Silicatein and Calcium Carbonate

Lab Books

Silicatein

15/08/17

Resuspension of the primers with pure water :

o.17.017 : 240µL

o.17.018 : 275µL

o.17.019 : 244µL

o.17.020 : 275µL

o.17.021 : 218µL

o.17.022 : 265µL

o.17.023 : 243µL

o.17.024 : 233µL

o.17.025 : 221µL

o.17.026 : 300µL

o.17.027 : 295µL

o.17.028 : 274µL

Storage in -20°C.

16/08/17

Annealing of Anderson promoter. Tube o.17.027 et tube o.17.028

in a 1.5mL eppendorf tube :

- 50µL of o.17.027
- 50µL of o.17.028

Incubation 5min at 95°C.

Cooling overnight.

17/08/17

PCR

1. Pbad

o.17.017

o.17.018

Tm : 50°C

2. P0015	3. P0015
o.17.019 |	o.17.020
o.17.021 |	o.17.021
Tm : 51.2°C	Tm : 51.2°C

Dilution of primers to 1/10 to get a work solution.

Dilution of the DNA solution to 1/50.

PCR solution :

- 19µL of water
- 1µL of DNA
- 2.5µL of Forward Primer
- 2.5µL of Reverse Primer
- 25µL of Master Mix

Cycle :

Initial denaturation : 95°C 3min;

|Denaturation : 95°C 30s;

x30 { |Annealing : X°C 30s;

|Elongation : 72°C 1min;

Final elongation : 72°C 5min;

Gel migration :

2% gel with 5µL of SybrSafe

Failed ladder	Empty	1	2	3	Ladder
__	__	__	__	__	__

18/08/17

PCR purification

Kit Qiagen :

- 5 volume of PB buffer for 1vol of PCR product;
- Pour the solution in the column;
- Centrifuge at 13Krmp for 60s;
- Discard the flow-through;
- Add 750µL of PE Buffer;
- Centrifuge at 13Krmp for 60s;
- Discard the flow-through;
- Centrifuge at 13Krmp for 60s;
- Place the column in a 1.5mL eppendrof tube;
- Add 50µL of EB buffer in the column;
- Let it still for 1min;
- Centrifuge at 13Krmp for 60s;

DNA concentration of sample :

1 : 23.0ng/µL;

2 : 38.1ng/µL;

3 : 17.0ng/µL.

Resuspension of DNA obtain from Delf :

- Cut the filter paper with the DNA;
- Put it in an 1.5mL eppendorf;
- Add 100µL of Water;
- Leave it for 10 min at room temperature.

Silicatein Delft : 2.4ng/µL

Silicatein Ompa Delft : 1.5ng/µL

19/08/17

Do everything on ice unless told otherwise:

- Thaw competent cell on ice for 10/15 min;
- Pipette 50µL of competent cell in cold 1.5 mL eppendorf tube;
- Pipette 5µL of resuspended DNA/ same quantity of water for the control;
- Incubate on ice for 30min;
- Heatshock at 42°C for 45s;
- Add 950µL of LB media;
- Incubate at 37°C shaking at 300rpm for 1h;
- Pipette 100µL on half a plate and spread it;
- Centrifuge the remaining at 3000 rpm/3min;
- Remove 800µL of supernatant;
- Resuspend the cell in remaining media;
- Pipette it and spread it on the other half of the plate;

- Incubate overnight;
- Take a colony and do an overnight liquid culture and a striking for a plate culture.

→ Growth on all the plate, even the control.

21/08/17

Colony PCR with the two transformation and the control, plus a PCR control.

PCR solution :

- 19µL of water;
- 1µL of a colony;
- 2.5µL of Forward Primer;
- 2.5µL of Reverse Primer;
- 25µL of Master Mix.

1. Silicatein Delft transformed. From a striked colony.
2. Silicatein Ompa Delft transformed. From a striked colony.
3. Negative Ctrl Transformation.
4. PCR Negative Ctrl.

