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# Efficient production of recombinant IL-21 proteins for pre-clinical studies by a two-step dilution refolding method

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ABSTRACT

Produced by $CD4^+$ helper T cells and natural killer T (NKT) cells, interleukin-21 (IL-21) performs broad regulatory functions on B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells and NKT cells. Targeting IL-21 to enhance the immune system has attracted great interests in the development of vaccination, anti-infection and anti-tumor therapies. Administration of IL-21 in pre-clinical models is however limited by relatively high expense of the recombinant IL-21 protein. Here, we report a rapid and cost-effective method to produce IL-21 using *Escherichia coli* (*E. coli*) by introducing a novel two-step dilution strategy for refolding. The method has been validated to produce milligrams of human IL-21, human IL-21/IL-4 chimera and mouse IL-21 with high bioactivities and low endotoxin, mostly suitable for *in vitro* and *in vivo* pre-clinical studies.



## 1. Introduction

Interleukin 21 (IL-21) is a recently discovered type I cytokine and classified in the family, which includes IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15, all share common gamma chain subunit in their receptors [1]. The production of IL-21 is limited to $CD4^+$ T and natural killer T (NKT) cells but the expression of its receptor has been detected on multiple cellular components of the innate and adaptive immune system as well as certain non-hematopoietic cells such as endothelial cells [2]. IL-21 induces B cell proliferation and differentiation [1] and maintains the germinal center response for antibody affinity maturation [3,4]. IL-21 enhances $CD4^+$ T cell proliferation and regulates the development and maintenance of IL-17 producing helper T (Th17) [5,6] and follicular helper T (Tfh) cells [7,8]. IL-21 acts synergistically with IL-15 to promote the proliferation and cytotoxicity of $CD8^+$ T cells [9], and prevents $CD8^+$ T cell exhaustion during chronic infection [10–12]. IL-21 enhances the maturation and promotes the cytolytic function of nature killer (NK) cells [2,13].

The pleiotropic immunostimulatory functions suggest that IL-21 function may be used, or manipulated, for vaccinations, anti-tumor and anti-infection therapies. Indeed, in reported phase II clinical trials on renal cell carcinoma and melanoma, recombinant human IL-21 demonstrated an acceptable safety profile and encouraging activity in its use as a single agent [14]. Though promising, investigating the therapeutic applications of recombinant IL-21 *in vivo* in pre-clinical animal models is limited by relatively high cost of commercial resource of recombinant IL-21. For example, 1 mg recombinant human or mouse IL-21 from Pepprotech (Rocky Hill, NJ, USA) will cost 5200 US dollars, which makes it more desirable to develop an efficient method to produce recombinant IL-21 for pre-clinical *in vivo* studies.

Recombinant IL-21 proteins expressed in *Escherichia coli* (*E. coli*) show similar activities with those produced from mammalian systems [15] and have been used in clinical trials [14]. Here we describe an optimized method for rapid and cost-effective production of recombinant IL-21 using *E. coli*. Compared to previously reported methods [15–17], the step to refold denatured IL-21 proteins solubilized from inclusion bodies (IBs) is modified by using a sequential dilution and dialysis procedure, which helps to achieve high yield, cut down the running time and reduce the cost of reagents. In addition, a simple and efficient strategy to remove endotoxin is incorporated in the method, rendering the feasibility of the final products to be directly used for *in vitro* and *in vivo* studies.

## 2. Materials and methods

### 2.1. Expression of IL-21

The cDNAs for human IL-21 without the secretion signal (NM_021803), mouse IL-21 without the secretion signal (NM_021782) and human IL-21/IL-4 chimera [15] were synthesized by GeneArt (Invitrogen, Carlsbad, CA, USA) and cloned into the pET-28(a) expression vector, with hexaHistidine tag fused at the N-terminus. Rosetta competent cells (Novagen, Madison, WI, USA) were transformed with IL-21 expression vectors and cultured at 37 °C in LB media with 50 μg/mL Kanamycin. When the optical density (OD) at 600 nm of the cell culture reached 0.6–0.8, 1 mM isopropylthio-β-galactoside (IPTG) was added to induce protein expression and the cell culture was incubated for 3 h.

