Cloning and expression of the *Peltigera membranacea lec-2* gene

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2012

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Abstract

In order to study LEC-2 protein, the DNA of the lichen *Peltigera membranacea* was extracted. It was used to synthetize by PCR *lec-2* gene using specific primers designed with *Nco*I and *Eco*RI recognition sites. The *lec-2* PCR product was then purified using column before to be double digested using *Nco*I and *Eco*RI-HF restriction enzymes. A gel extraction was then used.

Plasmids pET28a was isolated using spin column method and double digested using *Nco*I and *Eco*RI restriction enzymes.

Plasmids en PCR product was then ligated together using T4 DNA ligase. A first chemical transformation was effected to insert the plasmid into TOP10 competent cells.

The plasmids were then removed from TOP10 competent cell to BL21(C43) competent cells by Sheeba S. Manoharan. IPTG was then added to induce the expression of *lec-2*.

Samples were taken every hour to identify the production peak of *lec-2* for a potential important production.

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Introduction

Lectins are sugar binding proteins that recognize and bind specifically and reversibly to a sugar using carbohydrate recognition domains. They are generally glycoproteins and the proteins can associate that give lectins the capacity to agglutinate cells.

Lectins are present in most eukaryote cells like plants, mammalians or lichens. In lichens, it has been shown that lectins present high variability in their amino acid sequences that let us think that they would be under strong positive selection (see Manoharan et al., in impress). They may play a role in co-evolution, in ways to recognize partners or escape recognition.

A lichen is a symbiotic entity composed of a mycobiont (fungus) and a photobiont (alga or cyanobacterium). Lichens are very resistant and can survive in hostile environnement (high and low temperature, dry or poor environnement, etc...).

Peltigera membranacea is a lichen composed of a heterotrophic acomycete as a mycobiont and a photosynthetic cyanobacterium from genus *Nostoc* as photobiont. In this lichen, lectin serves as a good example of coevolution. These observations were firstly characterized with *lec-1*, which is differentially expressed in thalli, rhizines and apothecia, different tissues composing the lichen (see Miao et al., 2012). .
A new lectin gene has been recently reported in *P. membranacea*, called *lec-2*. It is characterized by a high level of polymorphism due to a positive selectio.

Materials and Methods

Lichen DNA isolation

The *Peltigera membranacea* samples were collected in Iceland. The DNA used was from a collection database built by the University of Iceland. DNA id 74A was used in this study and was collected in Keldur (63° 49´ 21´´ N, 20° 04´ 30´´ W).

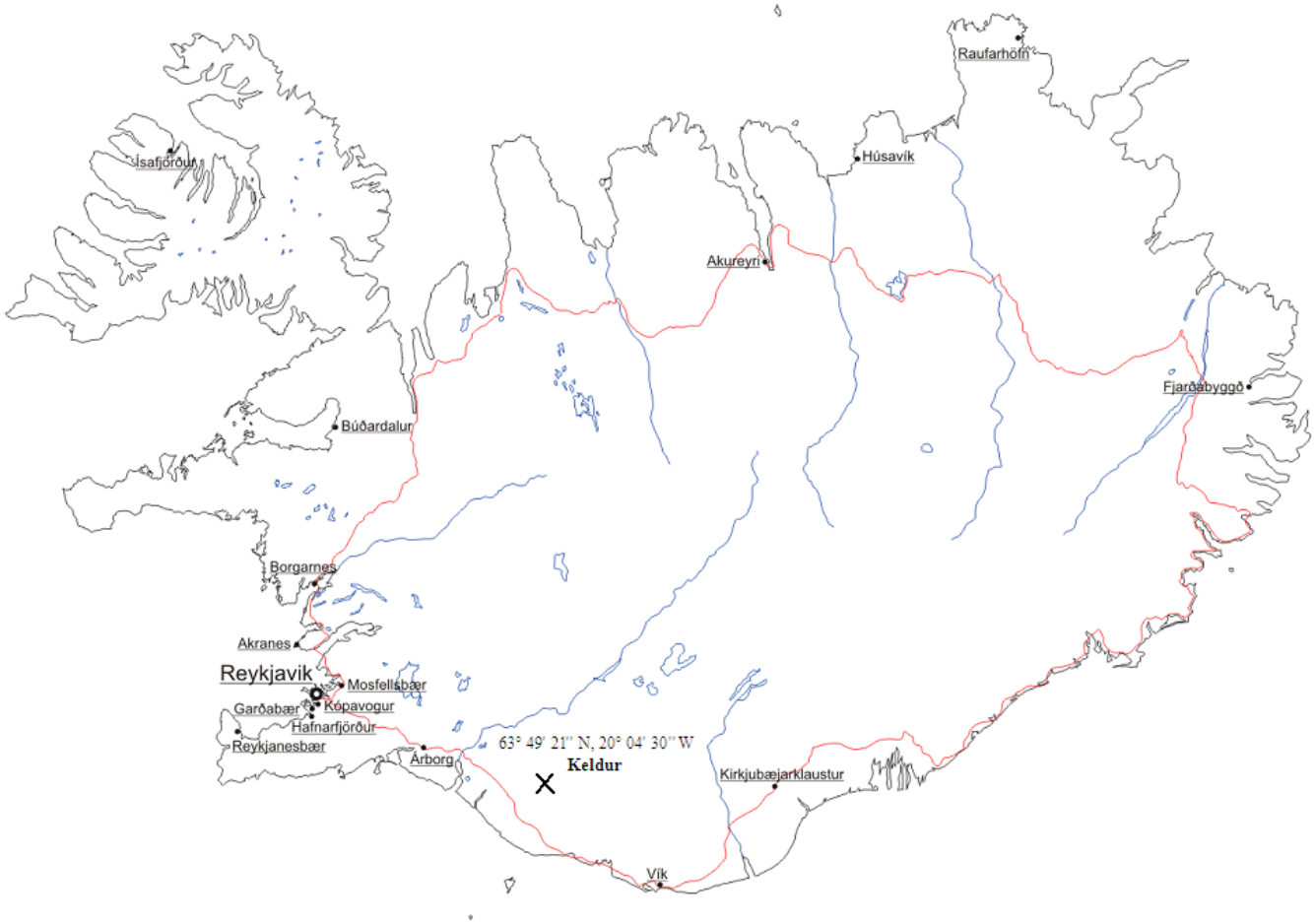


Figure 1: Map of Iceland with Keldur localization (Daniel Dalet)

Identification and screening of *lec-2* by PCR

The *lec-2* gene was identified in a database of contigs from the on-going *P. membranacea* whole genome project (see Manoharan et al., in impress). The genome was sequenced by commercial companies via the Roche 454 and the Illumina/Solexa methodologies.

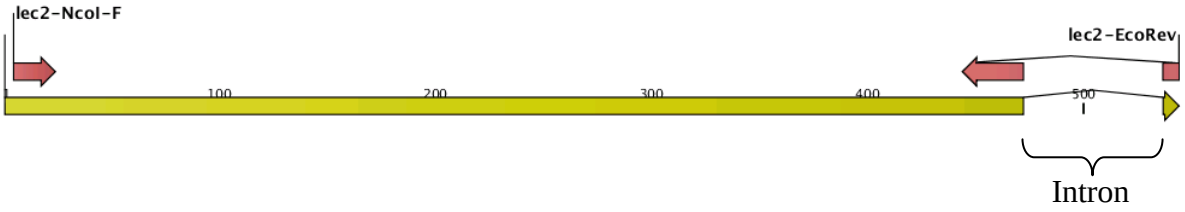


Figure 2: Map of *lec-2* gene and its primers

Polymerase Chain Reaction (PCR) – Amplification and colony PCR

To amplify *lec-2* and analyze the transformed colonies, PCRs was performed using the DNA Engine Tetrad® 2 - Peltier Thermal Cycler.

Details of primers used:

Primer name	Primer sequence	Melting Point (°C)
Lec-2-NcoI-F	5´-GCA TAC CCA TGG CCA GCC AGT GGA TTT TTC TGG AGC-3´	64.0
Lec2EcoRev	5´-CCG CGA ATT CCT AGT TTT CAA TTA CCG CTG TAA TCA CTG GAT CGG AA-3´	67.7

note: Lec2EcoRev primer was designed so as to span the intron.

The colony PCR was effectued using Lec2-NcoI-F and Lec2-EcoRev primers and T9 DNA from *P. membranacealichen* was used as a positive control.

Digestion using restriction enzymes

Double digestion of *lec-2* PRC product :

*Nco*I restriction enzyme.

5´ ... C|C A T G G ... 3´
3´ ... G G T A C|C ... 5´

*Eco*RI-HF restriction enzyme.

