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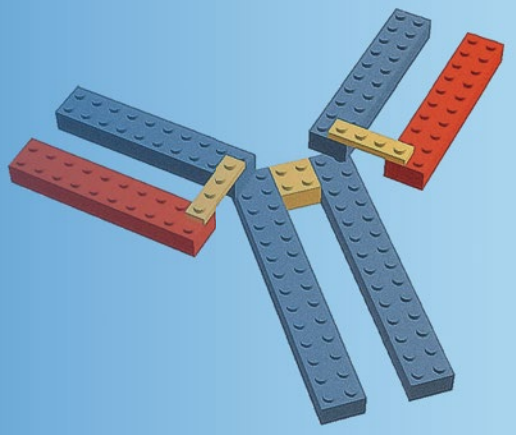

Vincent Ossipow
Nicolas Fischer *Editors*

Monoclonal Antibodies

Methods and Protocols

Second Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Edited by

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Preface

Over the past decades, monoclonal antibodies (monoclonals) have become invaluable for basic research, diagnostics, clinicians, and thousands of patients suffering from severe afflictions. Monoclonals are widely used laboratory reagents, and it is fair to say that the availability of a good monoclonal has led to increased understanding of the target biology as many experimental approaches, ranging from classical western blots to chromatin immnunoprecipitation assays, are enabled. Beyond basic research, therapeutic monoclonals are increasingly used as drugs and will account for over 50 billion USD in sales in 2013, and this figure is forecast to grow at double-digit rate, higher than any other therapeutic class. Since OKT3 (Muromonab-CD3, Johnson & Jonson/Ortho Biotech), the first monoclonal approved for human use, 34 other monoclonals have been approved. This is the tip of the iceberg, as it is estimated that over 400 monoclonals are in clinical development worldwide.

This brisk success is explained by several factors. First and most importantly, their specificity and low off-target toxicity often provide monoclonals with an exceptional "therapeutic window," i.e., the ability to dose them effectively with acceptable side effects. Monoclonals are also embraced because of their prolonged half-life and, more generally, as their pharmacokinetic properties are more predictable than for other classes of drugs. Consequently, the rate of success for the clinical development of monoclonals is significantly higher than for small molecule drugs or for other biologicals.

With the progress of genetic and protein engineering, academic labs and the biopharmaceutical industry have advanced many novel antibody formats and antibody-based approaches. These comprise multi-specific antibodies, fragments, antibody–drug conjugates, antibodies with enhanced effector function, and so on. However, despite these evolutions, being able to generate high-quality monoclonals against carefully selected epitopes remains the absolute foundation for subsequent improvements. Equally important is the meticulous characterization of candidate monoclonals. We actually contend that because of the greatly expanded toolkit for improving monoclonals, epitope selection and biochemical characterization has now become even more important for generating well-differentiated monoclonals. One can directly witness this notion at the bench with antibodies raised against the same target that perform very differently in various assays but also in the clinic, where monoclonals against different epitopes can exert very different responses in patients (e.g., the three anti-CD20 monoclonals Rituximab, Ofatumumb, and GA-101 by Roche, Genmab/GSK, and Roche respectively).

The purpose of this new edition of "Monoclonal Antibodies, Methods in Molecular Biology" is to offer modern approaches to indeed generate high-quality monoclonals against carefully selected epitopes, and meticulously characterize them. With a few exceptions, we deliberately concentrated on the basic IgG format. All the key steps from antigen generation to some final applications are described in these 36 chapters and should provide the reader with multiple useful methods to generate an appropriate monoclonal. We divided the book into four parts corresponding to four distinct objectives. Part I covers monoclonal

antibody generation, Part II deals with monoclonal antibody expression and purification, Part III presents methods for monoclonal antibody characterization and modification, and Part IV describes some applications of monoclonal antibodies. For each Part we strived to balance "must-have" protocols and recent innovative approaches, all "debugged" in the author's laboratories. In Part I, we included, for instance, protocols for the generation of monoclonals using natural sources such as mouse, rabbit, or immortalized human B-cells, but also in vitro selection methodologies such as phage and yeast display as well as antibody repertoire mining by deep sequencing. In Part II, several approaches are proposed for downstream purification of IgG as well as some alternative formats that should satisfy different requirements and downstream uses. In Part III, epitope mapping with various astute technologies such as phage- or bacterial display or extensive mutagenesis are well covered, as well as strategies for examining the primary sequence and structural and physicochemical properties of monoclonals. The latter are often overlooked but are important, as experiments performed with aggregated or unstable monoclonals can lead to erroneous conclusions. In Part IV, we provide some examples of use of monoclonals including immunofluorescence, crystallization chaperoning and the generation of solid-state arrays.

By no means is our selection of protocols exhaustive, a task impossible within the context of such a book. On the other hand, some topics are covered in more than one chapter, providing alternatives for the readers to select the most appropriate method for her/his use. We hope that our protocol choice will fulfill its intended goal of covering the crucial initial steps of monoclonal antibody generation and characterization with state-of-the art protocols.

Geneva, Switzerland — *Vincent Ossipow*
Plan-les-Ouates, Switzerland — *Nicolas Fischer*

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Part I

Antibody Generation

Chapter 1

Antigen Production for Monoclonal Antibody Generation

Giovanni Magistrelli and Pauline Malinge

Abstract

The quality of the target antigen is very important in order to generate a good antibody, in particular when binding to a conformational epitope is desired. The use of mammalian cells for recombinant protein expression provides an efficient machinery for the correct folding and posttranslational modification of proteins. In this chapter, we describe a process to rapidly generate semi-stable human cell lines secreting a recombinant protein of interest into the culture medium. Simple disposable bioreactors that can be used in any standard cell culture laboratory enable the production of recombinant protein in the multi-milligram range. The protein can be readily purified from the culture supernatant by immobilized metal affinity chromatography. In addition, by inserting a tag recognized by a co-expressed biotin ligase, the protein can be biotinylated during the secretion process. This greatly facilitates the immobilization of the protein for assay development or for antibody isolation using in vitro selection technologies.

