Laboratory Exercise

Overexpression, Immunodetection, and Site-directed Mutagenesis of *Anabaena* Sp. PCC 7120 Flavodoxin: A Comprehensive Laboratory Practice on Molecular Biology

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Abstract

Recombinant protein expression and site-directed mutagenesis of target genes have demonstrated an increasing importance in the fields of molecular biology, biochemistry, biotechnology, and medicine. By using the flavodoxin of the model cyanobacterium *Anabaena* sp. PCC 7120 as a laboratory tool, we designed a comprehensive laboratory practice encompassing several well-established molecular biology techniques and procedures in order to fulfill two main objectives: (1) overexpression and immunodetection of *Anabaena* flavodoxin in recombinant *Escherichia coli* cell extracts, and (2) site-directed mutagenesis of the *Anabaena* flavodoxin gene *isiB*. This lab practice provides undergraduate students the possibility to perform by themselves several essential techniques in the field. With the aid of professors, students are stimulated to think, to interpret, and to discuss the results based on what they had learned in previous theoretical courses. © 2018 by The International Union of Biochemistry and Molecular Biology, 00:000–000, 2018.

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Keywords: *Recombinant protein expression; protein immunodetection; site-directed mutagenesis;* Anabaena *flavodoxin*

Introduction

Cyanobacteria are oxygen-evolving photosynthetic prokaryotes found in virtually all ecosystem habitats on Earth. As primary producers, these organisms are major participants in global carbon cycles, playing key roles in both food chain dynamics and biogeochemistry of oceans and freshwaters. In addition, many cyanobacteria are capable of fixing atmospheric dinitrogen in the absence of combined nitrogen sources, providing the largest fraction of total nitrogen fixation in the open ocean [1, 2].

In cyanobacteria and other oxygenic photosynthetic organisms, the small mobile iron-sulfur containing protein ferredoxin (Fd) transfers one electron from photosystem I (PSI) to the flavoenzyme ferredoxin-NADP$^+$ reductase (FNR) in the photosynthetic electron transfer chain. Thus, a substantial fraction of photoreduced Fd is employed for NADPH biosynthesis through NADP$^+$ reduction catalyzed by FNR. NADPH provides the reducing power required for the regenerative step of the Calvin cycle and for other biosynthetic pathways. In addition, reduced Fd delivers electrons to other acceptors, including enzymes involved in nitrogen and sulfur assimilation, amino acid biosynthesis, fatty acid and secondary metabolisms, reductive activation of enzymes by the ferredoxin-thioredoxin reductase system, among other physiological processes [3].

Under iron limited conditions, cyanobacteria and certain algae replace metalloprotein Fd by another soluble electron carrier that contains flavin mononucleotide (FMN)

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as cofactor instead [2Fe-2S] cluster, flavodoxin (Fld) [4]. Fld properties as redox shuttle largely match those of Fd, being able to replace the metalloprotein in many reactions. Hence, Fld replaces Fd during the transfer of one electron from PSI to FNR in the photosynthetic electron transfer chain [5].

Traditionally, induction of Fld expression in diatoms and marine cyanobacteria has been used as a molecular indicator of iron limitation in phytoplankton [6]. However, since several years ago, microbial Flds have awakened the interest of the scientific community in order to take best advantage of their key roles in bacterial physiology. Thus, cyanobacterial Fld has become in a particularly interesting protein in the field of biotechnology [7–9]. Tobacco (*Nicotiana tabacum*) plants expressing cyanobacterial Fld in chloroplasts displayed increased tolerance to multiple sources of stress, including redox-cycling herbicides, extreme temperatures, high irradiation, water deficit, and UV radiation. Cyanobacterial Fld was able to mediate plant Fd-dependent reactions *in vitro*, including NADP+ and thioredoxin reduction [7]. Additionally, *Sinorhizobium meliloti* overexpressing cyanobacterial Fld induced alfalfa (*Medicago sativa*) root nodules with increased accumulation of starch, delayed senescence, and enhanced tolerance to salinity stress [8, 9]. On the other hand, since Fld functions as an essential protein in certain clinically relevant pathogens such as *Helicobacter pylori*, this bacterial flavoprotein has been considered a promising therapeutic target for novel antimicrobial drug development [10].

The *Anabaena* sp. PCC 7120 Fld is a well-characterized protein, easily overproduced and purified in laboratory [11, 12]. Here, we describe a comprehensive laboratory practice encompassing several well-established molecular biology techniques and procedures which include preparation of *Escherichia coli* competent cells, chemical transformation, recombinant protein overexpression, SDS-PAGE, Western blotting, PCR amplification, mini-prep isolation of plasmid DNA, plasmid digestion using restriction enzymes, and agarose gel visualization. This molecular biology practice has been designed, and is being currently imparted to fourth/final-year undergraduate students of the Degree in Biotechnology at University of Zaragoza, Spain, as part of the course Microbial Biotechnology. The practice is intended to students with previous theoretical background in microbiology, biochemistry, molecular biology, and genetic engineering, as well as basic skills and safety knowledge on laboratory scenarios (https://estudios.unizar.es/estudio/ver?id=125). In four 3-hr laboratory sessions, the students integrate several techniques routinely used to DNA manipulation, recombinant expression, and biochemical characterization of proteins.

