*i*CapTag™

Protein Purification Using *i*CapTag™

EXAMPLES OF PROTOCOLS & RESULTS

IMAGINE DESIGN INNOVATE DISCOVER

*i*CapTag™

Welcome to Your Discovery

iCapTag™

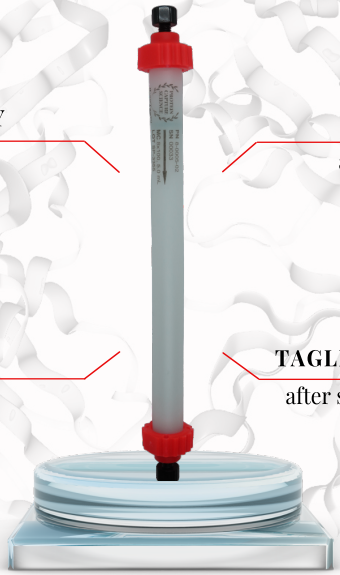
PROTEIN CAPTURE SCIENCE

HIGH PURITY

95-99%

EASY TO USE

and reusable resin



SINGLE STEP

purification

RESULTS IN TAGLESS PROTEIN

after small pH change

PROTEIN CAPTURE SCIENCE

www.proteincapturescience.com

Follow the Future

SINGLE PLAFORM FROM R&D TO MANUFACTURING

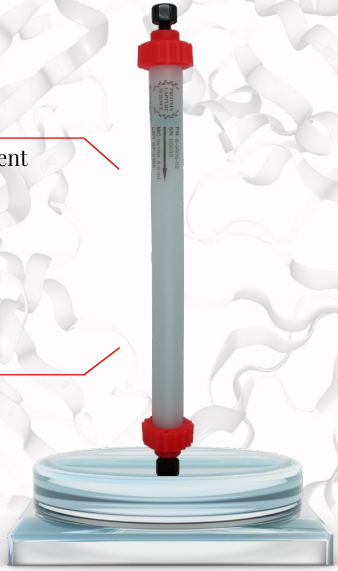
iCapTag™

SAFE

for the enviorment

RECYCABLE

materials



PROTEIN CAPTURE SCIENCE

www.proteincapturescience.com

Protect the Environment

SINGLE PLAFORM FROM R&D TO MANUFACTURING

*i*CapTag™

Welcome to Your Discovery

Protein Purification Using *i*CapTag™

EXAMPLES OF PROTOCOLS & RESULTS

Contents

1. Background

1.1 The *i*CapTag™ Technology

The purification of recombinant proteins at laboratory and manufacturing scales represents a substantial difficulty in the development of new therapeutic proteins. At laboratory scales, researchers rely on affinity tags to rapidly purify diverse new targets. These approaches cannot be used for clinical applications however, due to the potential immunogenicity of the tags and the difficulty of removing the tags in a manufacturing setting. The result is a divided world of protein purification, in which tag-based methods dominate in the laboratory, but complex non-tag methods must later be developed for manufacturing. The disconnect between these methods can significantly delay drug development and approval, and in some cases can stop promising therapeutics from ever being commercialized. The result is untreated diseases, high drug costs, and slow market entry for new lifesaving medicines.

To address these issues, we developed the *i*CapTag™ (intein Capture Tag), a self-removing tag-based technology (**Figure 1**). It is a single platform that is designed to bridge all phases of biopharmaceutical development from early R&D through manufacturing. It is a promising, patented, and disruptive affinity tag technology that is based on a type of protein known as an 'intein'. The disruptive advantage of the intein is its ability to provide a self-cleaving tag for simple protein purification, where the tag is removed during the purification process. This unique combination of features provides strong advantages for applications in pure research and drug discovery, while also providing a platform for use in biopharmaceutical manufacturing. Moreover, this product can be used with standard equipment and buffers that are commonly observed in both research and manufacturing.

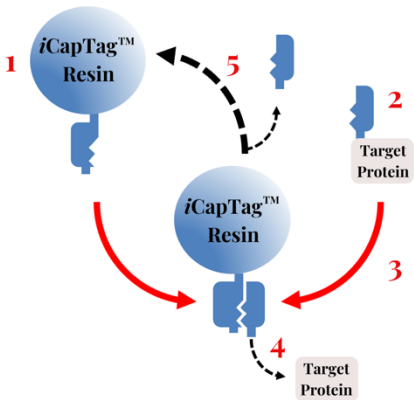


Figure 1: Diagram of the *i*CapTag™ system steps**. [1]** Prepare and equilibrate a column containing *i*CapTag™ resin. **[2]** Prepare a protein that includes the modified Npu^{C} domain in expression system of choice. **[3]** Run clarified feed through the *i*CapTag™ column. **[4]** Adjust pH in a column to initiate cleaving and elute. **[5]** Regenerate column for reuse or storage.

1.2 Inteins and Split Inteins

An Intein (Intervening protein) is a naturally produced protein sequence located within a larger host protein. Inteins naturally splice their flanking peptide bonds but can be modified to cleave only one end. Some inteins are naturally split into two proteins, which are non-functional when separate but can self-assemble to form a functional whole (**Figure 2**). These complete inteins then self-cleave in a predictable way, where the C-terminal cleaving reaction is conveniently induced by a small shift in buffer pH.

The *i*CapTag™ system uses a split intein that has been engineered to exhibit rapid cleaving, where the cleaving rate is highly sensitive to pH. The C-terminal segment of the intein acts as the affinity tag and is fused to the N-terminus of the target protein. The N-terminal intein segment acts as the affinity ligand and is covalently bound to the *i*CapTag™ resin. Strong and highly selective affinity between the intein segments allows simple purification of the target protein, and once purified, the buffer pH is adjusted to trigger self-cleavage of the tag.

The intein cleavage rate can also be controlled by temperature, where lower temperatures (*e.g.*, 4°C) decrease the rate of cleavage reaction, while higher temperatures (*e.g.*, 37°C) increase this rate. We recommend running the cleavage reaction at room temperature (20°C to 22°C) or higher.

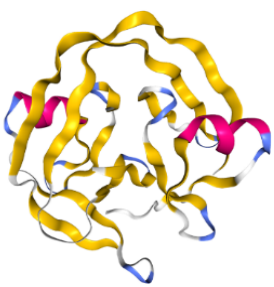


Figure 2: Image of self-assembled Nostoc punctiforme (Npu) intein, a main component of patented *i*CapTag™ technology, which can self-assemble from N- and C-terminal segments (referred to here as Npu^N and Npu^C) to form the self-cleaving Npu^{NC} complex (PDB ID 4QFQ).

