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Naturally split intein *Npu* DnaE mediated rapid generation of bispecific IgG antibodies

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ABSTRACT

High product purity, preserving natural IgG architecture, and excellent production efficiency are highly desirable in bispecific antibody manufacturing. We have reported a platform called Bispecific Antibody by Protein Trans-Splicing (BAPTS) to synthesize BsAbs with natural human IgG structure and no chain mispairing. In the method, two antibody fragments carrying different target-specificities are separately expressed in mammalian cells and subsequently fused to form BsAbs by utilizing the trans-splicing property of the split intein *Npu* DnaE. The hinge region of antibody, a region with less functional impact, is selected for conjugating the two fragments. The method involves the following steps: (i) constructing five plasmids coding antibody components; (ii) separately expressing and purifying two antibody fragments A and B. Fragment A contains one Fab, "Knobs-into-Holes" mutations in the CH3 domain and *NPU* DnaE^{C}. Fragment B contains another Fab and *NPU* DnaE^{N}; (iii) mixing of fragments A and B under permissive reducing conditions *in vitro* to enable trans-splicing reaction; (iv) removing the reductant to allow re-oxidation of disulfide bonds; (v) isolating BsAb product from unreacted precursors by affinity chromatography. The method allows correct assembly of two heavy and two light chains to form bispecific IgG antibodies in natural structure with no synthetic linkers. No chain mispairing was observed in the product by UPLC-MASS. In addition, the observed kinetics and low reaction activation energy confirmed that the trans-splicing is thermodynamically favored reaction. The BAPTS technology is feasible for industrial applications.

1. Introduction

While therapeutic monoclonal antibodies (mAbs) are widely used to treat single-target human diseases, there is growing evidence showing that bispecific antibodies (BsAbs) have enhanced therapeutic effects in many cases. For example, a bispecific antibody may act as a bridge to recruit immune cells to tumor cells [1]; simultaneously antagonize two receptors [2]; increase specificity for cells expressing both antigens [3]; serve as a transport mechanism to shuttle an antibody across the blood-brain barrier [4]; and efficiently neutralize virus [5]. Unfortunately, generating BsAbs, from original IgG structure with desired antigen specificities, is not a trivial task.

Chain mispairing and low purification efficiency are two main challenges. Bispecific antibody production is complicated because human antibodies with IgG structure are multi-domain proteins. Each BsAb with native IgG structure consists of two different heavy and two different light chains linked by disulfide bonds. Four different chains have to be assembled correctly to form a BsAb with two different antigen-binding regions. Many methods have been developed to produce BsAbs for industrial application, including the use of quadroma approach [6], a common light chain [7], Fab arm exchange [8], domain crossovers [9], *in vitro* re-oxidation [10,11], dual-acting Fab [12], half-antibodies [13] and an orthogonal Fab interface [14]. Each of these approaches has advantages but also limitations such as immunogenicity, poor pharmacokinetic properties, no general applicability, time-consuming, lacking post-translational modification, or needing complex protein engineering.

Recently, we reported a generic technology platform for generating bispecific IgG antibodies, "Bispecific Antibody by Protein Trans-Splicing (BAPTS)" [15]. BAPTS technology enables correct chains

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assembly without using of any synthetic linkers. The key process of BAPTS is a protein trans-splicing reaction of *Npu* DnaE split intein. *Npu* DnaE spilt intein consists of a 102 a.a. N-terminal split fragment (Int^N) and a 36 a.a. C-terminal split fragment (Int^C) [16]. Trans-splicing in BAPTS is thermodynamically favored reaction. At 37 °C and under appropriate reducing conditions, 90% of the substrates are consumed within 25 min. Even at low temperature (4 °C), the reaction is completed within two hours. The reaction efficiency, kinetics rate constant and activation energy were reported previously. This report focuses on the BAPTS methodology including reaction condition optimization and trouble-shooting.

2. Materials

2.1. Cells

HEK293E (American Type Culture Collection, CRL-10852).
CHO-S (Thermo Fisher Scientific, R80007).