5´ ... G|A A T T C ... 3´
3´ ... C T T A A|G ... 5´

Double digestion of pET28a plasmid :

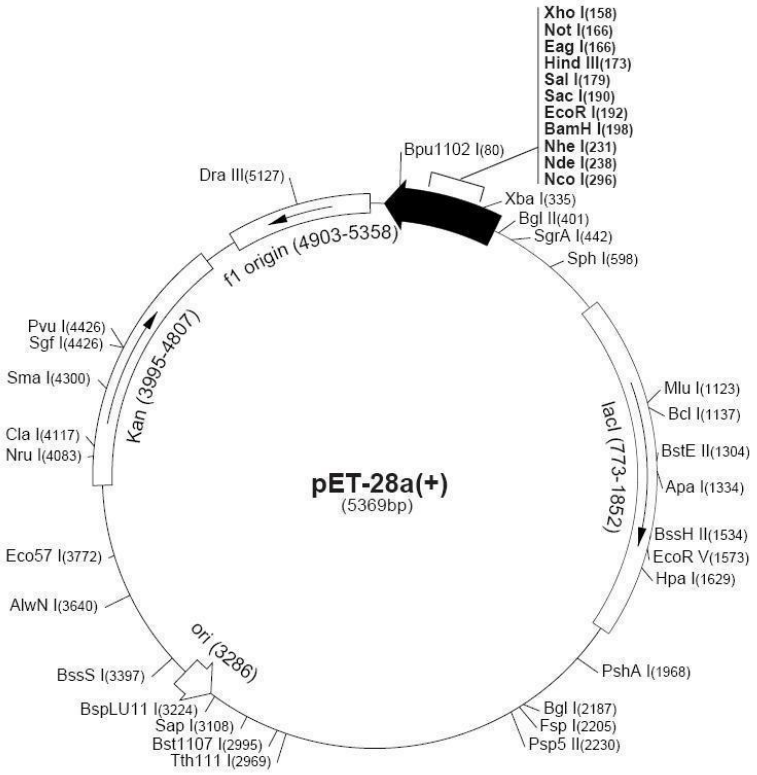
*Nco*I restriction enzyme.
*Eco*RI restriction enzyme (same recognition site than *Eco*RI-HF).

Plasmid isolation from *Escherichia coli* (map vector pET28a)

Plasmid pET28a was used as a vector for the *lec-2* insert.

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His• Tag coding sequence	270-287
T7• Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His• Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.



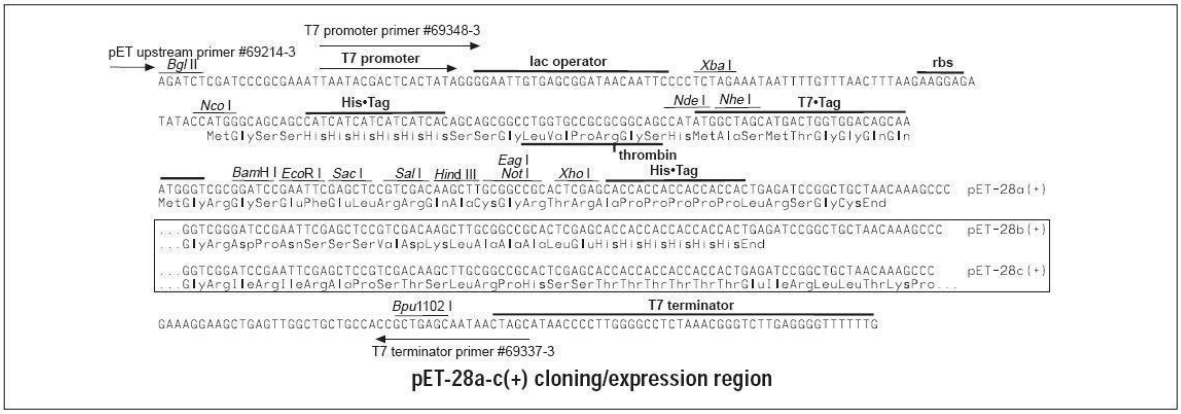


Figure 3:Cloning vector pET28a(+). Circular map, including sequence landmarks and detailed cloning and expression region.

Spin column purification of *lec-2* PCR product and gel extraction

The column purification of the *GenElute*™ kit was used (see appendix).

To purify *lec-2,* a gel extraction was performed. Using a commercial kit "GenEluteTM" with crystal violet to see the DNA under UV light. We used a 0,8% agar gel with Crystal

Violet, then , after the electrophoresis, we cut the DNA band and used the kit to isolate the DNA from this band in Elution Buffer.

Ligation

In order to ligate the cut vector pET28a with the *lec-2* insert, a 1:5 mole proportion vector/insert was used with T4 DNA ligase and incubate at room temperature for 30 min (see appendix).

Transformation

Two different kinds of competent cells was used for transformations.

TOP10 competent cells whose genotype is:
F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

BL-21(C43) whose genotype is:
F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) [75, 76].

Results

For all DNA electrophoresis, the 1 kb DNA Ladder from NEB was used :

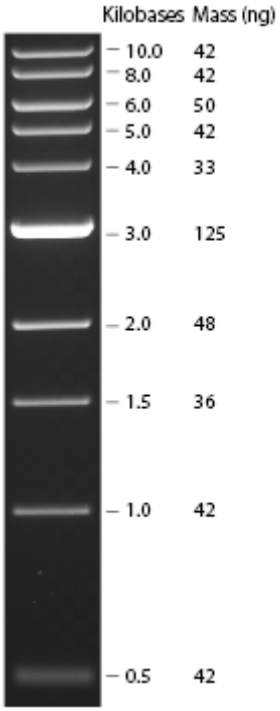


Figure 4: 1 kb ladder

lec-2 PCR from lichen genomic DNA

After amplifying genomic DNA using Lec-2-NcoI-F and Lec2EcoRev primers, a gel was run in order to verify the presence of *lec-2* PCR product.

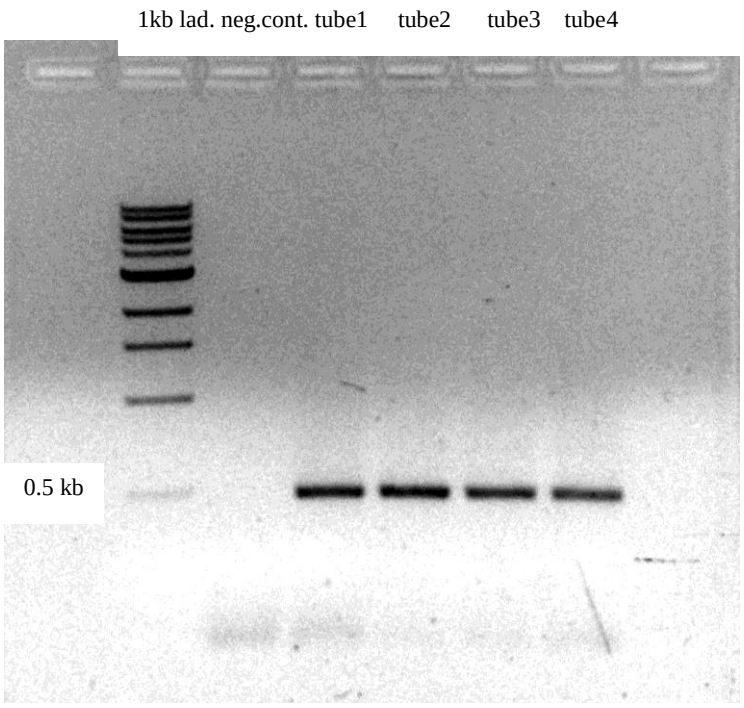


Figure 5: 1% agarose gel electrophoresis of *lec-2* PCR product 11/10/12

Digestion and purification of *lec-2* PCR product

After PCR, the lec-2 PCR product was double digested using *Nco*I and *Eco*RI-HF restriction enzymes and then spin column purification was performed followed by a gel extraction. The following picture show the electrophoresis result:

