NOTEBOOK 1

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF BlcR

iGEM TU Delft 2022

SPYKE
DETECT TO PROTECT

TU Delft

Introduction

Agrobacterium tumefaciens produces the IclR-type family member BlcR, an allosteric transcription factor. BlcR contains two distinct binding sites; the DNA binding domain, which interacts with the *blc* operator sequence, and the effector binding domain which detects the effector molecule Gamma-Hydroxybutyric Acid (GHB) or the GHB analogue succinic semialdehyde (SSA), that allow this protein to bind to both DNA and GHB (or an analogs of GHB such as SSA). In this module we aim to produce, purify and characterize BlcR.

To characterize the association and dissociation of the transcription factor BlcR to its DNA binding sequence we used Electrophoretic mobility shift assays (EMSA). Electrophoretic mobility shift assays (EMSA) are commonly employed for the analysis of nucleic acid/ protein interactions with a native gel system. A gel shift assay can allow us to determine the binding affinity of BlcR to the DNA. Since our protein of interest is an allosteric transcription factor, we also determined the binding affinity of BlcR to its effector SSA, a GHB analog.

Date: 09/06/2022

Experimentalist(s): Robin

Protocols: Transformation using heat shock, Agar plates

Transformation

Agar plates with chloramphenicol were prepared. Transformation of iGEM part BBa_K1758376 in *E. coli* cells DH5**α** strain was done with heat shock. The transformed cells are spread out over the agar plates, and left upside down overnight at 37 ºC.

Date: 14/06/2022

Experimentalist(s): Robin

Protocols: Inoculation

Inoculation

Inoculation was done of the BBa_K1758376 plasmid. Chloramphenicol is used as an antibiotic.

Date: 16/06/2022

Experimentalist(s): Robin

Protocols: Glycerol stock

Glycerol stock

Glycerol stock was made of BBa_K1758376.

Date: 22/06/2022

Experimentalist(s): Robin

Protocols: Transformation using heat shock, Agar plates

Transformation

Agar plates with ampicillin were prepared. The plasmid pET-11a-BlcR (BBa_K4361106) was transformed into two different *E. coli* strains: DH5***α*** and BL21(DE3) following the heat shock protocol. The agar plates are left overnight at 37 ºC.

Date: 23/06/2022

Experimentalist(s): Robin

Protocols: Inoculation

Inoculation

Inoculation of one colony of DH5***α*** and BL21(DE3) was done in LB-Amp medium and samples were left overnight in a incubator at 37 ºC.

Date: 24/06/2022

Experimentalist(s): Robin

Protocol: Glycerol stock

Glycerol stock

Glycerol stock of pET11a-BlcR in DH5***α*** was made.

Date: 27/06/2022

Experimentalist(s): Robin, Brent, Haroun, Martina & Allegra

Protocol: LB medium, LB agar

LB medium and LB agar were prepared and autoclaved.

Date: 28/06/2022

Experimentalist(s): Robin

Protocol: Inoculation

Inoculation

10 µL Ampicillin was added to 10 mL autoclaved LB broth. One colony of the transformed cells was dipped in LB broth and left overnight in a shaker 180 rpm 37 ºC following the inoculation protocol.

Date: 29/06/2022

Experimentalist(s): Robin

Protocol: Protein purification

Protein purification

Overnight culture with BL21(DE3) cells was transferred to 5 L Erlenmeyer flask with 1L LB medium and 1 mL of ampicillin. Cells were grown at 37 ºC at 180 rpm. After 5 hours OD was checked. OD of 1.2 was measured. Cells were diluted with 600 mL of LB and 400 mL of culture and grown again until OD of 0.67. Cells were induced with 1 mL of 1M IPTG to have an end concentration of 1 mM of IPTG. The cells were left overnight at 18 ºC and 180 rpm. This was all done following the protocol of protein purification.

Washing buffer (50 mM Tris-HCl, 5 mM imidazole, 300 mM NaCl, pH 7.5l) and elution buffer (50 mM Tris-HCl, 200 mM imidazole, 300 mM NaCl, pH 7.5) for protein purification are prepared for the protein purification of BlcR [1].

Date: 30/06/2022

Experimentalist(s): Robin

Protocol: Protein purification

Protein purification of BlcR

Protein purification of BlcR was done following the protocol of protein purification of BlcR. Samples were stored at 4 ºC.

Date: 04/07/2022

Experimentalist(s): Robin, Martina

Protocol: SDS page

SDS PAGE

A 10 % precast Tris-Bis SDS PAGE from Thermo Fisher was used to analyze the protein samples from the purification. The buffer used was 1x NuPASE MES SDS running buffer and the gel ran for 30 mins at 200V. The staining used was SimplyBlue safe stain for 20 mins and discolored with Milli-Q 3x refreshed.

RESULTS

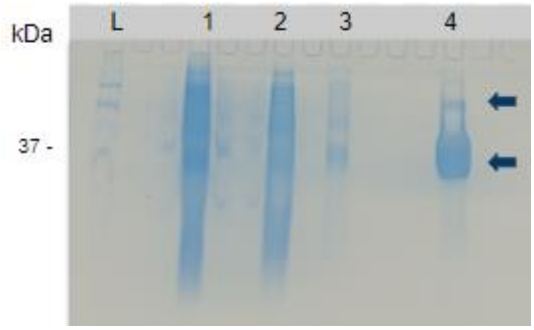

Figure 1. SDS-PAGE for visualization of protein production BlcR. 1= supernatant, 2= flowthrough of the column, 3= wash of column, 4= elution of column.

From the SDS PAGE we could see abundant bands corresponding to the size of BlcR in the elution lane.

Date: 05/07/2022

Experimentalist(s): Martina

Protocol: Nanodrop

Nanodrop

With the nanodrop, check the concentration of the purified BlcR. First, calibrate the machine with Milli-Q. Then do a blank measurement with an elution buffer and lastly load the sample on the machine. Do not forget to wipe the machine between each measurement.

RESULTS

The concentration of the protein was checked with Nanodrop.

The extinction coefficient of BlcR (monomeric) was determined using the ExPASy ProtParam tool: Giving an extinction coefficient ϵ_{280} = 14500 M^{-1} cm^{-1}.

Sample	Concentration (uM)	260/280	A_{280} 10mm
BlcR	1.6	0.61	1.424

From this we can calculate [BlcR] = A_{280} / ϵ_{280} x 10^6 = 98 uM

Date: 07/07/2022

Experimentalist(s): Robin

Protocol: Buffer exchange; Dialysis

Buffer exchange; Dialysis

To prepare for the protein dialysis the protein solution was first concentrated with a Vivaspin 20, 30,00 MWCO membrane from the sartorius. The protein solution was added to the membrane and centrifuged at 4 ºC, 4000 rpm for 5 minutes. After 5 minutes the solution was resuspended. This process was repeated 3 times until the volume was reduced from 15 mL to 7 mL.

For the dialysis, a Slide-A-Lyzer 10K Dialysis Cassettes 10,00 MWCO from Thermo Scientific was used. The protocol of Buffer exchange; Dialysis is used.

Preparation SDS sample buffer:

SDS sample buffer was prepared.

First stock solution for the different components were prepared following this scheme:

Table 1. Stock solutions for SDS sample buffer

Component	concentration	weight (g)	volume (mL)
Tris-HCl pH 6.8	1M	6.057	0.05
Bromophenol Blue	0.1 %	0.001	1
β-Mercaptoethanol	14.3 M	5.59	5

SDS sample buffer 4x stock 10 mL solution was prepared following this scheme:

T**able 2.** Preparation SDS sample buffer

Component	concentration	volume/weight
Tris-HCl pH 6.8	1M	2.5 mL
ddH2O	-	0.5 mL
SDS	-	1 g
Bromophenol Blue	0.1 %	0.8 mL
Glycerol	40 %	4 mL
β-Mercaptoethanol	14.3 M	2 mL
Total volume	-	10 mL

Date: 08/07/2022

Experimentalist(s): Allegra

Protocols: Nanodrop

Nanodrop

The extinction coefficient of BlcR (monomeric) was determined using the ExPASy ProtParam tool: Giving an extinction coefficient ϵ_{280} = 14500 M^{-1} cm^{-1}.

Sample	Concentration (uM)	260/280	A_{280} 10mm
BlcR	1.6	0.61	0.023

From this we can calculate [BlcR] = A_{280} / ϵ_{280} x 10^6 = 1.6 uM

Date: 12/07/2022

Experimentalist(s): Martina, Brent

Protocol: Protein production

BlcR production

The glycerol stock of DH5α with plasmid pET11a-T7tag-6xHis-TEV-BlcR (BBa_K4361106) was revived by scraping some culture from the frozen stock, suspending it in water, and plating it on LA-Amp.

After 7 hours of incubation at 37°C, some colonies were scraped with a toothpick, which was placed in a 15 mL LA-Amp and incubated overnight, shaking, at 37°C.

Date: 13/07/2022

Experimentalist(s): Brent

Protocols: Protein production,

Protein production

The 15 mL overnight culture was divided between two flasks containing 1 L LB-Amp, which were incubated at 37°C at 130 rpm.

Date: 14/07/2022

Experimentalist(s): Martina, Brent

Protocols: Protein production

Protein production

Washing buffer (50 mM Tris-HCl, 5 mM imidazole, 300 mM NaCl, 10% glycerol, pH 7.5) and elution buffer (50 mM Tris-HCl, 200 mM imidazole, 300 mM NaCl, 10% glycerol, pH 7.5) were found in literature for the protein purification of BlcR [1].

Date: 19/07/2022

Experimentalist(s): Martina

Protocol: Transformation

Transformation

Transformation of pET11a-BlcR (BBa_K4361106) into BL21(DE3) was carried out following the heat shock protocol. The cells were then plated on pre-prepared agar plates with ampicillin and incubated at 37°C overnight.

Date: 20/07/2022

Experimentalist(s): Robin, Martina

Protocol: SDS-PAGE, Protein production

SDS-PAGE

The SDS page was done according to the SDS page protocol. The SDS page was run with the BlcR fraction after concentrating and dialysis. The running buffer was diluted 20 times, gel kits were already made from Thermo fisher. The buffer used was 1x NuPAGE MES SDS running buffer and the gel ran for 70 mins at 200V. The staining used was Coomassie Brilliant Blue stain for 20 mins and discolored with Milli-Q 3x refreshed.

Nanodrop

With nanodrop the concentration of the protein was checked.

Inoculation

A 10 mL tube of LB wit ampicillin was prepared. With a toothpick, scrape one colony and dip the stick in the tube. Leave the tube shaking overnight at 37°C and 130 rpm, with the lid only loosely closed to allow oxygen exchange following the inoculation protocol.

Ampicillin stock was prepared by diluting 1g of ampicillin in 10 mL of MilliQ. It was mixed thoroughly till the powder dissolved, aliquoted into 1 mL samples and stored at -20°C.

0.6 g of IPTG were added to 10 mL of Milli Q. The solution was thoroughly mixed till IPTG completely dissolved. The solution was aliquoted into 2mL working samples and stored at -20°C.

RESULTS

SDS-PAGE

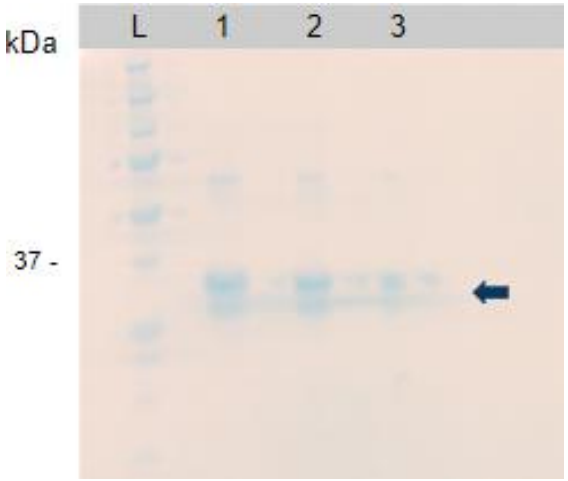

Figure 2. SDS PAGE BlcR production after buffer exchange from protein elution buffer 50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole, into 50 mM HEPES pH 7.2 buffer.

Bands at the right height around 33 kDa were visible. But the concentration of the protein was too low for some experiments therefore a new production round was started.

Nanodrop

The concentration of the protein was checked with Nanodrop. The protein concentration is calculated with the

The extinction coefficient of BlcR (monomeric) was determined using the ExPASy ProtParam tool: Giving an extinction coefficient ϵ_{280} = 14500 M^{-1} cm^{-1}.

Table 4. Nanodrop results of BlcR sample

Sample	Concentration (uM)	260/280	A_{260} 10mm
BlcR	1.6	1.47	0.024

From this we can calculate [BlcR] = A_{280} / ϵ_{280} x 10^6 = 1.6 uM

Date: 21/07/2022

Experimentalist(s): Martina

Protocol: Glycerol stock, Protein production

Glycerol stock

1 mL from the cultivation was added to a 2 mL tube, together with 50% glycerol. The sample was then frozen with liquid nitrogen and stored at -80°C.

2 x 1L of LB wit ampicillin were prepared and then transferred into sterile 5 L flasks. 5 mL of the previous cultivation were added to each flask. The flasks were left shaking at 37°C and 130 rpm for roughly three hours. In the meantime, OD600 was periodically measured.

RESULTS

Flask 1 OD600= 0.68
Flask 2 OD600= 0.72

IPTG induction

After desired OD600 was reached, the cultivations were placed on ice as per protocol for 30 mins. Afterward, 1 tube of IPTG stock was poured into each flask. The flasks

were then placed in the incubator overnight, shaking at 130 rpm and 18°C, as per protocol.

Date: 22/07/2022

Experimentalist(s): Martina

Protocol: Protein production

Protein production

The protocol for production and purification was followed. The cultures were weighted before centrifugation (each was around 813g). After centrifugation, the supernatant was discarded and the pellet was weighted (each was around 5g).

The lysis buffer was prepared according to protocol, by mixing 100 mL of washing buffer and 1 mL of protease inhibitor cocktail. Washing buffer (50 mM Tris-HCl, 5 mM imidazole, 300 mM NaCl, pH 7.5l) and elution buffer (50 mM Tris-HCl, 200 mM imidazole, 300 mM NaCl, pH 7.5) for protein purification are prepared as found in literature for the protein purification of BlcR [1].

The protein purification was carried out according to protocol, though some slight changes were made with respect to the previous protocol: instead of sonication, a cell disruptor was used. And in order to get rid of the DNA, DNAse and Magnesium chloride were added and let act for 15 minutes approximately. The protein purification was carried out with gravity columns and nickel beads at 4°C, in the cold room.

Date: 25/07/2022

Experimentalist(s): Brent

Protocol: Buffer exchange; Dialysis, SDS-PAGE, Nanodrop

Buffer exchange; Dialysis

The purified protein was split into three fractions: 2 of 11 mL and one of 1 mL. Each fraction was dialyzed for 24 hours according to the protocol. The 11 mL samples were both dialyzed with 50 mM HEPES pH 7.5 storage buffer; the 1 mL sample was dialyzed with 20 mM MOPS, 2.5 mM Mg, pH 7 buffer.

SDS PAGE

The samples taken from various stages of the BlcR purification process were visualized on a 10 % Bis- Tris SDS-PAGE from Thermo Fisher according to the SDS-PAGE protocol.

Nanodrop

The Protein concentration in the elution buffer is checked with nanodrop.

RESULTS

SDS PAGE

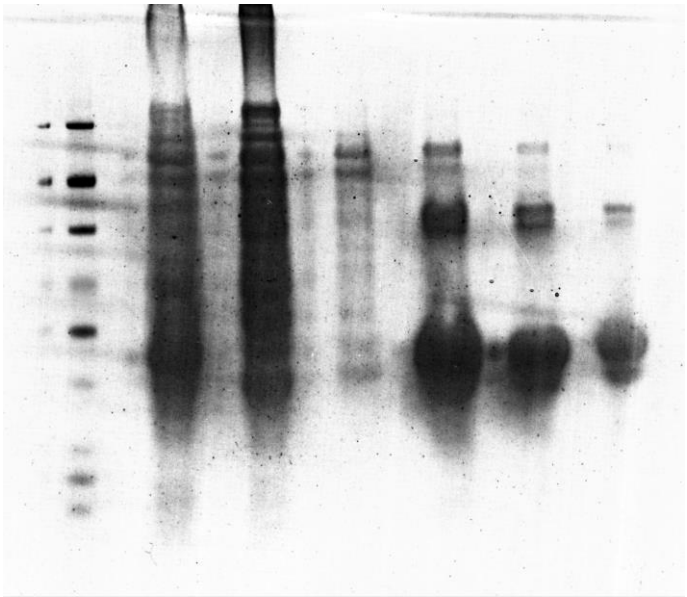

Figure 3. SDS Page of protein purification. Loaded next to the ladder: clear lysate, column flow through, column wash, 10 µL elution, 5 µL elution, 2.5 µL elution. µL

The gel showed abundant protein of an appropriate size in the elution.