2.2. Reagents

pCEP4 Mammalian Expression Vector (Thermo Fisher Scientific, V04450).
Endo-free Plasmid Maxi Kit (Omega, D6926-03).
25-KD polyethyleneimine (PEI) (Polysciences, 23966-1).
Freestyle 293 medium (Gibco, 12338018).
SFM4 HEK293 medium (Hyclone, SH30521.02).
Valproic acid (VPA) (Sigma-Aldrich, PHR1061-1G).
Sodium dihydrogen phosphate anhydrous (NaH_2PO_4) (Sigma-Aldrich, 17844).
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) (Sigma-Aldrich, 71633).
Sodium chloride (NaCl) (Sigma-Aldrich, 31434).
Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, P5655).
Potassium chloride (KCl) (Sigma-Aldrich, 746436).
Hydrochloric acid (HCl) (Sigma-Aldrich, 30721).
Sodium hydroxide (NaOH) (Sigma-Aldrich, 30620).
Citric acid (Sigma-Aldrich, C7129).
Sodium citrate (Sigma-Aldrich, C7254).
Tris (hydroxymethyl) aminomethane (Tris) (Sigma-Aldrich, 252859).
Dithiothreitol (DTT) (Sigma-Aldrich, D0632).
Imidazole (Sigma-Aldrich, I2399).
Bis (2 – hydroxyethyl) iminotris (hydroxymethyl) methane (Bis-Tris) (Sigma, B4429).

2.3. Chromatographic columns

HiScreen Capto L (GE Healthcare, 17547814).
HisTrap FF (GE Healthcare, 17531901).
HiScreen MabSelect SuRe (GE Healthcare, 28926977).

2.4. Equipment

Sorvall ST 16 Centrifuge (TermoFisher Scientific, 75004240).
NanoDrop One Microvolume UV–Vis Spectrophotometer (TermoFisher Scientific, ND-ONE-W).
ÄKTA avant 150 (GE Healthcare, 28976337).
pH meter (Mettler Toledo, FE28).
Slide-A-Lyzer™ G2 Dialysis Cassettes, 10 K MWCO, 15 mL (TermoFisher Scientific, 87731).
Milli-Q advantage A10 water purification system (Millipore, ZMQS5VF01).

2.5. Buffers

Capto L and MabSelect affinity chromatography binding buffer: 20 mM sodium phosphate, 500 mM NaCl, pH 7.4.
Capto L and MabSelect affinity chromatography elute buffer: 0.1 M sodium citrate, pH 2.8.
Neutralizing buffer: 1 M Tris-HCl, pH 9.0.
Splicing buffer: 10 mM Tris-HCl, 0.5 M NaCl, pH 7.9.
Dialysis buffer: 10 mM Tris-HCl, 500 mM NaCl, pH 8.0.
HisTrap affinity chromatography binding buffer: 20 mM Bis-Tris, 500 mM, NaCl 30 mM imidazole, pH 6.5.
HisTrap affinity chromatography elute buffer: 20 mM Bis-Tris, 500 mM NaCl, 300 mM imidazole, pH 6.5.
PBS buffer: 150 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4.

3. Experimental design

3.1. The principle of BAPTS

The BAPTS (Bispecific Antibodies by Protein Trans-Splicing) is a generic technology platform that generates bispecific antibody with correct assembly of two heavy chains and two light chains, derived from possibly any existing or new antibodies, without using any additional linkers [15]. As shown in Fig. 1, antibody molecule is divided into fragments A and B at hinge region where less functional impact may occur. The two fragments carrying necessary intein components are expressed in mammalian cells separately, so there is no light/heavy chains mispairing. The “Knobs-into-holes” Fc engineering method is adopted in fragment A construction, enhancing correct heavy chains hetero-dimer formation at about 97% rate [17]. In addition, there are distinct physical-biochemical properties between homo-dimers and hetero-dimers in BAPTS assembling system, due to the asymmetric structure of fragment A. Residual homo-dimers can be efficiently removed with Capto L affinity chromatography, eliminating heavy/heavy chain mispaired contaminants. Semi-purified fragment A and fragment B are conjugated by *Npu* DnaE split intein’s autocatalytic PTS (Protein Trans-splicing) ability to form a BsAb. The product BsAb is purified efficiently through affinity and optional ion exchange purification steps. The un-reacted fragment A with His-tag is removed by Ni-Sepharose capturing. The BsAb product is captured by MabSelect affinity column and un-reacted fragment B is separated for its lack of affinity to Protein A.