Cycle :

Initial denaturation : 95°C 3min

x30 { |Denaturation : 95°C 30s
|Annealing : 51.8°C 30s
|Elongation : 72°C 3min

Final elongation : 72°C 5min

Gel migration 1%

Ladder	Empty	1	2	3	4
—	—	—	—	—	—

22/08/17

Transformation. Do everything on ice unless told otherwise → growth on all the plate, even the control.

23/08/17

Resuspension of Silicateinβ + RBS + P0015;

Centrifuge the tube for 3/5 sec at 3000rpm;

Add 100µL/ng to reach final concentration of 10ng/µL;

Vortex briefly;

Incubate at 50°C for 20min;

Briefly vortex and centrifuge;

Store at -20°C.

24/08/17

Colony PCR with the two transformation from the 22/08 and the control, plus a PCR control.

PCR solution :

- 19µL of water;
- A colony picked with a toothpick;
- 2.5µL of Forward Primer;
- 2.5µL of Reverse Primer;

- 25µL of Master Mix.

1. Silicatein Delft transformation 1. From a striked colony
2. Silicatein Ompa Delft transformation 1. From a striked colony
3. Negative Ctrl transformation 1.
4. Silicatein Delft transformation 2
5. Silicatein Ompa Delft transformation 2
6. Silicatein Delft Ctrl 1, transformation 2
7. Silicatein Delft Ctrl 2, transformation 2
8. PCR Negative Ctrl

Cycle :

Initial denaturation : 95°C 3min

|Denaturation : 95°C 30s

x30 { |Annealing : 51.8°C 30s

|Elongation : 72°C 3min

Final elongation : 72°C 5min

Gel migration 1%

Ladder 1 2 3 4 5 6 7 8 Aya Aya Ladder

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Transformation :

Sample : Silicatein Delft, Silicatein Ompa Delft, Controle. → Chloramphenicol

Sample : Aya's, Aya's controle → kanamycin

25/08/17

- Resuspension of Silintaphin-1 + RBS + P0015;
- Centrifuge the tube for 3/5 sec at 3000rpm;
- Add 100µL/ng to reach final concentration of 10ng/µL;
- Vortex briefly;
- Incubate at 50°C for 20min;
- Briefly vortex and centrifuge;
- Store at -20°C.

Resuspension of Pasteur silicatein :

Add 15µL to get a 18µL with a concentration of 10ng/µL

26/08/17

Test of precision of the different iGEM pipette in the lab :

https://docs.google.com/spreadsheets/d/1uQRsf8QE5ZlBULMAteCwDEmwZNn10ev4l7PDxfCS8BE/edit#gid=0

27/08/17

Liquid culture of 3 colony of Silicateinα from the third transformation, one from the growth test of the second transformation of silicatein and one from the first negative control of the second transformation.

Each colony is peaked and resuspended in 10 mL of LB medium with chloramphenicol.

28/08/17

Miniprep 2mL of yesterday culture.

Nanodrop results :

1. First colony : 114.5ng/µL;
2. Second colony : 142.1ng/µL;
3. Third colony : 29.7ng/µL ugly results;
4. Growth test of normal silicatein from second transformation : 108ng/µL;
5. Growth test of the negative control from the second transformation : 101.6ng/µL.

PCR the results of the miniprep

PCR solution :

- 19µL of water;
- 1µL of DNA;
- 2.5µL of Forward Primer;
- 2.5µL of Reverse Primer;
- 25µL of Master Mix.

Cycle :

Initial denaturation : 95°C 3min

x30 { |Denaturation : 95°C 30s
|Annealing : 61.5°C 30s
|Elongation : 72°C 1min

Final elongation : 72°C 5min

Gel migration 1% for 20min

Ladder 1 2 3 4 5 6 Ctrl Ladder

__ __ __ __ __ __ __ __ __ __ __ __

29/08/17

Do a 1/10 dilution of yesterday miniprep and do a new PCR.