Key words CELLine bioreactor, Protein purification, IMAC, Single site biotinylation, Episomal vector, Semi-stable cell line

1 Introduction

Having access to pure antigen in sufficient quantities is a first and important step in the development of a monoclonal antibody (mAb). As access to the native protein is usually limited, recombinant protein expression and purification is a method of choice to generate the antigen, although alternative approaches, such as the use of cells or DNA for immunization, can also be envisaged [1–3]. When the objective is to generate antibodies that are biologically active or recognize the antigen in its native state, it is particularly important to generate a properly folded protein that closely mimics the structure of the native protein [4].

Three types of cells are commonly used for recombinant protein production: bacteria (*Escherichia coli*), insect cells and mammalian cells. The choice of an expression system can be guided by the structure and origin of the protein to be expressed, the presence of disulfide bonds or if posttranslational modifications are important for its functionality. The recombinant protein can be expressed

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either in the cytoplasm or can be secreted in the culture medium by the addition of an appropriate signal peptide. In addition, the antigen can be expressed as a fusion with a peptide or a protein that can increase its solubility [5–10]. Protein tags, such as the Fc of an antibody or a hexahistidine sequence, are also routinely included at one extremity of the protein to facilitate purification using affinity chromatography. All these available options enable the design and testing of a large number of expression constructs.

In this chapter we describe the use of an episomal expression system for the rapid generation of semi-stable human cell lines secreting a recombinant protein of interest into the medium [11]. We have found this approach very versatile and it often allowed for the successful expression of soluble recombinant protein in the milligram range, even for challenging eukaryotic proteins. This system combines several elements that facilitate and accelerate the overall process.

First, the gene encoding the protein of interest is cloned into an expression vector capable of autonomous episomal replication that can be maintained within cells without integration into the genome. The vector also contains a puromycin resistance gene for efficient selection of transfected cells and for the establishment of semi-stable pools of cells harboring the expression vector. The multi-cistronic design of the expression construct enables the simultaneous expression of multiple proteins. The protein of interest is secreted into the medium and carries a hexahistidine tag for single step purification and an AviTag™ for single site biotinylation. The internal ribosome entry site (IRES) enables the co-expression and secretion of biotin ligase (LsBirA) driving site-specific biotinylation and enhanced green fluorescent protein (EGFP) expression for easy monitoring of the transfection efficiency and the expression within a cell pool [12]. Single site-biotinylation on the AviTag™ facilitates protein immobilization and detection using streptavidin based reagents [13]. Second, the use of two-compartment bioreactors (CELLine) allow for a straightforward scale-up of production using equipment found in any standard cell culture laboratory [14]. The benefit of this system is that the two compartments are separated by a membrane that allows nutrients and waste products to exchange. In this way serum-containing medium can be used in the main compartment to support optimal cell growth and viability, without contamination of the cell compartment with bovine serum albumin (BSA) and bovine IgG, facilitating the purification process. In addition, the protein can be recovered from the culture in a small volume avoiding the need for concentration steps before purification. Finally, an optimized protocol for affinity purification via immobilized metal ion affinity chromatography (IMAC) enables efficient purification of the recombinant protein in a single step from the mammalian cell culture supernatant.

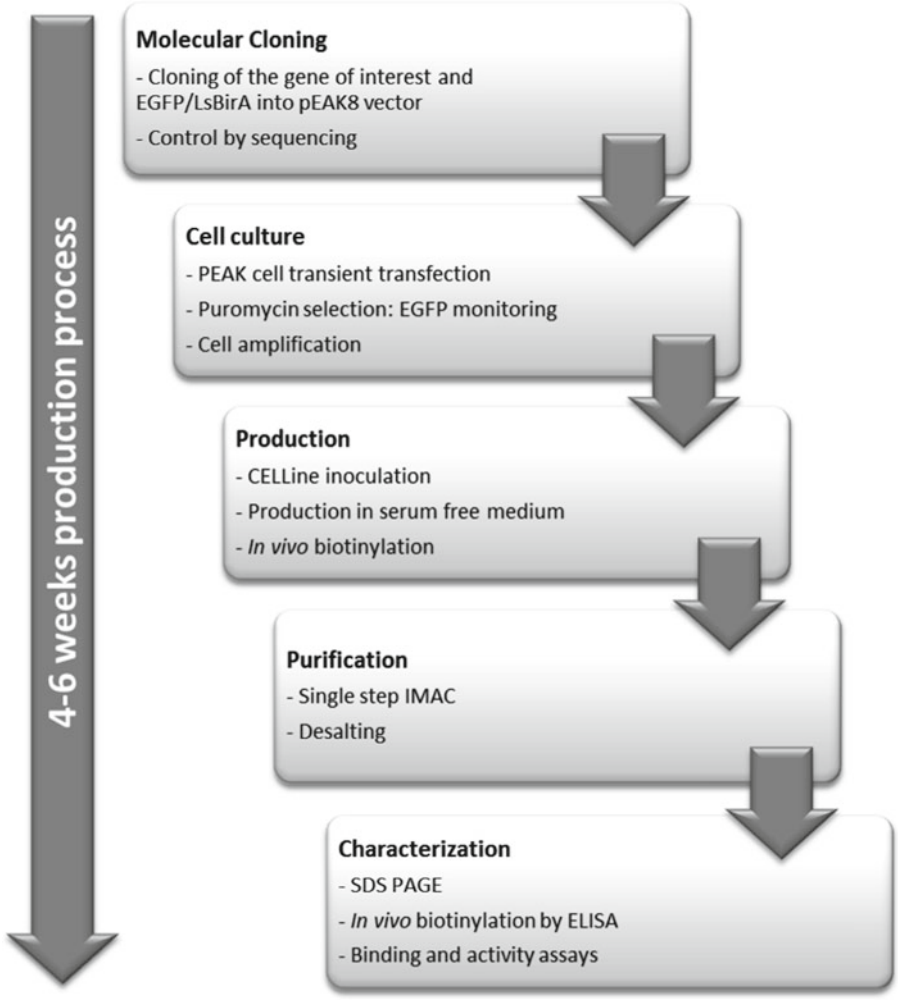

Fig. 1 Schematic representation of the overall process

Over 20 human and mouse recombinant proteins have been expressed and purified using this approach, greatly facilitating the production and characterization of monoclonal antibodies. The overall process is schematically represented in Fig. 1.