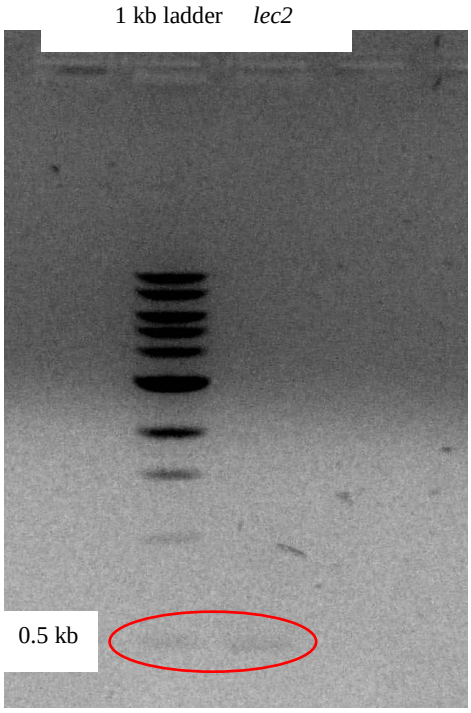
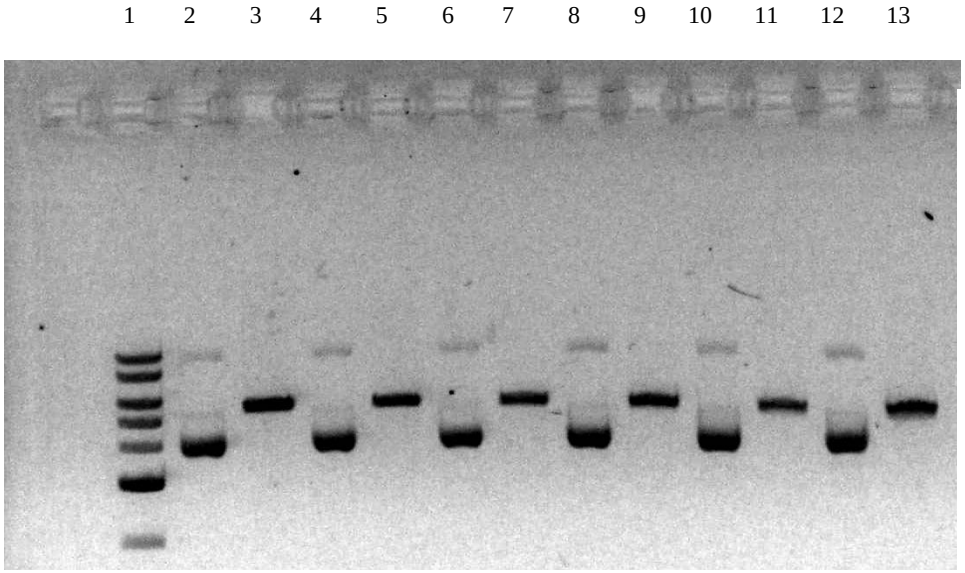


Figure 6: 1% agarose gel electrophoresis of gel purified *lec-2* PCR product 17/10/2012

Simple and double digestion of pET28a plasmid

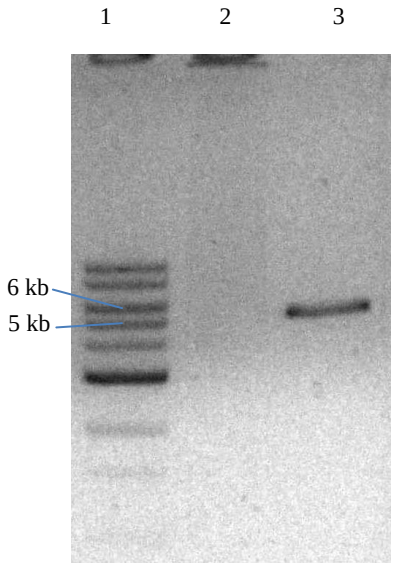
In order to verify the presence of an *Eco*RI restriction site in pET28a plasmid that was isolated, a simple digestion using *Eco*RI restriction enzyme was performed:



1: 1 kb ladder
2: undigested plasmid n°1
3: digested plasmid n°1
4: unidig. plasmid n°2
5: dig. Plamid n°2
6: unidig. plasmid n°3
7: dig. Plamid n°3
8: unidig. plasmid n°4
9: dig. Plamid n°4
10: unidig. plasmid n°5
11: dig. Plamid n°5
12: unidig. plasmid n°6
13: dig. Plamid n°6

Figure 7: 1% agarose gel electrophoresis of pET28a vector digested by *Eco*RI enzyme

The positive results obtained permitted to proceed to the double digestion of pET28a plasmid using *Ecor*RI and *Nco*I restriction enzymes:



1: 1 kb ladder
2: undigested plasmid n°3
3: double digested plasmid n°3

Figure 8: 1% agarose gel electrophoresis of pET28a vector double digested by *Eco*RI and *Nco*I enzymes

Transformation of competent cells

For chemical transformation (see annexe) with TOP10 competent cells, 4 different combinations was used in 2 different dillutions. The results were as follows:

Transformation of TOP 10 competent cells results:

Specificity	Dillution	Nomber of colonies
Vector uncut	1 : 10	> 200
Vector uncut	1 : 100	193
Vector cut	1 : 10	6
Vector cut	1 : 100	2
Insert + Vector - ligase	1 : 10	2
Insert + Vector - ligase	1 : 100	0
Insert + Vector + ligase	1 : 10	17
Insert + Vector + ligase	1 : 100	0

To verify the presence of *lec-2* insert, a colony PCR was performed for 7 colonies from the 17 obtained, using Lec2-NcoI-F and Lec2-EcoRev primers. We then ran a gel to confirm the amplification of lec-2 insert.

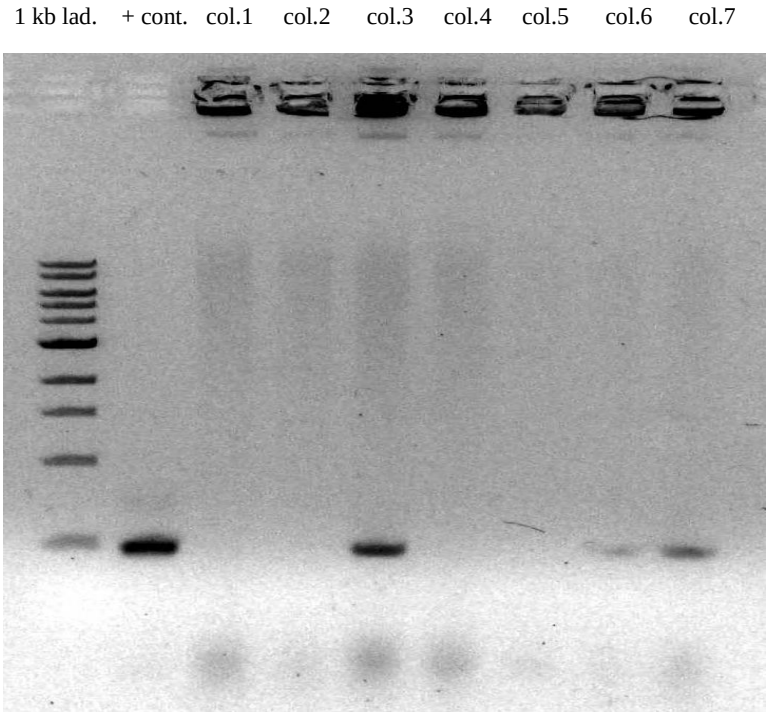


Figure 9: 1% agarose gel electrophoresis of colony PCR using Lec2-NcoI-F and Lec2-EcoRev primers

This electrophoresis confirms the presence of *lec-2* insert in colonies 3, 6 and 7.

To be sure of the presence of *lec-2* insert in colonies 3, 6 and 7, a double digestion of plasmid from these colonies was performed using *Eco*RI and *Nco*I restriction enzymes:

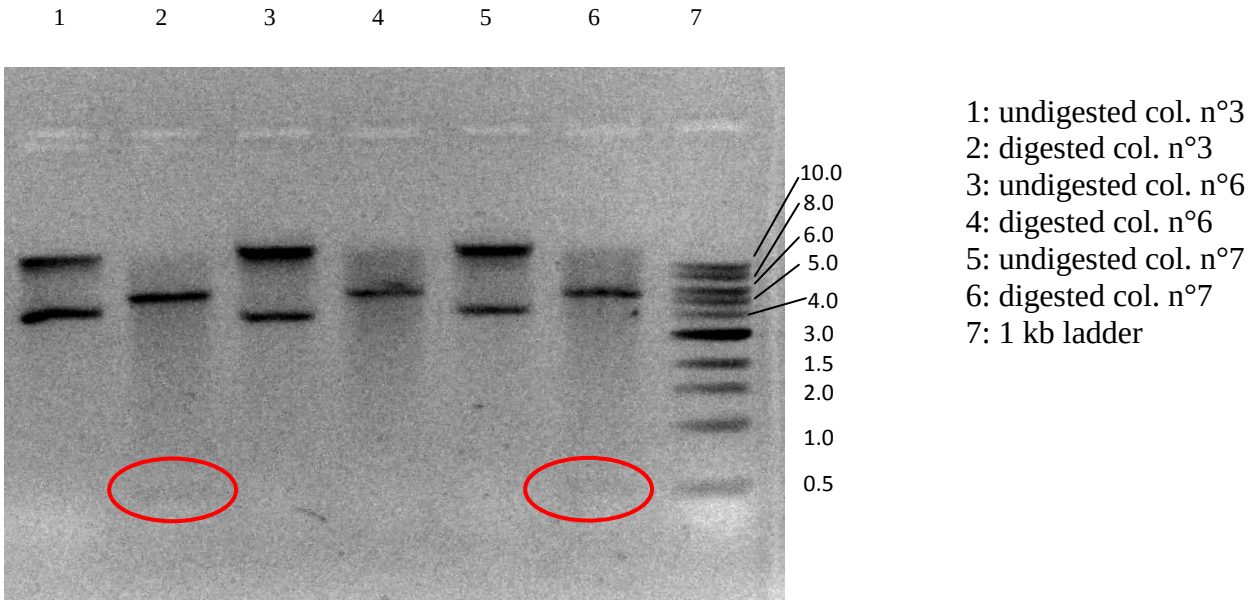


Figure 10: 1% agarose gel electrophoresis of double digestion of plasmids from colonies 3, 6 and 7 using *Eco*RI and *Nco*I enzymes

The presence of *lec-2* insert is confirmed in colonies 3 and 7.

