N-terminal amino acid of the target protein.

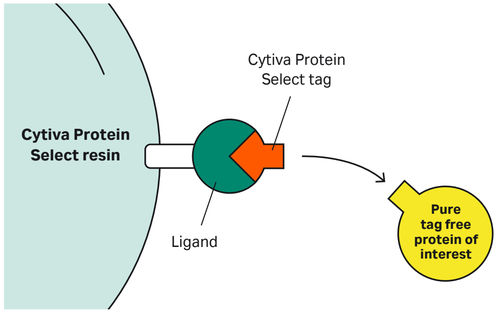


Cleavage site:



4. Tag-free protein elution

After self-cleavage, your pure, tag-free protein elutes with no residual tag amino acids.



Cleavage mechanism *cont.*

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	Question	Answer
2	How do the first three amino acids at the N-terminus affect the cleavage kinetics and cleavage time for proteins?	Hydrophobic and aromatic amino acids in the second position will increase the rate of cleavage. Small, nonpolar, or negatively charged amino acids in the first position will decrease the rate of cleavage. Proline amino acids located in first and/or second position halts the cleavage. The cleavage table shows the expected cleavage rates for different combinations of the first two N-terminal amino acids of the target protein. Combinations labeled with a plus sign (+) have a faster cleavage rate than combinations labeled with a minus sign (-). The amino acid at the third position also affects cleavage rate, although to a lesser extent. It is the combined properties of the first three amino acids that is defining the cleavage kinetics. For proteins whose first 3 amino acids are expected to have a slow cleavage rate, one might consider mutating some of these amino acids or inserting extra amino acids at the N-terminus to increase the cleavage rate. Inserting Ala-Phe-Val or Phe-Arg-Val at the N-terminus has shown fast cleavage for the proteins we have tested. The duration of the hold step is protein dependent. Four hours is recommended for the first trial. Overnight incubations may be required for slow-cleaving targets, especially if cleavage is performed at lower temperatures.
3	What initiates the cleavage?	The cleavage occurs after the tagged protein binds to the resin. The reaction is spontaneous and does not require additional excipients or stimuli.

First amino acid of target protein

Second amino acid	Charged		Small		Hydrophobic	
	Glu	Arg	Ser	Ala	Leu	Phe
Glu	-	-	-	-	-	+
Arg	-	+	+	-	+	+
Ser	-	+	+	-	+	+
Ala	-	+	+	-	+	+
Leu	-	+	+	+	+	+
Phe	+	+	+	+	+	+

Cleavage mechanism *cont.*

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	Question	Answer
4	Does the self-cleavage require reducing conditions or any special condition?	**No,** the cleavage reaction is spontaneous and does not require additional excipients or stimuli.
5	Does cleavage occur only after the sample loading and wash step?	**No,** The cleavage will start as soon as the tagged protein is bound to the resin. The instructions recommend a maximum of 30 min sample loading and wash time, but one should do sample loading and wash as fast as possible to minimize loss of target protein. If you have a slow-cleaving protein, you might still have reasonable recovery with sample loading and wash lasting longer than 30 min.
6	What is cleaving the tag? Do we need to use special reagents or buffer while the tagged protein is bound on the resin?	**No,** you do not need special buffers or reagents for the purification process. The tag is self-cleaving, and the cleavage reaction is spontaneous and does not require additional excipients or stimuli. You can use one buffer for the whole purification process. Suggested buffers: • MES (pH 6.0 to 6.5) • Phosphate (pH 6.5 to 7.5) • Tris (pH 7.5 to 9.0)
7	Why doesn't the Cytiva Protein Select tag work on the C-terminus?	The tag binds to the ligand on the resin, then the tag-ligand complex folds and forms a self-cleaving complex. Cleavage occurs between the last amino acid of the tag and the first N-terminal amino acid of the target protein. This process cannot happen if the tag is at the C-terminus of the target protein.
8	Is there protease linked to the column?	**No,** there is no protease involved in the system.
9	How do post-translational modifications (PTMs) affect the cleavage yield?	PTMs can potentially affect the cleavage rate. In our experiments with purification of the glycosylated receptor-binding domain (RBD) of the SARS-CoV-2 spike protein, the protein cleaved as expected. See data file.