Nanodrop

The extinction coefficient of BlcR (monomeric) was determined using the ExPASy ProtParam tool: Giving an extinction coefficient ϵ_{280} = 14500 M^{-1} cm^{-1}.

Table 5. Nanodrop results of BlcR sample

Sample	Concentration (uM)	260/280	A_{260} 10mm
BlcR	1.6	1.23	1.247

From this we can calculate [BlcR] = A_{280} / ϵ_{280} x 10^6 = 86 uM

Date: 26/07/2022

Experimentalist(s): Martina

Protocol: EMSA

EMSA

A troubleshooting round for EMSA was carried out according to protocol but with some slight changes. The quantities of BlcR were increased gradually.

Stock solution of 300 nM of BlcR is sued. And the concentration of DNA is remained constant at 27 nM.

The samples were incubated at 37 °C for 45 mins, and consequently loaded in the pre-ran gel. No dye was needed. The ladder was prepared by mixing in a 1:1 ratio of DNA ladder and glycerol. Samples were loaded as follows: ladder, 0x, 0.5x, 1x, 2x. The gel was run at 100V for 45 mins. After running the gel was stained with SYBR Safe for 15 mins and visualized with gel doc.

RESULTS

The samples did not migrate trough the gel.

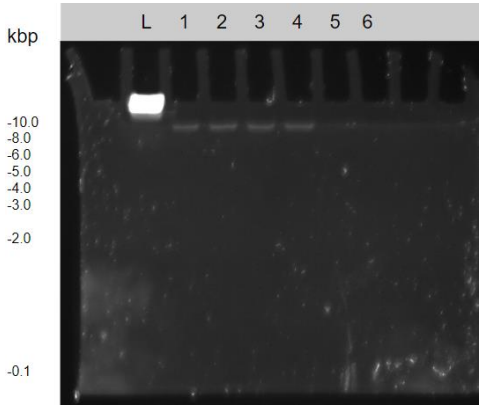

Figure 4. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

Date: 27/07/2022

Experimentalist(s): Martina

Protocol: EMSA

EMSA

The mix for the gel was prepared according to protocol. The gel was cast and let polymerize for at least 2 hrs.

The samples were prepared according to the table above and incubated for 30 mins at 37°C. The ladder was prepared by mixing in a 1:1 ratio of DNA ladder and glycerol. The gel was run at 100V for 45 mins. After running the gel was stained with SYBR Safe for 15 mins and visualized with gel doc.

RESULTS

When imaged with the gel doc, some bands were visible the ladder was however not very well defined.

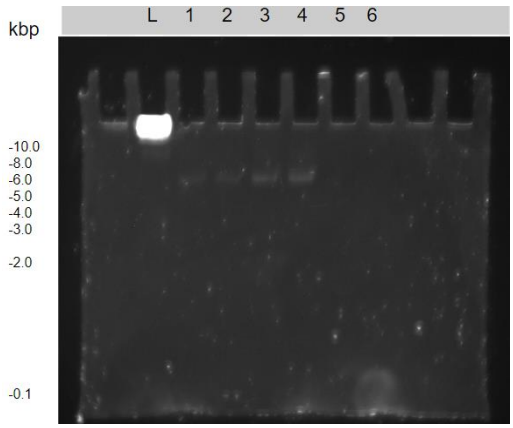

Figure 5. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

Date: 28/07/2022

Experimentalist(s): Martina, Brent

Protocol: EMSA, Buffer exchange; Dialysis

EMSA 1

The mix for the gel was prepared according to protocol [2]. The gel was cast and let polymerize for at least 2 hrs.

The samples were prepared according to the table above and incubated for 30 mins at 37°C. The pH of the master mix was 7-8. The ladder was prepared by mixing in a 1:1 ratio of DNA ladder and glycerol. The gel was run at 100V for 45 mins. After running the gel was stained with SYBR Safe for 15 mins and visualized with gel doc.

RESULTS

The sample did not migrate trough the gel, this is especially visible with the ladder.

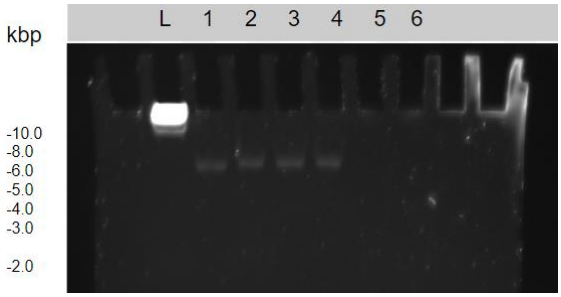

Figure 6. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

Buffer exchange; Dialysis

The dialysis of BlcR into 50 mM HEPES, pH 7.2 storage buffer and 20 mM MOPS, 2.5 mM Mg, pH 7. In both samples participation was visible. The two resulting dialyzed protein samples were centrifuged at max speed for 10 minutes to collect the supernatant, containing the soluble BlcR.

Date: 29/07/2022

Experimentalist(s): Martina

Protocol: EMSA

EMSA

The gel mix was prepared according to protocol [2]. After cooling down for 30 mins at room temperature, APS and TEMED are added and the mix is cast in the appropriate

casting apparatus and let polymerize for at least 2 hours. Gels are then wrapped in wet tissue paper and conserved at 4°C over the weekend.

Date: 01/08/2022

Experimentalist(s): Martina, Brent

Protocol: EMSA, SDS-PAGE

EMSA

The gel mix was prepared according to protocol [2]. After cooling down for 30 mins at room temperature, APS and TEMED are added and the mix is cast in the appropriate casting apparatus and let polymerize for at least 2 hours.

The pH of the master mix of Batch 1 is between 3 and 4 and the pH of the master mix of Batch 2 is between 7 and 8. Samples were incubated for 1 hour (circa) at 37°C. The gel was run at 120 V for 1 hour. The gel was stained with SYBR Safe for 15 mins and visualized with the gel doc.

RESULTS

The gel was visualized with the gel doc. In the case of the samples with pH 3-4, nothing was detected. Most likely the samples diffused as the pH of the running buffer was different therefore it led to diffusion. In the case of the samples with pH 7-8, bands were visible. A similar image to the previous run (29/07) gel was obtained. Since the gel was run for longer, the bands were lower than the one previously obtained and more bands of the ladder were visible. The samples still did not migrate well trough the gel.

Next time: running it at a higher voltage (i.e. 200 V for 1 hour)

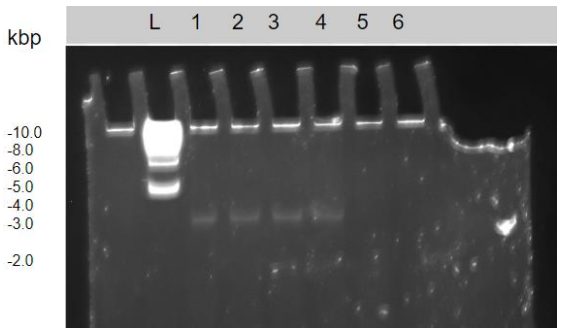

Figure 7. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

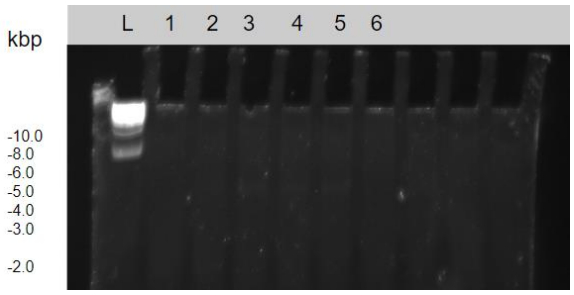

Figure 8. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

SDS-PAGE

To check the quality of the BlcR solution obtained from dialysis, an 10 % Bis-Tris SDS-PAGE gel from Thermo Fisher was run according to the protocol to compare a sample of the purification eluent with various fractions of the protein solutions.

RESULTS

The gel showed only weak staining, but a band corresponding to BlcR is clearly visible in the supernatant of the 50 mM HEPES storage buffer dialysis (lane 2) and the supernatant of the 20 mM MOPS 2.5 mM Mg buffer dialysis (lane 5). Some soluble protein was recovered by resuspending (lane 3) but not as much as the original supernatant. The second resuspension did not recover any protein (lane 4).

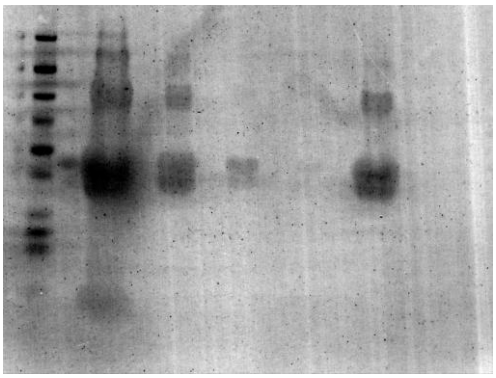

Figure 9. SDS-PAGE to visualize BlcR after dialysis into 50 mM HEPES, pH 7.2 (lane 1-3) and 20 mM MOPS, 2.5 mM Mg (lane 5).

By NanoDrop the concentration of protein was checked for the supernatant of HEPES dialysis and the supernatant of MOPS+Mg dialysis:

Table 6. BlcR concentrations in different buffers.

Sample	Concentration	Molarity	Volume
HEPES dialysis supernatant	0.35 g/L	12 µM	30 mL
MOPS dialysis supernatant	0.5 g/L	17 µM	1 mL

Date: 02/08/2022

Experimentalist(s): Martina

Protocol: EMSA

EMSA

The gel mix is prepared according to protocol [2]. After cooling down for 30 mins at room temperature, APS and TEMED are added and the mix is cast in the appropriate casting apparatus and let polymerize for at least 2 hours. The gels are then wrapped in wet tissue paper and conserved at 4°C overnight.

The master mix was prepared by mixing 15 µL of binding buffer with 0.75 µL of DTT and 0.75 µL of BSA. 5 working samples of the master mix were made and stored at -20°C.

The pH of the master mix of both batches is between 7 and 8. Samples were incubated for 45 mins (circa) at 37°C. The ladder was prepared by diluting in a 1:1 ratio the purple 2-log ladder and glycerol 50%. 3 µL of loading dye were added to each sample to aid the loading. The gel was pre-ran at 120 V for 20 minutes and, after loading the samples, it was run at 180 V for 1 hour. The gel was stained with SYBR Safe. After staining, one of the two gels was unstained in water, and both were visualized with the gel doc.

RESULTS

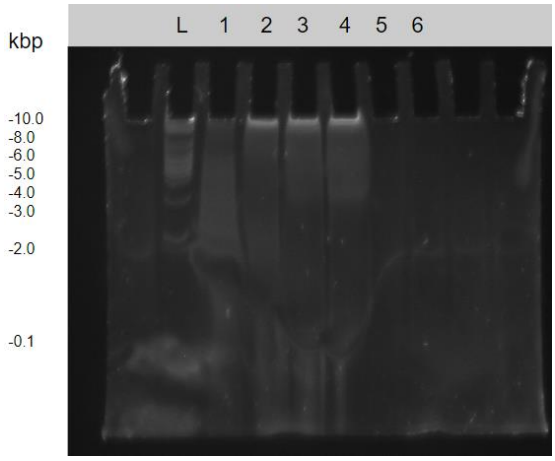

Figure 10. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

Date: 03/08/2022

Experimentalist(s): Robin, Martina

Protocol: Bradford assay, EMSA

Bradford assay

To determine the concentration of BlcR after dialysis from the production performed on the 22nd of July a Bradford assay was performed.

A stock of 10 mg/mL of BSA was 5 times diluted to get a new stock of 2 mg/mL. 9 standard dilutions of BSA were made. We have one unknown, the BlcR in solution. The amount of working reagent was calculated with the following formula:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume of working reagent required.

(9 + 1) x 2 x 2mL = 40 mL

To have a little bit more 42 mL of the working reagent was created. This was done by mixing 41.18 mL of reagent A with 0.82 mL of reagent B (50:1).

The Bradford assay was performed following the Bradford assay protocol.

EMSA

The gel mix is prepared according to protocol [2]. After cooling down for 30 mins at room temperature, APS and TEMED are added and the mix is cast in the appropriate casting apparatus and let polymerize for at least 2 hours. The gels are then wrapped in wet tissue paper and conserved at 4°C overnight

After some further calculations, two batches of samples were prepared according to the protocol in the following way:

The pH of the master mix of both batches is between 7 and 8. One batch of samples was incubated for 45 mins (circa) at 37°C. The second one was incubated at room temperature (20°C) for 45 mins still. The ladder was prepared by diluting in a 1:1 ratio the purple 2-log ladder and glycerol 50%. The gel was pre-ran at 120 V for 20 minutes and, after loading the samples, it was run at 180 V for 1 hour. The gel was stained with SYBR Safe. After staining the gels were visualized with the gel doc.

RESULTS

The first gel, with the samples incubated at 37°C showed an increase in the intensity of the bands where the protein has been retained. However, no decrease in intensity for the free DNA bands was visible and some diffusion was still present, most likely due to the gel composition.

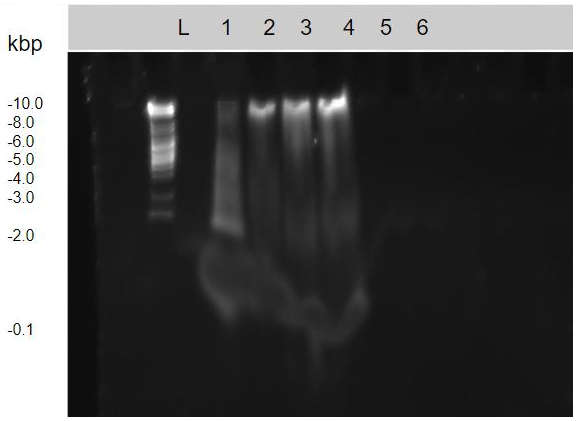

Figure 11. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

The second gel showed the same pattern. The only difference detected was in the staining as the concentration of SYBR Safe was lower.

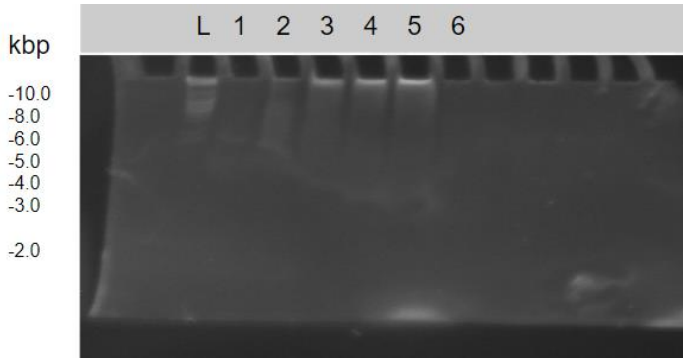

Figure 12. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 nM BlcR, 2: 50 nM BlcR, 3: 100 nM BlcR, 4: 200 nM BlcR, 5: 300 nM BlcR.