Experimental Practice

Objectives and Flowchart

The practice has been designed as a small research project with two main objectives: (1) overexpression and immunodetection of *Anabaena* Fld in recombinant *E. coli* cell extracts, and (2) site-directed mutagenesis of the *Anabaena* Fld gene *isiB*. As part of a brief pre-lab introductory visual presentation at the beginning of the first session of the practice, information about the objectives, background, flowchart (Fig. 1), and safety issues were explained to the students. It is advisable that the students understand that the practice has two distinguishable independent objectives, and the experiments that will carried out to fulfill these objectives will be perform at the same time in each

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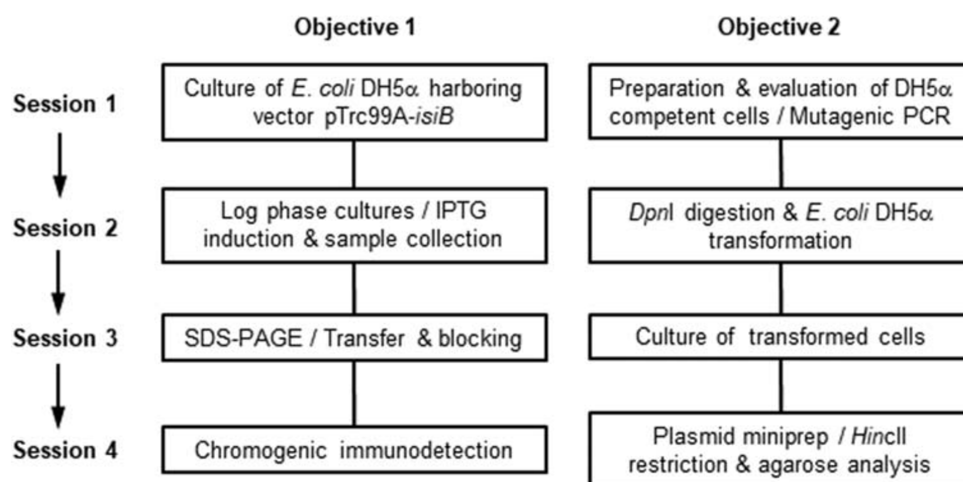

FIG 1 *Flowchart for the laboratory practice. The practice has been designed to integrate, in four 3h laboratory sessions, several of the most routinely used molecular biology techniques in order to fulfill two objectives, (1) overexpression and immunodetection of* Anabaena *Fld in recombinant* E. coli *cell extracts, and (2) site-directed mutagenesis of the* Anabaena *Fld gene* isiB.

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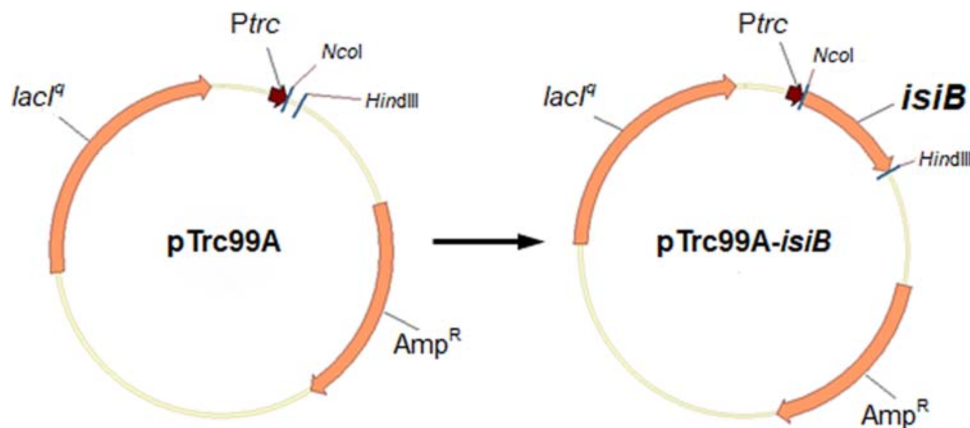

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FIG 2 *Cloning of* Anabaena *flavodoxin gene (*isiB*) into the* NcoI - HindIII *sites of the IPTG-inducible expression vector pTrc99A. [Color figure can be viewed at wileyonlinelibrary.com]*

practice session, a fact that commonly occurs in research labs. It is recommended that the students work in pairs.

Background

Anabaena (*Nostoc*) sp. PCC 7120 (hereafter *Anabaena*) is a filamentous, nitrogen-fixing, heterocyst forming cyanobacterium widely used as a model organism for studying several prokaryotic and eukaryotic processes, including oxygenic photosynthesis, nitrogen fixation, cell differentiation, multicellularity in prokaryotes, heterocyst pattern formation, and evolution of chloroplasts. *Anabaena* genome has been sequenced and its data are freely accessible (http://genome.microbedb.jp/CyanoBase). The *Anabaena* chromosome harbours only one gene for Fld, denote as *alr2405* or *isiB*. Fld from *Anabaena* is a single polypeptide chain which shows a Mr of 19.500 Da in SDS-PAGE and contains one FMN per molecule of protein. This flavoprotein contains 169 amino acid residues and it shows an isoelectric point of 3.8 [11].