PCR solution :

- 19µL of water;
- 1µL of DNA;
- 2.5µL of Forward Primer;
- 2.5µL of Reverse Primer;
- 25µL of Master Mix;

Cycle :

Initial denaturation : 95°C 3min

x30 { |Denaturation : 95°C 30s
|Annealing : 61.5°C 30s
|Elongation : 72°C 1min

Final elongation : 72°C 5min

Gel migration 1% for 20min

Ladder 1 2 3 4 5 6 Ctrl

Ladder

__ __ __ __ __ __ __ __ __ __ __ __

PCR directly the samples send by Delft.

PCR solution :

- 19µL of water;
- 1µL of DNA;
- 2.5µL of Forward Primer;
- 2.5µL of Reverse Primer;
- 25µL of Master Mix;

Cycle :

Initial denaturation : 95°C 3min

|Denaturation : 95°C 30s

x30 { |Annealing : 61.5°C 30s

|Elongation : 72°C 1min

Final elongation : 72°C 5min

Gel migration 1% for 20min

The first is normal silicatein, the second is the silicatein with Ompa domain.

30/08/17

Purification of yesterday PCR.

Mix 1 volume of PCR product with 5 volume of PB buffer.

Poor in a column and centrifuge 45s at 13k rpm.

Discard the flow-through, keep the column and the tube.

Add 750µL of PE buffer.

Centrifuge 45s at 13k rpm.

Discard the flow-through.

Centrifuge 45s at 13k rpm.

Place the column in a new 1.5mL tube.

Add 50µL of EB buffer in the column and let it still for 1min

Centrifuge 1min at 13k rpm.

Silicatein : 41.5ng/µL

Silicatein + Ompa : 17.6ng/µL

GoldenGate :

Silicateinα

pSB4K5 : 1,34µL

Pbad : 1,40µL

Silicateinα : 1,30µL

P0015 : 1,88µL

T4 ligase : 1µL

Bsa1 : 1µL

10X T4buffer : 2µL

10X Bsa1 buffer : 2µL

Water : 8,09µL

Silicateinα + Ompa :

pSB4K5 : 1,34µL

Pbad : 1,40µL

Sil+Ompa : 6,24µL

P0015 prime : 0,84µL

T4 ligase : 1µL

Bsa1 : 1µL

10X T4buffer : 2µL

10X Bsa1 buffer : 2µL

Water : 4,19µL

Cycle :

37°C 10min

80x |16°C 5min

|37°C 5min

37°C 10min

80°C 10min

65°C 10min

12°C ∞min

31/08/17

Transformation.

1/09/17

Put 3 colony in Liquid LB media for overnight culture.

2/09/17

Miniprep 2mL of the 3 cultures

Transfer 2mL of each culture in 2mL tube

Centrifuge at 8000rpm for 2min to pellet the cells, remove the supernatant

Use of the thermofisher GeneJET Plasmid Miniprep Kit

Ressuspend with 250µL of resuspension solution and vortex

Add 250µL of lysis solution and invert the tubes 6 times

Add 350µL of Neutralization solution and invert the tubes 6 times.

Centrifuge 5 min at 12k rpm.

Transfer the supernatant in a column

Add 500µL of wash solution and centrifuge for 60s at 12k rpm

Discard the flow-through

Repeat the two last step.

Centrifuge empty column for 1 min at 12k rpm

Transfer the column in a new tube.

Add 50µL of Elution Buffer and incubate 2min

Centrifuge 2min at 12k rpm

Throw the column and store the tube in freezer.

Store remaining cell in the fridge

4/09/17

Start new cultures with the cell in the fridge :

Take 100µL and put it in 5mL of LB + Kanamicyn.

8/09/17

Start new cultures with the previous one :

Take 100µL and put it in 5mL of LB + Kanamicyn.

9/09/17

Test silicate production :

Start new culture with 3 tubes for each colony.

Wait for the beginning of log phase.

Induce with 1% of Arabinose 1X

Wait 4hours;

Add silicate standard to a concentration of 60µM;

Wait overnight.