Bradford assay

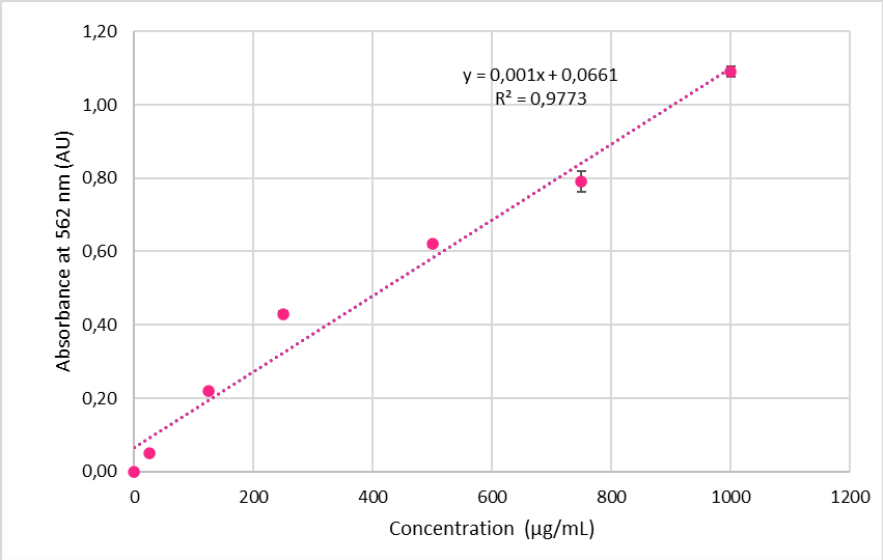

Figure 13. Bradford assay calibration curve for protein concentration determination in 50 mM HEPES, pH 7.2.

Table 7. Average and corrected absorbance at 562 nM for different BSA samples for calibration curve of bradford assay.

Concentration ug/mL	A_{562} corrected	Variance σ
1000	1,06	0,042
750	0,78	0,014
500	0,6	0,028
250	0,44	0,01
125	0,22	0,01
25	0,05	0
0	0	0

The formula to calculate the protein concentration is y = 0.001x + 0.066. With this formula, the concentration of BlcR is calculated.

Table 8. Protein concentration determination for BlcR in 50 mM HEPES, pH 7.2 buffer.

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} corrected	Concentration ug/mL
BlcR	0,81	0,85	0,8315	0,6215	555,4

A concentration of 555.4 ug/mL corresponds to:
555.4 / 70000 ug/umol = 0.0079 umol/mL = 8 uM

Bradford assay MOPS buffer:

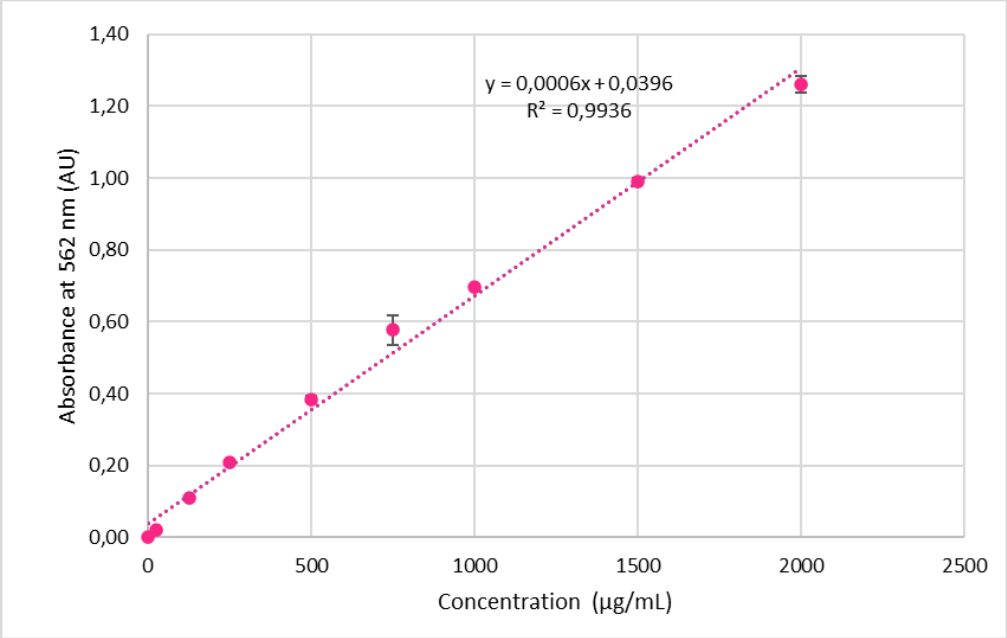

Figure 14. Bradford assay calibration curve protein concentration determination. In 20 mM MOPS, 2.5 Mg, pH 7 buffer

Table 8. Average and corrected absorbance at 562 nM for different BSA samples for calibration curve of bradford assay.

Concentration ug/mL	A_{562} corrected	Variance σ
2000	1.26	0.023
1500	0.99	0.007
1000	0.70	0.002
750	0.58	0.042
500	0.38	0.008
250	0.21	0.006

125	0.11	0
25	0.021	0.001
0	0	0.001

The formula to calculate the protein concentration is y = 0.0006x + 0.0396. With this formula, the concentration of BlcR is calculated.

A concentration of 1180 ug/mL corresponds to:
1180 / 70000 ug/umol = 0.0079 umol/mL = 17 uM

Table 9. Protein concentration determination for BlcR in 20 mM MOPS, 2.5 mM Mg, pH 7 buffer

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} corrected	Concentration ug/mL
BlcR	0.89	0.86	0.88	0.75	1.180

Date: 08/08/2022

Experimentalist(s): Martina

Protocol: EMSA

EMSA

Two batches of samples were prepared following the protocol [2]. Stock solution of 12 µM BlcR is used. Both batches of samples were incubated for 45 mins at 37°C. The ladder was prepared by diluting in a 1:1 ratio the purple 2-log ladder and glycerol 50%. After loading the samples, the gel was run at 200 V for 30 mins. The gel was stained with SYBR Safe for 20 mins. The gel was then washed with water for 10 minutes 3 times. After this process, the gels were visualized with the gel doc.

RESULTS

Bands with increasing intensity were visible in the upper part of the gel, which leads to thinking that BlcR bound DNA. Some father bands are visible, which should indicate the free DNA but they are not as defined as expected. The ladder also stretched out quite well.

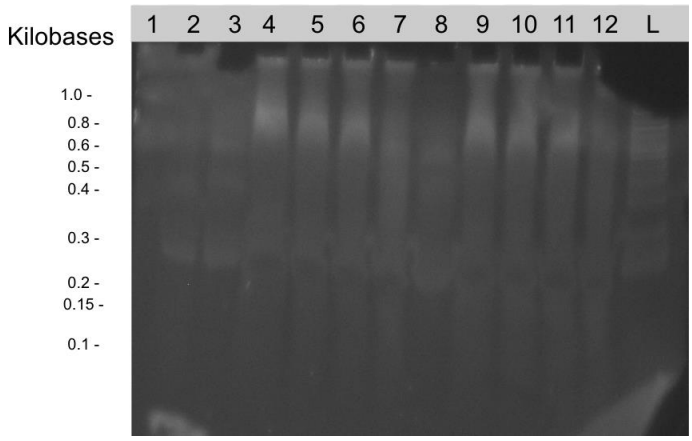

Figure 15. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 1 µM BlcR, 2: 1.5 µM BlcR, 3: 2 µM BlcR, 4: 2.5 µM BlcR, 5: 3 µM BlcR. 6: 3.5 µM BlcR, 7: 4 µM BlcR, 8: 4.5 µM BlcR, 9: 5 µM BlcR, 10: 5.5 µM BlcR, 11: 6µM BlcR, 0µM BlcR

Date: 09/08/2022

Experimentalist(s): Robin

Protocol: Inoculation

Inoculation

A toothpick was used to pick a colony from a transformed BL21DE3 E. coli with the production plasmid for BlcR. Inoculation was done in 10 mL LB with ampicillin. And the samples were left overnight at 37 °C in the shaker. This was done twice.

Date: 10/08/2022

Experimentalist(s): Robin, Martina

Protocols: EMSA (from iGEM Bielefeld 2015), protein purification

Protein production

The storage buffer for BlcR was made. 2L of HEPES pH 7.2 and 2L of HEPES pH 7.2 with 100 mM NaCl. The pKa of HEPES is 7.5 and the pKa of Tris is 8.1. If you want to maintain the pH at 7.5, HEPES is better than Tris. If you want to maintain it at 8.0, Tris is better.

The 10 mL from the inoculation of BL21DE3 cells were transferred to two different 5L Erlenmeyer flasks filled with 1 L of LB medium and ampicillin. The Erlenmeyer flasks

are incubated at 37 °C, 180 rpm. After 3 hours the OD was first checked. an OD of 0.35 and 0.23 was measured. The flasks were again incubated. After 1 more hour, an OD of 0.81 and 0.73 was measured. The cells were put on ice for 30 minutes and induced with IPTG. The flasks were incubated overnight at 18 °C, 180 rpm.

The necessary buffers and components for the EMSA protocol [3] were prepared as following:

- 5X EMSA buffer (100 mL, 100 mM Na_2HPO_4, 375 mM KCl, 25% glycerol, pH 8)
  → 1.42g Na_2HPO_4
  → 2.8g KCl
  → 25 mL glycerol
  → Milli-Q to 100 mL

- EMSA running buffer (20 mM Na_2HPO_4 adjust to pH 8)
  → make 500 mL Na_2HPO_4 stock (7.1g in 500 mL of Milli-Q)
  → dilute 5x (200 mL of stock solution + 800 mL of Milli-Q)

- 2.5% agarose gel
  → dilute 2.5g of agar in 100 mL of running buffer
  → microwave till powder has dissolved
  → pour gel and let it solidify

- 25 mM $MgCl_2$ stock
  → 238 mg of $MgCl_2$ in 100 mL of Milli-Q

- 2.5mM EDTA
  → 73 mg EDTA in 100 mL Milli-Q, adjust pH to 8 for the powder to dissolve

EMSA

The samples were prepared according to protocol, as follows:

Component	Sample 1	Sample 2	Sample 3
Cy3 DNA (10 nM)	5 µL	5 µL	5 µL
BlcR (1 uM)	-	2.5 µL	5 µL
EMSA 5X	4 µL	4 µL	4 µL
$MgCl_2$	2 µL	2 µL	2 µL

EDTA	0.8 µL	0.8 µL	0.8 µL
BSA	1 µL	1 µL	1 µL
Salmon Sperm	1 µL	1 µL	1 µL
Nuclease Free Water	6.2 µL	3.7 µL	1.2 µL

The samples were then incubated for 15 mins at room temperature, in the dark as Cy3 labeled DNA is photosensitive. After incubation, the ladder and samples were loaded in the gel as follows: ladder, sample 1, sample 2, and sample 3. The gel ran for 80 mins at 4°C (in the cold room).

Results

The Cy3 labeled DNA appeared bright and visible on the gel. Only one line of bands was visible, probably due to a too low concentration of BlcR. Will repeat the protocol, with a higher protein concentration.

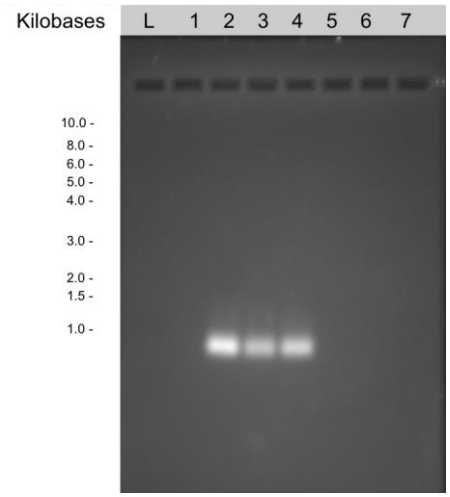

Figure 16. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 µM BlcR, 2: 0.1 µM BlcR, 3: 0.25 µM BlcR.

Date: 11/08/2022

Experimentalist(s): Robin, Martina

Protocols: SDS PAGE, EMSA

SDS PAGE

The protein purification of BlcR was done following the protocol. This time the french press was used. 20 µL DNase (20 U/ uL). After the production cycle the product was analyzed with a precasted 10 % Bis-Tris SDS-PAGE from ThermoFisher.

EMSA

The samples were prepared according to protocol [3], as following:

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Cy3 DNA (10 nM)	5 µL	5 µL	5 µL	5 µL	5 µL
BlcR (1 uM)	-	1 µL	2.5 µL	4 µL	5 µL
EMSA 5X	4 µL	4 µL	4 µL	4 µL	4 µL
$MgCl_2$	2 µL	2 µL	2 µL	2 µL	2 µL
EDTA	0.8 µL	0.8 µL	0.8 µL	0.8 µL	0.8 µL
BSA	1 µL	1 µL	1 µL	1 µL	1 µL
Salmon Sperm	1 µL	1 µL	1 µL	1 µL	1 µL
Nuclease Free Water	6.2 µL	5.2 µL	3.7 µL	2.2 µL	1.2 µL

The samples were then incubated for 15 mins at room temperature, in the dark as Cy3 labeled DNA is photosensitive. After incubation, the samples were loaded in the gel from left to right: sample 1, sample 2, sample 3, sample 4, and sample 5. The gel ran for 80 mins at 4°C (in the cold room).

RESULTS

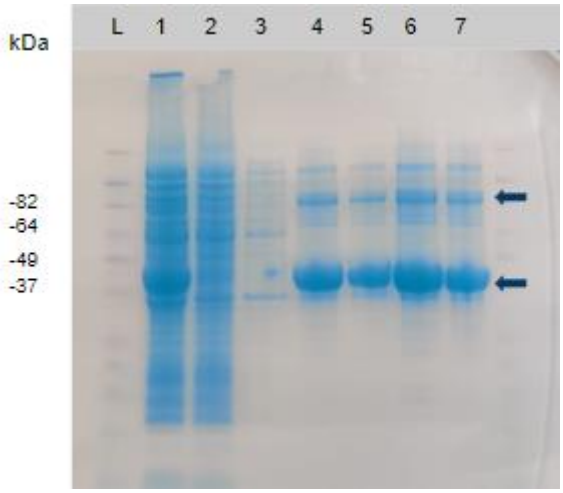

Figure 17. SDS-PAGE to visualize the production of BlcR. 1: clear lysate, 2: column flow trough, 3: column wash, 4: elution 1 10 uL, 5: elution 1 5 uL, 6: elution 2 10 uL, 7: elution 2 5 uL

In the elution fractions 4 - 7 abundant bands around 37 kDa are visible. This corresponds to the size of the monomer of our protein of interest. Between 82 and 64 kDa are strong bands visible which could correspond to the dimer of BlcR.

Only one line of bands was visible. What was clear however was a clear decrease in the brightness intensity of the bands, from left to right.

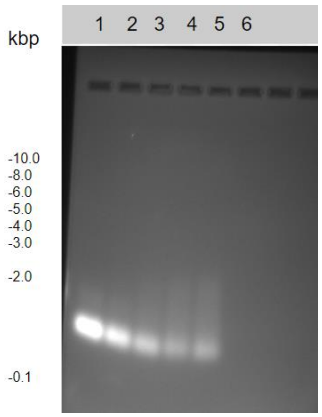

Figure 18. EMSA gel, DNA concentration remained constant at 27 nM, while the BlcR concentration was increased gradually 1: 0 µM BlcR, 2: 0.05 µM BlcR, 3: 0.1 µM BlcR. 4: 0.15 µM BlcR, 5: 0.25 µM BlcR.

Date: 15/08/2022

Experimentalist(s): Robin

Protocols: Buffer exchange; Dialysis

Buffer exchange; Dialysis

Dialysis was done overnight to exchange buffers. The elution buffer (300 mM NaCl, 300 mM imidazole, 50 mM Tris-HCl pH 7.2) was exchanged for a storage buffer (50 mM HEPES, 100 mM NaCl, pH 7.2) and MOPS storage buffer.

Date: 16/08/2022

Experimentalist(s): Robin

Protocols:Buffer exchange; Dialysis. SDS PAGE

Dialysis of BlcR

Dialysis was done to exchange buffers. The protein was dialysed into four different buffers. The elution buffer (300 mM NaCl, 300 mM imidazole, 50 mM Tris-HCl pH 7.2) was exchanged for a storage buffer (50 mM Tris pH 7.2), and storage buffer (50 mM HEPES pH 7.2).

SDS PAGE

Precasted 10 % Bis-Tris SDS PAGE from Thermo Fisher of the dialysis into 20 mM MOPS, 2.5 mM Mg, pH 7 and 50 mM HEPES 100 mM NaCl, pH 7.2 buffer.