A protein gel analysis has been then proceeded:

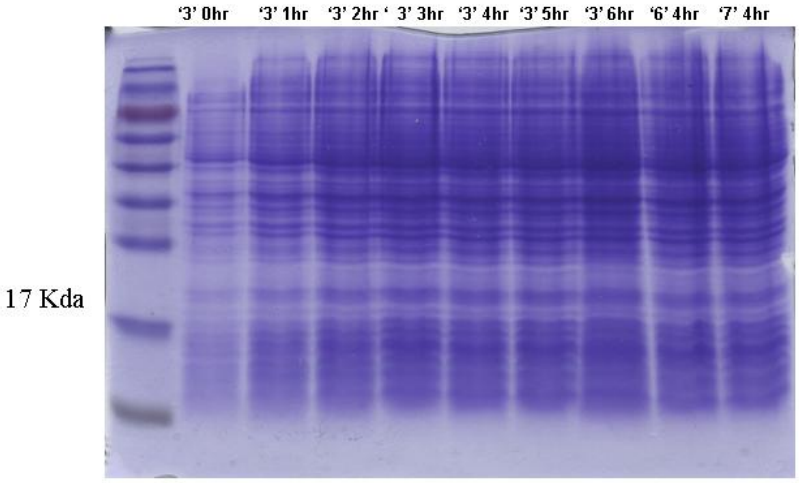


Figure 11: SDS-PAGE of TOP10 transformed competent cells from colonies 3,6 and 7

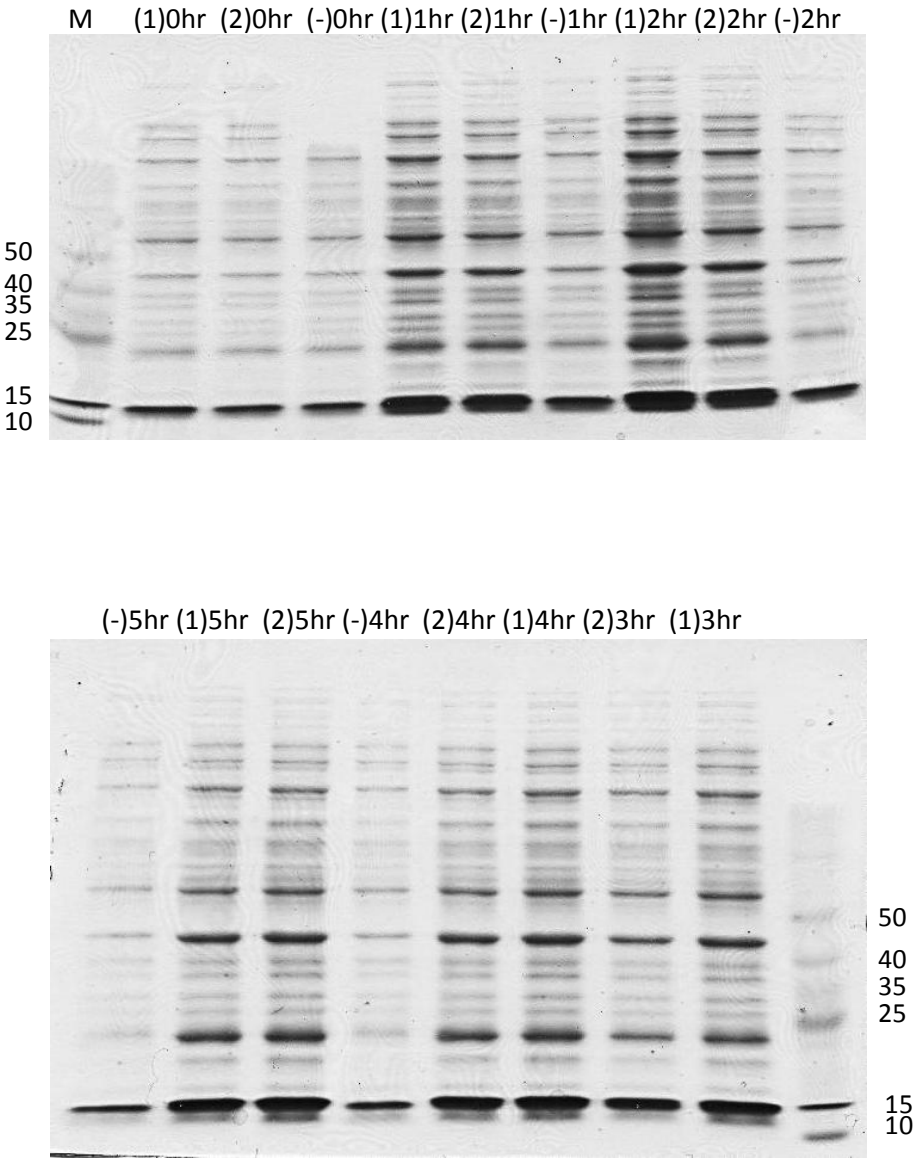
Modified plasmids was then isolated from colonie 3 and used to proceed a transformation in BL21(C43) competent cells which are enable to induce expression of T7 promoter and so LEC-2. The results of this transformation are as follows:

Transformation of BL-21(C43) competent cells results

Specificity	Dillution	Nombre of colonies
pET28a + *lec-2*	1 : 1	3
pET28a	1 : 1	63

In the next part, we will call 1 and 2 two colonies issue of pET28a+*lec-2* transformation and – one colonie issue or pET28a transformation.

The induction of LEC-2 after added IPTG was then performed. Absorbance at 600 nm was made each hour.



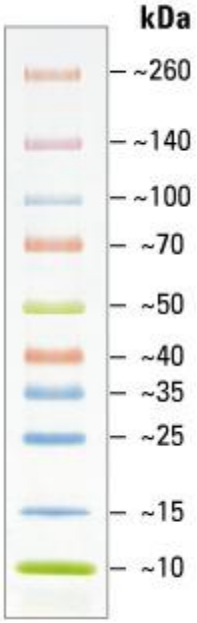


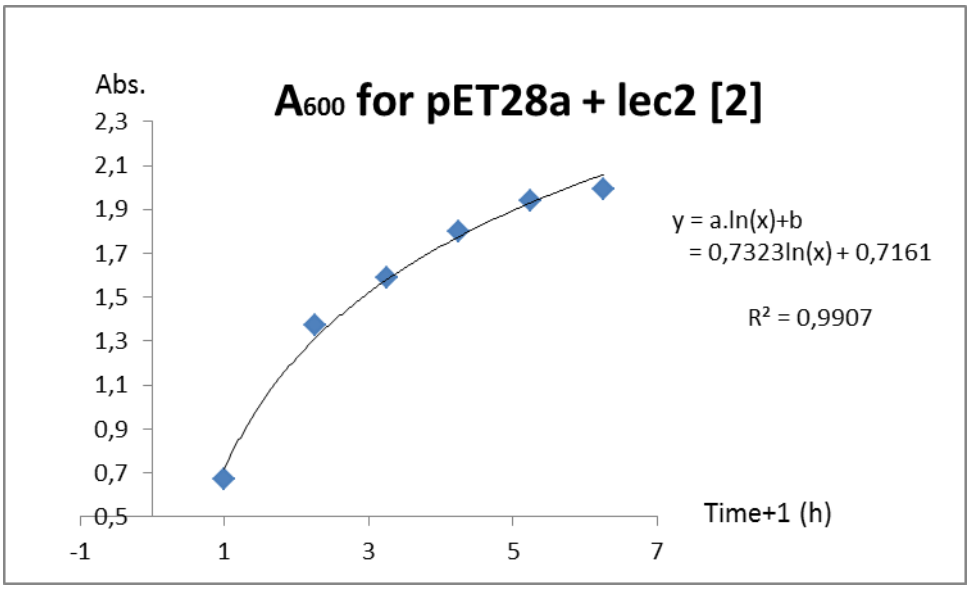
Figure 12: SDS-PAGE band profile of the Thermo Scientific Spectra Multicolor Broad Range Protein Ladder from Invitrogen.