1.3 Summary of *i*CapTag™ Advantages

Flexibility of Expression Systems

The *i*CapTag™ is compatible with commonly used expression systems, including mammalian and microbial hosts as well as cell-free *in vitro* translation systems.

Internal data has shown that the *i*CapTag™ does not substantially decrease the secretion of expressed target protein from mammalian hosts (*e.g.*, CHO and HEK293 derivatives), but the secretion signal sequence may need to be optimized for maximum expression. In general, this technology is ideal for rapid and simple purification of proteins that express and fold well.

Cleavage Rate Controllability & Predictability

The cleavage rate (k) of the target protein is controlled by a shift in buffer pH (typically pH 8.5 to pH 6.2), where a variety of buffers have been shown to be compatible with the reaction. Our recommended buffer is a mixture of AMPD and PIPES that has been titrated to the target pH values of 8.5 and 6.2. Other buffers can be used however, and we have demonstrated success with a wide range of buffers including, tris, bis-tris, bis-tris propane, 2-(N-morpholino) ethane sulfonic acid, and phosphate buffers.

A key breakthrough of this technology was the characterization of how the amino acids preceding the intein (-1/-2/-3) effect pH sensitivity and overall cleavage rate (**Figure 3**), allowing us to optimize the behavior of the intein for applications in research and manufacturing. Similarly, we have also characterized how the first two amino acids of the target protein (+1/+2) effect the cleaving rate, allowing a predictive model of cleavage rates for new proteins based on their initial amino acids. These observations allow simple optimization of cleaving via rational modification of the first or second amino acid of the target.

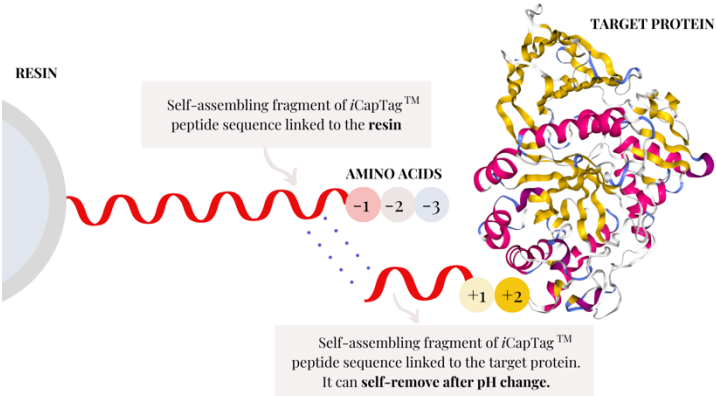


Figure 3: Critical amino acids that effect intein cleaving.

Neutrality to Other Buffer Additives

While pH has a strong influence on cleavage rate, we have found that the *i*CapTag™ is generally neutral to salts, sugars, and other buffer additives, as long as they do not unfold the intein complex during the binding, washing or cleaving steps of the protocol. Thus, salts, weak detergents and other stabilizing excipients can be safely added or adjusted to maximize purity, stability, and yield of the purified target. Notably, this includes common protease inhibitors used to decrease degradation of targets during purification, allowing this intein to be used in processes where protease inhibitors would otherwise make tag removal difficult or impossible.

Resin Regeneration and Sanitization

The *i*CapTag™ technology can be regenerated by a stripping buffer containing 150 mM phosphoric acid (pH ≤ 2.0). Additionally, the *i*CapTag™ can be sanitized with standard caustic buffer protocols without a loss of resin activity.

1.4 The *i*CapTag™ Protein Purification

Overview

The basic steps described in **Figure 4** can be used at both laboratory and large scale with appropriate adjustments. Examples of buffer compositions and running conditions for specific applications are provided in later sections of this manual. It should be noted that there is significant flexibility in how each purification is operated, where a gravity column purification (see **Section 5**) can be used to optimize incubation times, elution conditions and optimal buffer components.

! Important Note: Before using the resin for the first time (loose or in a prepacked column), the resin should undergo initial stripping and activation with 150 mM phosphoric acid (at pH ≤ 2).

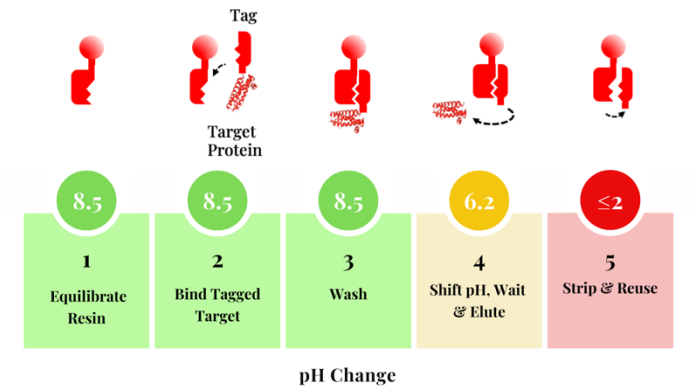


Figure 4: Steps of protein purification using the *i*CapTag™ technology after the initial stripping of the resin at the pH ≤ 2.

1. Background

1 After the resin is stripped, equilibrate a column with pH 8.5 buffer.

2 Add clarified feed containing the tagged target protein to the column at pH 8.5. The *i*CapTag™ will immobilize the target protein onto the resin. Note that initial clarification of a lysate/cell culture supernatant is performed using centrifugation followed by filtration to remove cell debris, DNA, *etc*. Commonly, in a filtration step 0.45 µm membrane filter attached to 50 mL syringe is used followed by 0.2 µm membrane filter attached to 50 mL syringe or a disposable vacuum filter.

3 Wash the column with pH 8.5 buffer to remove the rest of the unbound impurities.

4 Adjust the column buffer to pH 6.2 to accelerate cleavage and incubate the resin to allow the cleaving reaction to proceed. The optimal cleaving time can be roughly predicted using **Table 1**, or precisely measured via a gravity column optimization (see **Section 5**). Finally, elute the now tagless target protein.

5 Strip the cleaved *i*CapTag™ from the resin with an acid wash.

🔄 Repeat from Step 1 or prepare resin for 📦 storage.

2. Construction of Tagged Proteins

The *i*CapTag™ must be added to the N-terminus of the target protein, similarly to a conventional tag, where the final asparagine residue of the *i*CapTag™ tag immediately proceeds the first amino acid of the target protein (**Figure 5**). The resulting fusion protein can be expressed from the vector of choice by the end user.