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### 2.2. Harvest of inclusion bodies, removal of endotoxin and solubilization

Cultured cells were pelleted by centrifugation (4000 *g*, 15 min) and resuspended in the lysis buffer (0.1 M Tris–HCl, pH 7.5, 1 mM EDTA) at 4 °C. Lysozyme (1.5 mg/g of pellets) was added and incubated for 30 min. After sonication and incubation in the buffer A (0.5 M NaCl, 10 mM EDTA, 2% Triton X100) for 30 min, inclusion bodies (IBs) were pelleted by centrifugation (30,000 *g*, 10 min) and resuspended in the buffer B (PBS pH 7.4, 1% Triton X-114). Endotoxin was removed by repeating the following cycle for three times: incubation (4 °C, 30 min), heating (56 °C, 10 min), centrifugation (30,000 *g*, 10 min) and collection of pellets [18]. Processed IB pellets were washed with PBS, resuspended in the solubilization buffer (PBS pH 7.4, 6 M guanidine hydrochloride (Gdn-HCl)) and stirred at room temperature till solution came clear. After centrifugation (30,000 *g*, 10 min) to remove insoluble debris, the solution of denatured proteins was filtered (0.45 μm) and loaded into Nickel-affinity chromatography (Qiagen, Valencia, CA, USA). The column was washed with 40 volumes of pre-chilled buffer C (PBS pH 5.3, 8 M urea, 0.1% triton X114) to further remove endotoxin [19]. After wash with 20 volumes of the buffer D (PBS pH 5.3, 8 M urea) to remove the detergent, purified denatured IL-21 proteins were eluted by the buffer E (PBS pH 3.2, 8 M urea).

### 2.3. In vitro refolding of IL-21

After concentration to 10 mg/mL (approximately 500 μM), denatured IL-21 proteins were added into 50 volume of the refolding buffer (PBS pH 8.5, 1 M Gdn-HCl, 500 μM GSSG, 5 mM GSH, 0.7 M L-Arg) by drop wise (0.5 mL/min) under rapid stirring. After incubation for 6–12 h, 50 volumes of the buffer F (PBS pH 8.5, 0.7 M L-Arg) were added by drop wise (0.5 mL/min) and incubated for 6–12 h. L-Cystine (1.6 mM) was then added to stop disulfide shuffling. Denaturant and L-Arg were removed by dialysis against PBS. After filtration to remove the precipitation, protein solution was concentrated to an appropriate volume. His-tag was cleaved by incubation with Presscion protease (GE healthcare, Pittsburgh, PA, USA) for overnight.

### 2.4. Purification by gel filtration

IL-21 proteins were further purified by gel filtration through Superdex 200 column (GE healthcare) and AKTA FPLC system (GE healthcare) with a flow rate of 0.6 mL/min.

### 2.5. SDS-PAGE and western blotting

SDS-PAGE was performed with commercial Nu-PAGE system (Invitrogen, Carlsbad, CA, USA). Western blotting was performed with monoclonal antibody of human IL-21 (3A3-N2, ebioscience, San Diego, CA, USA) and horseradish peroxidase-conjugated goat anti-mouse Fc fragment antibody (Invitrogen). Signal detection was preformed with Supersignal west pico substrate (Pierce, Rockford, IL, USA). Recombinant human IL-21 from a commercial supply (Peprotech) was used as a control. Images were analyzed using the Quantity-One software (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.6. Evaluation of endotoxin levels

Endotoxin levels were measured with Limulus amebocyte lysate (LAL) detection kit (GeneScript, Piscataway, NJ, USA) according to the manufacturer's introductions.