Large-scale production and column packing

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	Question	Answer
1	Can the resin be used in GMP production?	In the future, support and products for scaling-up to clinical and commercial scale will be available including regulatory support files and ligand leakage kit. Larger resin containers (1 L, 5 L, and 10 L) will eventually be available for packing in larger columns, such as HiScale™ or AxiChrom™ chromatography columns.
2	How do I scale up using a flow rate of 600 cm/h and sample load and wash time of 30 min? If your sample isn't highly concentrated, it will take much longer than 30 min to load and wash the column. What column design would work best in this case?	We recommend using wide columns with a bed height of 10 cm. The Instructions for Use recommends a flow rate of 600 cm/h during sample load and wash for up to 30 min. This is a strong recommendation for the first trial; however, for slow-cleaving target proteins, the flow rate during sample loading and washing can be decreased (e.g., to 400–500 cm/h), which will extend the time for sample load and washing. In such cases, increasing bed height is an option.

Licensing

	Question	Answer
1	Will there be a licensing fee for using the product?	**No,** there will not be any license or royalty fee for using this product.

Ligand

Back to FAQ topics

	Question	Answer
1	What is the nature of the ligand? Is it a small molecule, peptide, something else?	Cytiva Protein Select technology uses a protein ligand produced in *E. coli*.

Ligand leakage

	Question	Answer
1	What is the ligand leaching detection method for Cytiva Protein Select resin?	More information on ligand leaching (including method for testing) will be provided in the future.
2	Do you have a toxicity study on the ligand and the tag?	**No,** but we have done a toxicity risk assessment. The results so far suggest that the ligand and the tag are unlikely to have toxic effects.
3	Will there be an ELISA kit for the ligand?	**Yes,** more information on ligand leakage, ligand toxicity, etc., will be provided in the future.

Multiple tags

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	Question	Answer
1	Will a higher-molecular weight solubility tag (GST or MBP) in front of the Cytiva Protein Select tag influence the interaction between the Protein Select tag and the ligand on the resin? That is, is it necessary to remove the added tag before binding to the ligand?	Although we have not tested this, it should be OK to have an MBP or GST tag upstream of the Cytiva Protein Select tag. It should still bind and cleave (a potential issue with GST is however its propensity to dimerize, which would affect the cleavage). You might also consider adding a linker (several amino acids) between the two tags to reduce the risk of steric hindrance.
2	Regarding adding a signal peptide or dual tag upstream of the Cytiva Protein Select tag, is there a limit to the size? Could you add a sequence of 90–100 aa, for example? And how would that affect binding or cleavage efficiency?	We are not aware of a size limitation of a dual tag or a signal peptide upstream of the Cytiva Protein Select tag. Things to consider are potential interactions of a dual tag and a suitable linker between the tags to allow a certain degree of movement for the different parts of the fusion protein. Any effects of an additional tag on binding and cleavage could be tested against a single-tagged control protein. We have seen minor effects on cleavage rate (slightly slower), with an additional 40 aa affinity tag attached via a (GGSG)-linker.
3	Can a small tag, such as a His- or FLAG tag, be cloned between the Cytiva Protein Select tag and the target protein?	We have tested proteins with His-, Strep-tag® II, and FLAG tags upstream of the Cytiva Protein Select tag. They bind to the resin and cleave as expected. If the additional tag is placed between the target protein and the Cytiva Protein Select tag, the system will try to cut between the Cytiva Protein Select tag and the additional tag. The target protein will still have the additional tag in this case. In contrast, if the additional tag is placed upstream of the Cytiva Protein Select tag, this would still generate tagless target protein (i.e., the system tag is always cut at the C-terminus).
4	Do you think the Cytiva Protein Select tag could help solubilize the protein?	The tag is rather small (about 4 kDa), so it will not help solubilization. You can place a solubilization tag upstream of the Cytiva Protein Select tag. You might also consider adding some linker amino acids between the tags.
5	Will adding a FLAG or histidine tag upstream of the Cytiva Protein Select tag prevent or affect capture by the ligand?	**No.** It is OK to have a FLAG or histidine tag upstream of the Cytiva Protein Select tag. We have tested proteins with other tags upstream of the Cytiva Protein Select tag and they worked well.