Example expression plasmids containing the electronic DNA sequence of the tag can be downloaded from the Protein Capture Science website at www.ProteinCaptureScience.com.

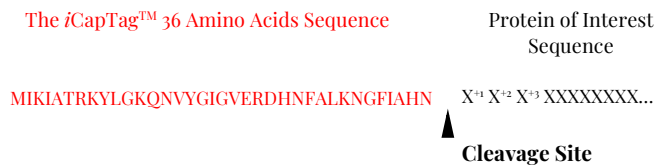


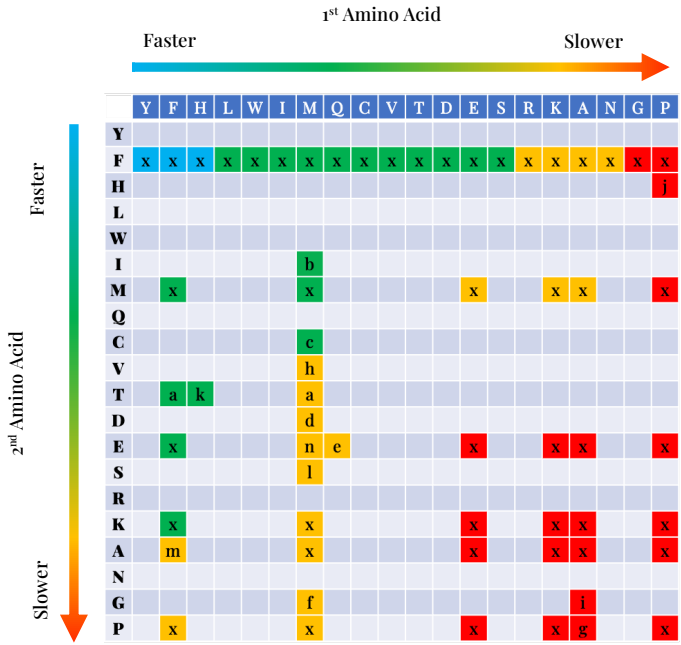
Figure 5: Structure of tagged protein showing the location of the cleavage site and X^{+1}, X^{+2}, and X^{+3}. Note that the methionine (M) at the beginning of the tag is added when *E.coli* expression system is used but it can be omitted when mammalian expression systems are used instead.

For secreted proteins, the secretion signal sequence would precede the *i*CapTag™ sequence. The first two amino acids of the protein of interest have a significant impact on the cleaving rate. In general, aromatic residues tend to accelerate cleaving while smaller residues significantly slow cleaving and proline abolishes cleaving. For proteins of interest that have unacceptably slow cleaving, amino acids can be added or modified at the N-terminus of the target to provide more optimal cleaving rates under desired conditions.

A partial guideline on the observed effects of combinations of first and second amino acids is included in **Table 1**. However, for specific products, we recommend directly observing cleavage using a gravity column, as described in **Section 5** of this manual.

2. Construction of Tagged Proteins

Table 1: Guidance on experimentally observed cleavage rates for combinations of first and second amino acids (X^{+1}/X^{+2}). In the upper grid, color-coded boxes indicate experimentally verified cleaving rates for he indicated 1st and 2nd amino acids. Lowercase letters within the grid correspond to experimental target proteins in the table, where the first three amino acids ($X^{+1}/X^{+2}/X^{+3}$) for each cleaved target protein are given. Rough cleavage rates are indicated by color code, where blue is the fastest, followed by green, yellow, and red, where the latter indicates slow overnight cleaving.



Examples of the First Three Amino Acids of Purified Proteins			
x	eGFP (modified +1/+2/N)	h	Pre-pro-FVII (M/V/S)
a	G-CSF (modified +1/T/P)	i	Pro-FVII (A/G/G)
b	Streptokinase (M/I/A)	j	Mature FVII (P/H/R)
c	Mature IFN (M/C/D)	k	RCNA (H/T/I)
d	Pro-tPA (M/D/A)	l	TrxA (M/S/D)
e	Mature tPA (Q/E/I)	m	NPRT (F/A/S)
f	Pro-EPO (M/G/V)	n	DNAJ (M/E/L)
g	Mature EPO (A/P/P)		

Very rapid cleaving (possible losses during wash steps)

80-90% cleaved in 5 hours at room temperature

80-90% cleaved overnight at room temperature

< 50% cleaved overnight at room temperature

2.1 Example of Plasmid for *E. coli* Expression

When designing the tagged fusion protein, it is important that the *i*CapTag™ amino acid sequence be joined directly to the first amino acid (X^{+1}) of the protein of interest, with no additional amino acids added. For this reason, the *i*CapTag™ is typically added using Gibson assembly or a similar method that does not rely on added restriction sites between the tag and protein of interest.

Example plasmids for *Escherichia Coli (E. coli*; see **Figure 6**) or mammalian expression can be downloaded from our web page at https://www.proteincapturescience.com.

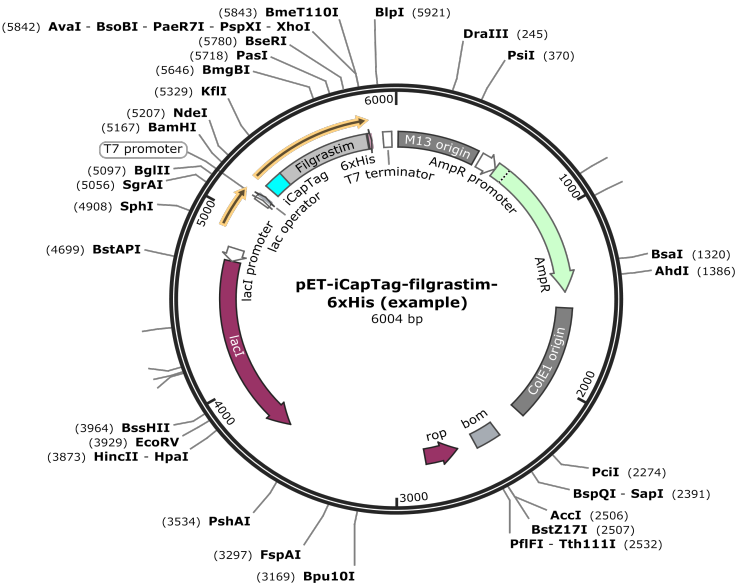


Figure 6: Example plasmid for expression of his-tagged (for detection) Filgrastim with *i*CapTag™ in *E. coli*. Figure generated with SnapGene® software (from Insightful Science; available at snapgene.com).