To overexpress the protein in the laboratory, the *isiB* gene was previously cloned into the *Nco*I - *Hin*dIII sites of the pTrc99A vector (Fig. 2). This expression vector carries the strong inducible *trc* promoter, the *lacZ* ribosome-binding site, the multiple cloning site of pUC18 and the *rrnB* transcription terminators [13]. Additionally, the *lacI^q* allele of the LacI repressor gene was added to the vector in order to ensure efficient repression of the *trc* promoter before induction of recombinant expression with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The *lacI^q* gene contains a mutation in its own promoter that enhances *lacI* expression by ten folds [14]. Unlike T7 promoter systems, which require the use of T7 RNAP-containing strains, the *trc* promoter vectors may be used with any *E. coli* strain as host for recombinant expression. In this laboratory practice, we used the expression system pTrc99A/DH5α to overproduce the *Anabaena* Fld, which yielded high levels of recombinant expression in only 2 hr after IPTG induction.

F2

Mutagenesis has crucial importance in the fields of molecular biology, biochemistry, and biotechnology. The ability to quickly and specifically mutate an amino acid residue in a protein of interest plays a pivotal role in structural, mechanistic, and functional studies, as well as in protein engineering of commercial enzymes [15]. Several site-directed mutagenesis methods have been developed to date [16]; however, a simple, quick and easy three-step procedure developed by Stratagene Corporation, known as "QuikChange", has been extensively used. QuikChange works by using a pair of complementary primers with the desired mutation included. In a round of PCR cycles, these primers anneal to the template DNA (i.e. the target gene cloned into a plasmid vector) and replicate the entire plasmid DNA, thereby introducing the mutation and generating mutant DNA products with a strand break (nick). The resulting DNA pool of PCR (mutant and parental plasmids) is then treated with *Dpn*I enzyme in order to destroy the parental methylated DNA from the newly synthesized unmethylated mutant DNA. Finally, the digested mixture is transformed into *E. coli* competent cells, where the nick is ligated by host repair enzymes (Fig. 3).

F3

In this practice, we used the QuikChange site-directed mutagenesis procedure to generate a single-amino acid mutant of *Anabaena* Fld. Specifically, the aspartic acid residue (D) located at the position 150 of the polypeptide chain was substituted by lysine (K). Substitution of amino acids was allowed by the replacement of the natural G<u>A</u>C codon by <u>A</u>A<u>G</u>. The replacement of the natural codon in the *Anabaena* Fld mutant D150K generated a new restriction site into the *isiB* gene for the endonuclease *Hinc*II (Supporting Information). Hence, effective mutagenesis was easily confirmed by *Hinc*II restriction of the pTrc99A vector harboring the D150K *isiB* mutant (Fig. 4) and subsequent visualization on agarose gels.

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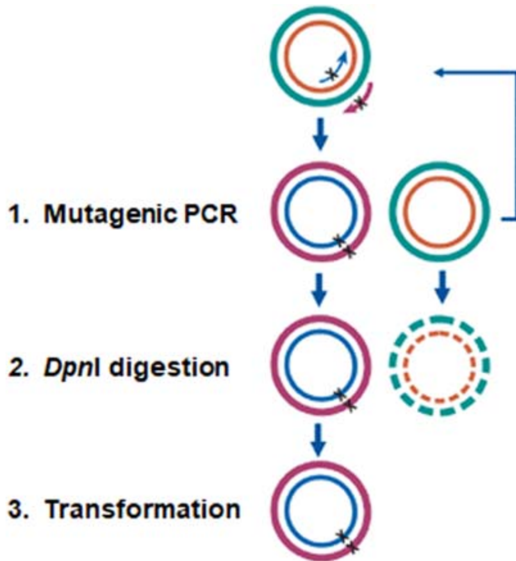

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FIG 3 *Overview of the QuikChange side-directed mutagenesis method. Mutant-strand synthesis by PCR using mutagenic primers was followed by* DpnI *digestion of both parental methylated and hemimethylated DNA vectors. Transformation of the double-stranded nicked DNA molecules into* E.coli *cells resulted in nick repair. The figure has been adapted from the QuikChange II side-directed mutagenesis kit user manual (Agilent Technologies). [Color figure can be viewed at wileyonlinelibrary.com]*

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Safety Issues and Precautions

Lab coats were worn during all laboratory experiments. Use of gloves was recommended to manipulate biological material including microbial cultures, enzymes, antibodies, DNA. Special precautions and use of gloves and goggles were required handling certain reagents such as acrylamide, sodium dodecyl sulphate solution (SDS, 20%), ammonium persulfate solution (APS, 10%), tetramethylethylenediamine (TEMED), isopropyl-β-D-thiogalactopyranoside solution (IPTG, 1M), β-mercaptoethanol, 3,3′,5,5′-tetramethylbenzidine (TMB), dioctyl sodium sulfosuccinate (DOSS), hydrogen peroxide solution 30%. All of these substances are toxic by inhalation or ingestion and may cause irritation in contact with skin or eyes. Professors alerted the students to keep hands away from face, eyes, mouth, and body while using these and other chemicals or biological material. Students handled high voltage power systems during SDS-PAGE, Western blotting, and agarose gel electrophoresis. Hence, power supply was turned off and the electrical leads were disconnected before manipulate electrophoresis gels and blot. All used materials were properly disposed after the experiments.