### 2.7. Bioactivity assay

Ba/F3 is a murine IL-3 dependent pro-B cell line and has been widely used in drug discovery [ref]. Coding sequences for human IL-21R (huIL-21R) and mouse IL-21R (msIL-21R) were cloned by PCR from human or mouse cDNA libraries respectively, sequenced to match NM_021798 and NM_021887, and cloned into a bicistroinc GFP-expression retroviral vector [20]. $GFP^+$ Ba/F3-huIL-21R and Ba/F3-msIL-21R were sorted by fluorescence-activated cell sorting using BD Influx cell sorter (BD, NJ, USA) and were cultured in the full RPMI-1640 media [21] in the presence of human IL-21 or mouse IL-21 respectively in a humidified chamber with 5% $CO_2$ at 37 °C. For [$^3$H]-labeled thymidine incorporation assay to measure the cell proliferation, Ba/F3-huIL-21R or Ba/F3-msIL-21R cells were starved in the cultured media without cytokine for 18 h and then were incubated for 72 h with the addition of 1 μCi/well [$^3$H]-thymidine (Perkin Elmer, Waltham, MA, USA) for the last 18 h of culture in the presence of cytokines with indicated concentrations. Cells were harvested onto filter mats (Perkin Elmer) and incorporated radioactive nucleic acids counted using a Top Count NXT Scintillation Counter (Packard Biosciences, Meriden, CT, USA). Results were expressed as mean count per minute (CPM) for triplicate cultures.

### 2.8. Statistical analysis

Statistical analysis was performed with the Student's t test (for comparison between 2 groups) GraphPad Prism software (GraphPad, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

## 3. Result

### 3.1. High refolding efficiency using a new two-step dilution strategy

Eukaryotic gene expressed in the prokaryotic *E. coli* expression system may lead to the formation of IBs, which is the case for IL-21 [16]. Solubilizing the IBs in proper denaturants (usually urea or Gdn-HCl) and refolding the denatured proteins into their native states are necessary to examine the function of IL-21 in pre-clinical studies. In previously published methods for the IL-21 refolding, Asano R. et al. reported a dialysis strategy which achieved a high refolding efficiency but the time and reagent cost were high. The step-wise dialysis took 6–12 h for each of six dialysis steps (36–72 h in total). And a volume of 5 L dialysis buffer for 10 mg of IBs was required for each step (30 L in total) [16]. Another refolding strategy reported by Bondensgaard K. et al. [15] and Lee C. et al. [17] using a dilution method, although only took 12–24 h and consumed 100 mL of the refolding buffer, reached a low refolding efficiency [17].

After designing a new strategy for the refolding process, we developed a new method to efficiently produce recombinant IL-21 proteins with a low endotoxin level, thus suitable for *in vitro* and *in vivo* pre-clinical studies (Fig. 1A). To compare our method with the other three previously published methods for the refolding efficiency, 1 mL of denatured human IL-21 proteins (10 mg/mL) was refolded using different methods. The products were concentrated to 1 mL, same volume as before refolding, which allows the side-by-side comparison to evaluate the refolding efficiency. As shown in the SDS-PAGE gel (Fig. 2), the step-wise dialysis method reported by Asano R. et al. [16] achieved a higher refolding efficiency (50%) than those using two different dilution methods (20% [15] and 7% [17], respectively). Our method, also applying a new two-step dilution strategy for refolding (Fig. 1A), yielded the same if not higher refolded proteins (52%) as the method by Asano R. et al.

The human IL-21 proteins refolded using the two-step dilution strategy have been proven stable. Precipitation, usually due to incorrect refolding [22], was not observed when the sample was kept at 4 °C for one week or after a −80 °C freeze-thaw cycle. Importantly, the same two-step dilution strategy for refolding was used to produce mouse IL-21 and the IL-21/IL-4 chimera, a human IL-21 derivative with higher bioactivity than the wildtype human IL-21 [15] (Fig. 3).

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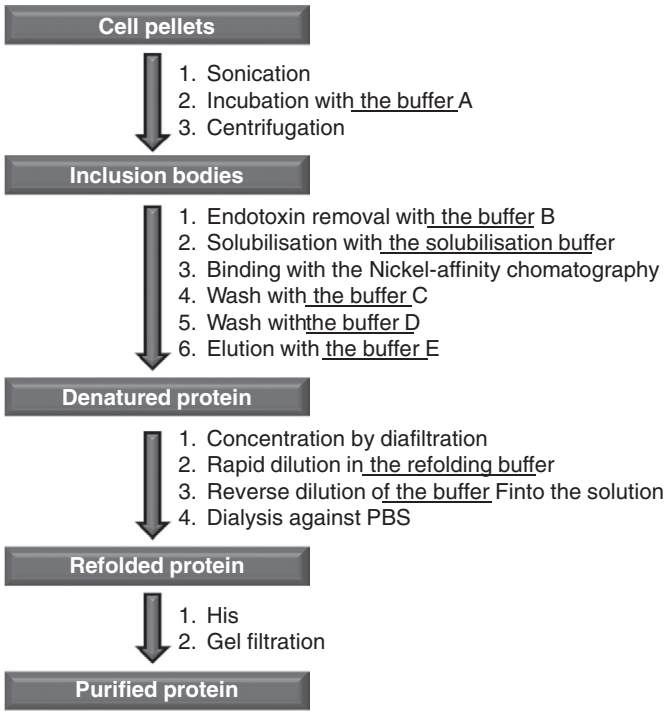
A