RESULTS

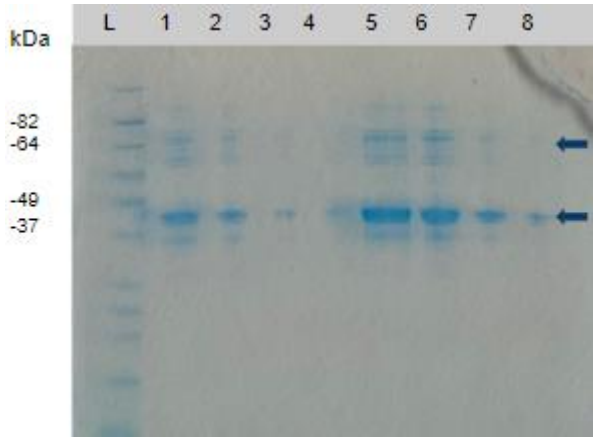

Figure 18. SDS-PAGE after dialyzing BlcR into 50 mM HEPES, 100 mM NaCl, pH 7.2 and 20 mM MOPS, 2.5 mM Mg, pH 7. 1: MOPS 15 uL, 2: MOPS 10 uL, 3: MOPS 5 uL, 4: MOPS 2.5, 5 HEPES 15 uL, 6: HEPES 10 uL, 7: HEPES 5 uL, 8 HEPES 2.5 uL.

Bands are visible around 37 kDa and between 64 and 82 kDa corresponding to the size of the monomer and dimer of BlcR. There is still some contamination, we decided to further purify with Size Exclusion

Date: 17/08/2022

Experimentalist(s): Robin

Protocols: SDS PAGE

SDS PAGE

Precasted 10 % Bis-Tris SDS PAGE from Thermo fFisher of the dialysis into 50 mM Tris-HCl, pH 7.2 and 50 mM HEPES, pH 7.2 buffer was done.

Bradford assay

A Bradford assay was done with the protein sample dissolved in 50 mM HEPES, 100 mM NaCl, pH 7.2.

Results

SDS PAGE

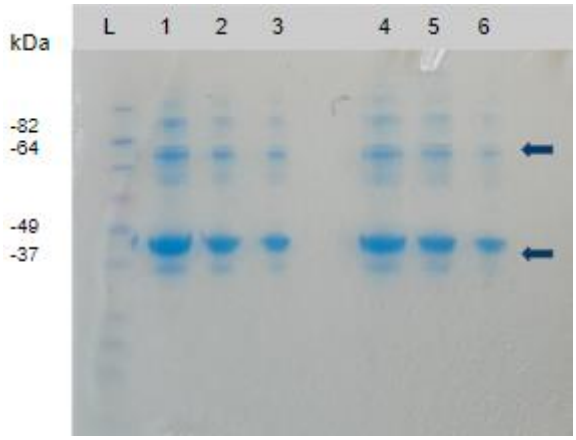

Figure 19. SDS-PAGE after dialyzing BlcR into 50 mM HEPES, pH 7.2 and 50 mM Tris-HC, pH 7. 1: HEPES 15 uL, 2: HEPES 10 uL, 3: HEPES 5 uL, 4: HEPE 2.5, 5 Tris-HCl 15 uL, 6: Tris-HCl 10 uL, 7: Tris-HCl 5 uL, 8 Tris-HCl 2.5 uL.

Bands are visible around 37 kDa and between 64 and 82 kDa corresponding to the size of the monomer and dimer of BlcR. There is still some contamination, we decided to further purify with Size Exclusion

Bradford assay

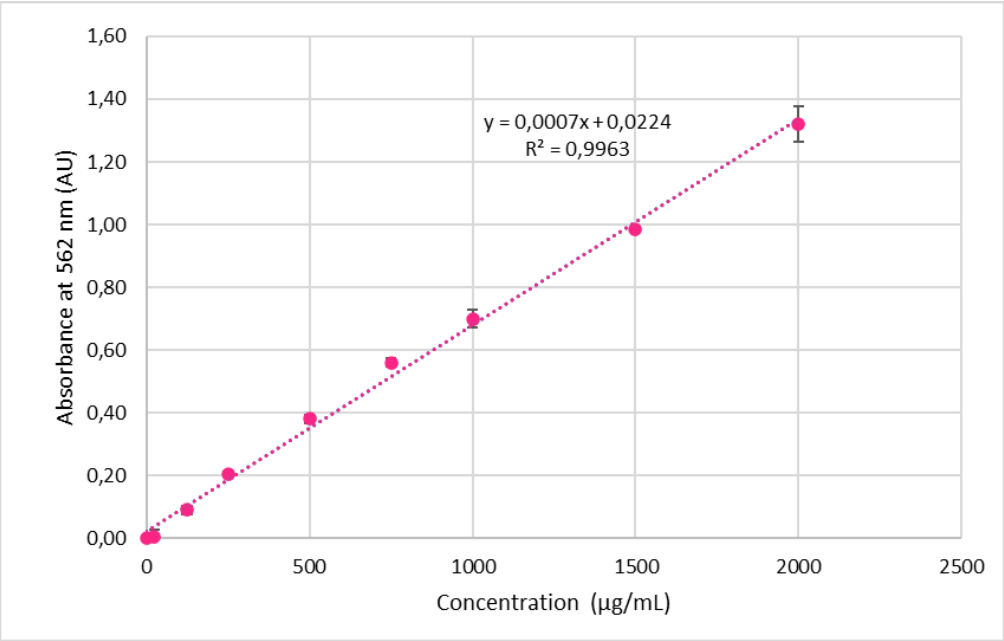

Figure 20. Bradford assay calibration curve protein concentration determination in 50 mM HEPES, 100 mM NaCl, pH 7.2 buffer.

Table 10. Average and corrected absorbance at 562 nm for different BSA samples for calibration curve of bradford assay in 50 mM HEPES, 100 mM NaCL, pH 7.2 buffer.

Concentration ug/mL	A_{562} nm average & corrected	Variance σ
2000	1.32	0.057
1000	0.99	0.007
1500	0.70	0.028
750	0.56	0.014
500	0.38	0.014
250	0.21	0.007
125	0.09	0.014
25	0.005	0.021
0	0	0

The formula to calculate the protein concentration is y = 0.0007x + 0.0224. With this formula the concentration of BlcR is calculated.

Table 11. Absorbance at 562 nm and corresponding concentration of BlcR.

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} avg corrected	Concentration ug/mL
BlcR	1.01	1.05	1.03	0.80	1110

A concentration of 1180.4 ug/mL corresponds to:
1110.4 / 70000 ug/umol = 0.0158 umol/mL = 16 uM

Date: 22/08/2022

Experimentalist(s): Robin

Protocols: Buffer exchange; Concentrator tube

Buffer exchange; Concentrator tube

For the size exclusion the storage buffer (50 mM HEPES, 100 mM NaCl, pH 7.2) is changed for another storage buffer (50 mM HEPES, 200 mM NaCl, 10 % glycerol, pH 7.2). This is done by using a concentrator tube. The sample is concentrated to a volume of 2.2 mL

Date: 23/08/2022

Experimentalist(s): Robin

Protocols: Size exclusion

Size Exclusion

The column, HiPrep™ 16/60 sephcercyl™ s-200 HR, for the size exclusion is washed with 2x the column volume (120 mL) milli Q and 1x the buffer. Akta machine was prepared for size exclusion by cleaning the machine with ethanol and milliQ and equilibrating the machine with the storage buffer of BlcR: 50 mM HEPES, 200 mM NaCl, 10 % glycerol, pH 7.2.

Date: 24/08/2022

Experimentalist(s): Robin

Protocols: Size exclusion

Size Exclusion

Size exclusion was done following the size exclusion protocol. Sample was injected in a 2 mL volume.

RESULTS

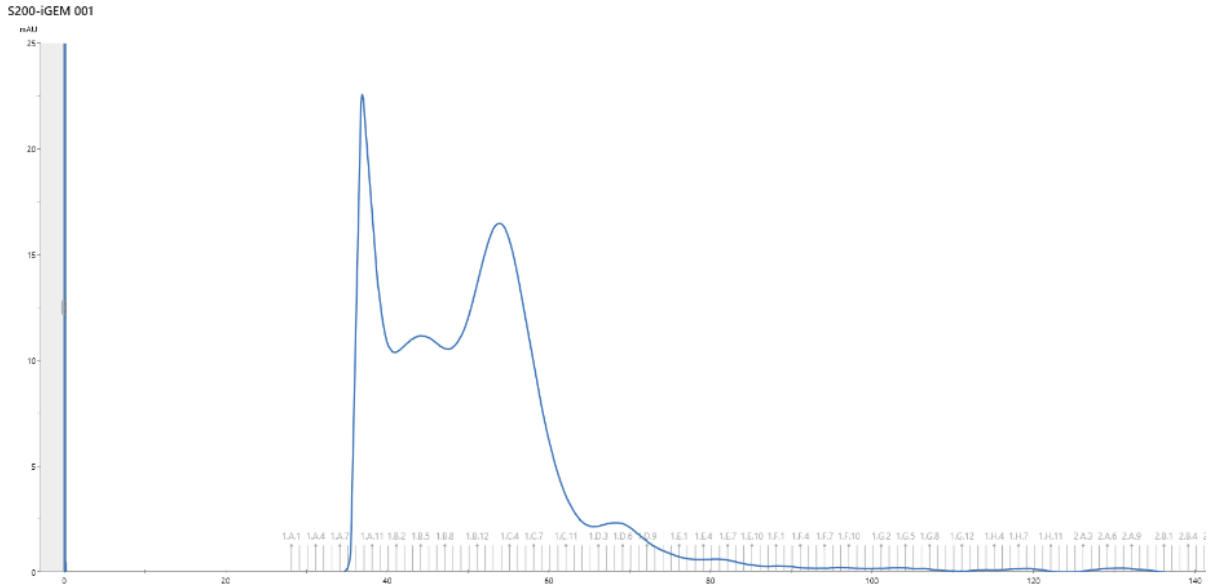

Figure 21. Absorbance at 280 nm plotted against column volume (HiPrep™ 16/60 sephcercyl™ s-200 HR). Elution pattern of gel filtration with BlcR sample in 50 mM HEPES, 200 mM NaCl, 10 % glycerol.

The elution pattern of the size exclusion. The absorbance at 280 nm is plotted against the column volume. When we look at the elution pattern we see two different big peaks. One around 40 and one around 60 mL. We decided to put a fraction on gel from the big peak around 40 mL (A11), one from the smaller peak around 45 mL (B5), and several from the large peak at 60 mL (C1, C4, C5, C9), to check if it indeed contained our protein of interest.

Date: 26/08/2022

Experimentalist(s): Robin, Brent

Protocols:

ϵ_{280} = 14500 M^{-1} cm^{-1}.

Table 12. Nanodrop results BlcR sample

Sample	Concentration (mg/ml)	260/280	A_{260} 10mm
BlcR	0,08	0,82	0,0765

From this we can calculate [BlcR] = A_{280} / ϵ_{280} = 5.27 µM.

Date: 26/08/2022

Experimentalist(s): Allegra, Robin

2 µM stock BlcR:
100 µl stock: 38 µl BlcR + 62 µl MilliQ

2.5 nM stock Cy3 DNA:
1 ml stock: 2.5 µl Cy3 DNA + 997.5 µl MilliQ

1 µg/µl stock salmon sperm:
100 µl stock: 10 µl of salmon sperm + 90 µl MilliQ

Date: 29/08/2022

Experimentalist(s): Robin, Brent

Protocols: Nanodrop, Protein production

Nanodrop

All the tubes containing pure BlcR, C1-C9, are combined. The absorbance at 280 nm of the solution is measured with Nanodrop in double. The average of the two measurements is 0.042. The concentration of BlcR is calculated according to the following formula: [BlcR] = A_{280} / ϵ_{280} with ϵ_{280} equals ϵ_{280} = 14500 M^{-1} cm^{-1}.

[BlcR] = 0.042 / 14500 = 29 x 10^{-7} M = 2.9 µM

Table 13. Nanodrop results BlcR sample

Sample	A_{260} 10mm
BlcR	0.049
BlcR	0.035

The BlcR is aliquoted, 100 µL and 200 µL, in liquid nitrogen and stored in -80 ℃.

Protein production

BL21(DE3) with pET-11a-BlcR (BBa_K4361106) was inoculated from plate colonies in 10 mL LB-Amp for overnight incubation.

Date: 30/08/2022

Experimentalist(s): Allegra, Brent

Protocols: Agarose gel, Protein production

Agarose gel

Cy3, scrambled DNA and the Wild Type were run using a 0.8% agarose gel. We used 5 µL of Sybr Safe, and every DNA sample had a concentration of 5 µM.

Protein Production

Culture of BL21(DE3) with pET-11a-BlcR (BBa_K4361106) was scaled up to 2L. After 3 hours, both cultures were around OD=0.7. IPTG was added to a final concentration of 0.5 mM to induce the production of BlcR, and cultures were left incubating at 18°C overnight.

RESULTS

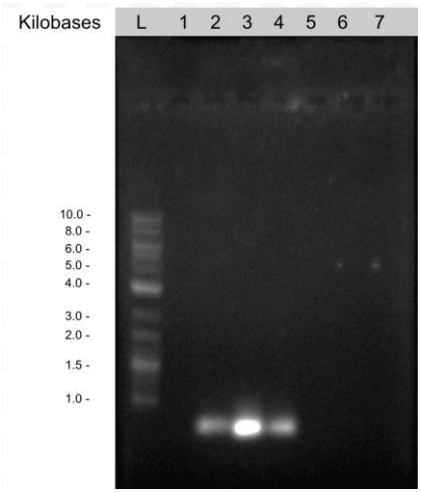

Figure 22. Agarose gel to visualize different DNA oligos (5 µM): Cy3 (2), Scrambled (3), and WT (4).

Date: 30/08/2022

Experimentalist(s): Allegra, Brent

Protocols: Agarose gel, protein production, His Tag purification

EMSA

The samples were prepared according to protocol, as following:

Table 14. Sample preparation scheme for EMSA study.

Component	Sample 1	Sample 2	Sample 3	Sample 4	Final Concentration
Cy3 DNA	2 µL				2.5 nM
Scrambled DNA		2 µL			2.5 nM
WT (old)			2 µL		2.5 nM
WT (new)				2 µL	1.25 nM
EMSA 5X	4 µL	4 µL	4 µL	4 µL	1x
$MgCl_2$	2 µL	2 µL	2 µL	2 µL	2.5 mM
EDTA	0.8 µL	0.8 µL	0.8 µL	0.8 µL	0.1 mM
BSA	1 µL	1 µL	1 µL	1 µL	1 µg/µl
Salmon Sperm	1 µL	1 µL	1 µL	1 µL	1 µg/µl
Nuclease Free Water	9.2 µL	9.2 µL	9.2 µL	9.2 µL	

Stock Scrambled DNA 25nM:

Initial concentration of 100 µM, we wanted 25 nM in 1000 µL.
5 µM: 1 µL Scrambled DNA (100µM) + 19 µL MilliQ
1000 µL = 5 µL of Scrambled DNA (5µM) + 995 µL of MilliQ

Stock WT DNA old 25nM:

Initial concentration of 100 µM, we wanted 25 nM in 1000 µL.
5 µM: 1 µL WT DNA old + 19 µL MilliQ

1000 µL = 5 µL of WT DNA old (5µM) + 995 µL of MilliQ

Stock WT DNA new 25nM:

Initial concentration of 100 µM, we wanted 25 nM in 1000 µL.
5 µM: 1 µL WT DNA new (100µM) + 19 µL MilliQ
1000 µL = 5 µL of WT DNA new (5µM) + 995 µL of MilliQ

The gel ran as follows: Ladder (L), Cy3 (2), Scrambled (3), and WT old (4), WT new (5) for 80 min at 80V.

Protein Purification

Overnight induced BL21(DE3) cultures were lysed by cell disruption and the lysate was subsequently clarified according to the production & purification protocol.

HisPur Ni-NTA beads were regenerated and washed according to the HisPur Ni-NTA user instructions. The clarified lysate was purified by Ni-NTA affinity chromatography according to the His-Tag purification protocol. The beads were eluted two times.