3. Protein Expression in *E. coli*

The following steps were followed for expression of *i*CapTag™ tagged streptokinase protein in *E. coli* BL21(DE3). This protocol can be adapted as needed for larger volumes or alternate *E. coli* strains.

3.1 Reagents and Solutions

Table 2: Reagents used during protein expression in *E. coli.*

<table>
<tr><th>Reagent</th><th colspan="3">Composition</th></tr>
<tr><td>LB medium
(starter culture media)</td><td colspan="3">10 g NaCl
5 g yeast extract
10 g tryptone
Add deionized water to 1 L
-Autoclave-
Store liquid medium up to 6 months at room temperature</td></tr>
<tr><td rowspan="2">Terrific Broth
(growth media)</td><td colspan="2">prepare separately</td><td rowspan="2">Autoclave separately, mix at <60°C and store up to 1 week at 4°C</td></tr>
<tr><td>6 g tryptone
12 g yeast extract
2 mL glycerol
Add deionized water to 450 mL</td><td>1.155 g KH_2PO_4
6.270 g K_2HPO_4
Add deionized water to 50 mL</td></tr>
<tr><td>100 mg/mL Ampicillin Stock
(antibiotic)</td><td colspan="3">100 mg ampicillin sodium salt
Dissolve in deionized water in a total volume of 1 mL
Sterilize using 0.22 µm syringe filter
Store frozen in a sterile 1.5 mL microtube at -20°C</td></tr>
<tr><td>1 M IPTG Stock
(T7-lac inducer)</td><td colspan="3">238 mg IPTG
Dissolve in deionized water in a total volume of 1 mL
Sterilize using 0.22 µm syringe filter
Store frozen in a sterile 1.5 mL microtube at -20°C</td></tr>
<tr><td>pH 8.5 Lysis Buffer</td><td colspan="3">100 mM AMPD
100 mM PIPES
200 mM NaCl
Adjust pH to 8.5
Prepare 5 mL</td></tr>
</table>

3.2 Expression of Target Protein

1 Place a colony of a protein expression strain with your plasmid into 2 mL[1] of LB media. Add 100 μg/mL ampicillin.
2 Incubate for 16–24 hr at 37°C while shaking.
3 Mix 25 mL of prewarmed Terrific Broth medium with 250 μl of the overnight culture and add 100 μg/mL ampicillin.
4 Incubate while shaking at 37°C, periodically monitoring optical density at a wavelength 600 nm (OD 600).
5 When OD 600 reaches 0.6 to 0.8 (typically about 3 hr) allow culture to cool and remove from shaking.
6 Induce protein expression via IPTG addition to a final concentration of 0.5 mM. Grow cells for 3 hr, at 37°C while shaking.[2]

3.3 Harvest Cells

7 Centrifuge harvested cells for 15 min at 6000 × g, at 4°C and discard supernatant.
8 Resuspend pellet in 5 mL of pH 8.5 lysis buffer.[3]
9 Lyse resuspended cells[4] via sonication on ice with 30 seconds pulses of 5 to 7 W, separated by 60 seconds cooling on ice. Repeat for 8 to 10 cycles. Avoid foaming and keep tubes on ice.
Diagnostic: Collect a Whole Lysate [WL] sample.[5]
10 Clarify the lysate by centrifugation for 15 min and 12,000 × g, 4°C and collect supernatant.
Diagnostic: Collect a Clarified Feed [CF] sample.

---

[1] Volumes are representative of those used in the sample procedure and can be scaled appropriately as needed.
[2] Target proteins cloned using T7-lac control often overexpress and may aggregate. If target protein solubility is a problem, altering the growth time to 20 hr at 16°C after IPTG addition may help.
[3] See buffers and solutions **Section 3.1**.
[4] Depending on the expression level and solubility of the target protein, it may only be necessary to use a fraction of the harvested cells for the described purification. Under such circumstances, the resuspended cell pellet can be divided into fractions and frozen for later use. In this case, the cell suspension was aliquoted into five 1.0 mL fractions.
[5] For diagnostic steps, transfer 20 μl of the sample to a clean microcentrifuge tube and mix immediately with an equal volume of 2 × SDS-PAGE loading buffer. Heat immediately for 5 min at 95°C.

4. Example of Mammalian Protein Expression

The following were the steps followed during an example protein expression of *i*CapTag™ tagged epoetin alfa in a serum-free medium using the Expi293™ expression host. The *i*CapTag™ can be used in other hosts using appropriate expression vectors and protocols.

4.1 Reagents and Solutions

Table 3: Tips for transfection and mammalian expression.

	Tips
Expression Media	Expression was performed in Gibco™ Expi293™ expression media
Plasmid	An appropriate volume of high-quality plasmid DNA for transfection was prepared
Transfection Enhancer	Transfection was performed using ExpiFectamine™ from Gibco™

4.2 Growth, Transfection, and Protein Expression

1. Seed chosen cells in expression media; subculturing and expanding to a desired volume.
2. For the final subculture, allow cell density to reach around $5x10^6$ cells/mL then dilute to $2.5x10^6$ cells/mL.
3. Mix plasmid DNA with partially diluted transfection enhancer and incubate at room temperature for 15 minutes.
4. Gradually introduce a plasmid mix to a culture until the concentration of plasmid in a cell culture is 1 µg/mL.
5. Incubate cells at 37°C in an 8% CO_2 incubator for 7 days with shaking.
6. After 7 days of incubation, centrifuge cells to recover clarified cell culture supernatant.
7. Adjust pH to 8.5.
   - Diagnostic: Collect a Clarified Feed [CF] sample.

5. Protein Purification Using *i*CapTag™ in a Gravity Column

5.1 Buffers and Solutions

Table 4: Recommended buffers and solutions for operation of *i*CapTag™ resin.

Buffer	Composition	pH[6]	Volume Recommended[7]
pH 8.5 Column Buffer	20 mM AMPD[8] 20 mM PIPES[9] 200 mM NaCl[10]	8.5	20 CV + 10 CV (see notes below)[11]
pH 8.5 Wash Buffer	20 mM AMPD 20 mM PIPES 500 mM NaCl[10]	8.5	10 CV
pH 6.2 Elution Buffer	20 mM AMPD 20 mM PIPES 200 mM NaCl[10]	6.2	10 CV + 2 CV
*i*CapTag™ Acid Stripping Solution	150 mM H_3PO_4	≤ 2.0	20 CV + 1 CV
Storage Solution	20% Ethanol		20 CV

5.2 Prepare for Protein Purification Step

1 While the feed is clarifying, prepare a clean 10 mL gravity-flow chromatography column and plug the bottom (outlet) of the gravity column (*e.g.,* BioRad gravity column, Cat#7311550).