Pre-cleavage

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	Question	Answer
1	Does the cleavage start within the expression system?	**No,** it does not. Cleavage starts only after your target protein is bound to the resin (after which a self-cleaving complex is formed). Cleavage will not occur during expression or sample preparation.

Protein constructs

	Question	Answer
1	Are there any sequences that can serve as positive or negative controls?	**Yes.** We have been using human IL-1ß for positive and negative controls. The mature sequence contains a proline in the second position, which binds but cleaves poorly, so it can be used as negative control. The positive control has a proline-to-phenylalanine point mutation, which cleaves relatively fast.
2	If the tag sequence is being used in a mAb, does it matter if it is on the light chain or heavy chain?	This must be empirically tested. Note also that the kinetics of the cleavage reaction are likely to be different from that of monomeric proteins and will likely need some optimization.
3	What is the impact of free cysteine?	Addition of cysteines to the media or the protein of interest should not impact binding and cleavage activity.
4	You observed that hydrophobic/aromatic amino acids in the second position may increase the rate of cleavage. Is this increase enough to cause a problem — for example, loss of a lot of target protein in the wash due to the speed of the reaction?	It could happen, especially during a lengthy sample application. An optimization of the purification method would be needed to increase the yield. For example: screening for conditions (pH, salt, temperature) that minimize cleavage rate and/or a concentration of the sample to reduce the overall loading/wash time.

Protein constructs *cont.*

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	Question	Answer
5	Do you have data on how this system works with multimeric proteins?	We don't have much data on multimeric proteins. The effects are probably dependent on the nature of the protein — e.g., whether it is a homo- or hetero- multimer or whether all monomers are tagged. If the protein is a heterodimer and only one monomer is tagged, for example, the system should work. If you're purifying a homo-multimer and all monomers are tagged, it's possible you'll have heterogeneous proteins after purification. For example, if the protein is a homodimer and both monomers are tagged, there are several scenarios that can happen during the process: 1. One of the tags could bind to the resin ligand and be cleaved off during the hold step. The protein in question would then diffuse and bind with the second tag to the resin ligand, which would also be cleaved off at some point. You would, in this case, get a homodimer with both tags on the monomers cleaved off. 2. One of the tags could bind to the resin ligand and be cleaved off during the hold step. The protein could then come off the column during elution, because no ligand would be available to bind the second tag. In this case, you would get a heterodimer: one monomer with tag and one monomer without tag. 3. Both tags on each monomer could bind to the resin during sample application and be cleaved off during the hold step. In this case, you would get a homodimer with both tags on the monomers cleaved off.
6	You mentioned the importance of amino acids in positions 1 and 2. What about the effect of the amino acid at position 3 at the N-terminus of the target protein?	Position 2 has the largest impact on cleavage rate, followed by position 1. Position 3 also has an impact, but to a lesser extent — i.e., position 3 is not equally important to consider. That said, it is the combined properties of the three amino acids (positions 1–3) that define the cleavage kinetics.
7	Will the performance of the Cytiva Protein Select resin be hindered with a 60 kDa protein of interest?	**No,** a protein of 60 kDa will not hinder the performance of the resin.
8	If the first 3 amino acids of target protein contain sequences that might halt or slow the cleavage process and we don't want to mutate the target protein, can we add a linker sequence (that includes all the recommended amino acids for efficient cleavage) between the C-terminus of the tag and the N-terminus of the protein?	**Yes,** one can insert amino acids between the C-terminus of the Cytiva Protein Select tag and N-terminal of protein. Cleavage will be after the last amino acid of the tag (Asn). Inserting Ala-Phe-Val or Phe-Arg-Val has shown fast cleavage for the proteins we have tested.