B

| Refolding Method | Refolding Efficiency | Time Expense (hours) | Reaction Volume (mL) |
|---|---|---|---|
| Step-wise dialysis [16] | ~50% | 36-72 | 5000 |
| Dilution [15] | ~20% | 12-24 | 100 |
| Dilution [17] | <10% | 12-24 | 100 |
| Two-step dilution | ~50% | 18-36 | 100 |

**Fig. 1.** Summary of the new production method (A) workflow of the production procedure. (B) Comparison of the refolding efficiency, the time and reagent cost between the published methods and the new method.

*3.2. Low time and reagent cost using the new two-step dilution strategy for refolding*

The published refolding method using the step-wise dialysis for the IL-21 production [16] had a similar high refolding efficiency of more than 50% as our two-step dilution strategy (Fig. 2). However, the six-step protocol took 6–12 h to dialyze and consumed 5 L of dialysis buffer for each step [16]. In total, 36–72 h and 30 L of dialysis buffer are required for the step-wise dialysis method. In contrast, the newly designed two-step dilution method, when used to process same amount of denatured proteins for refolding, requires only two dilution steps, followed by a dialysis against PBS (Fig. 1A). Each dilution step took 6–12 h to dilution and consumed a small volume of 50 mL of dilution buffer for each step. To summarize, the running time is cut down to 18–36 h and the consumption of the reagent is dramatically reduced by about 50 fold by our new two-step dilution strategy for refolding (Fig. 1B).

*3.3. Incorporation of the endotoxin-removal step during the purification*

Denatured human IL-21 proteins were firstly purified by the Nickel-affinity chromatography, followed by the refolding step using our new two-step dilution strategy to restore the native conformation. After a further purification by gel filtration, a purity of 95% was achieved, demonstrated by the FPLC profile (Fig. 4). However, a significant level of endotoxin was detected in this already purified IL-21 product (data not shown), which was a common contamination from recombinant proteins expressed from the *E. coli* system. IL-21 proteins containing this level of endotoxins, predominantly in the form of lipopolysaccharide (LPS), are not suitable for investigating its immunological functions since the undesirable false results might be caused by LPS, which induces broad effects on immune cells *in vitro* and *in vivo* [23]. Triton X-114 has been reported to effectively extract LPS through phase separation [24]. To remove the endotoxin contamination, Triton X-114 was added before IB solubilisation and in the washing buffer during the Nickel-affinity chromatography (Fig. 1A). The endotoxin-removal steps reduced the endotoxin levels in the final products below 1 EU/mg of IL-21 protein (data not shown), which is far below the commercial grade for recombinant proteins at 1 EU/μg of protein.

*3.4. Validated bioactivity of recombinant IL-21 proteins*

In addition to the high purity of the recombinant protein shown in the FPLC profile (Fig. 4), the recombinant human IL-21 proteins, with or without His-tag, were both specifically recognized by an anti-human IL-21 monoclonal antibody in western blotting, same as the recombinant human IL-21 from a commercial supply (Fig. 5A). The recombinant human IL-21 protein without His-tag, showing bands slightly below the