---

[6] Adjust pH with NaOH.

[7] Does not include volume required for system priming, pump washes, etc.

[8] 2-amino-2-methyl-1,3-propanediol

[9] 1,4-piperazinediethanesulfonic acid

[10] NaCl concentrations in the Column, Wash and Elution Buffers can be adjusted as desired. The concentrations in **Table 4** are recommended for initial experiments.

[11] CV or Column Volume is the total used volume in the column, *e.g.*, the 5mL *i*CapTag™ prepacked column is filled with 5 mL of resin, so the CV is 5mL. The amount of column buffer necessary to operate that column is 30 CV, so the volume will come out to 150 mL.

5. Protein Purification Using *i*CapTag™ in a Gravity Column

2 Resuspend the 50% slurry of loose *i*CapTag™ intein resin and pipet 2.0 mL into the empty column. Allow the resin to settle for at least 15 min. Verify that the settled resin bed volume measures 1.0 mL.

3 Remove the bottom plug/cap and allow the 20% ethanol solution to drain through until the liquid level reaches the top of the resin bed.

! Important Note: Do not allow the resin to dry out.

! Important Note: If this is the first time the resin is being used, apply 20 CV of Acid Stripping Solution and allow it to drain until the liquid level reaches the top of the resin bed. Cap the bottom of the column and wait for 1 hour at room temperature (20 to 22°C). Stripping can be performed multiple times if it is suspected that some tag remains on the resin.

1 Equilibrate the column by applying 20 column volumes (CV) of pH 8.5 Column Buffer.[12] Apply buffer to the top of the wall of the gravity column to avoid mixing/disturbing the packed resin bed.

2 Replace the bottom outlet plug/cap until ready to begin the purification protocol. The resin should be used as soon as possible after equilibration to avoid microbial contamination.

3 Apply the full volume of the clarified feed from the final step of **Sections 3.3** or **4.2**. Collect the flowthrough in a 15 mL conical tube.

Diagnostic: Collect a Flow Through [FT] sample.[13]

4 Optional: Reapply the flowthrough to the upper reservoir of the column and allow it to flow through the column a second time. Collect the flowthrough in a 15 mL conical tube.

Diagnostic: Collect a Flow Through [FT2].

5 Wash the column with 10 CV of pH 8.5 Column Buffer. Collect the flowthrough in a 15 mL conical tube.

Diagnostic: Collect a Wash phase pH 8.5 [W] sample.

[12] When adding a buffer/solution to the column, allow the liquid to drain through the resin and out into a clean collection tube (or waste as appropriate), until the liquid level just reaches the top of the resin bed.

[13] For all diagnostic steps, transfer 20 µl of the sample to a clean microcentrifuge tube and mix immediately with an equal volume of 2 × SDS-PAGE loading buffer. Heat immediately for 5 min at 95°C.

6 Wash the column with 10 CV of pH 8.5 Wash Buffer to increase purity as needed.

7 Apply 10 CV of pH 6.2 Elution Buffer and allow it to flow through the column until the liquid level is at the top of the resin bed. Collect the flowthrough in a 15 mL conical tube.

Diagnostic: Collect a Wash phase pH 6.2 [ΔpH] sample.

8 Cap the bottom of the column and add 1 CV of pH 6.2 Elution Buffer to the top of the resin bed. Cap the top of the column and incubate at room temperature. Be sure to note the current time.

Diagnostic: Take time-point resin samples[14] [RS] by resuspending resin in the Cleaving Buffer, taking a 20 µl sample of the resin suspension, and boiling it in an equal volume of 2x SDS-PAGE loading buffer. Recommended time points for the initial experiment are: 0, 0.5, 1, 2, and 5 hr.

9 After 5 hr[15] have elapsed, elute the cleaved, purified protein from the column. Carefully remove the top and bottom caps from the column sequentially. Allow the contents to drain into a collection tube until the liquid level reaches the top of the resin bed.

10 Collect an additional elution fraction by adding 1 CV of pH 6.2 Elution Buffer to the top of the column and collecting the flowthrough in a fresh tube. Repeat for up to 4 elution fractions.

Diagnostic: Collect Elution [E#] samples.

Diagnostic: Collect a post-elution resin sample [RS_f] by adding 1 CV of pH 6.2 Elution Buffer, resuspending resin, collecting a 20 µL sample of the resuspended resin and boiling it in an equal volume of 2x SDS-PAGE loading buffer.

11 After purification is complete, allow 20 CV of Acid Stripping Solution to flow through the column.[16]

12 Cap the column at the bottom to stop flow, keeping in mind that the column should never run dry.

13 Add an additional 1 CV of Acid Stripping Solution to the column and mix well with a pipet. Cap column and allow the column to sit 1 hr at room temperature.

---

[14] To take a 'Time-point Resin Sample', use a wide-bore or cut pipet tip to resuspend the resin bed in the 1 CV of elution buffer within the column by pipetting up and down until a homogenous suspension is reached. Take sample from slurry.

[15] Incubation time will depend on the amino acids in the X^{+1} and X^{+2} positions.

[16] It is critical that enough acid stripping solution is used. Any remaining *i*CapTag™ bound to the column will reduce the effects of future purifications.

14 After incubation, allow the 1 CV of Acid Stripping Solution sitting in the column to flow through until the liquid level reaches the top of the bed.

15 (For storage) Wash the column by applying 20 CV of 20% (v/v) ethanol and allow the solution to drain out to a waste container until the liquid level reaches the top of the resin bed. See **Section 8** for storage considerations.

-or-

16 (To reuse resin) Wash the column by applying 20 CV of column buffer and allow the solution to drain out to a waste container until the liquid level reaches the top of the resin bed. Return to Step 5 above.

Use SDS-PAGE to analyze all diagnostic samples. See **Figure 7.**

Commonly, high-throughput screening or gravity column(s) are used to analyze and better understand behavior of a target protein during protein purification. Examples of key questions to troubleshoot protein purification method are collected in **Tables 8 and 9** (see **Chapter 9**).