RESULT

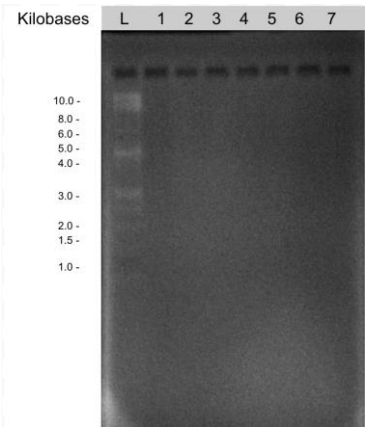

Figure 23. EMSA agarose gel with only DNA (25 nM). Ladder (L), Cy3 (2), Scrambled (3), and WT old (4), WT new (5).

Date: 01/09/2022

Experimentalist(s): Martina, Robin, Brent

Protocols: EMSA, SDS PAGE, Protein production

EMSA

2.5% agarose gel was prepared by mixing 2g of agar in 80 mL of EMSA running buffer, as per protocol [3]. The samples for EMSA were prepared following the scheme:

Table 15. Sample preparation scheme for EMSA study.

Component	Sample 1	Sample 2	Sample 3
Cy3 DNA (25 nM)	5 µL		
Scrambled DNA (25 nM)			5 µL
WT (new) (25 nM)		5 µL	
EMSA 5X	4 µL	4 µL	4 µL
$MgCl_2$	2 µL	2 µL	2 µL
EDTA	0.8 µL	0.8 µL	0.8 µL
BSA	1 µL	1 µL	1 µL
Salmon Sperm	1 µL	1 µL	1 µL
Nuclease Free Water	6.2 µL	6.2 µL	6.2 µL

The second batch was composed in the following way:

The samples were incubated at room temperature in the dark for 15 mins. They were consequently loaded in two different gels, both in this order: ladder (1), Cy3 DNA (2), wild type DNA (3) and scrambled DNA (4). The gel was run for 80 mins at 80 V.

RESULTS

The different DNA oligos were visible on the EMSA gel.

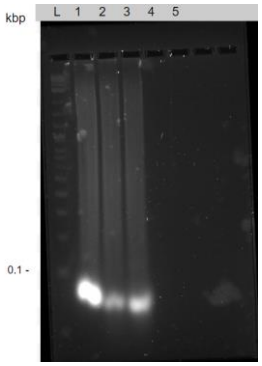

Figure 24. EMSA study: ladder (1), Cy3 DNA (2), wild type DNA (3) and scrambled DNA (4).

SDS-PAGE analysis of purification

Different fractions of the purification process were analyzed by SDS-PAGE. Precast 10 % Bis-Tris SDS PAGE from Thermo Fisher and MES running buffer.

RESULTS

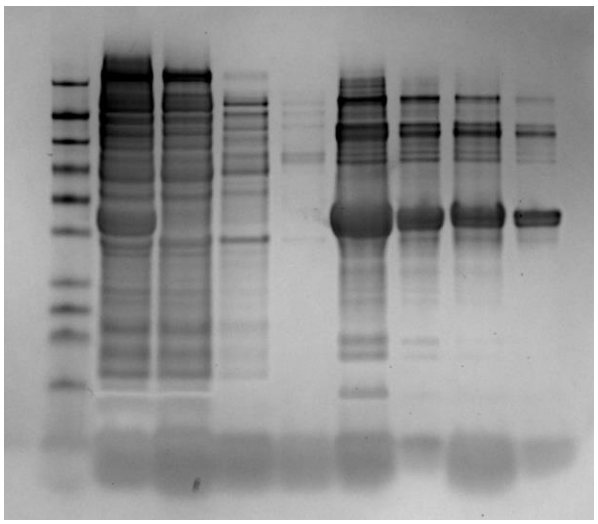

Figure 25. SDS-PAGE with different samples from BlcR production. Samples are loaded in the following order: Ladder, clear lysate, column flow trough, column wash 1, column wash 2, 15 µL protein elution 10 µL protein elution, 5 µLprotein elution, 2.5 µL protein elution.

Both elutions show significant amounts of a protein of around 40 kDa, but also significant amounts of contamination with other proteins.

Date: 02/09/2022

Experimentalist(s): Robin

Protocols: Buffer exchange; concentrator tube

BlcR buffer exchange

The two elution fractions containing BlcR were combined and buffer-exchanged by centrifugation in Amicon® Ultra-15 Centrifugal Filter Units (MWCO = 10 kDa), using storage buffer (50 mM HEPES, 200 mM NaCl, 10% glycerol, pH=7.2), following the buffer exchange protocol.

Date: 06/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

0.8% and 2.5% gels were prepared by mixing respectively 1.6g and 2.5g with 200 mL of EMSA running buffer. The mixture was then microwaved and casted.

Cy3 DNA :
initial concentration: 5 µM; desired concentration: 100 nM
→ 1 µL of Cy3 DNA stock solution + 199 µL of nuclease free water

BlcR for 50% binding:
initial concentration: 28 µM; desired concentration: 11 µM
→ 10 µL of protein (newest batch) + 15 µL of nuclease free water

BlcR for 90% binding:
initial concentration: 28 µM; desired concentration: 15 µM
→ 10 µL of protein (newest batch) + 9 µL of nuclease free water

DTT:
desired concentration: 50 mM
→ mix 771 mg of DTT powder in 100 mL of MilliQ

EMSA running buffer (20 mM Na_2HPO_4, adjusted to pH 8 with HCl):
initial concentration: 100 mM; desired concentration: 20 mM
→ 200 mL of buffer + 800 mL of MilliQ

EMSA 5x binding buffer (prepared as per the AptaVita protocol)[2]:
→ PBS 10x: 250 µL
→ $MgCl_2$ 1M: 2.5 µL
→ KCl 1M: 30 µL
→ Glycerol 80%: 156.25 µL
→ MilliQ: 61.25 µL
→ Total: 500 µL

The samples were prepared as follows, by adding different concentrations of BlcR, so that different bindings can be expected:

Table 16. Sample preparation scheme for EMSA study.

Component	Sample 1	Sample 2	Sample 3	Final Concentration
Cy3 DNA (100 nM stock)	2 µL	2 µL	2 µL	10 nM
BlcR (11 uM)		2 µL		1.1 µM
BlcR (15 uM)			2 µL	1.5 µM
EMSA 5X	4 µL	4 µL	4 µL	1x
$MgCl_2$	2 µL	2 µL	2 µL	2.5 mM
EDTA	0.8 µL	0.8 µL	0.8 µL	0.1 mM
BSA	1 µL	1 µL	1 µL	1 µg/µl
Salmon Sperm	1 µL	1 µL	1 µL	1 µg/µl
DTT	2 µL	2 µL	2 µL	5 mM
Nuclease Free Water	9.2 µL	10.4 µL	9.2 µL	

RESULTS

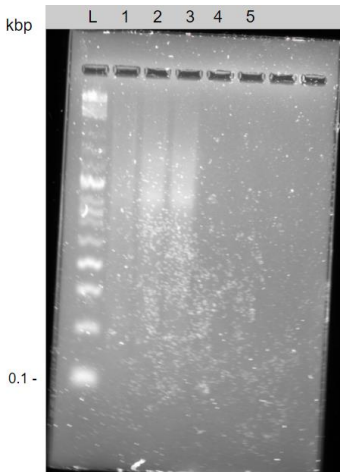

Figure 26. EMSA study. In all samples a DNA concentration of 10 nM is used. BlcR concentration increases from 0 to 15 uM: 1: 0 uM BlcR, 2: 10 uM BlcR, 3: 15 uM BlcR

Date: 07/09/2022

Experimentalist(s): Allegra, Martina

Protocols: Gel preparation, EMSA

EMSA

The samples were prepared as following the scheme on 06/09/2022, by adding different concentrations of BlcR.:The gel was made with 2.5% agarose in the EMSA running buffer (20 mM Na_2HPO_4, 375 mM KCl, 25 % Glycerol). The gel ran for 80 mins at 80 V.

RESULTS

No visible bands on the gel were visible.

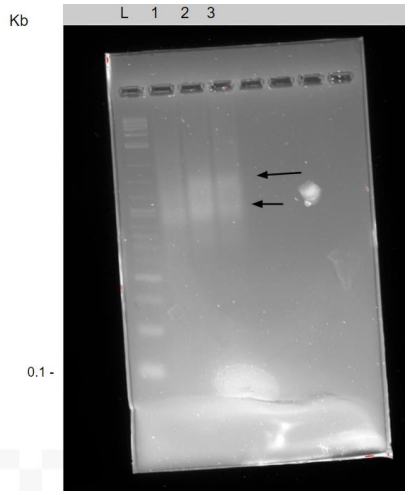

Figure 27. EMSA study. In all samples a DNA concentration of 10 nM is used. BlcR concentration increases from 0 to 1.5 uM: 1: 0 uM BlcR, 2: 1.1 uM BlcR, 3: 1.5 uM BlcR

Date: 08/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

The samples were prepared as follows, by titrating BlcR while keeping the DNA concentration constant. gel was a 2.5% agarose gel prepared with EMSA running buffer (20 mM Na_2HPO_4, 375 mM KCl, 25 % Glycerol):

Table 17. Sample preparation scheme for EMSA study.

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Final concentration
Cy3 DNA (100 nM stock)	2 µL	2 µL	2 µL	2 µL	2 µL	10 nM
BlcR (11 uM)		1 µL				0.8 µM
BlcR (11			2 µL			1.1 µM

uM)						
BlcR (11 uM)				3 µL		1.3 µM
BlcR (15 uM)					2 µL	1.5 µM
EMSA 5X	4 µL	4 µL	4 µL	4 µL	4 µL	1x
$MgCl_2$	2 µL	2 µL	2 µL	2 µL	2 µL	2.5 mM
EDTA	0.8 µL	0.8 µL	0.8 µL	0.8 µL	0.8 µL	0.1 mM
BSA	1 µL	1 µL	1 µL	1 µL	1 µL	1 µg/µl
Salmon Sperm	1 µL	1 µL	1 µL	1 µL	1 µL	1 µg/µl
DTT	2 µL	2 µL	2 µL	2 µL	2 µL	5 mM
Nuclease free water	7.2 µL	6.2 µL	5.2 µL	4.2 µL	5.2 µL	

The samples were loaded in the gel as follows:The gel ran for 80 mins at 80 V. Afterward, it was stained with SYBR Safe (5 µL of SYBR Safe in 50 mL of running buffer) for 30 mins. Finally the gel was visualized with UV.

RESULTS

Only one line of bands was visible, therefore the gel was inconclusive.

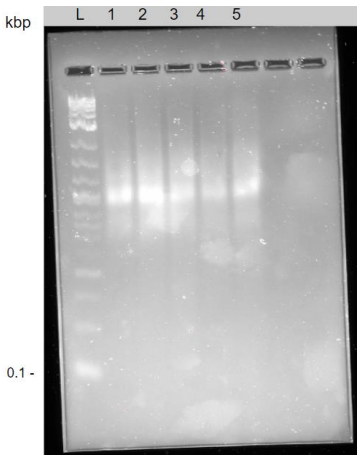

Figure 27. EMSA study. In all samples a DNA concentration of 10 nM is used. BlcR concentration increases from 0 to 1.5 uM: 1: 0 uM BlcR, 2: 0.8 uM BlcR, 3: 1.1 uM BlcR, 4: 1.3 uM BlcR, 5: 1.5 uM BlcR.

Date: 12/09/2022

Experimentalist(s): Brent

Protocols: Protein production

Protein production

E. coli BL21(DE3) with pET-11a-BlcR (BBa_K4361106) was inoculated into 15 mL LB-Amp according to the production protocol.

Date: 13/09/2022

Experimentalist(s): Robin, Brent

Protocols: TEV digestion, Protein production

TEV digestion

The BlcR solution produced by buffer exchange on 02/09 was treated with TEV protease to cut off the His Tag from the protein. A BlcR TEV protease solution was left overnight.

Protein production

The 15 mL culture of E. coli BL21(DE3) with pET-11a-BlcR (BBa_K4361106)was divided over three shake flasks with 1L LB-Amp each, incubated, induced with IPTG, and incubated again according to the production protocol.

Date: 14/09/2022

Experimentalist(s): Robin, Brent

Protocols: Size exclusion, Protein production, His-tag purification

Size exclusion

Size exclusion was done following the size exclusion protocol. The solution with BlcR was injected twice with an injection volume of 2 mL. The buffer that was used for size exclusion was the storage buffer of BlcR, 50 mM HEPES, 10 % glycerol, 200 mM NaCl, pH 7.2.

RESULTS

For the first run of size exclusion we got the elution pattern. The absorbance at 280 nm is plotted against the column volume. From the first time size exclusion we knew that our protein eluted around 60 mL. When we look at the elution pattern we see two different big peaks. One around 40 and one around 60 mL. We decided to put a fraction on gel from the big peak around 40 mL (A9), one from the smaller peak around 45 mL (B5), and several from the large peak at 60 mL (C1-D1), to check if it indeed contained our protein of interest.

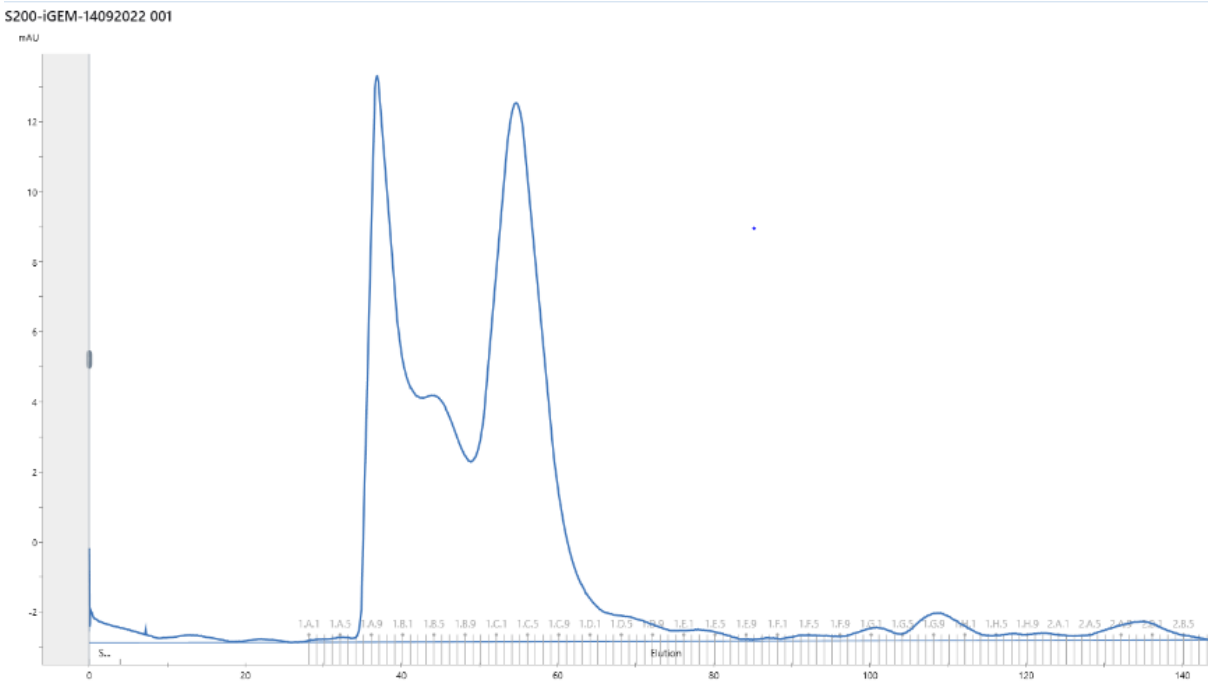

Figure 22. Absorbance at 280 nm plotted against column volume (HiPrep™ 16/60 sephcercyl™ s-200 HR). Elution pattern of gel filtration with BlcR sample in 50 mM HEPES, 200 mM NaCl, 10 % glycerol.

For the sercond run of size exclusion we got the elution pattern. The absorbance at 280 nm is plotted against the column volume. From the first time size exclusion we knew that our protein eluted around 60 mL. When we look at the elution pattern we see two different big peaks. One around 40 and one around 60 mL. We decided to put a fraction on gel from the big peak around 40 mL (A12), and several from the large peak at 60 mL (B12-C12), to check if it indeed contained our protein of interest.