Figure 13: SDS-PAGE of BL-21 (C43) competent cells inducing *lec-2*

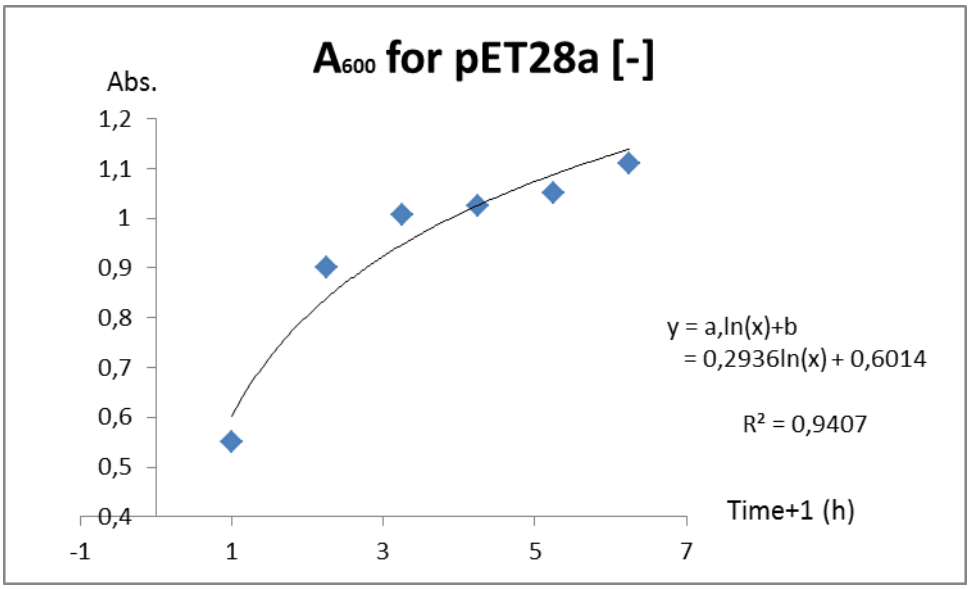
Because the protein concentration was not the same for every sample, another SDS-PAGE electrophoresis was performed with a normalized protein concentration in each sample.

Measures of Absorbance during expression :

		Time	A_{600}	Volume loaded on gel (15/A)	Product conc.
pet28(a)+lec2 [2]	0 hr	11:45	0,62	24,2	15
pet28(a)+lec2 [2]	1 hr	13:00	1,123	13,4	15
pet28(a)+lec2 [2]	2 hr	14:00	1,336	11,2	15
pet28(a)+lec2 [2]	3 hr	15:00	1,504	10,0	15
pet28(a)+lec2 [2]	4 hr	16:00	1,656	9,1	15
pet28(a)+lec2 [2]	5 hr	17:00	1,756	8,5	15



		Time	A_{600}	Volume loaded on gel (15/A)	Product conc.
pet28(a) [4]	0 hr	11:45	0,55	27,3	15
pet28(a) [4]	1 hr	13:00	0,9	16,7	15
pet28(a) [4]	2 hr	14:00	1,007	14,9	15
pet28(a) [4]	3 hr	15:00	1,026	14,6	15
pet28(a) [4]	4 hr	16:00	1,05	14,3	15
pet28(a) [4]	5 hr	17:00	1,109	13,5	15



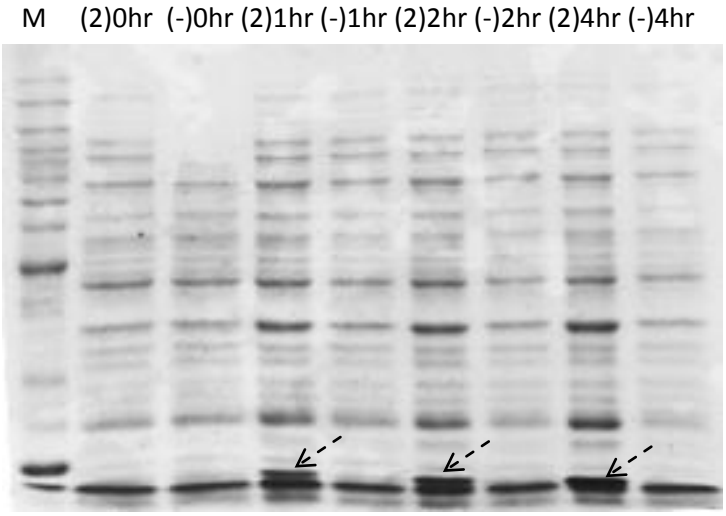


Figure 14: SDS-PAGE of BL-21 (C43) competent cells inducing *lec-2* with normalized concentration

This SDS-PAGE gel shows clearly the induction of LEC-2 in colonie 2 samples. The test permits to think that we should use 4h incubation for a bigger production.

Discussions

In this study, the *lec-2* gene was synthetised from genomic DNA of *P. membranacealichen* and cloned to obtain BL21(C43) cells able to over produce LEC-2.

This results are the needed base to begin a larger production of LEC-2 and proceed to its characterisation.

References

Miao, V.P.W., Manoharan, S.S. 2012. *Expression of lec-1, a mycobiont gene encoding a galectin-like protein in the lighen Peltigera membranacea*. Symbiosis. **57**: 23-31

Manoharan, S.S., Miao, V.P.W., Andrésson Ó.S. In impress. *LEC-2, a highly variable galectin-like genes in the lichen Peltigera membranacea.*

Appendix

Polymerase Chain Reaction (PCR) – Amplification and colony PCR

PCR mixture (add Taq polymerase in last):

Product	Volume (µL)
10X PCR buffer	2,5
25mM $MgCl_2$	1,5
2mM dNTP Mix	2,5
10µM F primer	0,75
10µM R primer	0,75
DNA / Colonies	2,5
M. Q. H2O	14,3
Taq polymerase	0,2
Total	**25**

Apply this thermocycle :

94°C – 2 min
94°C – 20 sec
59°C – 20 sec x33
72°C – 40 sec
72°C – 10 min
12°C – ∞

Colony PCR:

1. Use the standard PCR reaction (25 µL)
2. To each PCR tubes containing 5.5 µL of water add a small amount of colony. To do this, use a fine yellow/red pipette tip attached to a pipette (set 1 µL to avoid addition of air into the PCR reaction) and pipette up and down to mix. The amount of cells should be small, just a touch will do, the small amount required to fill the end of the opening is sufficient. Sufficient mixing will result in complete cell lysis and high yields.
3. Add the PCR mixture to the tubes containing the colonies on ice. For multiple samples, make a large master mix and aliquot in each PCR tube (also on ice).

Double digestion of lec-2 insert

In a tube:

NEB buffer 4	8 µL	1X
DNA	X	6237.7 ng
10u/µL NcoI enzyme	5 µL	50 u
20u/µL EcoRI-HF enzyme	5 µL	100 u
10mg/mL BSA	0.9 µL	0.9 µg

Bring volume up to 80 µL with deionized water.

Split in 4 tubes conaining 20 µL each.

Incubate at 37°C for 3h.

NEB buffer 4

20 mM Tris-HCl
10 mM magnesium acetate $Mg(CH_3COO)_2$
50 mM potassium acetate CH_3CO_2K
1 mM dithiothreitol (DTT)
(pH 8.3 at 25 °C)

Double digestion of *pET28a* plasmid

1. In a tube:

NEB buffer EcoRI	4 µL	1X
DNA	X	2500 ng
10u/µL *NcoI* enzyme	2 µL	20 u
10u/µL *EcoRI* enzyme	2 µL	20 u

2. Bring volume up to 40 µL with deionized water.
3. Split in 2 PCR tubes conaining 20 µL each.
4. Incubate at 37°C for 3h.
5. Heat inactivate at 65°C for 20 min.