5.3 Example SDS-PAGE Diagnostic Results

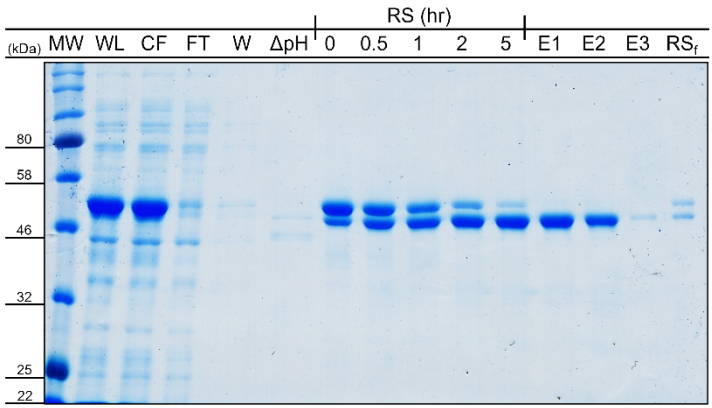


Figure 7: Example of diagnostic results for a purification of streptokinase protein using *i*CapTag™ in a gravity column.

Legend:

1 Standard Protein Ladder [MW]
2 Whole Lysate [WL]
3 Clarified Feed [CF]
4 Flow Through sample [FT]
5 Wash phase at pH 8.5 [W]
6 Wash phase at pH 6.2 [ΔpH]
7 Time-point resin samples [RS (hr)] at 0, 0.5, 1, 2, and 5 hours
8 Elution fractions 1, 2 and 3 [E1, E2, and E3]
9 Post-elution resin sample [RS_f]

6. Operation of Columns via FPLC

Table 5: Quick sheet for flow specifications for currently available *i*CapTag™ resin pre-packed columns after an initial stripping of the resin is performed.

Phase	Equilibrate	Bind	Wash	ΔpH	Cleaving Incubation	Elution	CIP[17]/ Storage
1 mL Column Bed Height: est. 2.33 cm; Internal Diameter: 0.726 cm Maximum Pressure: 2.8 bar (40 psi); Maximum Flow Rate: 2 mL/min							
Duration	10 CV	Feed[18]	15 CV	5 CV	3-16 hr[19]	5 CV	10 CV + 10 CV
Flow Rate [mL/min]	1	0.25	1	1	0	0.5	1
Buffers[20]	A1	N/A	A2	B1	N/A	B1	B2:10 CV ST:10 CV
Outlet Fractions	Waste	1 CV	1 CV	1 CV	-	1 CV	B2:1 CV ST: N/A
5 mL Column (16x26 mm) Bed Hight: est. 2.42 cm; Internal Diameter: 1.57 cm Maximum Pressure: 2.8 bar (40 psi); Maximum Flow Rate: 10 mL/min							
Duration	10 CV	Feed	10 CV	5 CV	3-16 hr	5 CV	10 CV + 10 CV
Flow Rate [mL/min]	5	1.25	5	5	0	2.5	5
Buffer	A1	N/A	A2	B1	N/A	B1	B2:10 CV ST:10 CV
Outlet Fractions	Waste	1 CV	1 CV	1 CV	-	1 CV	B2:1 CV ST: N/A
5 mL Column (8x100 mm) Bed Hight: 10 cm; Internal Diameter: 0.8 cm Maximum pressure: 2.8 bar (40 psi); Maximum Flow Rate: 2 mL/min							
Duration	10 CV	Feed	15 CV	5 CV	3-16 hr	5 CV	10 CV + 10 CV
Flow Rate [mL/min]	1.5	1.25	1.5	1.5	0	1	1.5
Buffer	A1	N/A	A2	B1	N/A	B1	B2:10 CV ST:10 CV
Outlet Fractions	Waste	1 CV	1 CV	1 CV	-	1 CV	B2:1 CV ST: N/A

[17] CIP – Cleaning in Place.
[18] Apply the entire volume of the clarified feed, or the maximum necessary to saturate column.
[19] Dependent on X^{+1} and X^{+2} of target protein (See **Table 1**).
[20] Listed in **Table 6.**

Table 6: Buffers preparation for FPLC usage.

Type of Buffer	Column Buffer	Wash Buffer	Elution Buffer	Stripping Buffer	Storage Solution
	A1	A2	B1	B2	ST
Composition	20 mM AMPD 20 mM PIPES 200 mM NaCl	20 mM AMPD 20 mM PIPES 500 mM NaCl	20 mM AMPD 20 mM PIPES 200 mM NaCl	150 mM H_3PO_4	20% Ethanol Solution
pH	8.5	8.5	6.2	≤2.0	

6.1 Sample Procedure for 1 mL Pre-packed Column

This sample protocol is based on the buffer designations and compositions given in **Table 6**. By default, chromatography should be performed at room temperature (20°C to 22°C)

1. Connect pre-packed column to FPLC.
2. Before first operation of column, perform an initial stripping step with the acid stripping solution (B2).
3. Equilibrate with 10 mL[21] of buffer A1, applied at 1 mL/min, draining the outlet to waste.
4. Load the clarified feed at 0.25 mL/min (residence time of 4 minutes). Collect the flowthrough for analysis.
5. (Optional) Apply 10 mL of buffer A1 at 1 mL/min to wash feed impurities from column. Collect flowthrough for analysis.
6. Apply 15 mL of buffer A2 at 1 mL/min to wash additional impurities from column as needed. Collect flowthrough for analysis.
7. Add 5 mL of buffer B1 at 1 mL/min to adjust the internal pH of the column to 6.2. This can be directly monitored if your FPLC device has a pH flow cell.
8. Stop flow through column for an appropriate length of time to allow for sufficient cleavage of *i*CapTag™. The length of time will

[21] For a 1 mL column 1 CV equals 1 mL. Buffer volumes can be directly scaled with relative column size while flow rates are scaled as directed in **Table 5**.

depend on the protein and could range from a short time (less than 2 hours) to overnight.[22]

9 Elute cleaved proteins with 5 mL of buffer B1 at 0.5 mL/min and collect fractions for analysis.

10 Regenerate the column with 10 mL of buffer B2 at 1 mL/min.

! Important Note: Do not allow column to sit in acid for an extended period. Either go back to step 3 if you plan on immediately reusing it or prepare for storage.

11 (For storage) apply 10 mL of 20% ethanol solution before capping and removing from FPLC.

Use SDS-PAGE to analyze all diagnostic samples. See **Figure 8**.