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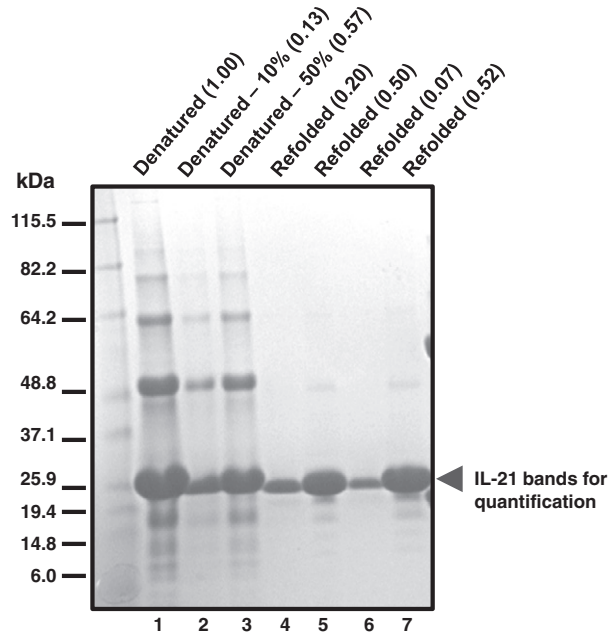


**Fig. 2.** High refolding efficiency using a new two-step dilution strategy. SDS-PAGE gel showing denatured human IL-21-His proteins (lane 1–3) before refolding and refolded human IL-21-His proteins (lane 4–7). Proteins were quantified using Quantity One software. The ratios relative to the denatured protein (Lane 1) were calculated and reflected the refolding efficiencies. Lane 1, denatured protein. Lane 2, denatured protein (1:5 dilution). Lane 3, denatured protein (1:2 dilution). Lane 4, refolded proteins using the dilution method [15]. Lane 5, refolded protein using the step-wise dialysis method [16]. Lane 6, refolded proteins using the dilution method [17]. Lane 7, refolded proteins using the new two-step dilution method.

band of 19.4 kDa of the protein ladder, had a similar protein weight as the commercial product. The recombinant human IL-21 protein with His-tag was bigger in size.

The produced recombinant IL-21 proteins were then tested for bioactivities in an IL-21-dependent cell proliferation assay. The concentration of recombinant IL-21 produced was quantified by Bradford assay based on BSA standard curve (data not shown) and comparable to the IL-21 protein purchased from the commercial supply. SDS-PAGE showed similar band sizes between home-made and commercial human IL-21

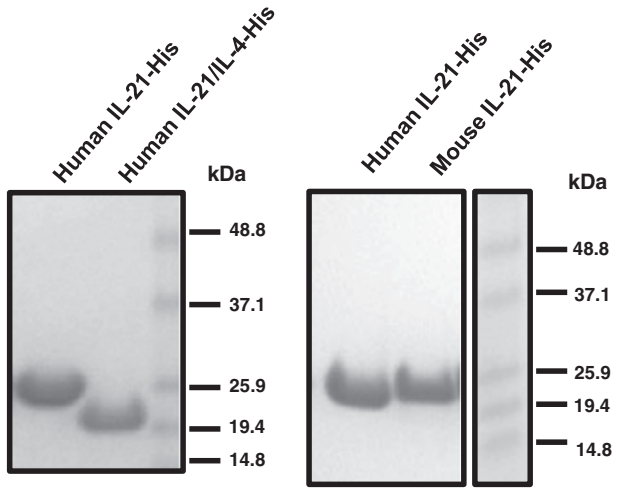


**Fig. 3.** The production of human IL-21/IL-4 and mouse IL-21 proteins using a new two-step dilution strategy. SDS-PAGE gel showing refolded human IL-21/IL-4-His protein (left panel) and mouse IL-21-His protein (right panel) using the new two-step dilution method. Human IL-21-His protein was used as a control.