Materials and Pre-Lab Preparations

SDS-PAGE and Western blotting were carried out using Mini-PROTEAN electrophoresis systems provided by Bio-Rad (U.S.A.). Rabbit polyclonal antiserum against *Anabaena* Fld was produced by Aragon Institute of Health Sciences (Spain). *Pfu Turbo* DNA polymerase was provided by Agilent (U.S.A.). Oligonucleotides used in site-directed mutagenesis were synthetized by Invitrogen (U.S.A.). Restriction endonucleases *Dpn*I and *Hinc*II were purchased from Fermentas (U.S.A.). Plasmid mini-preps were carried out using the GenElute plasmid miniprep kit of Sigma-Aldrich (U.S.A.). Plasmid DNA concentrations were determined using a NanoVue Plus spectrophotometer of Biochrom (United Kingdom). Agarose gels from DNA electrophoresis were stained with SYBR Safe DNA gel stain (Invitrogen) and processed with a Gel Doc 2000 Image Analyzer (Bio-Rad).

In order to save time and ensure total execution of all programmed experiments, it is recommended that the professors and/or technical assistants prepare some solutions,

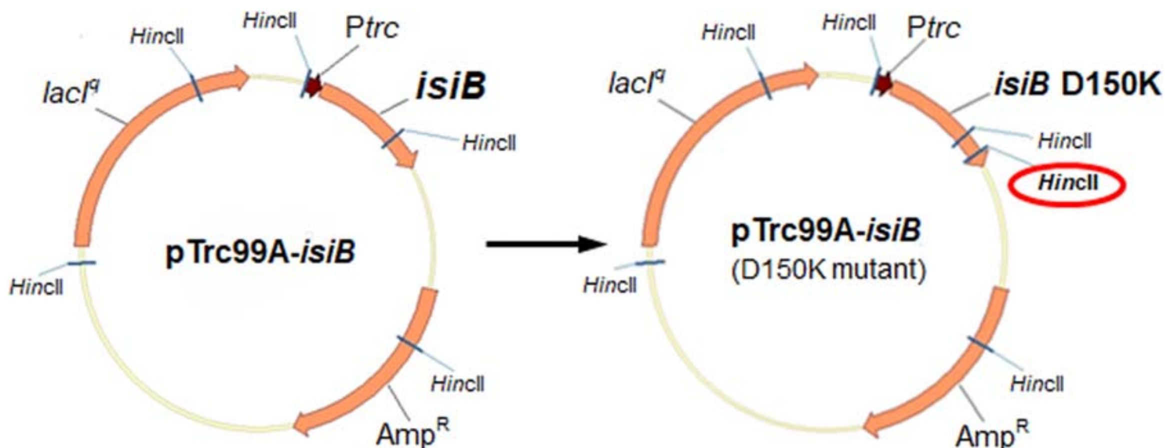

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FIG 4 *Replacement of the natural codon GAC by AAG in the* Anabaena *Fld gene* isiB *generated a new restriction site for the endonuclease* HincII*. Effective mutagenesis was confirmed by* HincII *restriction of the pTrc99A vector harboring the D150K* isiB *mutant. [Color figure can be viewed at wileyonlinelibrary.com]*

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buffers, and other materials prior to the beginning of the practice sessions. Pre-lab materials were prepared according to the procedures described in Supporting Information.

Methods

Preparation and Evaluation of E. coli DH5α Competent Cells

Chemically competent cells of *E. coli* strain DH5α were prepared and transformation efficiency was evaluated. Briefly, a single colony of *E. coli* DH5α grown overnight (O/N) at 37°C in LB agar was inoculated in 10 mL of LB broth using 50 mL Falcon tube and incubated O/N at 37°C with vigorous shaking. About 2 mL of this O/N culture was used to inoculate 200 mL of fresh LB broth using 1 L Erlenmeyer flask. Culture was incubated at 37°C with vigorous shaking until optical density at 600 nm achieved a value of 0.35–0.45. Then, Erlenmeyer flask was put on ice for 10 min. Cells were collected by centrifugation at 4,000 rpm during 10 min and gently resuspended (by pipetting) in 40 mL of ice-cold sterile buffer A for chemically competent cells (see Supporting Information). Cells were collected by centrifugation and gently resuspended in 2 mL of ice-cold sterile buffer B for chemically competent cells. Aliquots of 150 µL competent cells were dispensed to sterile ice-cold Eppendorf tubes and store at −80°C. Transformation efficiency (TE) was determined by transforming one aliquot of competent cells with 0.5 µg of pTrc99A-*isiB* plasmid. Briefly, the aliquot of competent cell was thawed on ice for 5 min, mixed with 5 µL of vector pTrc99A-*isiB* (100 ng/µL), and incubated on ice for 10 min. Then, each aliquot of DNA-competent cells was exposed at 42°C during 90 sec and immediately transferred back to ice for 2 min. Next, heat shock exposed cells were suspended in 800 µL of LB medium and incubated at 37°C during 30 min with mild shaking. Recuperated cells were centrifuged, resuspended in 50 µL of LB and plated on ~~LBAmp~~. Inoculated plates were incubated O/N at 37°C. TE = number of colonies counted on the plate/µg of plasmid DNA transformed/total dilution of DNA before plating. Competent cells were optimal for use if TE ≥ 10^6 colonies/µg.