3.2. General considerations

Several expression vectors for antibodies are readily available for the Fragments A and B of “BAPTS”. A general workflow to obtain expression vectors includes the following steps: (I) identifying the sequences of VH and VL regions of the parental antibodies, (ii) obtaining codon-optimized versions of the VH, VL and *Npu* DnaE coding regions, human IgG1 Fc region (including the “Knobs-into-Holes” mutations) and human Fab constant regions, (iii) cloning all coding regions in expression plasmids (e.g., pCEP4; Invitrogen), (iv) preparing five expression plasmids (pCEP4-CD3-Lc, pCEP4-CD3-Knob Fc, pCEP4-IntC-Hole Fc, pCEP4-HER2 VH-CH1-IntN, pCEP4-HER2-Lc) by conventional plasmid synthesis and purification methods.

3.3. Plasmid construction

Using CD3 × HER2 BsAb as an example, pCEP4 (Invitrogen) vector is selected for secretion expression with HEK293E cells growing in suspension. In this paper, the kappa light chain signal peptide (M D M R A P A G I F G F L L V L F P G Y R S) was adopted in the expression cassette for secreting [18]. Otherwise, it is possible to adopt other signal peptides. As shown in Fig. 2, “Knobs” (S354C, T366W) mutations are

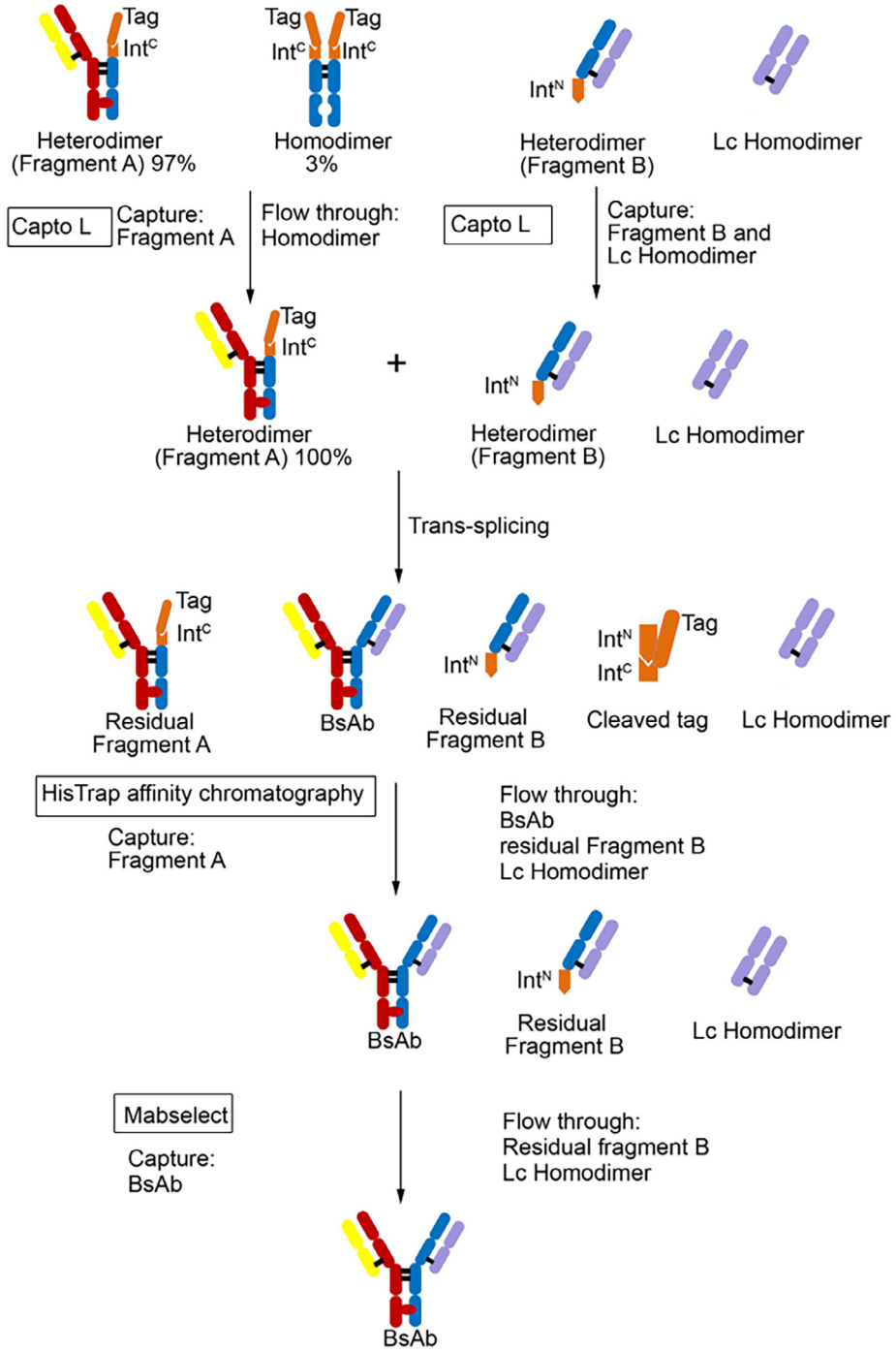

Fig. 1. Schematic of the process for BsAb expression and purification using BAPTS. Three steps were involved, the first was the expression and Capto L affinity purification of the fragments A (anti-CD3) and B (anti-HER2) respectively; the second was trans-splicing of the fragments A and B *in vitro*; and the last was removal of residual fragments A and B via His tag affinity chromatography and MabSelect affinity chromatography.

introduced into anti-CD3 heavy chain, and "Holes" (S349C, T366S, L368A, Y407V) mutations are introduced into an Fc fragment ("Hole" Fc). *Npu* DnaE^N is cloned downstream the CH1 domain of anti-HER2 Fab, and *Npu* DnaE^C is cloned upstream the "Hole" Fc domain. There is no modification in anti-CD3 and anti-HER2 light chains. Each plasmid (pCEP4-CD3-Lc, pCEP4-CD3-Knob Fc, pCEP4-Int^C-Hole Fc, pCEP4-HER2 V_H-C_{H1}-Int^N, pCEP4-HER2-Lc) is purified using the Endo-free Plasmid Maxi kit (Omega Bio-Tek) following manufacturer's instructions.