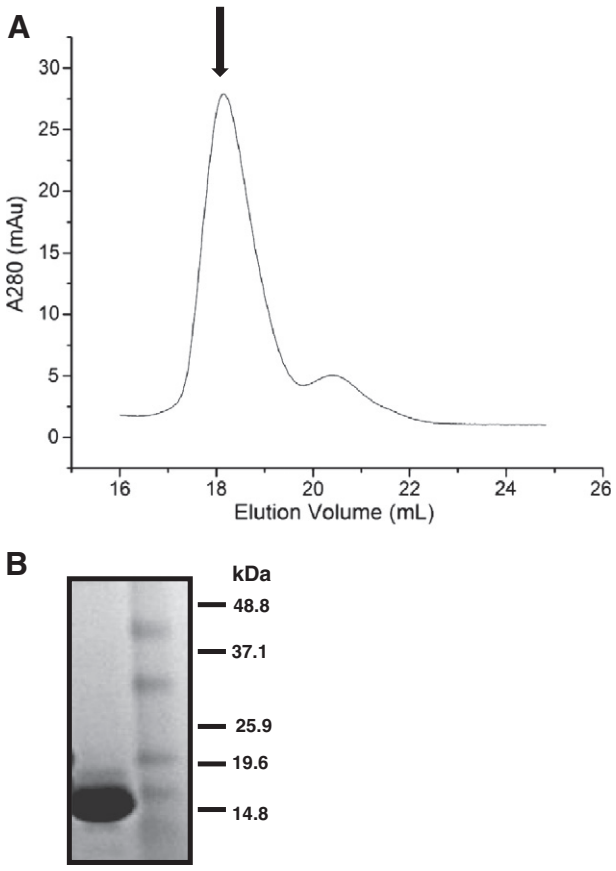


**Fig. 4.** High purity of the human IL-21 protein. (A) Profile of Gel filtration. The IL-21 fraction was indicated by the arrow. (B) SDS-PAGE gel showing the purified human IL-21 protein (without His-tag).

protein at the concentration of 0.1 mg/mL (Fig. 5B). In various concentrations tested, home-made and commercial human IL-21 displayed similar bioactivities to induce the proliferation of Ba/F3-huIL-21R cells (Fig. 5C). Home-made human IL-21 undergone one −80 °C freeze-thaw cycle remained a similar bioactivity (Fig. 5C). The bioactivity of recombinant mouse IL-21 protein was also validated by inducing the proliferation of Ba/F3-msIL-21R cells to the similar levels as commercial mouse IL-21 protein at various concentrated tested (Fig. 5D). Interestingly, human IL-21 with a His-tag fused to the N-terminus showed a reduced bioactivity (Fig. 5E), suggesting the step for the His-tag cleavage is necessary to produce intact IL-21 for pre-clinical studies.

## 4. Discussion

Compared to previously published methods to produce IL-21, the method reported here demonstrates a major improvement for the refolding process.

Theoretically, an effective refolding strategy needs to ensure that the unfolded and flexible proteins, which have been solubilized in denaturant, can refold into the native conformation once the denaturant is removed. In light of the size (15–20 kDa) of IL-21 and its structural characteristics containing two disulfide bonds [15], denatured IL-21 conformation may experience the intermediate state during the refolding process. Therefore, a high refolding efficiency was achieved by the step-wise dialysis strategy [16]. The rapid dilution, as another commonly used method for refolding, although overcomes the high time and reagent cost for the step-wise dialysis, led to misfolding and aggregation and significantly reduced the refolding efficiency [15,17].

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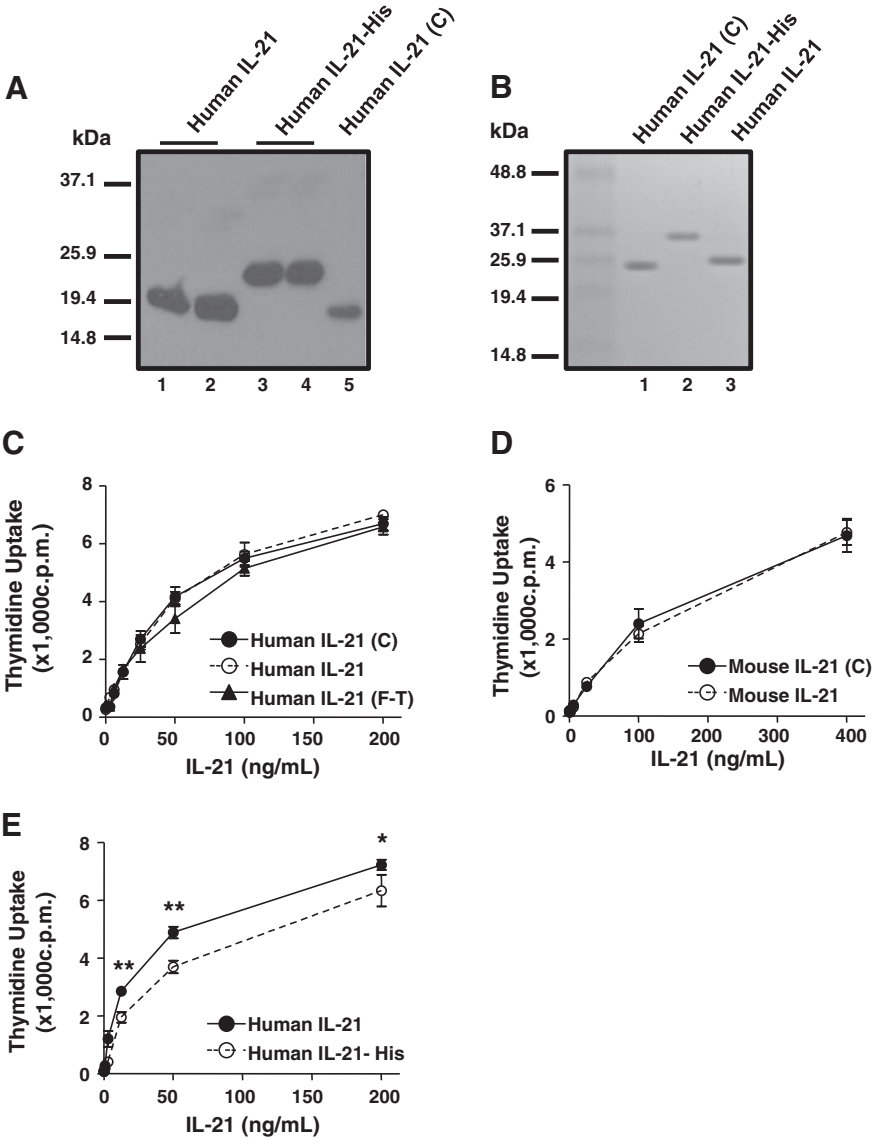