Overexpression of Anabaena Fld in E. coli

A single colony of *E. coli* DH5α harboring vector pTrc99A-*isiB* was inoculated in 10 mL of ~~LB~~ broth ~~supplemented with ampicillin 50 µg/mL (LBAmp)~~ and incubated O/N at 37°C with vigorous shaking. About 0.2 mL of this O/N culture was used to inoculate 10 mL of fresh ~~LBAmp~~. Cultures (in duplicate) were incubated at 37°C with vigorous shaking until optical density at 600 nm achieved a value of 0.5–0.7 (~ 2 hr). At this time, 1 mL aliquots of both culture replicas were taken aseptically, and next, one of the two cultures was supplemented with 1 mM of IPTG. Both cultures were further incubated for 2 hr. At this time, 1 mL samples of both cultures were taken. All samples were properly identified and centrifuged at full speed for 5 min. Cell pellets were washed once with 0.9% NaCl solution and conserved at −20°C. Since the execution of the entire overexpression experiment last > 3 hr, which is the total duration of a practice session, a professor or technical assistant performed the inoculation of cultures previously to the beginning of the practice session.

SDS-Polyacrylamide Gel Electrophoresis

Each pair of students prepared two identical polyacrylamide gels and carried out two simultaneous SDS-PAGEs with identical samples. After proteins were separated, one gel was stained with Coomassie blue and the other gel was transferred to a PVDF membrane for Western blotting.

SDS-PAGE was carried out using Mini-PROTEAN 3 cell (Bio-Rad), according to the instruction of the manufacturer. Resolving gel (15%) was prepared by mixing the following components: diH_2O (1.4 mL), Tris-HCl 1.5 M, pH 8.8 (4.5 mL), acrylamide/bis-acrylamide 30% solution (6.0 mL), SDS 10% (0.060 mL), APS 10% prepared fresh by the students (0.040 mL), and TEMED (0.020 mL). The gel mixture was carefully poured into the glass plate sandwich up to ~ 1.0 cm below the bottom of the comb. Prior to polymerization, the gel surface was overlaid with 2–3 mm of isopropanol layer. Isopropanol is immiscible in the gel mixture and helps to remove bubbles at the top of the gel, ensuring a perfect horizontal interface between the resolving and stacking gels, but also prevents oxygen diffusion that inhibits polymerization while keeps the polymerized resolving gel from drying out. After polymerization of the resolving gel was complete, isopropanol was removed and the gel surface was rinsed with diH_2O. Stacking gel was prepared by mixing the following components: diH_2O (2.8 mL), Tris-HCl 0.5 M, pH 6.8 (0.5 mL), acrylamide/bis-acrylamide 30% solution (0.7 mL), SDS 10% (0.020 mL), APS 10% prepared fresh (0.020 mL), and TEMED (0.020 mL). Immediately after the gel mixture was carefully poured into the glass plate sandwich, a clean comb was inserted into the gel solution. Trapping air bubbles under the comb was avoided.

During the times required for complete polymerization of resolving (~30 min) and stacking gels (~15 min), the protein samples were prepared. Frozen pellets of recombinant *E.coli* DH5α collected before and after IPTG induction were suspended in 50 µL of 0.9% NaCl solution and the same amount of 2× loading sample buffer. Samples were incubated for 10 min at 95°C to lyse cells and denature proteins. Then, samples were centrifuged by 2 min at full speed in order to deposit insoluble cell debris. Soluble proteins, including recombinant *Anabaena* Fld, remained in the supernatant.

After polymerization of the stacking gel was complete, the combs of both gel cassette sandwiches were gently removed. Prior to the loading of samples, the stacking gel wells were rinsed with running buffer using a blunt hypodermic needle attached to a syringe, in order to remove unpolymerized acrylamide. Up to 15 µL of each protein

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sample was loaded, following a predetermined order, into the wells of the staking gel using a micropipette. A proper protein molecular weight marker (Sigma M3913) was also loaded in each gel. Gels were run at 35 mA per gel (two gels simultaneously at 70 mA) until the bromophenol blue was discharged from the resolving gel to the running buffer.

Coomassie Blue Gel Staining

For Coomassie blue staining, the staking gel was carefully separated with a clean cutter and discarded. The resolving gel was submerged in Coomassie brilliant blue staining solution and incubated at room temperature with mild shaking during 30 min. Previous to the staining, the orientation of gel was marked by cutting a little piece on the top left-hand corner. After the staining, the stain solution was removed, the excess of stain was rinse with diH_2O, and the gels were submerged in destain solution O/N with mild shaking. Bands of proteins were visualized and photographed by using a Gel Doc 2000 Image Analyzer (Bio-Rad).