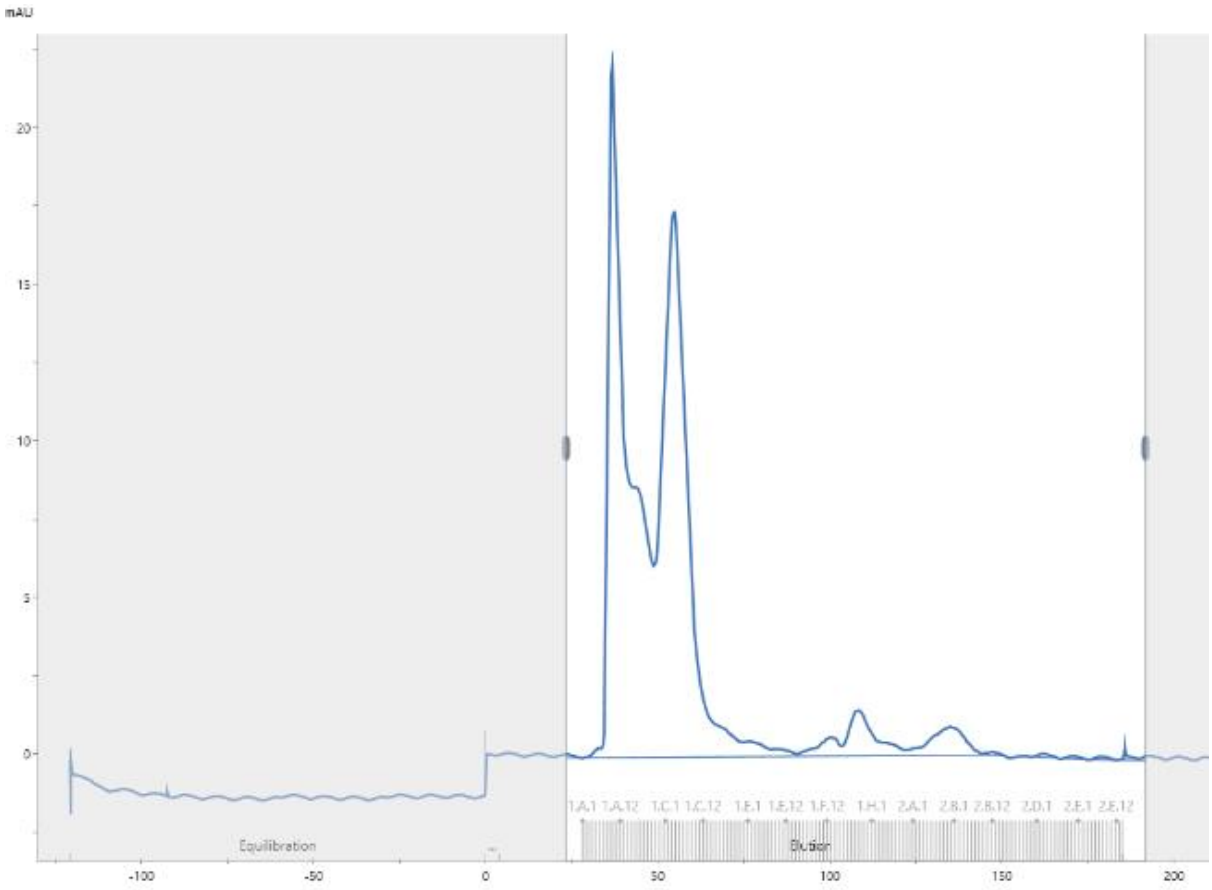

Figure 23. Absorbance at 280 nm plotted against column volume (HiPrep™ 16/60 sephcercyl™ s-200 HR). Elution pattern of gel filtration with BlcR sample in 50 mM HEPES, 200 mM NaCl, 10 % glycerol.

Protein Production

After incubating overnight, the 3L of culture was lysed according to the production protocol.

The clarified lysate was immediately purified by His-tag chromatography with TEV elution. According to the purification protocol, Ni-NTA beads were washed, incubated with clarified lysate, and the flow-through was removed by column elution. The beads with immobilized protein were washed 2 times by column elution and 2 times by centrifugation.

In addition to the regular purification protocol, 100 µL of concentrated TEV protease was added to the bead suspension to cleave the His-tags from the BlcR, and left to incubate overnight, rotating, at 4°C.

Date: 15/09/2022

Experimentalist(s): Robin, Brent

Protocols: SDS-PAGE, Nanodrop, His-Tag purification

SDS-PAGE

Based on the elution pattern from size exclusion we decided to put different fractions on gel. The fractions that correspond to the peaks are chosen to analyze with 10 % Bis-Tris SDS PAGE from Thermo Fisher.

1st run: A9, B5, C1, C2, C4, C8, C12, D1
2nd run: A12, B10, B12, C1, C2, C6, C8, C12

Nanodrop

Nanodrop of the protein solution of the combined fractions with pure BlcR is determined with Nanodrop

Protein purification

After incubating with TEV protease overnight, the bead suspension was centrifuged and the supernatant was removed and filtered through an empty column (called TEV digestion 1).

The beads were resuspended in the wash buffer, and again 100 µL of concentrated TEV protease was added and the mixture was incubated, rotating, at 4°C.

After 7 hours, the process was repeated to remove the liquid (called TEV digestion 2) and start another digestion.

RESULTS

SDS-PAGE

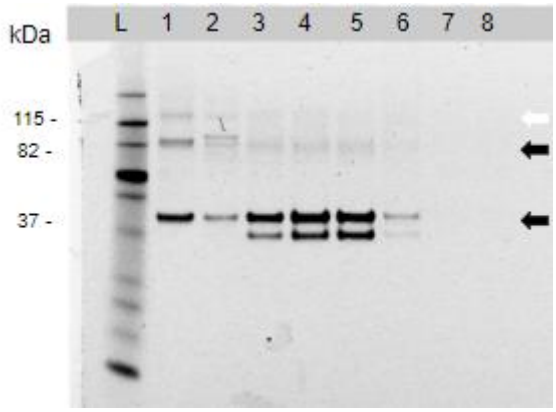

Figure 24. SDS-PAGE after gel filtration with BlcR protein sample. Next to the ladder: 1: A9, 2: B5, 3: C1, 4: C2, 5: C4, 6: C8, 7: C12, 8: D1.

The difference between the BlcR with His Tag and without His Tag is visible on the SDS PAGE around 37 kDa. In the first and second lane you can still see another protein around 115 kDa.

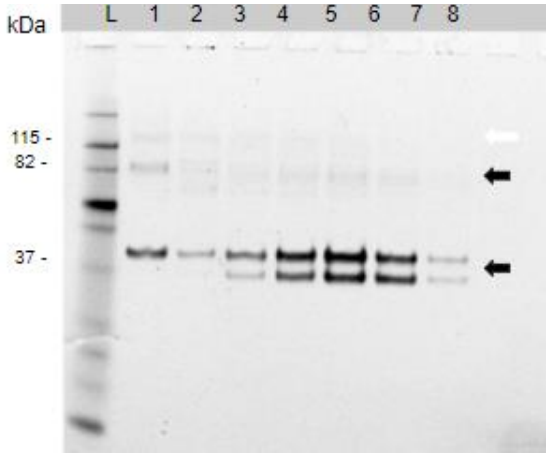

Figure 25. SDS-PAGE after gel filtration with BlcR protein sample. Next to the ladder: 1: A12, 2: B10, 3: B12, 4: C1, 5: C2, 6: C6, 7: C8, 8: C12.

The difference between the BlcR with His Tag and without His Tag is visible on the SDS PAGE around 37 kDa. In the first lane you can still see another protein around 115 kDa.

From both gels we decided to combine the fractions C1-C6 since in both runs they contain the most pure and concentrated BlcR.

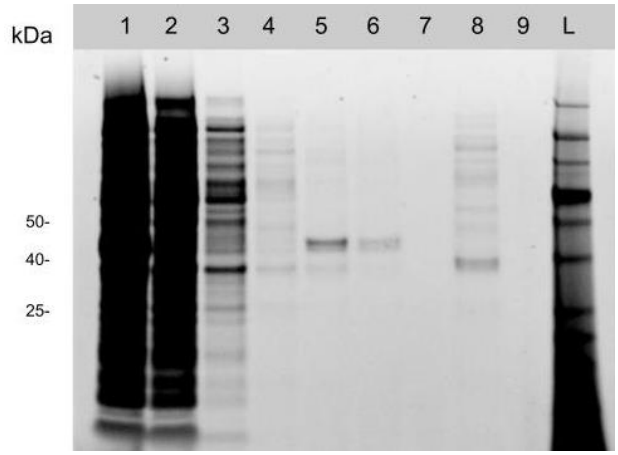

Figure 26. SDS-PAGE after protein production and purification cycle. 1: clarified lysate, 2: column flow-through, 3-6: wash 1-4, 8: TEV digestion 1, L: BenchMark Prestained Protein Ladder.

The SDS-PAGE with samples from the new production and purification cycle showed low effectivity in elution with TEV protease. A second elution step with imidazole will be done.

Nanodrop

following formula: [BlcR] = A_{280} / ϵ_{280} with ϵ_{280} equals ϵ_{280} = 14500 M^{-1} cm^{-1}.
[BlcR] = 0.042.5 / 14500 = 29 x 10^{-7} M = 2.9 µM

Table 18. Nanodrop results of BlcR sample.

Sample	A_{260} 10mm
BlcR	0.044
BlcR	0.041

Date: 16/09/2022

Experimentalist(s): Brent

Protocols: His-tag purification, SDS-PAGE

His-tag purification

The third and final TEV-digestion elution was removed by centrifuging the bead suspension and filtering the supernatant.

Subsequently, any remaining protein was eluted from the beads by addition of Elution buffer (50 mM Tris-Hcl, 200 mM Imidazole, 300 mM NaCl, pH 7.5), following the procedure in the purification protocol.

SDS-PAGE

To analyze the whole purification process, a Precasted 10 % Bis-Tris SDS-PAGE from Thermo Fisher was used to visualize the different fractions, according to the SDS-PAGE protocol.

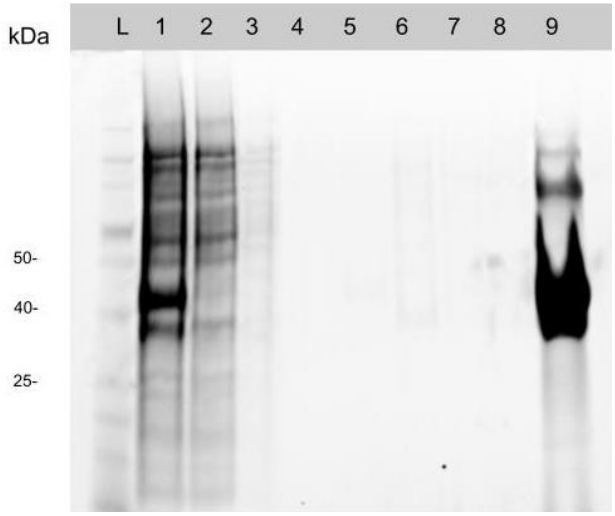

Figure 27. SDS-PAGE gel with fractions of the protein purification process. L: ladder, 1: clarified lysate, 2: column flow-through, 3-5: wash 1-3, 6-8: TEV elutions 1-3, 9: imidazole elution.

Date: 19/09/2022

Experimentalist(s): Martina, Brent

Protocols: EMSA, Buffer exchange; concentrator tube

Buffer exchange; Concentrator tube

8 mL of the purified BlcR produced by SEC on 14/09 and 12 mL of the imidazole elution from 15/09 were both dialyzed into 50 mM Tris, 300 mM NaCl, 0.5 mM EDTA, pH=7.5 using Slide-A-Lyzer cassettes, following the buffer exchange protocol.

EMSA

4% non-denaturing polyacrylamide gel was prepared as follows: 1x TAE, 30 % acrylamide-bis. Before casting the gel add: 15 µL TEMED and 75 µL APS. Cast the gel between the two glass plates and let it polymerize for 1 hour.

The samples were prepared as follows, according to the EMSA protocol.

1) Binding buffer
2)

Table 19. Components binding buffer for EMSA study

Component	Quantity (ul) for 10 samples	Final concentration
Tris-Cl, pH 8 [20mM]	20	2mM
EDTA [2.5Mm]	16	0.2mM
DTT [20mM]	20	0.2mM
Potassium Glutamate [120 mM]	20	12 mM
BSA	40	4 ug/ul
Glycerol [20%]	20	2%

3) Sample preparation: one gel with 25 nM DNA and one gel with 10 nM DNA

Table 20. Sample preparation scheme for EMSA study of BlcR.

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Final concentration
Cy3 DNA (100 nM stock)	4 µL	4 µL	4 µL	4 µL	4 µL	25 nM / 10
BlcR (11 µM)	-	1 µL	2 µL	3 µL	4 µL	from 0.78 uM to 1.6 uM
Binding buffer	13.6 µL	13.6 µL	13.6 µL	13.6 µL	13.6 µL	1x
Nuclease free water	5.4 µL	4.4 µL	3.4 µL	2.4 µL	1.4 µL	

The samples were incubated for 15 mins at room temperature in the dark. After incubation, the samples were loaded in the gel as follows: ladder, sample 1, sample 2, sample 3, sample 4, sample 5. The gel was run at 120V for 40 mins.

RESULTS

Two sets of bands were clearly visible. The upper band corresponds to the bound fraction whereas the lower band corresponds to free DNA.

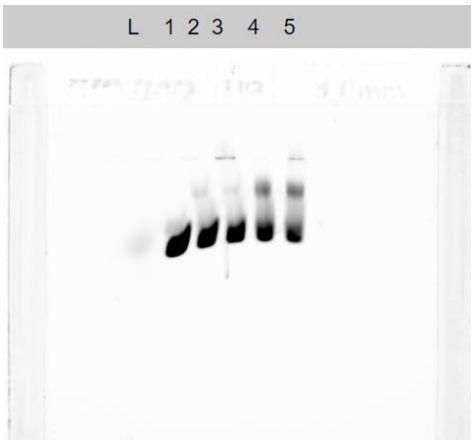

Figure 28. EMSA study for the binding of BlcR to its DNA binding sequence. DNA concentration remained constant (25 nM) and BlcR concentration increased gradually from 0.78 to 1.6 uM.

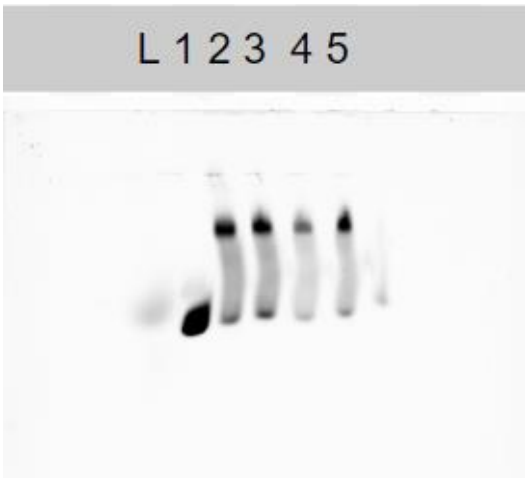

Figure 29. EMSA study for the binding of BlcR to its DNA binding sequence. DNA concentration remained constant (10 nM) and BlcR concentration increased gradually from 0.78 to 1.6 uM.

Date: 20/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

Gel was prepared following the EMSA protocol. Sample preparation was done following the scheme in Table 20. The samples were incubated for 15 mins at room temperature in the dark. After incubation, the samples were loaded in the gel as follows: ladder, sample 1, sample 2, sample 3, sample 4, sample 5. The gel was run at 90V for 40 mins.

RESULTS

The gel resulted in two clear and distinguishable sets of bands, of different intensities. The lower set decrease in intensity, meaning that the fraction of free DNA decreases as the protein concentration is increased. The upper set of bands, on the other hand, increases in intensity meaning that BlcR is binding to the DNA.

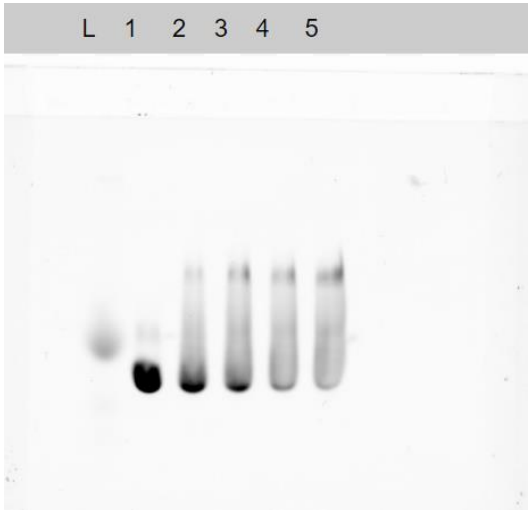

Figure 30. EMSA study for the binding of BlcR to its DNA binding sequence. DNA concentration remained constant (25 nM) and BlcR concentration increased gradually from 0.78 to 1.6 uM.