NEB buffer EcoRI

1X Composition:

50mM Tris-HCl
10mM $MgCl_2$
100mM NaCl
0.02 % Triton X-100
0.1mg/mL BSA
(pH 7.5 at 37°C)

10X PCR buffer

100 mM Tris-HCl
500 mM KCl
15 mM MgCl2
0.01% (w/v)
(pH 8.3 at 25 °C)

Plasmid isolation

Day 1: Pick a single colony from a selective plate and inoculate a culture of 4 ml LB medium containing the appropriate selective antibiotic. Incubate overnight at 37°C with vigorous shaking.

Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.

Day 2:

1. Pour culture into 1.5 ml microcentrifuge tube and spin 1 minute at 10,000 rpm in a table-top microcentrifuge. Pour off media and resuspend pelleted bacterial cells in 250 µl Buffer P1, using p1000 blue tip to resuspend cells. No cell clumps should be visible after resuspension of the pellet.

2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.

Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.

To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3.

4. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge.

5. Pour the supernatant from step 4 into a spin column.

6. Centrifuge for 60s at 14,000 rpm. Discard the flow-through.

7. Wash spin column by adding 0.70 ml Buffer PE and centrifuging for 1min at 14,000.

8. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

9. Place the spin column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB or water to the center of each spin column, let stand for 2 min at room temperature, and centrifuge for 1 min at 14,000 rpm.

10. Determine plasmid concentration using NanoDrop spectrophotometer. Write DNA concentration on side of tube and store DNA at -20C.

Buffers composition:

LB-medium (400mL):

1. In 300mL deionized water, dissolve:
    - 4 g peptone
    - 2 g bacto yeast extract
    - 4 g NaCl
2. Adjust the pH of the medium to 7.0 using 1N NaOH and bring volume up to 400mL with deionized water.
3. Autoclave on liquid cycle for 20 min at 15 psi. Allow solution to cool to 55°C, and add 20mg kanamycin (50µg/mL).
4. Store at room temperature or +4°C.

Buffer P1

50 mM Tris HCl pH 8.0
10 mM EDTA
(I use 2. 5 ml of 100X Tris-EDTA buffer from Sigma #T-9285 in 50 ml water)
100 ug.ml RNASE A

RNase A
Dissolve RNASE A powder in H_2O to give 100 mg/ml
Boil 5 min to kill DNase
Aliquot and store at –20 deg.

Buffer P2 (200mM NaOH, 1% SDS):

1M NaOH	10 ml
10% SDS	5 ml
Water	35 ml

Buffer N3

3.0 M KOAc, pH 5.5 (29.5 g/ 100ml water, adjust pH with glacial acetic acid)

Buffer PE

75% EtOH.
25 mM NaCl,
5 mM Tris-Hcl, pH 7.5

Buffer EB

10 mM Tris·Cl, pH 8-8.5

Column purification of PCR products

All centrifugations (spins) are at 12,000 – 16,000 x *g*

1. Insert a GenElute plasmid mini spin column (with a blue o-ring) into a provided collection tube, if not already assembled. Add 0.5 ml of the Column Preparation Solution to each mini spin column and centrifuge at 12,000 x *g* for 30 seconds to 1 minute. Discard the eluate.

Note: The Column Preparation Solution maximizes binding of the DNA to the membrane resulting in more consistent yields.

2. Add 5 volumes of Binding Solution to 1 volume of the PCR reaction and mix. For example, add 500 mL of Binding Solution to 100 mL of the PCR reaction. Transfer the solution into the binding column. Centrifuge the column at maximum speed (12,000-16,000 X*g*) for 1 minute. Discard the eluate, but retain the collection tube.

3. Replace the binding column into the collection tube. Apply 0.5 ml of diluted Wash Solution /PE buffer to the column and centrifuge at maximum speed for 1 minute. Discard the eluate, but retain the collection tube.

Note: Be sure to add ethanol to the Wash Solution Concentrate prior to first time use. See Preparation Instructions.

4. Replace the column into the collection tube. Centrifuge the column at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Discard any residual eluate as well as the collection tube.

5. Transfer the column to a fresh 2 ml collection tube. Apply 50 µL of Elution Solution/EB buffer or water to the center of each column. Incubate at room temperature for 1 minute.

Note: When eluting with water, make sure that the pH of the water is between 5.5 and 8.5. Elution may also be performed using the Elution Solution diluted 10-fold with water.

6. To elute the DNA, centrifuge the column at maximum speed for 1 minute. The PCR amplification product is now present in the eluate and is ready for immediate use or storage at – 20 °C.

GenElute™ Gel Extraction Procedure

Spin Procedure for Agarose Gels

All centrifugations (spins) are performed at 12,000 to16,000 x *g* (See Appendix I to convert *g*-force to rpm).

1a. **Excise band.** Excise the DNA fragment ofinterest from the agarose gel with a clean, sharp scalpel or razor blade. Trim away excess gel to minimize the amount of agarose.

2a. **Weigh gel.** Weigh the gel slice in a tared colorless tube.

3a. **Solubilize gel.** Add 3 gel volumes of the Gel Solubilization Solution to the gel slice. In other words, for every 100 mg of agarose gel, add 300 mL of Gel Solubilization Solution. Incubate the gel mixture at 50-60 °C for 10 minutes, or until the gel slice is completely dissolved. Vortex briefly every 2-3 minutes during incubation to help dissolve the gel.

Note: To adequately dissolve a gel with an agarose concentration greater than 2%, it is necessary to increase the ratio of the Gel Solubilization Solution volume to the gel weight to 6:1.

4a. **Prepare binding column.** Preparation of the binding column can be completed while the agarose is being solubilized in step 3a. Place the GenElute Binding Column G into one of the provided 2 ml collection tubes. Add 500 µL of the Column Preparation Solution to each binding column. Centrifuge for 1 minute. Discard flowthrough liquid.

Note: The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.

5a. **Check the color of the mixture.** Once the gel slice is completely dissolved (step 3a) make sure the color of the mixture is yellow (similar to fresh Gel Solubilization Solution with no gel slice) **prior** to proceeding to the following step. If the color of the mixture is red, add 10 mL of the 3 M Sodium Acetate Buffer, pH 5.2, and mix. The color should now be yellow. If not, add the 3 M Sodium Acetate Buffer, pH 5.2, in 10 mL increments until the mixture is yellow.

6a. **Add isopropanol.** Add 1 gel volume of 100% isopropanol and mix until homogenous. For a gel with an agarose concentration greater than 2%, use 2 gel volumes of 100% isopropanol.

7a. **Bind DNA.** Load the solubilized gel solution mixture from step 6a into the binding column that is assembled in a 2 ml collection tube. It is normal to see an occasional color change from yellow to red once the sample is applied to the binding column. If the volume of the gel mixture is >700 mL, load the sample onto the column in 700 µL portions. Centrifuge for 1 minute after loading the column each time. Discard the flowthrough liquid.

Note: Do not be alarmed if the flow-through has changed color.

8a. **Wash column.** Add 700 mL of Wash Solution (diluted from Wash Solution Concentrate G as described under Preparation Instructions) to the binding column. Centrifuge for 1 minute. Remove the binding column from the collection tube and discard the flow-through liquid. Place the binding column back into the collection tube and centrifuge again for 1 minute without any additional wash solution to remove excess ethanol. Residual Wash Solution will not be completely removed unless the flow-through is discarded before the final centrifugation.

9a. **Elute DNA.** Transfer the binding column to a fresh collection tube. Add 25 µL of Elution Solution to the center of the membrane and incubate for 1 minute. Centrifuge for 1 minute. For efficient recovery of intact plasmid DNA, preheat the elution solution to 65 °C prior to adding it to the membrane. Eluting at 65 °C improves plasmid DNA recoveries by 2 to 3-fold. Yields of large linear DNA fragments (>3 Kb) can also be increased by up to 20% by preheating the elution solution to 65 °C.

Note: To increase the concentration of the eluted DNA, the volume of Elution Solution may be reduced to 25 mL. Yields are ~25% lower when eluting with 25 mL as opposed to 50 mL.

Ligation of pEt28a vector and lec-2 insert

1. In ice, mix in a tube:

pET28a vector (100 ng)	X
Lec-2 insert (500 ng)	Y
10X Tango Buffer	2 µL
0.5 mM ATP	5 µL
5u/µL T4 DNA ligase	1 µL

2. Bring volume up to 20 µL with deionized water.
3. Gently mix by pipetting up and down.
4. Incubate at room temperature for 30 min.
5. Heat inactivate at 65°C for 5 min.
6. Keep in ice, transfert in PCR tubes.
7. Store at -20°C.