2 Materials

All reagents and kits must be used and stored as described by the manufacturer instructions. Experiments using cells are performed under a sterile hood and chemical reagents are manipulated under a fume hood when necessary.

2.1 Molecular Cloning

1. pEAK8 plasmid (Edge Biosystems).
2. pVector IRES EGFP and pVector IRES LsBirA IRES EGFP plasmids (available upon request from the authors).
3. *Hin*dIII, *Eco*RI, *Xba*I, *Mfe*I, and *Nhe*I restriction enzymes and appropriate buffers (New England Biolabs) (*see* **Note 1**).
4. Rapid DNA Dephos & Ligation Kit (Roche).
5. E-Gel® Agarose gels and power-pack (Invitrogen).
6. E-Gel® 1 Kb Plus DNA ladder (Invitrogen).
7. MinElute® PCR purification Kit (Qiagen).
8. MinElute® Gel Extraction Kit (Qiagen).
9. QIAprep Spin Miniprep Kit (Qiagen).
10. XL1-Blue competent *E. coli* cells.
11. Ampicillin (AppliChem).
12. Terrific Broth (TB) medium (Invitrogen): Dissolve 47 g of TB powder in 1 L of demineralized water; add 4 mL of glycerol; autoclave for 15 min at 121 °C.
13. LB-Amp agar plates: Dissolve 10 g of Bacto™ Peptone (Becton Dickinson), 10 g of NaCl, 5 g of Bacto™ Yeast (Becton Dickinson), and 15 g of Bacto™ Agar (Becton Dickinson) in 950 mL of deionized H_2O; adjust the pH to 7.5 by adding 10 mM NaOH; adjust the volume of the solution to 1 L. Sterilize by autoclaving for 20 min at 121 °C and let cool in a water bath at 55 °C for 30–60 min; add ampicillin at a final concentration of 100 μg/mL; distribute 20 mL of medium per petri dish and let solidify at room temperature (RT). Store the plates at 4 °C.
14. Shaking Incubator.
15. UV imaging system.
16. Temperature controlled water bath.
17. Scalpel.
18. Thermomixer.
19. NanoDrop® ND-1000 Spectrophotometer (Witec AG) or equivalent spectrophotometer.
20. Sequencing primers: Forward primer 5′ TGCGATGGAG TTTCCCCACACTG 3′ and Reverse primer 5′ CACCCGGG CAGACCTGAGGAAGAGATG 3′.

2.2 Cell Culture, Transfection, and Selection

1. Transformed human embryo kidney monolayer epithelial cells (PEAK cells; Edge Biosystems) (*see* **Note 2**).
2. Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich).
3. Fetal Bovine Serum (FBS; Sigma-Aldrich).
4. L-glutamine solution 200 mM (Sigma-Aldrich).

5. Gentamicin solution 50 mg/mL (Sigma-Aldrich).
6. Complete DMEM: DMEM supplemented with 10 % of FBS; 2 mM L-glutamine; 50 μg/mL of gentamicin.
7. 6-Well cell culture plates (Multidishes Nunclon™ Δ Surface, Nunc).
8. ThermoForma Steri-Cycle CO_2 incubator (Thermo Scientific).
9. TransIT®-LT1 transfection reagent (Mirus).
10. Puromycin dihydrochloride solution 10 mg/mL (Sigma-Aldrich).
11. Fluorescence microscope (Axiovert 40CFL; Zeiss).
12. FACS (FACSCalibur; BD Biosciences).
13. Phosphate Buffered Saline (PBS; Sigma-Aldrich).
14. Bovine Serum Albumin (BSA; Sigma-Aldrich).
15. Vi-CELL XR counter (Beckman Coulter) or equivalent cell counter.
16. Dimethyl sulfoxide (DMSO; Sigma-Aldrich).
17. Freezing medium: FBS; 10 % DMSO.
18. Biobanking and cell culture cryogenic tubes (Nunc).
19. Cool-box: CoolCell® Cryopreservation cell freezing container (VWR).

2.3 Protein Production

1. Serum-free medium: 293 SFM II liquid medium (Gibco).
2. CELLine 1000 bioreactor (CL1000; Integra) (*see* **Note 3**).
3. Biotin solution 5 mM (Avidity).
4. TC Flask 175 CM2 SI Filter cap (Thermo Scientific).
5. TC Flask 80 CM2 SI Filter cap N (Thermo Scientific).

2.4 Protein Purification and Desalting

1. Stericup filter unit with a 0.22 μm filter (Millipore).
2. Imidazole powder (Sigma-Aldrich).
3. Binding/Washing buffer: PBS; 20 mM imidazole; pH 7.4; filtered on a Stericup filter unit.
4. Elution buffer: PBS; 400 mM imidazole pH 7.4; filtered on a Stericup filter unit.
5. Ni-NTA Superflow Cartridge, 5 mL (Qiagen).
6. ÄKTA Prime chromatography system (GE Healthcare) or equivalent.
7. PrimeView 5.0 software (GE Healthcare).
8. 5 mL Polystyrene round-bottom tubes (BD Falcon™).
9. Amicon filter device: Ultra-4 PLTK Ultracel-PL membrane (Millipore) (*see* **Note 4**).
10. Siliconized tubes (Sigma-Aldrich) (*see* **Note 5**).

2.5 Protein Characterization

2.5.1 Proteins Analysis by Gel Electrophoresis

1. Electrophoresis Novex mini-cell (Invitrogen).
2. NuPAGE® 4–12 % Bis-Tris Mini Gel (Invitrogen).
3. NuPAGE® MES SDS Running Buffer (Invitrogen).
4. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).
5. NuPAGE® LDS Sample Buffer (Invitrogen).
6. β-Mercaptoethanol (Sigma Aldrich) (*see* **Note 6**).
7. Power supply (Fischer Scientific).
8. SimplyBlue™ SafeStain (Invitrogen).