Protein constructs *cont.*

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	Question	Answer
9	The Cytiva Protein Select tag ends with Ala-Ser-Asn. What happens if we have the same sequence at or close to the N-terminus of our protein? Would we see unexpected cleavage?	Having an Ala-Ser-Asn sequence in your protein will not affect the cleavage position. After the tag binds to the ligand on the resin, the tag-ligand complex folds and forms a self-cleaving complex, wherein cleavage occurs after Asn of the Ala-Ser-Asn in the tag.
10	Is there any limitation regarding the use of further tags upstream of the Cytiva Protein Select tag? For example, how does size play a role (e.g., MBP tag)? Any recommendation here? Which tags have worked best so far?	We have mainly tested epitope tags like polyhistidine, FLAG-tags, and Twin-Strep-tag®. The main effects we have seen from using additional tags upstream of the Cytiva Protein Select tag are slightly slower cleavage kinetics, larger mass-shifts on SDS-PAGE (makes it easier to see the cleavage), and some protection from proteolytic cleavage for some test proteins.
11	If we have some small and nonpolar or negatively charged amino acids in the first, second, and third position of the protein, will the cleavage be total after 24 h?	**No,** some uncleaved protein will likely remain bound to the resin. If you want to improve the cleavage kinetics, you could also consider adding two additional amino acids (such as Ser-Leu). We have also tested for predicted protein domains and shifted the start of the domain (e.g., +1 or -1 from the intended start). In this way the sequence will still be "native".
12	Does the size of the target protein affect the binding or cleavage efficiency?	The first three amino acids have a larger impact than the size of the protein. Very large proteins could potentially have sterical hindrance during binding to the resin.
13	Does the system work with PEGylated proteins?	It should work with PEGylated proteins, but it's not something we have explored.
14	What are the protein sizes tested so far?	We have tested proteins between 15 and 80 kDa.
15	Your data file says the first 3 amino acids of the protein are critical for self-cleavage efficiency. Do you have examples of the most optimal amino acid sequences?	In our tests, proteins that have Ala-Phe-Val or Phe-Arg-Val as the first 3 amino acids showed fast cleavage rate.

Purification conditions

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	Question	Answer
1	The data file says that 1 M is the highest NaCl concentration recommended for the buffer; what is the lowest NaCl concentration recommended?	Salt is not required for binding or cleavage, but a good starting point could be to add 150–300 mM NaCl to minimize unspecific interactions and increase purity.
2	Do you have experience using this affinity tag and resin with proteins at low expression levels — for example, 100 mg/L and a 200 L scale? We're wondering about loading time and the risk of losing protein of interest at flow-through.	Cytiva Protein Select needs a shorter sample load and washing phase because of the self-cleaving activity, so it's best to avoid low-expressed proteins unless you can add a concentration step before binding. For example, you could concentrate mammalian cell feeds by ultra-filtration to increase sample load. Concentration can also be done using other techniques than ultra-filtration, including ion-exchange chromatography.
3	You recommend 4 h or overnight for the hold step. What would happen if we were to leave it 1 or 2 days?	Most of the cleavage reaction occurs at the beginning of the hold step, so a hold time of 1 or 2 days can increase cleavage, but the size of the effect depends on the protein. The outcome would also depend on whether the target protein is stable during that time.
4	At 50°C or 60°C, is it possible to have efficient self-cleavage?	We don't have data on cleavage at 50°C or 60°C. Theoretically, cleaving is faster at these temperatures, but one has to consider the stability of the column hardware, resin, and the target protein.
5	Do you have any recommendations for batch purification?	Because tag cleavage occurs after the tagged protein is bound to the resin, we recommend fast sample application and wash. Batch purification is slower than running over a packed column and can potentially cause more loss of target protein. That is why we recommend purification using a packed column.
6	Is the sample loading and wash time limited to a maximum of 30 min?	The 30 min sample loading and wash time is a guideline to minimize risk of losses. However, the maximum loading and wash time depends on the cleavage kinetics of your target molecule. The impact of loading time is lower for a slow-cleaving protein.
7	What is the yield in percent?	The percent yield depends on several factors, including the first three amino acids of the target protein (which affect cleavage rate), the sample loading and washing time, and the hold time.