Western Blotting

Western blotting was carried out using Mini-Trans-Blot cell (Bio-Rad), according to the instruction of the manufacturer. The stacking gel was discarded, and the resolving gel was transferred to a membrane of PVDF. Briefly, the PVDF membrane was activated by submerging in methanol during 15 sec; next, it was carefully washed with diH_2O during 5 min and finally was equilibrated with transfer buffer for 5 min. A sandwich of gel and PVDF membrane was carefully made, placing in this order: foam pad, filter paper, polyacrylamide gel, PVDF membrane, a second sheet of filter paper, and finally a second foam pad. Air burbles between layers of the sandwich were removed by carefully rolling a serological pipette over the surface of the sandwich without apply excessive pressure to prevent damaging the membrane and gel. After that, the sandwich was placed into the cassette holder, which was properly placed in the transfer tank. The gel side of the cassette holder was put facing the cathode (−), and the membrane side facing the anode (+). Then, transfer was carried out during 40 min at 400 mA.

After transfer was complete, the blot membrane was carefully removed from the sandwich and placed in a clean container with the side of transferred proteins facing up. Blot membrane was submerged in TBS and washed for 5 min using an orbital shaker with mild agitation. Next, blot was submerged in blocking solution (skim milk 5% in TBS, prepared fresh before use by the students) and incubated O/N at 4°C. Then, blot was washed three times with skim milk 0.5% in TBS (TBSM, prepared fresh) and incubated with primary antibody solution (rabbit anti-*Anabaena* Fld polyclonal antiserum diluted 1:800 in TBSM) during 60 min at room temperature with mild agitation. Next, blot was washed three times (10 min each) with TBS containing 0.1% Tween 20 (TBST, prepared fresh). After washings, blot was incubated with secondary antibody solution (anti-rabbit IgG-peroxidase conjugate diluted 1: 5,000 in TBSM) during 60 min at room temperature with mild agitation. Then, blot membrane was washed three times (10 min each) with TBST and equilibrated with phospho-citrate buffer for 5 min. Chromogenic immunodetection of recombinant *Anabaena* Fld was achieved by incubating the blot membrane in a proper soluble chromogenic substrate prepared fresh by the students just before use (0.015 mL of hydrogen peroxide solution 30% mixed with 30 mL of phospho-citrate buffer, pH 5.3, and 10 mL of ethanol absolute containing 20 mg of TMB and 80 mg of DOSS). Blot membrane was incubated in the substrate mixture for few seconds until color development. Reaction was stopped by washing the membrane in several changes of diH_2O.

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out according to the QuikChange method (Agilent). Mutagenic oligonucleotide primers (mutated nucleotides are highlighted) D150Kup: 5′-GATGAAGATAATCAATCT**AAG**TTAACAGACGATCGC-3′, and D150Kdw: 5′-GCGATCGTCTGTTAA**CTT**AGATTCATTATCTTCATC-3′ were previously designed by professors in order to replace the natural codon GAC by AAG, thereby substituting residue D150 by K in the *Anabaena* Fld amino acid sequence. A PCR reaction mixture containing 37 μL of diH_2O, 5 μL of 10X *Pfu Turbo* reaction buffer, 2.5 μL of each 10 μM oligonucleotide primers, 1 μL of 25 mM dNTP mix, 1 μL of vector pTrc99A-*isiB* (100 ng/μL), and 1μL of *Pfu Turbo* DNA polymerase (2.5 U/μL) was cycled according to the following parameters: one denaturation cycle at 95°C for 30 sec; 25 cycles of denaturation (95°C, 30 sec), annealing (55°C, 1 min), and extension (72°C, 10 min); plus a final extension cycle of 72°C during 12 min. Following temperature cycling, amplification reaction was conserved at 4°C O/N and students proceeded with subsequent steps of site-directed mutagenesis in the next practice session (Fig. 1).

Amplification reaction was directly treated with *Dpn*I restriction endonuclease during 1 hr at 37°C using the following reaction mixture: 17 μL of PCR product, 2 μL of 10X *Dpn*I reaction buffer, and 1 μL of *Dpn*I (10 U/μL). As control of *Dpn*I restriction, 50 ng of vector pTrc99A-*isiB* were digested with 10 U of *Dpn*I under the same conditions.

After *Dpn*I restriction was complete, 1μL of each sample (Mutagenic PCR product and vector pTrc99A-*isiB*, both treated with *Dpn*I) was used to separately transform 150 μL aliquots of DH5α competent cells by heat shock at 42°C. After heat shock exposure, cells were suspended in 800 μL of LB medium and incubated at 37°C during 30 min with mild shaking. Recuperated cells were centrifuged, resuspended in 50 μL of LB and plated on LBAmp. Inoculated plates were incubated O/N at 37°C.

Mutagenesis Verification

Two colonies of *E. coli* DH5α harboring the expected vector pTrc99A-*isiB* D150K mutant were separately inoculated in

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10 mL of LBAmp broth and incubated O/N at 37°C with vigorous shaking. Plasmid was isolated by using the GenElute plasmid miniprep kit (Sigma-Aldrich) according to the instructions of manufacturer. The concentration of purified vector was determined by using a NanoVue Plus spectrophotometer (Biochrom).