10/10/17

Dye the cell :

Add 1/1000 of Rhodamine 123

Wait 10 min

Wash the cells :

Centrifuge the cells 2 min at 14000rpm

| Remove the supernatant

5X | Add PBS

| Centrifuge the cells 2 min at 14000rpm

Make microscope slide with the cells :

Put a drop of water on the slide

Scratch the pellet with a tip and stir it in the droplet.

Put the cover slip

Pass quickly in the burner to seal it.

19/09/17

Observe the slide under the fluorescence microscope with the GFP setting for the rhodamine.

21/09/17

Revive cell from the glycerol stock.
With a loop scratch some stock and spread it on a kanamicyn plate.
Incubate overnight

22/09/17

Start new cultures with revived cells :
Take some cells with a loop and put it in 5mL of LB + Kanamicyn.

23/09/17

Test silicate production :
Start new culture with 3 tubes for each colony, 1 tube of each colony not induced without silicate, 1 DH5α induced with silicate and 1 DH5α not induced without silicate.
Wait for the beginning of log phase.
Induce with 1% of Arabinose 1X
Wait 4hours
Add silicate standard to a concentration of 60µM
Wait overnight

25/10/17

Dye the cell :

Add 1/1000 of Rhodamine 123

Wait 10 min

Wash the cells :

Centrifuge the cells 2 min at 14000rpm

| Remove the supernatant

5X | Add PBS

| Centrifuge the cells 2 min at 14000rpm

Make microscope slide with the cells :

Put a drop of water on the slide

Scratch the pellet with a tip and stir it in the droplet.

Put the cover slip

Pass quickly in the burner to seal it.

26/09/17

Observe the slide under the fluorescence microscope with the GFP setting for the rhodamine.

Calcium Carbonate

15.08.2017

Making Calcium Competent Cells JF1 with this protocol :

Day 1

1. Streak out frozen glycerol stock of bacterial cells (Top10, DH5α, etc.) onto an LB plate (no antibiotics since these cells do not have a plasmid in them). Work sterile. Grow plate overnight at 37°C.

Day 2

1. Autoclave:

   1 L LB (or your preferred media)
   1 L of 100 mM CaCl2
   1 L of 100 mM MgCl2
   100 mL of 85 mM CaCl2, 15% glycerol v/v
   4 centrifuge bottles and caps Lots of microfuge tubes

2. Chill overnight at 4°C:
   100 mM CaCl2
   100 mM MgCl2 85 mM CaCl2, 15% glycerol v/v
   Centrifuge rotor
3. Prepare starter culture of cells Select a single colony of E. coli from fresh LB plate and inoculate a 10 mL starter culture of LB (or your preferred media – no antibiotics). Grow culture at 37°C in shaker overnight.
4. Notes:
   • You will have extra CaCl2 and MgCl2. These solutions can be saved and reautoclaved for the next time you make competent cells.
   • You can also substitute other media like SOB, 2xYT, etc. for the LB if you prefer.

• All glassware should be detergent free. Presence of detergent reduces competency of cells.

Day 3

1. Inoculate 1 L of LB media with 10 mL starter culture and grow in 37°C shaker. Measure the OD600 every hour, then every 15-20 minutes when the OD gets above 0.2.

2. When the OD600 reaches 0.35-0.4, immediately put the cells on ice. Chill the culture for 20-30 minutes, swirling occasionally to ensure even cooling. Place centrifuge bottles on ice at this time.

IMPORTANT NOTES:

• It is important not to let the OD get any higher than 0.4. The OD should be carefully monitored and checked often, especially when it gets above 0.2, as the cells grow exponentially. It usually takes about 3 hours to reach an OD of 0.35 when using a 10 mL starter culture.

• It is also very important to keep the cells at 4°C for the remainder of the procedure. The cells, and any bottles or solutions that they come in contact with, must be pre-chilled to 4°C.