Purification conditions *cont.*

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	Question	Answer
8	What sample preparation method should I use for a target protein expressed in *E. coli*?	Suspend expression *E. coli* pellet/cells in binding buffer (1 g cells per 5–10 mL buffer) prior to lysis (e.g., homogenization or sonication). Clarify the sample by centrifugation (we recommend starting with 25000 × g for 20 min). Immediately before applying it to the column, centrifuge and/or filter the sample through a 0.22 µm or a 0.45 µm filter.
9	The buffer recommended for Cytiva Protein Select resin is MES, Tris or PBS with pH 6–9, but how about adding low-concentration salt to the buffer to reduce nonspecific reactions, especially during sample application?	It is always good to have some salt to reduce nonspecific reactions. We recommend 20–50 mM buffer compound supplemented with up to 1 M salt (preferably NaCl), at a pH range of 6 to 9, which means one can use 0 to 1 M salt.
10	How efficiently does the tag bind to the ligand during sample application? At the maximum recommended sample application of ~600 cm/h, is it possible that some of the sample might end up in the flowthrough?	The affinity between the tag and the ligand is very high, and we see fast binding of tagged protein to the resin. A small amount of cleaved protein will be in the flowthrough and wash (this is also dependent on the first three amino acids of the protein, which affect the speed of cleavage).
11	If I purify protein from cell culture supernatant with a large sample volume and sample loading takes a long time, is there a risk that parts of the protein will be cleaved and elute before loading of the column is complete?	We recommended that sample application including wash step are completed within 30 minutes. This is because if the target protein is fast cleaving, some loss of cleaved protein will occur during sample application. Consider sample concentration before purification.
12	Can we perform the purification at 4°C?	**Yes,** you can run the purification at 4°C (such as in a cold room) but cleavage will be slower, so you may need to increase the time for the hold step.
13	Why do the His-tagged and Cytiva Protein Select tagged proteins look like they're different sizes after purification (on the SDS-PAGE gel shown in the data file)?	In the data file we showed a comparison of Cytiva Protein Select tag vs His-tag with TEV protease. The purified protein from the Cytiva Protein Select tag does not contain extra amino acids, while the one from His-tagged protein contains residual amino acids after TEV cleavage. We confirmed both molecular weights by LC-MS. See data file.
14	How do you prevent tag cleavage during the binding and wash steps?	The cleavage will start once the tagged protein is bound to the resin. This is why we recommend the sample loading and wash are done as fast as possible.
15	What is the composition of the elution buffer? Is it necessary to exchange elution buffer for dialysis or size exclusion chromatography, for example?	The protein will elute in the buffer that was used for wash after sample loading. There is not a dedicated elution buffer.