1. Synthesize codon-optimized V_H, V_L, *Npu* DnaE^N, *Npu* DnaE^C coding regions, human IgG1 Fc region (including the "Knobs-into-Holes" mutations) and human Fab constant regions. Overlap PCR is used to connect genes together.
2. *Npu* DnaE^N is cloned downstream the CH1 domain of anti-HER2 Fab.
3. *Npu* DnaE^C is cloned upstream the "Hole" Fc domain.
4. "Knob" Fc domain is introduced into anti-CD3 Heavy chain.
5. There is no modification in anti-CD3 or anti-HER2 light chains.
6. Preparing five expression plasmids by conventional plasmid synthesis and purification methods.
7. Determine the concentration endotoxin level of the five plasmids.

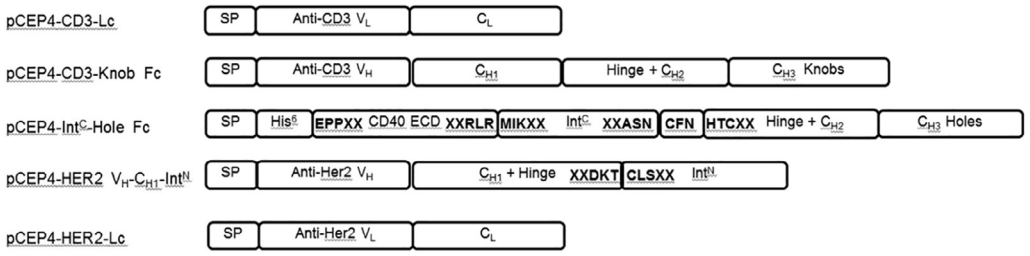

Fig. 2. Design of five cotransfect vectors. The fragments A was produced by transient expression from three expression plasmids (pCEP4-CD3-Lc, pCEP4-CD3-Knob Fc, pCEP4-Int^C-Hole Fc), and the fragment B was produced by transient expression from two expression plasmids (pCEP4-HER2 V_H-C_{H1}-Int^N, pCEP4-HER2-Lc).

Low endotoxin level (< 1 EU/μg) is required for further experiments.

3.4. Parental antibody fragments production

BAPTS requires sufficient amounts (milligrams) of starting materials for generating BsAb. Transfection of HEK293E or CHO-S cells is performed according to the published procedures for transient or stable expression cells [15,19], although transient transfection is focused in this report. For transient transfection, cell culture supernatant was taken for analysis or processing either at one week post-transfection or when the cell viability is under 50% [20,21]. Fragment A and B are purified by Capto L affinity chromatography (GE Healthcare) from filtered cell culture supernatants following product descriptions. The supernatants are loaded on a Capto L equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4). Elution of antibodies is achieved with a 20 column volume (CV) linear gradient from 0% to 100% 0.1 M sodium citrate, pH 2.8. The eluate is immediately pH-neutralized with 1 M Tris-HCl, pH 9.0. The eluted Fragments A and B are dialyzed overnight against splicing buffer (10 mM Tris-HCl, 0.5 M NaCl, pH 7.9). Protein concentrations are determined by absorbance at 280 nm. We separately purified about 20 mg fragment A (anti-CD3) and 80 mg fragment B (anti-Her2) from 1 L cell culture supernatant by Capto L.