3. (Spin #1) Split the 1 L culture into four parts by pouring about 250 mL into ice cold centrifuge bottles. Harvest the cells by centrifugation at 3000g (~4000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 4. Decant the supernatant and gently resuspend each pellet in about 100 mL of ice cold $MgCl_2$. Combine all suspensions into one centrifuge bottle. Make sure to prepare a blank bottle as a balance. 5. (Spin #2) Harvest the cells by centrifugation at 2000g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 6. Decant the supernatant and resuspend the pellet in about 200 mL of ice cold $CaCl_2$. Keep this suspension

on ice for at least 20 minutes. Start putting 1.5 mL microfuge tubes on ice if not already chilled. 7. (Spin #3) Harvest the cells by centrifugation at 2000g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. At this step, rinse a 50 mL conical tube with ddH2O and chill on ice. 8. Decant the supernatant and resuspend the pellet in ~50 mL of ice cold 85 mM CaCl2, 15% glycerol. Transfer the suspension to the 50 mL conical tube. 9. (Spin #4) Harvest the cells by centrifugation at 1000g (~2100 rpm in the Beckman GH-3.8 rotor) for 15 minutes at 4°C. 10. Decant the supernatant and resuspend the pellet in 2 mL of ice cold 85 mM CaCl2, 15% glycerol. The final OD600 of the suspended cells should be ~ 200-250. 11. Aliquot 50 µL into sterile 1.5 mL microfuge tubes and snap freeze with liquid nitrogen. Store frozen cells in the -80°C freezer.

16.08.2017

Transformation of J61002, pBS4K5, pBS3C5 in to Dh5a competent cells.

1ul J61002;

1ul pBS4K5;

1ul pBS3C5.

Transformation with protocol:

1. Resuspend DNA in selected wells in the Distribution Kit with 10ul dH2O. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
2. Label 1.5ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5 tubes in a floating foam tube rack.
3. Thaw competent cells on ice: This may take 10-15 min for 260ul stock. Dispose of unused competent cells. Do not refreeze unused cells, as it will drastically reduce transformation efficiency.

4. Pipette 50 ul of competent cells into 1.5 ml tube: 50ul in a 1.5ml tube per transformation. Tube should be labeled, pre-chilled, and in floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.
5. Pipette 1ul of resuspended DNA into 1.5ml tube: Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
6. Pipette 1ul of control DNA into 1,5ml tube: Pipette 1ul of 10pg/ul control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
7. Close 1.5ml tubes, incubate on ice for 30 min: Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
8. Heat shock tubes at 42^{o}C for 45 seconds.
9. Incubate on ice for 5 min.
10. Pipette 950 LB media to each transformation.
11. Incubate at 37 ^{o}C for 1h, shaking at 200-300rmp.
12. Pipette 100ul of each transformation onto petri plates.
13. Spin down cells at 3000rmp for 5 min and discard 800 of the supernatant. Resuspend the cells in the remaining 100ul, and pipette each transformation onto petri plates.
14. Incubate transformations overnight(14-18 hr) at 37 ^{o}C.
15. Pick single colonies: grow up cells and miniprep, also make glycerol stocks.
16. Count colonies for control transformation.

17.08.2017

Making Dh5a competent cells.

18.08.2017

Miniprep Results

J61002	A 231,5 ng/ul
	B 290,8 ng/ul
	C 230,3 ng/ul

pBS4K5	A 93,8 ng/ul
	B 148,7 ng/ul
	C 123,4 ng/ul

pBS3C5	A 253,4 ng/ul
	B 192,7 ng/ul
	C 204,4 ng/ul

19.08.2017

Fast digestion and Ligation of pBS3C5 and insert part.

Fast digestion protocol:

1. Prepare the reaction mixture at room temperature in the order indicated:

Component	Volume		
	Plasmid DNA	Unpurified PCR product	Genomic DNA
Water, nuclease-free	15ul	17ul	30ul
10X Fast digest@ Buffer or 10X Fast digest@ Green Buffer	2ul	2ul	5ul
DNA	2ul (up to 1ug)	10ul (~ 2ug)	10ul (5ul)
Fast Digest Enzyme	1ul	1ul	5ul

2. Mix gently and spin down.
3. Incubate at 37^{o}C in a heat block or water thermostat for 5 min.
4. Inactivate the enzyme(optional).