**Fig. 5.** Validation of the bioactivities of the recombinant proteins. (A) Western blot showing produced human IL-21 (Lane 1, 2), produced human IL-21-His (Lane 3, 4) and human IL-21 from the commercial supply (Peprotech) (Lane 5) blotted with the anti-human IL-21 antibody. (B) SDS-PAGE gel showing 1 μg human IL-21 from the commercial supply (Peprotech) (Lane 1), 1 μg produced human IL-21-His (Lane 2) and 1 μg produced human IL-21 (Lane 3) used in the bioactivity assay. (C–E) Ba/F3-huIL-21R (C, E) or Ba/F3-msIL-21R (D) was cultured in the presence of human IL-21 (C, E) or mouse IL-21 (D) respectively at indicated concentrations. [$^3$H]-labeled thymidine was added for the last 18 h of culture. Incorporated radioactivities were measured and presented as mean ± standard deviation. C.P.M.: count per minute. (C): proteins from the commercial supply (Peprotech). (F-T): −80 °C freeze-thaw cycle. **: p value < 0.01. *: p value < 0.05.

We inherit the recipe of the refolding buffer with 1 M Gdn-HCl in a proper redox condition (GSSG and GSH) with additives (L-Arg), which has been shown to be highly efficient environment for the formation of native disulfide-bond within IL-21 [16]. Instead of using a step-wise dialysis in buffers reducing Gdn-HCl from 6 M to 1 M [16], we chose to dilute denatured IL-21 proteins in a drop-wise manner directly into the refolding buffer containing 1 M Gdn-HCl (Fig. 1A). This process also limits the protein concentration under 10 μM and prevents undesirable protein aggregations meanwhile the concentration of denaturant (Gdn-HCl) was retained. An obvious benefit using the new two-step dilution strategy is to significantly reduce the time and reagent cost (Fig. 1B). The new two-step dilution strategy is applicable to produce various IL-21 and its derived proteins we have tested (Fig. 3).

The rapid and cost-effective method has successfully produced milligrams of human IL-21, human IL-21/IL-4 chimera and mouse IL-21 with superb bioactivities and low endotoxin, suitable for many *in vitro* and *in vivo* pre-clinical studies. It may also be able to be applied to produce other proteins, especially cytokines with similar characteristics to IL-21.

## Contributions

Z.C. performed most experiments, analyzed the data and wrote the manuscript. Y.C. supervised the study and edited the manuscript. Y.A.L. helped on the experiments and edited the manuscript. D.Y. supervised the study, designed experiments and wrote the manuscript.

## Competing financial interests

The authors declare no competing financial interests.

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