Purification conditions *cont.*

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	Question	Answer
16	Can wash buffers be applied to remove unwanted impurities?	**Yes,** after sample loading you can use different buffer(s) for washing out unwanted impurities.
17	Can we use protease inhibitors in buffers?	**Yes.** Pefabloc and EDTA worked well in our purification.
18	What is the pH dependency of the cleavage reaction?	The cleavage is rather insensitive to pH at range 6–9. For some proteins, switching from, say, MES pH 6.2 buffer for sample loading to Tris pH 8.5 for washing buffer might increase the cleavage rate, but this is of course protein dependent.
19	Can you comment about nonspecific binding on the resin?	We have not seen any particular issue with nonspecific binding, but it is always a good idea to include some salt in the buffer to reduce nonspecific binding.
20	Are the concentrations listed in the "Resin stability and compatibility" section of the Instructions for Use* for operation or for storage? And are they upper limits, or could a higher concentration of these buffers be used?	The "Resin stability and compatibility" section of the Instructions for Use* lists the buffers and additives that Cytiva Protein Select resin can be stored in for 1 week. Some of these are not suitable for operation. During purification we suggest these buffers: • MES (pH 6.0 to 6.5) • Phosphate (pH 6.5 to 7.5) • Tris (pH 7.5 to 9.0) It is recommended to use 20 to 50 mM of buffer compound supplemented with up to 1 M salt (preferably NaCl), at a pH range of 6 to 9.
21	Does cleaving rate impact the protein purity?	**No,** cleaving rate does not impact purity of eluted protein.
22	How do you prove traceless cleavage?	The eluted protein has no traces of tag amino acids, confirmed by mass spectrometry analysis.

* Cytiva Protein Select resin: Affinity Chromatography Resin Instructions for Use. Cytiva, 29711002 AA: V19; 2023:6

Purified proteins

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	Question	Answer
1	In the data file you showed mass spec data on the scaffold protein. Have you characterized the analysis artifact peak?	**Yes,** it is an acetonitrile adduct (+42) and likely a neutral loss of $H_2$0 (-18).
2	Does the protein stay active once purified with Cytiva Protein Select technology?	**Yes.** Protein activity is retained after purification with Cytiva Protein Select technology. Data published by researchers at Cytiva and AstraZeneca† showed that SARS-CoV-2 spike protein RBD has a high degree of binding to RBD monoclonal antibodies (measured by biolayer light interferometry) when purified by immobilized metal affinity chromatography (IMAC) or Cytiva Protein Select resin. They also showed results on an artificial bispecific T-cell engager (BiTE) designed to link T cells with cells expressing epithelial cell adhesion molecule. When purified with IMAC or Cytiva Protein Select resin, the BiTE showed similar CD4+ and CD8+ T-cell activation profiles, as well as cytotoxic activities (characterized using a viability assay monitoring real-time changes in cellular impedance)†.

† Clifford R, Lindman S, Zhu J, *et al.* Production of native recombinant proteins using a novel split intein affinity technology. *J Chromatogr A.* 2024;1724:464908. doi:10.1016/j.chroma.2024.464908

Resin

	Question	Answer
1	What is the base matrix of the resin and why was this base matrix chosen?	The resin is based on an established high-flow agarose matrix with a particle size of ~ 60 µm. This is one of Cytiva's modern affinity purification resins. We chose it based on its resolution and scale up capabilities.
2	What are the formats available for Cytiva Protein Select resin?	Cytiva Protein Select resin is available in 25 mL, 100 mL, and 500 mL packs of bulk resin and in HiTrap 1 mL and 5 mL columns (HiTrap Protein Select columns), which are well suited for research and process development work. Instructions are supplied for packing Cytiva Protein Select resin in Tricorn columns. You can see all formats on the Cytiva Protein Select shop page. In the future, support and products for scaling up to clinical and commercial scale will be available, including regulatory support files (RSFs) and ligand leakage kits. Larger resin containers (1 L, 5 L, and 10 L) will eventually be available for packing in larger columns, such as HiScale or AxiChrom chromatography columns.

Signal peptide

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	Question	Answer
1	Can any signal peptide be used in combination with the Cytiva Protein Select tag? Other than removing the initial methionine of the tag, are there signal peptide constraints or limitations?	In principle, any signal peptide can be used with the Cytiva Protein Select tag. We have tested just a few. Removal of the tag's initial methionine is not required.