[22] For particularly fast cleaving proteins, it may be of benefit to operate the column in a flow mode during the cleaving step. This can be accomplished by introducing buffer B1 at a rate such that the column residence time is ~1 hour and collecting fractions for at least three column volumes.

6.2 Example of SDS-PAGE Diagnostics

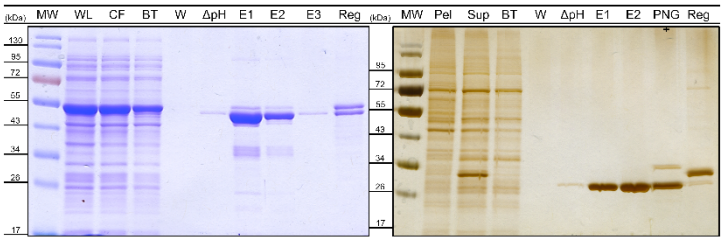


Figure 8: SDS-PAGE results for FPLC purification. Left – *E. coli* expressed streptokinase. Right – scFv expressed in Expi293™ cells.

Legend:

1 Standard protein ladder [MW]
2 Whole Cell Lysate [WL]
3 Clarified Feed [CF]
4 Breakthrough [BT]
5 Cell Pellet [Pel]
6 Cell Culture Supernatant [Sup]
7 Wash phase at pH 8.5 [W]
8 Wash phase at pH 6.2 [ΔpH]
9 Elution fractions # [E1, E2, *etc.*]
10 PNGaseF for detecting N-glycosylation [PNG]
11 Final flow-through for regeneration [Reg]

7. Characteristics of Bulk Resin

Specific parameters related to the *i*CapTag™ resin are shown in **Table 7**.

Table 7: Important characteristics of the *i*CapTag™ resin.

Parameters	Requirements[23]
Ligand Density	15 ± 2 g/L
Capacity DBC Q_{10}[24]	>7 g/L (no bed compression; based on GFP test target protein)
Cleavability	95% or higher cleavage in 5 hours at room temperature
Base Bead	Hardened agarose (6% cross-linked agarose)
Particle Size, d_{50v}[25]	~90 µm
Recommended Maximum Operating Flow Velocity	300 cm/h in a 5 cm diameter x 10 cm bed height column using aqueous buffer
Maximum Back Pressure	40 psi
Typical Working Temperature	18 °C[26] to 37 °C
Chemical Stability	Resin is initially stripped at pH ≤ 2.0 followed by buffers with the pH between 6.2 and 8.5. Neutral to salts, sugars, and other buffer additives, including protease inhibitors. Stable under standard caustic sanitization protocols.
Resin Packing	50% slurry in storage solvent
Storage	18% ± 2% Ethanol, 4 °C to 8 °C

! Important Note: Do not freeze the *i*CapTag™ resin.

[23] Specific parameters of the resin are included in the Certificate of Analysis based on the lot number of the resin.
[24] Dynamic binding capacity at 10% breakthrough.
[25] Median particle size of the cumulative volume distribution.
[26] While the recommended operating temperature is around room temperature (20 °C), there may be situations in which cleavage occurs too rapidly at higher temperatures. In this case the temperature can be decreased to as low as 4 °C to suppress cleavage during the binding and pH shift phases. Do not freeze.

8. The *i*CapTag™ Columns and Resin Storage

! Important Note:

- Do not allow columns or resin to dry out.
- Do not freeze resin.

📦 Storage Steps:

1. Before storing columns or resin, wash with 10 CV of Acid Stripping Solution.
2. Wash with 20 CV of 20% ethanol.
3. For gravity columns allow 20% ethanol to drain until liquid reaches the top of the resin and seal the column.
4. For longer term storage add 1 CV of 20% ethanol and resuspend resin. Remove resin suspension from the column and move it to an appropriate airtight container.
5. Store at 4°C to 8°C.

When removing from 📦 storage, be sure to equilibrate with column buffer before applying clarified feed.

9. Troubleshooting

Table 8: Common indications, causes and solutions detected.

Tagged target protein not detected		
Possible Cause	Indication	Solution
Low expression of target protein.	SDS-PAGE and/or western blot shows minimal or absent band at expected size for the precursor in [WL].	Adjust and optimize medium, expression conditions, and induction conditions required by the target protein.
Target or precursor *i*CapTag™ -target fusion is fully or partially insoluble.	Significant reduction in precursor band intensity going from [WL] to [CL].	Consider adding a solubility tag or stabilizing agents.
Thermal/chemical denaturation during lysis.		Reduce lysis stringency (shorten sonication cycles/lower power/reduce total cycles).
Incomplete lysis or extraction of protein from host cells.	Significant reduction in precursor from [WL] to [CL]. This is typically accompanied by a decrease in all band intensities in the [CL].	Increase lysis stringency (longer sonication cycles/higher power/increase total cycles/use chemical lysis methods).
Weak or incomplete binding of the precursor fusion protein		
Possible Cause	Indication	Solution
Insufficient contact time with *i*CapTag™ resin.	[CL] and [FT] contain similar quantities of the precursor, while little can be observed in [RS], resin samples cumulated at the time zero to hr(s) timeframes.	Increase residence time by slowing flow rate, or by recycling the flowthrough onto the column for multiple passes.
Specific interaction between *i*CapTag™ intein segment and target protein.		In-solution, verify that tagged protein binds to resin.
Proteolytic degradation of *i*CapTag™ domain.	See above; multiple or smeared bands appear below precursor in resin sample(s) cumulated at specific timeframes; from time zero to hr(s).	Add protease inhibitors to lysate, and/or minimize delay from lysis to binding stage; most degradation occurs while precursor sits in lysate.
Excessive tagged target protein exceeds column capacity.	[CL] and [FT] contain similar quantities of the precursor, but purified protein is liberated from column after cleaving.	Increase column bed volume or decrease amount of material loaded.

(Continues next page)

9. Troubleshooting

Cleavage of intein before elution phase		
Possible Cause	Indication	Solution
Lysate overwhelms buffering capacity of binding buffer; lowering pH and accelerating cleaving.	Cleavage products observed in [FT], [Wash] samples, and [RS], resin samples (from time zero to hr(s)) show significant cleaving.	Verify diluted lysate is buffered to pH 8.5.
Binding and washing time are excessive relative to cleaving rate at pH 8.5.	Cleavage products observed in [FT] and [Wash] samples, [RS], resin samples cumulated at the time zero to hr(s) timeframes shows significant cleaving; binding and washing phase take longer than 90 min.	Scale down process; decrease temperature during binding or perform parallel purifications; verify that column flow rate is not significantly impeded by fouling; verify that lysate is fully clarified before loading.
Target's N-terminal peptide sequence accelerates the intein cleaving reaction at pH 8.5.	Band at expected size for cleaved product is significantly more abundant in [FT] and [Wash] SDS-PAGE samples than [CL] and [FT].	Reduce process time; reduce scale; bind/wash at 4°C; or if possible, add three amino acids Met-Phe-Asn (MFN) leader to N-terminal of target protein to ensure rapid and controllable cleaving.