2.5.2 Capture ELISA for Biotinylation Analysis

1. Tween 20 (Sigma-Aldrich).
2. PBST: PBS; 0.05 % Tween 20.
3. PBST–BSA: PBST; 1 % BSA.
4. StreptaWell transparent 96-well microplate (Roche).
5. Antigen specific mouse monoclonal antibody (*see* **Note 7**).
6. Horseradish Peroxidase (HRP)-coupled goat anti-mouse IgG antibody (Jackson ImmunoResearch).
7. Penta-His HRP Conjugate (Qiagen).
8. 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich).
9. Sulfuric acid H_2SO_4 2 N (Sigma-Aldrich).
10. Microplate reader.

3 Methods

3.1 Molecular Cloning

The molecular cloning approach relies on the simultaneous ligation of two DNA fragments into the episomal pEAK8 vector: (a) a DNA fragment encoding the recombinant protein to be expressed; (b) a DNA fragment containing either one or two IRES for the co-expression of EGFP alone or EGFP and LsBirA. The IRES containing expression cassettes are easily retrieved by restriction enzyme digestion from existing plasmids. The cloning strategy to generate the final expression constructs is schematically described in Fig. 2.

3.1.1 Preparation of the DNA Fragment Encoding the Protein of Interest

1. Generate a DNA fragment encoding the protein of interest, either by gene synthesis or by PCR amplification. Include a leader sequence for secretion into the medium as well as a hexahistidine tag at the N or C-terminus. An AviTag™ sequence can also be included if single site biotinylation is desired. Also include *Hin*dIII and *Nhe*I restriction sites at the 5′ and 3′ ends of the DNA fragment, respectively (*see* **Notes 8** and **9**).
2. Digest the DNA fragment with *Hin*dIII and *Nhe*I: Mix 5 μL of NEBuffer 2 10×; 0.5 μL of BSA 100×; 1 μg of DNA fragment;

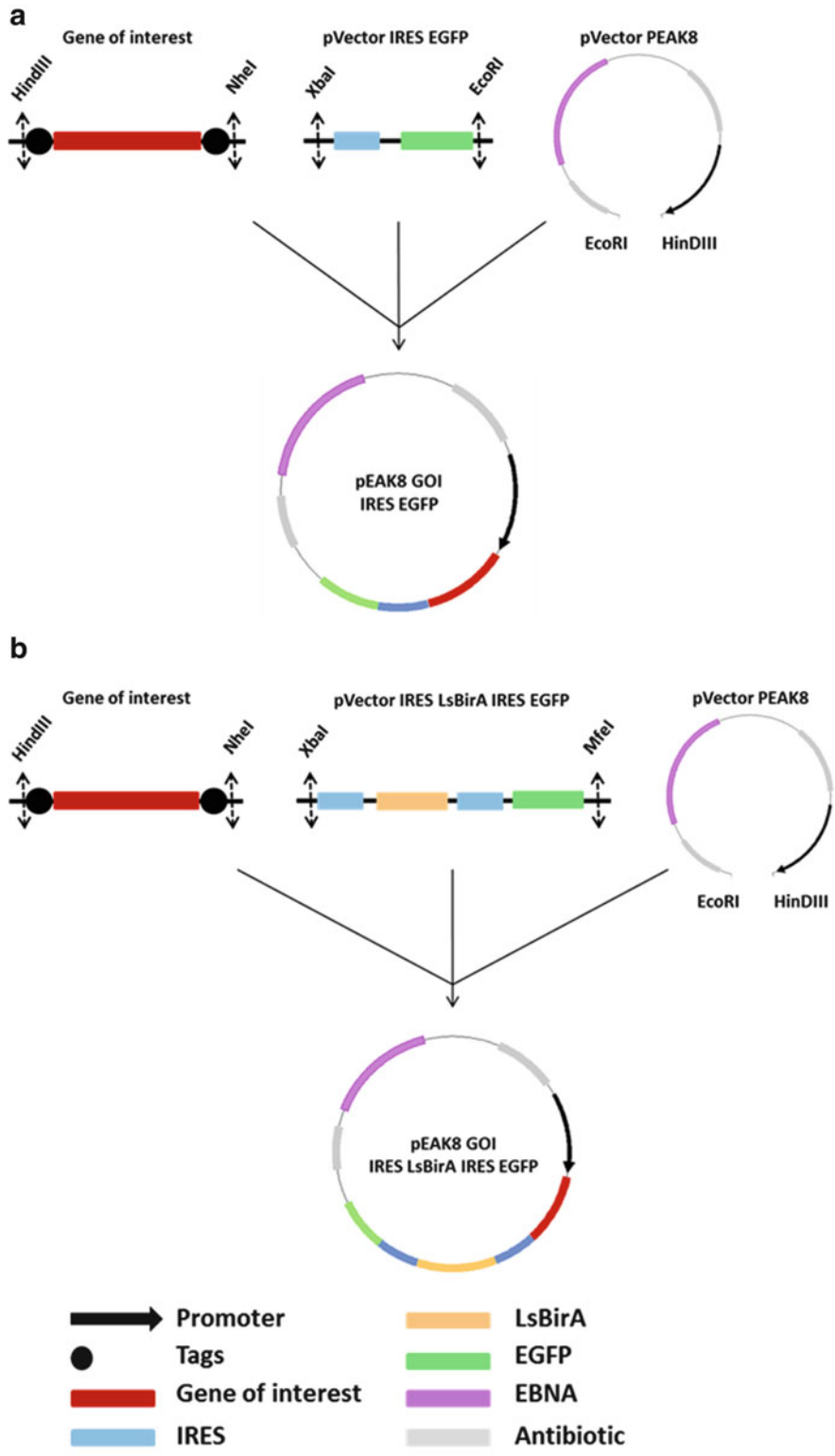

Fig. 2 Cloning process. (**a**) Bi-cistronic construct: gene of interest co-expressed with EGFP; (**b**) Tri-cistronic construct: gene of interest co-expressed with LsBirA and EGFP. GOI: Gene of Interest

10 U of each restriction enzyme in a final volume of 50 µL adjusted with sterile water.

3. Incubate the reaction for 2 h at 37 °C.
4. Purify DNA using the MinElute® PCR Purification Kit as follows.
5. Add 5 volumes of PB buffer to 1 volume (50 µL) of the digestion reaction.
6. Apply the sample to MinElute column and centrifuge for 1 min at full speed in a micro centrifuge.
7. Discard flow-through and wash the column with 750 µL of reconstituted PE buffer.
8. Centrifuge for 1 min at full speed and discard the flow-through. Centrifuge the column at full speed for two additional minutes to dry the filter membrane.
9. Transfer the column to a 1.5 mL tube, add 10 µL of EB buffer to the filter membrane, wait for 1 min, and centrifuge the column for 1 min at full speed to elute DNA.
10. Measure DNA concentration using a Nanodrop (R).