1. Transfection of HEK293E is performed according to published transient transfection procedure [20].
2. Harvest cell culture supernatant by centrifugation at 1000 rpm at 4 °C for 20 min, followed by 0.22 μM filtration.
3. Capto L affinity chromatography is performed according to the procedures suggested by the manufacturer in the product descriptions.
4. The solution containing purified fragment A or B is dialyzed overnight against trans-splicing buffer (10 mM Tris-HCl, 0.5 M NaCl, pH 7.9) using Slide-A-Lyzer GD2 Dialysis Cassettes (ThermoFisher Scientific).
5. The dialyzed fragments A and B are at −80 °C before trans-splicing reaction.

3.5. Trans-splicing reaction

Since our previous report [15], we have been further investigating the impact of dithiothreitol (DTT) concentration, incubation time and temperature on the trans-splicing reaction. The optimal DTT conditions was optimized to 0.5 mM. At this DTT concentration, about 90% of substrates can be converted to product within 2 h, even at the low temperature of 4 °C. For *Npu* DnaE mediated protein trans-splicing reaction, 10 μM of the fragment A is mixed with 10 μM of the fragment B in splicing buffer (10 mM Tris-HCl, 0.5 M NaCl, pH 7.9). Freshly prepared DTT is added to a final concentration of 0.5 mM. After an incubation at 37 °C for 2 h, aliquots are removed and quenched by the addition of H_2O_2 and SDS-PAGE sample buffer without reducing agent. As shown in Fig. 3, the yields of the trans-splicing reactions are

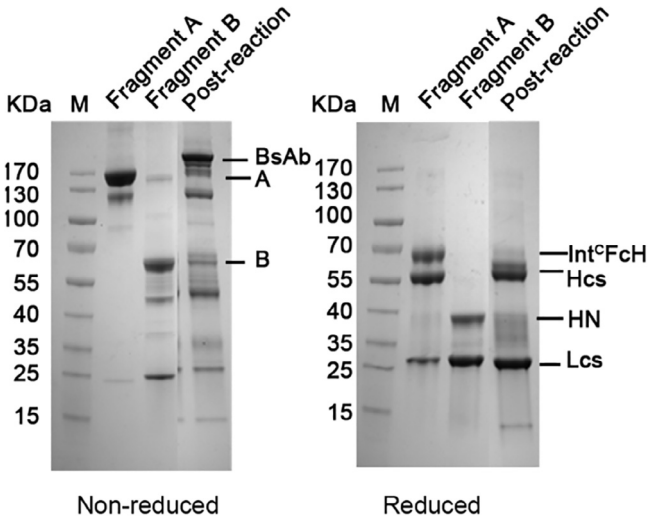

Fig. 3. Determining catalytic trans-splicing reaction between fragment A and B at 37 °C in the presence of 0.5 mM DTT for 2 h by Coomassie Brillant Blue-stained SDS-PAGE. BsAb, bispecific antibody; A, fragment A; B, fragment B; Int^{C}FcH, *Npu* DnaE^C-Hole mutated Fc; Hcs, heavy chains; HN, V_H-C_{H1}-Int^N; Lcs, light chains.

determined from the intensities of the fragment A bands by Coomassie Brillant Blue-stained SDS-PAGE. We also observed some other impure bands showing increase or decrease besides of disappearance of reaction substrate fragments A and B (Non-reduced), Int^{C}FcH and HN (Reduced). The band between 40 KDa and 55 KDa represented dimeric light chain and the band at 25 KDa represented the monomeric light chain. The cleaved *Npu* DnaE^N bands appeared at 15 KDa. Alternative reducing agents (e.g., 2 – mercaptoethylamine (2 – MEA), tris (2 – carboxyethyl) phosphine (TCEP)) can also be used for trans-splicing. However, the conditions described in this article are optimized specifically using DTT at 0.5 mM.