Incomplete cleavage of intein		
Possible Cause	Indication	Solution
Insufficient time elapsed for cleaving reaction or slow cleavage kinetics.	Low recovery of cleaved product in [E] samples; [RS] resin samples show minimal change in precursor-cleaved product ratio over time; [RS_f] sample shows prominent precursor band.	Increase incubation time; reduce elution buffer pH to <6.2; increase incubation temperature to 37°C; add 'MFN' leader to N-terminal of target protein.

Cleaved product not recovered in elution		
Possible Cause	Indication	Solution
Cleaved product aggregates and remains on column.	[RS_f] shows prominent cleaved band remaining on resin after elution, and [E] shows only a faint band.	Ensure that the native target is soluble in elution buffer; mild surfactants and/or stabilizing agents can be introduced in a secondary elution buffer as needed.

(Continues next page)

9. Troubleshooting

Poor protein purity		
Possible Cause	Indication	Solution
Co-purified products.	Nonspecific bands appear in [RS] and [E] samples.	Add EDTA, salts, and/or mild surfactants during wash phase to disrupt target protein interaction with host-cell contaminants; if co-purifying species is a suspected chaperone, then addition of ATP to elution buffer can help.

Low fractional recovery of target protein		
Possible Cause	Indication	Solution
Expression titer and/or scale exceeds resin binding capacity.	Purification appears successful, although precursor quantity in [FT] and [CL] appears similar or equal.	Scale down expression or scale up volume of resin bed.
Significant product losses during purification.	See incomplete binding/ rapid cleavage/incomplete cleavage described above.	See incomplete binding/rapid cleavage/incomplete cleavage described above.

Table 9: Step-by-step analysis of protein(s) using gravity column.

EQ. at pH 8.5	**pH 8.5**	**Equilibrate (EQ.) resin and CCL to pH 8.5 to prevent cleaving during load and wash**	**Clarified Cell Lysates (CCL) pH 8.5**
LOAD CCL SAMPLE		**Early flowthrough, are you binding intein-protein complex to the resin?**	**Flow Through Start (FT1) pH 8.5**
		Late flowthrough, is the column saturated?	**Flow Through End (FT2) pH 8.5**
WASH		**Did you clean the resin enough? There should be no protein(s) (in late wash)**	**Wash (W) pH 8.5 (end of wash period)**
EQUILIBRATION at pH 6.2 (or 6.0-6.5)	**pH 6.2**	**Is your protein cleaving too fast? There should be none or very little cleaved protein**	**Shift of pH Followed by Sample Equilibration pH6.2 (CEQ) (after 1 CV)**
WAIT & LET PROTEIN CLEAVE WHILE SMALL RESIN SAMPLES ARE COLLECTED OVER TIME		**Cleaving Optimization**	**Resin Sample 0h (RS-0)**
			Resin Sample 1h (RS-1)
			Resin Sample 3h (RS-3)
			Resin Sample 5h (RS-5)
ELUTION		**Collect cleaved protein. Almost all cleaved protein should be in the first 2 CVs of the elution step.**	**Elution #1 (Ex1) – sample collected below the column**
			Elution #2 (Ex2)
			Elution #3 (Ex3)
			Elution #4 (Ex4)
STRIP	**pH≦2**	**Do you have uncleaved and/or aggregated target protein on the resin?***	**Post-elution Resin Sample (RS_f)**
		Do you have uncleaved and/or aggregated target protein on the resin?**	**After Resin is Stripped with 1 CV Buffer**

* - For gravity column or bulk binding take resin sample during stripping

** -For FPLC take a sample during stripping

10. References

1. Cooper, M. A.; Taris, J. E.; Shi, C.; Wood, D. W., A convenient split-intein tag method for the purification of tagless target proteins, *Current Protocols in Protein Science*, **2018**, 91, pp. 5.29.1–5.29.23

2. Prabhala, S.V.; Mayone, S.A.; Moody, N.M.; Kanu, C.B.; Wood, D.W., A convenient self-removing affinity tag method for the simple purification of tagless recombinant proteins, *Current Protocols*, **2023**, 3 (10), e901.

3. Expi293™ Expression System: Structural Biology and Inducible Expression Modules. Gibco User Guide, 2020. https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0007814 _Expi293_ExpressionSystem_UG.pdf (accessed October 2, 2021)

4. Lee, Y.Z.; Kuo, J.H.; Sue, S.C.; Crystal structure of native Npu DnaE split intein, PDB ID 4QFQ, https://www.rcsb.org/structure/4qfq (accessed July 3, 2020)

5. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N., Bourne, P. E., The Protein Data Bank. *Nucleic Acids Research*, **2000**, 28(1), 235–242. https://doi.org/10.1093/nar/28.1.235

Protein Purification Using *i*CapTag™
Examples of Protocols & Results

Cover imagery courtesy of Canva.com.
Interior and cover design: Dr. Izabela Gierach.
Produced in the United States of America
Fourth Edition
2023

How to reference this manual? Example: Protein Capture Science. Protein Purification Using *i*CapTag™, Examples of Protocols & Results. Protein Capture Science, 2023. https://www.proteincapturescience.com (accessed 2023-04-02).

Additional references: Note, that some of the parts of the manual refer to specific peer-reviewed publications. Therefore, please reference those publications directly.

iCapTag™

PROTEIN CAPTURE SCIENCE

PROTEIN CAPTURE SCIENCE

A NEW BIOTECH PLATFORM FOR YOU

SHOP NOW

SINGLE PLAFORM FROM R&D TO MANUFACTURING

NOW AVAILABLE

TROUBLESHOOTING KIT

3 X 1 ML

NOW COMBINED

1.5 ML OF BULK RESIN

PROTEIN CAPTURE SCIENCE

www.proteincapturescience.com

SINGLE PLAFORM FROM R&D TO MANUFACTURING


PROTEIN
CAPTURE
SCIENCE