3.1.2 Preparation of the DNA Fragments Encoding EGFP and LsBirA

1. Digest either the plasmid pVector IRES EGFP (for co-expression of EGFP) or pVector IRES LsBirA IRES EGFP (for co-expression of EGFP and LsBirA) with *Xba*I/*Eco*RI and *Xba*I/*Mfe*I respectively: Mix 5 µL of NEBuffer 4 10×; 0.5 µL of BSA 100×; 1–5 µg of plasmid; 10 U of each restriction enzyme in a final volume of 50 µL adjusted with sterile water.
2. Incubate the reaction for 2 h at 37 °C.
3. Load the digested vector product and 10 µL of E-Gel® 1 Kb Plus DNA ladder on an E-Gel® 1.2 % (*see* **Note 10**).
4. After migration, extract the band corresponding to the IRES-EGFP or IRES-LsBisA-IRES-EGFP expression cassette (the expected size are approximately 1,500 bp and 3,000 bp, respectively).
5. Purify the band containing vector DNA using the MinElute® Gel Extraction Kit as follows.
6. Using a scalpel cut the bands from the gel and add 3 volumes of QG buffer for 1 volume of gel.
7. Incubate at 50 °C during 10 min under agitation with a thermomixer to dissolve gel slice.
8. Apply the sample to a MinElute column.
9. Centrifuge for 1 min at full speed. Discard the flow-through.
10. Wash the column with 750 µL of reconstituted PE buffer. Discard the flow-through and centrifuge the column at full speed for 1 additional minute to dry the filter membrane.

11. Transfer the column to a 1.5 mL tube, add 20 µL of EB buffer to the filter membrane, wait for 1 min, and centrifuge the column for 1 min at full speed to elute DNA.
12. Measure DNA concentration using a Nanodrop (R).

3.1.3 Preparation of Digested pEAK8 Vector

1. Digest the pEAK8 vector with *Hin*dIII and *Eco*RI: Mix 5 µL of NEBuffer 2 10×, 0.5 µL of purified BSA 100×, 1 µg of pEAK8 vector DNA, 10 U of each enzymes in a final volume of 50 µL adjusted with sterile water.
2. Incubate the reaction for 2 h at 37 °C.
3. Add 1 µL of Alkaline Phosphatase provided with the Rapid DNA Dephos & Ligation Kit and incubate for 15 additional minutes at 37 °C.
4. Load the digested vector product and 10 µL of E-Gel® 1 Kb Plus DNA ladder on an E-Gel® 1.2 % (*see* **Note 11**).
5. After migration, extract the band containing vector DNA using the MinElute® Gel Extraction Kit as described in Subheading 3.1.2.
6. Measure DNA concentration using a Nanodrop (R).

3.1.4 DNA Ligation

1. The two digested DNA fragments are co-ligated into the digested and dephosphorylated pEAK8 vector (*see* **Note 12**): Mix 2 µL of DNA dilution Buffer 5×, 50 ng of vector and 20–50 ng of each insert in a final volume of 10 µL adjusted with sterile water (*see* **Note 13**).
2. Add 10 µL of T4 DNA Ligation Buffer 2× and 1 µL of T4 DNA Ligase.
3. Incubate the reaction mix for 15 min at RT.
4. Transform the ligation products and negative control into chemically competent XL1-blue *E. coli* by adding directly 50 µL of cells to the ligation mix (*see* **Note 14**).
5. Mix gently and incubate for 30 min on ice. Heat the cells in a 42 °C water bath for 1 min and then replace them for 2 min on ice.
6. Add 500 µL of TB medium and incubate for 1 h at 37 °C under agitation at 1,250 rpm.
7. Spread 1/10 of the transformed bacteria on LB-Amp agar plates.
8. Incubate the plates overnight at 37 °C in a static incubator.
9. Prepare plasmid DNA from individual colonies using any standard kit such as QIAprep Spin Miniprep Kit.
10. Control the sequence of the inserts by DNA sequencing using the forward and reverse primers located upstream of the gene of interest and downstream of the EGFP sequence, respectively.

3.2 Cell Transfection and Selection

PEAK cells are cultured in complete DMEM in a static incubator at 37 °C, 5 % CO_2, and humidified atmosphere (*see* **Note 15**).

1. Plate 2×10^5 cells per well in 6-well plates, in 2 mL of complete DMEM medium per well.
2. After 24 h, transfect cells using the TransIT®-LT1 transfection reagent. For each well mix: 97 µL of DMEM; 3 µL of transfection reagent; 2 µg of vector DNA. Mix the reagents as follows.
3. Add the transfection reagent to DMEM and incubate at RT for 5 min.
4. Add this mix to the DNA and incubate at RT for 30 min.
5. Gently add, drop by drop, 100 µL of the mix to each well of the 6-well plate.
6. Mix gently the medium by carefully rocking the plate.
7. One day after transfection, evaluate transfection efficiency by monitoring EGFP expression by fluorescence microscopy or by FACS (*see* **Note 16**).
8. 1–2 days after transfection, add 0.5 µg/mL puromycin to the medium for selection and semi-stable clone generation.
9. Culture and propagate until an adherent layer of cells resistant to puromycin is obtained (*see* **Note 17**). Propagate the selected semi-stable cells in puromycin containing medium until enough cells for cryopreservation and seeding of culture flasks for amplification and production are obtained (*see* Subheading 3.3).
10. Cryopreserve the semi-stable cell lines: prepare aliquots of 1×10^7 cells in 1 mL of freezing medium.
11. Place cryotubes in the cool-box and incubate at −80 °C for 1–2 h (*see* **Note 18**).
12. Transfer cryotubes in liquid nitrogen for long-term storage.