Tag

	Question	Answer
1	How big is the Cytiva Protein Select tag?	The Cytiva Protein Select tag is roughly 4 kDa in size. It is made of 36 amino acids. For more information about this tag, visit this page, and fill in the form. The sequence of the tag will be sent by email to you immediately.
2	What is the affinity of the Cytiva Protein Select tag to the resin?	The affinity between the Cytiva Protein Select tag to the ligand on the resin is very high. Measurements of surface plasmon resonance (SPR) signals on a Biacore instrument show very high affinity and an extremely low dissociation rate.
3	How can I obtain the sequence of Cytiva Protein Select tag?	Visit this page and fill in the form. The sequence of the tag will be sent by email to you immediately.

Upstream

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	Question	Answer
1	Have there been instances of the tag cleaving in the cell culture broth (via protease or cofactor)?	**Yes,** it can happen. In some cases, reducing temperature for storage or reducing the expression temperature helps.
2	If the tag is self-cleavable, what is the stability of the tag inside prokaryotic and eukaryotic cells?	The self-cleavage can only occur after binding to the resin. Of course there is the risk that the tag itself can be cleaved by other proteases in the sample, e.g., if the sample is stored for an extended time at room temperature.
3	Can you recommend cell culture conditions?	**No,** as this will be very dependent on the protein of interest and the expression system.
4	How do users determine titer of their cell culture when using this technology?	That depends on the protein of interest (POI). We suggest that the titer determination is done on the POI or on additional tags, if used.
5	What vector type do you suggest for the plasmid?	Any vector should be fine — or at least, we have yet not found any compatibility challenges to date.
6	Are yeast or insect cell expression systems suitable for the Cytiva Protein Select system?	We have extensive experience using *E. coli* and mammalian cell expression systems (CHO and HEK) for this technology, but not yeast or insect cells. We think it is likely that yeast and insect cell expression would be successful, but we haven't tested it.
7	Could you share details such as titer and yield about the upstream process? Is there a difference in performance between His tag and Cytiva Protein Select tag in protein expression?	The answer depends on the expression system and protein. We have tested the expression levels of native proteins and His-, FLAG-, and Strep-tagged proteins in comparison to the Cytiva Protein Select tag. In those studies, we did not see an impact on expression levels by adding Cytiva Protein Select tag.
8	Does the size of the Cytiva Protein Select tag relative to that of the target protein affect expression or activity?	In the proteins we've tested so far, we haven't seen any major effect of the tag on target protein expression levels in bacterial or mammalian systems, nor on the functionality after purification and tag removal.
9	Which signal peptides did you use successfully?	We have successfully used Ig kappa, CD36, and IL-2 signal peptide sequences.
10	Are antibodies to the Cytiva Protein Select tag available?	No antibodies to the Cytiva Protein Select tag are available now. They may be available in the future. In the meantime it is possible to add, e.g., a FLAG or His-tag upstream of the Cytiva Protein Select tag for detection.

Upstream *cont.*

Back to FAQ topics

	Question	Answer
11	Can we insert the tag into any expression plasmid?	**Yes,** you can add the tag sequence to your protein of interest and in your vector of choice. This can be done in many different ways, such as tag and gene synthesis, cloning of genes or inserts by PCR or restriction digestion, or Gibson assembly. You can also buy synthetic genes from external vendors.
12	Can you produce tagged protein in cell culture medium containing serum?	**Yes.** We have tried a few different media formulations, and we prefer serum free because it's less complex. But the system works with cell media that contain serum as well.
13	Would this work in cell-free systems?	We haven't evaluated that, but in theory it should work in a cell-free system.
14	Do you provide the DNA sequence for the Cytiva Protein Select tag?	The optimal DNA sequence will depend on the expression system and should be optimized together with the protein of interest. There are several codon optimization tools available to improve the rate of translation by overcoming limitations associated with host cell codon usage and the abundance of transfer RNA (tRNA). Although we have not seen an impact on the tag's performance based on DNA sequence, codon optimization has the potential to cause changes in the target protein's conformation and functionality. Many external vendors can produce plasmids optimized for yield based on amino acid sequence and expression system.

Viral clearance data

	Question	Answer
1	Do you have viral clearance data?	We will provide more information in the RSF during the next launch of large-scale formats of Cytiva Protein Select resin.