Since D150K mutation generates a new *Hin*cII restriction site, the expected mutated vector was digested during 90 min at 37°C with *Hin*cII using the following reaction mixture: 15 µL of diH_2O, 2 µL of 10X *Hin*cII reaction buffer, 2 µL of plasmid (200 ng/µL), and 1 µL of *Hin*cII (10 U/µL). As control of *Hin*cII restriction, 400 ng of vector pTrc99A-*isiB* were digested with 10 U of *Hin*cII under the same conditions. After restriction, the bands of DNA were separated in agarose 1% electrophoresis, stained with SYBR Safe DNA gel stain (Invitrogen) and analyzed with a Gel Doc 2000 Image Analyzer (Bio-Rad). GeneRuler 1kb DNA Ladder (Thermo Scientific) was used as DNA molecular weight marker.

Results

Overexpression and Immunodetection of *Anabaena* Fld in Recombinant *E. coli* Cell Extracts

Anabaena Fld was overexpressed in *E. coli* DH5α by using the expression vector pTrc99A harboring one wild-type copy of the *Anabaena* Fld gene *isiB*. Recombinant expression was induced by the addition of 1 mM IPTG and further incubation during 2 hr. Overexpression of the recombinant protein was confirmed by 15% SDS-PAGE and Coomassie blue staining, as shown in Fig. 5*A*. Immunodetection of the *Anabaena* Fld was carried out by Western blotting using a rabbit anti-Fld polyclonal antiserum as primary antibody. Direct visualization of Fld bands in blot membrane was achieved using anti-rabbit IgG-peroxidase conjugate and its chromogenic substrate TMB (Fig. 5*B*).

The results of these experiments from all the student pairs met the general expectation in most cases. Major pitfalls occurred during preparation of SDS-PAGE gels and transfer to PVDF membranes. Mistakes in the amount of gel components leading to incorrect polymerization, trapping of air bubbles into the acrylamide gels, and difficulties during sample application were commonly observed. Moreover, the preparation of SDS-PAGE gel and PVDF membrane sandwich to protein transfer were common pitfalls. Professors guided and followed the execution of these procedures in order to minimize common mistakes of students.

Site-Directed Mutagenesis of the *Anabaena* Fld Gene *isiB*

The wild-type *Anabaena* Fld gene *isiB* was mutated through substitution of residue D150 by K. Mutant D150K was obtained by using the QuikChange site-directed mutagenesis procedure developed by Stratagene (now part of Agilent Technologies). By this technique, the natural codon GAC corresponding to D150 was replaced by the mutated codon AAG by using mutated nucleotides containing the novel DNA sequence. Parental methylated wild-type DNA was completely digested by *Dpn*I and mutated *isiB* gene was easily verified by restriction with *Hin*cII, since the above mutation generated a new restriction site for this endonuclease. Thus, *Hin*cII restriction of pTrc99A-*isiB* yielded five DNA fragments of 1,886, 934, 917, 459 y 437 bp, while the same restriction of pTrc99A-*isiB* D150K mutant resulted in six bands of 1,886, 934, 827, 459, 437 y

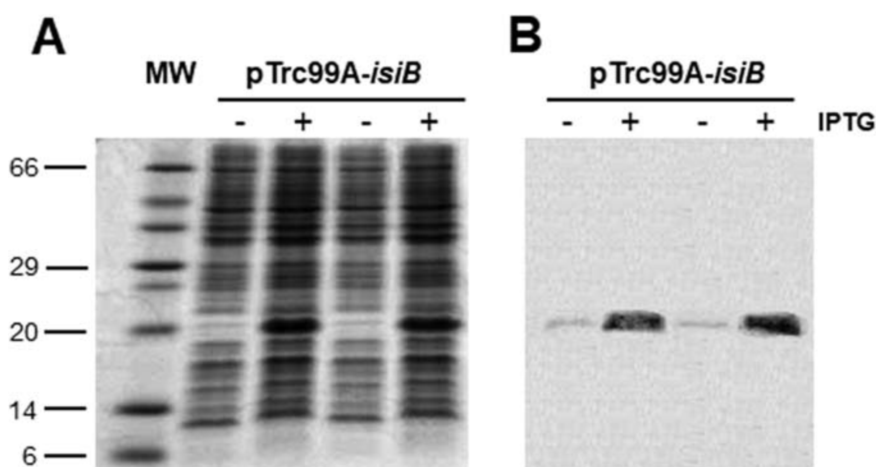

FIG 5 *Overexpression of* Anabaena *flavodoxin in* E. coli *DH5α and immunodetection of the recombinant protein in cell extracts. Log-phase growing* E. coli *DH5α cells harboring the expression vector pTrc99A-*isiB *were exposed to 1 mM IPTG (+) during 2h. An un-induced control (-) was also included by each pair of students in the overexpression experiment. Total cell extracts of both samples were separated by 15% SDS–PAGE in duplicate. (A) One gel was stained with Coomassie blue. (B) The other gel was electrotransferred to a PVDF membrane and challenged with anti-Fld antiserum. Molecular weights (MW) are indicated in KDa. Images represent the results obtained by four different pairs of students.*