3.3 Cell Amplification and Protein Production in CELLine Bioreactor (Fig. 3)

1. Amplify the semi-stable cells in DMEM containing 0.5 µg/mL puromycin in T75 or T175 flasks depending on the number of bioreactor that will be seeded (5×10^7 cells for each CELLine) (*see* **Note 19**).
2. Detach the cells from the flasks by vigorous manual shaking of the flask.
3. Count the cells using a Vi-Cell counter or equivalent.
4. For each CELLine to be seeded, centrifuge 5×10^7 cells at $500 \times g$ for 10 min.
5. Discard the supernatant and resuspend the cells in 10 mL of serum-free medium.
6. For bioreactor conditioning, add 200 mL of complete DMEM to the upper compartment of a CELLine and 10 mL of serum-free medium in the lower compartment (*see* **Note 20**).

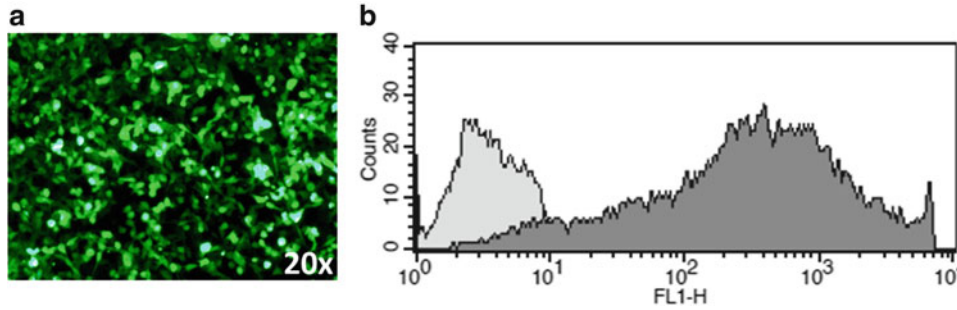

Fig. 3 (**a**) EGFP expression detected by fluorescence microscopy in PEAK cells; (**b**) Analysis of PEAK cell transfection by monitoring EGFP expression by FACS: control of untransfected PEAK cells (*light grey*); PEAK cells 48 h after transfection (*dark grey*)

7. Add the 10 mL cell suspension in the lower compartment and fill the upper compartment up to 700 mL with complete DMEM.
8. Add 5–50 μM of biotin in the upper compartment for in vivo biotinylation (*see* **Note 21**).
9. After 3 to 4 days of culture, add 200–300 mL of complete medium in the upper compartment to provide fresh nutrients to cells (Fig. 4).

3.4 Purification

1. Harvest cell culture supernatant after 7 to 10 days of production: recover with a pipette the medium from the lower compartment. Add fresh serum-free medium to wash the cell compartment and combine this wash fraction with the first harvest.
2. Clarify the harvest by centrifugation at 1,000 × *g* for 10 min.
3. Filter the clarified supernatant on a 0.22 μm Stericup filter unit to remove remaining cells and cell debris.
4. Add imidazole to the harvest to a final concentration of 100 mM.
5. Equilibrate the Ni-NTA chromatography column at RT with 5 column volumes of PBS and then 5 column volumes of Binding/Washing buffer at a flow rate of 2 mL/min using a peristaltic pump (*see* **Note 22**).
6. Load the harvest on the column at a flow rate of 2 mL/min (*see* **Note 23**).
7. Wash the column with Binding/Washing buffer (*see* **Note 24**).
8. At the same time, wash the ÄKTA Prime system and tubing with water and then corresponding inlet tubing with Binding/Washing buffer and Elution buffer. Insert 45 collection tubes into the ÄKTA fraction collector.

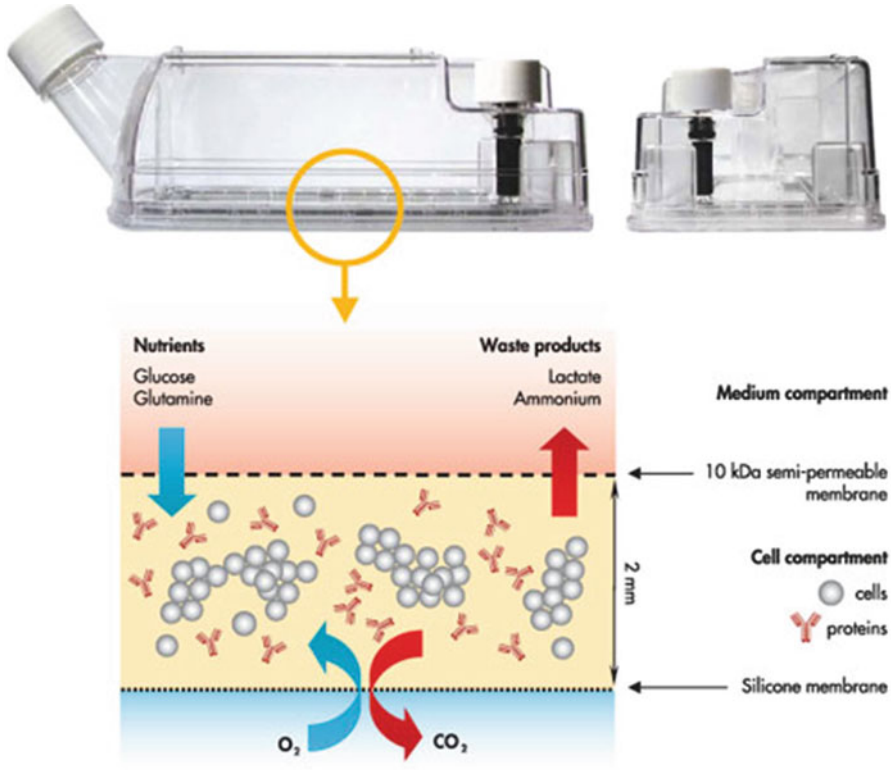

Fig. 4 CELLine—Two-compartment Technology (adapted from http://www.integra-biosciences.com)

9. Connect the column to the ÄKTA (*see* **Note 25**) and elute proteins applying the following parameters: flow rate of 2 mL/min, 30 mL imidazole gradient (from 20 to 400 mM of imidazole, i.e., from 100 % Binding/Washing buffer to 100 % Elution buffer), collect 1 mL fractions (45 fractions in total). The chromatogram displayed using the Prime View software shows the absorption at 280 nm, the buffer gradient as well as the collected fractions.
10. Pool fractions corresponding to the elution peak (*see* **Note 26**) (Fig. 5).