Experiment:

CARP12, CARP13 concentration = 50ng/ul;

Vector pBS3C5 concentration = 204 ng/ul;

Components:	CARP12	CARP13	pBS3C5	No enzymes	EcoRI	PstI
Water, nuclease-free	12ul	12ul	13ul	16ul	15ul	15ul
10X Fast digest@ Buffer	2ul	2ul	2ul	2ul	2ul	2ul

DNA	4ul	4ul	3ul	2ul	2ul	2ul
EcoRI	1ul	1ul	1ul	0	1ul	0
PstI	1ul	1ul	1ul	0	0	1ul
Total volume:	20ul	20ul	20ul	20ul	20ul	20ul

Gel purification:

CARP12 concentration =7,8 ng/ul

CARP13 concentration =10,4 ng/ul

Vector pBS3C5 concentration = 15,1 ng/ul

Ligation protocol :

1. Set up the following reaction in a microcentrifuge tube on ice:

Components:	Amount:
T4 DNA ligase Buffer(10X)	2ul
Vector DNA(4kb)	50ng
Insert DNA(1kb)	37,5ng
Nucleas-free water	Up to 20ul
T4 DNA ligase(HC)	1ul

2. Gently mix the reaction by pipetting up and down and microcentrifuge briefly.

3. For cohesive(sticky) ends incubate at 16^{o}C overnight or room temperature for 10 minutes.

4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2h (alternatively, high concentration T4 DNA ligase can be used in a 10-minute ligation).

5. Heat inactivate at 65 °C for 10 minutes.

6. Chill on ice and transform 1-5ul of the reaction into 50ul of competent cells.

Experiment:

Components:	CARP12	CARP13
T4 DNA ligase Buffer(10X)	2ul	2ul
Vector DNA(4kb	3.3ul	3.3ul
Insert DNA(1kb)	4.75ul	3.6ul
Nucleas-free water	8,95ul	10.1ul
T4 DNA ligase(HC)	1ul	1ul

13.09.2017

Transformation of CARP3 and CARP4 into BL21 and Dh5a.

CARP3 – len.662 bp, Vector pUC57

CARP4 – len. 1121 bp, Vector pUC57

Vector pUC57 – len. 2710 bp.

Material instruction for GenScript parts

Plasmids are delivered in vials with red caps. Store the vials at 4°C for short-term and frequent use, or at -20 °C for long-term storage.

- Keep the vial sealed until ready to use.

- Centrifuge at 6000 x g for 1 minute at 4^{o}C. Open the vial and add 20ul of sterilized water to dissolve the DNA.
- Close the led and vortex the vial for 1 minute. If necessary, heat the solution at 50 ^{o}C for 15 minutes to dissolve DNA.
- Transformation of the plasmid DNA can be performed directly after completion of the steps above. Verify the plasmid sequence after each subcloning and transformation.

	BL21/ 50 ul	Dh5a/50 ul
CARP3	1ul of plasmid DNA	1ul of plasmid DNA
CARP4	1ul of plasmid DNA	1ul of plasmid DNA

25.09.2017 – 20.10.2017

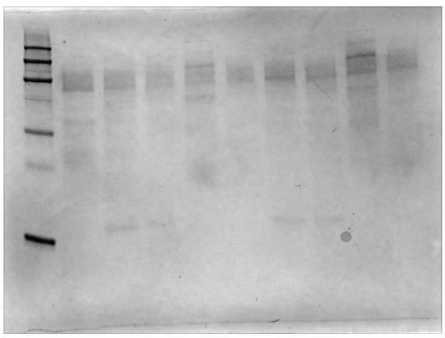
During last month I worked with protein induction, characterization, extraction and precipitation parts. I tried to find right conditions to increase all this parameters, by growing cells in different media rich on Ca^{2+}.