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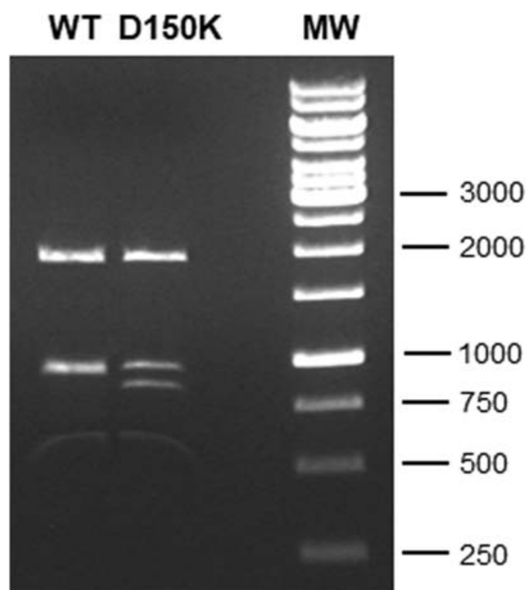

FIG 6 *Site-directed mutagenesis of the* Anabaena *flavodoxin gene* isiB. *A D150K mutant of* isiB *was obtained by the QuikChange site-directed mutagenesis procedure. The mutation generates a new restriction site for* Hinc*II endonuclease. After* Hinc*II restriction, the bands of DNA were separated by agarose 1% electrophoresis and stained with SYBR Safe DNA gel stain. Molecular weights (MW) are indicated in base pairs. Image represents the results obtained by a pair of students.*

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90 bp. In the agarose 1% gel, the bands of 934 and 917 bp as well as those of 459 and 437 bp migrated at the same position. Hence, in practice, the D150K mutation was easily confirmed by the appearance of the new 827 bp band in the gel (Fig. 6).

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Major student doubts during this part of the practice were observed in PCR mix preparation, and restriction with enzymes *Dpn*I and *Hinc*II. However, the student results commonly coincided with those expected.

Post-lab Evaluative Report and Questions

During each practice session, professors promoted join discussion of methods as well as the analysis of frequent pitfalls and probable results of the experiments. Despite experiments were carried out in pairs during lab sessions, each student prepared at home a written post-lab report which contributed to evaluate individual learning outcomes. The final evaluation of the practice took account of assistance to each practice session, the active participation in join discussion during lab sessions, the performance into the lab, the compliance with safety rules, and the quality of the post-lab report. All final laboratory reports were submitted to the professors at the most two weeks after the last lab session. In this individual written exercise, each student presented, analyzed, and discussed all the data collected during the lab sessions, including SDS-PAGE, Western blotting, and agarose electrophoresis photos, incubation times, optical density values. In addition, students analyzed and reflected in their individual reports the influence on the expected results of any change introduced in the original procedure as well as personal mistakes that could happen during the execution of the experiments. If the results obtained by a given pair of students did not correspond to the expected results, the members of this pair reflected in their respective reports both the observed and the expected results, and analyzed and discussed all the possible causes of the observed results. As part of the final report, the students must answered the following integrative questions based on the information given during the practice:

1. Explain why it is recommended that induction of recombinant protein expression take place at mid- to late-log phase of *E. coli* growth.
2. Describe the differences between resolving and stacking gels and define their respective uses during SDS-PAGE. Why SDS-PAGE is considered a denaturing protein electrophoresis?
3. Explain the importance of the blocking step in Western blotting. Why is it included 0.1% Tween 20 in the washing buffer used after antibodies exposure?
4. Explain the putative implications of use polyclonal antisera instead monoclonal antibodies as primary antibody in Western blotting?
5. Why is it use *Pfu Turbo* DNA polymerase in site-directed mutagenesis?

Learning Impact and Feedback

This lab practice on molecular biology provides undergraduate students the possibility to perform by themselves several essential techniques in the field. In four consecutive sessions of 3 hr, the students must carry out a set of experiments and procedures to fulfil two nonrelated research objectives of current relevance: (1) recombinant protein expression and antigen immunodetection, and (2) site-directed mutagenesis of target genes. With the aid of professors, students are stimulated to think, to interpret, and to discuss the results based on what they had learned in previous theoretical courses. The work in pairs stimulates enthusiasm and organization, and encourages the students to obtain better results. We have found that the students make better efforts to acquire greater practical experiences during laboratory sessions and they express an increased interest in the knowledge of biochemistry and molecular biology.

The impact of the practice in learning outcomes is measurable through different criteria, including: (1) the student analyses during join discussion of probable results before the execution of each technique, (2) the depth in student discussions of the practice results expressed in the individual laboratory reports, (3) the accuracy of the student responses to integrative questions, (4) the increased skills in the use of laboratory equipment, (5) the deeper understanding of laboratory protocols, and (6) the results of student surveys. As part of the feedback process, at the end of the course the students are encourage to answer anonymous evaluation questionnaires which include questions about the course program, laboratory practice organization, learning and skills acquired during the course, as well as professor performances. With the aid of these surveys, professors monitor the impact of teaching and course program on student learning outcomes and may reinforce weak points in the practice performance in order to allow the desired outcomes. After 11 school years of implementation and followed by around 350 undergraduate students in the University of Zaragoza, the surveys about this laboratory practice have resulted always in very positive evaluations from students, with an average score >4 points out of 5.

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