1. Determine the concentration of the fragments A and B stock solutions by A280 with specific extinction coefficients using NanoDrop. Protein concentrations are adjusted to 10 μM.
2. Per-warming fragments A and B at 37 °C for 10 min.
3. Add DTT stock solution to 10 μM fragment A to 1 mM final concentration.
4. Add equal volume of 10 μM fragment B into the 10 μM fragment A that with 1 mM DTT. The final concentration of DTT is 0.5 mM.
5. Incubate the mixture at 37 °C for 2 h, aliquots are removed and quenched by adding one tenth volume of 0.1% (V/V) H_2O_2 and SDS-PAGE sample buffer without reducing agent.
6. Determine the yield of the trans-splicing reaction using non-reducing SDS-PAGE.

Table 1
Troubleshooting reasons and advice.

Step	Problem	Possible reason	Solution
Expression of fragments from parental antibodies	No expression or low expression level of the antibody fragments	There are possible mistakes in five expression plasmids or endotoxin at high level	Confirm the five expression plasmids by DNA sequencing; Remove endotoxin of the plasmid solutions; and optimize the transfection conditions
	Fragments are impure.	The ratio of co-transfection plasmids is inappropriate	Optimize the ratio of three plasmids for fragment A, and two plasmids for fragment B
	No elution of antibody fragments on Capto L	The antibody fragments have strong binding ability to Capto L resins	Change the linear gradient to lower pH conditions
Trans-splicing reaction	No trans-splicing occurred	Final concentration of DTT is too low	Repeat trans-splicing reaction with freshly prepared DTT solution
	High residual of the fragments A or B still present in after the reaction	The incubation time was too short or temperature was too low or pH was inappropriate	Adjust pH after adding DTT. (Optimal conditions for trans-splicing are 2 h at 37 °C). Alternatively, an overnight reaction could be performed at 4 °C
Removal of the reductant	After removal of the reductant, the samples appear smear on Non-reducing SDS – PAGE	Denaturant of sample loading buffer can destroy cysteine bonds in the presence of residual reductant.	Samples can be stored for a longer period of time before use, to allow auto - oxidation of DTT and re-oxidation of the antibodies

3.6. Removal of the reductant

The removal of the reductant is an important step. Residual reductant left in the system may lead to a partial reoxidation of the antibodies, which results in poor quality and could potentially cause difficulty in downstream purification steps. Dialysis is a robust and scalable process. Hence, we choose overnight dialysis for reductant removal. Procedure is listed as following:

1. Dialyze the sample into reductant removing buffer (10 mM Tris-HCl 500 mM NaCl, pH 8.0) using a Slide-A-Lyzer GD2 (according to the manufacturer's instructions). Be careful not to pierce the membrane when using Slide-A-Lyzer cassettes, to avoid loss of the sample.
2. Dialysis is performed at 4 °C overnight, allowing sufficient time for complete removal of DTT.

3.7. BsAb purification

BsAb is purified from the reaction mix efficiently through affinity purification steps. The fragment A left from the reaction is removed by capturing on Ni-Sepharose affinity column through the His-tag. BsAb is isolated from the rest of reaction mixture by MabSelect affinity chromatography. Fragment B does not bind to MabSelect and is washed away.

1. Before purification, dialyze the sample into HisTrap affinity column binding buffer (20 mM Bis-Tris 500 mM NaCl 30 mM imidazole, pH 6.5) using a Slide-A-Lyzer G2 Dialysis Cassettes.
2. HisTrap affinity chromatography (GE Healthcare) is performed according to the standard protocol. Flow-through fraction containing BsAb product is collected.
3. The collected flow-through is further purified through MabSelect affinity chromatography (GE Healthcare). The antibody is eluted with a 20 CV linear gradient from 0% to 100% of 0.1 M sodium citrate at pH 2.8.
4. The eluate is neutralized immediately, dialyzed overnight against PBS, and filtered over 0.22-μM dead-end filter.
5. Determine the concentration of the BsAb solution by A280 with specific extinction coefficient using NanoDrop.
6. Store the BsAb solution at −80 °C.
7. Characterize the product using analytical methods such as size exclusion HPLC, SDS-PAGE, Ion mobility quadrupole time-of-flight mass and so on.

3.8. Hints for troubleshooting

In general, BAPTS is a straightforward method. Experiment troubleshooting advices are summarized in Table 1.

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Competing financial interests

Y.X, H.J, and J.Z are employees of Jecho Laboratories Inc. J.G. receives compensation from Jecho Biopharmaceuticals Co. Ltd. as an advisor. L.H. and J.Z. are employees of Jecho Biopharmaceuticals Co. Ltd.

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