Date: 21/09/2022

Experimentalist(s): Martina, Robin

Protocols: EMSA, SDS-PAGE

SDS-PAGE

To check the imidazole elution from 15/09 and the dialysis from 19/09, another precast 10 % Bis-Tris SDS-PAGE from Thermo Fisher following the SDS-PAGE protocol.

RESULTS

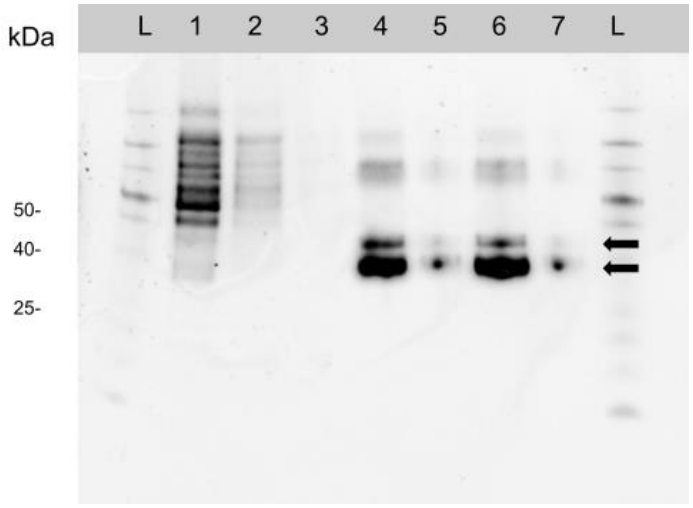

Figure 31. SDS-PAGE gel showing the elution of BlcR. 1: clarified lysate, 2: column flow-through, 3: wash 3, 4-5: imidazole elution, 6: elution after dialysis.

Date: 22/09/2022

Experimentalist(s): Rebecca

Protocols: Bradford assay

Bradford assay

Protein concentration was determined in elution buffer (50 mM Tris-Hcl, 300 mM NaCl, 300 mM Imidazole, pH 7.5) and new storage buffer (50 mM Tris-Hcl, 300 mM NaCl, 0.5 mM EDTA, pH 7.3) following the bradford assay protocol.

RESULTS

Bradford assay

Table 21. Average and corrected absorbance at 562 nm for different BSA samples for calibration curve of bradford assay in 50 mM Tris-HCl, 300 mM NaCL, 300 mM imidazole pH 7.2 buffer.

Concentration ug/mL	A_{562} nm average & corrected	Variance σ
2000	1,316	0,013
1500	1,076	0,014
1000	0,751	0,004

750	0,628	0,013
500	0,459	0,006
250	0,290	0,011
125	0,207	0,004
25	0,133	0,006
0	0,000	0,166

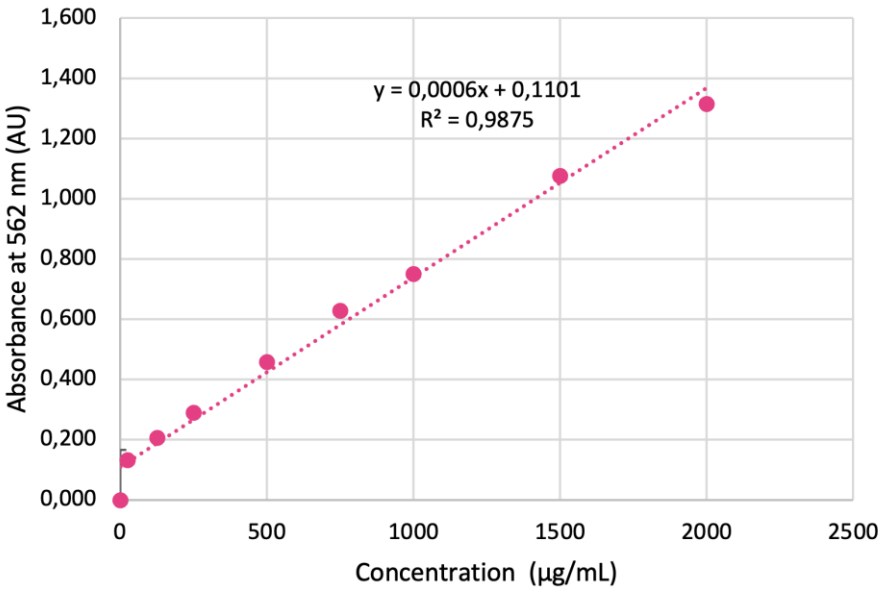

Figure 32. Bradford assay calibration curve.

The formula to calculate the protein concentration is y = 0.0006x + 0.1101. With this formula, the concentration of BlcR is calculated.

Table 22. Average and corrected absorbance at 562 nm for BlcR and corresponding concentration

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} avg corrected	Concentration ug/mL
BlcR	1,158	1,143	1,151	????	1734

The sample was diluted twice so the concentration is 1734*2 = 3468 um/mL. A concentration of 3468 ug/mL corresponds to: 3468 / 70000 ug/umol = 49.5 uM

Table 23. Average and corrected absorbance at 562 nm for different BSA samples for calibration curve of bradford assay in 50 mM Tris-Hcl, 300 mM NaCL, 0.5 mM EDTA, pH 7.5 buffer.

Concentration ug/mL	A_{562} nm average & corrected	Variance σ
2000	1,772	0,010
1500	1,481	0,021
1000	1,022	0,002
750	0,859	0,013
500	0,564	0,005
250	0,297	0,002
125	0,157	0,008
25	0,032	0,007
0	0,000	0,006

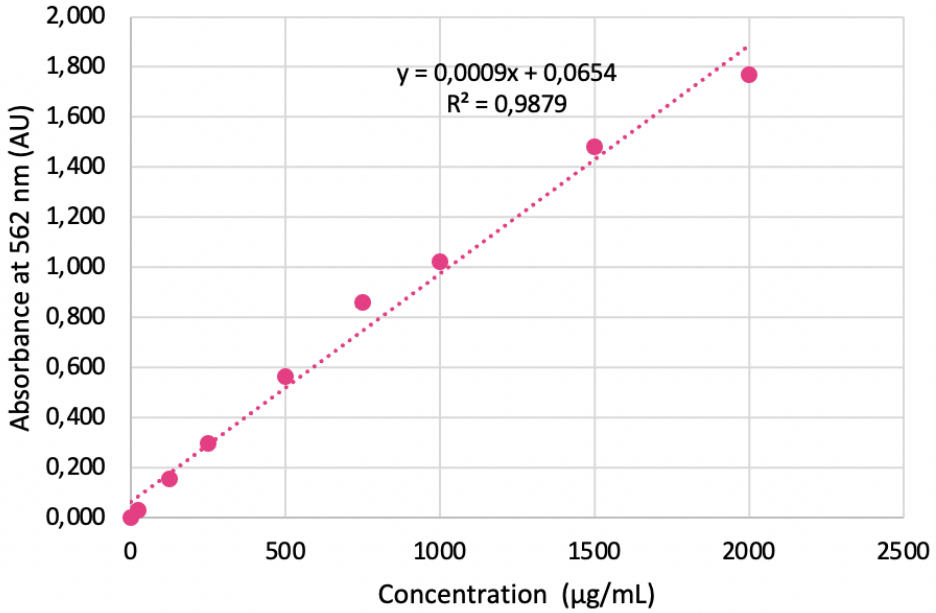

Figure 33. Bradford assay calibration curve.

The formula to calculate the protein concentration is y = 0.0009x + 0.0654. With this formula, the concentration of BlcR is calculated.

Table 24. Average and corrected absorbance at 562 nm for BlcR and corresponding concentration

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} avg corrected	Concentration ug/mL
BlcR	1,237	1,166	1,202	????	1262.4

The sample was diluted twice so the concentration is 1262.4*2 = 2524.7 um/mL. A concentration of 2524.7 ug/mL corresponds to: 2524.7 / 70000 ug/umol = 36.1 uM

Date: 24/09/2022

Experimentalist(s): Rebecca

Protocols: Bradford assay

RESULTS

Bradford assay

Table 25. Average and corrected absorbance at 562 nm for different BSA samples for calibration curve of bradford assay in 20 mM MOPS, 2.5 mM Mg, pH 7 buffer.

Concentration ug/mL	A_{562} nm average & corrected	Variance σ
2000	1,363	0,064
1500	1,079	0,067
1000	0,764	0,004
750	0,573	0,020
500	0,406	0,006
250	0,207	0,004
125	0,104	0,006
25	0,018	0,001
0	0,000	0,001

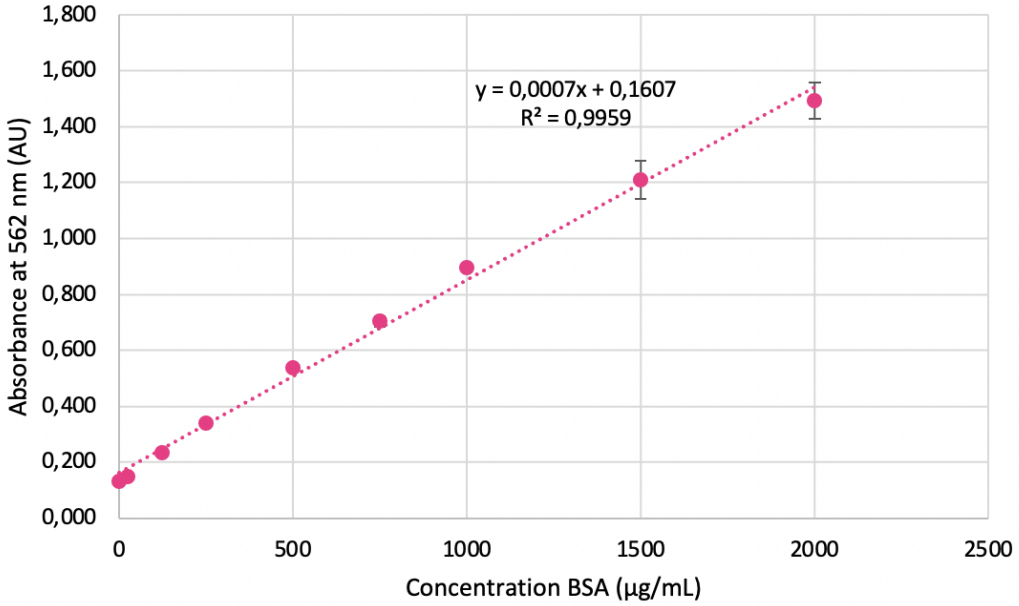

Figure 34. Bradford assay calibration curve.

The formula to calculate the protein concentration is y = 0.0007x + 0.1607. With this formula, the concentration of BlcR is calculated.

Table 26. Average and corrected absorbance at 562 nm for BlcR and corresponding concentration.

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} avg corrected	Concentration ug/mL
BlcR	1,102	1,024	1,078	0,946	1310.5

The sample was diluted twice so the concentration is 1310.5*2 = 2620.9 um/mL. A concentration of 2620.9 ug/mL corresponds to: 2620.9 / 70000 ug/umol = 37.4 uM

Date: 26/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

EMSA study was done to determine the dissociation of BlcR from its DNA binding sequence in presence of SSA, following the EMSA protocol.

Table 27. Sample preparation scheme for EMSA study of BlcR.

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Final concentration
Cy3 DNA (100 nM stock)	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	25 nM
BlcR (2.9 µM)	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	1.6 uM
Binding buffer	13.6 µL	13.6 µL	13.6 µL	113.6 µL	13.6 µL	13.6 µL	13.6 µL	13.6 µL	1x
SSA	-	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	from 0.64 nM to 0.01 mM
Nuclease free water	7.8 µL	4.8 µL	4.8 µL	4.8 µL	4.8 µL	4.8 µL	4.8 µL	4.8 µL	

The samples were incubated for 15 minutes at room temperature in the dark and consequently loaded on the gel. The gel was run at 90V for 40 mins.

RESULTS

While increasing the concentration of SSA in the sample, the lane corresponding to the free DNA in the gel would increase in intensity giving information on the dissociation. However, the first two samples were a bit out of line with the rest of the gel

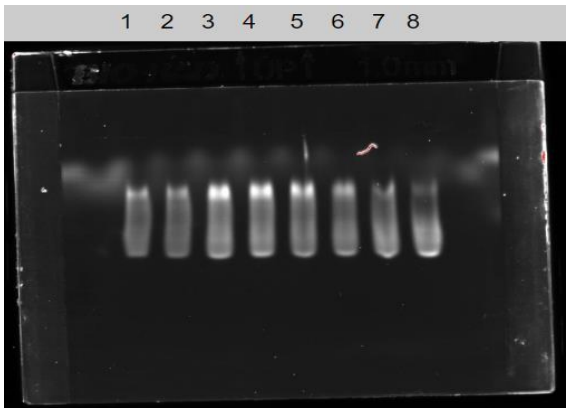

Figure 35. EMSA study for the characterization of BlcR dissociating from the 51-bp *blc* operator sequence in the presence of SSA. concentration of SSA is titrated from 0.64 nm to 0.01 mM.

Date: 27/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

EMSA gel and samples were prepared following the EMSA protocol.

In order to determine the binding affinity, the samples were prepared with different BlcR concentrations from 0 to 1.6 uM. The DNA concentration remained constant in all the samples (25 nM).

A second gel ran where the DNA and BlcR concentration stays constant at 25 nM and 1.6 uM. And the concentration of SSA varies in each sample from 64 nM to 1 mM, increasing 5x each time, therefore from left to right: 64 nM, 320 nM, 1.6 uM, 8 uM, 40 uM, 0.2 mM, 1 mM.

The samples were incubated at room temperature in the dark for 15 minutes and consequently loaded on the gel in the same order reported by the table. The gel was run at 90V for 40 mins.

RESULTS

Clear differences in intensity were detected between the different bands with increasing concentration. From this it was possible to calculate the binding affinity.

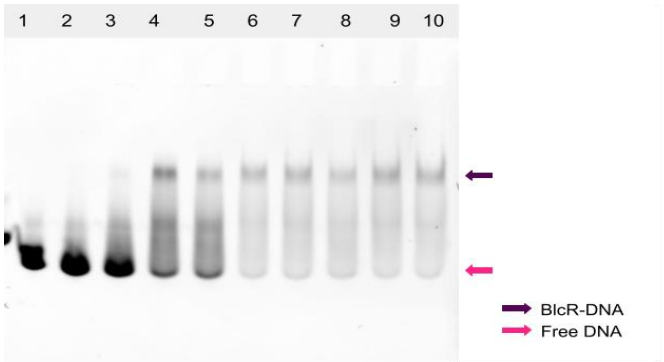

Figure 36. EMSA study for the characterization of BlcR binding to the 51 bp *blc* operator sequence. Concentration of Cy3-labeled DNA was maintained at 25 nM. Titration of dimeric BlcR from lane 1 to 10. 1: 0 μM, 2: 0.1 μM, 3: 0.25 μM, 4: 0.4 μM, 5: 0.5 μM, 6: 0.6 μM, 7: 0.7 μM, 8: 0.8 μM, 9: 0.9 μM, 10: 1 μM.

The band corresponds to free DNA increasing in intensity from left to right, corresponding to a increasing concentration of SSA, which leads to the dissociation of BlcR from the DNA.