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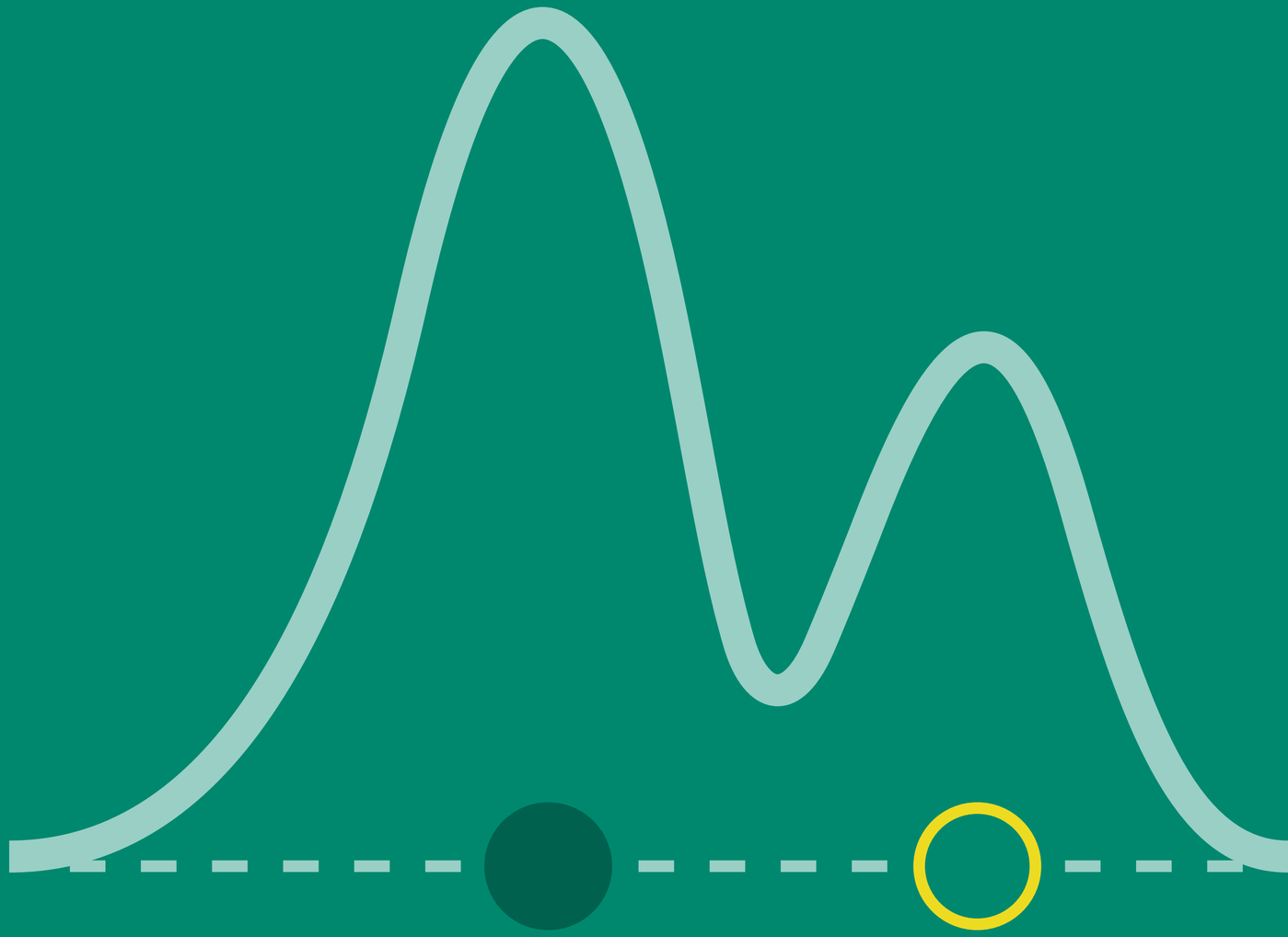
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