3.5 Desalting by Diafiltration (See Note 27*)*

1. Equilibrate the Amicon filter device with PBS (or an appropriate formulation buffer) by centrifugation at 2,000 × *g* for 4–5 min.
2. Apply the sample to the filter device and fill with PBS.
3. Centrifuge at 2,000 × *g* for 5–7 min, discard the flow-through, and fill the device with PBS to dilute the sample. Repeat this step 2–3 times to completely exchange elution buffer with PBS.
4. Recover the sample from the device by careful pipetting using a side-to-side sweeping motion for complete recovery.

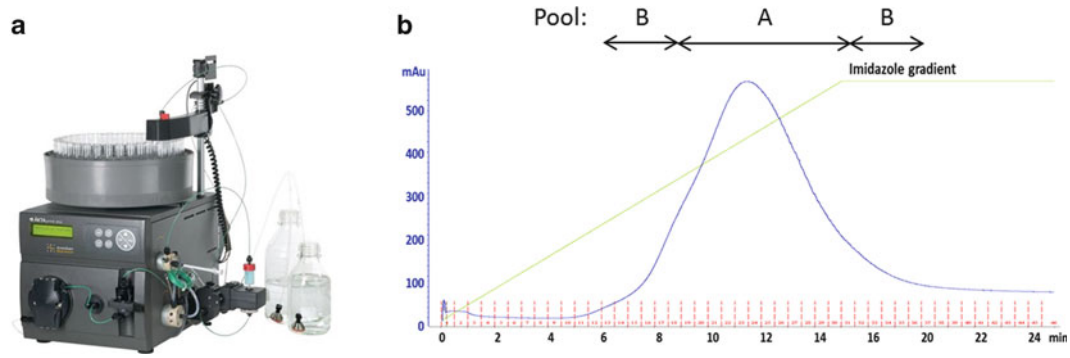

Fig. 5 (**a**) ÄKTA Prime system used for elution and fraction recovery; (**b**) Chromatogram of human IL-6R purification on a nickel chromatography resin, with fractions pooled to obtain a high (**a**) and a low (**b**) concentration batch

5. Aliquot the final product in siliconized tubes.
6. Quantify the purified protein by measuring the absorption at 280 nm using the Nanodrop (R) spectrophotometer.

3.6 Protein Characterization

3.6.1 Proteins Analysis by Gel Electrophoresis

1. Dilute 1 µg of purified protein sample into 20 µL of PBS (*see* **Note 28**).
2. Add 5 µL of LDS sample buffer, containing or not β-mercaptoethanol for loading in reducing or non-reducing conditions, respectively. A 1 mL reducing solution contains 960 µL of LDS sample buffer and 40 µL of β-mercaptoethanol.
3. Heat samples at 95 °C for 5 min for reducing conditions or 2 min for non-reducing conditions.
4. Load samples on the gel (*see* **Note 29**).
5. Start migration in MES running buffer at 180 V for 35 min.
6. After migration, recover the gel and wash with distilled water.
7. Add SimplyBlue™ SafeStain and stain the gel overnight (*see* **Note 30**).
8. Discard the SimplyBlue™, wash with distilled water and destain gel in distilled water under gentle agitation until bands appear (Fig. 6).

3.6.2 ELISA for Biotinylation Analysis (See **Note 31***)*

1. Dilute the purified proteins in PBS to a concentration between 1 and 5 µg/mL.
2. Add 100 µL of sample per well of a StreptaWell plate and incubate for 1 h at RT (*see* **Note 32**).
3. Wash each well three times with 200 µL of PBST.
4. Add 100 µL per well of antigen specific mouse monoclonal antibody diluted at 1 µg/mL or Penta-His HRP conjugate antibody diluted 1:2,000, in PBST–BSA (*see* **Note 33**).
5. Incubate for 1 h at RT.

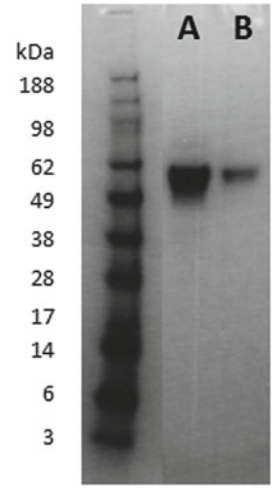

Fig. 6 SDS PAGE gel of purified human IL-6R in denaturing and reducing conditions on a 4–12 % Bis-Tris acrylamide gel. The two pools described in Fig. 5 were analyzed (Pool A and Pool B)

6. Wash each well three times with 200 µL of PBST.
7. For antigen specific antibody, add 100 µL per well of HRP-coupled goat anti-mouse IgG antibody diluted at 1:2,000 in PBST–BSA.
8. Incubate for 1 h at RT.
9. Wash each well three times with 200 µL of PBST.
10. Add 100 µL of TMB chromogenic substrate for revelation.
11. After 1 to 10 min of revelation time, add 100 µL of H_2SO_4 2 N to stop the reaction.
12. Measure the absorption at 450 nm on a microplate reader.

4 Notes

1. Specific reagents necessary for digestion reactions, such as BSA, buffers, are provided with enzymes.
2. PEAK cells stably express the EBNA-1 gene allowing for episomal replication. These cells are semi-adherent and can be easily detached from the flask by a vigorous shaking.
3. A CELLine is composed of two compartments separated by a 10 kDa semi-permeable membrane. Nutrients from the medium can migrate through the membrane to the cell compartment and waste products (lactate, ammonium) from cell culture in the lower compartment can diffuse to the upper compartment. A silicone membrane at the bottom of the bioreactor allows for gas exchange, as illustrated in Fig. 4.