Buffer Tango

1X Composition:

33mM Tris-acetate
10mM Mg-acetate
66mM K-acetate
0.1mg/mL BSA
(pH 7.9 at 37°C)

Chemical transformation

1. Remove tube of frozen competent cells from -80 °C and place on ice. Allow cells to thaw. Note: Keep cells chilled on ice to ensure high transformation efficiency.
2. Mix cells by flicking the tube gently, then remove 200µl per transformation into a sterile pre-chilled (on ice) tube.
3. Add 140 ng of DNA (in a volume no greater than 20µl) per 200ul cells. Quickly flick the tube several times to ensure the even distribution of DNA.
4. Immediately place tubes on ice for 30 minutes.
5. Heat shock the cells for 90 seconds in a water bath at **exactly** 42 °C. Do not shake.
6. Add 800µl of room temp (or 37 °C) SOC and incubate for 1 hour at 37 °C with shaking at 225 rpm.
7. Plate 100 µl of the transformation mix or an appropriate dilution (1 : 10 and 1 : 100) onto antibiotic plates.
8. Place plates in the 37 °C incubator and grow overnight 16h.
9. Store in 4°C before to pour plates.

SOC medium

2.0 g Bacto-Tryptone
0.5 g Bacto-Yeast extract
1 ml 1M NaCl
0.25 ml 1M KCl
1 ml 2M Mg stock (1M $MgCl_2$-6H_2O, 1M $MgSO_4$-7H_2O), filter sterilize
1 ml 2M Glucose, filter sterilize
Up to 100 ml ddH_2O

Add Bacto-Tryptone, Bacto-Yeast extract, NaCl and KCl to 97ml ddH_2O. Stir to dissolve. Autoclave and cool to room temp. Add 2M Mg stock and 2M Glucose, each to a final concentration 20mM. Filter the complete medium through a 0.2um filter. The pH should be 7.0.

LB agar-plates (400mL):

1. In 300mL deionized water, dissolve:
    - 4 g peptone
    - 2 g bacto yeast extract
    - 4 g NaCl
2. Adjust the pH of the medium to 7.0 using 1N NaOH.
3. Add 6g bacto agar.

4. Bring volume up to 400mL with deionized water.
5. Autoclave on liquid cycle for 20 min at 15 psi. Allow solution to cool to 55°C, and add 20mg kanamycin (50µg/mL).
6. Pour plates, then invert and store at +4°C in the dark.

Preparation and Transformation of TSS-Competent Cells

Preparation of Competent Cells:

1. Dilute an overnight culture of BL21 – C43 1:50 with LB broth.
2. Incubate at 37°C with shaking (at 200 rpm) until the cells reach early log phase (OD_{600} = 0.5).
3. While cells are growing, thaw 2X TSS on ice and dilute an appropriate amount 1:1 with sterile distilled water (100 µl of diluted TSS will be needed for each ml of cells). Chill on ice.
4. Place 1.0-ml aliquots of early log-phase cells into sterile 1.5-ml microcentrifuge tubes and pellet the cells by centrifugation at 4°C for 2 minutes.
5. Remove the supernatant with a sterile pipet tip and discard. Add 0.1 ml of the ice-cold 1X TSS and place the tubes on ice.
6. Gently suspend the cells by pipeting.
7. Proceed with the transformation protocol below.

Transformation of TSS-Competent Cells:

1. Add 50 ng of DNA to each tube of competent cells.
3. Flick the tubes to mix the cells and DNA and incubate the cells on ice for 10 minutes.
4. Heat shock the cells at 42°C for 90 sec.
5. Add 1 mL of LB broth.
6. Immediately incubate the cells at 37°C for up to 1 hour with shaking (at 200 rpm).
7. Plate the cells onto LB kanamycin plates and incubate overnight at 37°C.

<u>2X TSS (Transformation and Storage Solution for chemical transformation)</u>

- 85 % LB medium
- 10 % PEG (wt/vol, MW 8000)
- 5 % DMSO (vol/vol)
- 50 mM MgCl2 (pH 6.5)

Auto-induction: Studier method

1. At the end of day 1, streak plasmid-transformed BL21 bacteria from a frozen glycerol stock onto an LB + 1% glucose agar plate with an appropriate antibiotic. Place in a 37°C incubator overnight.
2. First thing in the morning on day 2, transfer a single colony from the fresh agar plate into 2 ml of ZYP-0.8G plus appropriate antibiotic in a 18 x 150 mm snap cap tube. Shake at 300 RPM at 37°C for 6-8 hours, until the culture is turbid but not saturated.
3. While the Inoculum Culture in step 2 is growing, prepare 6 2-liter baffled Erlenmayer flasks with 400 ml each of ZYP-5052 media plus appropriate antibiotic.
4. Towards the end of day 2, transfer 200 µl of the Inoculum Culture into each of the 6 2-liter flasks containing 400 ml ZYP-5052 + antibiotic. Shake at 300 RPM at 37 °C overnight.
5. First thing in the morning on day 3, cool the cultures by placing the Erlenmayer flasks containing the cultures ice buckets. Use flat, shallow ice buckets.
6. While the cultures are cooling, take a 1.0 ml sample from each flask and place in a labeled 1.5 ml microcentrifuge tube. Prepare a 1:10 dilution of each sample by transferring 0.1 ml of each into each of a second set of microcentrifuge tubes containing 0.9 ml of fresh ZYP-5052. Prepare an additional microcentrifuge tube containing 1.0 ml of fresh ZYP-5052 to use as a blank.
7. Measure the OD(600) of the 1:10 dilutions of each of the cultures and record the result in the Inclusion Body QC Database.
8. Pellet the bacteria in the remaining 0.9 ml by spinning the microcentrifuge at 1/2 of full speed for 2 minutes. Aspirate the supernatant.
9. Resuspend the bacteria in the microcentrifuge tubes in 1X SDS-PAGE reducing sample buffer (SDS-rSB) in two stages: (1) add 50 µl of SDS-rSB (or water?) to each of the pellets and vortex vigorously to resuspend the pellet; (2) add 400 µl of SDS-rSB to each of these suspensions, to bring the total volume of SDS-rSB to 1/2 of the culture volume. These samples will be used for subsequent analysis of total bacterial protein by SDS-PAGE.
10. Check to see that the chromosomal DNA in the samples has been thoroughly sheared by vortexing. Tell-tale signs of unsheared DNA are a “stringy” consistency that is observed when the tubes are opened or when a small volume is drawn up into a pipette tip. If residual unsheared DNA remains, it can be sheared by further vigorous vortexing. As a last resort, the DNA can be sheared by sonication for 2-3 minutes using a microtip sonicator at 50% duty cycle, with the samples cooled on ice.
11. Combine the contents of two of the Erlenmayer flasks into a single 1-liter centrifuge bottle, resulting in 800 ml of bacterial culture in the flasks. Similarly combine the contents of remaining flasks into 2 additional centrifuge bottles, yielding a total of three centrifuge bottles each containing 800 ml of culture. Equalize the volume of bacteria in the cultures by eye, transferring culture liquid from one or more bottles to one a bottle that may contain slightly less liquid. Balance the bottles on a pan balance by moving liquid with a pipette from heavier to lighter bottles.
12. Pellet the bacteria in the 1 liter bottles by spinning for 20 minutes at 5000 x *g* at 4°C.

13. While the bacterial cultures are in the centrifuge, prepare an SDS-PAGE gel for analysis of total protein. You should be able to prepare at least the separating portion of the gel before the centrifuge run is completed.
14. Decant the supernatants from the 1-liter centrifuge bottles into a large bucket or beaker, treat with a few hundred ml of 100% bleach, and then pour down the drain, rinsing with copious water.
15. Add 20 ml of resuspension buffer to each of the bacterial pellets in the 1-liter bottles. Resuspend to homogeneity by vigorous shaking on the platform rocker in the cold room. This can take up to 20-30 minutes.
16. While the bacteria is shaking in resuspension buffer, finish preparing your SDS-PAGE gel. In addition, set up a full 500-1000 ml beaker of water to boil for heating up your SDS-PAGE samples.
17. Run your SDS-PAGE gel.

ZY

- 10 g N-Z-amine AS (or any tryptic digest of casein, e.g. tryptone)
- 5 g yeast extract
- 925 ml water

20x NPS

Component	100 ml	1 liter	mol/liter
dd H_2O	90 ml	900 ml	-
$(NH_4)_2SO_4$	6.6 g	66 g	0.5 M
KH_2PO_4	13.6 g	136 g	1 M
Na_2HPO_4	14.2 g	142 g	1 M

- Add in sequence in beaker; stir until all dissolved.
- pH of 20-fold dilution in water should be ~6.75.

50x5052: (5052 = 0.5 % glycerol, 0.05% glucose, 0.2% α-lactose)

Component	100 ml	1 liter
Glycerol (weigh in beaker)	25 g	250 g

H_2O	73 ml	730 ml
Glucose	2.5 g	25 g
a-lactose	10 g	100 g

- Add in sequence in beaker, stir until all dissolved
- Lactose is slow to dissolve — may take two hours or more of stirring. Brief heating in a microwave can speed up dissolution of the lactose.