For the protein extraction I used BugBuster reagent and after with SDS-PAGE gel I checked a size of CARPs proteins. Also extracted proteins were checked for precipitation in different media with different Ca^{2+} concentration and seawater. After precipitated material was stained with Alizarin Red S staining-

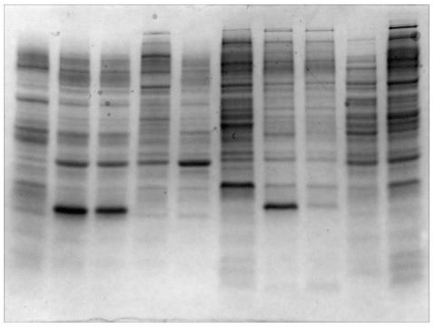
the organic dye Alizarin red S (ARS) will produce a pink to red stain on any carbonate that will react with dilute acid. The reaction between carbonates and acid is usually controlled (1 to 2 minutes at 25oC for thin sections) so that the more reactive minerals, such as calcite and aragonite, stain red but the less reactive ones, such as dolomite and siderite, remain unstained.

SDS-PAGE gels:

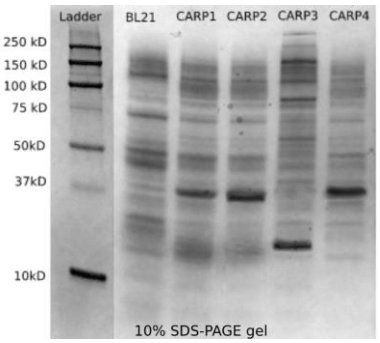
Aleks SDS gel kaleidoskop



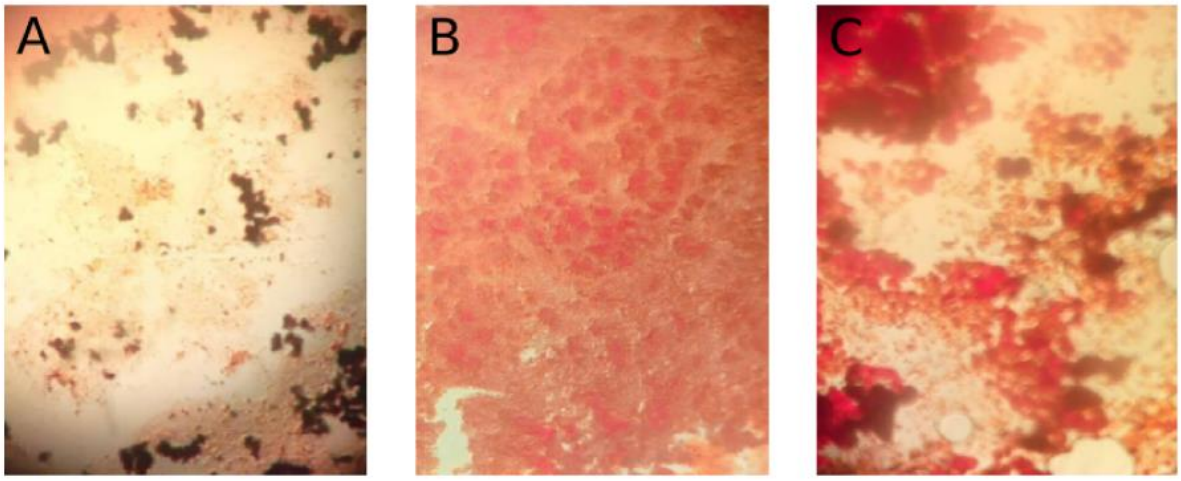
Aleks SDS page gel



Didn’t have right proteins size.



Succeed protein exprasion.



Alizarin Red staining for detection of calcium carbonate composites in the precipitated proteins: A) stained calcium carbonate powder - positive control, B) stained sample of BL21 extracted protein precipitation in CaCl 1M solution - negative control , C) stained sample of CARPs extracted protein precipitation in CaCl 1M solution.