4. The cutoff of the filter device must be chosen according to the size of the protein to desalt. This cutoff should be at least three times higher than the protein molecular weight to avoid product loss during this centrifugation process. The device exists also in different sizes according to the volume of sample to be centrifuged. During the successive centrifugations, store the flow-through in the event of protein loss during desalting.
5. Siliconized tubes must be used to avoid loss of protein on the inner wall of tubes. The mentioned tubes' caps are not locked. For freezing, keep tubes in a vertical position to avoid opening of the tube and product loss. Other locked tubes can be used such as the LoBind tubes from Eppendorf.
6. The β-mercaptoethanol must be manipulated under a fume hood.
7. In this chapter, a mouse primary antibody is used and so the secondary antibody is an anti-mouse antibody. If the primary antibody comes from another species, it is necessary to adapt the secondary antibody to the corresponding species.
8. The AviTag™ (GLNDIFEAQKIEWHE) from Avidity allows for a targeted enzymatic conjugation of a single biotin molecule on the tag using the biotin ligase. Here we describe the co-expression of the biotin ligase and in vivo biotinylation by addition of biotin in the production medium. The co-expression of several proteins can have a negative impact on the expression of the protein of interest. In this case, an in vitro biotinylation can be performed on the purified product using the biotin protein ligase kit from Avidity. A desalting step is necessary after in vitro biotinylation.
9. We highly recommend generating constructions, with tags at the N or at the C-terminus of the protein. In some cases, the position of the tags can influence protein expression, folding, functionality, but also tag accessibility for purification or biotinylation. By generating both constructs simultaneously, the impact of the tags is quickly identified, and protein is efficiently produced at the same time with the appropriate construction [15].
10. Maximum 1 μg of DNA can be loaded in one well to obtain a nice band. If different DNA samples are loaded on the same gel for purification, each sample must be separated by one empty well to avoid DNA contamination.
11. It is recommended to load in parallel 1 μg of non-digested vector to ensure that the vector was correctly digested.
12. *Nhe*I and *Xba*I are compatible sites; *Mfe*I and *Eco*RI are compatible sites.
13. 20 ng of insert is sufficient for the shorter insert (IRES-EGFP) and 50 ng are necessary for the longer insert (IRES-LsBirA-IRES-EGFP). Appropriate controls must be prepared during

the ligation such as the ligation mix with vector but without insert to obtain the background number of colonies corresponding to empty vector.

14. Untransformed competent cells must be spread on an LB agar dish to check the absence of contamination in the bacteria preparation.
15. PEAK cells are semi-adherent, avoiding the need for agitation and gassing. Therefore, no complicated fermentation system was necessary to produce the antigen. A CO_2 humidified incubator was sufficient for all cell culture.
16. Usually, between 70 and 90 % of the cells are transfected and are fluorescent under a fluorescence microscope or by FACS.
17. In some cases, the cells need a longer time for adaptation under puromycin and for growth to confluence. In this case, the medium can be exchanged before and usually, 5 to 10 days are necessary for selection.
18. The use of a cool-box allows for a slow freezing of cells, avoiding for the crystallization which could destruct cells. Different systems are available, containing either alcohol or a solid core. They provide a consistent and controlled decrease of the temperature at a rate of −1 °C per minute.
19. Usually, one flask T175 with cells at confluence is used to inoculate one bioreactor CL1000.
20. For a new CELLine bioreactor, it is necessary to humidify the membrane on both sides (cell and medium compartments) with serum-free medium and serum-containing medium in the lower and the upper compartment, respectively, before adding the cell suspension. No air should remain in the lower compartment after cell suspension inoculation; air bubbles can be eliminated by pipetting and tilting the CELLine. Additional information about the use of the CELLine bioreactor can be found at http://www.integra-biosciences.com/sites/celline_lit_e.html.
21. Due to its small size (244 Da), the biotin is added in the upper medium compartment and can diffuse across the CELLine membrane (cutoff 10 kDa).
22. We use a peristaltic pump for loading and washing steps as it allows for increased flexibility (for instance, several columns can be loaded in parallel). However, all purification steps (loading, washing, and elution) can also be performed on the ÄKTA system.
23. The flow-through must be stored until the end of the process in case of problems or if the capacity of the column is exceeded.
24. Usually, 3 to 5 column volumes are necessary to wash the resin after sample loading. The column must get back to its original blue color after washing.

25. To avoid insertion of air bubbles in the column and tubing during connection of the column to the ÄKTA, add binding/washing buffer on the top of the column before connecting the tubing. Artifacts in the elution can be caused by the presence of air bubbles in the system.
26. Fractions can be pooled in two different samples, one containing fractions from the high absorption region of the peak and one corresponding to the fractions of lower concentration (lower OD), as shown in Fig. 5b.
27. The desalting step can also be performed by gravity using gel filtration columns such as PD-10, NAP-10 or NAP-5 from GE Healthcare.
28. 1 ug of purified protein loaded per well of a NuPAGE gel is sufficient to obtain a clear band on the gel. To perform semi-quantification on gel, different amounts (5 μg to 0.1 μg) of a reference protein can be loaded in parallel. The concentration of the sample can be estimated by comparing the band intensities of the standards and the samples.
29. NuPAGE pre-casted gels are used for protein electrophoresis. After installation of the gel in the electrophoresis chamber, fill the buffer compartment with MES buffer, remove the comb, and wash wells by pipetting MES buffer into wells.
30. The staining can be shorter, for some proteins 2–3 h are sufficient to observe the bands on the gel.
31. The biotinylation of the protein can be verified by an ELISA. However, the result will confirm that biotinylated protein is present in the final sample but doesn't give information about the amount of biotinylated protein. The final product can contain biotinylated and non-biotinylated molecules. To evaluate the percentage of biotinylation, a pull-down assay using streptavidin-coated magnetic particles must be performed.
32. StreptaWell plates are pre-blocked, and no additional blocking step is required.
33. The concentration of the primary antibody can be adjusted according to the antibody used.

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