1 M $MgSO_4$

- 24.65 g $MgSO_4 \cdot 7H_2O$
- Water to make 100 ml

40% glucose (w/v)

Component	**100 ml**	**300 ml**
Glucose	40 g	120 g
H_2O	74 ml	222 ml

- Add glucose to stirring water in beaker; DO NOT ATTEMPT TO ADD WATER TO GLUCOSE!
- Stir until all dissolved — may take 45 minutes or more of stirring.

80% glycerol (v/v) (= 100% w/v)

- 100 g glycerol (weigh in beaker)
- 20 ml water

20% α-lactose (w/v)

Component	**100 ml**	**600 ml**
a-lactose	20 g	120 g

H_2O	87.5 ml	525 ml

- add lactose to stirring water in beaker
- stir until all dissolved -- may take 2 hours or more

1000x trace metals mixture (100 ml in ~50 mM HCl)

Prepare all metal stock solutions in ddH_2O, except for $FeCl_3$, which is dissolved in ~0.1M HCl, as noted in the table below. Combine the metal solutions as in the table below.

Component	Vol	MW	1x conc
H_2O	36 ml	-	-
0.1 M $FeCL_3$•$6H_2O$ (dissolved in ~0.1 M HCl = 100-fold dil of conc HCl)	50 ml	270.30	50 µM Fe
1M $CaCl_2$	2 ml	110.99	20 µM Ca
1M $MnCl_2$•$4H_2O$	1 ml	197.91	10 µM
1 M $ZnSO_4$•$7H_2O$	1 ml	287.56	10 µM Zn
0.2 M $CoCl_2$•$6H_2O$	1 ml	237.95	2 µM Co
0.1 M $CuCl_2$•$2H_2O$	2 ml	170.486	2 µM Cu
0.2 M $NiCl_2$•$6H_2O$	1 ml	237.72	2 µM Ni
0.1 M Na_2MoO_4•$2H_2O$	2 ml	241.98	2 µM Mo
0.1 M Na_2SeO_3•$5H_2O$	2 ml	263.03	2 µM Se

0.1 M H_3BO_3	2 ml	61.83	2 µM H_3BO_3

Autoclave the stock solutions of the individual metals, except 0.1 M $FeCl_3$ in 1/100 volume conc HCl.

A brief precipitate appeared upon addition of Na_2SeO_3, which redissolved rapidly

Store at room temperature

When making growth media, add the metals mix before NPS. If NPS is already present when 1000x metals mix is added, a precipitate forms which disperses but retains a light turbidity. If the metals are diluted to near their final concentration before NPS is added, the medium remains clear. The metals also precipitate and disperse or redissolve when added to ZY, a precipitate caused by yeast extract. Although apparently not a problem, the precipitate could be avoided by diluting the metals in the water before dissolving the yeast extract in making ZY.

Antibiotics

- Kanamycin (25 mg/ml in water, filter sterilize)
- Chloramphenicol (25 mg/ml in 95% ethanol, filter sterilize)
- Ampicillin (50 mg/ml in water, filter sterilize)

P-0.5G defined minimal medium for growth to saturation with little or no induction

- grow log-phase or saturated cultures for making freezer stocks and working stocks
- For all media, add 1 M $MgSO_4$ and 1000x metals mix before adding 20xNPS to avoid precipitate

Component	**50 ml**	**100 ml**	**200 ml**	**Final concentration**
ddH_2O (sterile)	~46.8 ml	~93.6 ml	~187.3 ml	-
1 M $MgSO_4$	50 µl	100 µl	200 µl	1 mM
10000x metals mix	5 µl	10 µl	20 µl	1
40% glucose	0.625	1.25 ml	2.5 ml	0.5%

	ml			
20x NPS	2.5 ml	5 ml	10 ml	1x
kanamycin (25 mg/ml)	0.2 ml	0.4 ml	0.8 ml	100 µg/ml

ZYP-0.8G

- Rich medium for growth with little or no induction
- Culture should go somewhat acid at saturation (slightly below pH 6)
- Collect cultures for freezer stocks well before saturation
- For all media, add 1 M $MgSO_4$ before adding 20xNPS to avoid precipitate
- Kanamycin is used at significantly higher concentrations (100 µg/ml) than is normally the case (25-40 µg/ml). Studier has found that in the T7 expression strains in these rich media, it does not provide adequate selection at the lower concentrations.

Component	**50 ml**	**100 ml**	**200 ml**	**400 ml**	**Concentration**
ZY	~46.5 ml	~93 ml	~186 ml	~372 ml	-
1 M $MgSO_4$	50 µl	100 µl	0.2 ml	0.4 ml	1 mM
40% glucose	1 ml	2 ml	4 ml	8 ml	0.8%
20x NPS	2.5 ml	5 ml	10 ml	20 ml	1x
kanamycin (25 mg/ml)	200 µl	0.4 ml	0.8 ml	1.6 ml	100 µg/ml

ZYP-5052 rich medium for auto-induction

- For all media, add 1 M $MgSO_4$ before adding 20xNPS to avoid precipitate

- Kanamycin is used at significantly higher concentrations (100 µg/ml) than is normally the case (25-40 µg/ml). Studier has found that in the T7 expression strains in these rich media, it does not provide adequate selection at the lower concentrations.
- We use 400 ml in a 2 liter baffled flask.

  Adequate aeration is essential to the performance of this media. Don't even think about using more than 20% of the nominal volume of the flask.

  Baffled flasks will give significantly better performance. You might obtain adequate results with non-baffled flasks, but I don't recommend it.

Component	200 ml	400 ml	500 ml	1 liter	Concentration
ZY	~186 ml	~372 ml	~464 ml	~928 ml	-
1 M $MgSO_4$	0.2 ml	0.4 ml	0.5 ml	1 ml	1 mM
50x 5052	4 ml	8 ml	10 ml	20 ml	1x
20x NPS	10 ml	20 ml	25 ml	50 ml	1x
kanamycin (25 mg/ml)	0.8 ml	1.6 ml	2 ml	4 ml	100 µg/ml

Resuspension Buffer

- 50 mM Tris-HCL
- 25% (W/V) sucrose
- 1 mM EDTA
- 0.1% (w/v) NaAzide
- 10 mM DTT (add fresh)

Protein extraction

1. In a tube centrifuge 500 µL of cell culture at 14.000 rpm for 1 min.
2. Pour off supernatant and resuspend the pellet in 100 µL of RSS.
3. Boil it for 5 min.

RSS:

100 mM Tris pH7
3% SDS (Sodium dodecyl sulfate) (vol/vol)
5% Glycerol (vol/vol)
2% 2-Mercaptoethanol (vol/vol)
10 mM DTT
5 mg Bromophenol Blue
Deionized water to 15 mL
(store at -20°C)

SDS-PAGE

1. Load the lower gel (or running gel) just after having prepared. Load slowly to avoid bubbles.
2. Add water to avoid reaction with oxygen.
3. Wait 15 min until it is gel.
4. Load the upper gel (or stacking gel) just after having prepared. Add the comb slowly.
5. Wait 5 min.
6. Add the samples.

Buffer A

1.5 M Tris/HCl (181.8g/L), pH 8.9

Buffer B

0.5 M Tris/HCl (60.6 g/L), 10 mL/L TEMED, ph 6.8

Buffer C

30 % acrylamide (30g/100mL), 2.6% bisacrylamide(0.8 g/100 mL)

Buffer Pn

1% ammonium persulfat (0.1 g/ 10 mL)

Buffer Pe

10% ammonium persulfat (1 g/10 mL)

Running gel:

Buffer A	2.25 mL
Buffer C	3.75 mL
dH_2O	2.95 mL
TEMED	12.5 µL
Pn	0.05 mL

Note: Pn has to be add in last because it launches the polymerization.

Stocking gel:

Buffer B	0.625 mL
Buffer C	0.375 mL
dH_2O	1.600 mL
Pe	0.060 mL

Sample preparation:

1. In a tube centrifuge 500 µL of cell culture at 14.000 rpm for 1 min.
2. Pour off supernatant and resuspend the pellet in 100 µL of RSS.
3. Boil it for 5 min.

RSS:

100 mM Tris pH7
3% SDS (Sodium dodecyl sulfate) (vol/vol)
5% Glycerol (vol/vol)
2% 2-Mercaptoethanol (vol/vol)
10 mM DTT
5 mg Bromophenol Blue
Deionized water to 15 mL
(store at -20°C)

1.