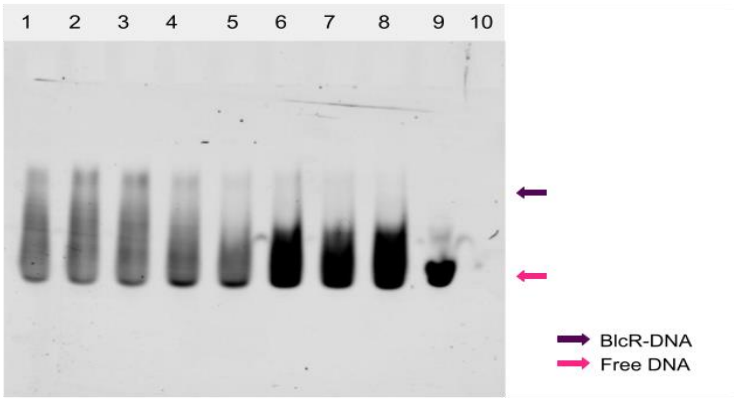

Figure 37. EMSA study for the characterization of BlcR dissociating from the 51-bp *blc* operator sequence in the presence of SSA. Concentration of Cy3-labeled DNA and BlcR were maintained at 25 nM and 1.6 µM, respectively. Titration of SSA from lane 1 to 9. 1: 0 nM, 2: 64 nM, 3: 320 nM, 4: 1.6 µM , 5: 08 µM, 6: 40 µM, 7: 0.2 mM, 8: 1 mM, 9: control with 25 nM DNA only.

Date: 27/09/2022

Experimentalist(s): Robin

Protocols: Size exclusion

Size Exclusion

Size exclusion was done with the protein purified on 15/09/2022. This time an injection volume of 5 mL was used.

RESULTS

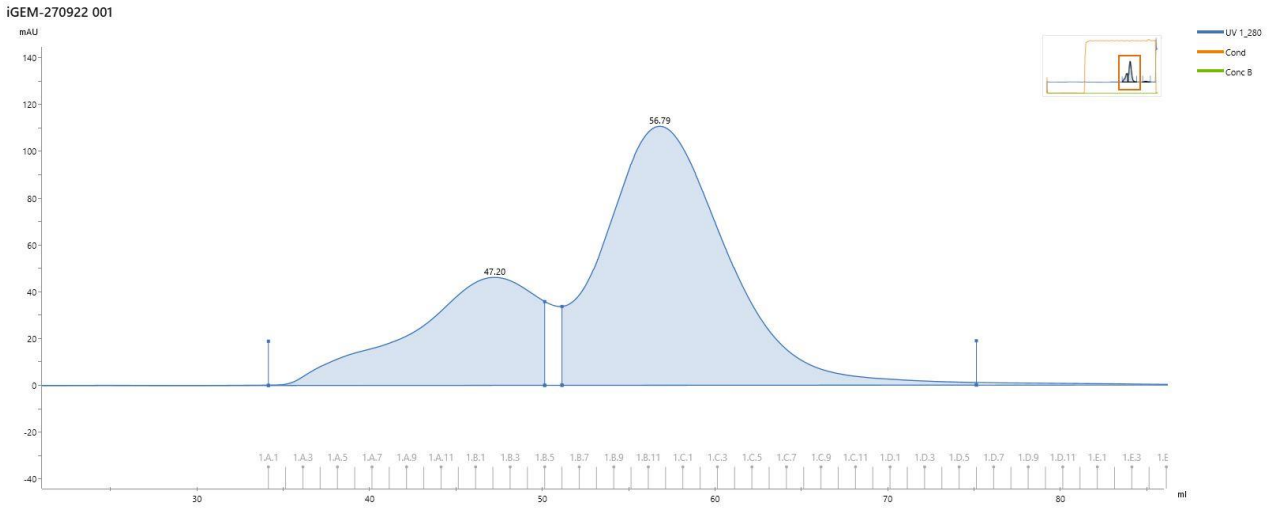

Figure 38. Absorbance at 280 nm plotted against column volume (HiPrep™ 16/60 sephcercyl™ s-200 HR). Elution pattern of gel filtration with BlcR sample in 50 mM Tris-HCl, 300 mM NaCl, 0.5 mM EDTA

From the Elution pattern of the size exclusion.We choose 9 fractions to analyze with SDS PAGE gel: B1, B3, B6, B7, B9, B11, C2, C5, C7.

Date: 28/09/2022

Experimentalist(s): Robin

Protocols: SDS-PAGE

SDS-PAGE

A SDS PAGE is done with a precast 10 % Bis-Tris SDS-PAGE from Thermo Fisher following the SDS-PAGE protocol. different fractions from the elution from size exclusion. The fractions that are put on gel are: B1, B3, B6, B7, B9, B11, C2, C5, C7.

RESULTS

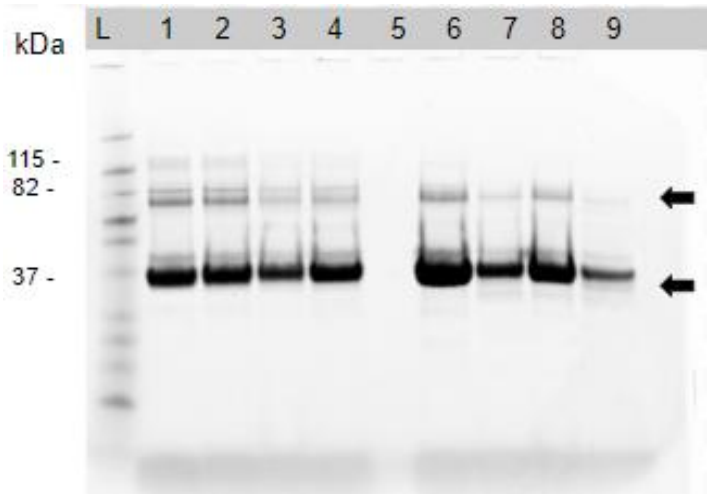

Figure 39. SDS PAGE after size exclusion. Different fractions from the Size exclusion are loaded:1 : B1, 2 : B3, 3 : B6, 4 : B7, 5 : B9, 6 : B11, 7 : C2, 8 : C5, 9 : C7.

From figure 29 we can see that BlcR is present in all samples, the bands around 82 and 37 kDa correspond to the dimer and monomer of BlcR. In fractions B2 and B3 (Figure 19, 1-2) another protein is visible around 115 kDa, this contamination is not visible in fraction B6 - C7, therefore the fractions B6 to C7 are combined. The concentration of the protein solution is checked with bradford assay.

Date: 29/09/2022

Experimentalist(s): Martina, Robin

Protocols: EMSA, Bradford assay

Bradford assay

The elution fractions B6 - C7 are combined and the concentration of the samples is measured using a Bradford assay.

EMSA

A new EMSA study was performed to examine the binding of BlcR to its operator sequence, following the EMSA protocol.

Table 29. EMSA sample preparation

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Final concentration
Cy3 DNA (100 nM stock)	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	25 nM
BlcR	-	0.7 µL	1.4 µL	2.8 µL	3.4 µL	4 µL	5.6 µL	6.8 µL	8 µL	10 µL	from 0.01 uM to 1.4 uM
Binding buffer	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	1x
Nuclease free water	14 µL	13.3 µL	12.6 µL	11.2 µL	10.6 µL	10 µL	8.4 µL	7.2 µL	6 µL	4 µL	

*for samples 1-8, a working solution of 0.29uM was made by diluting the initial stock 10 times.
The samples were then incubated at room temperature for 15 mins in the dark. The samples were consequently loaded on the gel, which was run at 90V for 40 mins.

RESULTS

EMSA

A big jump is visible between the concentrations of 0.08 uM and 1uM.

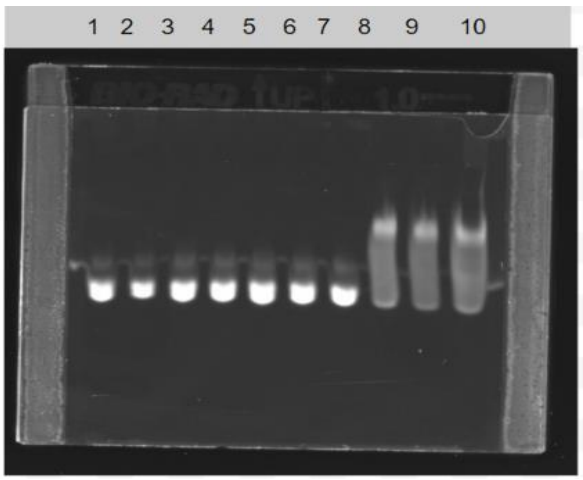

Figure 40. EMSA study for the characterization of BlcR binding to the 51 bp *blc* operator sequence. Concentration of Cy3-labeled DNA was maintained at 25 nM. Titration of dimeric BlcR from 0 uM to 1.4 uM. lane 8: 0.08 uM. lane 9: 1 uM

Bradford assay

Table 29. Average and corrected absorbance at 562 nm for different BSA samples for calibration curve of bradford assay in 50 mM Tris-Hcl, 300 NaCl, 0.5 EDTA, pH 7.5.

Concentration ug/mL	A_{562} nm average & corrected	Variance σ
2000	1,45	0,078
1500	1,18	0,035
1000	0,83	0,011
750	0,66	0,028
500	0,45	0,001
250	0,22	0,001
125	0,11	0,002
25	0,02	0,001
0	0,000	0,00

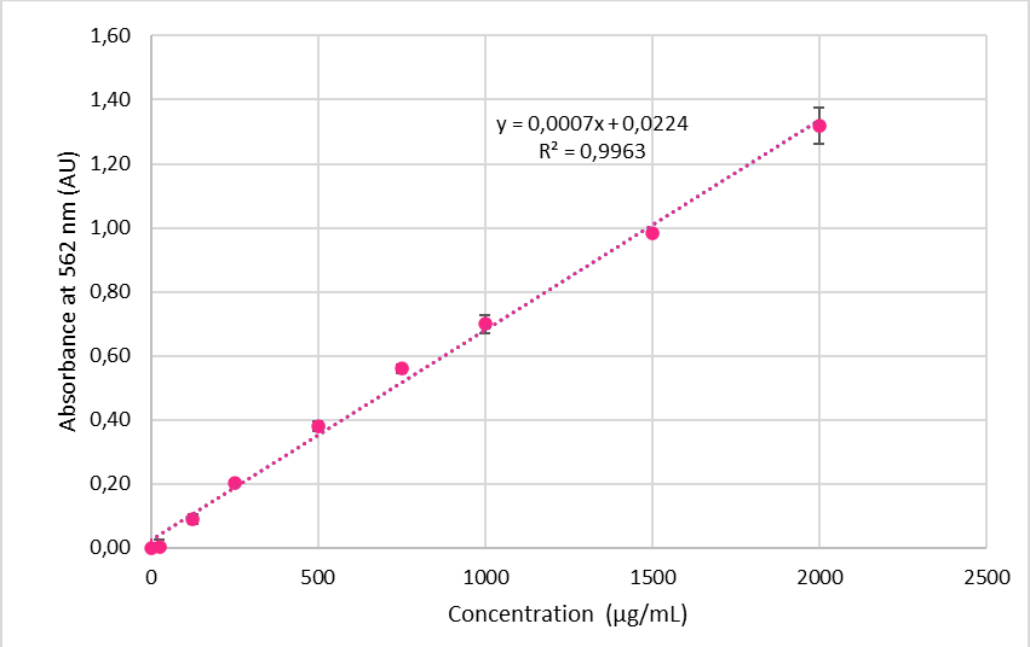

Figure 41. Bradford assay calibration curve

The formula to calculate the protein concentration is y = 0.0007x + 0.0224. With this formula, the concentration of BlcR is calculated.

Table 30. Average and corrected absorbance of BlcR sample and the corresponding concentration.

Sample	A_{562} nm (1)	A_{562} (2)	A_{562} avg	A_{562} avg corrected	Concentration ug/mL
BlcR	0.832	0.843	0.838	0.68	940.14

The concentration of 940.14 ug/mL corresponds to: 940.14 / 70000 * 1000 ug/umol = 13.4 uM.

Date: 30/09/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

One new EMSA reactions is performed to check the binding of BlcR to the DNA sequence in presence of ethanol (Table 32).

Table 31. EMSA sample preparation

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Final concentration
Cy3 DNA (100 nM stock)	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	25 nM
BlcR (2.9 uM)	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	1.6 uM
Binding buffer (10x)	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	1x
Ethanol (99%)	0.4 µL	1 µL	2 µL	4 µL	8 µL	-	from 2% to 40%
Nuclease free water	9 µL	7.4 µL	6.4 µL	4.4 µL	1.4 µL	9.4 µL	

The samples were loaded according to the table, from left to right. The gel was run at 90V for 40 mins, following the EMSA protocol.

RESULTS

From the results, it is clear that binding keeps happening, though with lower affinity as long as the concentration of ethanol is below 20%. At 40% we don't see any bindig happening anymore.

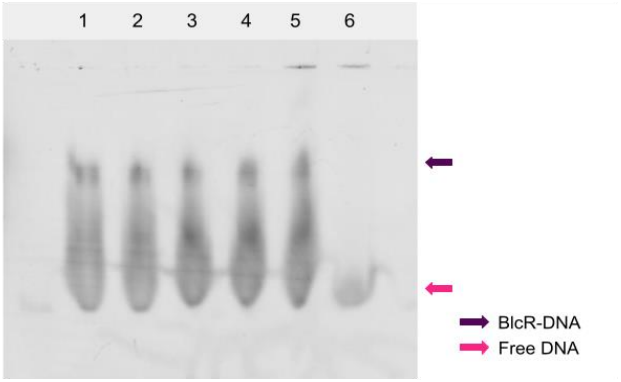

Figure 42. EMSA study for the characterization of BlcR binding to the 51-bp *blc* operator sequence in the presence of ethanol. Concentration of Cy3-labeled DNA and BlcR was maintained at 25 nM and 1.6 µM, respectively. Titration of ethanol from lane 1 to 6. 1: 0%, 2: 2%, 3: 5%, 4: 10%, 5: 20%, 6: 40% .

As the concentration of the protein increases, so does the binding. Accordingly, the amount of free DNA decreases. This is clearly visible from the gel.

Date: 03/10/2022

Experimentalist(s): Martina

Protocols: EMSA

EMSA

In order to determine the effect ethanol has on BlcR, the samples were prepared as follows:

Table 33. EMSA sample preparation

Component	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Final concentration
Cy3 DNA (100 nM stock)	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	25 nM
BlcR (2.9 uM)	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	4.6 µL	1.6 uM
Binding buffer (10x)	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	1x

Ethanol (99%)	-	1.5 µL	3 µL	6 µL	12 µL	18 µL	from 5% to 60%
Nuclease free water	18.4 µL	16.9 µL	15.4 µL	12.4 µL	6.4 µL	0.4 µL	

The samples were incubated for 1 hour on ice in the dark. Consequently, 4 µL of bromophenol and 6 µL of 80% glycerol were added to each sample. The samples were loaded on the gel and the gel was run for 40 mins at 90V.

RESULTS

The gel appeared slightly different from the previous one, however one trend could be clearly identified: as the concentration of ethanol increases, the binding decreases, as visible from the picture.

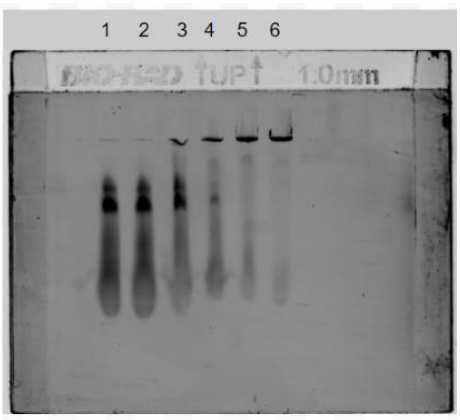

Figure 44. EMSA study for the characterization of BlcR binding to the 51-bp *blc* operator sequence in the presence of ethanol. Concentration of Cy3-labeled DNA and BlcR was maintained at 25 nM and 1.6 µM, respectively. Titration of ethanol from lane 1 to 6. 1: 0%, , 2: 5%, 3: 10%, 4: 20%, 4: 40% . 6: 60 %.

Literature

[1] Pan, Y., Fiscus, V., Meng, W., Zheng, Z., Zhang, L. H., Fuqua, C., & Chen, L. (2011). The Agrobacterium tumefaciens Transcription Factor BlcR Is Regulated via Oligomerization. *Journal of Biological Chemistry*, *286*(23), 20431–20440. https://doi.org/10.1074/jbc.m110.196154

[2]Adapted from iGEM TUDelft 2021 Apta Vita
https://2021.igem.org/Team:TUDelft/Experiments

[3] Adapted from iGEM Bielefeld 2015
https://2015.igem.org/Team:Bielefeld-CeBiTec/Protocols